Development of an in vitro myogenesis assay

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Department of Biomedical Engineering
College of Engineering
University of Arkansas
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by

Anna J Arnaud
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Thesis Advisor:

Thesis Committee:
Abstract

The objective of this study was to explore the interaction between mouse C2C12 cells and the extracellular matrix, particularly the process of myoblasts converting to myocytes. This study aimed to create a myogenesis assay that presents a process to effectively monitor the development of mouse C2C12 myoblasts into differentiated skeletal myotubes through detection of the protein MyoD. Myogenesis, the development of muscle tissue, occurs when muscle progenitor cells, myoblasts, fuse to form multinucleated myotubes, followed by cell fusion and resulting in a myofiber capable of contraction. An in vitro myogenesis assay would enable further research to efficiently test the effect of various growth factors and other parameters on skeletal muscle development, a field with numerous clinical applications. To test this, cells were seeded on both control substrates and wells coated with extracellular matrix. Cells were harvested at 3 and 7 days and tested for presence of MyoD. Analysis of PCR results showed negligible amounts of MyoD present in all experimental wells.
Introduction

Volumetric muscle loss can be defined as the loss of skeletal muscle resulting in a reduction or total loss of functionality\(^2\). Volumetric muscle loss is a common injury in the military, as improvised explosive devices have been used extensively in recent wars. Resulting soft tissue injuries and treatment of compartment syndrome in the extremities have left many veterans with loss of muscle functionality\(^14\). Volumetric muscle loss is also common in civilian life, as it can be caused by injuries such as vehicle accidents, gunshot wounds, and surgical excisions.

The replacement of tissue and promotion of muscle repair following volumetric muscle loss is a prominent challenge currently being investigated in tissue engineering. Lack of donor tissue limits the efficacy of procedures using muscle grafts to replace volumetric muscle loss, and the added challenge of donor-site morbidity has caused physicians and scientists to turn to tissue engineering as the most promising alternative to current free muscle transplant procedures\(^10\). Tissue engineering, as a field, seeks to either replace or improve biological tissues through the combined use of engineered materials, cells, and biochemical growth factors. Muscle tissue engineering is the branch of tissue engineering that focuses on the replacement of muscle tissue.

The process of muscular tissue formation is known as myogenesis. In order to understand adult myogenesis in fully developed skeletal muscle, it is necessary
to first understand embryonic myogenesis. As the body develops, satellite cells differentiate into embryonic myoblasts that divide rapidly in the presence of fibroblast growth factor. Following myoblast proliferation, myoblasts commit to differentiation, align and join to create myotubes, which are multi-nucleated fibers. Myotubes then mature into fully mature and functional skeletal muscle units known as myofibers. This process is illustrated in Figure 1.

![Figure 1. Illustration of myogenesis, the path from a satellite cell to a mature myofiber](image)

Various genetic factors are associated with the different stages of myogenesis, both adult and embryonic. For example, the myogenic regulatory factor family of basic helix loop helix transcription factors play a large role in the process of myogenesis, as they both activate the formation of muscle fibers and directly regulate muscle gene transcription. MyoD, a protein in the myogenic regulatory factor family, is necessary for both the initial conversion of a satellite cell to a skeletal myoblast and for regulation of the myoblast throughout myogenesis. MyoD self-activates transcription, inducing its characteristic stability in
commitment to myogenesis and its role in differentiation and repair of injured skeletal muscle\textsuperscript{13}.

The adult human body is capable of muscle regeneration, but only to a certain extent\textsuperscript{3}. Necrosis, or death, of muscle cells signals muscle tissue to begin the process of biological repair and muscle tissue regeneration. Muscle regeneration is a challenging process, as new muscle must contain vasculature sufficient to supply the new tissue with oxygen and innervation sufficient for the recovery of muscle functionality. Skeletal muscle, in comparison to cardiac and smooth muscle, is capable of a relatively large amount of regeneration due to cells known as myosatellite cells\textsuperscript{3}.

