CELL THERAPY PROCESS FOR VOLUMETRIC MUSCLE LOSS

A Major Qualifying Project submitted to the faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science

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ABSTRACT

Muscle injury therapy employs human primary satellite skeletal muscle cells seeded onto microthreads. However, the cell culture process prior to seeding that would assure maintenance of the myogenic potential has not been optimized. This cell line has both adherent and non-adherent cell populations; non-adherent population displaying a higher myogenic potential. The team was challenged with the optimization of growth and myogenic potential, either through the use of device or the optimization of the cell culture process.

The team focused on creating a design that was commercializable, user friendly, and optimized the production of non-adherent cells. In addition the team determined that this design must be safe for the user and for the cells, and must yield a myogenic potential of at least 30%. By using these objectives and constraints, the team developed a series of alternative designs, and determined that a process engineering approach of optimizing the medium was best suited to meet the client needs. For this project, the modified the concentration of growth factors in the current proliferation medium recipe to observe the effect of cell proliferation, myotube formation, and myogenic potential.

Initially, the team started with 23 treatments for preliminary testing of a cell count, proliferation, and differentiation percentages. The top ten treatments that produced the greatest results were verified using the same tests. From here, the top six treatments were chosen to conduct MyoD expression and myogenic potential assays. Based on these results the top two treatment mediums were determined and a cost-benefit analysis was conducted.

After conducting these assays, it was found that culturing the cells without EGF produced a less expensive and more beneficial process than that of the current medium. Compared to the control, the No EGF had a similar proliferation percent (31.8% ± 14.4 vs 34.4% ± 13.4). In addition, it had a greater population percentage of non-adherent cells (32.7% ± 9.6 vs. 23.3% ± 7.7) and almost doubled the myogenic potential (47.3% ± 6.5 vs. 27.1% ± 2.2). In addition, the cost-benefit analysis confirmed that this medium was nearly $1,000 less costly than the control to move forward with in a clinical setting of treating a wound defect of 15 cubic cm.

EXECUTIVE SUMMARY

I. INTRODUCTION

Human skeletal muscle is responsible for voluntary movement to complete basic daily functions [1]. However, injuries can often arise to the muscle. After these injuries, skeletal muscle has the unique ability to regain function, provided the muscle loss is not too high [2]. When injury occurs, the muscle first experiences inflammation and the fibers rapidly degrade. Following this, new tissue is formed from satellite cell proliferation and differentiation [2][3]. Finally, tissue remodeling occurs, which results in the alignment of myofibers and integration into the muscle [3]. However, in large-scale muscle injuries, the wound site is not able to regenerate muscle. As a result, a fibrin matrix is created, resulting in non-functional, collagen scar tissue [4].

Research is being conducted on human skeletal muscle derived cell-loaded fibrin microthread bundles to aid in functional tissue regeneration [4]. However, as the primary human skeletal satellite cells are cultured, the myogenic potential of these cells are lost. From previous research, culture of the satellite cells using a combination of growth factors leads to replication of cells in the non-adherent state. Further, it has been found that the myogenic potential of cells can be preserved over time by selectively subculturing the non-adherent cell population. Therefore, the goal of this project was to engineer a cell culture process to optimize the growth kinetics of satellite cells according to the proportion of cells in the non-adherent state.

To accomplish this, the concentrations of selected components of the cell culture medium were varied to determine their effect on the proportion of non-adherent satellite cells. The subsequent effect on myogenic potential was determined by inducing the cells to differentiate by culture in differentiation medium and calculating the myogenic index. An economic analysis was also performed to determine the cost-benefit of implementing the optimized process.

II. PROCEDURE

A. CELL CULTURE

1) Media Composition: Proliferation media is comprised of 54% Dulbecco's Modified Eagles Medium (DMEM) (Corning), 36% F12 Medium (Corning), 10% FCIII (Hyclone), 4mM glutamine (Cellgro), 1X penicillin/streptomycin (EMD), and various amounts of four growth factors (FGF2, EGF, HGF, and IGF). Different concentrations and combinations of these growth factors were used in each media condition to assess the effects on cellular growth. The differentiation media was made up of 58.2% DMEM, 38.8% F12, 2% Horse serum (Hyclone), 1% ITS, 4mM glutamine, and 1X penicillin/streptomycin (EMD).

2) *Subculturing Adherent and Non-adherent Cells:* Human primary satellite cells were obtained from a Worcester Polytechnic Institute frozen stock, and were grown at 37^{o}C with 5% CO_2 in proliferation media. Cells were seeded at 10,000 cells per well on Nunc 24-well plates and cultured.

B. CELL COUNTS

Through imaging and microscopy the percent of adherent to non-adherent cells were determined. Using a Zeiss inverted microscope, five images were taken after cells were cultured for three days. Images were taken at five identical spots in each well to standardize the process. The team gathered a total cell count for each condition using a hemocytometer.

C. BROMODEOXYURIDINE (BRDU) CELL PROLIFERATION ASSAY

BrdU incorporation assays were completed to determine the effect of each culture condition on cell proliferation rate. Cells were plated at 10,000 cells/well in a 24- well plate for each of the media conditions. After 48 hours 1µL of BrdU labeling reagent (Invitrogen) was added to each well. After an additional 24 hours, cells were fixed with 500 µL of formaldehyde (Sigma), permeabilized with 0.02% Triton X-100 (Fischer) and 1.5N HCl, and treated with -20^{o}C methanol. Primary antibodies, secondary antibodies and their concentrations were anti-BrdU 1:100 and Alexafluor-488 1:500, respectively (DSHB, Invitrogen). Nuclei were counterstained with 0.5 mg/ml Hoechst 33342 (CalBioChem). Images were taken using the same locations as previously described with a Zeiss inverted epi-fluor microscope and AxioVision Software.

D. IMMUNOCYTOCHEMISTRY FOR MYOSIN HEAVY CHAIN

Immunocytochemistry (ICC) for myosin heavy chain allows for identification of differentiated muscle cells. Cells were grown for 24 hours in each treatment media and then cultured in differentiation medium for 10 days. Cells were fixed using the same procedure as for the BrdU above, except the HCl treatment was eliminated. Myosin heavy chain was detected using the primary antibody MF20 at 1:500 dilution (DSHB) followed by secondary antibody Alexaflour-488 1:500 dilution (Invitrogen). Nuclei were counterstained with 0.5 mg/ml Hoechst 33342 (CalBioChem). Fluorescence was observed using a Zeiss inverted epi-fluor microscope and AxioVision Software.

E. IMMUNOCYTOCHEMISTRY FOR MYOGENIC POTENTIAL

To test for myogenic potential and visual recognition of myotubes, two primary and two secondary antibodies were used to target MyoD and myosin heavy chain in mirror wells. The primary and secondary antibodies and their concentrations were anti-MyoD1 antibody [5.2F] 1:1000 (Abcam) and MF20 1:500 (DSHB). Isotype-appropriate secondary antibodies labeled with Alexafluor-488 and Alexafluor-568 at a concentration of 1:1000 were also used. This assay was conducted at four time points: 0, 3, 5, and 10 days, to compare the percentage of cells expressing MyoD to the same percentage of late expressing myosin heavy chain. In addition, multinucleated myotubes were observed to measure myotube formation and fusion index.

III. RESULTS

A. CELL COUNTS

After the cells were cultured for three days in their respective media compositions the adherent and non-adherent cell populations were counted. As seen in Table 1 below, it was found that the +50% FGF2 and No EGF conditions showed to have a larger cell population. In addition, it was found that +50% FGF2, No EGF, -50% EGF, +50% EGF, and -50% HGF had a sizeable population of non-adherent cells which were all above that for control medium.

Table 1: Cell Count Data for Top 10 Treatments

	All GFs (Control)	Just FGF2	No FGF2	-50% FGF2	+50% FGF2	No EGF	-50% EGF	+50% EGF	-50% HGF	No IGF
Total Cell Count	78333 ± 2887	48333 ± 10408	53333 ± 11547	68333 ± 2887	71667 ± 20207	73333 ± 2887	65000 ± 5000	53333 ± 7638	36667 ± 2887	43333 ± 14434
Percent Non-Adherent	23.4% ± 7.7	21.7% ± 8.1	17.5% ± 8.8	24.6% ± 18.0	35.5% ± 7.8	32.7% ± 9.6	28.8% ± 9.6	36.2% ± 9.8	29.8% ± 13.1	26.8% ± 13.8

B. BRDU PROLIFERATION ASSAY

Cells were seeded at 10,000 cells per well and cultured for two days. BrdU labeling reagent was added at this time, and then cells were allowed to differentiate for 24 additional hours before the assay was completed. After completing the BrdU assay, it was observed that four treatment mediums displayed a BrdU positive cell percentage of greater than 30%. These treatments include the control of All GFs, +50% FGF2, No EGF and No IGF.

C. IMMUNOCYTOCHEMISTRY FOR MYOSIN HEAVY CHAIN

Cells were seeded at 10,000 cells per well, and cultured for three days in treatment medium. Cells were then placed into differentiation medium where they were allowed to differentiate for 10 days. After completing the assay, it was observed that three treatment media produced a percent differentiation of greater than 30%. These treatments include the control of All GFs, Just FGF2, and +50% FGF2. However, while these cells were highly differentiated, most of the cells produced only mononucleated myosin positive cells.

D. IMMUNOCYTOCHEMISTRY FOR MYOGENIC POTENTIAL

Using data from the cell count, BrdU, and ICC for myosin heavy chain, the team selected the top six treatments that yielded the best results. Cells were seeded at 10,000 cells per well for three days in treatment medium. Cells were then placed into differentiation medium and differentiated for 0, 3, 5, and 10 days and stained for either MyoD or myosin heavy chain. Cells that were stained for MyoD showed an increase in expression through Day 5. After Day 5, however, there was a drastic decline in MyoD expression in all treatments.

Figure 1 below displays the data obtained for cells stained to identify myosin heavy chain over a 10-day period. This data was used to calculate the myogenic potential through the use of a fusion index. As shown in the Figure, all treatments show a similar trend in differentiation, however two treatments, No EGF and +50% FGF2, maintained a high myogenic potential throughout the differentiation process. In addition, these treatments produced the greatest myogenic potential and were greater than the control.

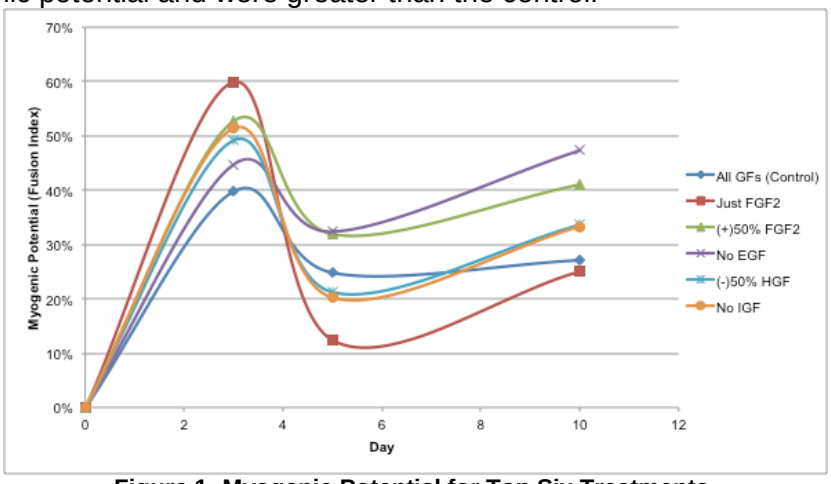

Figure 1: Myogenic Potential for Top Six Treatments.

IV. DISCUSSION

By analyzing the cell proliferation rate by cell count and BrdU incorporation, and differentiation of the cells, the team determined the top six treatments to test for myogenic potential. As previously mentioned, the non-adherent population is believed to be a leading

factor in preserving myogenic potential for satellite cell amplification *in vitro*. It was found that all of the treatments, with the exception of the No FGF2 treatment, yielded a higher percent non-adherent population than the currently used medium. Further, +50% FGF2 and No EGF also demonstrated a high cell count as well as a high percent non-adherent population.

In order to scale up this process for clinical use, cells need to demonstrate a high proliferation rate, and therefore treatments that produced a higher amount of BrdU positive cells were identified. Based on the BrdU results, only two conditions (+50% FGF2 and No IGF) showed greater proliferation than the medium used currently. In addition, the differentiation capability of the cells was determined through the ICC assay for myosin heavy chain. By analyzing this data, the top six treatments that provided a total cell count, non-adherent cell count, BrdU positive cell count, and differentiation percent were selected for further investigation. These treatments were the control of All GFs, Just FGF2, +50% FGF2, No EGF, -50% HGF and No IGF.

These treatments were then tested for the expression of MyoD and for their myogenic potential. MyoD is a transcription factor that is up-regulated during the early phases of differentiation. In analyzing this in conjunction with the expression of myosin, the myogenic potential was determined. Upon reviewing Table 1 and Figure 1, it can be observed that the two treatments that produced the greatest non-adherent population also produced the greatest and most consistent myogenic potential. This supports the previous hypothesis that the non-adherent population plays a key role in preserving the satellite cell myogenic potential.

A cost-benefit analysis was conducted on the two treatments that yielded the highest myogenic potential (+50% FGF2 and No EGF). This cost analysis was based on the amount of media needed to scale up from 500,000 cells to 30 billion cells (the amount needed to treat a 15 cubic cm wound). The media currently being used would cost roughly $118,500; the +50% FGF2 medium would cost roughly $132,500; and the No EGF medium would cost roughly $117,600. Not only was the No EGF treatment medium the least expensive, but it was also produced the greatest myogenic potential. In addition, further statistical analysis showed that this medium produced the most reproducible and reliable process, as indicated by the low standard deviations.

V. FUTURE RECOMMENDATIONS

With the No EGF treatment selected, there is further research that should be conducted to verify and further optimize this process. These include:

- Verifying the long term effects of the treatment medium on the cells
- Altering ambient oxygen levels and substrate surfaces
- Conducting a single cell contraction assay to verify the functionality of the cells
- Exploring the possibility of using a device to further optimize the process

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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

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AUTHORSHIP

All team members contributed to the writing of each chapter in this report. Each team member participated in individual as well as group writing, in addition to editing the entire report.

1. INTRODUCTION

Skeletal muscle is a large component of the human body and constitutes 40-45% of the human body mass. Unfortunately, injuries often arise to this tissue, primarily due to sports injuries, combat trauma, or motor vehicle accidents. However, skeletal muscle has a large capability to regenerate, largely in part due to progenitor satellite cells found in the muscle tissue. While these cells aid in muscle regeneration, they are unable to fully heal a large-scale injury wound due to scar tissue formation, leading to volumetric muscle loss (Turner and Badylak, 2012).

Currently, in the Page Lab is researching methods to reduce the volumetric loss of muscle. One method includes implanting cell-loaded microthreads into wound site. Through this process, some degree of skeletal muscle tissue and functionality can be regenerated with minimal scar tissue formation (Page et al., 2011). Unfortunately, the myogenic potential of satellite cells is lost with each successive passage, and in order to be used in a clinical setting, their needs to be a process to scale up the production of these cells. In addition, it has been found that both adherent and non-adherent populations exist in this cell culture, and it is believed that the non-adherent population is correlated to myogenic potential.

The goal of this project was to improve the myogenic potential and growth of human primary satellite cells. This was achieved by developing and optimizing a cell culture process, which improved the percent non-adherent cells and the myogenic potential. The cell culture medium was identified as an area for improvement. In order to meet the goal, a series of immunocytochemistry and cell counting assays were completed on various treatment mediums. These assays highlighted the cell proliferation, differentiation, total population, and the cellular potential to form functioning muscle tissue. By process engineering, the team was able to determine a final optimized medium and conduct a cost-benefit analysis.

The following chapters of the report contain a literature review outlining relevant information and literature pertaining to skeletal muscle and satellite cells; a project strategy outlining the objectives and constraints used to revise the client statement; alternative designs and methods used to determine the process engineering approach; preliminary testing and results; preliminary data verification; final design results and selection of a optimized medium; and finally the team's conclusions and further recommendations.

2. LITERATURE REVIEW

This project explored strategies to enhance the propagation and myogenic potential of human primary satellite cells *in vitro*. This chapter assesses the relevant background and literature on skeletal muscle tissue, skeletal muscle regeneration, engineering skeletal muscle, *in vitro* cell culture environments and medium components, non-adherent cells as well as different tests to monitor the growth of cells.

2.1 SKELETAL MUSCLE

There are three types of muscle found within the body: cardiac, skeletal, and smooth. The skeletal muscle is responsible for voluntary movement within the body. Without skeletal muscle, the human body would not be able to complete the most basic daily functions. Cardiac and smooth muscle are responsible for involuntary muscle contractions within the body (Shier, 2009). The following section discusses the skeletal muscle structure and describes the mechanism of how it functions.

2.1.1 SKELETAL MUSCLE HIERARCHY

The structure of skeletal muscles can be described as a decreasing hierarchal structure. The top-most layer, the fascia, is connective tissue that is composed of collagen. This connective tissue anchors the muscle to the bone. The next level in the muscle hierarchy is bundles of fascicles. Each fascicle contains bundles of muscle fibers, which are composed of myofibrils. Myofibrils contract when stimulated. Figure 1 below shows the structure of a skeletal muscle (Shier, 2009).

