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Cell Culture System to Enable a Therapy for Volumetric Muscle Loss

A Major Qualifying Project Report Submitted to the Faculty of Worcester Polytechnic Institute in partial fulfillment of the requirements for the Degree of Bachelor of Science By

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Abstract

Current skeletal muscle cell therapy systems have limited effectiveness due to poor proliferative capability. The cells are not functional enough to be implanted into a critical size muscle defect as a therapy for volumetric muscle loss. Altering *in vitro* factors, such as mitogens and oxygen tension, can have a favorable effect on the cells ability to retain myogenic potential. Individual system parameters were characterized through experimental testing of culture conditions, media composition, and cell kinetics. Our team designed, engineered, and validated a final scalable cell expansion system for proliferating myogenic cells *in vitro*.

Executive Summary

Introduction

There is presently a need for tissue engineering of muscle, since the current standard of treatment for muscle loss is ineffective. The main associated problem is the loss of functional muscle cells, limiting the contractile ability of the engineered muscle tissue [1].

Background

Volumetric muscle loss (VML) is the loss of large volume of skeletal muscle due to mostly vehicular accidents and battlefield injuries. Approximately 82% of American soldiers injured during battle have at least one extremity injury due to inability to treat the muscle loss [2]. Additionally, each year there are at least 20 million traffic related accidents resulting in extremity injuries [3]. The current standard of treatment is autologous tissue transfer. However this treatment has complications such as donor site morbidity, loss of functionality, and volume deficiency. In the case of VML, there are no healthy muscle fibers for the myoblasts to fuse with, resulting in non-functional dense scar tissue [4].

Mitogenic growth factors regulate cell behavior in terms of proliferation and differentiation by binding to cell surface receptors and signaling cellular pathways. These growth factors are critical for maintaining the mitotic activity of satellite cells, which are essential to the regeneration of muscle tissue. Epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), and fibroblast growth factor 2 (FGF-2) have shown to increase proliferation and delay differentiation in muscle precursor cells [5].

Methods

Our team considered the important objectives to be the ability to maintain myogenic potential, biocompatibility, reproducible, scalable, inexpensive, and quantifiable in decreasing order. The goal was to create a scalable system that exhibited a myogenic potential of at least 30%. In vitro experimentation determined if each growth factor was beneficial to the proliferation and myogenic potential of the cells by removing one growth factor per cell culture sample. Oxygen tension was characterized by culturing cells with and without growth factors in ambient oxygen (~19%) versus 5% oxygen to more closely mimic native muscle tissue. Furthermore, adherent and non-adherent cells, which have been shown to exhibit myogenic potential until senescence, were plated separately and stained for 5-bromo-2'-deoxyuridine in order to proliferation rate in relation to cell behavior.

In order to quantify the functionality of muscle cells grown *in vitro*, the myogenic potential is measured. Common tests used for quantifying biological data are immunocytochemical (ICC) assays. In order to test for functional muscle, neural cell adhesion molecule (NCAM) was utilized.. This is because NCAM is present when cell fusion occurs during the initial stages of myoblast differentiation [6].

Design Alternatives

The process focuses on scale-up for alternative designs to apply the characterized parameters that improve myogenic potential. The necessary functions of the design are that it must retain both the adherent and non-adherent cell populations, provide high surface area to media volume ratio, facilitate media exchange, and prevent the cells from terminally differentiating. More specific aspects are that it must maintain a myogenic potential of at least 30% and provide a greater surface area to media volume than $75cm^2$: 8mL. Three possible

alternatives were realized to address the predetermined functions: roller bottles, multi-layer flasks, and microcarrier systems.

Results

Staining for NCAM allowed for determination of the myogenic potential under different culture conditions. In a population with no IGF-1, the myogenic index was 21.47 ± 1.02%. When compared to the 31.43 ± 1.59% of the positive control, there is a p-value of 0.002. In a population cultured in 5% oxygen tension, the myogenic index was 34.56 ± 0.13%. When compared to a population at 19% oxygen, there was a p-value of 0.026. In a population without FGF-2, the cell surface area was measured to be 28781.67 ± 4425.36 μm^2. This had a p-value of 0.031 when compared to the positive control.

Final Design and Validation

In order to apply the results of the experiments that address the factors that influence the culture system, a final design for the scale-up was chosen through evaluation of the design and cost. The roller bottle was chosen, as it is capable of meeting the objectives and constraints of the system while being most cost effective. Through the use of a roller bottle, the designed system applies the determined growth factor concentrations to a low oxygen environment while maintaining both adherent and non-adherent populations in the roller bottle scale-up system.

Discussion and Conclusion

The results of these experiments showed the effect FGF-2 and IGF-1 have on the cell culture of human muscle cells when compared to the Page lab concentrations. They also showed that lowering the oxygen tension has a positive effect on the myogenic potential of the culture. There was no statistically significant difference for the characterization of adherent and non-

adherent populations; however, further research needs to be done on the proliferation percentage overtime from the separate populations. In future experiments it would be useful to test new growth factor concentrations such as testing pairs to see what effect one growth factor has on another one.

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1. Introduction

Tissue engineering is "the combination of cells, engineering, materials, and suitable biochemical and physiochemical factors to improve or replace biological functions" (Griffin, 2009). As the field of tissue engineering progresses, new technologies and techniques are developed that allow for advancements in medicine. There is currently a need for tissue engineering of muscle (Nerem, 1995). The current standard of treatment for muscle loss is not efficient because it has a complication rate of 36 percent (Suh, 2004). Muscle loss can occur in people who have suffered traumatic injuries such as car accidents, military injuries, and sports injuries (Yong, 2004). A new therapy is needed that will allow for integration of new muscle tissue and stimulate proper regeneration, without causing donor site morbidity. For this reason, a tissue engineering approach is needed to allow muscle to be produced *in vitro* from a very small biopsy that will not cause additional problems to the patient.

Recently, tissue engineers have been trying to develop ways to grow functional muscle tissue in order to implant it into patients with volumetric muscle loss (VML). The main problem encountered is the loss of functional muscle cells, limiting the contractile ability of the muscle tissue (Shefer, 2006). Engineered tissue does not function as well as healthy muscle tissue in the body and is not currently effective as a therapy. The proliferative capability of human muscle cells decreases with age, making it difficult to achieve a scalable culture that maintains the necessary myogenic potential (Shefer, 2006). Another issue is the integration of other body tissues, such as vasculature and nervous tissue. Without the integration of these tissues, necrosis of the implanted tissue will occur, coupled with an inflammatory response (Levenberg, 2005).

Research on tissue engineering of muscle started as early as the 1980s (Bischoff, 1986). Many potential solutions have been tested, but they lack efficacy. One development that has been

a focus is based on the idea of autologous myoblast implantation (Qu, 1998). Unfortunately, the injected cells displayed a poor survival rate. Ninety-five percent of the cells died within 48 hours (Qu, 1998). In this case, it could be beneficial to culture the cells *in vitro* since replicating *in vivo* conditions can be a challenge. *In vitro* factors, such as mitogens, oxygen tension, and plating density, can play a large role in how the cells behave and proliferate (Kook et al., 2008, Linkhart et al., 1981). Furthermore, most of the research being done is using animal models, as well as immortalized cell lines. The clinical application associated with these approaches is limited because cell lines are capable of maintaining differentiation potential during extended *in vitro* culture, which is not the case for primary cells (Rothman, 1992). Muscle cells from animal models behave differently than human primary muscle cells, and the results will not translate to humans (Linkhart et al., 1981).

Up to this point, there is still much research that needs to be done before tissue-engineered muscle has a clinical application. There has been little focus on *in vitro* cell culture systems for proliferation and differentiation of human primary muscle cells. In addition, there has been limited research on how different environmental factors and media components will affect the proliferation and differentiation potential of human muscle cell cultures. Concentrations of mitogens present in the media will affect the growth kinetics of the cells; and manipulation of these concentrations has potential to produce favorable proliferation and differentiation. Oxygen tension and substrate surface properties could also affect cell growth and the state of the cells. If the cells are maintained in the progenitor state and terminal differentiation is prevented, then the loss of myogenic capability will be reduced. Finally, in order to produce a high, clinically relevant number of cells to be used in a therapy for volumetric muscle loss, an *in vitro* system would have to be large-scale and efficient.

This report focuses on designing an *in vitro* cell culture system that selectively amplifies the myogenic cells in a human primary muscle cell population. In order to have research relevance, the amount of cells with contracting functionality present in the culture must be improved. The culture conditions will be characterized by employing combinations of differential adhesion, mitogenic stimulation, media components, ambient conditions, and plating densities. The team is designing the system to be scaled up so that it could eventually have industrial application and be used as a therapy for volumetric muscle loss.

2. Background

This section will discuss the reason behind the need of a cell therapy system and will then review the anatomy and physiology of skeletal muscle in detail. Advances in current culture techniques will then be discussed as well as composition in media and the recent advances that have been made. Finally, the section will close with techniques that are currently in use for the gathering of data such as quantification assays.

2.1 Clinical Need

Injuries and diseases are the primary cause of muscle degeneration. Severity of muscle degeneration ranges from sport related injuries to large muscle defects caused by trauma to skeletal muscle. Muscles are injured everyday through exercise, however, in the case of minor contusions, strains, or lacerations—this accounts for 55% of muscle injuries—skeletal muscle is able to regenerate itself under endogenous mechanisms (Quintero et al, 2009). It is important to note, however, that the process of self-regeneration is not 100% efficient. When tissue is torn, some scar tissue forms to aid the healing process at the cost of restoring native functionality and mechanical properties (Turner & Badylak, 2011).

The two types of muscle degenerations that have previously been discussed are causes of muscle atrophy. Muscle atrophy is referred to as the wasting or loss of muscle tissue (Dugdale et al, 2012). There are numerous causes of muscular atrophy such as aging, stroke, polio, malnutrition, etc. The two leading causes of muscular atrophy are volumetric muscle loss and muscular dystrophy and both of these conditions are dangerous if not treated properly (Dugdale et al, 2012). Muscular dystrophy is a genetic condition and symptoms of this condition may appear as early as two to three years of age while others have adult onset (MDA, 2009). More than a million individuals in the United States are affected by muscular dystrophy and most die by the time they reach 35 due to respiratory infections and cardiomyopathy (MDA, 2009).

Neuromuscular diseases affect the nerves that control voluntary muscles initiating muscular dystrophy. This is caused by mutations in the gene that encodes the cytoskeletal protein dystrophin (Nowak and Davies, 2004). Communication between nerves and muscle deteriorates, and as a result muscles weaken and eventually waste away. There are over 40 neuromuscular diseases that cause muscular dystrophy. In 2011, the Muscular Dystrophy Association provided over $38.1 million to the research for the treatment of this condition. In the same year, $22.9 million were given for the professional and public health education about muscular dystrophy (MDA, 2012). The funds spent on the research and education of muscular dystrophy show the need to develop a therapy for the treatment of the disease.

Extensive damage to the muscle caused by trauma is also known as volumetric muscle loss. Some injuries are so severe that the body cannot repair itself under endogenous mechanisms because fibrotic tissue takes over to speed up the healing process. Complete rupture or even complete elimination of muscle fibers is mostly due to motor vehicle accidents or ballistic wounds (Grogan et al, 2011). For volumetric muscle loss cases, there are very few alternatives therefore better treatment options are needed. When there is more than 20% volumetric muscle loss, the natural regeneration process will fail resulting in the formation of non-functional scar tissue (Turner & Badylak, 2011). Scar tissue takes over the wound site in order to promptly aid the healing process. In cases of extensive traumatic injury of skeletal muscle, the likely outcome is amputation of the wound site. Most of the injuries that these amputations are performed for are from combat-related injuries in the military (Fischer, 2010). Civilians can also sustain extensive damage to extremities from motor vehicle accidents, but more funding is being given for the treatment of soldiers. Each year, there are 20-50 million people who are involved in vehicular accidents resulting in extremity injuries (Hettrich and Browner, 2012). These injuries can lead to

compartment syndrome and ultimately to loss of limbs. Compartment syndrome is a condition of the limbs where there is insufficient blood supply to the vessels and nerves because of the increased pressure within the compartments of the limb (Tiwari et al, 2002). If the area affected by the condition is not treated within a short period of time, 6-12 hours, muscles will be severely affected ultimately requiring amputation. Compartment syndrome affects approximately 200,000 Americans each year (McQueen, et al, 2000). Other form of trauma to skeletal muscle include battlefield extremity injuries accounting for 82% of combat wounds, furthermore, since the last three U.S. military operations, there have been 1700 amputations performed due to extensive battle injury to the extremities (Fischer, 2013). Without reconstructive surgery, the number of total amputations would be much higher; however, patients who have gone through reconstructive surgery often select amputation later on in their lives. They prefer to have their extremity amputated as opposed to living their lives with a weak and disabled extremity, which can also be the source of chronic pain (Grogan et al, 2011).

The current gold standard, with limitations, to treat severe volumetric muscle loss is autografts, where muscle tissue is taken from a donor site in the body and transplanted to the injury site. Autotransplantation is becoming more prevalent in procedures with need of functional tissue because there is no risk of disease transmission, and implants tend to generate a minimal immune response (Vang, 2006). On the other hand, there are also problems that can still arise from autologous grafts such as donor site morbidity and loss of functionality and volume deficiency (Suh, 2004). Donor site morbidity plays an important role because the procedure creates a second surgical site to remove the amount of muscle needed. Grafts can also get rejected or the patient will have a limited restoration of the muscles that were involved in the surgery because there is no full recovery (Vang, 2006). The overall complication rate of

autologous tissue transfer is 36%. While most complications can be treated, some implants fail completely and this is the case of 6% of the individuals that undergo this treatment leading to a 1% mortality rate (Suh, 2004).Causes of the graft failure range from venous thrombosis to necrosis of native tissue (Suh, 2004) The last two complications are the main reason there is a clear need for an in vitro cell therapy system for volumetric muscle loss using human skeletal muscle cells (Grasman et al, 2012).

The development of an *in vitro* cell therapy system will greatly increase the integration of the graft to the host body. In turn, this could decrease necrosis and graft failure. There have been advances in culture and formation of skeletal muscle predominantly in the past decade. Most research being done is on the targeting and improving of the individual processes of progenitor cell proliferation, mobilization, and differentiation (Alberto-Rossi et al, 2010). It is important to understand that primary human skeletal muscle cell culture is not efficient enough to produce skeletal muscle for the treatment of volumetric muscle loss because at the moment there are some aspects of the process that are not fully understood such as being able to retain a non-adherent cell population throughout multiple passages. One reason for this problem is that cell culture is not done on a large enough surface area where large quantities of cells can grow because it is not economically feasible (Rafiq et al, 2013). Another problem with the culture is trying to mimic the conditions of the human body. Conditions inside the body are difficult to simulate because it is an environment that efficiently changes and adjusts because of satellite cells and signaling pathways that orchestrate the regulation of muscle repair (Allbrook, 1981). In order to achieve fully functional skeletal muscle, the myogenic index *in vitro* must be comparable to that of the human body. The myogenic index is helpful because it gives an idea of how efficiently muscle fibers can be created. The most efficient *in vitro* system has only been

able to reach approximately 25% myogenic potential (Lee et al, 2000). By reaching a fusion index over 30%, a volumetric muscle loss therapy could be developed in the future to treat different types of muscular atrophy.

2.2 Anatomy and physiology of skeletal muscle

It is important for the anatomy and physiology of the muscle to be understood as the importance of volumetric muscle loss therapy is addressed. It is also necessary to understand the essential components of skeletal muscle and the regeneration process that plays a role in the damage and repairing of the muscle as well. Before the regenerative process can be fully comprehended, the anatomy of the muscle must come into play.

There are three types of muscles within the human body; but in terms of volumetric muscle loss therapy, the focus will remain on the skeletal muscle. Skeletal muscle's most notably characteristic properties that vary it from other muscle types are its striations and multinucleated cells (Fox, 2011). The multinucleated tendency is a result of the fibers being formed from the fusion of several myoblast cells, which are mononucleated and spindle-shaped. The striations of the muscle appear from the presence of sarcomeres, which are the functional units as they repeat along the muscle. A sarcomere is a segment of the muscle that creates the banded look of the muscle, between thin dark lines that form the borders of the sarcomere called Z-lines. The Z lines are a result of the overlapping of the thin myofilaments of adjacent sarcomeres. Actin and myosin myofilaments are overlapped on one another in a repeating array to compose the sarcomere. Components between the Z-lines consist of the I-bands, which are isotropic, meaning "same," and light colored, and A-bands, which are anisotropic, meaning "not the same" and darker in color. Isotropic means that it contains only Actin while anisotropic contains both Actin and myosin. The A-bands result from where the myofilaments overlap and the I-bands result

from the areas where they do not. To better understand the muscle and its regeneration, it can be viewed from large scale to small.

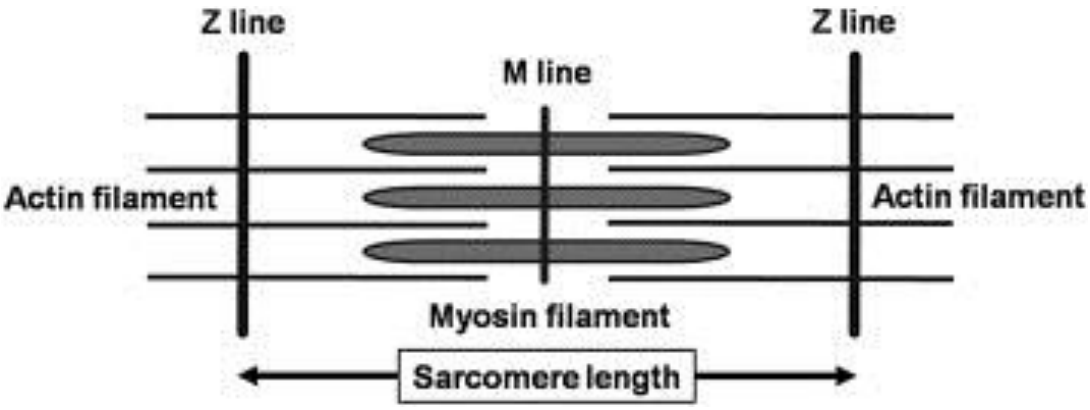


Figure 1: Sarcomere/Basic Unit of Muscle (Tomioka 2009)

The skeletal muscle is composed of individual fibers, or myofibers, that are able to contract when they are stimulated. With the muscle tissue, the fibers contain layers of connective tissue that cover and protect the fibers and fascicles of the muscle. The outermost layer of the muscle, the epimysium, is made of fibrous connective tissue proteins with the tendons that extend around the muscle. Muscle contains type I collagen as its main component, as well as type III collagen as a minor component (Light, 1984). This outer layer is often seen as an outer sheath of the muscle. Connective tissue from the epimysium extends further into the body of the muscle, separating it into various columns called fascicles. Each fascicle is encased in another connective tissue sheath known as the perimysium. Working down from the individual fascicle, the perimysium is composed of many fibers known as myofibers that again are sheathed similarly to the muscle and fascicles in extracellular matrix (ECM). The ECM is important as it provides and maintains the structural framework of the muscle. The fibers are surrounded by sarcolemma, a thin plasma membrane, and enveloped by a final thin connective layer, the endomysium. The fibers are then composed of myofilaments that lead to striations (Fox 2011).

Knowing the anatomy and physiology of muscle is important in the understanding of volumetric muscle loss and the resulting regeneration for various degrees of injury.

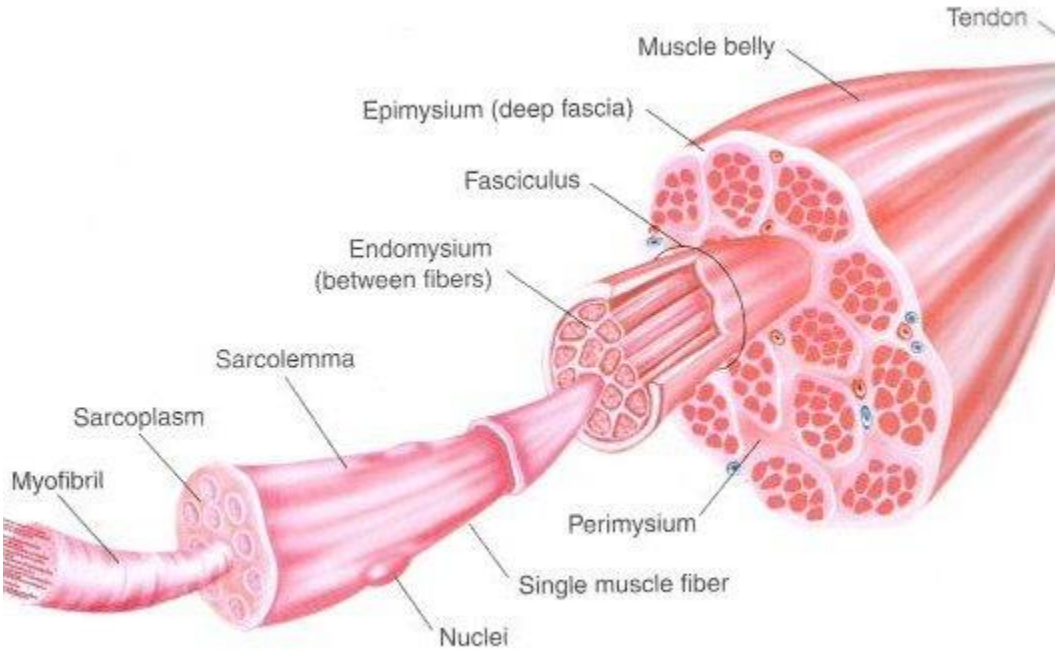


Figure 2: Breakdown showing individual muscle components (Peppel 2009)

As the anatomy and physiology of the muscle are outlined, a microscopic view becomes important to recognize. The muscle itself is a result of many differentiated cells known as myocytes. Before a cell differentiates into a myocyte, it is first a satellite cell. These are cells that have potential to differentiate into various cells within muscle lineages. They are undifferentiated cells that reside in a quiescent state between the basal lamina and the plasma membrane of each fiber. These cells, and their activation, proliferation/migration, and differentiation, are essential in the growth and regeneration of muscle. The need for regeneration is a daily occurrence, but the degree to which it is necessary varies with the injury. Typical small injuries that occur on a more common basis such as simple cuts and tears can regenerate on their own without the need

for autograft or assistance. With smaller scale injuries that do not result in volumetric muscle loss, regeneration is possible naturally by the body. Aside from small, daily regenerations from exercise and similar acts, the need for a therapy for volumetric muscle loss is a common result of sports injuries that cause the muscle to shear, results in tearing of the myofibers and connective tissues. To address this, the process is typically characterized by destruction, repair, and remodeling (Huard and Peng, 2004).