Myosatellite cells, as with all human skeletal muscle cells, develop embryonically from the paraxial mesoderm, specifically the somites. Throughout development, they become part of the dermomyotome\textsuperscript{7}. After the embryonic dermomyotome differentiation of primary muscle fibers, populations expressing transcription factors for myosatellite cells exist within the fetal skeletal muscle until a period late in the development of the fetus, at which time the myosatellite cells are produced\textsuperscript{7}. The signaling pathway throughout this fetal development of myosatellite cells is not well understood and is under investigation by several groups\textsuperscript{9,12}. 
In a fully developed adult, myosatellite cells are quiescent and are found between the basal lamina and the sarcolemma of the muscle fibers with which they are associated. The quiescent state persists until the myosatellites receive an activation signal indicative of injury to muscle tissue. This signal is transmitted from the plasma membrane via sphingosine-1-phosphate, a signaling sphingolipid that incites the myosatellite cell to enter the cell cycle. Mechanical factors such as stretch can also lead to the activation of myosatellite cells via the release of myogenesis stimulants such as hepatocyte growth factor or Follistatin, a molecule that inhibits the negative regulation of myogenesis caused by Myostatin. Yet another mode of myosatellite cell activation is that of growth factors secreted by the microenvironment. Specifically, fibroblast growth factors have been shown to trigger MAPK signal cascades, which are prerequisite to myosatellite cell activation.

Upon activation by muscle injury, myosatellite cells travel to existing muscle fibers, which they will adhere to in effort to repair damaged fibers and form new fibers. Activated myosatellite cells proliferate rapidly and differentiate into myoblasts, at which stage they divide several times prior to forming multinucleated myofibers. Due to the terminally differentiated nature of existing muscle fibers present in adult skeletal muscle, myosatellite cells are solely responsible for muscle regeneration following injury.
Volumetric muscle loss describes damage to skeletal muscle that results in the loss of more tissue than the body is capable of regenerating on its own\textsuperscript{2}. As muscle tissue engineering seeks to solve this problem through creation of artificial muscle and promotion of muscle regeneration, it is necessary to further understand the effects of growth factors and other biochemical substances on myogenesis. Exclusive use of animal models presents challenges with regard to both time and cost, which leads to the need for an \textit{in vitro} assay for use in preliminary testing.

As stated previously, MyoD is a protein of the myogenic regulatory factor family that serves as a marker of myoblast commitment to myogenesis\textsuperscript{13}. When investigating potential tissue engineering methods for promotion of muscle regeneration, it is possible to use MyoD as a screening tool. An increased concentration of MyoD indicates greater myoblast commitment to myogenesis, and thus greater muscle regeneration. This study aimed to profile the expression of MyoD to create an \textit{in vitro} myogenesis assay for use in the development of muscle regeneration therapies.

**Methods**

In order to test for adequate profiling of MyoD expression in an \textit{in vitro} assay while simultaneously investigating the effect of extracellular matrix (ECM) on C2C12 mouse myoblast cells, six different experimental parameters were tested. Cells were seeded on a control substrate, a substrate coated with 1 mg ECM per
mL water, and a substrate coated with 10 mg ECM per mL water to investigate the effect of the ECM on myoblast differentiation. Cells were collected at 3 and 7 days for each of the substrate conditions. All experimental cells underwent RNA purification, reverse transcription, and polymerase chain reaction to test for presence of MyoD.

**Muscle harvest**

Following the sacrifice of mature Sprague-Dawley rats, all tibialis anterior, biceps femoris, and gastrocnemius muscles were harvested and rinsed in deionized water to remove hair and debris. Muscle tissue was then stored in scintillation vials and frozen at -20°C until enough tissue was harvested to proceed to decellularization.

**Muscle decellularization**

All muscle tissues were removed from -20°C and submerged in a room temperature solution of 1% sodium dodecyl sulfate (Amresco) in 1X pH 7.4 phosphate buffered saline (Life Technologies). The scintillation vials were sealed and left on a rocking plate. The solution was replaced every 24 hours until the solution became colorless. The length of this process was approximately 4 days. Muscle tissues were then submerged in deionized water, which was exchanged every 6 hours for 24 hours. Muscle tissue was left submerged overnight in a 1X pH 7.4 phosphate buffered saline solution. The following day, the muscle was rinsed 3 times in deionized water and left in water overnight. At this point, the
muscle was completely decellularized and all that remained was the ECM. The
difference in original and decellularized muscle is shown in Figure 2.

![Figure 2](image.png)

**Figure 2.** Rat bicep femoris before (left) and after (right) decellularization using
1% sodium dodecyl sulfate in 1X phosphate buffered saline solution

**Preparation of ECM**

ECM was then ground into a fine powder using a mortar and pestle. One piece
of decellularized muscle tissue at a time was placed into the mortar, after which
liquid nitrogen was poured over the tissue. The pestle was then used to powder
the tissue, after which the powder was added to a clean scintillation vial. ECM
powder was stored at 4°C until later use.