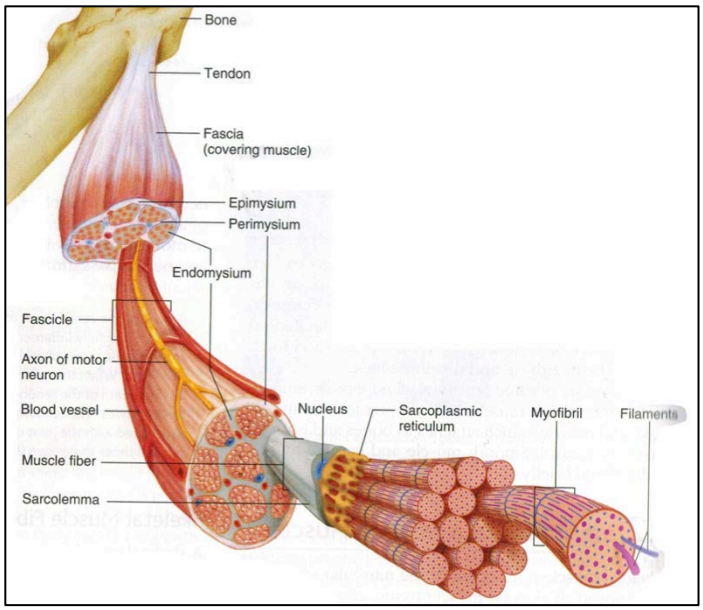

FIGURE 1: DIAGRAM INDICATING THE STRUCTURE OF SKELETAL MUSCLE (SHIER, 2009)

2.1.2 MYOCYTE STRUCTURE AND FUNCTION

Each myofibril, or myocyte, contains several sarcomeres. One sarcomere represents one functional unit. Each sarcomere contains several actin (thin) and myosin (thick) filaments. These filaments are stimulated when calcium ions are transported to them from the sarcoplasmic reticulum (SR). The SR surrounds each of the myofibrils and transmits an electrical signal, which is created at the neuromuscular junction. Once stimulated, the myosin heads attach to the actin and slide over one another, known as the Sliding Filament Theory. This shortens the sarcomere and, as a result, contracts the muscle (Shier, 2009).

2.2 SKELETAL MUSCLE REGENERATION

After injury, skeletal muscle is unique in the sense that it has the ability to regain complete functionality provided that the volumetric tissue loss from the injury is not too high (Collins, 2006). Regeneration relies on primary muscle satellite cells throughout the healing process which consists of three phases: 1) Inflammation, 2) Tissue formation, and 3) Tissue remodeling.

Inflammation is the first phase of skeletal muscle regeneration. This phase begins immediately after the injury followed by the process of necrosis of the injured myofibers and an inflammatory response (Turner and Badylak, 2012). When the skeletal muscle is initially injured, the myofibers rupture and release proteases that then lead to rapid degradation of the fibers. In

order to concentrate the proteases and isolate the damaged region, a cytoskeleton is created. When the proteases are released into the wound site, macrophages are signaled which induces the inflammatory response. Macrophages are important in skeletal muscle regeneration because they are responsible for the inflammatory response and they also help to support cell proliferation later in the healing process (Brunelli and Rovere-Querini, 2008). As the macrophages flood the site of the ruptured myofibers, they interact with the nearby satellite cells to promote chemotaxis, or movement of the satellite cells from a region of high cell concentration to low concentration. The macrophages also release TNF-α and IL-1β. These increase phagocytosis of the damaged tissue (Grefte, 2007). After 24 hours, another group of macrophages begin secreting anti-inflammatory factors and cytokines that increase cell proliferation and begin the process of tissue reformation.

Tissue formation consists of satellite cell proliferation and differentiation, which leads to the formation of new skeletal muscle tissue. Figure 2 shows this process.

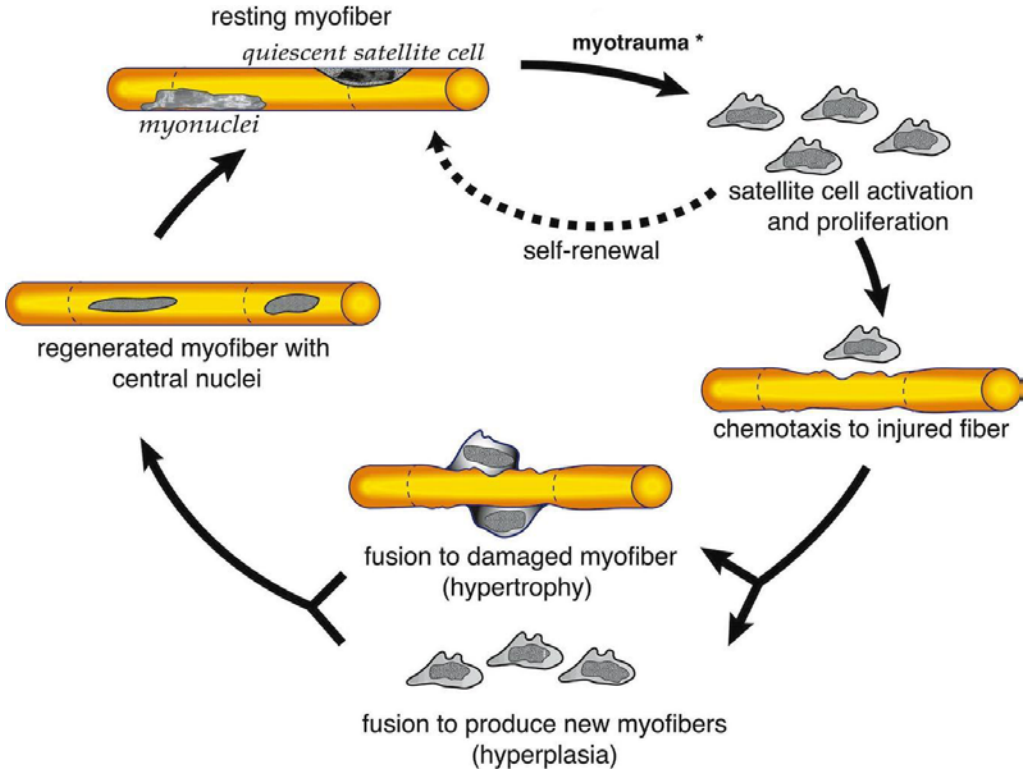

FIGURE 2: SKELETAL MUSCLE REGENERATION (SHI AND GARRY, 2006)

Satellite cells are quiescent when residing between the basal lamina and sarcolemma. There is limited gene expression, however this can become activated by trauma or myo-degenerative disease (Le Grand and Rudnicki, 2007). In looking at the figure above, one can observe that upon the arrival of the second macrophage population at the end of the

inflammatory phase, chemotactic factors, such as growth factors FGF, HGF, IGF-I, IGF-II, TFG-β, and IL-6 (Charge and Rudnicki, 2004), are released in order to attract quiescent satellite cells if the basal lamina has been damaged (Ciciliot and Schiaffino, 2010).

During activation, the cycling myoblasts start to co-express the paired-box transcription factors Pax7 and Pax3, and the myogenic regulator factors Myf5 and MyoD. These myoblasts will continue to divide and differentiate to form myocytes, leading to the downregulation of Pax7 and expression of myogenin. The differentiated myocytes fuse together to form multinucleated myofibers. Protein markers can be labeled and play an important role in satellite cell development, as it is needed for the differentiation of skeletal myoblasts. Myogenin is also important for the formation of myotubes and fibers as it downregulates the Pax7 gene, which is responsible for continuing myoblast growth and delaying differentiation (Le Grand and Rudnicki, 2007). When myoblasts differentiate, they express myosin protein. A diagram of satellite cell development into myofibers, as well as the factors and proteins present during these phases can be seen in Figure 3. These factors and proteins are important as they may have the potential to be used in assays as an early marker for the development of myofibers.

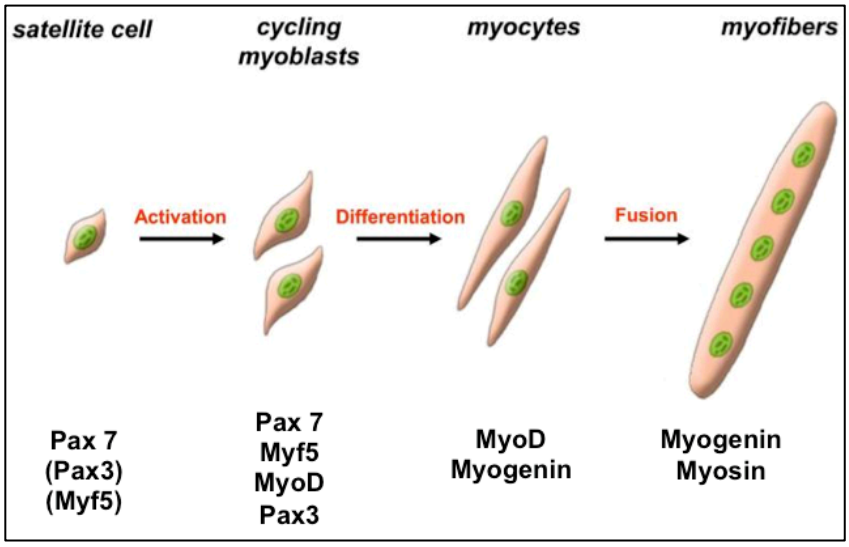

FIGURE 3: FLOWCHART OF HUMAN MYOGENESIS WITH RESPECTIVE EXPRESSED PROTEINS (LE GRAND AND RUDNICKI, 2007)

During the healing process, satellite cells from nearby muscle fibers migrate to the damaged tissue where they proliferate in order to rapidly repopulate the myofiber. Towards the end of the proliferation cycle, the satellite cells either differentiate into myoblasts or they return the quiescent state for future regeneration purposes. The cells that differentiate either bind together to form new myofibers or fuse to damaged myofibers to help the repairing process (Brunelli and Rovere-Querini, 2008). This phase generally occurs 24 hours after the initial injury.

Tissue remodeling is the final phase of skeletal regeneration. The newly formed skeletal myofibers are not completely matured at this point and therefore need to enter the remodeling phase. This process consists of revascularization, reinnervation, and alignment so that the new muscle can fully integrate with the surrounding muscle (Turner and Badylak, 2012).

In order to reestablish the supply of nutrients and oxygen to the new tissue, revascularization must occur early in the process otherwise the new myofibers cannot survive. For survival, myoblasts need to be within approximately 150 μm of a blood supply. This is the maximum thickness that the nutrients and oxygen can diffuse through. Once the vascular connection is established, a neural connection, or reinnervation, must occur. These neuromuscular junctions are responsible for the uniform contraction of a muscle. If connections are not made as the tissue is formed or soon after, the muscle will develop atrophy, resulting in further injury. The last phase of remodeling is the alignment of the myofibers in order to fully integrate them into the preexisting muscle. First, the basal lamina is repaired. Next, the myofibroblasts replace the temporary matrix created during the tissue formation phase (Turner and Badylak, 2012). The myofibroblasts allow for complete maturation of the newly remodeled tissue, thus creating a fully functional regenerated muscle tissue.

For small-scale injuries, fibroblasts infiltrate the wound site, and deposit collagen to create scar tissue that bridges the gap between the functional myofibers. For these injuries, the collagen aids in promoting muscle formation. However, for larger scale muscle injuries, there is an overwhelming fibrotic response. Fibroblasts quickly deposit collagen faster than myogenesis can occur. This causes scar tissue to form dense caps over the muscle fibers, preventing any myoblasts from fusing to the tissue to close the wound. This lack in fusion between the myoblasts and existing muscle results in volumetric muscle loss occurs (Turner and Badylak, 2012).

2.3 ENGINEERING SKELETAL MUSCLE TISSUE

Skeletal muscle tissue engineering could provide treatment to muscle diseases including muscular dystrophy and spinal muscular atrophy, as well as situations in which there is a loss of muscle tissue, including traumatic injuries. In order to engineer skeletal muscle tissue, several requirements must be met. First, the myofibrils must be in parallel alignment with the myosin and actin filaments. There also must be acetylcholine receptors within the tissue, which assists in creating forces within the muscle. In addition, there should be an intracellular calcium storage, which aids in muscle contraction (Liao, 2009). If this tissue is to be implanted into the body, it

must be biocompatible, integrate into the native tissue, and be vascularized and innervated to ensure viability (Vandenburgh, 2002).

In order to be used as a model, there needs to be a standardized, easy, reproducible, low-cost, and fast method to culture the cells. It also must be engineered using recognized cell lines, such as human primary satellite cells or C2C12s, to control the variability and mutations of testing gene function. In order to test the function of the tissue, it needs to be able to respond to mechanical and electrical stimuli (Khodabukus, 2007). Mechanical stimulation through applying stretch forces and pressure during myogenesis on the tissue influences gene expression, protein synthesis, and RNA/DNA content. It also promotes the parallel alignment within the myotubes needed. To develop more functional skeletal tissue and a higher differentiated population, electrical stimulation is used to imitate nerve stimulation during myogenesis. A static magnetic field can also aid in muscle differentiation and help the parallel orientation within the myotubes as well (Liao, 2009).

There are two common practices used to engineer skeletal muscle tissue. The first method, *in vitro* cell therapy, involves taking a biopsy to obtain the muscle cells, and then culturing them in a bioreactor. Upon differentiation, these cells are then reimplanted into the body. The other method, *in vivo* cell therapy, involves the cells to be obtained and grown *in vitro*. These cells are then seeded onto a transport matrix, where they are then implanted into the body, where they eventually differentiate into myotubes (Liao, 2009).

2.4 CLINICAL NEED

Injuries to the musculoskeletal system are common, and often arise from sports/exercise-induced injuries, combat trauma, or motor vehicle accidents. About 10%-55% of sustained sports injuries are muscle related, and are primarily contusions or strains. While these injuries are common and can lead to volumetric muscle loss, there are few clinical studies that have been conducted to treat this (Turner and Badylak, 2012).

For smaller scale injuries (tears, lacerations, contusions), there is not a significant loss of tissue. This is primarily due to the progenitor satellite cells found in the muscle tissue. The more severe the injury, however, the more difficult it is for the muscle to repair itself. If more than 20% of the muscle tissue is lost or damaged, the tissue will not be able to regenerate, and instead non-functional scar tissue will form (Turner and Badylak, 2012)..

The current standard of care is to use a graft of vascularized tissue to aid in the restoration of the tissue. Surgery can also aid in restoring function, however the tissue is not

regenerated and it may lead to alterations in the biomechanics of the implant site, as well as the donor site (Turner and Badylak, 2012).

A tissue engineering approach of using satellite cells to aid in regeneration could offer a solution to volumetric muscle loss. Currently, the Page Lab has been researching methods to combat this problem using autologous cell therapy. This research includes implanting cell-loaded microthread bundles into a skeletal muscle wound site. It has been found that this aids in the reformation of functional skeletal muscle and reduces the amount of scar tissue and collagen present in the wound (Page et al., 2011).

While this method has been successful when tested on a mouse, there are *in vitro* limitations. In order to treat a human-sized wound, the amount of cells needed must be scaled up. Therefore, an abundance of cells need to be available. Unfortunately, the myogenic potential of human primary satellite cells is lost with each passage. In order to use this type of cell therapy for humans, a process to optimize the cellular growth and expansion must be defined.

2.5 CELL CULTURE MEDIUM AND ENVIRONMENT

When culturing cells in a controlled environment, there are several factors that can directly affect their growth. It is important to understand that each of these components can work together to best mimic an *in vivo* like environment, making the cell culture as close to normal human cells as possible. The following sections discuss different medium components and ways to create an *in vitro* cell culture environment.

2.5.1 RECREATING SKELETAL MUSCLE TISSUE ENVIRONMENT

In order to successfully grow skeletal muscle cells in culture, similar biological conditions must be mimicked. Cells are grown in incubators that are kept at 37°C which is the same temperature of the human body. Further, the amount of oxygen and carbon dioxide in the cell culture system is also monitored. Too much or too little of these compounds could result in cell death. Generally, cells are grown in 15-20% O_2 and 5-10% CO_2 (Wolf, 2010). It is also important to monitor the pH of the cell culture as it directly relates to how the cells are growing. If the pH is too acidic, it can be assumed that a lot of cell growth has occurred resulting in cell waste, making the medium acidic and change colors. A pH that is too high or too low can directly affect the cells in the culture and could potentially damage them (Whitefleet-Smith, 2012).

2.5.2 BASIC MEDIUM COMPONENTS

When culturing cells it is essential that they obtain the proper amount of nutrients to support optimal cell growth, which is dictated by the medium used. Thus, it is vital that the medium has the proper components. Many basic elements and ions can be found in most mediums to support the cells. These include sodium, potassium, calcium, magnesium, chloride, phosphorus, iron, and zinc as well as some vitamins and amino acids. These are present to allow the cells to perform normal functions. In order to promote cell growth, other components such as glucose, antibiotics, and serum are added to the medium. Glucose provides a sugar source for the cells while the antibiotics help to prevent any contaminations that may occur throughout the cell culture process. Lastly, serum is added to the medium as it contains a lot of valuable properties. In general, serum has a lot of nutrients and various growth promoting components and can buffer toxic substances in the medium to neutralize them. Further, serum contains a large amount of peptide hormones and growth factors that greatly promote healthy cell growth (Wolf, 2010).

2.5.3 GROWTH FACTORS

There are four different growth factors that will be considered in this study: Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), Hepatocyte Growth Factor (HGF) and Insulin-like Growth Factor (IGF). Each one of these growth factors plays an important role in cell growth and differentiation. EGF has been known to stimulate RNA, DNA and protein synthesis, as well as increase the cell uptake of nutrients, within mammalian skin cells (King, 1985). FGF has the unique role of regulating cell migration, differentiation and proliferation (Ornitz, 2001). On the other hand, HGF regulates cell growth and cell movement (HGF, 2012). Lastly, IGF maintains and regulates the normal physiology within an organism and at the cellular level; it promotes cell proliferation and prevents apoptosis, also known as cell death (Clemmons, 2012). These mitogens can be used to aid in the growth of human primary satellite cells, and are currently being used in the Page Lab.