2.2.1 Muscle Regeneration

The regeneration process begins with an injury that exposes the intracellular contents to the extracellular environment. Typically, injuries occur due to the tearing of myofibers and connective tissues. An injury is the breaking up of the myofibrils into individual sarcomeric units, disrupting the continuous nature of the sarcolemma, as well as the disruption of the mitochondria and the sarcoplasmic reticulum (Carlson, 1989). Regeneration is often characterized by hematoma formation, muscle tissue necrosis, degeneration, and inflammatory cell infiltration. As a result of this, the activation of calcium-dependent protease leads to the contraction band of cytoskeletal proteins forming to prevent complete destruction of the myofibers at the site of injury. After this, the chemotactic recruitment of neutrophils and macrophages occurs. These neutrophils and macrophages are able to release cytokines to amplify, or suppress depending on the stage, the inflammatory response. They are used to digest necrotic fibers and cellular debris by phagocytosis that result from the injury. With the amplified inflammatory response and signals from the environment, the recruitment of satellite cells begins (Charge, 2004). Once the quiescent satellite cells are exposed to the signals from the inflamed environment, the activation of their proliferation and removal from quiescence is characterized by the expression of Myf5 and MyoD, two myogenic regulatory factors (Charge, 2004). As the cells are recruited, there are two phenotypes of macrophages that promote different behaviors in

the precursor, satellite, cells. The M1 phenotype phagocytose the necrotic tissue while promoting satellite proliferation. The M2 phenotype remodels the tissue further along, promoting proliferation, growth, and differentiation (Turner & Badylak 2011).

After the inflammation period, the satellite cells begin to migrate to the site of injury to begin the repair phase of regeneration as the muscle precursor cells begin to proliferate. When the cells exit the cell cycle, they begin to differentiate into myoblasts. With this, the myoblasts that are a result of differentiated satellite cells begin to fuse with myofibers that have necrosed in an effort to prevent complete degeneration of the non-functional fibers that are already there. When this is not possible, the myoblasts mature and fuse to form functional myotubes. While the differentiation and fusion are occurring, there is also proliferation of fibroblasts that produce the ECM necessary for the muscle to form on as a scaffold. The fibroblasts respond to the site of inflammation and are located in close proximity of the satellite cells and muscle fibers. The cells proliferate and differentiate as a means of creating an ECM that encapsulate the muscle fibers. After migration to the site of injury, fibroblasts differentiate into myofibroblasts and deposit ECM proteins, resulting in collagen deposits and connective tissue formation which promotes tissue fibrosis (Burks, 2011). In an in-vitro study, fibroblasts have the potential to form an ECM on which muscle may form. Due to environmental factors, fibroblasts are observed to proliferate with a quicker population doubling time than satellite cells. Unlike *in vivo* where it is naturally regulated¸ when compared side by side, a culture of the two cells would show a greater rate of proliferation for fibroblasts. If the fibroblasts are allowed to over proliferate, dense scar tissue forms. In native muscle, the over proliferation of fibroblasts does not occur. On a smaller scale, the dense fibrous connective tissue that remains is not always harmful to the individual. When the injury leads to greater generation of scar tissue and overgrowth of connective tissue, it

impedes the regeneration and leads to incomplete restoration of functionality. Once remodeling is complete, the satellite cells that did not differentiate migrate between the newly formed basil lamina and membrane of the new muscle and reenter a quiescent state as with the mature muscle (Peng and Huard, 2004).

In the event of an injury of a larger scale or degenerative disease, there is a need for a large quantity of cells and matrix to replace what is lost. A volumetric muscle loss therapy becomes necessary for increased regeneration and functionality of the injured muscle.

2.3 Cell Cycle and Culture Conditions

When satellite cells are activated, they enter the cell cycle and progress through myogenesis which is the formation of muscle tissue. The cell cycle is a process of events through which cells go through that involve division and duplication. The final result of the cycle is known as a daughter cell, which is an independent split from the original cell that contains its own nucleus and duplicated DNA. The cycle of cell division consists of four phases known as Gap 1 (G1), Synthesis (S), Gap 2 (G2), and Mitosis (M). The first phase, G1, is beginning of division where the cell begins to grow and enter the S phase. This second phase is characterized by the replication of the cell's DNA. Once DNA is replicated, the cell moves on the G2 phase where it continues to increase in size until division. As the cell reaches the final phase, the M phase, growth stops. With this, the cell divides in two, each with their own DNA and nucleus, into two independent cells (Cooper, 2009).

In order to differentiate, the cell must exit from the cell cycle which occurs before the S phase. Signal pathways that regulate cell activity drive proliferation and differentiation; and for differentiation to occur, the proliferation pathway must be inhibited. Throughout the cell cycle, myogenic transcription factors are expressed sequentially in order to regulate specific stages of

myogenesis. These transcription factors initiate the transcription of a specific gene regulating cell behavior. Upon inhibiting proliferation, myoblasts express myogenic transcription factors, such as MyoD and Myf-5; and the increased expression of MyoD and muscle-specific genes induces differentiation. Subsequent transcription factor expression of myogenin initiates fusion of differentiated myoblasts into multinucleated myotubes. Once terminally differentiated, cells cannot reenter the cell cycle (Wood, 2013).

The increased expression muscle-specific genes are essential to myoblast differentiation; however, mitogenic growth factors can suppress differentiation by promoting proliferation within myoblasts through mitogen-activated protein kinase (MAPK) pathways (Kitzmann, 1998). More specifically in mammalian cells, mitogens activate the extraceullular signal regulated protein kinase (ERK) cascades, ERK1 and ERK2, in order to induce transcription of cyclin D1 which is required for entry into the S phase of the cell cycle (Robinson, 1997).

2.3.1 Mitogenic Growth Factors

Mitogenic growth factors regulate cell behavior in terms of proliferation and differentiation by binding to cell surface receptors signaling cellular pathways. In order to control cellular activity, growth factors either promote or inhibit proliferation by promoting cells to exit or enter quiescence, respectively. Mitogenic growth factors are critical for maintaining the mitotic activity of satellite cells and are essential to the regeneration of muscle tissue. Furthermore, mitogens can be released from cells through signals to surrounding cells as a paracrine mechanism or to itself as an autocrine mechanism (Hayashi et al., 2000).

Insulin-like Growth Factor (IGF-1) promotes myoblast proliferation. Upon differentiation, IGF-1increases hypertrophy by promoting differentiation into myocytes and fusion into multinucleated myotubes. Studies have shown mice lacking IGF-1 and IGF-2 expression undergo limited hyperplasia and are unable to produce sufficient muscle mass to

function. Alternatively, as the result of up regulating IGF-1 expression, muscle regeneration and hypertrophy significantly increased (Duan, Gao, & Ren, 2010). Under normal oxygen supply, 19% ambient oxygen, IGF-1 expression promotes proliferation in cells; however, in the presence of a reduced oxygen environment simulating native muscle oxygen tension, IGF-1 promotes myoblast differentiation (Duan, Gao, & Ren, 2010). In an experimental study investigating the effect of hypoxia on the myogenic capacity of insulin-like growth factors, 300ng/mL of IGF-1 was shown to have a direct increase to the differentiation of C2C12 cells at normoxia while having an inhibitory effect to differentiation at hypoxia. Furthermore, 300ng/mL of IGF-1 was shown to increase proliferation rate of C2C12 cells at normoxia and nearly triple the proliferation rate at hypoxia as shown in Figure 3 below. Data are expressed as mean ± standard deviation for a sample, n=3, and statistical differences are noted as * for $p<0.05$ and ** for $p<0.01$ (Ren et al., 2010).

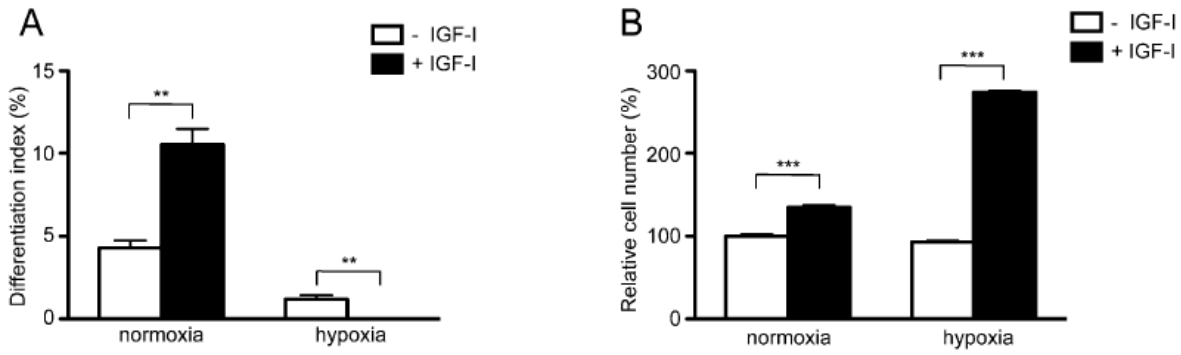


Figure 3: The Effect of Media with and without IGF-1 on the Proliferation and Differentiation of C2C12 Cells at Normoxia and Hypoxia (Ren et al., 2010)

Hepatocyte Growth Factor (HGF) induces quiescent cells to enter the cell cycle causing DNA synthesis and promoting cell proliferation by activating these quiescent cells to enter the S-phase of the cell cycle (Gal-Levi et al, 1998). Upon satellite cell proliferation, the cell stimulates further production of HGF with potential of increasing local concentrations of HGF. As the

concentration of HGF increases, there is potential for the cell to return to quiescence and resultant production of myostatin due to high concentration of HGF (Yamada, 2010). Furthermore, HGF significantly enhances cell proliferation when used in conjunction with Fibroblast Growth Factor-2 (FGF-2) (Deasy et al., 2001). The mitotoic effect of FGF-2 and the FGF family can be further enhanced by using the growth factor in combination with the optimal concentration of 10ng/mL of HGF (Sheehan, 1999). In an experiment study investigating the role of HGF, myoblast fusion was demonstrated to decrease from 30% to 0% as HGF concentration increased from 0ng/mL to 100ng/mL. At the maximum concentration of HGF, there were no multinucleated myotubes present in the culture, and the cell number had triple in comparison to 0ng/mL of HGF as shown in Figure 4 below. Data are expressed as mean ± standard deviation for a sample, n=4. There are statistical differences between means denoted as different letters (a, b, c, and d) for $p<0.05$ (Hayashi, 2000).

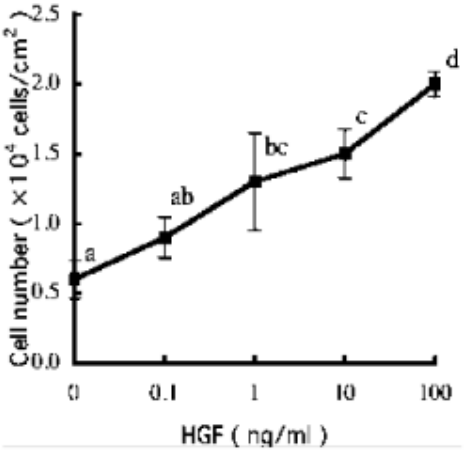


Figure 4: Dose-Dependent Effect of Recombinant Human HGF on Proliferation of Myoblasts (Hayashi, 2000)

Fibroblast Growth Factor-2 stimulates proliferation and migration within cells and can lead to an abundance of satellite cell-like myoblasts instead of terminally differentiated myotubes. Researchers have suggested that FGF-2 may exhibit an indirect inhibition to differentiation and is often used in combination with other growth factors in order to form fused

myotubes. Naturally in the body, FGF-2 is stored in the extracellular matrix and is released during the inflammatory stage of muscle regeneration *in vivo* prompting the first satellite cells to begin proliferating (Husmann, 1996).

Epidermal Growth Factor (EGF) stimulates cell proliferation through up regulating the intake of nutrients into cells. Furthermore, EGF has potential application in organ regeneration and wound healing as it enhances the proliferation and migration of cells (Wells, 1999). There is potential for increased proliferation and differentiation capability of cells as the EGF binding to the EGF-receptor activates mitogen-activated protein kinases (Jeong, 2013). EGF has been show to increase the proliferative capable of myogenic cells as in one experimental study where cells cultured in EGF exhibited an 85% proliferation rate compared to 77% without EGF. With the presence of EGF, more cells entered the cell cycle and proliferated (Deasy et al., 2002).

2.3.2 Media Composition

Ham's F-12 and Dulbecco's Modified Eagle Medium can be combined to provide cells with the essential nutrients, all 13 essential amino acids, and seven non-essential amino acids. This combination provides high amino acid content with a highly enriched nutrient content. FetalClone III (FCIII) serum is used in proliferation media within cell culture by providing the cells with essential components including glucose, growth factors, nutrients, vitamins, and hormones. Proliferation media is supplemented with IGF-1, HGF, FGF-2, and EGF to further preserve the mitotic activity of the cell population. A supplement of L-glutamine is added to the media drives proliferation and cellular activity (Huard & Jankowski, 2004). In order to induce differentiation, the media is transferred in media containing 2% adult horse serum and 1% insulin, transferrin, and selenium (ITS) (Huard & Jankowski, 2004). Low-serum media provides cells with a necessary supplement of ITS. Insulin is a necessary hormone for cells to progress through the cell cycle, transferrin provides iron to the cells, and selenium helps cells protect

against oxidative stresses (Van der Valk, 2010).The removal of mitogens prevents signaling of the MAPK pathways and results in expression of muscle specific genes for differentiation (Robinson, 1997).

2.4 Measuring Myogenic Potential

Many different assays exist that allow researchers to quantify data and produce workable results. In order to quantify the functionality of muscle cells grown *in vitro*, the myogenic potential is measured. Researchers identify antigens specific to functional myocytes and perform targeted assays to determine the percentage of functional cells present.

2.4.1 Neural Cell Adhesion Molecule Immunocytochemistry Assay

Common tests used for quantifying biological data are immunocytochemistry (ICC) assays. This involves using antibodies to target and mark a desired antigen. The presence of the antigen can be visualized by using fluorescent tags on the antibodies along with fluorescence microscopy. This is straightforward for extracellular antigens, but in order to tag intracellular antigens, the antibodies need to cross the cell membrane. For this to be possible, the cells must first be permeabilized using a detergent, such as Triton X-100 or Tween-20 (Gaster et al 2001). More specifically, ICC can be used to identify cells that have differentiated by targeting antigens unique to the differentiated cells. In order to visualize contractile muscle cells, researchers need to target antigens that exist only in differentiated myotubes. A common antigen to target is neural cell adhesion molecule (NCAM) (Gaster et al. 2001). NCAM is a satisfactory differentiation marker because it is expressed relatively early (within four days), allowing it to be stained for after less time (Rutishauser 1988). The figure below shows human skeletal muscle cells stained for NCAM. When used in conjunction with DAPI, researchers are able to visualize functional myotubes as multiple nuclei aligned on NCAM positive cells.

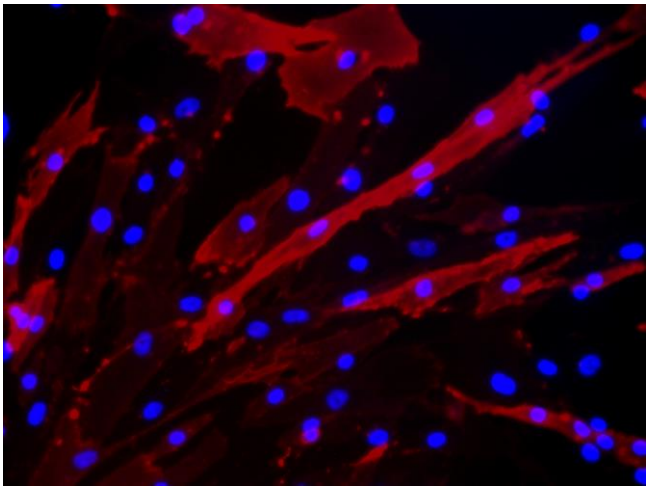


Figure 5: NCAM staining of human skeletal muscle

2.4.2 Desmin Staining

Another assay that can be used to visualize differentiation is desmin staining. Desmin is one of the first proteins expressed during the early stages of differentiation. It has been shown that desmin is expressed before MyoD expression (Costa et al. 2004). This expression will disappear over time making it more difficult to accurately analyze the percentage of differentiation in the culture (Cooper et al. 2004). Desmin is located all over myotubes, but tends to be at the highest concentration in the Z line.

2.4.3 Nuclear Staining

In order to calculate the myogenic index, one must first be able to visualize the nuclei of the cells present in the culture. A common technique involves using a fluorescent nuclear stain, 4', 6-diamidino-2-phenylindole (DAPI). DAPI is a blue-fluorescent nucleic acid stain that associates with the adenine-thymine cluster and allows for visualization of the DNA in the nucleus of a cell (Invitrogen 2006).

2.4.4 Calculating the Myogenic Index

Once the cells are stained, the myogenic index can be calculated. In the Page lab, the myogenic index is calculated using the following formula:

$$Myogenic\ Index = \frac{Number\ of\ aligned\ nuclei\ within\ NCAM\ positive\ cells}{Total\ number\ of\ nuclei}$$

This formula is appropriate because differentiated and fused myotubes will have a high expression of NCAM. Therefore, if a cell tests positive for NCAM, and contains multiple nuclei, it is counted as a myogenic cell. The myogenic index is a ratio of the myogenic cells to the total number of cells.

2.5 Measuring Proliferation

One way of measuring cell proliferation is to perform a 5-bromo-2'-deoxyuridine (BrdU) assay. This assay uses specific antibodies to insert fluorescent tags into cells that are dividing, by binding with newly synthesized DNA. BrdU is an analogue to the nucleoside thymidine (Waldman et al. 1988). In culture, BrdU will replace thymidine in the newly forming DNA during the S-phase of the cell cycle. Because the population doubling time for human skeletal muscle cells is around 40 hours (see Appendix B), the BrdU labeling agent should be incorporated in the culture for 48 hours. This is just enough time to allow all proliferating cells to go through only one complete cell cycle. The cells can be fixed and stained to visualize cell proliferation. Anti-BrdU antibodies can be added to the culture that will attach to the BrdU antigen. Then, a secondary antibody that targets the primary one can be introduced. This secondary antibody contains a fluorescent tag, which can be viewed under a fluorescence microscope, when excited by the appropriate fluorophore. Any fluorescence that is seen indicates a cell that has replicated its DNA and is proliferating (Kulldorff et al. 2000).

For comparison, a fluorescent nuclear counterstain can also be added to visualize the total number of cells in the culture. By comparing the fluorescence of the nuclear stain to that of the BrdU tag, the percentage of cell proliferation can be determined. By testing the adherent and non-adherent populations separately, it is possible to determine the significance of the non-

adherent cells, and their relationship to the cell cycle.

2.6 Recent Advances Volumetric Muscle Loss Therapy

There is a clear need for refined culture techniques of human skeletal muscle cells in order to further the development of a regenerative cell therapy process. Great improvements have been made for the understanding of the mechanisms that control cell proliferation and differentiation. A recent study showed that within a satellite cell population, there are slow-dividing and fast-dividing cells. These two populations have different characteristics and each population was studied. It was shown that slow-dividing cells possess long-term self-renewal ability whereas the fast-dividing cells tend to terminally differentiate after a couple doublings. The importance of this study was that the slow-dividing cell cultures showed an increased myogenic potential (Ono et al, 2012). Various types of substrate stiffness have shown to have an effect on the myogenic and regenerative potential of muscle cells. Research has demonstrated that there is a direct correlation between the stiffness of the substrate and the proliferation rate of the cells showing that a higher stiffness leads to proliferation (Engler et al, 2004).

Stem cell microenvironments tend to be hypoxic, a low oxygen level compared to ambient. It has been suggested that treating cells under hypoxic conditions will trigger response from satellite cells resulting in the adoption of a quiescent state where they proliferate at a faster preventing them from differentiating (Koning et al, 2011). What Koning and his colleagues hypothesized for this experiment was that hypoxia improves proliferation and differentiation of stem cells. Another way to maintain a muscle derived cell's stemness is to keep it in suspension or in their spheroid form. Several studies have tried to achieve this and have succeeded. A study done in Germany has come up with a protocol to culture "myospheres" using myopshere-growing media. This media was composed of Dulbecco's Modified Eagle's Medium (DMEM/F12, ratio was not specified), 20% Fetal Clone Serum (FCS), basic Fibroblast Growth

Factor (bFGF) 5 ng/ml, and 1% P/S (Wei et al, 2011). The media was kept at humidified of 5% CO_2 in air. Using this culture technique allowed the researchers to keep the cells in their spheroid condition for at least five months allowing the cells to go through over 40 population doublings (Wei et al, 2011). Their study concluded that myosphere-derived progenitor cells could be maintained proliferatively active in vitro for more than 20 weeks and passaged at least 18 times, despite an average donor-age of 63 years.

Much work has been done on the growth media composition and its components. The key components of the media are the growth factors such the ones that the Page Lab uses (EGF, IGF-1, HGF, and FGF-2). It has been shown that other growth factors such as Mechano Growth Factor (MGF) can further help the to induce the growth and hypertrophy in mechanically stimulated or damaged muscle. MGF or IGF-1Ec is a variant of the IGF-1 and was shown to have an increase by 30% in the myogenic potential of human myoblasts when compared to IGF-1 (Kandalla et al, 2011). Fusion indices have been increasing due to better culturing techniques and a study was done at the University of Pittsburg where a myogenic index of 25% was achieved from a female rat model compared to the rest of the preplates, which achieved no more than 15% myogenic index (Jankowski, 2004). The differentiation media was composed of DMEM containing 2% horse serum and 1% penicillin/streptomycin and containing different concentration of suramin (0, 1, 10, 100μl/ml). Suramin is an antifibrotic agent that improves muscle recovery by antagonizing transforming growth factor–beta1 (TGF-β1), a fibrotic promoter (Jankowski, 2004).

3. Project Strategies

The primary goal of this project is to design and optimize an *in vitro* cell culture system that selectively amplifies myogenic cells in order to extrapolate a prototype for the tissue engineering of contractile muscle tissue. The purpose of this chapter is to explain the process used in identifying and defining the objectives and constraints of the design. After evaluating the objectives and constraints, the group revised the initial client statement and outlined the project approach.

3.1 Design

In every design, the three stakeholders consist of the client, the user, and the designer. For this project, the client was Dr. Raymond Page, the users were researchers or graduate students experimenting with the application of functional muscle tissue engineering, and the designers consisted of Brishell Aquise, Kyle Bonaccorso, Jeffrey Ducki, and Andrew Lamb.

3.2 Initial Client Statement

The following statement is the initial client statement provided to the team by the client, Dr. Raymond Page. Based on the statement, the team shaped objectives and constraint necessary for reaching the desired goals.

> **"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."**

3.3 Objectives

In order to accomplish the project goals, the team designed the following objectives. The objectives were to be inexpensive, biocompatible, quantifiable, scalable, reproducible, and be able to maintain a myogenic potential of greater than 30%. Each of the objectives was listed in an Objectives Tree along with sub-objectives, as shown in Figure 6 below, and they were ranked in order of significance using a Pairwise Comparison Chart.