**Preparation of 12-well plate**

To test the effect of ECM on cell growth, 3 different powder concentrations were
used to coat the bottom of two 12-well plates (Greiner Bio-One). 1 mL of pure
deionized water was added to 8 wells, 1 mL of 1mg ECM/mL deionized water
was added to 8 wells, and 1 mL of 10 mg ECM/mL deionized water was added to
8 wells. All water was then allowed to evaporate overnight with the lids of the 12-
well plate turned slightly askew inside a running biosafety cabinet. Both 12-well plates were then sterilized using a traditional ethylene oxide sterilization cycle. Note: an additional 12-well plate with no water or ECM powder was also prepared and later seeded with cells purely for post-experiment cell density imaging purposes.

Figure 3. Well of 10 mg ECM powder per mL deionized water, post-evaporation of 1 mL solution

*Preparation of C2C12 cells*

C2C12 mouse myoblast cells (ATCC) were used in all cultures. Culture medium consisted of Gibco Dulbecco’s Modified Eagle Medium (DMEMF12) with 10% Gibco Fetal Bovine Serum, 1% Gibco GlutaMax L-Glutamine, and 0.1% Gibco Gentamicin reagent solution. 12 mL of culture media and one thawed vial of C2C12 cells were added to a T-75 flask (Greiner Bio-One) and incubated at 37°C. Media was replaced every 48 hours until cells reached 75% confluence, at which point cells were expanded to prevent premature differentiation. At 75% confluence, cells were passaged and split to two T-175 flasks. The 75%
confluent cells were treated with 0.25% trypsin-EDTA for detachment. Upon detachment, cells were removed from T-75 flask and centrifuged 5 minutes at 300G. Following centrifugation, supernatant was removed and 10 mL of new media was added to the cells. The cell pellet was broken up by pipetting the new media several times. 5 mL of evenly distributed cell and media mixture was added to each of the new T-175 flasks. An additional 12 mL of new media was added to each flask. Cells were allowed to reach 75% confluence, with media being exchanged every 48 hours.

**Seeding Cells**

Aiming to seed all C2C12 cells at 30% confluence, a concentration of 14,400 cells/cm$^2$ (55,000 cells/well) was utilized for all wells in 12-well plate. A total of 24 experimental wells and 4 imaging wells were seeded at 30% confluence and media was added for a total volume of 1 mL/well. Previously described detachment techniques were used. 55,000 cells/well were also added to 4 additional wells, to be used solely for post-experiment cell density imaging.

**Collecting Cells**

72 hours after seeding cells, 4 wells of each ECM powder concentration sample group (0 mg/mL, 1 mg/mL, 10 mg/mL) were collected. After media was removed from these 12 wells, 200 uL new media and 1 mL RNAprotect cell reagent (Qiagen) were added to each well and incubated at 37°C for 15 minutes. Following incubation, cells were fully detached. The contents of each of the 12
wells were transferred to a separate labeled 1.5 mL microcentrifuge tube (Qiagen) and stored at -80°C. Media was removed from 2 of the 4 wells seeded for imaging and 1 mL 4% paraformaldehyde (Sigma) was applied for 15 minutes to fix the cells. Paraformaldehyde was removed and the cells were left submerged in 1X pH 7.4 phosphate buffered saline at 4°C, where they were left until imaging. Used media was removed and 1mL new media was applied to the 12 experimental and 2 imaging wells that were not either fixed or detached.

7 days after the seeding of cells, the remaining 4 wells of each ECM powder concentration sample group were collected. After media was removed from these 12 wells, 200 uL new media and 1 mL RNaseq protect cell reagent (Qiagen) were added to each well and incubated at 37°C for 15 minutes. Following incubation, cells were fully detached. The contents of each of the 12 wells was transferred to a separate labeled 1.5 mL microcentrifuge tube (Qiagen) and stored at -80°C. Media was removed from the remaining 2 wells seeded for imaging and and 1 mL 4% paraformaldehyde (Sigma) was applied for 15 minutes to fix the cells. Paraformaldehyde was removed and the cells were left submerged in 1X pH 7.4 phosphate buffered saline at 4°C until imaging.

*Cell density imaging*

The 4 wells fixed for imaging were removed from 4°C and placed at room temperature. Phosphate buffered saline was removed. 700 uL DAPI nucleic acid stain was added to each well and left for 10 minutes. DAPI was then
removed and wells were rinsed 3 times with 1X pH 7.4 phosphate buffered saline. Wells were then imaged at 100X.