2.6 TESTS FOR MYOCYTE CULTURE

In cell culture there is a need to observe how cells are growing and behaving in their environment. Being able to see how the cells react to different induced changes is crucial to further research as well as understanding their behavior. There are several analytical techniques that can be used to observe how cells are growing and developing in a culture. The following is an overview of the main tests that will be utilized throughout the project.

2.6.1 BROMODEOXYURIDINE (BRDU) CELL PROLIFERATION ASSAY

The ability to see if cells are dividing is an essential cell culture technique that allows for the observation of how well cells are dividing. By labeling DNA that is synthesized, one can easily detect the formation of new cells. The BrdU Cell Proliferation Assay uses 5-bromo-2'deoxyuridine (BrdU) to label newly synthesized DNA. This is achieved by adding BrdU to the cell culture and allowing it to incorporate into DNA as the cells go through mitosis as BrdU is a nucleoside analogue for thymidine. Once cells undergo the S-phase of the cell cylce, the BrdU will be incorporated into the new cell allowing it to be detected. Once cells have been cultured for a defined time period, immunocytochemistry procedures can be completed to highlight the new cells. First, they are fixed and a primary antibody is added. This primary antibody, also known as Anti-BrdU antibody, binds to the BrdU that is now found in the DNA. Next, a secondary fluorescent antibody is added and correspondingly binds to the primary antibody. Finally, cells are stained with a Hoechst dye to identify the nuclei present. After this is completed, the cells that have divided will now fluoresce as seen in Figure 4B. Therefore, the cells that have not divided and do not contain BrdU will not fluoresce when observed under a fluorescence microscope. The total cell population can be seen in Figure 4A. Further, this assay easily allows for the observation of how cells are behaving and dividing in different environments (Ambady, 2012b).

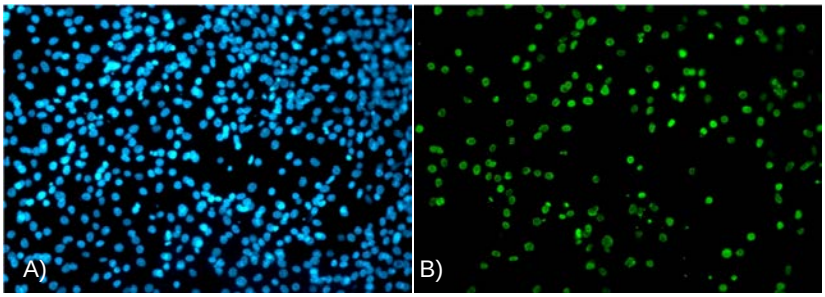

FIGURE 4: A) LIVE C2C12 CELLS STAINED WITH HOECHST AT 20X MAGNIFICATION. B) PROLIFERATING C2C12 CELLS WITH SECONDARY FLUORESCENCE ANTIBODY AT 20X MAGNIFICATION (OBER, 2012)

2.6.2 IMMUNOCYTOCHEMISTRY (ICC)

Similar to the BrdU assay previously described, immunocytochemistry (ICC) assays can also be used to target any marker in the cell as it goes through differentiation. Specifically, antibodies can be used to target myosin heavy chain. This technique detects myosin in the cells present. Myosin is a result from cells successfully forming differentiated myotubes. These are known to be signs of muscle cell development. In order to detect the myosin, a similar procedure as the BrdU assay is applied. This procedure labels the myosin by using a primary

antibody to bind to the myosin. Once the primary antibody is added, the secondary fluorescent antibody is added and correspondingly binds to the primary antibody. Once this is complete, the cells that express myosin will fluoresce when placed under a fluorescence microscope, as seen in Figure 5. This assay allows for the observation of the development of muscle cells, which can be extremely useful when trying to culture a single muscle cell to perform quantitative analysis on (Ambady, 2012c).

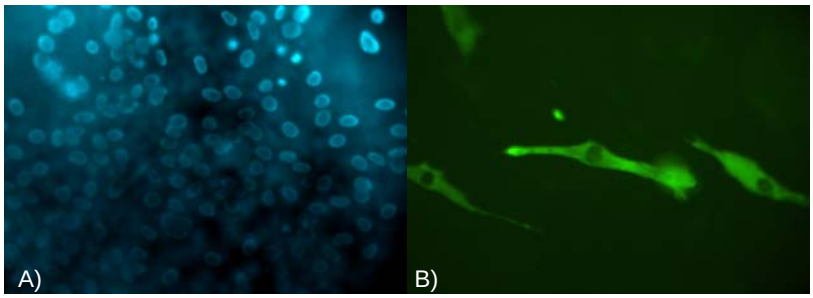

FIGURE 5: A) MUSCLE CELLS STAINED WITH HOECHST AT 10X MAGNIFICATION. B) C2C12 CELLS WITH SECONDARY FLURESCENCE ANTIBODY AT 40X MAGNIFICATION (OBER, 2012)

2.7 NON-ADHERENT CELLS

When cells are cultured using a specific combination of growth factors, there is a population of adherent cells, in which the cells attach to the surface of the flask, and non-adherent cells, in which the cells remain in suspension. As the cells start to replicate, there is a large amount of these non-adherent cells. In many cell culture studies, the non-adherent cell population is completely removed when the medium is changed in the culture. Only a few of these cells, however, are dead. As the non-adherent cells are flushed out with each media change, there is little research done on the efficacy of these cells, and whether or not they have the capacity to produce skeletal muscle tissue. Overall, this loss of cells can contribute to a lesser amount of cells obtained in the culture (Personal Communication, September 4, 2012).

However, Gharaibeh et al. have developed a protocol to obtain skeletal muscle cells that adhere rapidly or slowly from murine skeletal muscle. This preplating technique involves isolating the skeletal muscle cells based on their adhesion characteristics. The cells that adhere rapidly consist of fibroblast cells. After two hours, the non-adherent cells are passaged and re-plated into a new flask. This process is repeated four more times over the following four days to produce six different cell populations. The slowly adhering cells contain muscle-derived stem cells, which can be used to engineer skeletal muscle tissue (Gharaibeh, 2008).

In the Page Lab, when feeding cells, they are placed into a tube, centrifuged, and half of the medium is aspirated. Following this, 4 mL of fresh medium is inserted, re-suspended, and then fed into the flask. After a couple of days when cells need to be subcultured, the entire culture is trypsinized and counted. In the past, the Page Lab would take the non-adherent cells only and passage them forward. What they discovered is that these cells are somewhat consistent with a highly mitogenic population. If just the adherent population is put into a culture with the same medium for growth, another non-adherent population will grow. Overall, this is evidence that these cells are highly myogenic, and when these cells are discarded the viability is decreased (Personal Communication, September 4, 2012).

2.8 COST ANALYSIS

As discussed earlier, medium is an essential part of cell culture. The components of the medium provide the necessary nutrients that aid in cell growth. In addition to the base medium and serum, growth factors are often supplemented to the medium in order to enhance the proliferative ability of human primary satellite skeletal muscle cells. While these components may provide added benefits to the cell culture, they can be costly. Below in Table 1, is a breakdown of the cost of the current medium composition that is used in the Page Lab to grow these cells. Based on the cost from various companies, the cost to make 50mL of medium is $6.77.

TABLE 1: COST BREAKDOWN OF EACH MEDIUM COMPONENT, AND CALCULATED COST TO MAKE 50 ML OF MEDIUM.

Component	Amount/50mL	Commercial Price		Price/50mL of Medium
		Amount	Price	
DMEM	25 mL	6 X 500 mL	$115.46	$0.96
F12	18 mL	6 X 500 mL	$98.67	$0.59
FCIII	5 mL	12 X 500 mL	$153.42	$0.13
Glutamine	0.02922 g	100 g	$87.92	$0.03
Growth Factors				
FGF2 (4ng/ml)	20 µL	10ug solid	$80.00	$1.60
HGF (2.5ng/ml)	12.5 µL	10ug solid	$195.00	$2.44
IGF (5.0ng/ml)	5 µL	50ug solid	$195.00	$0.98
EGF (10ng/ml)	2.5 µL	500ug solid	$195.00	$0.05
			Total Cost:	**$6.77**

3. PROJECT STRATEGY

This chapter outlines the project strategy used to develop and define the project, based on the engineering design process. The team identified and prioritized the project's objectives, constraints, and revised the client statement. Through this process, the team was able to better understand the needs of the client versus their desires to ultimately outline a final strategy.

3.1. INITIAL CLIENT STATEMENT

Before the project was able to advance, the design team had to understand and decipher between the client's desires and needs. The client statement received was broad and not focused on a specific problem; therefore, it required revision. The text below is the initial client statement provided by Dr. Raymond Page:

> "Currently, the laboratory uses extruded fibrin microthreads with human skeletal muscle derived cells seeded onto the surface and transplanted into SCID mouse skeletal muscle injury models to study the effect of various cell derivation and culture methods on functional tissue regeneration. One of the limitations in primary human skeletal satellite cell and myoblast culture *in vitro* is the loss of myogenic potential with each subsequent passage. Some of this loss may be attributed to contamination of fibroblasts in the culture, and therefore selective amplification of the fibroblasts over the myoblasts, given the culture conditions employed. The laboratory has recently discovered a culture system based factors and selective cell adhesion characteristics that enable the propagation of mostly myogenic cells which on specific growth are capable of differentiating into myocytes upon removal of mitogens from the culture medium.
>
> In order to apply this technology at large and economical scale, the detailed growth factor dependent and substrate physical/biosignaling properties must be defined, and optimized. The goals of this project are:
>
> - Design an *in vitro* cell culture system that selectively amplifies myogenic cells by employing combinations of differential adhesion, selective mitogenic (growth factor) stimulation and controlling ambient oxygen.
> - Determine the growth kinetics and cell purity, and differentiation potential of the myogenic cell population with increased time *in vitro* according to individual system design parameters. The myogenic potential (measured by the fusion index) for the cells at the end of the culture period must be at least 30%.
> - Employ individual parameter optimization results to engineer, build, and test a final ex vivo myogenic cell expansion system.
> - Extrapolate (scale up) prototype system to enable replacement of 20 ml of muscle tissue from cell derived from a 200 µl skeletal muscle biopsy."

3.2. OBJECTIVES AND FUNCTIONS

After interviewing the client, the team was able to construct a list of objectives and functions. This kept the team on task and allowed them to focus on the realistic goals.

3.2.1. OBJECTIVES

Objectives are goals and sub-goals that should be sought out in the final design. They are measureable and defined. At the end, they can be achieved in the expected time frame. After developing a list of objectives, the group met with the client to be sure the list of objectives highlighted the key tasks or needs of the problem.

An objectives tree was then created in order to organize the objectives. Higher order objectives are located further left while sub-objectives are located further right. As shown in the objectives tree in Figure 6, this project has three main objectives: commercializable, optimize non-adherent cell population, and user friendly.

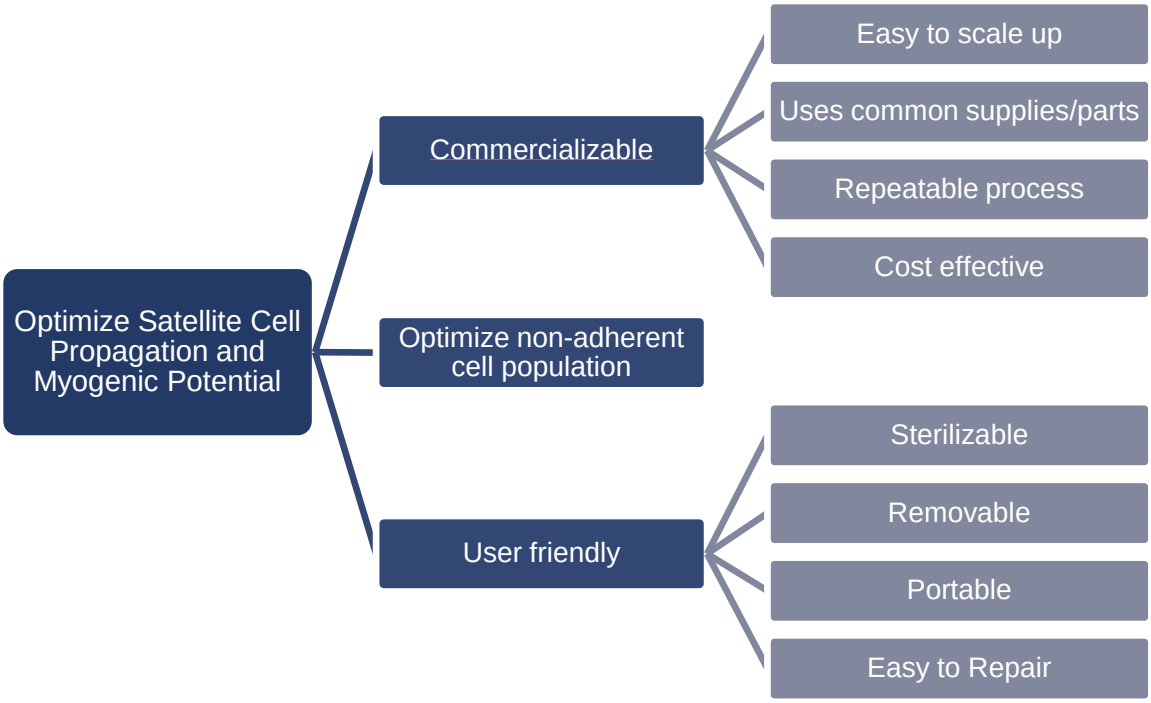

FIGURE 6: OBJECTIVES TREE

The process needs to have commercial potential. Specifically, the design needs to be easy to scale up. The team is performing the tests and assays on a small scale; however, to implement this process, it needs to be used on a larger scale. First, the process should be made from common supplies, or anything that can be easily purchased or found in a lab. This will also

help to keep the product inexpensive. In addition, the process must be repeatable to allow for consistent results to be obtained. The final product must also be cost effective.

The developed product also needs to optimize the non-adherent cell population. There are multiple ways to accomplish this goal including, but not limited to, changing medium components such as growth factors and medium nutrients.

In order for the product to be user friendly, it needs to be easy to integrate into a typical cell culture lab setting. One of the key aspects to consider is sterilization, because when culturing cells, bacteria can easily contaminate and destroy cell life. Therefore, the limitations of sterilization tools like an autoclave must be considered. In addition, the design must follow aseptic techniques. Similarly, the device needs to have the capability of being moved from one setting to another, thus it should be easily removable and portable. In the event that the product breaks, the user should be able to easily repair it.

From the list of objectives, the team was able to create a pairwise comparison chart in order to rank the importance of each objective. The objectives that are more important receive a score of 1, while if they are of equal importance, each receives a score of 0.5. Overall, the objectives are ranked based their total score. The team's completed pairwise comparison chart is shown in Table 2 below. As observed, the most important objectives are for the process to be sterilizable and to optimize the non-adherent cell population. Other important objectives include ensuring the process is cost-effective, easy to scale up, and repeatable.

TABLE 2: PAIRWISE COMPARISON CHART

	Easy to Scale Up	Common Parts	Repeatable Process	Cost Effective	Optimize Non-Adherent Pop	Sterilizable	Removable	Portable	Easy to Repair	**Total**	**Rank**
Easy to Scale Up		1	0.5	0.5	0	0	1	1	1	**5**	**4**
Common Parts	0		0	0	0	0	1	1	1	**3**	**6**
Repeatable Process	0.5	1		0.5	0	0	1	1	1	**5**	**4**
Cost Effective	0.5	1	0.5		0.5	0	1	1	1	**5.5**	**3**
Optimize Non-Adherent Pop	1	1	1	0.5		0.5	1	1	1	**7**	**2**
Sterilizable	1	1	1	1	0.5		1	1	1	**7.5**	**1**
Removable	0	0	0	0	0	0		1	1	**2**	**7**
Portable	0	0	0	0	0	0	0		0	**0**	**9**
Easy to Repair	0	0	0	0	0	0	0	1		**1**	**8**

3.2.2. CONSTRAINTS

If the process does not meet the prescribed limitations, the process fails. Using client interviews and research, the team was able to create a list of constraints, shown below.

- The entire project must remain under the budget of $524.
- The entire project must be completed in 28 weeks.
- The medium components must be compatible with the cells being used.
- The team is limited to human primary satellite cells (Page et al., 2011).
- The final product must be safe for the user.
- The final product must be sterilizable or create a sterile environment to inhibit cell contamination.
- The myogenic potential for the cells at the end of the culture period must be at least 30% (Page et al., 2011).

3.3. REVISED CLIENT STATEMENT

After interviewing the client and identifying the project's objectives and functions, the team was able to refine the initial client statement to better portray the client's needs. The team first removed unnecessary information and added in specific objectives to better define the scope of the project. The team identified that the culturing of the non-adherent cell population was the main focus. The text below is the revised client statement:

- "Research, design, and optimize a process for non-adherent cell growth and enable the production of myogenic competent cells. Improve the cell culture process through optimization of the medium components necessary for myogenic competent cells. Return a final, tested and verified design. Conduct a cost-benefit analysis of the final design."

4. ALTERNATIVE DESIGNS

The following outlines the team's various method options of achieving the project goal to improve the myogenic potential and growth of human primary satellite cells. In addition to the previously listed objectives and constraints, the team brainstormed various alternative designs to complete the project. After conducting a feasibility test, a final alternative design was chosen, and a list of functions and specifications were developed. Upon selecting the final design, a the team determined the project approach.