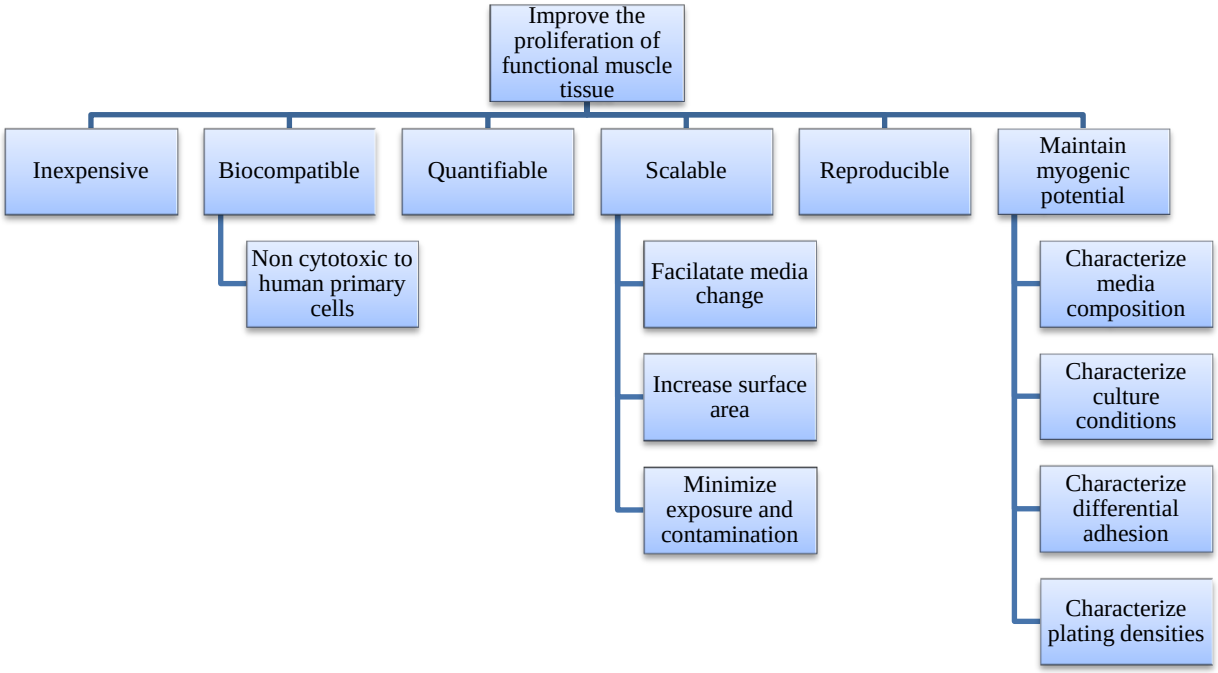


Figure 6: Objective Tree including objectives and sub-objectives.

Inexpensive: Muscle tissue engineering requires a cell culture system that better preserves the myogenic potential of cells through proliferation to large populations. There will not be a feasible commercial application for the system if the price of the process is not affordable by a combination of volumetric muscle loss patients and insurance providers. The feasibility of the cost will be determined based on the cost benefit analysis in selecting the final design.

Biocompatible: The cell culturing process uses human primary cells; and therefore all ambient conditions, substrates, and media compositions has to mimic the physiological environment of human primary muscle cells and be non-cytotoxic.

Quantifiable: The design needed to be quantifiable in order to measure myogenic index and compare the effect of design parameters by counting cells and calculating the myogenic potential of cells.

Scalable: In order to have commercial application, a final expansion system requires a high surface area, ease of media change, and elimination of exposure and risk of contamination. A scalable system reduces the necessary resources, expenses, size restrictions, and other facts that limit the feasibility of increasing the amount of cell within the culture process.

Reproducible: The specific protocol and cell culture system design had to be easily and feasibly reproducible in order to have application in improving functional muscle tissue engineering.

Maintain myogenic potential: Maintaining a myogenic potential of greater than 30% was significant in order to advance the clinical relevance of the process. Engineering muscle tissue with enough potential to fuse into myotubes after increased time *in vitro* is critical to creating research applications. In order to achieve this myogenic potential, sub-objectives of characterizing media composition, ambient conditions, differential adhesion, and plating densities needed to be completed.

Pairwise Comparison Charts, as shown in Table 1 below, rank objectives by comparison each of them against each remaining objective. If the objective is more important than the other, it receives a score of 1. If the objective is less important than the other, it receives a score of 0, and ties result in a score of 0.5.

Table 1: Pairwise Comparison Chart

	Inexpensive	Biocompatible	Quantitative	Scalable	Reproducible	Maintain Myogenic Potential	Score	Rank
Inexpensive	X	0	1	0.5	0	0	1.5	4
Biocompatible	1	X	1	1	1	0	4	1
Quantitative	0	0	X	0	0.5	0	0.5	5
Scalable	0.5	0	1	X	0	0	1.5	4
Reproducible	1	0	0.5	1	X	0	2.5	3
Maintain Myogenic Potential	1	1	1	1	1	X	5	2

After evaluating the objectives, the team decided the objectives ranked in decreasing significance are:

1. Optimize Myogenic Cells
2. Biocompatible
3. Reproducible
4. Scalable
5. Inexpensive
6. Quantifiable

3.4 Constraints

The team identified and compiled a list of constraints that the design could not violate. These constraints were expressed within the initial client statement and discussed with the Dr. Raymond Page before finalized:

- The cell culture process, media, and other components must be biocompatible with the human primary cells in use.
- The cell culture process must consist of sterile technique, sterile equipment, and fit inside the sterile biosafety cabinets and incubators.
- Incubator space limited the size of the system as the lab contained four incubators shared with several other project groups. The dimensions within the incubator were 2'x18"x19.5" split into three shelves of 7"x18"x19.5".

3.5 Revised Client Statement

After meeting with the client and reviewing the ranked objectives and constraints, the team revised the initial client statement to be as follows:

Design a biocompatible and quantifiable *in vitro* cell culture system that characterizes individual parameters of adherent and non-adherent cell kinetics by employing combinations of differential adhesion, media composition and supplements, ambient conditions, and plating densities. Characterize an environment to increase proliferation and selectively amplify myogenic cells in the culture to exhibit a myogenic potential of at least 30% in order to engineer a scalable cell expansion system with increased time *in vitro*.

3.6 Financial Approach

In order to ensure that the project spending is controlled and will stay within the total budget, potential expenses were estimated based on current pricing. The main expenses will be

on different media components to be used in the research. All of the media components are purchased from VWR International with the exception of the growth factors, which are purchased from PeproTech. The cost for making 500 mL of DF12 complete media with growth factors was calculated based on the prices, and is shown below.

Table 2: Cost breakdown for medium

Component	Amount/500mL	Commercial Price		Price/500mL
		Amount	Price	
DMEM	270 mL	500 mL	$16.34	$8.82
HAMSF12	180 mL	500 mL	$16.41	$5.91
FCIII	50 mL	500 mL	$164.98	$16.50
L-glutamine	0.1578 g	25 g	$60.18	$0.38
FGF-2 [4.0 ng/mL]	2.000 µg	500 µg	$720.00	$2.88
HGF [2.5 ng/mL]	1.250 µg	500 µg	$2,880.00	$7.20
EGF [10 ng/mL]	5.000 µg	500 µg	$195.00	$1.95
IGF-1 [5.0 ng/mL]	2.500 µg	100 µg	$195.00	$4.88
Total				**$48.52**

The total cost to make 500 milliliters of media is $48.52. Estimating that the research required about 2,000 milliliters, the total expense for media was around $194.08. Because the media components were given donated by the Page lab, the expense was not taken from our budget. There was also a $100 expense to cover lab materials being purchased by Worcester Polytechnic Institute. Some of these materials include pipets, micropipette tips, flasks, well plates, and conical tubes. In addition, a gas tank was purchased from AirGas for $160.35, and roller bottles were purchased from Thermo Scientific for $220.64. The total budget for this project is $624. The remainder of the budget was used for support expenses.

3.7 Experimental Approach

The experimental approach includes a description of the cell culturing methods, experiments, and the financial approach to the completion of the project. The experimental

approach identifies important challenges and the technical strategies in overcoming them in order to meet the project objectives.

3.7.1 Cell Culture Methods

The cells used in the project were hSkm, human primary muscle cells. The cells were taken from a skeletal muscle biopsy of a 58yr old woman, and cell line derivation was completed. In order to isolate the myogenic cells for culture, the Page Lab used a modified pre-plating technique removing the fibroblasts by selective adhesion. For cell culture, the DF12 complete media consisted of Dulbecco's Modified Eagle Medium and Ham's F12 at a 60:40 ratio containing 10% FCIII and 4mM L-glutamine. The media contained the growth factors: HGF, IGF-1, EGF, and FGF-2 at current Page Lab concentrations of 2.5ng/mL, 5.0ng/mL, 10ng/mL, and 4.0ng/mL, respectively.

Cell culture media often contains antibiotics in order to reduce the risk of contamination; however, the use of antibiotics can result in antibiotic resistant microorganisms or have a negative effect on cellular behavior (Valk et al, 2010). Since this media does not contain antibiotics to protect the culture from contaminants, the importance of sterile technique increased as the cell culture had no resistance to contaminants. The team had to incorporate sterile technique into every aspect of the subculturing process. Everything entering the hood was wiped with 70% ethanol. When opening containers and maneuvering inside the Biological Safety Cabinet, the team took care not to cross hands or non-sterile objects directly above open containers.

The team started cell culturing by thawing 500,000 hSkm 1201 P4 into 2 T75 flasks according to the protocol in Appendix A. Following thawing, the media was replaced every 48 hours with fresh media while retaining all the non-adherent cells, and the cells were passaged with 0.05% Trypsin each time the cell population reached 70% confluence, as described in

protocols in Appendix A. In order to retain the non-adherent cells, the media in active culture was pipetted into a 15mL conical tube, and the cells were pelleted using a Clinical 50 centrifuge. The pellet was broken up by repeat pipetting, resuspended in media, and replated in a new vessel, and adherent cells were passaged using 0.05% Trypsin to release cells. After trypsinization, non-adherent cells were pelleted, resuspended in media, and replated in a new vessel. The passage rate, type or size of vessel, and plating densities varied depending on the current experiment as described in Section 3.6.3 Experiments for Characterizing Individual System Parameters.

3.7.2 Quantification Process

A quantification process must be standardized with specific parameters in order for a design to be accurately evaluated and return statistically significant resu1lts. In order for results to be statistically significant, T-tests were used to analyze whether there was a statistically significant difference between results for a test group and a control group. If the null hypothesis was not rejected, conclusions were drawn concerning the individual system parameter isolated in the experiment. The team used an immunocytochemistry assay staining for NCAM to measure myogenic index and myogenic potential, as previously described in Section 2.4.1, after cells were plated into well plates and differentiated in DF12 complete media containing 2% HS, 1% ITS, and no growth factors. Furthermore, the team counted cells using a hemocytometer in order to determine proliferation rates. After performing the assays, the cells were imaged using a Zeiss inverted epi-fluor microscope and analyzed using AxioVision software. Three images were taken per stained well, and T-tests evaluated the myogenic indexes of each sample against the control and other samples for significant differences. Also, the magnification of the microscope was set at 20x for all images. In order to calculate the myogenic index precisely, myotubes were specifically defined as two or more NCAM positive nuclei fused into a multinucleated cell and

aligned in a central axis across the cell. The resultant myogenic index was calculated as previously described in 2.4.4 Calculating the Myogenic Index. When staining for NCAM and BrdU, one stained control removed the primary antibody and the other removed the secondary antibody. This displayed the non-specific staining that was ignored within the experimental groups. Finally, qualitative images taken during cell culture were analyzed with AxioVision software.

3.7.3 Experiments for Characterizing Individual System Parameters

In order to characterize individual system parameters, experiments were set up isolating growth factor combinations, adherent versus non-adherent cell behavior, and oxygen tension to determine the effect on myogenic potential of human primary cells. These experiments determined an effective environment for maintaining a myogenic potential above 30%. A final scaled-up expansion system incorporated the experimental results in order to apply effective culture system parameters.

Growth Factor Characterization Objective:

The primary objective in this study was to determine if each growth factor is beneficial to the culture system in increasing proliferation and myogenic potential. The secondary objective is to determine the magnitude of the effect of removing each individual growth factor compared to the others. This information will determine further growth factor characterization studies.

Hypothesis:

The removal of each growth factor should decrease the proliferation rate of cells since all the growth factors are used in order to preserve the mitotic capacity of cells and prevent them from leaving the cell cycle. Similarly, removing growth factors should decrease the number of non-adherent cells since they have been shown to preserve proliferative capability and myogenic potential.

Experimental Design:

The initial growth factor study consisted of making four separate complete proliferation media solutions by removing one growth factor while maintaining the current Page Lab concentrations for the remaining three. In addition, the study included a positive control containing the current Page Lab concentrations for all four growth factors and a negative control containing no growth factors. Six identical starting populations of 50,000 cells were plated into six T25 Nunc flasks in complete proliferation media each with the respective media as shown in Figure 7 below. Media was routinely changed, and cells were passaged at 70% confluence in order to characterize the effect of each growth factor on the adherent and non-adherent cell behavior. Cell counts were taken for adherent and non-adherent cell populations upon passage in order to calculate population doublings and compare proliferation rates between populations. After passaging, cells were plated at 20,000 cells per well in a 4 well plate, proliferated for 24 hours in proliferation media containing the specific growth factors, and switched to differentiation media. After differentiating for 4 days, the cells were stained for NCAM using Immunocytochemistry, and the myogenic index was calculated as described in calculating the myogenic index.

Process Flow Diagram:

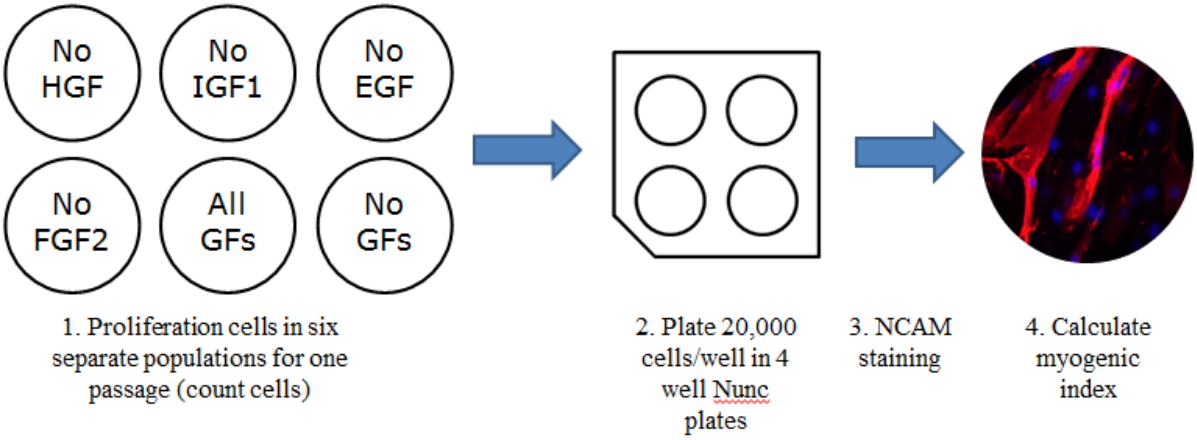


Figure 7: Growth Factor Characterization Study

Oxygen Tension Characterization Objective:

The objective of this experiment is to determine if low oxygen amplifies or reduces the effect of the growth factors. Furthermore, the study without the growth factors will determine if the oxygen tension has a direct effect on the proliferation and differentiation of human primary cells.

Hypothesis:

The hypothesis is that there will be a significant difference between proliferation, measured by an increased cell count, and differentiation, measured by NCAM staining, behavior of the native and ambient oxygen tension cultured cells (Kook et al., 2008).

Experimental Design:

In order to determine the effect of oxygen tension on cell behavior, two identical starting populations of cells will be plated at 250,000 cells per T75 Nunc flask in current Page Lab growth factor concentrations and will be cultured with routine media change and passaging using routine protocols as previously described in subculturing cells. One of the flasks will be cultured

in a low oxygen cylindrical chamber at 5% oxygen replicating native oxygen tension of skeletal muscle within the body. In order to control the oxygen level of the chamber, an air supply tank containing 5% CO_2, 5% O_2, and balance nitrogen was purchased from Airgas. Following each media change on days 3 and 6, the oxygen level was reset in the native oxygen chamber. The other flask will be cultured in an ambient oxygen tension incubator, at 19% oxygen. In addition, this experiement will contain an identical study as the previous except without growth factors. Upon passage on day 9, the cells will be seeded in 0.5mL of proliferation media containing the current Page Lab growth factor concentration at 20,000 cells per well and incubated for 24 hours at the respective oxygen tension. Next, the proliferation media will be removed, and the non-adherent cells will be pelleted and resuspended in 0.5mL of differentiation media containing 2% adult horse serum, 1% ITS, and no growth factors. After 4 days, the cells will be stained for NCAM using the protocol described in Appendix A, and the myogenic index will be calculated as described in Immunocytochemistry Assay.

Process Flow Diagram:

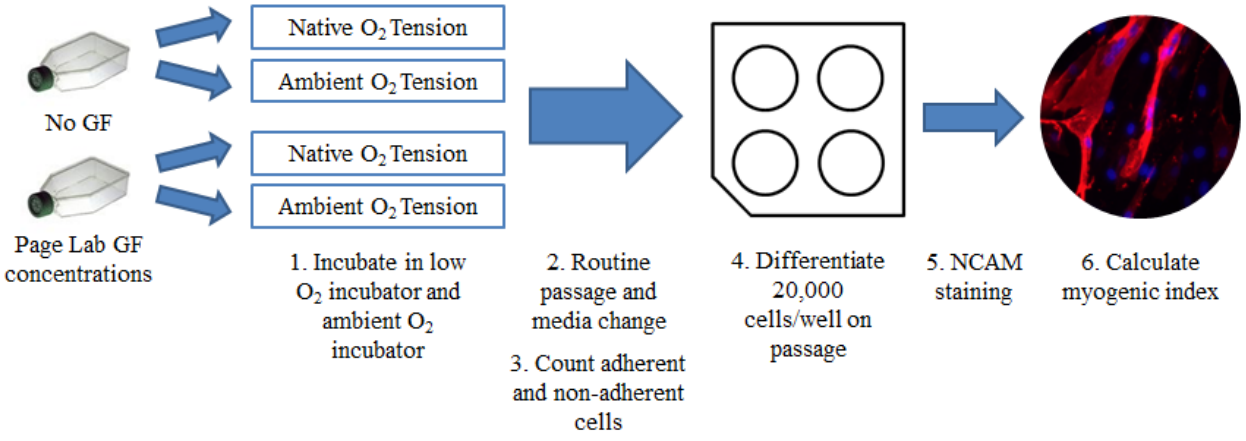


Figure 8: Oxygen Tension Characterization

Adherent Cell and Non-Adherent Cell Behavior Characterization Objective:

The objective of this study is to determine whether the non-adherent cell population exhibit a higher proliferation rate and are more often progressing through the cell cycle than the adherent cell population.

Hypothesis:

The non-adherent cells will exhibit a higher proliferation rate because the adherent cells have more cells that have terminally differentiated and fused.

Experimental Design:

In order to characterize the proliferation behavior of non-adherent cells, 60,000 cells were plated into a T25 Nunc flask and cultured until 60% confluence as shown in Figure 9 below. Upon passaging, 7,500 non-adherent cells and 7,500 adherent cells were plated separately into a 4 well Nunc plate. Each of the cell samples were plated in 0.5mL of proliferation media containing current Page Lab growth factor concentrations with 5µL of BrdU labeling agent for 48 hours. After 48 hours, the wells were stained for BrdU as previously described.

Process Flow Diagram:

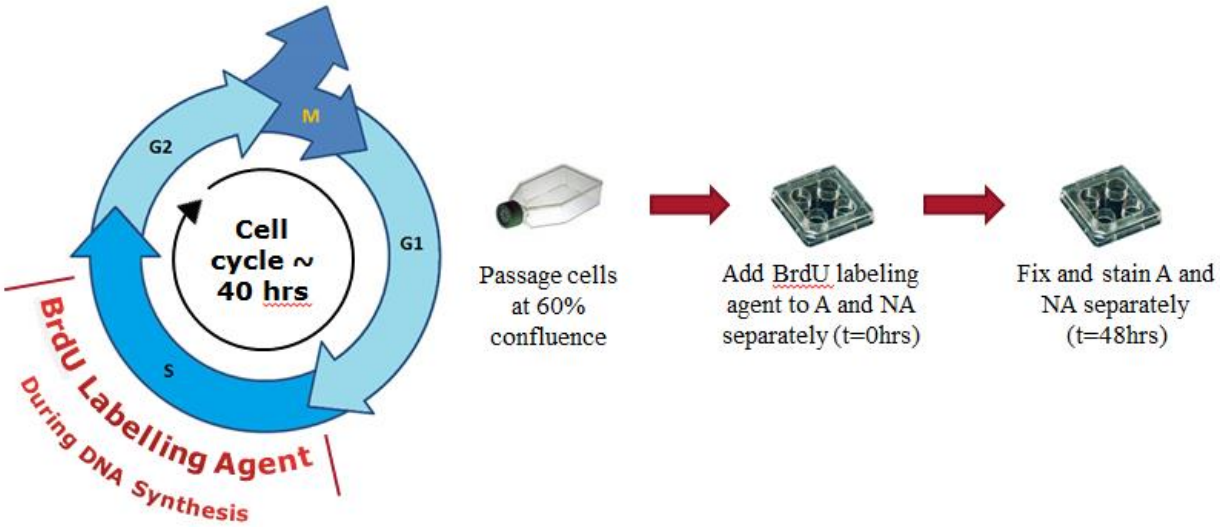


Figure 9: Adherent and Non-Adherent Behavior Characterization

4. Alternative Designs

With the client statement finalized and an approach determined, it becomes necessary to begin creating various designs in an effort to meet the constraints and objectives set forth. As there are many solutions that may be used to meet the demand of the client, it is important to understand the specific functions of the ideal design as a means of analyzing the multitude of options for completing the goal. Once designs and functions are determined, they are compared and analyzed for the best fit for the need of the client.

4.1 Need Analysis

Before creating and brainstorming conceptual designs, it is essential to create a list of functions and specifications that must be met. The means of achieving each function were determined in order to incorporate them into the design.

4.1.1 Design Functions

The functions that result from the client statement are compiled and analyzed to prioritize their importance and feasibility in relation to the conceptual and final design. The functions are as follows:

1. Retain non-adherent cell population
2. Provide a high growth surface area for scale-up
3. Increase ease of media exchange
4. Prevent cells from terminally differentiating

Retain non-adherent cell population: In order for the design to meet the expectations of the client and maintain a high myogenic potential, it is important to retain the non-adherent cell population as well as the adherent population. This function is necessary to reduce the loss of cells that positively contribute to the myogenic potential of the culture.

Provide a high surface area for scale-up: It is necessary for the system to be cultured in a vessel that provides high surface area in order to reduce the amount of supplies, time, and labor that goes into passaging the cells. This allows the cost of the system to be reduced dramatically.

Increase ease of media exchange: An important step in the cell culturing protocol is changing the media that the cells are suspended in. It is essential to be able to access and change the media in the system to continue culturing.

Prevent cells from terminally differentiating: It is necessary to maintain a population that has not begun terminally differentiating. The process and system must be able to avoid terminal differentiation of the cells in order to meet the needs of the client. When a cell terminally differentiates, it is no longer capable of proliferating. This leads to a lower number of functional muscle cells.

4.1.2 Design Specifications

Along with functions, there are also several specifications necessary for the design to meet in order for it to be successful. Through an analysis of the client statement and functions for the objectives determined, the following specifications were concluded:

1. Maintain a myogenic potential of 30% or greater
2. Provide at least 1000 cm^2 of surface area for scale up

Maintain a myogenic potential of 30% or greater: One of the most important objectives set forth by the client was the necessity of a high myogenic potential, maintaining a minimum of 30%. This is a benchmark that must be attained in order for the cells to be functional enough to treat volumetric muscle loss.