**RNA purification and reverse transcription**

All RNA purification was performed using the RNeasy Protect Cell Mini Kit from Qiagen. Following immediate stabilization of nucleic acids in experimental C2C12 cells using RNAprotect cell reagent as described above in the ‘collecting cells’ section, total RNA purification was performed. Stabilized experimental cells were removed from storage at -80°C and allowed to thaw in a water bath at 37°C. After thawing, the cells were centrifuged. The cell pellet was lysed and homogenized using a guanidine-thiocyanate buffer, thus inactivating RNAses to protect all existing RNA. The homogenized lysate was placed in a gDNA Eliminator spin column, which used in conjunction with a salt buffer resulted in removal of all genomic DNA. Ethanol was then added to RNA flow-through to allow RNA to bind, after which an RNeasy spin column was used to isolate RNA and eliminate all contaminants. RNA was then eluted in RNAse-free water. Reverse transcription of isolated RNA was then performed using the iScript cDNA Synthesis Kit (Bio-Rad).

**Polymerase Chain Reaction**

All polymerase chain reaction (PCR) procedures were carried out using MyoD1 primer (Bio-Rad) as a gene of interest, GAPDH primer (Bio-Rad) as a housekeeping gene, and SYBR Green (Bio-Rad) as a DNA-binding dye.
Results

Cell density imaging results are shown in Figure 4. There was a drastic increase in confluence of mouse C2C12 cells from 3 days to 7 days. Cells were initially seeded at 30% confluence, and imaged with DAPI for nucleic acid staining. All images were taken at 100X magnification.

Figure 4. Mouse C2C12 cells at 3 (left) and 7 (right) days following seeding at 30% confluence, imaged with DAPI for nucleic acid staining at 100X magnification

Analysis of PCR results showed negligible amounts of MyoD present in all experimental wells. The housekeeping gene, GAPDH, presented at 2000-2500 relative fluorescent units (RFU). PCR results showed there was no MyoD present in 11 of the 12 tested wells. The one well that contained MyoD presented at 1200 RFU.

Conclusion

Results from PCR showed a lack of MyoD in 11 of 12 experimental wells. The presence of GAPDH, the housekeeping gene, shows that there was indeed
enough genetic material collected and purified to glean results with PCR. The absence of MyoD could possibly be due to presence of Myf5 instead, a similar gene with functional redundancy\(^6\). The absence of MyoD also could indicate that the mouse C2C12 cells did not reach the confluence during the 3 to 7 days of incubation that was required for further differentiation and thus expression of MyoD.

Results showing no MyoD in any experimental wells present the possibility that the model used in this experiment was ineffective. Sources of error leading to an ineffective assay include but are not limited to media formulation and cell density. In a similar experiment by Kong et al., C2C12 myoblasts were treated with chloroquine and DNA calcium phosphate precipitates prior to plating in order to promote stable DNA transfections\(^6\). Cells were also switched to a differentiation medium consisting of low-glucose DMEM and 2% horse serum prior to differentiation\(^6\). Kong et al. used a similar seeding density to this experiment, differing by less than 1,000 cells/cm\(^2\), and allowed cultures a maximum of four days before harvest.

Several operational shortcomings also arose throughout the experiment, including but not limited to RNA purification and PCR techniques. Due to the inherent instability of RNA, storage at -80°C for several days prior to RNA purification could have affected the amount of RNA that was intact and available for isolation. During RNA isolation, trace amounts of phenol could have
remained in the RNA, thus introducing error into RNA quantification. Degradation of the DNA template from nucleases in the testing environment or contaminants present in the master mix could have contributed to downstream error in PCR.

Failure to detect MyoD effectively using this assay leads to a search for other methods of monitoring myogenesis. In future work, another method of screening for myogenesis using an in vitro system could be utilization of imaging devices to monitor myoblast differentiation. As myoblasts proliferate and differentiate, they align into a network of parallel, multinucleated myotubes. Through characterization of the surface topography of experimental wells, it is possible to quantify the degree of alignment and thus the degree of differentiation of myoblasts into myotubes.

Future experimentation using RNA purification and PCR would call for further investigation into media formulations and protocols to promote myogenesis and appropriate cell culture length. Significant presence of MyoD in similar studies was only noted after the use of specific differentiation mediums. In future work, there is also need for additional practice in RNA isolation and PCR techniques in order to reduce the likelihood of DNA degradation, contamination, and pipette error.
References


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