4.1. DESIGN ALTERNATIVES

After brainstorming, the team was able to develop four design alternatives that could be used in this project. Each design has unique characteristics, which are described below.

4.1.1. CELL CULTURE FILTER PLATE

The first alternative design was a Cell Culture Filter Plate. Based on the Filter Plate from Millipore, the purpose of this design is to improve the cell culturing process and to retain the non-adherent cells (EMD Millipore, 2012). As shown in Figure 7, the plate allows for the medium to be vacuumed out, while keeping all the adherent and non-adherent cells. Each of the 24-well receivers have their own port for medium distribution. The teardrop well design removes the chance of air bubbles, while the raised edges improve the sealing of the well to decrease contamination (EMD Millipore, 2012). While the current design is a 24-well plate, the plate can be reverse-engineered to be a single well plate. The disadvantages of this design include that it does not remove human error during medium changes and cell passaging and the plates are also too expensive for the budget available to the team.

FIGURE 7: CELL CULTURE FILTER PLATE (EMD MILLIPORE, 2012)

4.1.2. AUTOMATED HANDS FREE MEDIUM CHANGE

The second design utilizes gravity and a series of valves to move and measure medium throughout a culture system. The purpose of this design is to not have any human interaction during the cell culture process. Instead, a Programmed Logic Controller (PLC) operates the system. This is connected to three solenoid valves and one level sensor. The PLC can be programmed to turn these valves on or off after a certain time interval has passed. This allows for the culture system to be continuously cultured without the need of personnel in the lab. By having an automated system, the process is repeatable and the risk for human error is also greatly decreased. The only human interaction in this design is placing medium in the initial medium reservoir. After the programmed time span, the first valve will open and allow medium to flow into the chamber, which measures the amount of medium. When the fluid level reaches the level sensor, a signal is sent to the PLC and the first valve is shut off. Once the medium is measured, the second valve will open and medium will drain into the culture dish. At this point, the medium could be in the cell culture dish for an extended amount of time. However, once the PLC allows the next valve to open, the medium, which contains the non-adherent cell population will drain and be placed into either another cell culture dish or a waste container. A preliminary design of this can be seen in Figure 8 below.

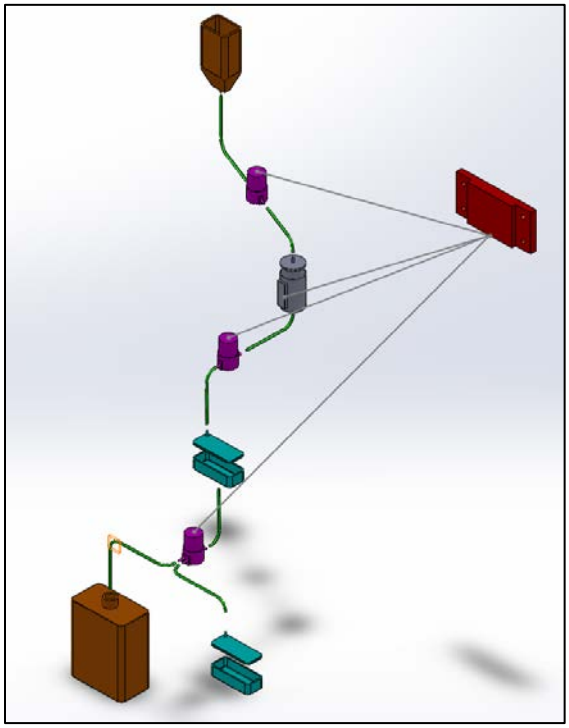

FIGURE 8: CAD DESIGN OF AUTOMATED HANDS FREE MEDIUM CHANGE DESIGN

4.1.3. CONTINUOUS MEDIUM FLOW

The third design alternative uses an ebb and flow/hydroponics based design. This design allows for a continuous fresh flow of medium throughout the system. In this system, the cells are plated onto a Petri dish and protected with a semi-permeable membrane that allows the medium to flow through, but keeps the cells in the plate. The dishes are placed in a closed container, which houses the medium and an overflow tube. When there is too much medium in the container, it goes through the overflow tube and into another container that just houses the medium. Using a pump, this medium is then pumped back to the upper container, providing a continuous cyclic flow of medium. With this design, the medium in the bottom container can easily be changed by removing this container and adding fresh medium. One of the anticipated challenges that this design faces is making sure that all of the medium (growth factors, antibiotics, etc.) flows through each Petri dish, instead of a direct path to the exit. One way to counter this is to make the upper container more narrow, and only allowing one row of Petri dishes, so the medium flows in one straight line. An initial sketch of this design alternative can be seen below in Figure 9.

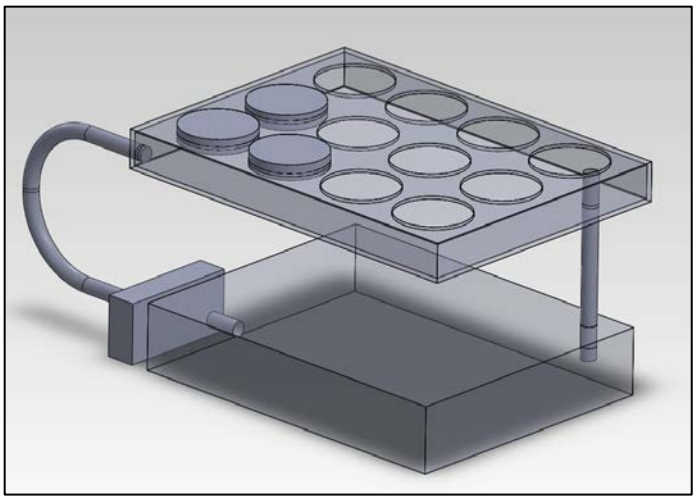

FIGURE 9: CAD DESIGN OF CONTINUOUS MEDIUM FLOW DESIGN

4.1.4. PROCESS ENGINEERING DESIGN

The fourth design alternative consists of analyzing the current process used to culture human primary satellite cells and highlight areas for improvement. Through this, the team identified that the culturing medium, primarily the growth factors within this medium, had the greatest effect on cell propagation. By modifying the medium through growth factor levels, the cells should reflect these changes in the cell growth and myogenic potential. The growth factor concentrations were modified by isolating each growth factor, removing one or two of the growth

factors, and then increasing and decreasing individual growth factor concentrations. The challenge of this method is that until testing, the team is not sure of how the addition or subtraction of growth factors may affect the growth or myogenic potential of the cells.

4.2. FEASIBILITY TESTING

Before determining a final design, the team needed to determine the feasibility of each of the aforementioned design alternatives. First, the cell culture filter plate design does not allow for easy transfer into another culture dish, nor does it reduce human error. This device is also not reasonably priced and for these reasons the team will not be moving forward with this design alternative. Next the continuous medium flow device was ruled out. It would be difficult to ensure that each individual plate will receive equivalent medium components. A membrane that would allow for medium to pass through feeding both the adherent and non-adherent populations would be difficult to fabricate and would not be made from common parts, which is an objective. Of the 3 device designs the automated hands free device seemed to be the most promising. With the ability to reduce human error through automated cell passaging, it also allows for co-culturing of both the adherent and non-adherent population. However a device such as this could be bulky and cumbersome. The logistics behind building a device of this magnitude would also be likely to expand outside the team's budget.

For the reasons listed above the team decided it would be best to move forward with the process engineering design. This is the simplest design and would be easiest to incorporate into the current laboratory techniques and tasks. It was determined that it was most beneficial for the client to focus on the process being used to culture these cells and optimize it. This design allows the co-culture of the adherent and non-adherent populations as well as the ability to isolate important factors that significantly impact the growth and myogenic potential.

4.3. FUNCTIONS AND SPECIFICATIONS

Through interviewing the client and brainstorming amongst the team, a list of functions and specifications were developed to aid in designing various options.

4.3.1. FUNCTIONS

The group created a functions-means chart shown in Table 3 below. The functions-means chart is a tool that compared the different options to satisfy the function. These means further allowed the team to brainstorm design alternatives that meet the needs.

TABLE 3: FUNCTIONS-MEANS CHART

Functions	Means					
Optimize myogenic potential	Alter growth factor concentrations	Alter serum to medium ratio	Alter passage times			
Optimize non-adherent cell growth	Alter growth factor concentrations	Alter serum to medium ratio	Alter passage times	Alter seeding density	Antibiotics	Glutamine

From the table above, the team identified two functions. The process must optimize the myogenic potential and non-adherent cellular propagation. When culturing human primary satellite cells, it is observed that there are both adherent and non-adherent cells. It is normally thought that the non-adherent cells are dead. However, in this culture the non-adherent cells are alive and have been shown to have a high potential for differentiation into the skeletal muscle cells. Having a high myogenic potential is essential. If cells do not exhibit 30% myogenic potential then they will be ineffective when used for cell therapy.

In addition to enhancing the myogenic potential, it is important to maintain and optimize the living cell population. Since cell growth can be affected by different medium components, the team must determine the best combination. Further, the team will determine the ideal passaging time and seeding density that will provide the optimized cell population. As it is thought that the non-adherent cell population is correlated to a high myogenic potential, this cell population will be optimized. For these reasons, the team must optimize the myogenic potential and cellular growth of the cultures.

Normally, when a cell culture is passaged, the cell population is split and divided. Once the cells are split, they are given new medium to provide nutrients for the culture. The team will continue to work with and passage the non-adherent cell population in order to optimize the myogenic potential in the total cell population. Thus, a cell population will develop into adherent skeletal muscle cells.

4.3.2. SPECIFICATIONS

A specification is a detailed description of a design. The following is a listing and explanation of the specifications the team was able to derive.

- **Medium components:**
    - *Proliferation Medium*: A 60/40 ratio of Dulbecco's Modified Eagle Media (DMEM) (Corning) and F12 (Corning) respectively is currently used as the base medium for culturing human primary satellite skeletal muscle cells (Page et al., 2011). DMEM is the most common medium used in cell culture. It is also inexpensive, allowing the team to remain under budget (Whitefleet-Smith, 2011). This medium is supplemented with 10% fetal clone III serum (FCIII) (Hyclone), 4mM glutamine (Cellgro), 1X penicillin streptomycin (EMD), and a proprietary cocktail of growth factors. Fetal clone III serum (FCIII) contains nutrients and natural growth factors that aid in the growth of healthy cells (Whitefleet-Smith, 2011). Glutamine provides sugar for the cell to use to allow it to perform its normal functions as well as glycolysis (Whitefleet-Smith, 2011). Antibiotics, specifically penicillin/streptomycin will be used to help reduce the chance of any bacterial contamination that arises due to human error (Whitefleet-Smith, 2011). Four growth factors (FGF2, EGF, IGF, and HGF) will be used in different concentrations to understand their effect on the proliferation rate and myogenic potential of the cells. These results will be used to understand the minimum amount of growth factors that return the optimal results and reduce cost. Specifically, FGF2 is used to produce a non-adherent cell population (Personal Communication, September 4, 2012).
    - *Differentiation Medium:* Similar to the proliferation medium, a 60/40 ratio of DMEM and F12 will be used as the base medium. This medium will be supplemented with 2% horse serum (Hyclone), 1% ITS (Hyclone), 4 mM Glutamine, and 1X penicillin streptomycin. The primary difference between this medium and proliferation medium is that it lacks mitogens, and therefore induces differentiation of the myoblasts. A detailed recipe for each of these mediums can be found in Appendix B.
- **Cells**:
    - Human primary satellite cells will be used, as these are the type of cells that are being used for autologous cell therapy to reduce volumetric muscle loss.

4.3 PROJECT APPROACH

The team developed a four-step process to complete this project. These steps included cell culturing, preliminary testing, assessing the proliferation rate and myogenic potential, and

developing a cost-benefit analysis of the final design. As seen in Appendix A, a Gantt chart was created to plan and organize the steps necessary to complete the project.

4.3.1 CELL CULTURE

The first part of the projected included getting a greater understanding of the cell culture process that is currently used in the Page Lab. To do this, members of the team toured the laboratory and learned how the current process is carried out and identified areas of improvement. The team also obtained the medium recipes for proliferation and differentiation of the cells, which can be found in Appendix B.

Upon learning the proper cell culture techniques, the team began culturing human primary satellite cells by seeding 500,000 cells into T75 culture vessels. The cells were cultured until they became 60% confluent. If any contamination was observed in the culture, the plates were immediately discarded. After thawing, the cells underwent one successful passage before any plating for testing began. The Cell Culture Protocols used in this project can be found in Appendix C.

4.3.2 PRELIMINARY TESTING

Growth factors play a key role in the growth of the cells; however their exact contribution on cell growth and myogenic potential still needed to be understood. The team derived 23 different medium treatments to test through the removal of one, two or three growth factors, or by increasing or decreasing the concentration of single growth factor. The cells were seeded onto 24-well plates using the different treatment mediums and then assessed by counting the adherent and non-adherent cells. In addition, a BrdU assay and an ICC for myosin heavy chain were conducted to determine the proliferation rate and the percent differentiation into myotubes. After observing the results from these tests, the medium concentrations that provided the optimal proliferation of non-adherent cells and highest differentiation percentage were used for further testing.

4.3.3 TESTING FOR PROLIFERATION RATE AND MYOGENIC POTENTIAL

In order to determine which medium would yield the highest proliferation and amount of differentiated cells, ICCs for BrdU and myosin heavy chain were repeated for validation. A cell count was also conducted to determine the total cell count and the percent of non-adherent cells. Since the non-adherent cell population is believed to be more myogenic as well as providing other assets during culture, it is important to find a medium that contains a high percentage on non-adherent cells. From these three sets of data the team narrowed the

medium down again and tested for myogenic potential.

An ICC assay for myogenic potential was conducted on differentiated muscle cells. Cells were grown in the previously determined growth factor medium concentrations, switched to differentiation medium, and then tested. The mirror cultures were stained for both MyoD and myosin heavy chain. Cells were then determined to be differentiated or non-differentiated, to be part of a multinucleated myotube, or to contain MyoD. The MyoD to myosin heavy chain ratio provides data to determine if MyoD can be used as an early marker for myogenic potential. In addition, fusion index calculations were completed to determine which treatment medium contains the greatest percentage of myogenic potential.

4.3.4 COST-BENEFIT ANALYSIS

Finally, a cost-benefit analysis was conducted to compare the costs of the current medium against the final medium composition. The cost to grow the amount of cells to treat a 15 cubic cm wound was calculated, and the amount of medium to grow these cells was determined. This was used to decide if the chosen treatment medium provided the added proliferative and myogenic benefits necessary. The cost change was then used to determine if the added benefits from the medium outweighed the additional costs, or potential reduced cost.

4.4. PROCESS ENGINEERING APPROACHES

The major component in the medium that affect the growth and myogenic potential of satellite cells is the concentration of growth factors. By identifying the contribution of each growth factor to the cell propagation, the medium can be optimized. Initially, 23 different growth factor concentrations were used in optimizing the medium. Each of these treatments is detailed in Appendix D. Briefly, the medium currently used in the Page Lab was used as a control, which can be found in Appendix B. As there are four growth factors present in the medium, the team decided to create 23 different combinations of growth factors by removing a single growth factor from the medium, removing two growth factors from the medium, and removing three growth factors from the medium. In addition, the team also created medium that doubled the concentration of a single growth factor or decreased a single growth factor concentration by fifty percent. The concentrations for the remaining growth factors remained the same as the control, thus only changing one parameter at a time. It is predicted that EGF and FGF2 will have the greatest effect on the growth and myogenic potential, therefore when these are removed, a significant decrease in the cell population should be noted.

After conducting a cell count, BrdU cell proliferation assay, and ICC for myosin heavy chain assay, the top ten medium treatments that produced the best results were carried over for future testing. A cell count, BrdU, and ICC for myosin heavy chain were completed on these treatments again to verify the previous results. The top six treatments that resulted in the most positive results were then used for further testing. An ICC for MyoD and myosin heavy chain was conducted on these treatments, and then fusion index calculations were conducted to determine which treatment yielded the highest myogenic potential. A cost-benefit analysis was conducted on any treatment that produced a high myogenic potential in order to select a final medium. A visual representation of the process engineering approach can be seen in Figure 10 below.