Provide 1000 cm^2 of surface area for scale up: For the process to improve upon current practices and prove feasible, the design must utilize a scale-up system. Currently, cells are cultured in a flask or dish with a surface area of $75cm^2$. In a scale-up, it is necessary increase the surface area, so the system requires less time and labor. Reduced time and labor will reduce the cost of the system.

4.1.3 Functions and Means Analysis

To analyze the functions and decide on various means, a Functions-Means chart is utilized. With each function, possible means are determined that are capable of meeting the given functions. The following chart analyzes and displays the relations between the functions and means.

Table 3: Functions-Means Chart

Functions	Means		
Retain non-adherent cell population	Centrifuge media and resuspend pellet	Media filter	Sedimentation
Provide a high surface area for scale-up (>1000 cm^2)	Roller bottle	Multi-tray vessel	Microcarrier system
Facilitate media exchange	Communal vent and fill port	Media filter	Larger culture vessel
Prevent cells from terminally differentiating	Passage cells at 60%	Increase surface area through expandable plates	Low oxygen tension

Using the above chart, the possible means of meeting the specific functions are displayed and are used to create conceptual designs for the cell culture system. Various means are combined to find the dominant aspects for several designs that meet the needs of the client most

accurately. For the system to function properly, it must be able to retain both the non-adherent and the adherent cell populations in the culture. To allow for this, manual media exchange can be utilized. Using a centrifuge to pellet the non-adherent cells at the bottom of a 15 mL conical tube, they can be separated from the old media and resuspended in fresh media before being returned to the system. A related function is the facilitation of media exchange. A possible means of this function is the use of a communal port system to allow for the manual exchange of media. The scale-up system may use a connected port or tube that is capable of draining the media all at once. If this is done in a manual and controlled manner, then both populations will be maintained. To allow for the exchange, the system would also require a single fill port to provide media to the entire system.

It is also important for the system to be able provide a high surface area, so that it requires less labor and time. There are several methods of scale-up currently on the market. One is a roller bottle. This involves using a round container that is spun on a roller mill, to provide media to all the cells on the inner cylinder. Another means is the multi-tray vessel. This scale-up approach increases the surface area of the system using a shelf-like container for the cells. A third means of increasing the surface area is a microcarrier system. This system allows for cell adhesion on small particles that are suspended in the media, increasing the overall surface area.

The final function that is addressed with several means is preventing terminal differentiation. A possible means is being able to passage the culture when the confluence reaches approximately 60%. The confluence of 60% prevents the cells from differentiating as they would at a higher density. This allows for an earlier passage, not allowing a confluence that supports differentiation or fusion. Another means is increasing the surface area. Greater surface area permits higher cell counts before the confluence for passaging is reached. A final means of

deterring terminal differentiation is decreasing the oxygen tension of the system. A lower oxygen tension creates a hypoxic system that is supportive of proliferation instead of differentiation while still sub culturing at less than 60% confluence (Kook 2008).

4.2 Conceptual Designs

In process design, there are many aspects to a design that come into play. Various studies were designed to characterize the different parameters of the system, including growth factor concentrations, oxygen tension, and plating densities. With the characterizations that result, a proposed combination was added to a scale-up system to complete the design. Meeting the functions of the system, the ideal means were chosen and the following scale-up options that are capable of applying specific test results were created.

4.2.1 Media Filter Culture Dish

A media filter culture dish can be used to prevent non-adherent cells from being pipetted into the supernatant that is aspirated from the culture dish. This functions exactly as a 100mm diameter cell culture dish; however, the non-adherent cells are retained without a centrifugation step or extra time spent retaining the non-adherent cells. The design of the media filter culture dish is shown below. Media is removed from within the section filtered by a microfilter, and media containing cells is added into the large section of the plate.

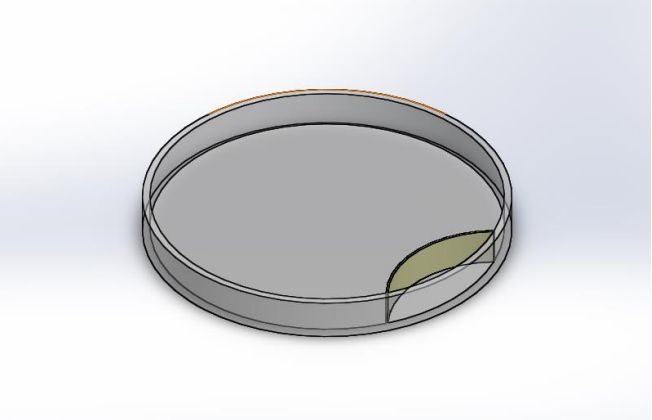


Figure 10: Media Filter Culture Dish Solidworks Model

4.2.2 Roller Bottle

One of the proposed scale-up methods is the roller bottle. The roller bottle is a long cylindrical tissue culture treated polystyrene bottle that is continuously rolled in a horizontal axis at a designated rate in an incubator. The media is placed in the bottle through an individual opening. As the bottle slowly rolls, the cells attach. The cells suspended in the media moves along the surface allowing for a greater surface area to use a smaller volume of media. With the single opening, there is minimal exposure to the external environment as it is covered with a fastened cap with a sterile filter. The surfaces of the bottles are ridged to increase the surface area. The spinning makes the culture dynamic, which allows for better gas exchange and prevents the formation of a gradient (Rafiq 2013).

Figure 11: Roller Bottle (Park, 2008)

4.2.3 Multi-tray vessel

Another possible scale-up design is the multi-tray vessel. The multi-tray scale-up uses several layers to utilize surface area in a smaller, confined space. Using an enlarged flask, several layers are added within its walls that are connected in the bottom corner for media removal and addition. Though further media is necessary, there are several positive aspects of the system. There is no need for electrical assistance as it lays flat in an incubator. Also, the flask does not need a lot of space within an incubator. For media exchange, there is only one opening that is securely covered by a filtered cap. Media is filtered through a connecting port and is able to be collected and added from a single chamber, making exchange time efficient.

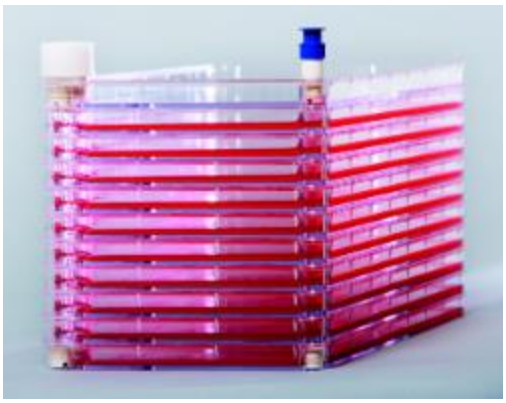


Figure 12: Multi-Layer Flask (Thermo Scientific)

4.2.4 Microcarrier system

A third conceptual design option for the scale-up is a microcarrier system. The system uses small particles that are placed in a bioreactor to promote adhesion. The particles in the bioreactor are mixed in the given media with the suspended cells, promoting cell-particle adhesion. The system increases the surface area by using particles as the adhesive surface. The number of particles can be controlled, which allows for manipulation of the system. Other benefits of the system result from the ability to control the scale-up system. Factors such as shear strength, particle seeding concentration, and continuous passaging allow for the design to be modified for the specific pilot study results (Tashiro et al., 2012). As such factors are able to be controlled, the system becomes more efficient. The seeding concentration allows for controlled population growth and density while the continuous passage allows the population to remain within constant media without exposing the cells to a bare environment or unnecessary chemicals. Also, with the varying physical properties of cells, the shear strength may be controlled to prevent unwanted stress.

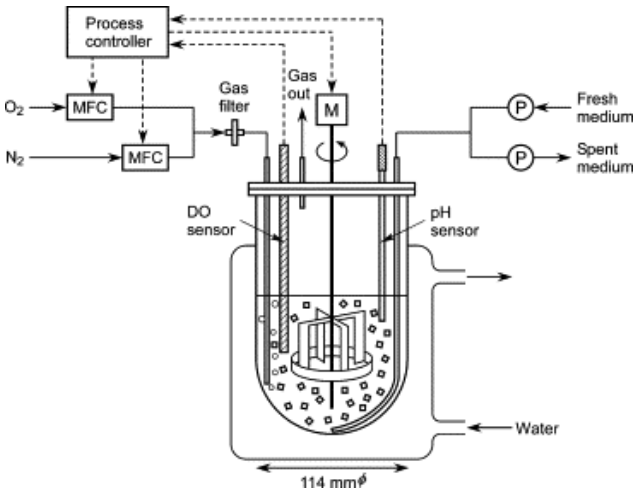


Figure 13: Bioreactor/Microcarrier System (Yamaji, 2006)

4.3 Selection of Final Design

After creating three conceptual scale-up designs, a final design was chosen to best fit the need of the client. Analyzing the relationship between the costs and benefits with each, as well as analyzing the designs in relation to objectives and constraints, a final design was selected.

4.3.1 Cost-Benefit Analysis

In order to assist in the selection of a final design, a cost-benefit analysis was performed to determine which design alternative has the best benefit to cost ratio. The ideal design would best meet all of the desired criteria and would cost the least. Benefits of using a roller bottle include having a high surface area (850-1750 cm^2), which allows for reduced time and labor. The media can be changed easily without losing the non-adherent cell population and the risk of contamination is the same as a standard cell culture flask. The cost for a case of twenty roller bottles is between $150 and $425 depending on the size (VWR International). This is relatively cheap compared to other scale-up options. It also requires a motorized device to spin the bottle. The multi-tray vessel is beneficial because it allows for a large amount of surface area (632-6320 cm^2) in a small total volume, the largest being 2000 milliliters. It requires the same media volume ratio, but it does not have to be motorized. The media can be changed easily through one

port. The cost for a case of six of these multi tray vessels ranges from $380 to $1500 depending on the size and number of chambers (VWR International). Finally, the microcarrier system allows for a higher surface area based on the seeding density of the microparticles in the bioreactor. The ability to control the various factors of the system makes it advantageous for modifying the reactor to fit the desired culture conditions. The cost of a bioreactor is around $230 and collagen microparticles are around $120 for 10g. The estimated total cost of this system is around $350.

Based on the cost-benefit analysis, the roller bottle is the best option. It has the cheapest price per surface area and performs all the other desired functions.

4.3.2 Design Evaluation Matrix

A Design Evaluation Matrix was used to analyze the various conceptual designs in relation to the previously determined objectives and constraints, three being the highest and zero being the lowest.

Table 4: Design Evaluation Matrix

	Optimize Myogenic Potential	Scalable	Biocompatible	Quantifiable	Repeatable	Inexpensive	*Sterile*	*Accessible*	*Compatible with Cells*	**Total**
Roller Bottle	2	3	3	3	3	2	Y	Y	Y	16
Multi-Tray Vessel	2	3	3	3	3	1	Y	Y	Y	15
Microcarrier Vessel	2	3	3	3	3	0	Y	N	Y	14

Following the evaluation matrix, it was found that the Roller Bottle scale-up system is the most feasible design alternative. Though the three designs coincided with most of the

objectives, the Roller Bottle best fit the objectives as it has the same effectiveness but is less effective.

5. Experimental Results

Before a final conclusion can be drawn and a final system design implemented, it is necessary to gather the results of the various characterization experiments designed. The results were used to determine the best design solution for the previously stated client statement. The following data results from the previously mentioned characterization experiments.

5.1 Growth Factor Characterization

To understand the impact the individual growth factors had on the cell culture, the population was split into six separate populations, each exposed to differing media lacking a different growth factor. Using the complete proliferation media with current Page Lab growth factor concentrations as a positive control, each population was exposed to the complete media without the concentration on one of the growth factors. Flask 1 contained media that excluded HGF while Flask 2 excluded IGF-1. The third and fourth flasks excluded EGF and FGF-2 respectively. The negative control population was exposed to complete media without growth factors.

5.1.1 Passaging

The various populations were allowed to proliferate for one passage. Once the population reached approximately 60% confluence, the population was passaged and replated at a lower density of 20,000 in 4-well plates. The following images correspond with the experimental groups at the first passage.

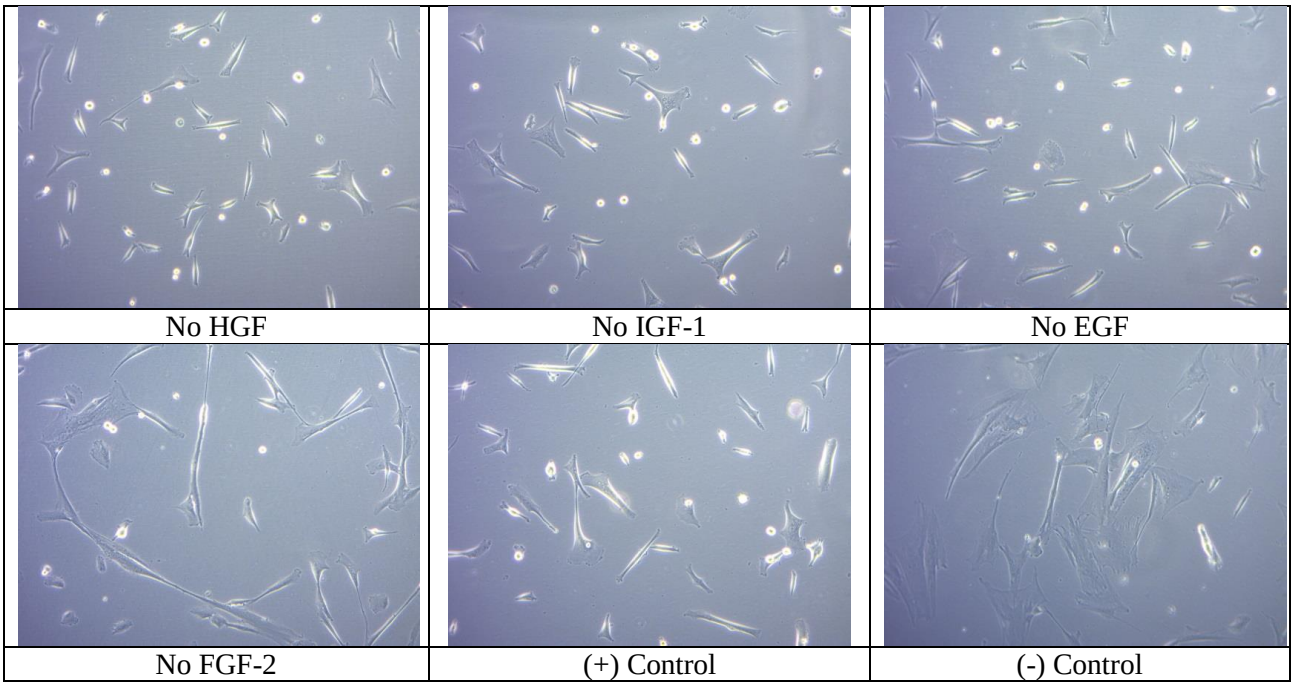


Figure 14: GF Characterization Passage 1

5.1.2 Adherent Vs Non-Adherent Cell Counts

After allowing proliferation for a passage, the both the adherent and non-adherent populations of each group were counted and compared to show the effects on the ratio of the two.

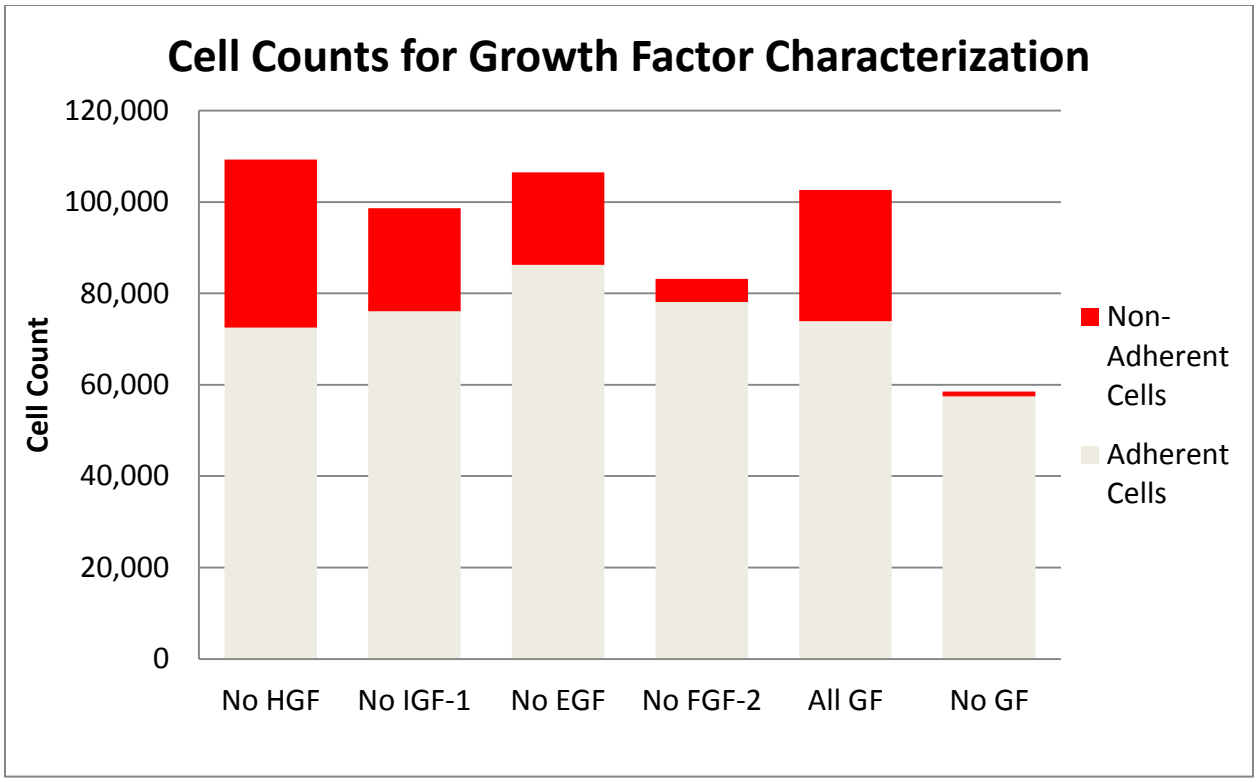


Figure 15: Cell Counts for Growth Factor Characterization Study After 1 Passage

5.1.3 Immunocytochemistry and Myogenic Potential

Following a passage in the respective media for each experimental group, the populations were seeded at 20,000 cells into four well plates, allowing for two days of proliferation. After two days, the media was exchanged for differentiation media, as previously mentioned. Differentiation media was exchanged every two days over a course of eight days of differentiation. The groups were stained and imaged using Immunocytochemistry. The blue stain expresses the nuclei of the cells while the red expresses the NCAM.

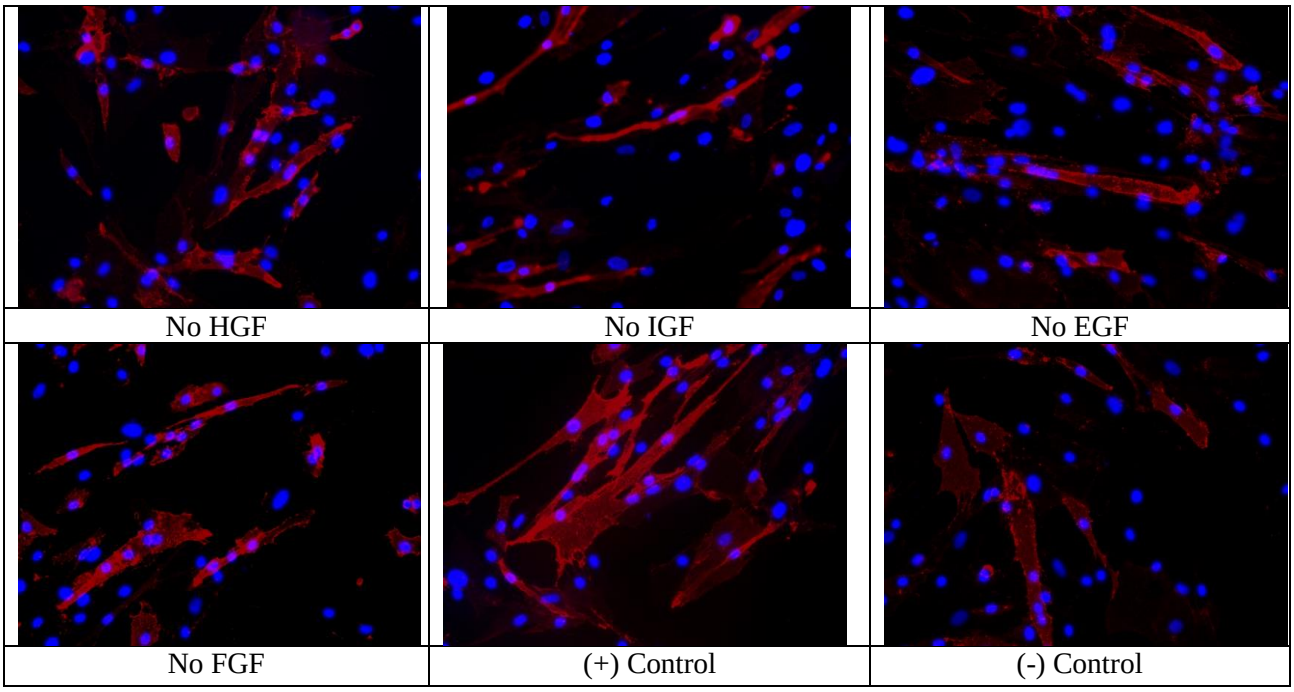


Figure 16: NCAM Staining

Utilizing the fluorescent images for each of the experimental groups, along with both control groups, results were found through imaging software. Using the total number of nuclei and the NCAM positive nuclei, the myogenic potentials of each group were determined. The variance in the mean myogenic index among the groups is expressed in the following graph.

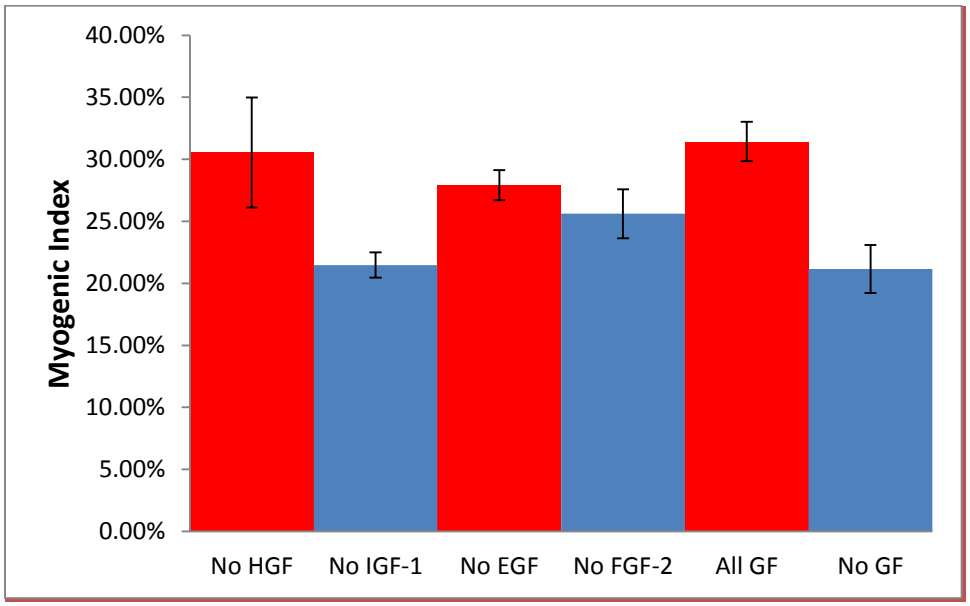


Figure 17: Growth Factor Characterization

In the cell population that was exposed to media that excluded HGF while following standard concentrations for the remaining growth factors, a mean myogenic index of 30.54 ±4.43% was found. Using media that neglected IGF-1 over three passages, a mean myogenic index of 21.47 ± 1.02% is the result. Removing EGF allowed for a mean myogenic index 28.91 ± 1.21%. The final experimental group without FGF-2 followed with an index of 25.61 ± 1.97%. The two controls also were analyzed to find average myogenic indices. The positive control gave a myogenic index of 31.43 ± 1.59% while the negative was 21.16 ± 1.94%. The following chart portrays the final results of the growth factor characterization.

Condition	Mean Myogenic Index (with SEM)
No HGF	30.54 ±4.43%
No IGF-1	21.47 ± 1.02%
No EGF	28.91 ± 1.21%.
No FGF-2	25.61 ± 1.97%
(+) control	31.43 ± 1.59%
(-) control	21.16 ± 1.94%

Table 5: Table of Average Myogenic Potential

With the results that are concluded from the experiment, a statistical analysis was used to determine significance. In relation to the negative control group, it was found that the group without EGF was statistically different with a p-value of 0.026. The no IGF-1 group was statistically different from the positive control with a p-value of 0.002. The two controls were statistically different with p-values of 0.006.

5.1.4 Cell Surface Area

To determine the extent to which cells are stressed, the surface area of each imaged cell were determined by means of imaging software. Using the average surface area for each group, the effect each individual had on the elongation and stress of cells amongst groups were found.

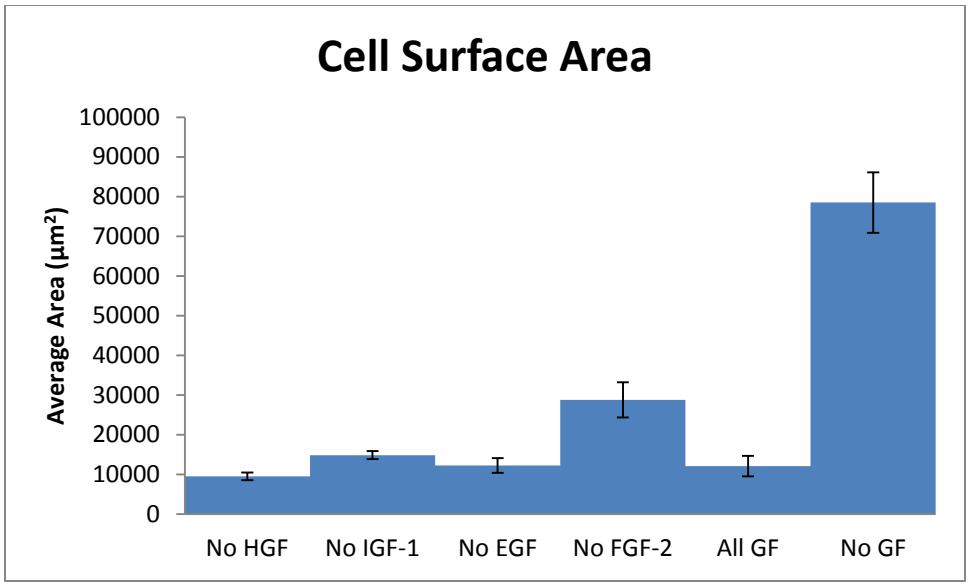


Figure 18: Average Cell Surface Area for the Removal of Each Individual Growth Factor

For the analysis of surface area, the only statistically significant result was the no FGF-2 group in relation to the positive control. All other comparison had p-values of higher than 0.05.

5.2 Oxygen Tension Characterization

To characterize the difference culturing cells in the ambient oxygen conditions of a incubator and a low oxygen environment with 5% O_2, the study was conducted with identical populations being cultured in both environments. After two passages in their respective conditions, the experimental and control groups were imaged prior to passage.

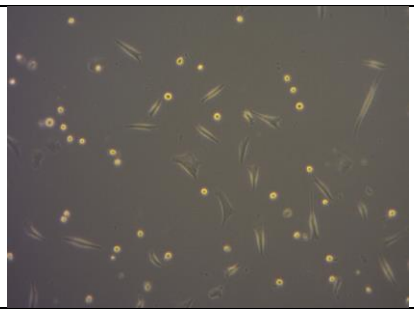
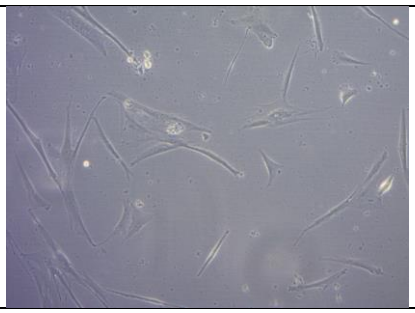
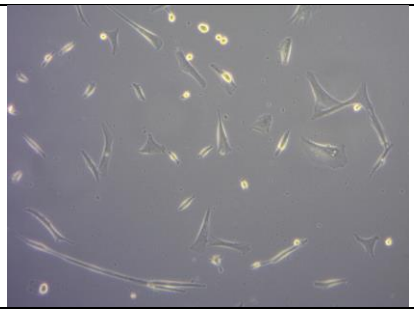
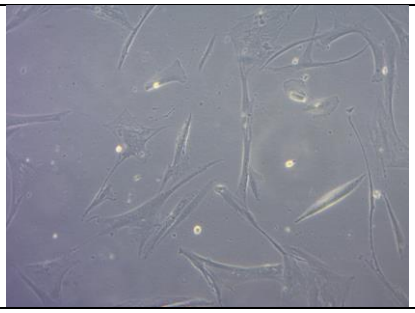
	
5% O_2 with GF	5% O_2 without GF
	
Ambient O_2 with GF	Ambient O_2 without GF

Figure 19: Qualitative Images for Oxygen Tension Characterization

With the qualitative data presented in the images at passaging, the low oxygen tension shows a higher retaining of non-adherent cells when cultured in growth factor media. The control in ambient oxygen with growth factor had both adherent and non-adherent cells as well. Both non-growth factor groups were showed to be stressed as represented by the elongated shape of the cells.

5.1.1 Adherent Vs Non-Adherent Cell Counts

After allowing proliferation for a passage, the both the adherent and non-adherent populations of each group were counted and compared to show the effects on the ratio of the two.

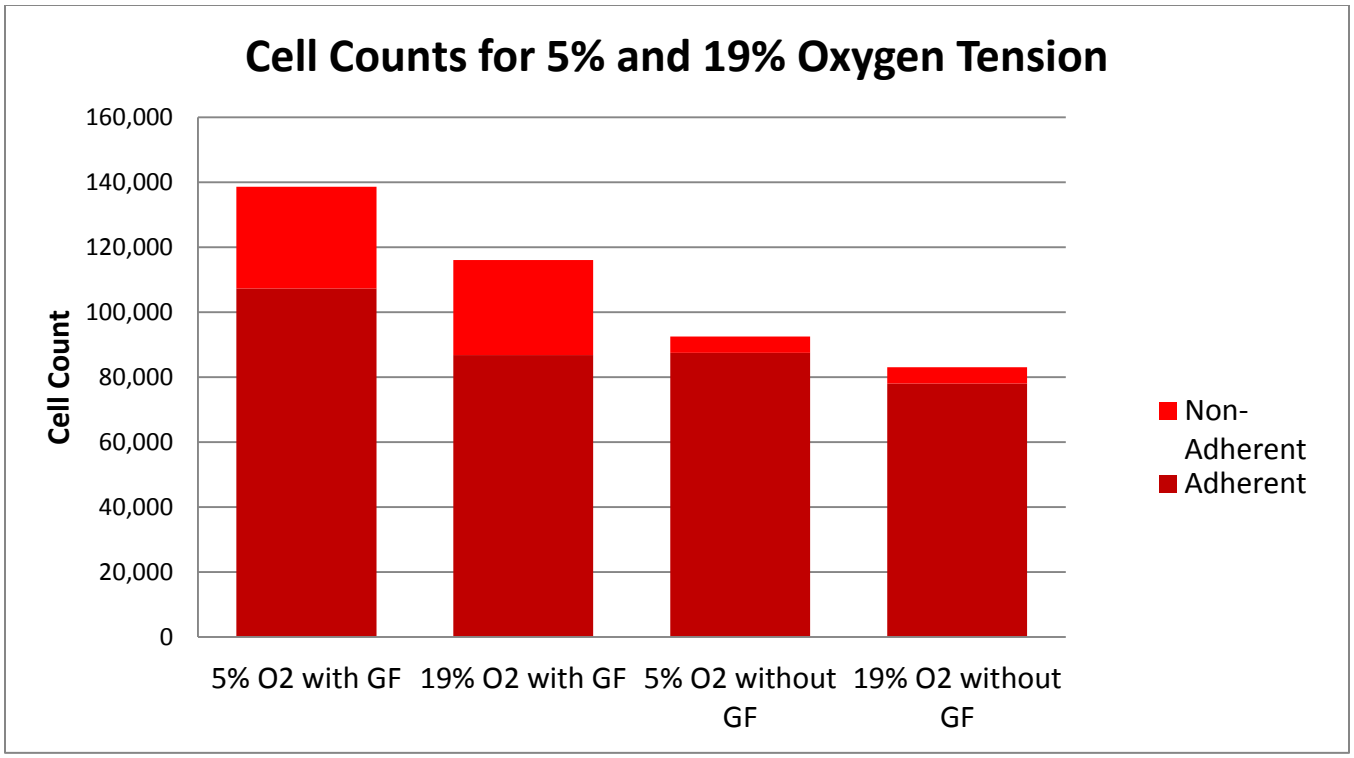


Figure 20:Cell Counts for Oxygen Tension Characterization

5.2.2 Myogenic Potential

Aside from the qualitative data, the growth factor culture in low oxygen had approximately 111,250 cells that were adherent and 19,375 non-adherent cells. The ambient growth factor group had 83, 750 adherent cells and 31,250 non-adherent cells. For the non-growth factor groups, the count for non-adherent cells were both too low to accurately count, proving to be less than 5,000 cells each. The low oxygen group without growth factors had 97,500 cells while the ambient oxygen contained 105,000 adherent cells. Following the cells counts, the groups were stained for NCAM and the myogenic potential was calculated.

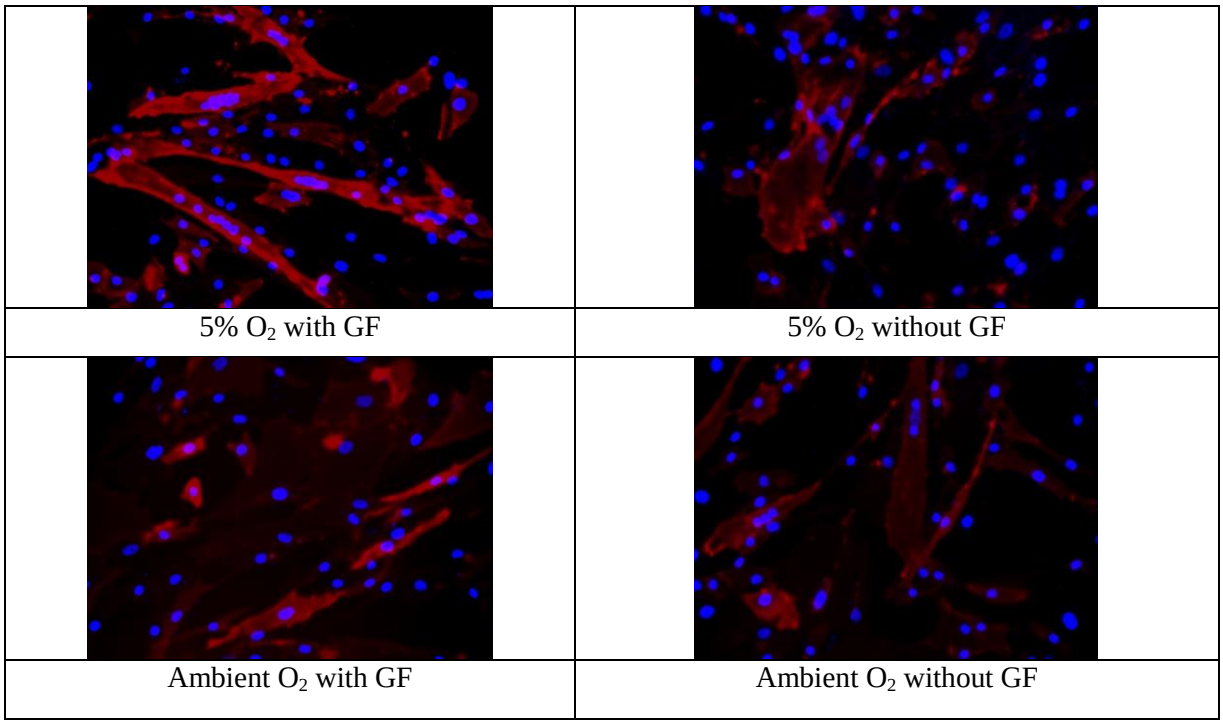


Figure 21: Immunocytochemistry Staining for NCAM for Oxygen Tension Characterization

Fluorescent microscopy was used to image the four groups after staining for NCAM. The blue represents the nuclei while the red stains NCAM. With the images, the myogenic indexes were calculated.

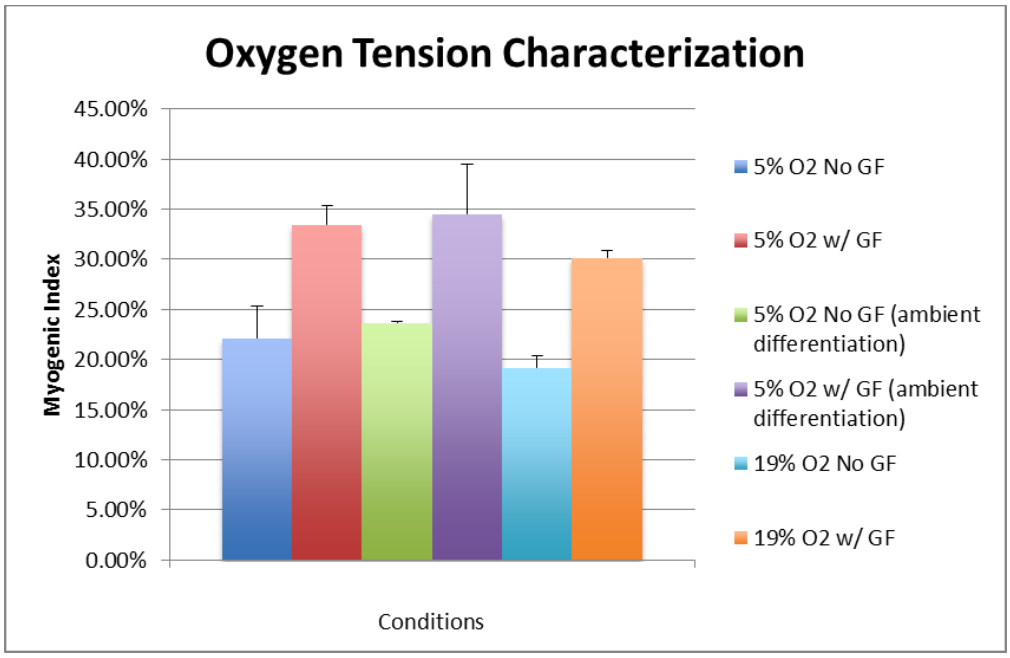


Figure 22: Myogenic Indices for Oxygen Tension Characterization

The low oxygen environment with growth factors had a myogenic index of 33.47 ± 1.93% while the ambient conditions with growth factors had an index of 30.16 ± 1.26%. The non-growth factor group in low oxygen has a myogenic index of 22.14 ± 3.21%. The group without growth factors in ambient oxygen had a myogenic index of 19.14 ± 0.65%. Indexes were also found for the low oxygen proliferated groups that were differentiated in ambient oxygen.

Table 6: Mean Myogenic Indices for Oxygen Tension Characterization

Condition	Mean Myogenic Index (with SEM)
5% O2 w/ GF	33.47 ± 1.93%
5% O2 w/o GF	22.14 ± 3.21%
5% O2 w/ GF (ambient differentiation)	34.56 ± 0.13%
19% O2 w/ GF	30.16 ± 1.26%
19% O2 w/o GF	19.14 ± 0.65%
5% O2 w/o GF (ambient differentiation)	23.66 ± 4.91%

With the results of the oxygen tension characterization, a statistical significance was found between 5% O_2 with growth factors and ambient oxygen with growth factors having a p-value of 0.026. Resulting in a p-value of 0.002, there is also a difference between ambient oxygen with growth factors and ambient oxygen without growth factors showing significance.

5.2.3 Cell Tension

To determine the effect of oxygen concentration on the cell tension, the surface area of the imaged cells were found. With a less stressed cell being characterized as having a smaller surface area, the following chart shows the results of the oxygen tension experiment as it relates to the state of stress for the cells.

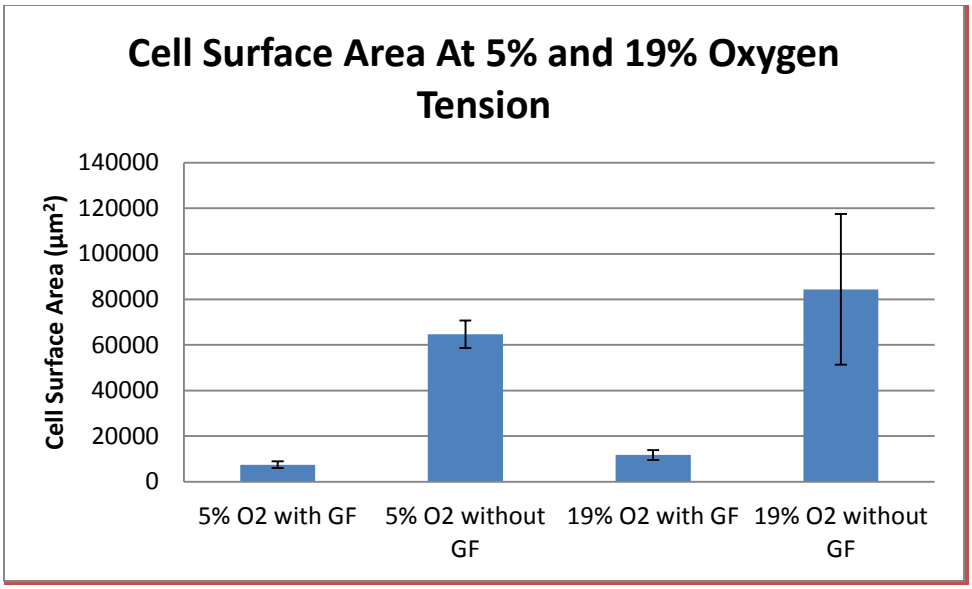


Figure 23: Average Cell Surface Area At 5% and 19% Oxygen Tension

With a p-value of 0.012, the low oxygen with growth factors proved significantly different than the low oxygen without growth factors.

5.3 Adherent and Non-Adherent Behavior Characterization

In determining whether the non-adherent cell population has a higher rate of proliferation over the adherent population, a characterization study that utilizes BrdU staining is used. It is also used to determine which progresses through the cell cycle at a more progressive rate. Cells of the same population and passage were placed into four well plates at a density of approximately 7,500 cells per well. The cells were separated into three groups. One well contained only the adherent population. Another well contained only the non-adherent population. The final group contained a combination of both populations. BrdU staining was used to stain the nuclei that are present in a proliferative state while all nuclei are stained with Hoechst.

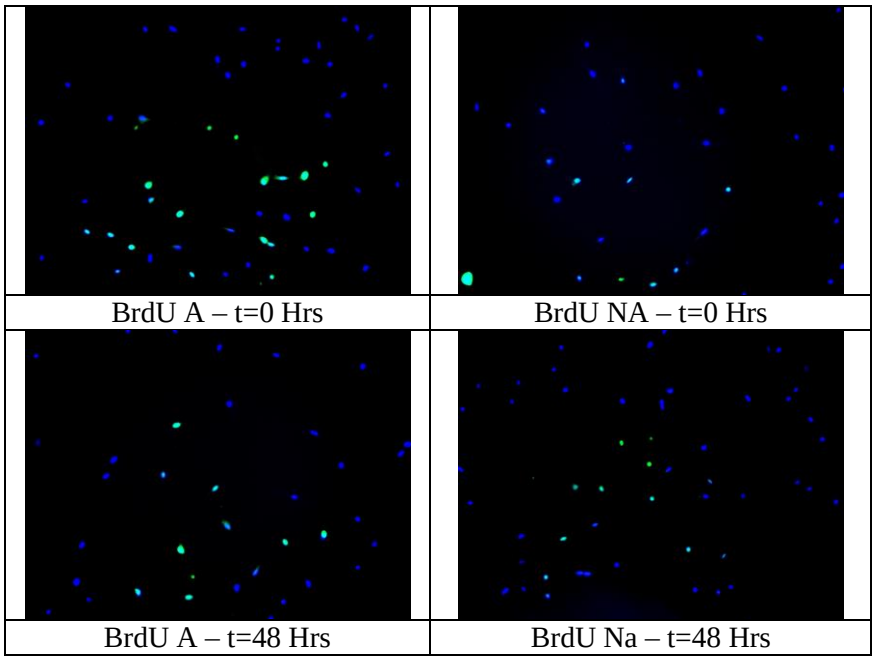


Figure 24: BrdU Assay for t=0 and t=48 hrs

5.3.1 BrdU Labeled at t=0 hours

When the population was initially split into the three groups, the BrdU labeling agent was added. The labeling agent was first added at t=0 hours. After forty-eight hours, the BrdU quantification assay was followed to obtain the percentage of cells proliferating.