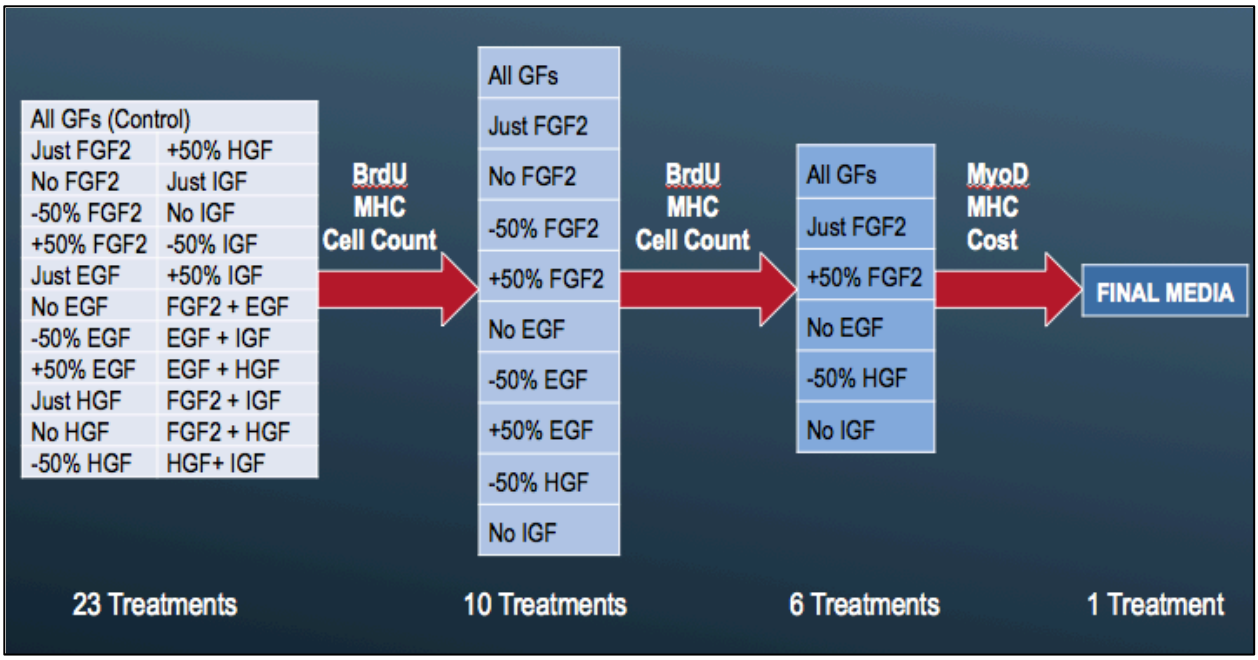

FIGURE 10: ENGINEERING PROCESS AND EXPERIMENTAL PLAN TO NARROW DOWN TREATMENT MEDIUMS

5. PRELIMINARY TESTING AND RESULTS

Preliminary testing was an important step in determining the direction of this project. This chapter outlines results gathered from the approach described in Chapter 4. It also discusses these results and explains how they were interpreted. Furthermore, the key conclusions that can be made from this data, its impact on decisions, and future testing are discussed. Together, the team was able to create correlations between the non-adherent cell population and proliferation rate as well as percent differentiation.

As there were 23 different medium treatments, it would be highly expensive and tedious to test each treatment for myogenic potential. Therefore, preliminary testing on each of the treatments was conducted. The ratio of adherent to non-adherent cells was calculated to help determine the non-adherent contribution to the formation of myotubes. The proliferation of the cells was also verified using an ICC for BrdU. Finally, the differentiation of cells was determined using an ICC for myosin heavy chain expression. The process and timeline for preliminary testing is outlined below.

5.1 PROCEDURES AND TIMELINE

After the first passage following the thawing of the human primary satellite cells, 4 mL aliquots of the different medium cocktails were created. Using the template found in Appendix D, 5,500 cells were plated into each well of a 24-well plate and grown in the different medium conditions. Cells will be plated into three, 24-well plates: one for cell counting, one for the ICC for BrdU assay, and one for the ICC for myosin heavy chain. Figure 11 depicts the timeline for completing the aforementioned preliminary testing.

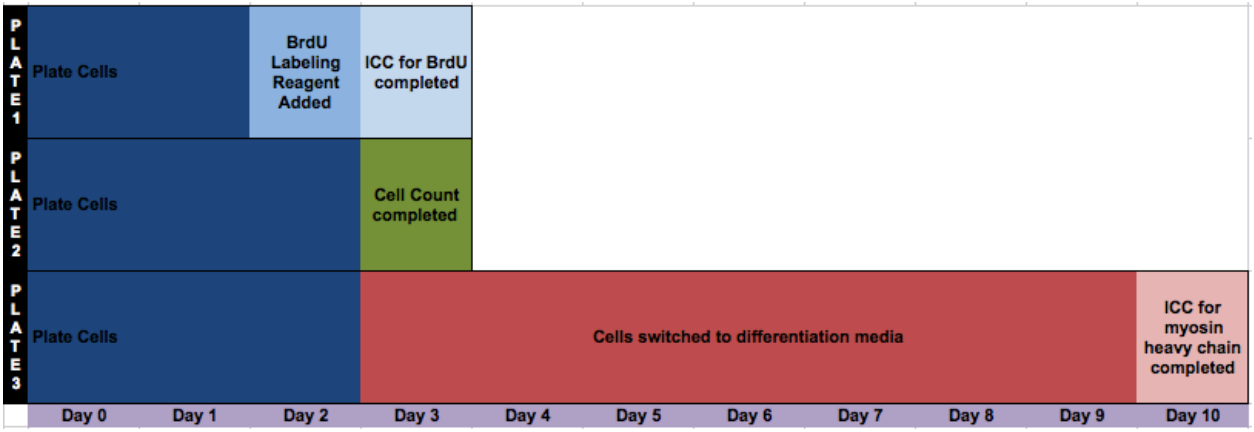

FIGURE 11: TIMELINE FOR PRELIMINARY TESTING

In order to standardize the results that were obtained, five representative images were taken in each well. This was to reflect the overall cell population within each well. As cell growth occurred, the central location became very confluent while the outer locations were less. The images were taken and data was gathered at the same locations as seen in Figure 12 below.

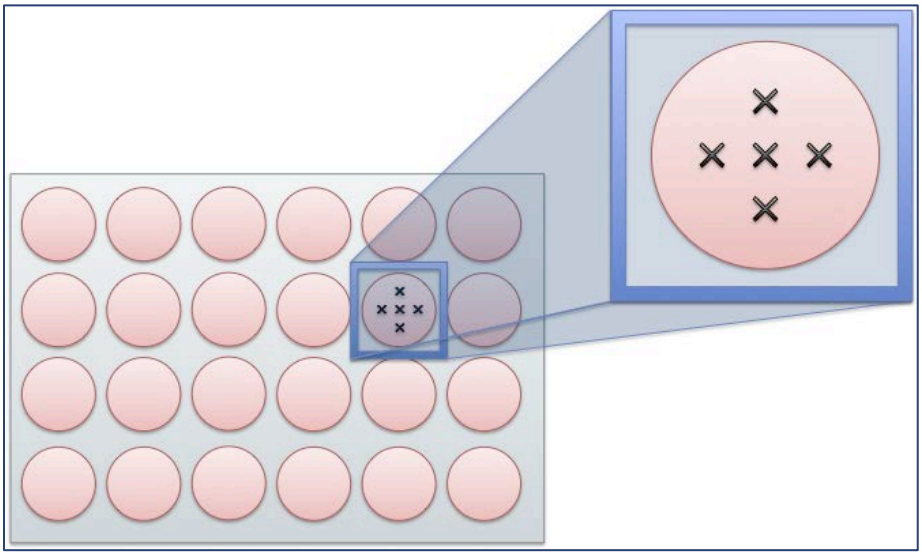

FIGURE 12: CELL IMAGING LOCATIONS FOR STANDARIZATION OF RESULTS

5.2 ICC FOR BRDU

After the cells in the control medium treatment became 40% confluent, the BrdU reagent was added to each well and 24 hours later, the assay was conducted. The assay was completed within 24 hours because human skeletal muscle satellite cells have a 36-hour full cell cycle, and conducting it at his time shows if the treatment medium increases or decreases the proliferation rate. This assay works by incorporating the Bromodeoxyuridine nucleotide analog into the newly synthesized DNA during the S phase of the cell cycle. The procedure for the BrdU testing can be found in Appendix E. After the 24-hour incubation period of the BrdU reagent, cells were fixed with methanol-free formaldehyde, and rinsed twice using DPBS with divalent cations (Ca++ and Mg++). The nuclei of these cells were then permeabilized using 0.2% Triton-X-100, and then rinsed with DPBS twice. Cells were then treated with 1.5N HCl, rinsed with DPBS, and incubated with anti-Brdu antibody diluted 1:100 in PBS + 0.05% Tween-20. After incubation, Alexa Fluor 488 was diluted to 1:500 and added to each well. Cells were then stained with 0.5µg/mL Hoechst 33342. After washing the stain with DPBS, the cells were observed using a Zeiss inverted epi-fluor microscope and imaged using Axiovision software. All nuclei fluoresced blue, while the proliferating cells fluoresced green. The ratio of green

(proliferated cells) to blue (total cells) indicates the proliferative potential of the cells in the given treatment medium.

Once the cells in the 23 treatments were stained, the percent proliferation for each condition was calculated. It was found that nine treatments were significantly higher than the control: +50% FGF2, No EGF, -50% IGF, +50% EGF, +50% IGF, Just HGF, -50% HGF, FGF2 + HGF, FGF2 + IGF. These nine treatments displayed a proliferation rate that was higher than the control. This was required to improve and optimize the current cell culture process by producing a higher population of cells.

5.3 NON-ADHERENT CELL COUNT

While the cells were being fixed for the BrdU assay, a cell count of adherent and non-adherent cells was conducted. The supernatant containing the non-adherent cells was centrifuged and counted. The adherent cells that remained on the plate were trypsinized, centrifuged, and counted. Using this quantitative data, the ratio of non-adherent cells to total cells was established.

After the cell count of the adherent and non-adherent cell populations were conducted, the team analyzed the total cell count as well as percent of non-adherent cells for all 23 treatments. It was found that three treatments had a total cell count around or higher than the control: +50% FGF2, -50% EGF, and +50 EGF. Further, seven treatments had a non-adherent population of higher than 35%: +50% FGF2, No EGF, -50% EGF, +50% EGF, No IGF, -50% IGF, -50% HGF. Note that the three treatments that had a high cell count also had a high non-adherent population. The data for all 23 treatments is not shown, however, the highlights of this data just described can be found in Table 4, below.

TABLE 4: PRELIMINARY CELL COUNT HIGHLIGHTS

	All GFs (Control)	+50% FGF2	No EGF	-50% EGF	+50% EGF	No IGF	-50% IGF	-50% HGF
Total Cell Count	49000	47000	28750	43500	52750	15000	14500	20750
Percent Non-Adherent	34.7%	38.4%	40.2%	41.2%	36.5%	35.3%	35.7%	35.9%

5.4 ICC FOR MYOSIN HEAVY CHAIN

Simultaneously, as the cell count was completed, differentiation medium was added to the plate intended for ICC for myosin heavy chain testing. After 1 week of differentiation, this assay was completed. This procedure is explained in detail in Appendix E. Cells were fixed in methanol-free formaldehyde, permeabilized using 0.2% Triton-X-100, and rinsed using DPBS with divalent cations. Blocking solution (3% FBS) was added to each well at 150 µL per well, and incubated. The primary antibody MF20 (Developmental Studies Hybridoma Bank), diluted at 1:500 in DPBS/0.05% Tween-20, was added, incubated for 30 minutes, aspirated, and rinsed with DPBS three times, for three minutes per wash. Following the aspiration of the DPBS, Alexa Fluor 488 conjugated secondary antibody (Invitrogen) diluted at 1:500 in DPBS/0.05% Tween-20 was added to each well and then the plate was incubated for 30 minutes. After the incubation period, each well was rinsed three times with DPBS, stained with 0.5µg/mL Hoechst 33342, and rinsed and stored in DPBS. Each well was then observed using a Zeiss inverted epi-fluor microscope and imaged using Axiovision software. The cell nuclei were stained blue, while the differentiated cells that expressed myosin fluoresced green. From this data, the differentiation percentage was determined.

Finally, once the ICC for myosin heavy chain data was collected, the percent of differentiated cells were calculated. From analyzing the results it was found that five treatments had higher than 20% differentiation: +50% FGF2, Just FGF2, Just IGF, FGF2 + IGF, -50% HGF. By having a high differentiation into myotubes, the number of cells that can be used in a clinical setting is increased. It is necessary for this percentage to be optimized to ensure that the maximum number of cells is carried through differentiation and into clinical applications.

5.5 PRELIMINARY DATA CONCLUSIONS

After comparing the results from all three tests, the teamed selected the top ten treatments that exhibited a combination of an increased proliferation, differentiation, or non-adherent cell population. A correlation was established between the percent of non-adherent cells and these results. Although some treatments showed a high proliferation rate over a 24-hour period, after three days they did not exhibit the highest cell counts or non-adherent population. Therefore, +50% IGF, Just HGF, and FGF2 + HGF were eliminated from further testing. Similarly, -50% IGF was removed from further testing because it exhibited a low differentiation percentage. FGF2 + IGF was also rejected because it displayed a very low non-adherent cell population which is important for myogenic potential. The top ten treatments

selected for further testing and validation were: Control, +50% FGF2, No FGF2, +50% EGF, No IGF, Just FGF2, -50% FGF2, No EGF, -50% EGF, -50% HGF.

6. PRELIMINARY DATA VERIFICATION

This chapter discusses the verification process in the growth of the human primary satellite skeletal muscle cells. The cell culturing procedure used in the Page Lab is tested and verified through analyzing the results described in each of the sub-sections below. Through the confirmation of these results, the team can further develop the optimization of cell proliferation and differentiation.

6.1. CULTURING OF HUMAN PRIMARY SATELLITE CELLS

The verified process for culturing human primary satellite cells is divided into more detailed descriptions in the sections below. These sub-sections include an overview of this cell line, the medium combination process, how to thaw cells, and specifics related to the culture of adherent and non-adherent cells.

6.1.1. HUMAN PRIMARY SATELLITE CELLS

The cells used in this project were derived from a human skeletal muscle primary satellite cell line. This cell line was chosen because they are a cell type that can be used for autologous cell therapy. The cells were obtained from a Worcester Polytechnic Institute frozen stock, and were cultured in an incubator at 37^{o}C with 5% CO_2.

6.1.2. MAKING MEDIUM

Cells need to be exposed to a healthy, nutrient-rich environment in order to remain viable. In this project, proliferation medium of 54% Dulbecco's Modified Eagles Media (DMEM), 36% F12, 10% FCIII, 4 mM glutamine, and 1X penicillin/streptomycin was used as a base medium. Growth factors (EGF, FGF2, HGF, and IGF) were also incorporated into the proliferation medium. These concentrations varied based on the medium treatment that was used. Differentiation of the primary cells was induced by replacing the proliferation medium with differentiation medium. This medium contained 58.2% DMEM, 38.8% F12, 2% Horse serum, 1% ITS, 4 mM Glutamine, and 1X penicillin/streptomycin.

Initially, antibiotics were not used in the testing of these cells; however various bacterial contamination issues occurred during the beginning phases of testing. While sterile techniques were used throughout the culturing process, there was an increased risk of contamination due to the shared workspace within the laboratory. As a result, a small amount of antibiotics were

used in an attempt to reduce this risk. After the incorporation of these antibiotics, no more contamination issues were observed.

6.1.3. THAWING CELLS

To preserve cellular function and viability, large cell harvests are generally frozen through the process of cryopreservation. In order to use the cells at later time, they need to be thawed through a sensitive process. To restart the cell line, one must quickly thaw the vial to avoid high levels of toxicity in the cells as a result of the dimethyl sulfoxide (DMSO) they were suspended in during the freezing process. The vial must be diluted with medium almost immediately, to diminish the effects of the DMSO. The cryopreservation and thawing processes can be found in Appendix C.

6.1.4. SUBCULTURING ADHERENT AND NON-ADHERENT CELLS

It is commonly thought that cells that are non-adherent, in a cell population that is known to be adherent, are dead. However, some preliminary observations have shown that both of these cell populations exist as healthy cells, when culturing human primary satellite skeletal muscle cells. When observing these cells in culture, doublings can be seen indicating that these non-adherent cells are still alive. When subculturing these cells, the non-adherent cell population is not discarded; rather, both adherent and non-adherent are passaged into a new culture together.

6.2. ICC FOR BRDU

Following the same procedure described in section 5.1, cells were stained to detect the nuclei as well as proliferating cells. The ratio of proliferated cells to total cells counted indicates the proliferated potential of the cells in the given treatment medium. Cells were allowed to proliferate for forty-eight hours in treatment medium. BrdU labeling reagent was then added, and after 24 hours cells were fixed and stained. Figure 13, below, displays the proliferative cells for the ten treatment mediums.

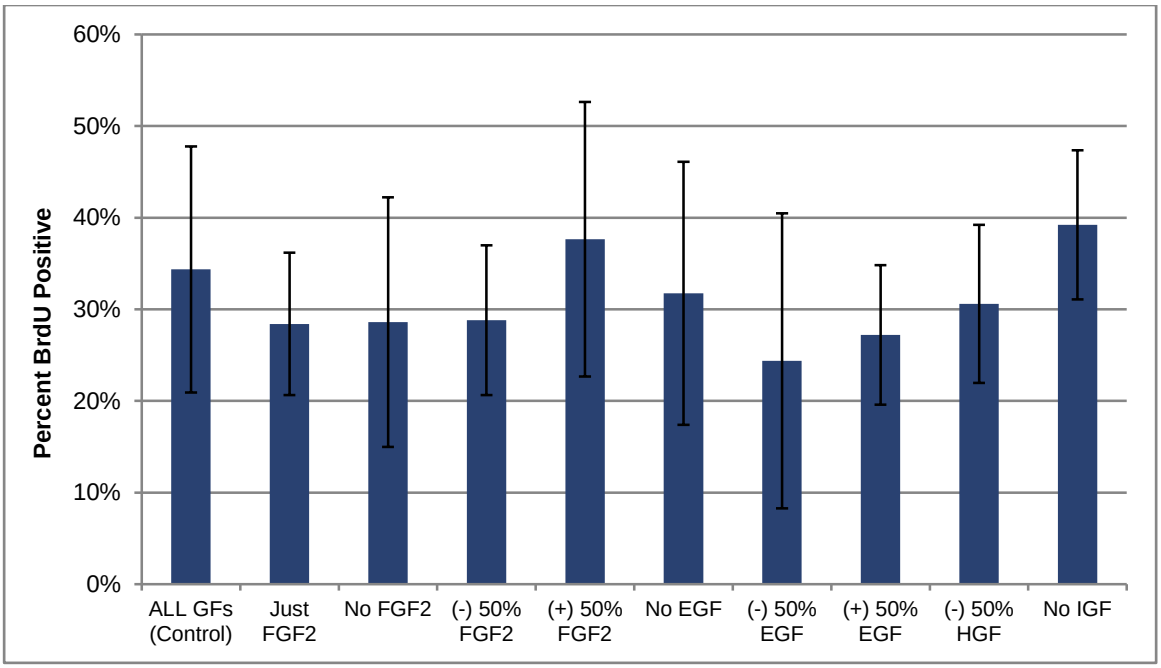

FIGURE 13: PERCENT BRDU POSITIVE CELLS AFTER 24 HOURS OF TOP TEN TREATMENTS

From Figure 13, above, it can be observed that four treatment mediums that were distinctively above 30% proliferation. These treatments include the control of All GFs, +50% FGF2, No EGF and No IGF. These treatments show potential to maintain a high proliferative state while maintaining the potential for a high cell count or percent differentiation. Representative images of these four treatment mediums can be seen below in Figure 14. Note that the total cell population is colored blue while the BrdU positive cells are depicted in red.