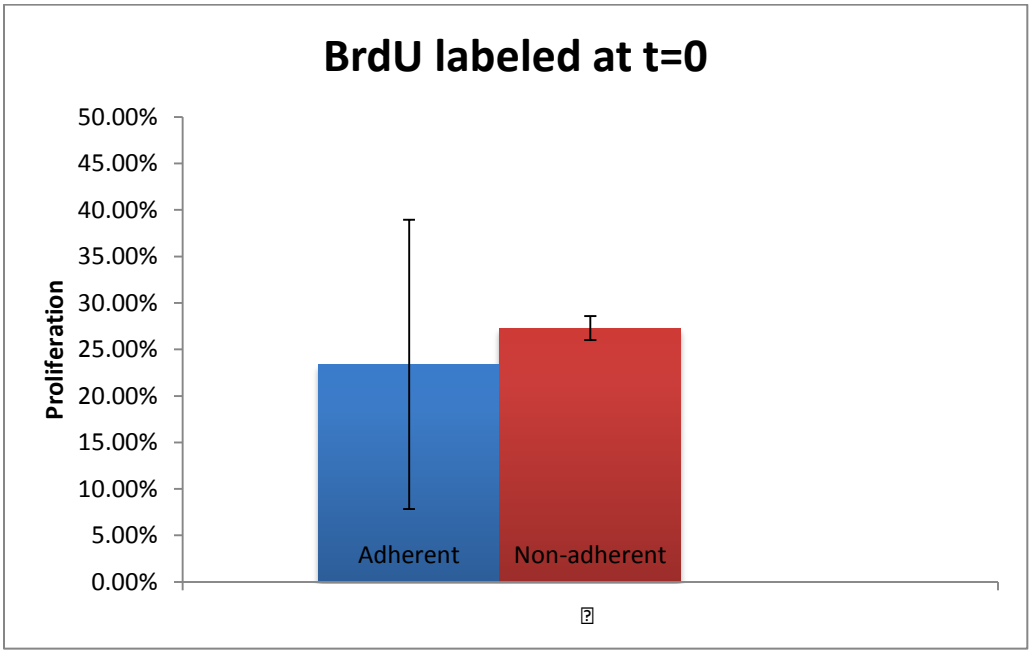


Figure 25: Graph of BrdU Labeled at t=0 hrs

For the groups that were stained at t=0 hours, vary results were found. For the group that contained only the adherent cells from the previous population, approximately 23.39 ± 15.54% of the cells were in a proliferative state on average. The mean proliferation percentage of the non-adherent cells was found to be approximately 27.28 ± 1.29%.

Table 7: Table of BrdU Labeled at t=0 hrs

BrdU labeled at t=0	
Population	Mean Proliferation % (with SEM)
Adherent	23.39 ± 15.54%
Non-adherent	27.28 ± 1.29%

5.3.2 Adherent Cells BrdU Labeled at t=48 hours

After forty-eight hours of proliferation, the original all adherent population left from the original split into an adherent and non-adherent population. With these populations, 7,500 cells per well were again seeded for the adherent, non-adherent, and the mixed populations. At t=48 hours, the labeling agent was again added. The cells labelled another 48 hours before staining. Each population gave way to a varied mean proliferation percentage.

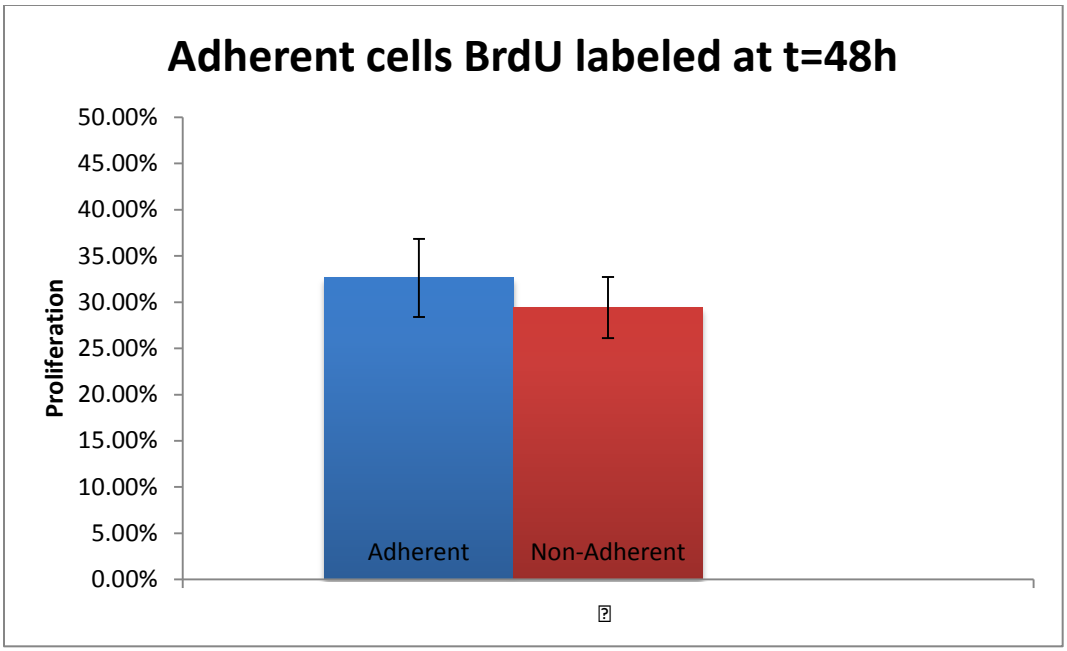


Figure 26: Graph of Adherent cells BrdU at t=48 hrs

The results of the final BrdU assay ranged among the three groups. For the adherent cells of the t=48 hours population, the mean proliferation was 32.63 ± 4.22%. The non-adherent was slightly lower with a mean proliferation percentage of 29.40 ± 3.31%.

Table 8: Table of Adherent Cells BrdU Labeled at t=48 hrs

Adherent cells BrdU labeled at t=48h	
Population	Mean Proliferation % (with SEM)
Adherent	32.63 ± 4.22%
Non-Adherent	29.40 ± 3.31%

6. Discussion

This chapter will identify the significance of the results, as well as the limitations of what can be concluded from the results. It takes a critical look at the uniqueness of this project, and also determines the impact that the project could have on a number of societal factors.

6.1 Results Analysis

It is necessary to analyze the results in order to confirm or reject the hypotheses of each of the tests. To analyze the results, t-tests were performed in order to quantify the significance. The team determined if there were any significant differences between the test groups.

6.1.1 Growth Factor Characterization

The team looked at the results for the myogenic indices obtained from our growth factor characterization study. It was hypothesized that the removal of any of the growth factors would decrease the myogenic potential of the culture. Based on this, the only growth factor that caused a statistically significant change when removed from the culture was IGF-1. The average size of the cells was also measured before the cells were differentiated. This showed that the removal of FGF-2 caused a significant difference in the cell size. The cells became more stressed and resembled the population with no growth factors. There were limitations encountered when performing the growth factor characterization study. Due to the number of samples, some of the standard errors were relatively large.

6.1.2 Oxygen Tension Characterization

Cells that were passaged at low and ambient oxygen were compared to see if the condition produced significant results. After staining for NCAM, it was shown that there was a significant difference in myogenic potential between the 5 percent oxygen population and the ambient oxygen population. Both populations contained growth factors in Page lab concentrations. When cultured at lower oxygen tension, the culture maintained a higher

myogenic potential. There was no significant correlation between the level of oxygen tension and the average surface area of the cells.

6.1.3 Adherent Versus Non-adherent Behavior Characterization

The group analyzed the results from the BrdU study in order to determine if there is a statistically significant conclusion that can be drawn between the non-adherent state of the cells and the proliferation. It was hypothesized that the well with non-adherent cells would have the higher proliferation percentage, and the well with adherent cells would have the lower proliferation percentage. No statistical difference was identified between the different populations. Therefore, there was no significant difference in proliferation for any of the wells. These results were limited by the sample size that was tested, which did not allow these results to produce a significant conclusion.

6.2 Impact Analysis

In the group's pursuit for an innovative way to culture human primary skeletal muscle cells for prolonged periods of time while maintaining a high myogenic index, it was also important to analyze the impacts the cell culture system might have on multiple areas within our society and the world in general. The following section will address the economical, environmental, political, and ethical impacts the system could have in today's world. It is also paramount to understand the societal influence, health and safety issues as well as the sustainability and manufacturability of the cell culture system the group has designed. The group took into consideration every one of the topics keeping in mind that technology is always moving forward and if a designed product negates this progression, it cannot be considered a successful product.

6.2.1 Economics

One of the first impacts to consider is the economic impact our cell culture system has in our current society. The cell therapy industry is a market where there is significant demand. Our system is part of a process to successfully treat volumetric muscle loss using a biopsy. Enabling a therapy for volumetric muscle loss will change the industry and investors could see substantial revenue from this in the future. According to an editorial from futuremedicine.com, the cell therapy industry had global sales of $410 million back in 2008 and that number is predicted to increase to $5.1 billion by the end of this year (Mason, 2011).

6.2.2 Environmental

Our design does not directly affect the environment, however, if the tools used during this process are not disposed of according to the guidelines set by our institution, those by products could potentially end up harming the environment. Because there are non-renewable and non-biodegrable products being used throughout the whole process, taking precaution by reading guidelines and safety protocols before disposing of those materials will minimize the effect on the environment. Furthermore, the purpose of the scale-up is to reduce the flasks' surface area to media ratio. This would also minimize the effect our final design would have on the environment.

6.2.3 Societal Influence

It was previously mentioned that the cell therapy industry is a market where demand for treatments of different diseases is increasing while the treatments themselves are not being developed as fast. For instance, in 2009 it was estimated that approximately 2.5 million patients in the United States could have benefited from a cell-based therapy. Because of the need for newly developed therapies, only 6,000 patients were able to receive an advanced cell-based therapy (Mason, 2009). We hope that our cell culture system will further the development of a

new cell-based therapy system for volumetric muscle loss. This would have an enormous impact in society as millions of patients could see themselves benefited from a volumetric muscle loss cell therapy.

6.2.4 Political Ramifications

Our cell culture system will most likely not have any political ramifications, as these procedures are performed in-vitro in a closed environment. Since this type of treatment is relatively new, developing nations and even some developed nations might not see this treatment as a feasible one at the moment so it is not expected for our system to have a big impact on the global market.

6.2.5 Ethical Concern

Because the origin of the primary muscle skeletal cells the group used for this experiment was from a biopsy, we believe there are minimal ethical concerns. This type of research was done with the purpose of enabling a therapy for volumetric muscle loss. Fetal-Clone III is composed of processed bovine serum and this may raise ethical concerns as to how the serum was harvested from the fetus (Thermo Scientific, 2013).

6.2.6 Health and Safety Issue

This cell culture system has the potential to further the progress to enable a therapy to treat volumetric muscle loss. Individuals suffering from trauma to extremities will see themselves benefitted as well as individuals suffering from muscular dystrophies and compartment syndrome. This cell-based therapy would negate the need for autografts that has limitations such as donor site morbidity, loss of functionality, and volume deficiency. Cell-based therapies could have the potential to create more jobs in the industry while improving the lives of millions of patients. Along with Pharmaceuticals, biologics, and medical devices, cell therapies could become the fourth pillar of healthcare (Mason, 2009).

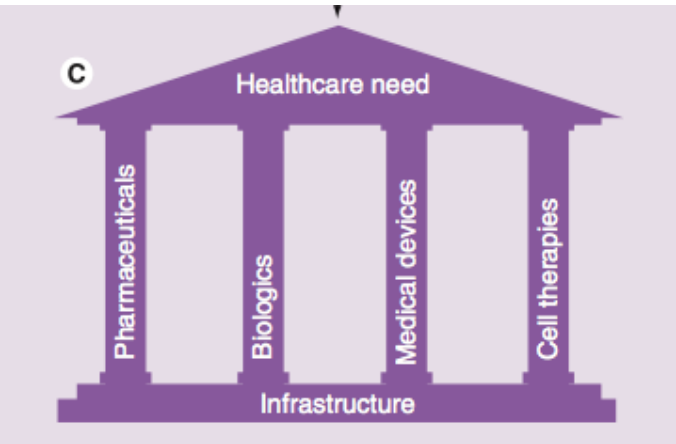


Figure 27: Pillars of Healthcare (Mason, 2009)

6.2.7 Manufacturability

The group designed a cell culture system to more effectively grow primary skeletal muscle cells while maintaining a high myogenic index under specific conditions. The conditions under which our final system was designed are fairly simple to replicate, however, they are very specific and steps need to be followed carefully.

6.2.8 Sustainability

The group utilized medium components such as Fetal-Clone III serum which derives from bovine serum. There are individuals who are opposed to the use of a fetus for medical purposes and do not agree with the purpose of our research. What we are trying to do is help people whose lives have been affected by an accident or disease. All components of our cell culture systems were used following the protocols and guidelines of out institution. We hope our product will give people an opportunity to experience a different way of life than the one they currently know.

Chapter 7: Final Design and Validation

Upon completion of the experiments characterizing individual system parameters, the experimental results were analyzed and applied to a final *in vitro* scale-up system. The final cell culture system will incorporate effective oxygen tension, growth factor concentrations, and cell kinetics into a biocompatible, quantifiable system that improves the proliferation rate of human primary muscle cells while maintaining a myogenic potential of greater than 30%.

7.1 Cell Culture

In order to assess the final design of the scaled cell culture system, assays will be performed using hSkm-1201 cells as previously mentioned in Cell Culture Methods. After a population of 500,000 cells was retrieved from the Page Lab, cells were proliferated in complete proliferation media containing current Page Lab growth factor concentrations in T75 Nunc flasks in order to generate enough cells for testing. Upon passaging at 60%, cells were replated in T75 flasks for further proliferation, plated at specific densities with set conditions for experimental testing, or frozen at 500,000cells/mL stored in liquid nitrogen in order to maintain a stock of cells.

7.2 Assessment of Roller Bottle Scale-Up

The feasibility, cost, and effectiveness of the use of a roller bottle for scale up will be determined by testing a dynamic culture in a 1050cm^2 Nunc roller bottle compared to a controlled, static culture in a T75 Nunc flask. In order to perform this testing, 1050cm^2 Nunc roller bottles were purchased from VWR. A roller bottle and flask will be cultured simultaneously in proliferation media with current Page Lab growth factor concentrations for one

passage with the surface areas, media volumes, and cell plating densities listed in the table below.

Table 9: Surface Area, Media Volume, and Cell Numbers for Scale-Up Testing

	Roller Bottle	Flask
Surface Area (cm^2)	1050	25
Media Volume (mL)	180	3
Cell Plating Density (total cells)	1,260,000	30,000

Both populations will be cultured for one passage with routine media exchange every 3 days. When cells are ready to be trypsinized, adherent and non-adherent cell populations will be counted.

7.3 Testing the Final Design

The final design was tested for scalability by comparing cell culture populations in a $1050cm^2$ Nunc roller bottle and a $T25cm^2$ Nunc flask. Cells were plated at the same cell density in terms of cells/cm^2 in the roller bottle and flask. At t=0, 1,260,000 and 30,000 cells were plated in the roller bottle and flask, respectively, and continued for one passage. The roller bottle was placed on a roller mill rotating at 1rpm inside the incubator for 24 hours in order for cells to seed. For the remaining duration of the cell culture, the roller mill spun at 2rpm. Media was routinely changed every 3 days, and on t=9days, the roller bottle and flask were passaged and non-adherent and adherent cell populations were counted using a hemocytometer. The resultant counts are shown in Table 14 below. The roller bottle yielded 74.26% that the equivalent surface area, $1050cm^2$, of $T25cm^2$ flasks yielded.

Table 10: Cell Counts for Final Design Scale-Up Testing

	1050cm^2 Roller Bottle	T25cm^2 Flask
Adherent Cells	2,274,355	73,500
Non-Adherent Cells	788,424	24,700
Total Cells	3,062,779	98,200

7.4 Design of Scaled Up System

The roller bottle system was scaled up for potential application in generating a large cell population to fill a critical size defect. The current system and the final design system were scaled up to generate a cell population of at least 1,000,000,000 cells for regeneration of a muscle defect of 15cm^3. Figure 16 below contains a flow chart displaying the materials and cell numbers used in each passage.

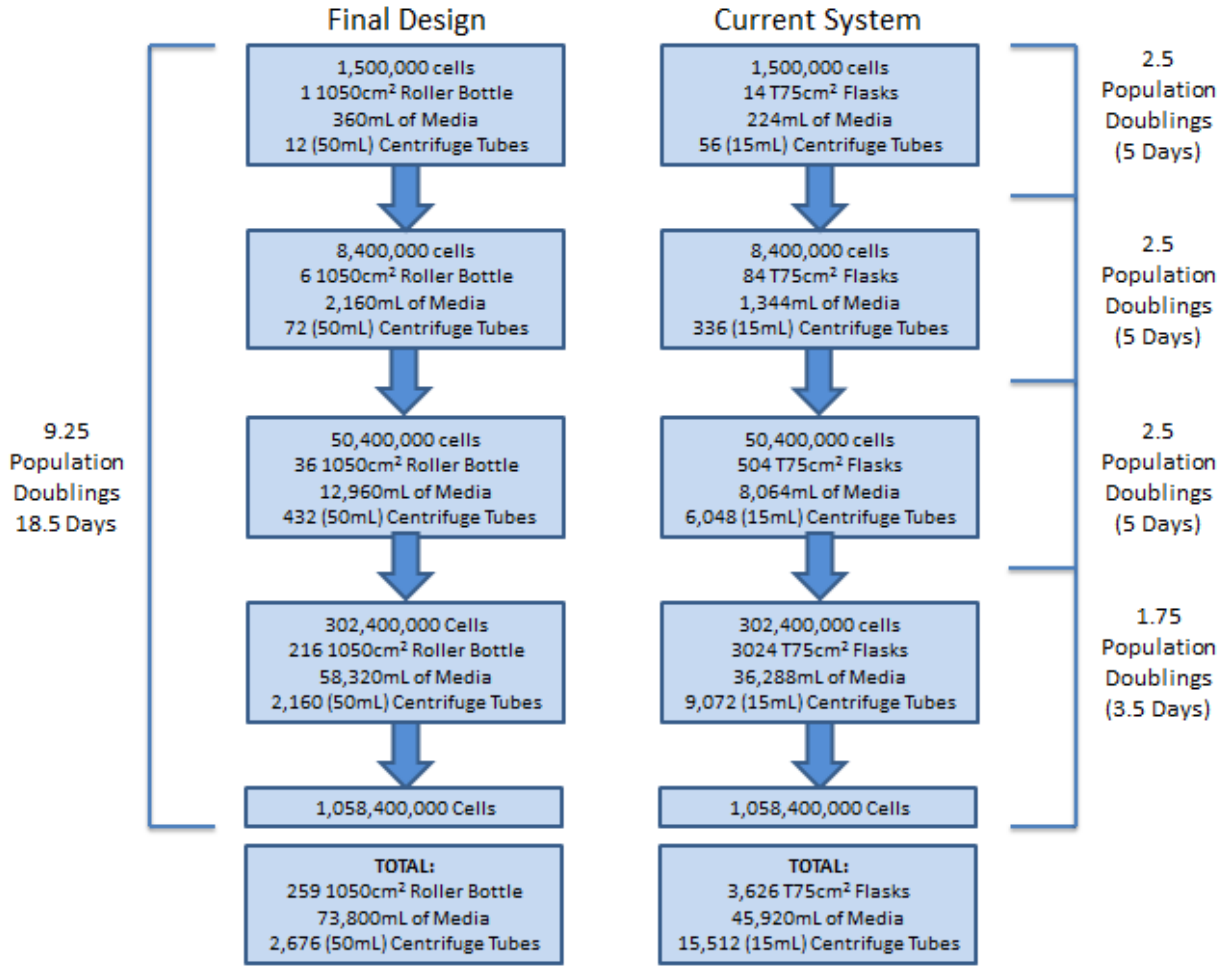


Figure 28: Flow Charts Comparing the Final Design System and the Current Cell Culture System

In addition to the use of 1050cm^2 roller bottles, the final scaled up system also applies the parameters of oxygen tension and growth factor combinations in order to improve the benefits of the system. The removal of HGF from the proliferation media reduces the cost of 500mL by $7.20 while providing statically similar results to the proliferation media with all current Page Lab growth factor concentrations. Furthermore, the results of the oxygen tension characterization suggested that 5% oxygen tension improves the proliferation rate of the cell population. Oxygen tension and the dynamics of the roller bottle culture, including rpm, media volume, and plating densities can be further characterized in future work to increase the proliferation rate of cells within the roller bottle to cells Based on these parameters, a cost-benefit analysis was performed comparing the current system with the scalable final design.

7.5 Cost-Benefit Analysis of Final Design

The cost-benefit analysis comparing the current cell culture system with the final design of the improved system evaluated cost of materials, media, media components, and labor is shown in the table below. Culture materials are based on pricing from VWR International for Nunc vessels and Celltreat centrifuge tubes. Nunc T75cm^2 flasks and 1050cm^2 cost $3.59 and $11.00, respectively (VWR International). Celltreat 15mL and 50mL centrifuge tubes cost $0.21 and $0.24, respectively (Celltreat Scientific Products). Labor costs for media exchange were calculated as $2,210.14 for 118 hours for media change in the current system versus $468.25 for 25 hours in the final system. This calculation was based on 10 minutes to change media in 6 flasks and 10 media to change media in 2 roller bottles. Labor costs for passages were calculated as $7,548.19 for 403 hours for passaging within the current system versus $1,217.45 for 65 hours in the final system. This calculation was based on 20 minutes to passage 3 flasks and 10 media to

change media in 2 roller bottles. The average salary used to calculate lab technician labor cost was $18.73 per hour (Chron, 2014).

Table 11: Cost-Benefit Analysis of the Roller Bottle System Versus the Current Cell Culture System

	1050cm^2 Roller Bottle System	**T75cm^2 Flask**
Media	$6,098.83 ($41.32/500mL without HGF)	$4,456.08 ($48.52/500mL)
Labor ($18.73/hr)	$1,685.70	$9,758.33
Culture Vessel	$2,849	$13,017.34
Centrifuge Tubes	$642.24	$3257.32
Total:	**$11,275.77**	**$30,489.07**
mL of Media	73,800	45,920
Number of Culture Vessels	259 Roller Bottles	3626 T75 Flasks
Number of Centrifuge Tubes	2,676 50mL	15,512 15mL
Total Time	18.5 days	18.5 days

In generating a population of at least 1,000,000,000 cells, the roller bottle system costs $11,275.77 in comparison with $30,489.07 for the current T75cm^2 flask system. The final scaled up system can more effective generate large cell population without significant differences in maintaining myogenic potential.

8. Conclusion

Upon completion of the characterization experiments and the analysis of the results, it was concluded that there is an effect on the cell culture of human muscle cells in relation to growth factor concentration and the presence of an adherent and non-adherent population. From the results portrayed by the growth factor characterization, it is possible to conclude that the removal of one or more of the standard growth factors decreases the overall myogenic potential in relation to the current complete media and growth factor standards set forth by Page Lab. In relation to myogenic potential, there is no statistically significant difference among the various growth factor concentrations. The largest qualitative impacts were seen with the removal of FGF-2 and EGF, where minimal change occurred with the lack of HGF. The exclusion of FGF-2 lead to stressed cells and early differentiation. This was confirmed by measurements of cell surface area, which proved that there was a statistical difference in the cell size compared to the positive control. There is minimal difference when comparing the positive control and the experimental group with media that excluded HGF. From the growth factor characterization experiment, the designed system using media that contains IGF-1, EGF, and FGF-2 has no significant difference from the current system. The removal of HGF produces the same effect but reduces the overall cost of the system.

Based on the results from the oxygen tension characterization experiment, it can be concluded that lower oxygen tension has a positive effect on the myogenic potential of a culture of human skeletal muscle cells. This along with a roller bottle scale-up system can produce favorable myogenic cultures, while allowing for reduced labor and material cost.

In future experiments to further address the system design, there are several recommendations that should be taken into consideration. When designing and completing the

characterization experiments, it is essential to have multiple passages so that the cells can be exposed to the variable conditions for an extended period of time. This will allow researchers to see the long-term effects of the altered culture conditions. After the culturing adjustments, it would also be useful to test other growth factor concentrations based on the qualitative results of the growth factor characterization. Finally, looking further into the relationship between the adherent and non-adherent population is important to better design the system. With the proposed design and further investigation into the future recommendations, a cell-based therapy system for volumetric muscle loss progresses further down the path towards clinical application.

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Appendix A: Protocols

NCAM Immunocytochemistry

(Biomedical Engineering Department, Worcester Polytechnic Institute, 2013)

Materials

PBS (MediaTech Cat # 21-040-CM, VWR Cat # 45000-448)

Tween-20

DPBS+ Dulbeco's phosphate buffered saline with Ca/Mg, MediaTech Cat # 21-030-CV , VWR, Cat. # 45000-430)

FBS (use blocking FBS, stored at -20C), or any bovine serum, or serum derived from species of the secondary antibody

Alexafluor-488 conjugated anti-mouse IgG (Invitrogen), or Alexafluor-568 conjugated anti-mouse IgG (Invitrogen)

Antimyosin antibody (mouse IgG), MF20 from Developmental Studies Hybridoma Bank, stored at 4 C

Fomaldehyde (3.7% in DPBS+)

PBS with 0.05% Tween-20 (PBS/Tween)

Cells grown in 24-well plates

Glycerol DNase, RNase, protease free (Shelton Scientific, Cat # IB15672, VWR Cat # IB15762)

Hoechst 33342 (EMD Cat # 382065-100MG, VWR Cat # 80056-706)

Procedure

1. Aspirate culture medium and wash cells with DPBS for 1 min.
2. Aspirate DPBS and add 500 µl/well 3.7% formaldehyde and let stand for 10 min.
3. Aspirate formaldehyde and add 1.0 ml/well PBS
4. 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.
5. Aspirate blocking solution 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.
6. Aspirate primary antibody and wash 4X with PBS for 3 min each wash.
7. Aspirate PBS and add secondary antibody (diluted 1:500 in PBS/Tween) at 150 µl/well and incubate for 30 min.
8. 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.

9. 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.

DAPI staining

Counterstaining Adherent cells for fluorescence microscopy
(Biomedical Engineering Department, Worcester Polytechnic Institute, 2013)

Sample Preparation

Use appropriate fixation protocol. DAPI staining is normally performed after all other staining. Note that fixation and permeabilization of the sample is not necessary for counterstaining with DAPI.

Counterstaining Protocol

1. Equilibrate the sample briefly with phosphate-buffered saline (PBS).
2. Dilute the DAPI stock solution to 300 nM in PBS. Add approximately 300 µL of this dilute DAPI staining solution to the coverslip preparation, making certain that the cells are completely covered.
3. Incubate for 1-5 minutes
4. Rinse the sample several times in PBS. Drain excess buffer from the coverslip and mount. It is recommended to use a mounting medium with an anti-fade reagent.
5. View the sample using a fluorescence microscope with the appropriate filters.

Feeding Cells

(Biomedical Engineering Department, Worcester Polytechnic Institute, 2013)

Feeding cells refers to removing cell culture medium and replacing it with fresh cell culture medium.

NOTE: Pre-warm medium to 37°C in a water bath prior to adding it to the cells.

1. Remove half the volume of medium from the culture and put into 15 mL conical tube.
2. Centrifuge at 200G for 6 minutes in order to pellet cells at the bottom.
3. Aspirate old medium using a Pasteur pipet attached to a vaccum trap. (Be careful to avoid aspirating the pellet of cells)
4. Resuspend the pellet in fresh medium (same volume that was removed).
5. Using serological pipet, return cell suspension to the culture dish.

Passaging Cells

(Biomedical Engineering Department, Worcester Polytechnic Institute, 2013)

When cells are 60-70% confluent, remove medium with serological pipet and put into 15 mL conical tube. Wash cells with 1X DPBS(-) (without Ca^{++}/Mg^{++}) by adding and then aspirating PBS.

Confluent: Refers to the area of cell coverage on the plate. If cells are covering

~ 70% of the surface of the plate, they are 70% confluent.

NOTE: Use ½ the volume of PBS to rinse as there was medium in the dish (e.g., a 100 mm dish is fed 10 mL medium, therefore use 5 mL PBS per rinse).

 Trypsinize cells by adding 0.05% trypsin EDTA (~ 3 mL for a 100 mm plate) for 3-5 mins.

Plates may be returned to the incubator while trypsinizing.

NOTE: Dilute trypsin stock 1:5 in sterile DPBS(-) to make a working solution of

0.05% to add to the cells.

 Add fresh media to plate, pipette cells up and down to resuspend and break up clumps of cells.

NOTE: Serum in culture medium inactivates trypsin.

 Add a volume of resuspended cells to fresh medium in a new plate (e.g., add 2.5 ml cell suspension to 7.5 ml fresh medium in a new 100 mm dish = a 1:4 split/passage). Alternately, seed a specific number of cells in the total volume prescribed for a particular culture dish.

 Check growth each day; passage as necessary.

Cell Counting

(Biomedical Engineering Department, Worcester Polytechnic Institute, 2013)

For most experiments, it is important to use a consistent plating density (the number of cells added to the culture per area of the culture dish they are plated on). Rather than plating an arbitrary volume of cell suspension as described in “Passaging Cells” above, it is necessary to count the number of cells per unit volume in the cell suspension, and determine the volume of cells to plate to achieve the desired plating density. Below is a depiction of the counting area of a typical hemocytometer. The cells are trypsinized and resuspended as described above. A small volume of cells (~ 12 microliters) is added to the counter at the indentation in the bottom glass and allowed to fill the area by capillary action under the coverglass. Do not force-fill the counting chamber.

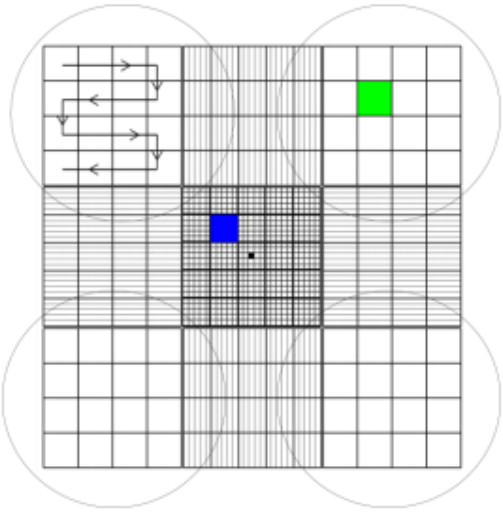


Figure 1
Count cells in the four large corner squares as indicated (see arrows)

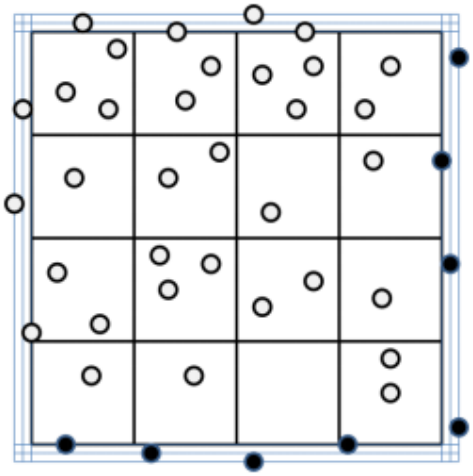


Figure 2
Include cells on the left and top edges
Exclude cells on the right and bottom edges

1. Count all cells in the four corner sections (light colored squares below each consisting of 16 squares) as shown in Figure 1 and 2.

2. Divide the total by 4 to obtain the average number of cells per square.

3. The volume of each square is 100 nl (nano liters) or 1/10,000 ml. Calculate total count per ml as follows. Make sure to take into account the original dilution of cells (if applicable) and the dilution used during trypan blue staining.

4. Calculate the total cell count by multiplying the above figure with the total volume of cell suspension from which the sample was drawn.

To increase the cell density of the suspension or to concentrate the cells for freezing (see below), centrifuge the cell suspension at low speed (200g for 5 minutes) to pellet them. Gently aspirate the supernatant and resuspend the cell pellet in the appropriate medium at the appropriate volume.

Collagen Coating

(Biomedical Engineering Department, Worcester Polytechnic Institute, 2013)

1. Add 250 ul of collagen, poly L-lysine and gelatin stock solutions to the corresponding wells and close the lid.
2. Swirl the plate to ensure that the coatings cover the entire surface.
3. Allow coating to proceed for 30 minutes to 1 hour inside the hood.

4. After coating, aspirate the solutions from every well.
5. Add 500 µl sterile water to the poly L-lysine wells. This wash step is only for the poly L-lysine wells. DO NOT WASH THE OTHER WELLS. Aspirate and discard the water. Repeat the rinse two more times (for a total of 3 rinses).
6. Leave the plate with the lid open inside the hood.
7. Allow the wells to dry for 20-30 minutes inside the hood.

Appendix B: Lab Spreadsheet

Date	Population	Passage #	Confluence	Cell Count	Media (Diff/Prolif)	Notes
1/11/14	1	4		250,000	P	Thawed cells; Plated in two T75s
	2	4		250,000	P	
1/13/14	1		20%		P	Media Change (MC)
	2		20%		P	MC
1/15/14	1		30%		P	MC
	2		30%		P	MC
1/17/14	1		40%		P	MC
	2		40%		P	MC
1/20/14	1	5	80%	975,000	P	Over-confluent, reseeded at 200k
	2	5	70%	750,000	P	Reseeded at 200k cells
1/22/14	1		50%		P	MC
	2	6	50%	600,000	P(GF study)	Used for growth factor study
1/24/14	1		60%	750,000	Freeze	Poor cell quality; froze 500k cells
	2		30%		P(GF study)	MC
1/26/14	2		40%		P(GF study)	MC
1/28/14	2		50%		P(GF study)	MC
1/30/14	2	7	60%	78,000	P(GF study)	First GF study passage
2/1/14	2		20%		P(GF study)	MC
2/3/14	2		30%		P(GF study)	MC
2/5/14	2		40%		P(GF study)	MC
2/7/14	2		50%		P(GF study)	MC
2/9/14	2	8	60%	81,000	P(GF	Second GF

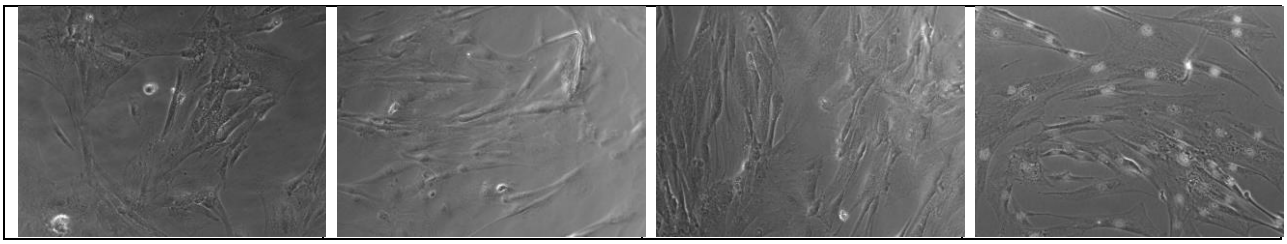
					study)	study passage.
2/11/14	2		10%		P(GF study)	MC
2/13/14	2		20%		P(GF study)	MC
2/16/14	2		40%		P(GF study)	MC
	3	4		500,000	P	Thawed cells; Plated in T75
2/18/14	2	9	50%	43,000	P(GF study)	Third passage into 4 well plates
	3		40%		P	MC
2/20/14						Made Differentiation Media
2/21/14	2		70%		D	Media Change to Diff. media
	3	5	60%	560,000	P	Split into two T75 flasks (3 + 4)
2/23/14	2				D	MC
	3		40%		P	MC
	4		30%		P	MC
2/25/14	2				D	MC
2/26/14	3	6	60%	570,000	P	BrdU study started
	4		40%		P	MC
2/27/14	2				D	MC
2/28/14	3		40%		P	MC
	4		50%		P	MC
						Got results for GF study and first part of BrdU
3/1/14	3		40%		P	MC
3/2/14	4	6	60%	500,000	Freeze	Froze 500k cells

Appendix C: Imaging

Growth Factor Characterization

No HGF:

Bright Field:



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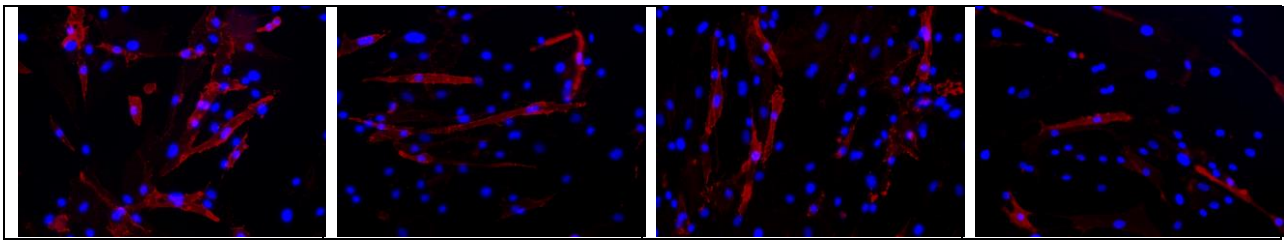

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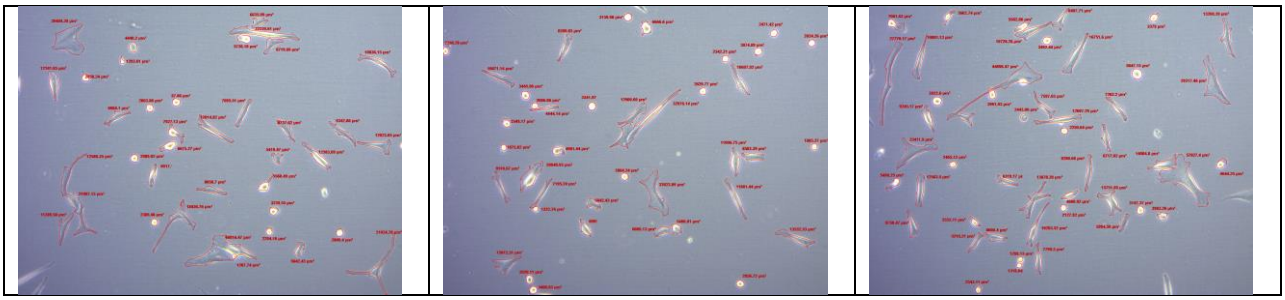

No IGF-1

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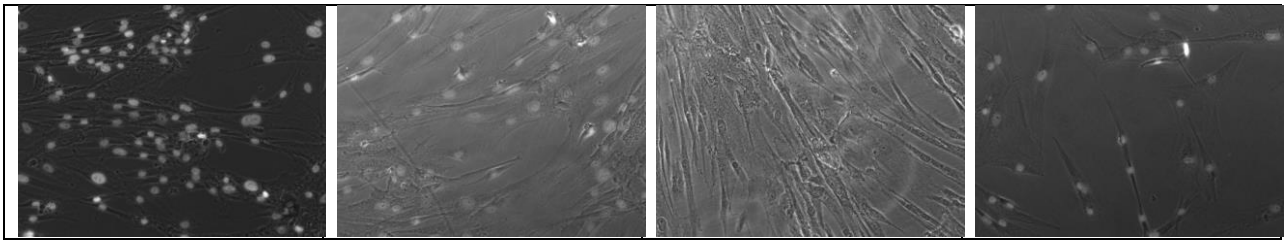

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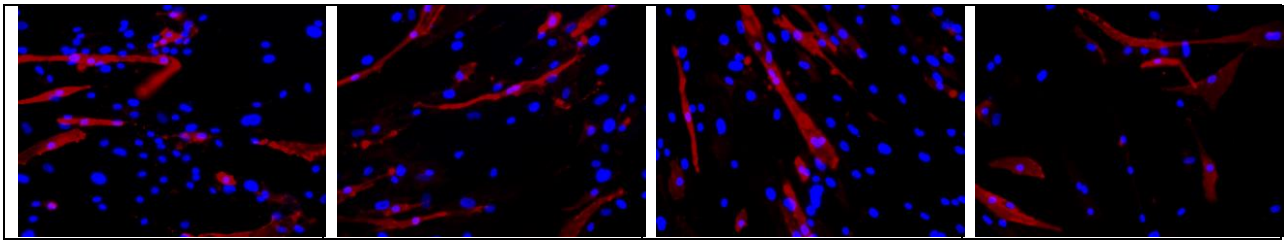

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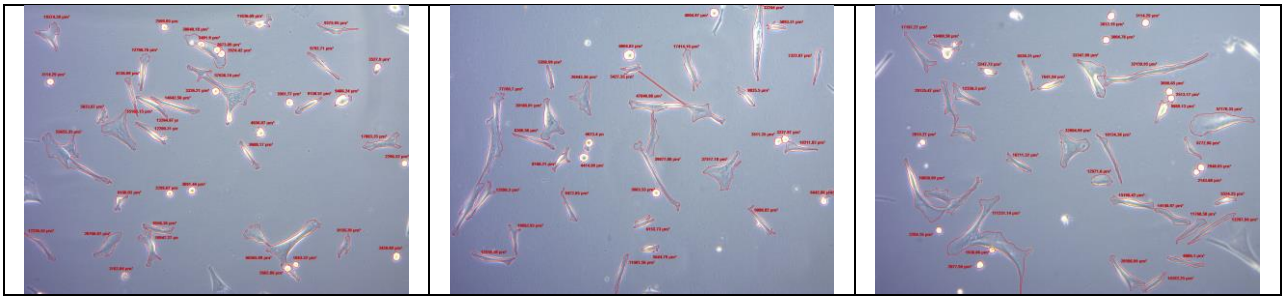

No EGF:

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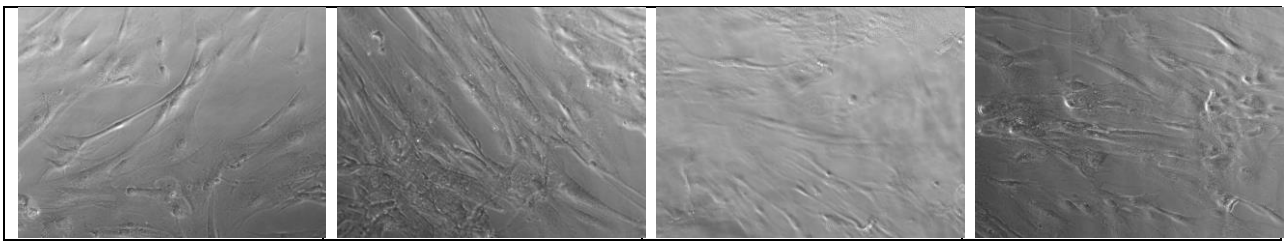

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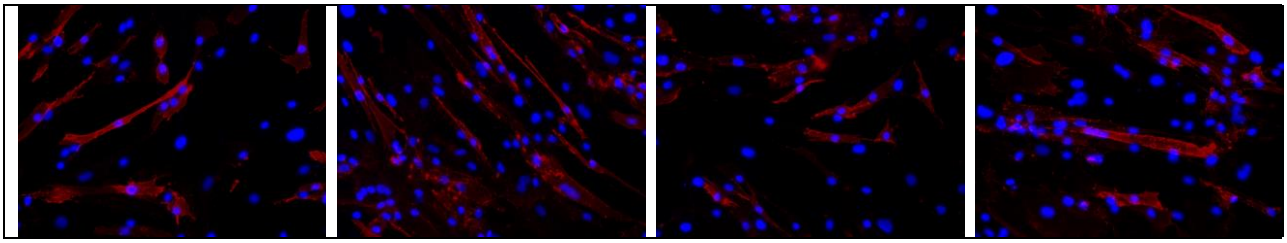

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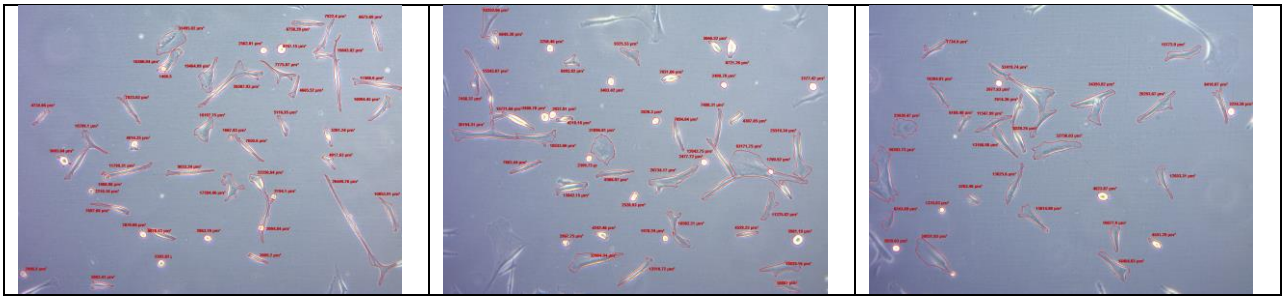

No FGF-2:

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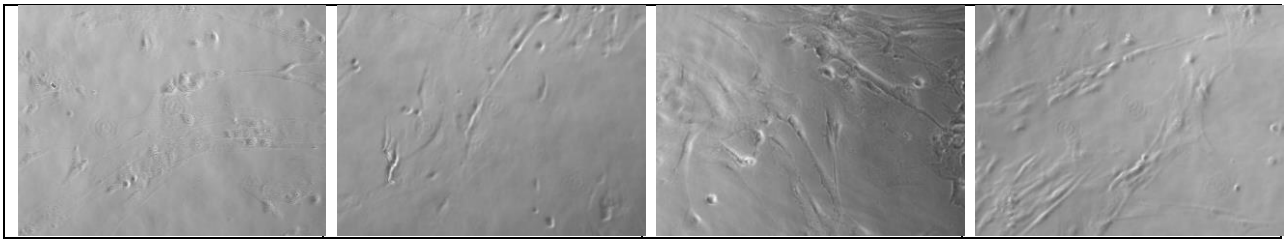

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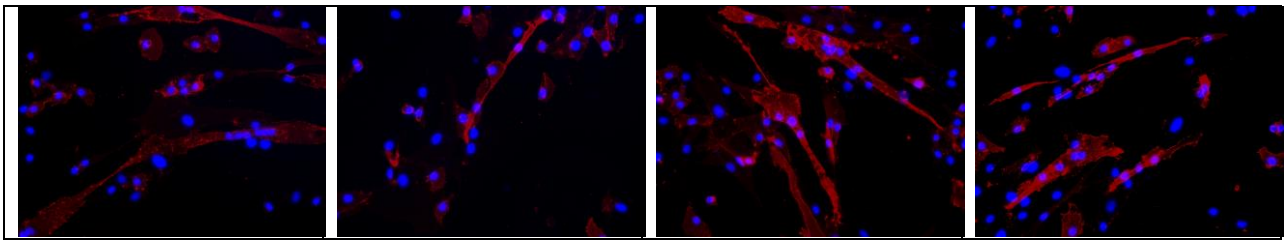