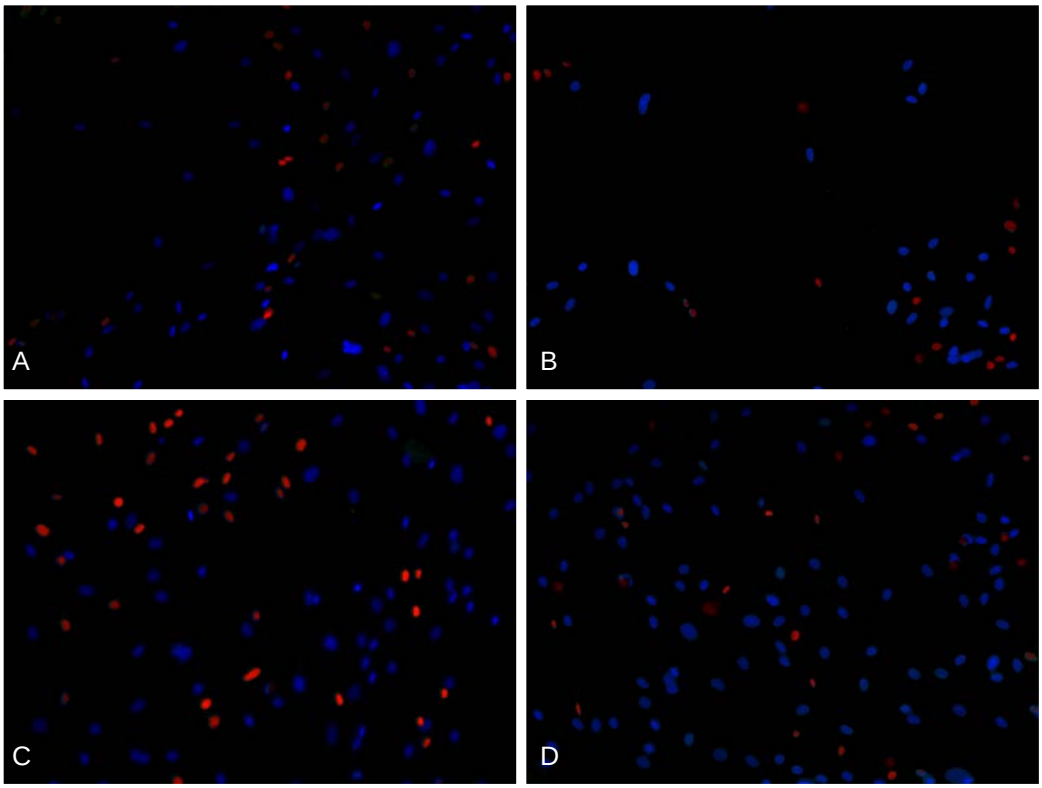

FIGURE 14: BRDU POSITIVE CELL (RED), TOTAL CELLS (BLUE) A) ALL GFS (CONTROL), B) +50% FGF2, C) NO EGF AND D) NO IGF

As seen from Figure 14, +50% FGF2 and No IGF show a substantially higher proliferation rate than the control of All GFs.

6.3. NON-ADHERENT CELL COUNT

As the BrdU assay was completed, a cell count of the adherent and non-adherent populations was conducted. The total cell population was calculated by adding the adherent and non-adherent cell populations. Table 5, below, displays these results.

TABLE 5: TOTAL AND PERCENT NON-ADHERENT CELL COUNTS OF TOP TEN TREATMENTS

	All GFs (Control)	Just FGF2	No FGF2	-50% FGF2	+50% FGF2	No EGF	-50% EGF	+50% EGF	-50% HGF	No IGF
Total Cell Count	78333 ± 2887	48333 ± 10408	53333 ± 11547	68333 ± 2887	71667 ± 20207	73333 ± 2887	65000 ± 5000	53333 ± 7638	36667 ± 2887	43333 ± 14434
Percent Non-Adherent	23.4% ± 7.7	21.7% ± 8.1	17.5% ± 8.8	24.6% ± 18.0	35.5% ± 7.8	32.7% ± 9.6	28.8% ± 9.6	36.2% ± 9.8	29.8% ± 13.1	26.8% ± 13.8

From the table above, it can be observed that three treatment mediums produced a cell count above 70,000 cells after 72 hours of proliferation: All GFs, +50% FGF2 and No EGF. It can also be seen that +50% FGF2 and No EGF created a higher percent non-adherent

population of 35.5% and 32.7% respectively. This is approximately 10% higher than the control of All GFs. The two treatment mediums of +50% FGF2 and No EGF, which produced the high total population as well as the high percent of non-adherent population, can be seen in Figure 15 compared to the Control.

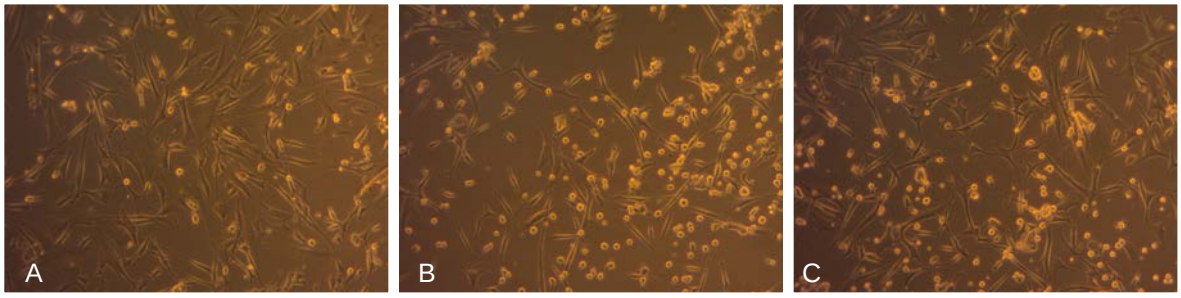

FIGURE 15: BRIGHT FIELD IMAGES OF TOTAL CELL POPULATIONS, A) ALL GFS (CONTROL) B)+50% FGF2, AND C) NO EGF

As seen from Figure 15, above, the +50% FGF2 and No EGF treatment mediums have a noticeably higher non-adherent cell population, as observed by the phase bright cells.

6.4. ICC FOR MYOSIN HEAVY CHAIN

When the ICC for BrdU assay and cell counts were conducted, cells were also placed into differentiation medium for 10 days. The procedure used was identical to that observed in Section 5.4. The blue stain indicated the cell nuclei, and green indicated the myosin heavy chain protein, which is expressed after differentiation. The data can be seen in Figure 16, below.

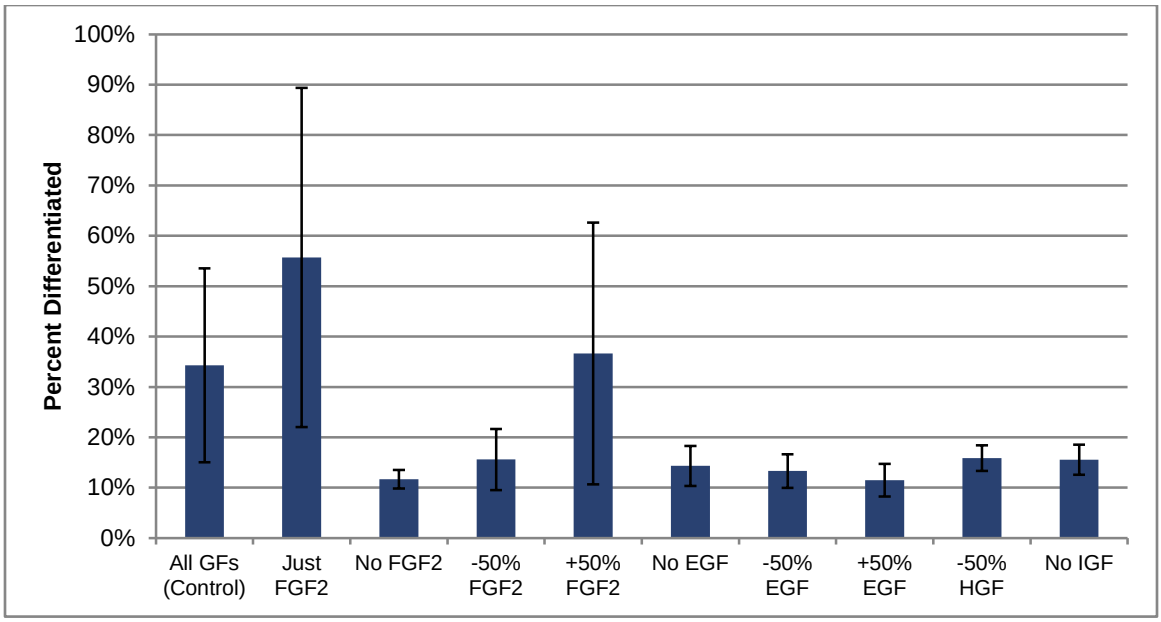

FIGURE 16: ICC FOR MYOSIN HEAVY CHAIN, AFTER TEN DAYS OF DIFFERENTIATION, FOR TOP TEN TREATMENTS

The data presented in Figure 16 shows three growth factor treatments that demonstrated a percent differentiation above 30%: All GFs, Just FGF2, +50% FGF2. However, it was noted that a high differentiation percentage does not indicate a high myogenic potential because some cells that differentiated were only a single cell and did not form multinucleated myotubes. Representative images of All GFs, Just FGF2 and +50% FGF2 can be seen in Figure 17, below. The total cell population was stained blue while the differentiated myosin positive cells were stained green.

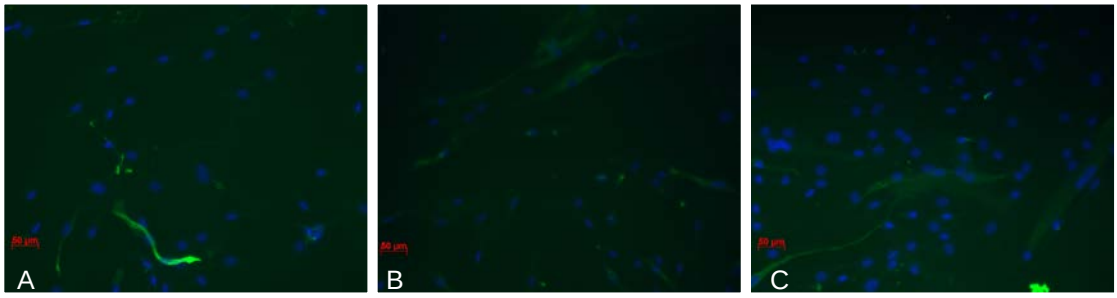

FIGURE 17: MYOSIN HEAVY CHAIN (GREEN), CELL NUCLEI (BLUE), A) CONTROL, B) JUST FGF2, C)+50% FGF2

As seen in Figure 17, above, Just FGF2 has a considerably higher percent differentiated cells when compared to the control where +50% FGF2 has a slight increase in differentiation.

6.5. PRELIMINARY DATA VERIFICATION DISCUSSION

Through analyzing the data in 6.2, 6.3 and 6.4, it was found that six treatment mediums showed potential to have a high myogenic potential by having a high proliferation, cell count, percent non-adherent population and percent differentiation. In addition to the control, +50% FGF2, No EGF and No IGF demonstrated a high proliferation rate, which was an important criteria to meet as the team determined that the cells must grow around the same rate as the currently used medium. Further, +50% FGF2 and No EGF also demonstrated a high cell count as well as a high percent non-adherent population. Since it is speculated that the non-adherent population may correlate to a high myogenic potential, these two treatments are especially of interest. Lastly, from the ICC for myosin heavy chain, it was found that Just FGF2 produced a high-differentiated population after ten days. In addition to these highlighted treatments, -50% HGF was also considered as overall it was slightly below the proliferation and had a higher percent non-adherent population compared to the All GFs treatment (control). Therefore, the top six treatments that were selected for further examination were the control of All GFs, Just FGF2, +50% FGF2, No EGF, -50% HGF and No IGF.

7. FINAL DESIGN AND RESULTS

This chapter discusses the myogenic potential and MyoD expression of the cells in the top six treatment mediums. From this data, the team can further determine if there is a correlation between MyoD expression and myogenic potential, as well as the effect of the non-adherent cell population on the consistency of myogenic potential throughout differentiation.

7.1. IMMUNOCYTOCHEMISTRY FOR MYOGENIC POTENTIAL

To confirm the myogenic potential of the differentiated muscle cells, an ICC assay for myogenic potential was conducted. Cells were grown in the treatment mediums until a confluence of 80% was reached. At this point, the differentiation medium was added, and differentiation was induced for 10 days before testing. To begin the ICC assay, mirror cultured cells were stained for MyoD with 5.2 MyoD Clonal Antibody (AbCAM) or MF20 antibody for myosin heavy chain (DSHB). Secondary antibodies Alexa Fluor 568 and Alexa Fluor 488 were used to make the MyoD and myosin heavy chain fluoresce red and green respectively. These two tests were run at four time points post differentiation: t=0, 3, 5, & 10 days. Wells were imaged using Leica DMIL LED microscope and IC Capture 2.1 software.

7.1.2 ICC FOR MYOD

MyoD expression was examined over these time points. If cells do not display MyoD, they will be considered to be going through the later stage of differentiation since MyoD is typically up regulated in the cell when differentiation is induced (De Angelis et. al., 1999; Portmann-Lanz, 2006). This test was also used to determine if MyoD could be used as an early marker for myogenic potential by comparing the MyoD expression results to the fusion index results seen in section 7.1.3. Results for MyoD expression over the four time points can be seen below in Figure 18.

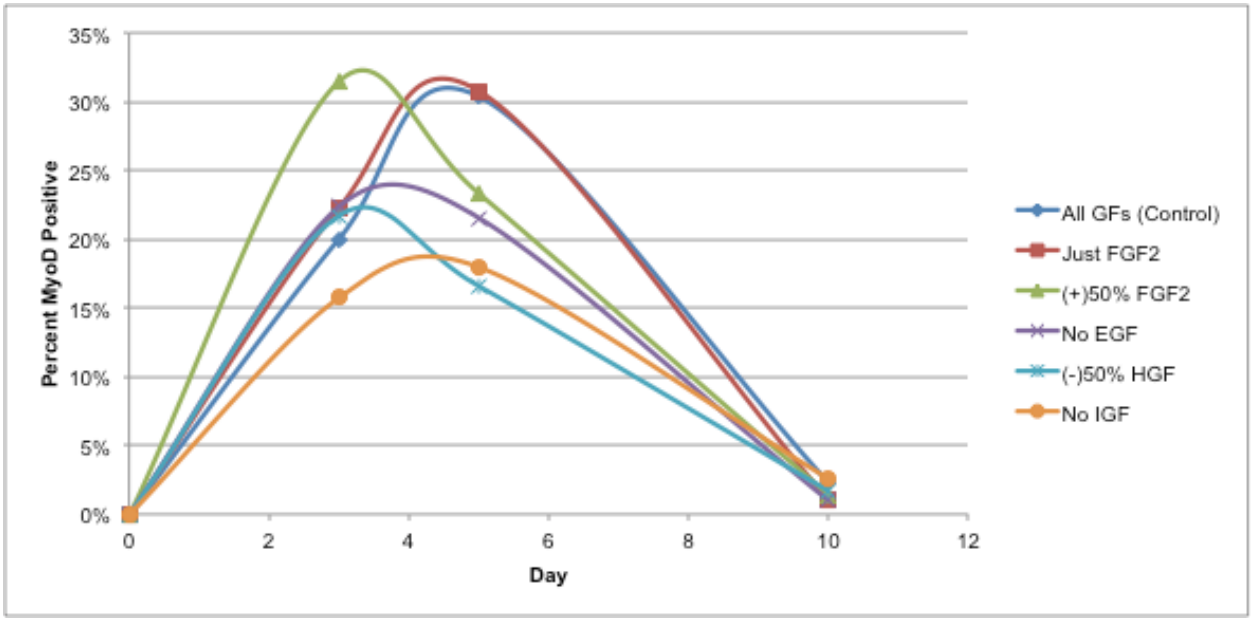

FIGURE 18: RESULTS OF MYOD EXPRESSION OVER 10 DAY PERIOD FOR THE TOP SIX TREATMENTS

As seen in the graph above, MyoD expression was relatively constant for all treatment mediums except for +50% FGF2. It is seen that MyoD is expressed at a high rate very early on in differentiation. Images for the All GFs, +50% FGF2, and No EGF treatment mediums at Day 5 can be observed in Figure 19. MyoD positive cells were stained red while the total cell nuclei were stained blue.

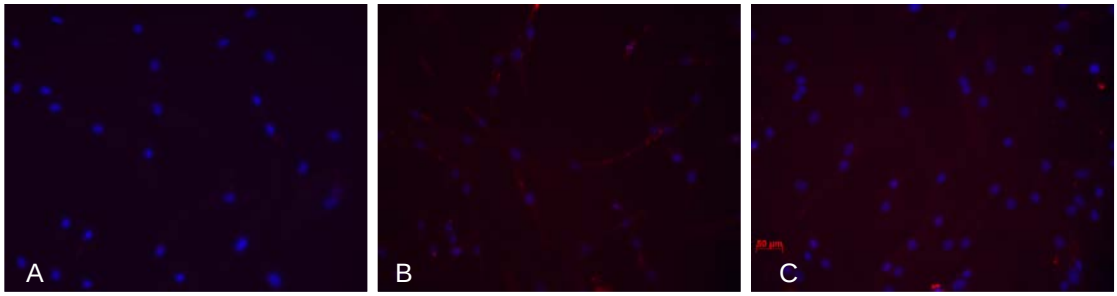

FIGURE 19: MYOD EXPRESSION AT DAY 5, MYOD (RED) AND TOTAL CELLS (BLUE), A) ALL GFS (CONTROL), AND B) +50% FGF2, C) NO EGF.

Note: Images are not as fluorescently bright as observed. This is because the imaging system used was switched over to a different camera prior to imaging this data set.

As seen in Figure 19, above, the +50% FGF2 treatment medium had a higher expression of MyoD, compared to the control, at an early stage in differentiation. From Figure 18, it can be seen that the overall trend of all treatment mediums is cells that are in a later stage

of differentiation, MyoD expression decreases, as expected. This data will be compared with the calculated fusion index to determine if there is a correlation so MyoD could be used as an early marker for myogenic potential.

7.1.3 FUSION INDEX & MYOGENIC POTENTIAL

Myogenic potential is an important characteristic of muscle cell differentiation. It can be described as the likelihood of the differentiated cells to form multinucleated myotubes and ultimately functioning muscle. To test for myogenic potential an ICC for myosin heavy chain was completed and a calculation was conducted. The same process aforementioned in section 6.4 was followed to complete this ICC for myosin heavy chain, staining for the myosin heavy chain with Alexa Fluor 488 (green) and nuclei with Hoechst (blue). The full protocol can be found in Appendix E.

Myogenic potential can be calculated using a mathematical equation called the fusion index. The Fusion Index, in Equation 1 below, is equal to the number of nuclei present in a myotube divided by the total nuclei present in the culture (Yang et al., 2010).

EQUATION 1: FUSION INDEX FORMULA

$$Fusion\ Index = \frac{Total\ number\ of\ nuceli\ inside\ myotubes}{Total\ nuceli}$$

An example of this calculation can be seen using the Figure 20 below as a sample group. The green in this image represents the differentiated cells and the nuclei are seen in blue.

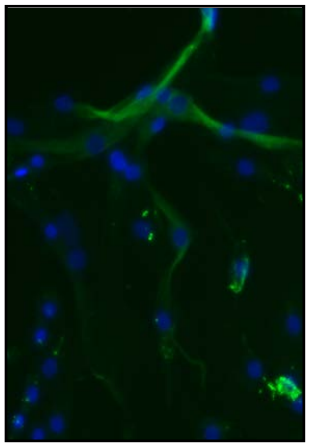

FIGURE 20: DIFFERENTIATED CELLS CONTAINING MULTINUCLEATED MYOTUBE FORMATION.

For example, from the image above, it can be determined that there are five nuclei present in myotubes, while 13 nuclei are contained in any differentiated cell. The total nuclei

present in Figure 20 were 35. This image displays a 14.3% myogenic potential in this cell population.

A calculation for fusion index was performed on each of the top six treatments to determine the myogenic potential over a 10-day period focusing on time points 0, 3, 5, and 10. This data is displayed in Figure 21, below.

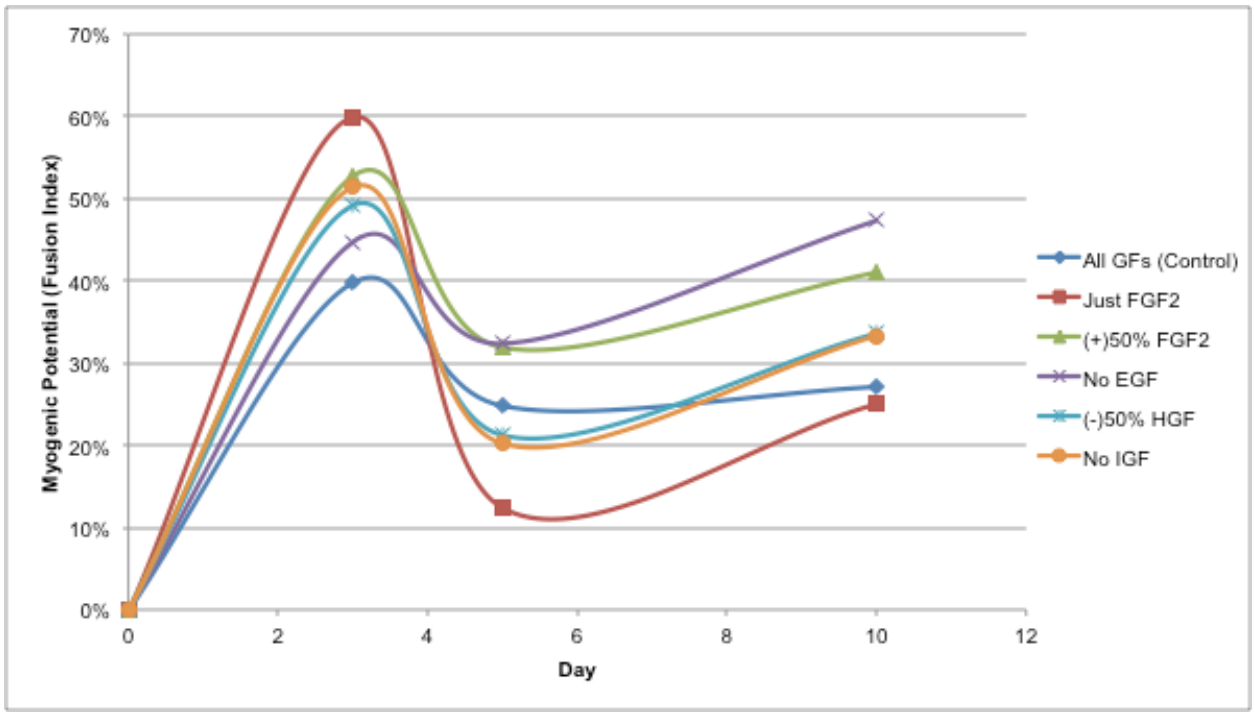

FIGURE 21: THE MYOGENIC POTENTIAL TRENDS OVER A 10 DAY TIME PERIOD POST DIFFERENTIATION OF THE TOP 6 TREATMENTS.

Figure 21, above, shows all six treatments, which follow a similar trend based on the myogenic potential calculated at all four time points. The +50% FGF2 and No EGF treatments display the highest myogenic potential over a 10-day time period. These two treatments also showed the most consistent myogenic potential throughout these ten days. It is important to note that these two treatments also showed the highest non-adherent cell populations. This supports the previously stated hypothesis that an increased non-adherent population increases the myogenic potential.

8. CONCLUSIONS AND RECOMMENDATIONS

8.1. COST-BENEFIT

Before a final medium was chosen, the team had to research the cost it would take to scale up for clinical applications. First, the amount of medium required to grow the amount of cells needed to treat a 15 cubic cm wound was determined. This was estimated to be around 30 billion cells. Next, the final cost to grow these cells in the prescribed medium amount was calculated. A starting cell population of 500,000 cells was used. It was assumed that cells would propagate with a 36-hour doubling time, and would be passaged into T-75 flasks every 72 hours. The final costs for the control and the top two treatment mediums that exhibited the highest myogenic potential are shown in Table 6 below.

TABLE 6: COST ANALYSIS OF CONTROL AND TOP TWO TREATMENT MEDIUMS

Component	Commercial Price		Control	+50% FGF2	No EGF
	Amount	Price	873.81 L of Medium	873.81 L of Medium	873.81 L of Medium
DMEM	6 X 500 mL	$115.46	$16,777.15	$16,777.15	$16,777.15
F12	6 X 500 mL	$98.67	$10,310.96	$10,310.96	$10,310.96
FCIII	12X 500 mL	$153.42	$2,271.91	$2,271.91	$2,271.91
Glutamine	100 g	$87.92	$524.29	$524.29	$524.29
Growth Factors					
FGF2 (4ng/mL)	10 ug solid	$80.00	$27,961.92	$41,942.88	$27,961.92
HGF (2.5ng/mL)	10 ug solid	$195.00	$42,641.93	$42,641.93	$42,641.93
IGF (5.0ng/ml)	50 ug solid	$195.00	$17,126.68	$17,126.68	$17,126.68
EGF (2.5ng/ml)	500 ug solid	$195.00	$873.81	$873.81	
		Total Cost:	**$118,488.64**	**$132,469.60**	**$117,614.83**

As shown in the table, +50% FGF2 adds an addition $13,980.96 to the total production cost while No EGF reduces the cost by $873.81 when compared to the control medium. After the cost was determined, the team looked into the overall results from all of the assays completed as seen in Table 7.

TABLE 7: COST-BENEFIT ANALYSIS OF CONTROL AND TOP TWO TREATMENT MEDIUMS

	Control	+50% FGF2	No EGF
Proliferation (%)	34.4 ± 13.4	37.6 ± 15.0	31.8 ± 14.4
Differentiation (%)	34.3 ± 19.3	36.6 ± 26.0	14.3 ± 4.0
Percent non-adherent (%)	23.3 ± 7.7	35.5 ± 18.0	32.7 ± 9.6
Myogenic Potential (%) (t=10 days)	27.1 ± 2.2	41.0 ± 5.9	47.3 ± 6.5
Cost to treat a 15 cm^3 wound	$118,488.64	$132,469.60	$117,614.83

As seen in the Table 7, the two treatment mediums have the same proliferation rate as the medium currently used, however both treatments yielded a higher non-adherent cell population. The two treatments with a higher non-adherent population also displayed a drastically higher myogenic potential than the control. This supports the theory that the non-adherent population correlates to a higher myogenic potential. One thing to note is the differentiation of the No EGF treatment medium. While this is low, it did form the most multinucleated myotubes. Differentiation in other treatment mediums may have had a higher differentiation percentage, however these cells were mononucleated. In addition, No EGF had a consistently lower standard deviation, which suggests that it provides a more reliable and repeatable process.

8.2. OVERALL CONCLUSIONS

Based on results, and the cost-benefit analysis, the team determined the No EGF treatment medium was the best option for the growth and differentiation into multinucleated myotubes. This medium maintains the same proliferation rate; however, it produces nearly a twofold increase in myogenic potential. One of the goals of this project was to optimize the myogenic potential of the culture to greater than 30%, which is met with the No EGF treatment. In addition, it reduces the cost needed to expand the cells for autologous cell therapy applications.

In addition, it was found that whenever the percent non-adherent cells increased in a treatment medium, the myogenic potential of those cells also increased. Therefore, these cells should not be discarded when culturing. This can be seen through the analysis of the gathered results. The treatment Just FGF2 had a low percent of non-adherent cells, whereas the No EGF treatment had one of the highest percent of non-adherent cells. In comparing their results in the final myogenic potential graph, you can see that although the Just FGF2 started with the highest

myogenic potential at day three, it's myogenic potential dropped drastically as time progressed. The No EGF treatment had a fairly high myogenic potential at day three, but it remained fairly constant with increasing time. This data supports the theory that the increased non-adherent cell population provides a higher myogenic potential over time.

Lastly, it was found that when cells are cultured in +50% FGF2, that MyoD was expressed early in differentiation. A correlation was made between cells grown within this treatment medium as they also exhibited a high myogenic potential. When clinical therapies are being conducting, a biopsy from the patient would be obtained. Those cells would then grow in our recommended No EGF treatment medium. To ensure that the population will still have a high myogenic potential, a small subculture would then be started in +50% FGF2 treatment medium. MyoD expression can then be examined to ensure that the cell culture will have a high myogenic potential since +50% FGF2 and No EGF have relatively the same myogenic potential.

8.3. FUTURE RECOMMENDATIONS

Further tests should be conducted with the final proposed medium to ensure the functionality and long-term effects of the cells in this medium.

- ***Single cell contraction assay****:* It is important to ensure that the medium chosen does not inhibit the cellular function. The myotube function can be tested through a single cell contraction assay. This bench top assay has not previously been tested on human primary satellite cells, but has been used successfully for cilliary muscle cells. The researchers state that it can be used for any slow growing, calcium induced contraction cells. After cells have differentiated they should be treated with a calcium free non-enzymatic dissociation buffer, followed by atropine and carbachol. The dissociation buffer removes the cells from the plate without disrupting their membranes. The atropine will allow the cell to shrink in size while the carbachol causes it to expand. These two chemicals together induce an environment in which the cells can be seen contracting. A way of measuring the contractile ability of these cells should be determined in order to compare the chosen treatment to the control medium. This protocol can be found in Appendix E.
- ***Long-term medium effects***: While this medium did exhibit a high myogenic potential, the longevity of this characteristic is unknown. In addition, the medium could extend the Hayflick Limit of this cell line. Therefore, long-term cell cultures should be conducted to

determine if any long-term effects are present, highlighting any positive or negative contributions.

- ***Altering culture surfaces and ambient oxygen:*** Cell culture environment has a significant contribution to cellular growth. It is possible that by altering the cell culture surface the adherent population will become smaller as it may not adhere. This could lead to a higher non-adherent population, and thus potentially a higher myogenic potential. Further studies researching the effects of oxygen levels should also be conducted. Lower oxygen levels mimic the *in vivo* environment; therefore by having a culture system that represents the *in vivo* environment more, cells may respond differently to treatment mediums.
- ***Alternative designs*****:** While the team decided to optimize the medium to meet the needs presented by the client, the three other alternative designs proposed in Chapter 4 should be extensively looked into. This could make the process of culturing these cells easier by eliminating some extent of human contact when passaging, and therefore reducing the risk of losing cells.

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APPENDIX

APPENDIX A: GANTT CHART

ID	Task Name	Duration	Start	Finish
1	Introduction	36 days	Thu 8/23/12	Thu 10/11/12
2	Literature Review	36 days	Thu 8/23/12	Thu 10/11/12
3	**Project Strategy**	**18 days**	**Thu 8/23/12**	**Mon 9/17/12**
4	Revised Client Statement	18 days	Thu 8/23/12	Mon 9/17/12
5	**Objectives**	**10 days**	**Thu 8/23/12**	**Wed 9/5/12**
6	Objective Tree	10 days	Thu 8/23/12	Wed 9/5/12
7	Constraints	10 days	Thu 8/23/12	Wed 9/5/12
8	Project Scope	10 days	Thu 8/23/12	Wed 9/5/12
9	Pairwise Comparison Chart	10 days	Thu 8/23/12	Wed 9/5/12
10	Functions	10 days	Thu 8/23/12	Wed 9/5/12
11	Means	30 days	Thu 8/23/12	Wed 10/3/12
12	Functions-Means Chart	30 days	Thu 8/23/12	Wed 10/3/12
13	Specifications	10 days	Thu 8/23/12	Wed 9/5/12
14	**Research + Results**	**94 days**	**Tue 10/23/12**	**Fri 3/1/13**
15	**Assays**	**60 days**	**Fri 12/14/12**	**Thu 3/7/13**
16	BrdU	60 days	Fri 12/14/12	Thu 3/7/13
17	ICC	60 days	Fri 12/14/12	Thu 3/7/13
18	FGF2 ELISA	60 days	Fri 12/14/12	Thu 3/7/13
19	Western Blot	60 days	Fri 12/14/12	Thu 3/7/13
20	Flow Cytometry	60 days	Fri 12/14/12	Thu 3/7/13
21	**Cell Culture**	**98 days**	**Tue 10/23/12**	**Thu 3/7/13**
22	Test Prof. Page's Process	98 days	Tue 10/23/12	Thu 3/7/13
23	**Optimize Media Components**	**60 days**	Fri 12/14/12	Thu 3/7/13
24	Growth Factors	60 days	Fri 12/14/12	Thu 3/7/13
25	Serum	60 days	Fri 12/14/12	Thu 3/7/13
26	Antibiotics	60 days	Fri 12/14/12	Thu 3/7/13
27	Glucose	60 days	Fri 12/14/12	Thu 3/7/13
28	**Optimize Cell Culture**	60 days	Fri 12/14/12	Thu 3/7/13
29	Seeding Density	60 days	Fri 12/14/12	Thu 3/7/13
30	Passaging Time	60 days	Fri 12/14/12	Thu 3/7/13
31	Process Verification	41 days	Thu 1/10/13	Thu 3/7/13
32	**Final Deliverables**	**90 days**	**Fri 12/14/12**	**Thu 4/18/13**
33	Final Paper	90 days	Fri 12/14/12	Thu 4/18/13
34	Final Presentation	90 days	Fri 12/14/12	Thu 4/18/13
35	Final Process Protocol	90 days	Fri 12/14/12	Thu 4/18/13

APPENDIX B: MEDIA RECIPES

Note: All media should be warmed prior to adding to cells. Cold media, from the refrigerator may kill your cells.

Human Primary Cell Proliferation Media

Component	Volume for 50 mL	Final Concentration
60% DMEM	25 mL	54%
40% F12	18 mL	36%
FCIII	5 mL	10%
FGF2	20 µL	n/a
IGF	5 µL	n/a
EGF	2.5 µL	n/a
HGF	12.5 µL	n/a
4mM Glutamine	0.02922 g	n/a
Final Volume	50 mL	100%

Human Primary Cell Differentiation Media

Component	Volume for 50 mL	Final Concentation
60% DMEM	29.1 mL	58.2 %
40% F12	19.4 mL	38.8%
Horse Serum	1 mL	2%
ITS	0.5 mL	1%
4mM Glutamine	0.02922 g	n/a
Final Volume	50 mL	100%

C2C12 Proliferation Media ("Cell Culture Manual-Detailed", 2010)

Component	Volume for 50 mL	Final Concentration
Penn Strep	0.5 mL	1%
Glutamax	0.5 mL	1%
FBS	5 mL	10%
50%/50% DMEM/F12	44 mL	88%
Final Volume	50 mL	100%

C2C12 Differentiation Media ("C2C12 Differentiation", 2012)

Component	Volume for 10 mL	Final Concentration
Horse Serum	200 µL	2%
ITS	100 µL	1%
Glutamax	100 µL	1X (2mM)
Penn Strep	100 µL	1X (1%)
50%/50% DMEM/F12	9.5 mL	95%
Final Volume	10 mL	100%

Freezing Media (“Cell Culture Manual-Detailed”, 2010)

Component	Volume for 10 mL	Final Concentration
DMSO	2 mL	20%
FBS	1 mL	10%
DMEM	7 mL	70%
Total Volume	10 mL	100%

1. Determine the total volume of media needed, then calculate the volume of each component which needs to be added to reach this total volume of media.
2. Pipet the required amount of media and componenets into a 50mL, 150mL, or 500 mL filter unit.
3. Filter sterilize the media into a clean, sterile container. Cap the containers and leave in the hood until you are finished.
4. Attach vacuum line to the Steriflip side-arm, turn on vacuum and continue pulling until all media has mostly filtered through. Turn off pump. Remove vacuum line when all media has been pulled into the lower tube (ignore bubbles).
5. Remove Steriflip filter and top tube then discard in biohazard trash. Cap the bottom (media containing) tube with the sterile cap provided in the Steriflip package.

(“Making Media”, B 2011)

APPENDIX C: CULTURE PROTOCOLS

THAWING CELLS

1. Place an empty 15mL conical tube in the hood, and then obtain a cryotube of the desired cells. Place the cryotube in the 37° C water bath to thaw quickly. While thawing label the conical tube with your initials, and transfer your warmed media to the hood along with other necessary supplies. Cells must not be left in the water bath any longer than needed for thawing (1-2 min).
2. When thawed, rinse the outside of the vial with ethanol and wipe dry, then place the tube in a rack in the hood. Make sure all ethanol has evaporated before opening the tube as ethanol is toxic to the cells!
3. Using a 1 mL pipet gently re-suspend the contents of your cryovial by pipetting up and down, without creating bubbles, then gently transfer entire suspension to the 15 mL conical tube. Very slowly add 10 mL of warm medium down the inside to the tube: one drop, then 2-3, then 4-6 drops, etc. with gentle hand mixing (tap tube on side) between each addition, for at least the first 5 mL. It should take 2-3 minutes to add all of the media.
4. Cap tightly, gently mix the cell suspension by slowly inverting the tube 2-3 times, then centrifuge for 5 minutes at around 1200 rmp. Re-wipe the tube with 70% EtOH (taking care not to disturb the cell pellet) and return to the hood.
5. Aseptically attach a sterile, glass, Pasteur pipet to the end of the vacuum line. Turn on the pump, uncap the tube and aspirate off as much medium as possible without disturbing the pellet.
6. Re-suspend the pellet in 2 mL of medium. Perform a cell and viability count. Determine the total live cells/mL, which will be used in the next step. If the cell number is already known proceed to step 8 and do not perform a cell and viability count.
7. Determine the volume of cell suspension ('V') that contains the appropriate # of live cells for the experiment. For subculturing, 200,000 cells are needed for a T75 flask.
8. Plate your cells in a T75 flask with a final total volume of 10 mL:
    a. Place a T75 flask in the hood and add 10 - V mL ('V' from step 7) of media down the back of the flask. This is the large surface that will be on the bottom, when you set the flask down so that the canted neck is pointing upward; example*:* If you will be adding 0.5 mL of cells, you must put 10.0 - 0.5 = 9.5 mL in the flask. *Note: if V is less than 0.1 mL use a full 10 mL of media.*
    b. Gently re-suspend the cells, then remove (use an appropriate size pipet) the required volume of cell suspension determined in step 7 and place it in the T75 flask by gently dispensing the liquid down the back of the flask. If you do not have enough cell suspension, add all that you have.
    c. Cap the flask tightly (vented flasks only), mix and distribute the cell/media mixture by gently rocking the flask to coat the entire bottom with cell suspension (hold horizontally, with the canted neck slanting up). When finished, place the flask in the incubator. Aspirate any excess cell suspension, then clean the vacuum line by rinsing with 70% EtOH, and discard cryovial in biohazard can.

("Thawing Cells", B 2011)

TRYPSINIZING, COUNTING AND PASSAGING ADHERENT CELLS

1. Take flasks out of incubator
2. Remove media with a serological pipette and place in a 15mL conical tube (These are the non-adherent cells!!)
3. Label tube for non-adherent cells (N) and fill with media (without growth factors).
4. Place aside until the adherent cells are in a conical tube as well. If adherent cells are not being typsonized, skip to step 7.
5. Once the non-adherent cells are removed, add 1 mL of 0.05% trypsin, hold horizontally (cell layer down) and gently rock the flask to cover the entire layer of cells with trypsin. Let sit for 30 seconds, gently rock flask, and then start looking at the cells by eye. Alternate gently rocking and observing until all of the monolayer has released and most of it has broken into small piece. As soon as most of the monolayer completely breaks apart proceed to the next step. (Note: it will be noticeable around the edges when the cells are coming off the plate)
6. Once most the cells are off the plate, add 5 mL of proliferation media (without growth factors) and vigorously wash the bottom surface of the flask, pipetting up and down. Squirt the suspension several times against the bottom of the flask, to break up all cell clumps. When you have a uniform suspension transfer it to a 15 mL conical tube and label it for adherent cells (A).
7. Spin down conical tubes for 5 minutes at 2.5-3 speed.
8. Aspirate off the medium, re-suspend the cell pellet in 5 mL of proliferation media (with growth factors). Again, pipet up and down until no clumps is visible. This is extremely important so that the cells are properly mixed. (Note: The pellet may be small and on the side of the tube).
9. Remove a 100 µL aliquot and place in an eppendorf tube. In the same tube add 100 µL aliquot of trypan blue. Close the tube and mix thoroughly by flicking and inverting.
10. Count cells in each of the four large corners in a hemocytomer by adding 10 µL of cell-trypan mixture to each side. Dead cells will appear blue while alive cells will appear normal.
11. Take an average of the four corners to obtain average cell count.
12. Multiply this value by 10,000 (volume of grid). Multiply again by 2 (dilution factor). And multiply again by how many mLs you suspended in. This value will be your total cell count.
13. Use the following formula to determine how many mls of cell suspension and media should go in the flask:

    V of cell suspension needed = (number of cells needed)/(number of total cells)

    V of media (with GF) needed = 5 mL (for T25) – (V of cell suspension needed)
14. Place the calculated amount of cells in a new T25 or T75 flask and then add the proper amount of media. Note: If a T25 flask is used the final volume should be 5mL while if a T75 flask is used the final volume should be 10mL.
15. Label flask with today's date, your initials, the cell line and media, then place in the incubator.
16. Aspirate any leftover culture. Discard the aspirator pipet and clean the aspirator line with 70% EtOH. Open aspirator line and turn off pump.

(“Trypsonizing and Plating of Adherent Cells”, B 2011)

Human Primary Cell Differentiation Media

Component	Volume for 50 mL	Final Concentation
60% DMEM	29.1 mL	58.2 %
40% F12	19.4 mL	38.8%
Horse Serum	1 mL	2%
ITS	0.5 mL	1%
4mM Glutamine	0.02922 g	
Final Volume	50 mL	

Procedure

1. Grow cells in proliferative media in 24-well plates at the desired cell density.
2. On the day of start of differentiation (D0), aspirate complete medium from wells.
3. Rinse once with 1X DPBS.
4. Add 500 µl differentiation medium per well.
5. Continue culturing cells until they differentiate and form myofibrils (usually 7 to 10 days).
6. Periodically check the wells for proliferation and change media if necessary. The cells may continue to proliferate for the first 3-5 days until they slow down and exit proliferation and start to differentiate.
7. Successful differentiation results in about 30 to 50% of the cells aligning and fusing to form multinucleated myofibrils.

<u>BrdU labeling of cells to determine cell proliferation or lack thereof</u>:
If you plan to determine the time point at which cells exit proliferation and start differentiating, follow the protocol below.

1. Add 50 ul of BrdU labeling reagent (Invitrogen, Cat # 00-0103) to 500 ul medium (about 1:10 final dilution) in 24-well plates.
2. Continue culturing cells for the required amount of time (2 hours to overnight incubation).
3. Fix the cells and save in DPBS at 4^{o} C until further analysis.
4. Follow BrdU or ICC protocol to determine stage at which cells exit proliferation.

(C2C12 Differentiation, 2012)

APPENDIX D: TESTING REGIMENS

Below is the template used when plating the cells onto the 24-well plates for preliminary testing. As there was an ICC for BrdU assay, cell counting assessment, and ICC for myosin heavy chain completed on each of the medium conditions, the plates were seeded in triplicate, so one plate was devoted to one specific assay.

PLATE A	1	2	3	4	5	6
A	CONTROL	CONTROL	Just FGF2	Just EGF	Just IGF	Just HGF
B	EGF + FGF2	FGF2 + IGF	No FGF2	No EGF	No IGF	No HGF
C	EGF + IGF	FGF2 + HGF	-50% FGF2	-50% EGF	-50% IGF	-50% HGF
D	EGF + HGF	IGF + HGF	+50% FGF2	+50% EGF	+50% IGF	+50% HGF

The well map below is the 10 treatments that an ICC for BrdU and ICC for myosin heavy chain were conducted on for verification of the first 23 treatment results. Since each of these treatments were completed in duplicate, only one plate was seeded with cells. Data obtained from this plate was compared with the results from the initial preliminary testing.

PLATE A	1	2	3	4	5	6
A	CONTROL	+50% FGF2	-50% FGF2	+50% EGF	-50% HGF	
B	CONTROL	+50% FGF2	-50% FGF2	+50% EGF	-50% HGF	
C	Just FGF2	No FGF2	No EGF	-50% EGF	No IGF	
D	Just FGF2	No FGF2	No EGF	-50% EGF	No IGF	

The following well map shows the 6 most promising treatments for cellular growth and myogenic potential. An ICC for MyoD was conducted on rows A and B, while an ICC for myosin heavy chain was completed on rows C and D. As this test was completed at four different time points (0, 3, 5, and 10 days), the four plates were seeded. Using the myosin heavy chain data, a fusion index was calculated.

PLATE A	1	2	3	4	5	6
A	CONTROL	Just FGF2	+50% FGF2	No EGF	-50% HGF	No IGF
B	CONTROL	Just FGF2	+50% FGF2	No EGF	-50% HGF	No IGF
C	CONTROL	Just FGF2	+50% FGF2	No EGF	-50% HGF	No IGF
D	CONTROL	Just FGF2	+50% FGF2	No EGF	-50% HGF	No IGF

APPENDIX E: ASSAY PROTOCOLS

ICC FOR BRDU ASSAY

Procedure

1. Add 1.0 µl of BrdU stock solution per ml of culture medium to cells being assayed and incubate for 24 hours or the time required by the experimental protocol.
2. Aspirate culture medium and wash cells in 2X in DPBS+.
3. Add 500 µL of methanol-free formaldehyde into each well. Incubate at room temperature for 10 minutes.
4. Aspirate formaldehyde, and rinse with DPBS+ 3X.
5. Add 500 µL of 0.2% Triton-X-100 to permeabalize the cells. Incubate for 10 minutes at room temperature.
6. Aspirate and rinse with DPBS+ 3X.
7. Aspirate PBS and add 1.5 N HCl (0.5 ml/well for 24-well or 0.25 ml/well for 48-well plate) and incubate at RT for 20 min
8. Wash 3x with PBS, 5 min each
9. Aspirate DPBS+ and add ice cold (-20C) methanol (1.0 ml/well for 24-well plate). Incubate for 10 min at -20°C
10. Aspirate methanol and wash with 1.0 ml PBS for 10 min (plates can be stored at 4C with PBS in wells if analysis is not to be done right away
11. Dilute anti-BrdU antibody 1:100 in PBS +0.05% Tween-20
12. Add antibody solution at 150 µl/well for 24-well plate or 75 µl/well for 48-well plate) and incubate at RT for 30 min
13. Aspirate antibody solution and wash 3X with PBS for 5 min each
14. Add fluorescent dye conjugated secondary antibody diluted 1:500 in PBS+0.05% Tween-20 (150 µl/well for 24-well plate or 75 µl/well for 48-well plate) and incubate at RT for 30 min
15. Wash 3X with PBS (**without Tween**).
16. Add 0.5 µg/ml Hoechst 33342 to last wash (stock is 1 mg/ml) and incubate for 10 min at RT
17. Aspirate Hoechst solution, wash w/PBS and add PBS (1 ml/well for 24-well or 0.5 ml/well for 48-well)
18. Cells are ready for observation by fluorescence microscopy. Plates can be stored at 4C wrapped in foil to protect from light.

("BrdU Cell Proliferation Assay", 2012)

ICC FOR MYOSIN HEAVY CHAIN ASSAY

Procedure

1. Aspirate culture medium and wash cells with DPBS for 1 min.
2. Add 500 µL of methanol-free formaldehyde into each well. Incubate at room temperature for 10 minutes.
3. Aspirate formaldehyde, and rinse with DPBS+ 3X.
4. Add 500 µL of 0.2% Triton-X-100 to permeabalize the cells. Incubate for 10 minutes at room temperature.
5. Aspirate and rinse with DPBS+ 3X.
6. Aspirate PBS and add blocking solution (PBS with 3% FBS or appropriate serum) at 150 µl per well and incubate at RT for 30 min.
7. Aspirate blocking solution and rinse with DPBS+ 3X.
8. Add 1 ml/well ice cold (-20C) methanol and let stand for 10 min.
9. Aspirate methanol and wash 1.0 ml/well DPBS+ 3X.
10. Aspirate DPBS+ and add primary antibody (1:500 dilution in PBS/Tween) at 150 µl per well and incubate at RT for 30 min. Secondary antibody-only controls will skip this step
11. Aspirate primary antibody and wash 4X with PBS for 3 min each wash.
12. Aspirate PBS and add secondary antibody (diluted 1:500 in PBS/Tween) at 150 µl/well and incubate for 30 min
13. Aspirate secondary antibody solution and wash 4X with PBS. If nuclear counterstain is desired, add Hoechst 33342 at 0.5 µg/ml in PBS to last wash and incubate for 10 min. Aspirate Hoechst and add 1 ml/well PBS.
14. Cells are ready for observation by fluorescence microscopy. Plates can be stored at 4C wrapped in foil to protect from light. If long term storage is desired replace PBS with PBS containing 0.1% Na-Azide to prevent bacterial growth.

("Myosin Heavy Chain Immunocytochemistry Protocol", 2012)

ICC FOR MYOD

Procedure

1. After cells are fixed in formaldehyde and the antigen is intracellular, permeabilize cells by incubating in 0.5 ml of 0.2% Triton X-100 in PBS for 20mins.
2. Wash each well once 0.5 ml PBS/Tween for 5 mins.
3. Aspirate PBS and add 0.5ml of blocking solution at room temperature for 15mins (blocking solutions: 3% FBS in PBS).
4. Wash once with 0.5ml PBS/Tween for 5mins.
5. Add ice cold Methanol and let stand for 10mins .
6. Aspirate methanol and was 3 times with PBS/tween.
7. Incubate with primary antibody (5.2F Mouse monoclonal MyoD Antibody, AbCam) diluted 1:1000 in PBS/Tween for 30 mins at RT.
8. Aspirate primary antibody ad as each well 3 times with 0.5ml of PBS/Tween for 5mins each.
9. Incubate secondary antibody (Alexa Fluor 568 anti-mouse) (at 4ug/ml) for 30 mins in PBS/Tween at RT.
10. Aspirate secondary antibody and was each well four times with 0.5ml PBS for 5 mins each
11. For counterstaining add 0.5ug/ml Hoechst 33342 (for nuclear antigens) to last wash (stock is 1mg/ml) and incubate for 10 mins at RT.
12. Wash two times with 0.5ml PBS for 5 mins each. Add0.1% NaAzide in PBS and store at $4^{o}C$.

NOTE: Do not counterstain with DAPI if nuclear antigens are to be detected. It is best to avoid counterstaining unless specific counts of positive counts of positive cells are to be obtained. In this case, counterstain with Hoechst only.

SINGLE CELL CONTRACTION ASSAY

Materials:

1. Differentiation Media
2. Calcium Free Non enzymatic cell dissociation buffer (Sigma C5914)
3. Carbachol (Sigma C4382)
4. Atropine (Sigma A0132)
5. Real Time Video

Methods:

1. Add 1mL Differentiation Media to the cells and allow 1 week for cells to differentiate.
2. Add 500uL calcium free non-enzymatic cell dissociation buffer (Sigma catalog number C-5914) and incubate at 37^{o}C for 30 to 40 mins
3. Add 500uL 1uM Atropine and let sit 5 mins
4. Add 500uL 10µmol/L Carbachol

Take Photomicrographs - the cross sectional surface area of cells when overlapped will show which cells contract

(Pang et al., 1993)