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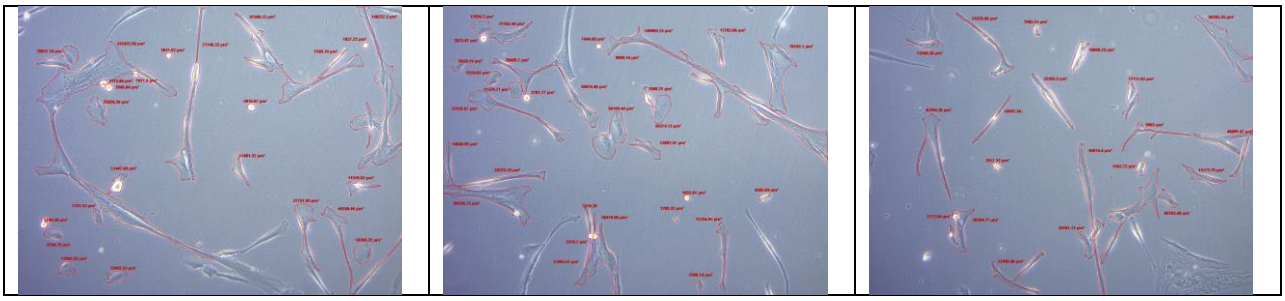

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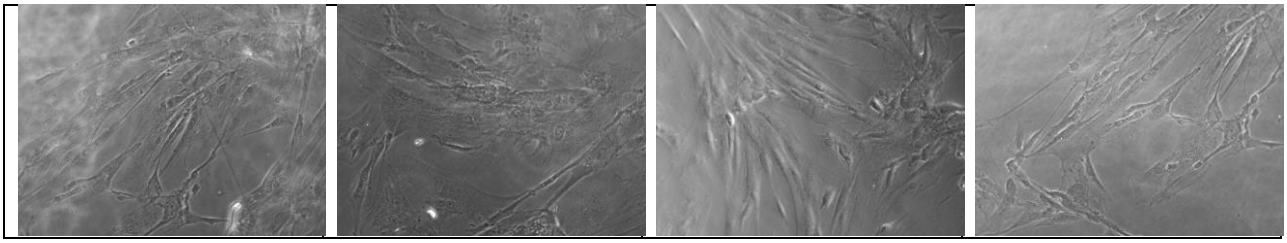

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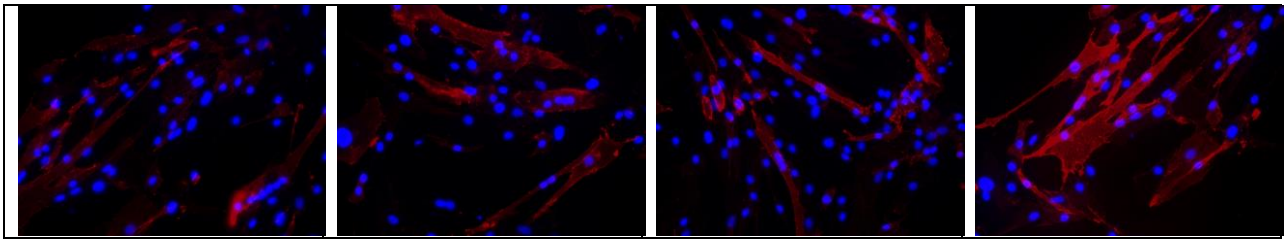

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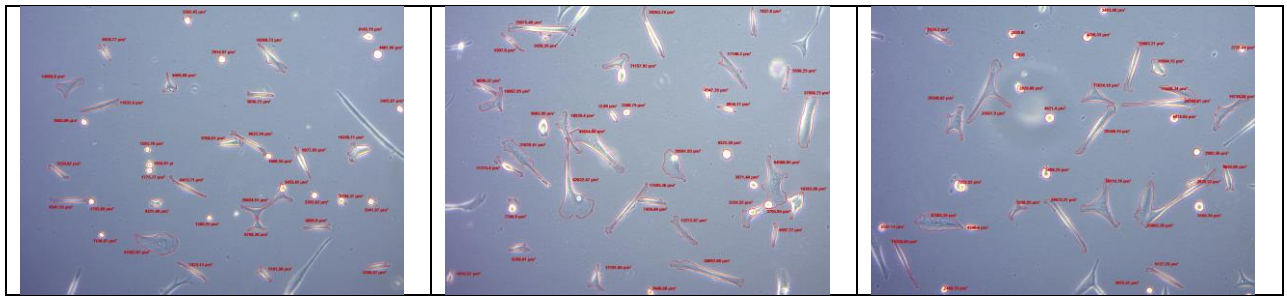

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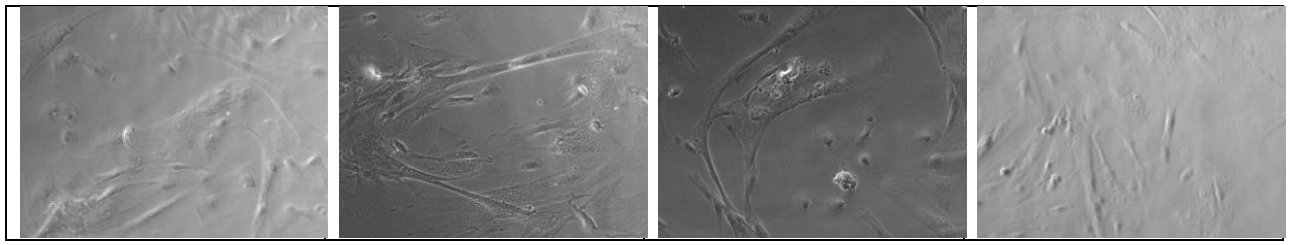

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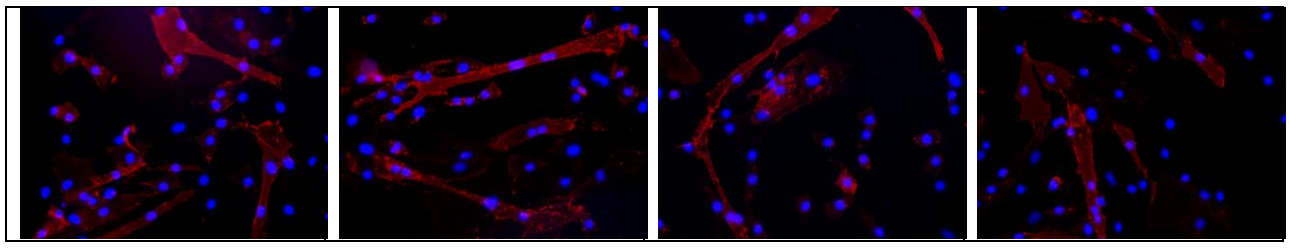

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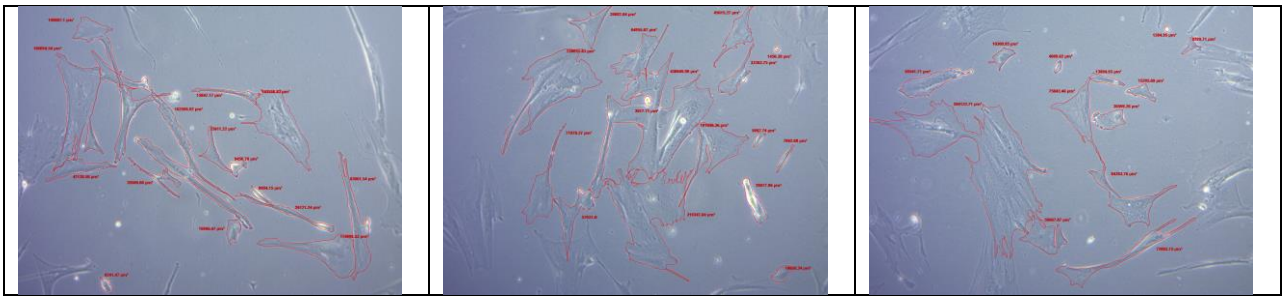

Oxygen Tension Characterization:

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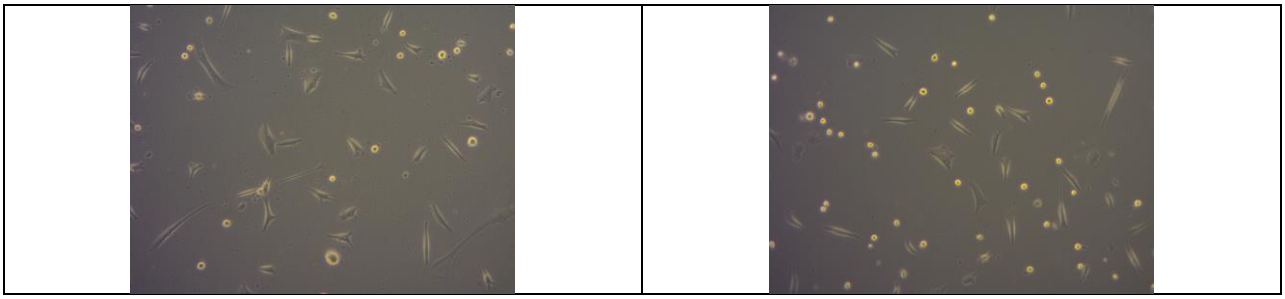
At passage:



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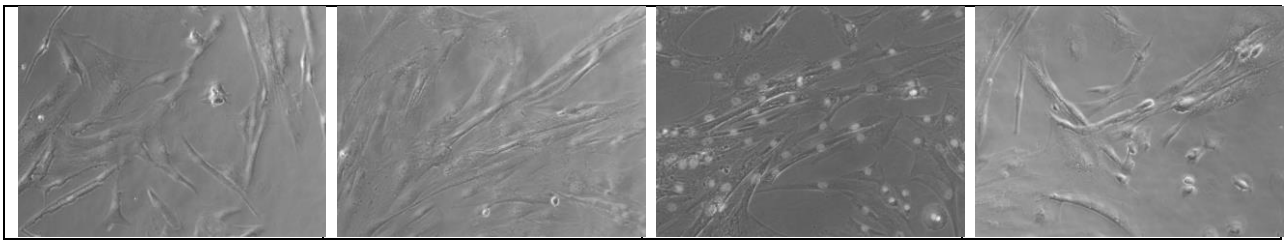

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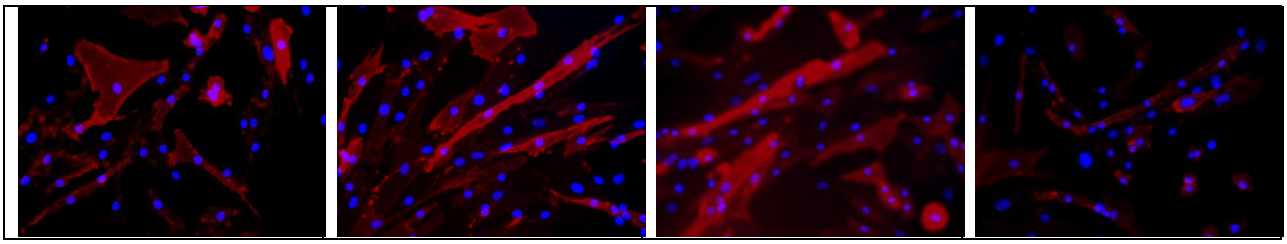
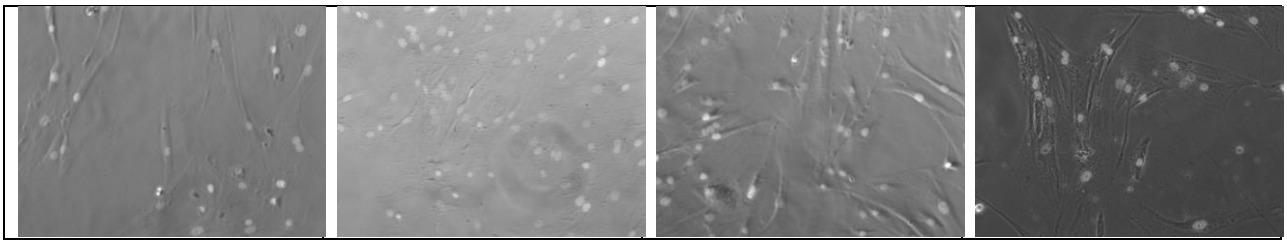


Surface Area:

5% O_2 w/o GF:

At passage:

Bright Field:



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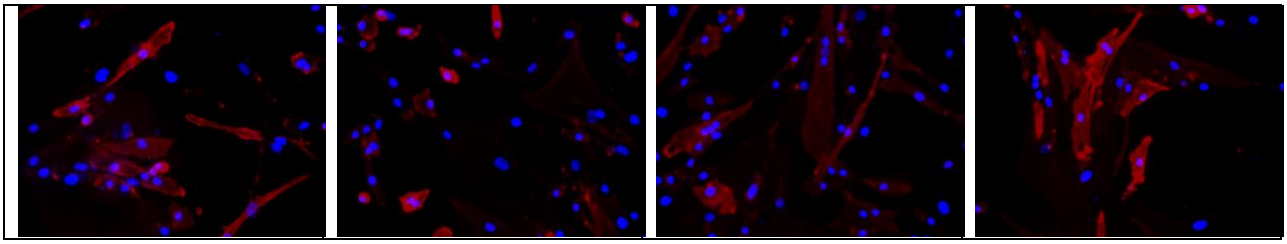

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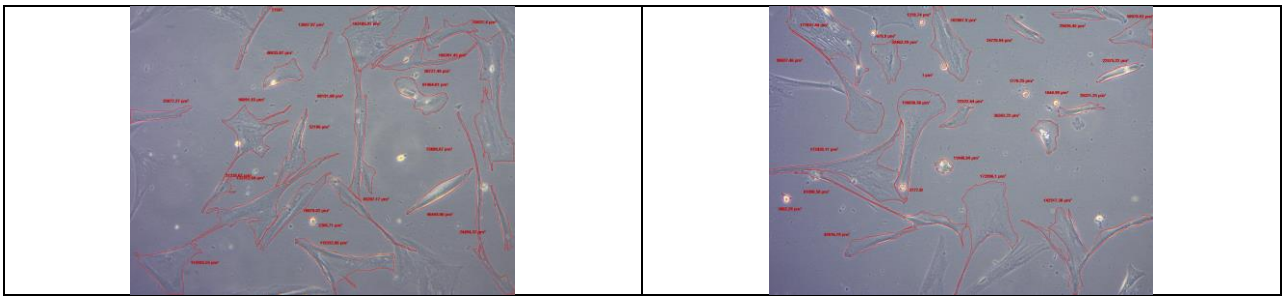

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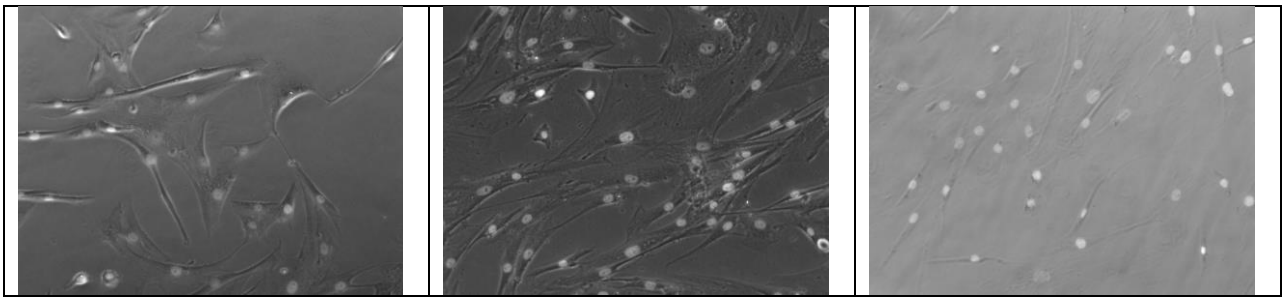
At passage:

Bright Field:



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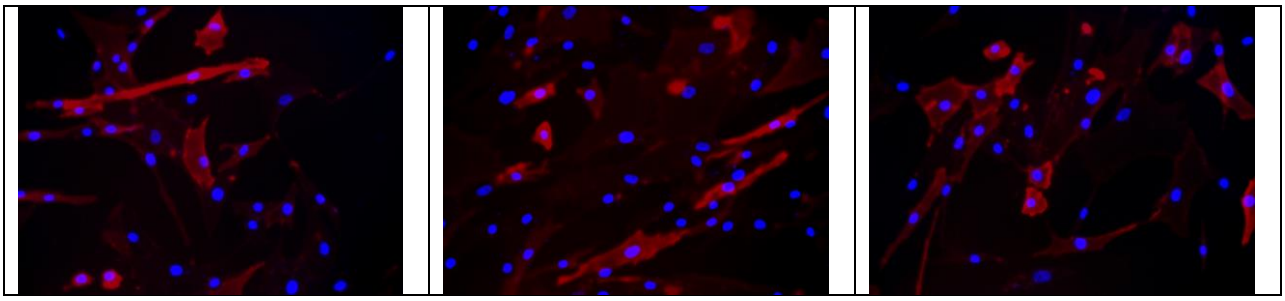

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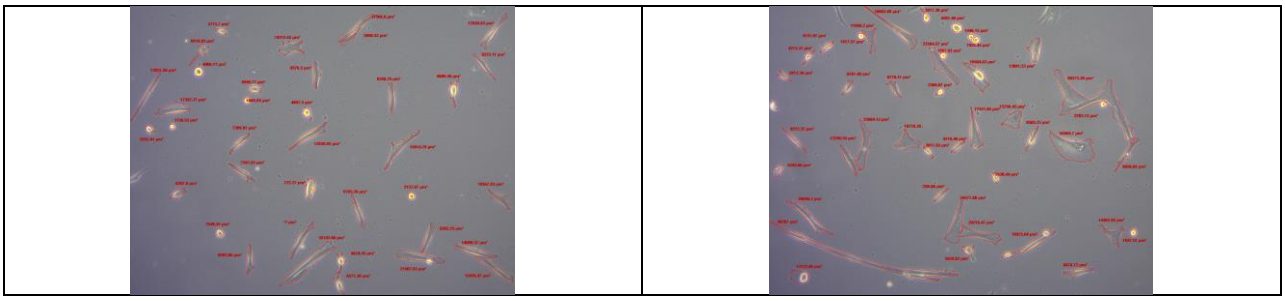
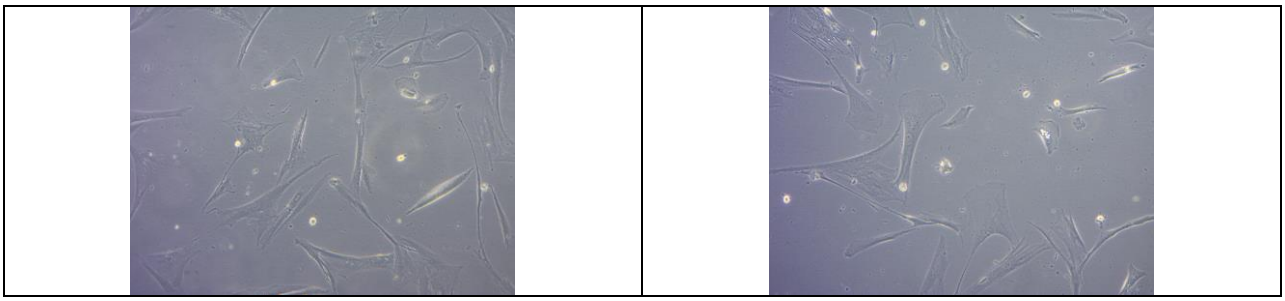


19% O_2 w/o GF:

At passage:



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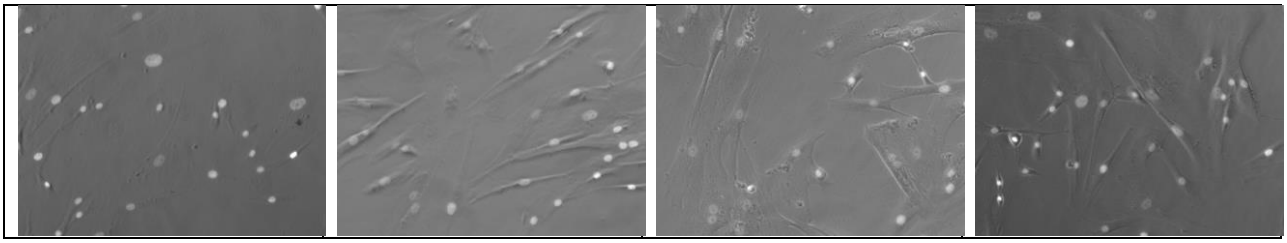

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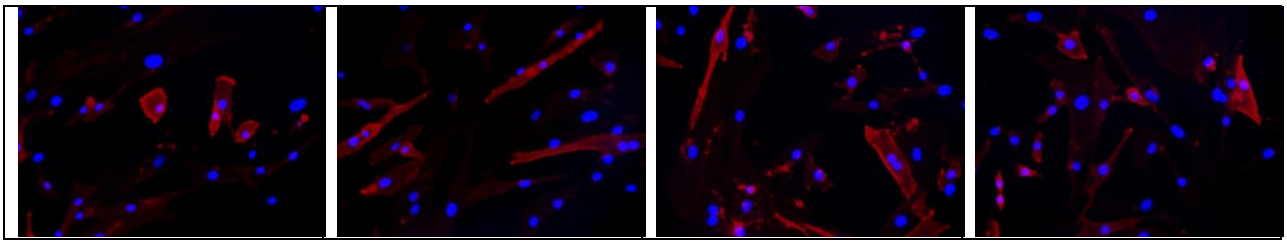

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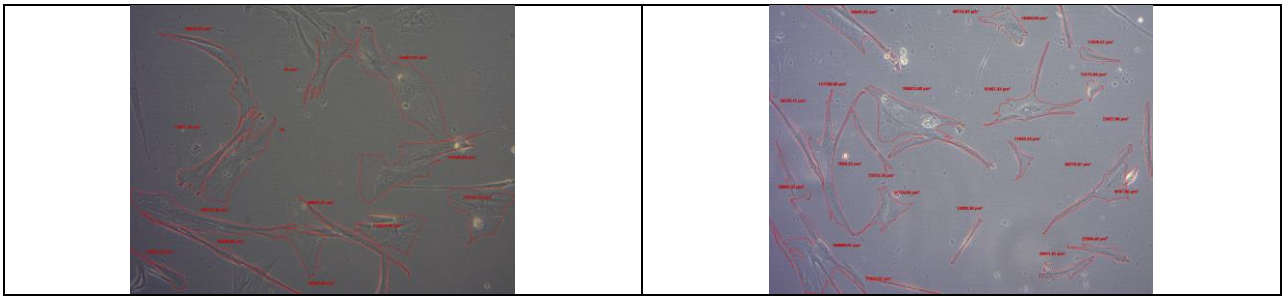
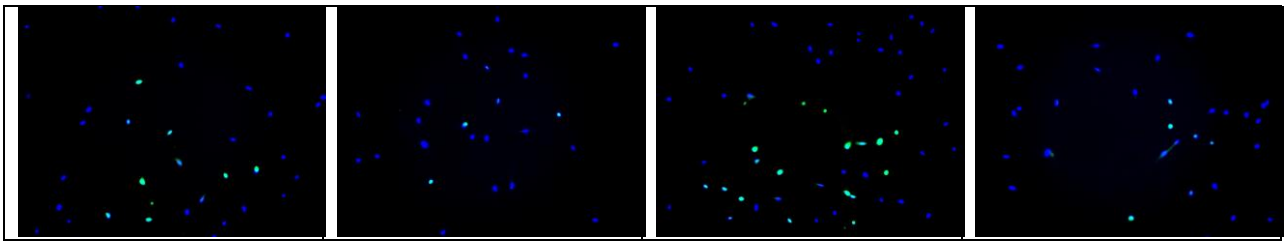


BrdU:

Adherent:



Non-Adherent: