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A high-content multiplexed screening platform for the evaluation and manipulation of force and fatigue of adult derived skeletal muscle myotubes in defined serum-free medium

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A HIGH-CONTENT MULTIPLEXED SCREENING PLATFORM FOR THE EVALUATION AND MANIPULATION OF FORCE AND FATIGUE OF ADULT DERIVED SKELETAL MUSCLE MYOTUBES IN DEFINED SERUM-FREE MEDIUM

by

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ABSTRACT

The overall focus of this project has two parts: First, was to develop a protocol utilizing serum-free media formulations and defined plating and culture techniques to create functional *in vitro* myotubes derived from adult skeletal muscle satellite cells. The second was to manipulate the inherent muscle parameters such as force output and fatigue of these myotubes by employing exercise regimes or by small molecule application.

The importance of serum-free medium use for *in vitro* cultures is becoming increasingly important in creating functional systems that can be validated for drug testing by the Food and Drug Administration (FDA). Also, the study of age related diseases as well as the potential for "personalized medicine" relies on the proliferation and maturation of satellite cells from adult derived tissue. For that purpose, a serum-free medium and culture system was designed to create mature striated myotubes in culture on a defined non-biological substrate N-1[3-trimethoxysilyl propyl] diethylenetriamine (DETA). These myotubes were evaluated by morphology, muscle specific protein expression, and by muscle functionality. After the thorough characterization of the resultant myotubes the functional output of the muscle was altered utilizing chemical means (creatine supplementation and PGC-1α agonists), chronic long term stimulation, and the use of PGC-1α deficient tissue.

In this thesis presentation the utility of the newly developed medium formulation to create myotubes from a variety of adult derived muscle sources will be shown. A protocol in which to exercise skeletal muscle in vitro to alter endurance was developed and employed to manipulate skeletal muscle. Finally, small molecules were tested to validate this system for drug

study use. This engineered system has the potential for high-throughput screening of drugs for efficacy and drug toxicity studies as well as general biological studies on muscle fatigue.

I would like to thank the members of the Hybrid Systems Lab especially Alec Simon Tulloch Smith, Sarah Najjar, Yunqing Cai, and Dr. Stephen Lambert without whom this endeavor would have taken much longer. I want to dedicate this work to my friends and family especially my mom and dad for all their love and support over the years.

Most Importantly I would like to praise my wife for all of her love, patience, and understanding. Thank you Lori for always being the solid rock upon which I could lean.

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LIST OF ABBREVIATIONS

aFGF: Acidic Fibroblast Growth Factor

BDNF: Brain Derived Nerve Factor

bFGF: Basic Fibroblast Growth Factor

CNTF: Ciliary Neurotrophic Factor

CT-1: Cardiotrophin-1

DETA: (3-Trimethoxysilyl propyl) diethylenetriamine

EGF: Endothelial Growth Factor

GDNF: Glial Derived Nerve Factor

IGF: Insulin-like Growth Factor

LIF: Leukemia Inhibitory Factor

MyHC: Myosin Heavy Chain

NT-3: Neurotrophin 3

NT-4: Neurotrophin 4

VEGF: Vascular Endothelial Growth Factor

CHAPTER ONE: INTRODUCTION

Skeletal muscle tissue engineering is an interdisciplinary field that incorporates cell biology and engineering to recapitulate fusion of individual mononucleated cells to generate functional multinucleated muscle. *In vivo* biological muscle involves a complex interaction between 3 main components: cells, extracellular matrix (ECM)(that consists of an array of proteoglycans, proteins, and glycosaminoglycans) and finally, signaling factors (Figure 1).[1] In vitro skeletal muscle engineering focuses on utilizing these three main components to recapitulate functional muscle for a variety of purposes including but not limited to grafting and drug discovery. Since the "origin" of skeletal muscle engineering in the 1970's [2-4] there have been considerable advancements in all three aspects of this field.

The original sources of progenitor cells were derived from the fetal tissue, typically of rodents or chick origin.[5-7] Typically the pectoral muscle or the hind limbs would be dissected and processed to release embryonic myoblasts that would be expanded and eventually fused to form myotubes or myofibers. This requires pregnancy of the maternal species as well as sacrifice to mother and offspring. While this provided a generous progenitor cell population that had relative ease of culturing *in vitro,* it had limitations. The cell source population has now expanded to include adult tissue derived skeletal muscle that provides ease of dissection and is more applicable to age related diseases.[8, 9] As well as, stem cells, both embryonic derived and induced pluripotent, which possess little to no invasive properties and also immortalized cell lines.[10-12] This thesis will focus on adult derived satellite cell skeletal muscle engineering.

The first medium formulations consisted of a simple base medium subsidized with a range of sera derived from prenatal animals, most commonly from fetal bovine subjects, ranging

from a concentration of 10-30% volume/volume. When these expanding cells have reached confluency a complete withdraw or minimalized withdraw of medium supplemented with low levels of horse or donkey serum resulted in myoblast fusion.[13-15] Since these initial formulations were introduced onto the biomedical scene there have been integral advancements in media compositions that further the *in vitro* myoblast fusion and maturation. For the future of drug development processes and drug discovery systems a defined serum free medium formulation system capable of being unbiased analyzed and interrogated is necessary to adhere to NIH and FDA initiatives and will be discussed further in this thesis.

In vivo skeletal muscle consists of muscle tissue, tendons, connective tissue, blood vessels and nerves. A connective tissue known as the epimysium surrounds the whole muscle. The whole muscle is composed of bundles of fibers known as fasciculi and are surrounded by a sublayer of connective tissue, the perimysium. Further investigation elucidates that each fasciculi consists of individual fibers surrounded by more connective tissue called endomysium. Each individual fiber consists hundreds or thousands of individual components known as myofibrils that make up the actual contractile functionality of skeletal muscle. Skeletal muscle is typically known as striated muscle due to the light and dark bands seen by light microscope. This is caused by the very orderly arrangement of the myosin and actin filaments that compose the contractile machinery within the myofibril. Single sarcomere consists of alternating actin and myosin filaments that allow for the Adenosine Tri-phosphate (ATP) motorhead located on the myosin filament to attach to actin filaments and provide contraction (Figure 2). In *in vitro* muscle the formation of this highly complex arrangement is indicative of functionally mature myofibrils (or myotubes).

Along with contractile machinery, myofibrils possess the same components as other cells, nuclei, enzymes, proteins, and mitochondria. The inherent strength of muscle and it's endurance are reliant on a few factors:[16-18]

1. Myosin ATPase activity
2. Succinic dehydrogenase (SDH)
3. Mitochondrial content (oxidative capacity)

Muscle with high ATPase activity and low mitochondria content and low SDH levels have rapid and forceful output but are rapidly fatigued (Type II fibers or fast-twitch). Conversely, muscle with low ATPase activity and high mitochondria content and high SDH levels possess less forceful and slower contraction but have high endurance levels (Type I fibers or slow-twitch). [16-18] The SDH, mitochondria levels, and Myosin ATPase activity can all be altered both *in vivo* and *in vitro* by a variety of factors. Exercise, both plyometric and endurance, can drive fiber conversion from slow to fast or vice versa as well as small molecule or drug applications that alter pathways responsible for mitochondria biogenesis or SDH production.[11, 19-22]

The development of an *in vitro* culture system consisting of functionally mature myotubes derived from adult skeletal muscle myoblasts grown in serum-free conditions is of paramount importance. Furthermore, the ability to measure contractile strength and endurance of this muscle provides a unique test bed for future drug discovery., The ability to measure changes to force and fatigue of muscle in response to exercise and drug application spawns a promising avenue by which to study exercise physiology, drug efficacy and toxicity, and utilizing adult based cells, the potential for personalized drug testing.

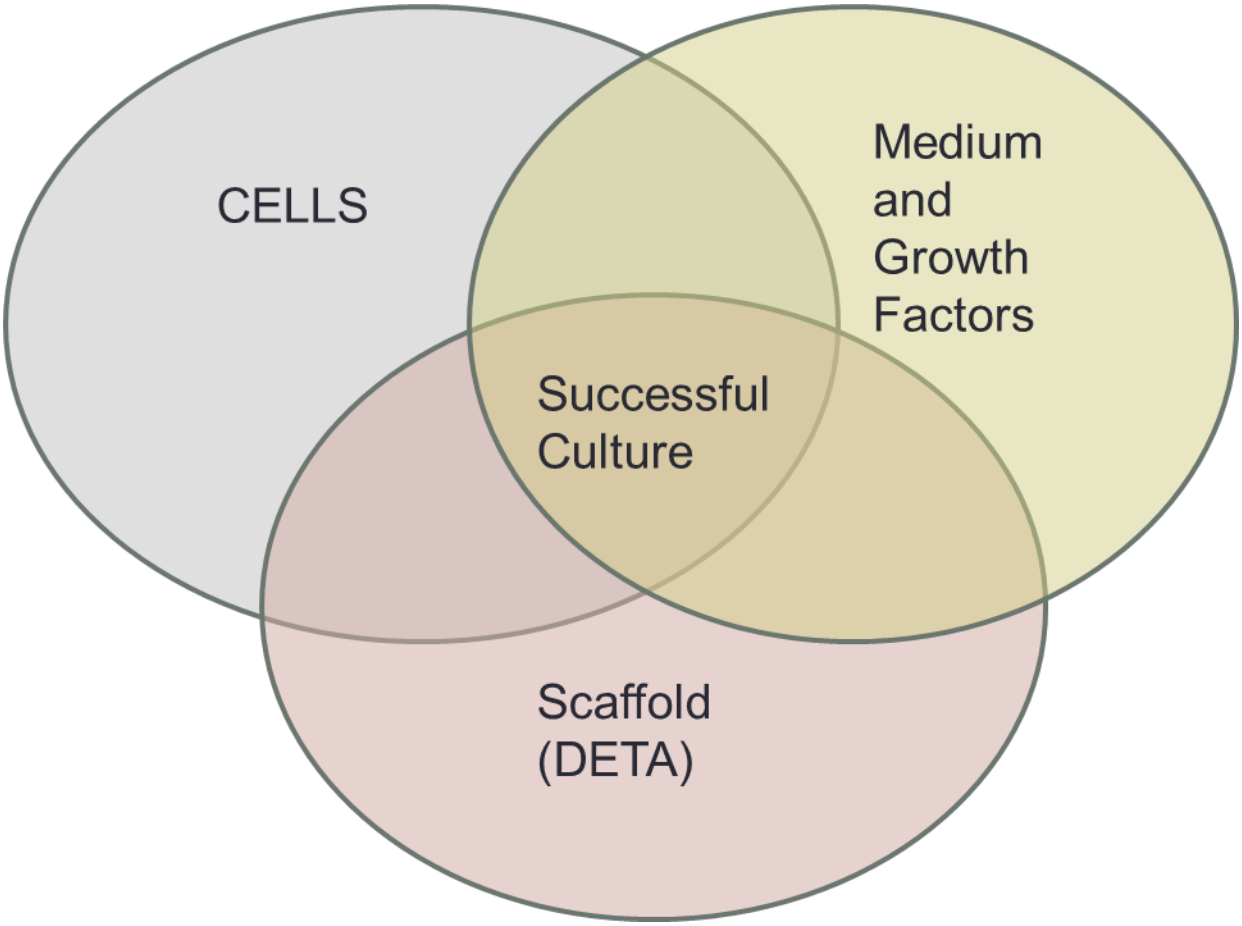


Figure 1: The tissue engineering paradigm

Adapted from (Makris, E.A., et al., *Repair and tissue engineering techniques for articular cartilage.* Nat Rev Rheumatol, 2015. **11**(1): p. 21-34)

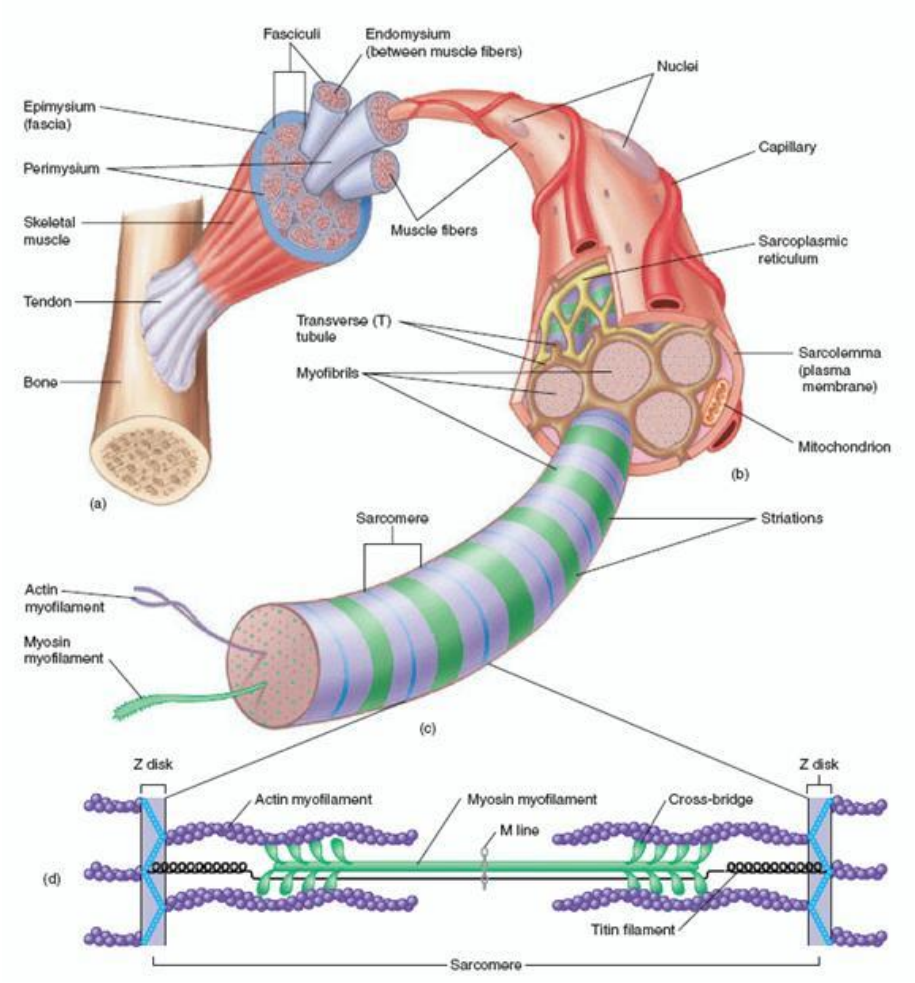


Figure 2: Muscle split into various components

Cited from (Baechle, T.R., et al., *Essentials of Strength Training and Conditioning*. 2008: Human Kinetics.)[23]

CHAPTER TWO: FUNCTIONAL MYOTUBE FORMATION FROM ADULT RAT SATELLITE CELLS IN A DEFINED SERUM-FREE MEDIUM

Introduction

Adult tissue-derived *in vitro* systems provide a unique modality to study physiologic and age related diseases. The study of adult skeletal muscle physiology and myopathies has especially benefitted from the ease of isolation of satellite cells which are adult muscle progenitor cells. Satellite cells are situated between the sarcolemma and basal lamina of muscle fibers. These normally quiescent cells, activated in response to exercise and pathological injuries, migrate to the site of damage where they proliferate, elongate, and fuse to each other, as well as to existing myofibers, to replace lost or damaged cellular material [24].

Currently, the prevailing dogma for the study of muscle related diseases and physiological phenomenon *in vitro* focuses on cells derived from adult animals grown in serum-containing medium formulations. Generally, animal-sera have been used in adult and embryonic muscle culture due to their ability to enable the rapid proliferation of muscle cells [13-15]. Once the cells have reached a confluent monolayer, the removal, or drastic reduction, of serum concentration triggers extensive myotube formation in the cultured cells. This has been the long established protocol due to cost efficiency, relative ease of use, and general effectiveness [25]. The drawback of this culture system is that the contents of animal sera have not been fully elucidated nor well characterized; there is also the batch to batch variability that occurs during serum production [26, 27]. Therefore, one cannot rule out the possibility of adverse stimulatory or inhibitory effects occurring as a result of its use. Consequently, the clinical applications of an *in vitro* model system that uses serum containing medium formulations are limited.

Previous work has detailed the development of a serum-free system that mimics the *in vivo* process of myotube formation and the physiological contractile properties of myotubes derived from fetal hind-limb satellite cells [28-30]. However, the use of embryonic muscle limits the applicability of these systems to the study of age related diseases and adult physiological processes such as Myosin Heavy Chain (MyHC) class switching.

To address this issue a culture system was developed where all aspects of the system were defined and controlled to provide a new model for the study of adult muscle physiology and disease. The process utilized satellite cells derived from adult rat hind-limbs, a defined serum-free media formulation, as well as a non-biological cell growth promoting substrate N-1[3-trimethoxysilyl propyl] diethylenetriamine (DETA). The triamine functionality of DETA is also a structural analog to spermidine, a growth factor known to promote cellular survival [31, 32]. This system exhibited physiological maturation (adult MyHC expression) and responded to physiological stimuli to provide a suitable model for the study of age related disease and muscle myopathies. Combined with previous work modeling neuromuscular junction formation [33-36] new functional assays could be developed from the adult cells derived from transgenics or disease related animals for more relevant *in vitro* disease models. Recent FDA/NIH initiatives for regulatory science require the use of serum-free media and as such, a new serum-free system for the study of adult derived skeletal muscle myotubes will be of paramount importance in the advancement of muscle biology and *in vitro* drug discovery.

Materials and Methods

Surface Modification and Characterization

Glass coverslips (VWR cat. nr. 48366067, 22×22 mm^2 No. 1) arranged in ceramic staining racks (Thomas Scientific, Swedesboro, NJ) were chemically cleaned and dried prior to surface modification. First, the coverslips were soaked in a solution of 50/50 methanol (VWR cat. nr. BJLP230-4) / hydrochloric acid (VWR cat. nr. EM1.00314.2503) for 2 hours and then rinsed thoroughly with DI water. The coverslips were then immersed in concentrated sulfuric acid (VWR cat. nr. BDH3072) for a minimum of 2 hours, rinsed as before and then boiled in deionized water for at least 30 minutes. After boiling the glass slides were placed in an oven set to 110°C and allowed to dry overnight. The 3-Trimethoxysilyl propyl diethylenetriamine (DETA), (United Chemical Technologies Inc. T2910) film was formed by the reaction of the cleaned and dried surfaces with a 0.1% (v/v) solution of the organosilane in freshly distilled toluene (VWR cat. nr. BDH1151). The DETA-toluene solution was prepared in a glove box (MBraun, Stratham, NH) under nitrogen atmosphere, and the reaction was carried out in the lab atmosphere in a dish covered with an inverted beaker. The dish containing the clean slides was heated to approximately 100°C for 30 minutes, and then allowed to cool to room temperature, when the slides were taken out of DETA solution, rinsed carefully three times with dry toluene and reheated in a fourth toluene rinse to approximately 100°C for 30 additional minutes. The DETA-coated slides were oven dried at 110°C for at least 2 hours prior to use or storage. Surfaces were characterized by static water contact angle measurements using a Rame-Hart Model 250 goniometer, and by X-ray Photoelectron Spectroscopy (XPS) using a VG ESCALAB 220i-XL spectrometer equipped with an aluminum anode and a quartz monochromator. The spectrometer was calibrated against the reference binding energies of a clean Ag sample. XPS

survey scans were recorded in order to determine the relevant elements (pass energy of 50 eV, step size of 1 eV). Si 2p, C 1s, N 1s, and O 1s high resolution spectra were recorded in order to determine the quality of the surfaces (pass energy of 20 eV, step size of 0.1 eV). The fitting of the peaks was performed with Avantage version 3.25 software, provided by Thermo Electron Corporation.

Skeletal Muscle Isolation and Serum-Free Medium

The left and right Tibialis Anterior (TA) were dissected from Adult Sprague-Dawley rats aged 6-12 months (Charles-River Laboratories). Briefly, rats were euthanized by inhalation of an excess of CO_2. This procedure complies with IACUC standards laid out by the Animal Research Council of University of Central Florida. The muscle was collected in a 15 ml conical tube and washed briefly in PBS to remove debris. The tissue was then minced into fine pieces and enzymatically dissociated in a 25 mg collagenase II (Worthington Biochemicals, LS004176), 1 mg dispase (Worthington Biochemicals, LS02104) solution in DMEM (Gibco, 11965) for 1 hour in a 37°C water bath at 100 rpm. Muscle was removed and triturated using fire polished glass pipettes. The suspension was plated on 100 mm uncoated dishes for 1 hour at 37°C, 100% relative humidity. The unattached cell suspension was collected and centrifuged at 300 g for 5 min at 4°C. The supernatant was removed and the pellet was re-suspended in proliferation medium (Table 1) and plated on DETA coverslips. The suspension was allowed to incubate for 45 minutes before additional medium was added. The cells were then serial plated twice on DETA coverslips to obtain a more pure population of myoblasts on the third coverslip. After 8 days the proliferation medium was slowly removed and replaced with differentiation medium

(Table 2) to promote cell alignment and fusion. The cells were maintained in a 5% CO_2 incubator and one half the differentiation media was changed every 3-4 days.

Table 1: Composition of serum-free growth medium for a 500 ml sample

Component	Company	Catalogue #	Quantity
Neurobasal	Invitrogen	21103049	250 ml
L15	Invitrogen	11415064	250 ml
aFGF	Invitrogen	13241-013	10ug
Antibiotic-antimycotic	Invitrogen	15240062	5 ml
Calcium chloride	Fisher	10035-04-8	250ug
VEGF	RND systems	293-ve-010	10ug
bFGF	RND Systems	3339-FB-025	20ug
CNTF	Cell Sciences	CRC 401B	20ug
NT-3	Cell Sciences	CRN 500B	10 ug
Nt-4	Cell Sciences	CRN 501B	10ug
GDNF	Cell Sciences	CRG 400B	10ug
BDNF	Cell Sciences	CRB 600B	10ug
CT-1	Cell Sciences	CRC 700B	10ug
LIF	Sigma	L5158	10ug
Vitronectin	Sigma	V0132	50ug

Table 2: Composition of serum-free differentiation media for 500 ml

Component	Company	Catalogue	Quantity
Neurobasal	Invitrogen	21103049	250 ml
L15	Invitrogen	11415064	250 ml
EGF	Invitrogen	53003018	50ug
IGF	Sigma	I2656	5ug

Immunocytochemistry

Coverslips were rinsed with PBS and fixed in 4% paraformaldehyde for 10 min. They were then washed in PBS, incubated in PBS supplemented with 1% bovine serum albumin and 0.1% triton X-100 (permeabilization solution) for 20 min, before being blocked for 30 min in the permeabilization solution + 5% donkey serum (blocking solution). The fixed cells were incubated in blocking solution overnight at 4°C with either a primary antibody against MyHC all classes (A4.1025, IgG, Developmental Studies Hybridoma Bank), diluted 1:10, a MyHC slow antibody (Sigma M8421), diluted 1:500, or a MyHC fast antibody (abcam ab91506), diluted 1:1000. Coverslips were washed with PBS and incubated with Alexafluor secondary antibodies (Invitrogen) diluted (1:400) in PBS for 2 hours at ambient temperature in the dark. After rinsing in PBS, the coverslips were mounted on glass slides using VectaShield + DAPI mounting media (Vector Laboratories H1200) and viewed on a confocal microscope (UltraVIEW™ LCI, PerkinElmer). For double staining with Pax-7 and Myo-D, cultures were processed for immunocytochemistry as just described. Next, cells were incubated overnight at 4°C with primary antibodies against Myo-D (abcam ab16148), diluted 1:1500, and Pax-7 (abcam ab34360), diluted 1:3000.

Patch Clamp Electrophysiology

Whole-cell patch clamp recordings of the mature myotubes were performed by Dr. Min Lin in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope as described previously[36]. Patch pipettes were prepared from borosilicate glass (BF150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller and filled with intracellular solution (in mM: K-gluconate 140, EGTA 1, $MgCl_2$ 2, Na_2ATP 2, phosphocreatine 5,

phosphocreatine kinase 2.4 mg, Hepes 10; pH = 7.2). The resistance of the electrodes was 6–8 MΩ. Voltage clamp and current clamp experiments were performed with a Multiclamp 700A amplifier (Axon Laboratories, Union City, CA). Signals were filtered at 2 kHz and digitized at 20 kHz with an Axon Digidata 1322A interface. Data recording and analysis were done with pClamp 8 software (Axon Laboratories). Membrane potentials were corrected by subtraction of a 15 mV tip potential, which was calculated using Axon's pClamp 8 program. Sodium and potassium currents were measured in voltage clamp mode using voltage steps from a −85 mV holding potential. Action potentials were evoked with 1 s depolarizing current injections from a −85 mV holding potential.

Statistical Analysis

30 random fields of view were analyzed across 3 independent experiments. The number of fields of view analyzed was deemed sufficient to provide an accurate representation of the mean as assessed by cumulative frequency analysis (data not shown).

Results

DETA surface Modification and Characterization

DETA is a spermidine analog that has been shown to promote the proliferation, maturation and, long term survival of an array of cell types [5, 31, 32, 35, 36]. Static contact angle and XPS analysis was used for validation of the surface modifications. Contact angles of 46° +/- 2° were shown to be consistent and reproducible across this study. XPS measurements for the ratio of N (1s) to Si (2p) of 1500 +/- 200 indicated that a reaction site limited DETA monolayer was formed on the coverslips.

Development of Serum-free Medium and Plating Technique

The starting medium formulation, surface modification and plating technique was developed previously by our lab to create a system for the development of myotubes derived from embryonic E18 rat hind-limbs[29]. However, this system was insufficient to enable the development of myotubes from satellite cells isolated from the TA of adult rats. The original medium formulation was altered by the addition of bFGF, which is well established as a promoter of muscle proliferation [5], L15 medium, which was previously shown to promote myoblast survival [29], and the addition of calcium, in the form of $CaCl_2$ [37]. This reformulated medium was sufficient to promote the proliferation of myoblasts. The plating technique was modified by introducing a serial plating protocol whereby the cell suspension was removed after 24 hours and re-plated on a new DETA coverslip [38]. After the second serial plating it was determined that the culture had a higher proportion of myoblasts and cell survival in the culture was increased.

After cell proliferation and culture purity were optimized, it was also necessary to alter the technique by which differentiation of myoblasts into myotubes was induced. The previously devised technique was to shock the cultures into differentiation by completely changing the growth medium to differentiation medium after 48 hours of proliferation [29]. It was observed that if this technique was employed, it resulted in complete cell death within 12 hours. Instead a gradual decline in growth factors was employed to enable, cell survival and maturation into multinucleated myotubes. 300 uL of growth medium was removed daily and replaced with an equivalent volume of a defined serum-free differentiation medium (Table 2) over the course of 5 days. This gradual reduction in growth factors resulted in robust cell survival and myotube formation. The combination of all these changes resulted in the establishment of a system by which mature myotubes could be routinely formed from adult satellite cells (Figure 3). Functional contractile *in vitro* myotubes tend to differ slightly from mature *in vivo* myofibers morphologically; they lack peripheral nuclei and, despite forming well-defined sarcomeric structures, they usually do not promote the formation of extensive and highly organized myofibrils. Therefore, for the purposes of this manuscript, these differentiated myogenic cells are referred to as functional myotubes, rather than myofibers.

Characterization of the Satellite Cells and Resultant Myotubes

Satellite cells express the transcription factor Pax-7. Cells of a myogenic lineage will also express the muscle specific marker Myo-D. There is some contention as to whether Pax-7 is expressed in quiescent versus activated satellite cells, nevertheless it is ubiquitously expressed in satellite cells as a whole [39-41]. As such, these markers were used to quantify the number and purity of muscle satellite cells isolated from the TA. Figure 4 shows the co-localization of Pax-7

and Myo-D in myoblasts expanding in proliferation medium after 2 days *in vitro* (DIV). Multiple coverslips were imaged, using cumulative frequency analysis, and the cultures were determined to be 60.15% +/- 10.39% positive for both Pax-7 and MyoD in the first plating, 71.36% +/- 6.3% for the second plating, and 94.96% +/- 3.85% after the final serial plating (Figure 4). This establishes that serial plating was needed to provide a relatively pure culture of proliferating myoblasts that promotes myotube formation in the serum-free medium. Figure 4a, panels D-F, also shows phase images of the myotubes from 12 DIV, indicating the continued purity of the culture.

Immunocytochemical Characterization of the Myotubes

After induction of myoblast fusion into myotubes the culture was characterized immunocytochemically. DIV 14 myotubes were stained with anti-MyHC antibody A4.1025 from the DSHB. MyHC is an essential element in the contractile apparatus of mature myotubes. Figure 6 shows highly striated muscle indicating mature myotubes with a highly developed contractile apparatus.

Characterizing Mature MyHC Expression

One of the limiting factors in the use of embryonic derived cultures is the limitation for those cultures to express more advanced isoforms of MyHC such as neonatal and/or adult. [42, 43] Figure 7 shows positive staining for both the mature MyHC slow isoform as well as the mature MyHC fast isoform. It was determined that approximately 73.89 ± 4.6% of myotubes at 12 DIV expressed the MyHC fast twitch isoform and that 10.67 ± 1.1% of those myotubes also

expressed the mature slow twitch isoform. This is in contrast to previous results indicating approximately 25% neonatal/adult conversion after 45 DIV for embryonic derived tissue. [33]

Electrophysiological Characterization

Muscle specific myoblasts were shown to fuse into MyHC positive myotubes. These myotubes were then tested to determine if the ion channels were functional utilizing whole cell patch clamp electrophysiology. DIV 14 myotubes were patched according to the described protocol and as can be seen in Figure 8 fired action potentials (APs) when stimulated. The myotubes expressed a mean inward current of -2998.7 +/- 307.9 pA with a mean outward current of 1816.3 +/- 202.2 pA indicating functional sodium and potassium channels and the APs had a mean amplitude of 46.7 +/- 5.8 mV (errors are expressed as +/- the SEM, with n = 9). The resting membrane potential of -59.9 +/- .8 mV measured in our system more closely mimics ideal *in vivo* potentials than previously reported membrane potentials of comparably aged *in vitro* rat myotubes in serum-containing medium. [44]

Discussion

The results described above document the development of a new serum free adult skeletal muscle culture system. This system furthers the evolution of physiologically relevant *in vitro* myotube models. Specifically, this manuscript documents the development of a medium formulation, surface composition and culture technique that results in myotubes that better recapitulates *in vivo* functionality and maturation by more rapid expression of adult myosin isoforms compared to embryonic tissue-derived myotube cultures.

Current *in vitro* muscle cultures derived from adult tissue primarily rely on animal sera for the division and growth of myoblasts. [45] The limitations of this approach are the variability of serum production and the lack of definition of the contents. A serum-free medium was developed, supplemented with a cocktail of defined growth factors supporting adult-derived myoblast proliferation and fusion as well as myotube maturation, both structurally and functionally. The completely defined nature of this medium formulation also makes it advantageous for high-content drug screening and *in vitro/in vivo* correlative studies. It could also be utilized in tissue engineering and regenerative medicine investigations by demonstrating new techniques for isolation and expansion of adult satellite cells that are capable of functional maturation.

Previously, studies conducted in our lab resulted in the development of a defined skeletal muscle myotube system derived from the hind-limbs of embryonic day 18 rat fetuses. [29] This culture system provided a starting point for studying myotube physiology in a serum-free environment, but was limited in its ability to reproduce *in vivo* mature muscle characteristics. This previous work has shown, and is supported by observations from our experiments, that MyHC class switching from an embryonic isoform to a more adult like phenotype occurs over a 45-50 day culture period, after which the culture will express ~25% neonatal/adult MyHC isoforms. [29] While the use of DETA provides for long term muscle cultures, up to a maximum of 6 months in our hands (data not shown), it would be more time efficient and cost effective to have myotube cultures that express adult MyHC isoforms at earlier time points.

We have shown here that skeletal muscle myofibers derived from the TA of adult rats undergo a more accelerated MyHC isoform switching than do their embryonic muscle

counterparts under these serum-free media conditions and using a defined organosilane surface. Figure 7 shows both mature MyHC fast-twitch fiber expression and MyHC slow-twitch fiber type expression by day 12 in culture. This equates to a 75% decrease in time to maturity over embryonic derived muscle in serum-free conditions. We have also shown that 73.89+/-4.6% of myotubes in culture at day 12 expressed the mature MyHC fast twitch isoform, and 10.67 +/- 1.1% of those also expressing the adult type slow twitch isoform; a 3-fold increase in mature MyHC expression over an embryonic-derived culture system. [29, 33, 46] The ratio of fast to slow twitch isoform expression in myotubes maintained for 12 DIV in serum-free conditions mirrors data attained using serum-containing media at equivalent time-points. [43]

It was observed that 31.0% +/- 19.1% of the 12 DIV myotubes contracted spontaneously at any one time, as well as approximately 70% of myotubes contracted under broad field stimulation. [47] Further studies have shown that this new culture technique and medium formulation allows for the interrogation of myotube contractile force and fatigue. [47] This system will allow for ease of isolation of tissue, reduction in culture cost for media, reduction in time to analysis, and increased relevance to mature muscle, making it a more representative culture system to study adult myopathies. This formulation is also compatible with neuronal culture, so it should facilitate multi cellular construct formation *in vivo*. Furthermore, the adult nature of the myoblasts used in this system facilitates the accurate investigation of adult myopathies, muscle regeneration and neuromuscular junction physiology. This opens new possibilities of directly culturing cells from transgenic animals after they began exhibiting their deficits or changes due to genetic manipulation.

Conclusion

In conclusion, this serum-free skeletal myotube culture system provides a model by which to investigate the role specific growth factors and drugs might play on adult physiological processes such as myotube maturation as well as disease processes associated with muscle regeneration including the muscular dystrophies.

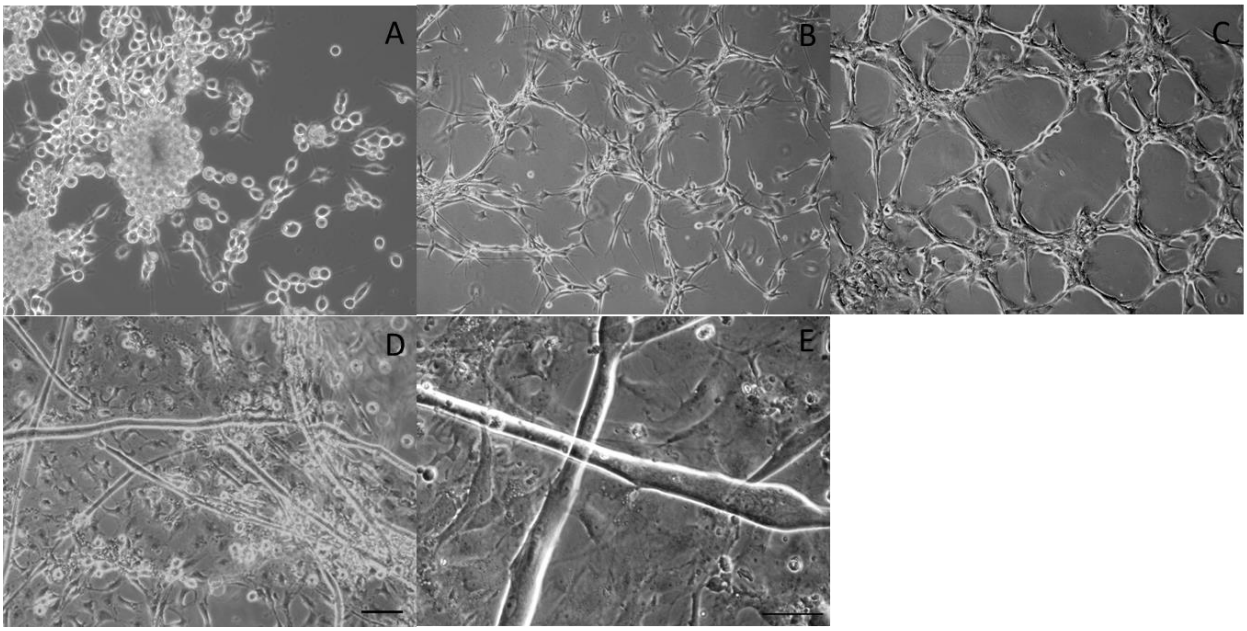


Figure 3: Phase images of myoblasts and myotubes derived from the Tibalias anterior of adult Sprague Dawley rats. Phase images of proliferating myoblasts at (A) 2 DIV, (B) 4 DIV, (C) 8 DIV. Phase images of fused myoblasts at 12 DIV (D,E). Scale Bar 50 µm, the scale bar in panel D pertains to panels A-D.

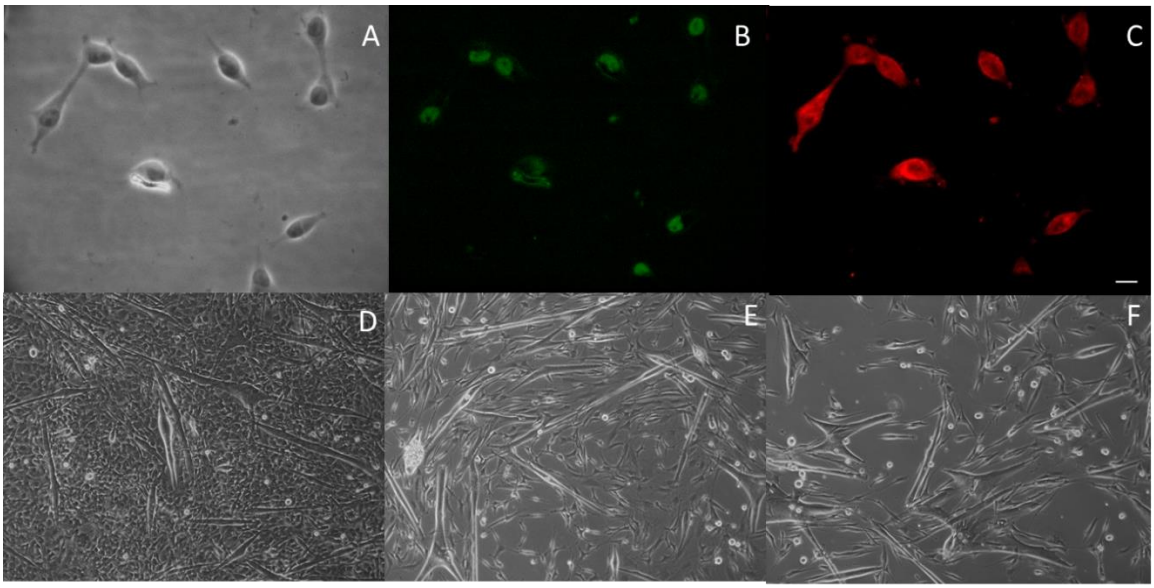


Figure 4: Pax-7 and Myo-D immunostaing of 2 DIV myoblasts derived from plate three of serial plating. (A) Phase image of myoblasts, (B) Pax-7 transcription factor staining, (C) Myo-D muscle marker staining. Phase images indicating the relative purity of myotube cultures from serial plating at 12 DIV. (E) Plate 1, (D) Plate 2, (F) Plate 3. Scale bar 10 µm.

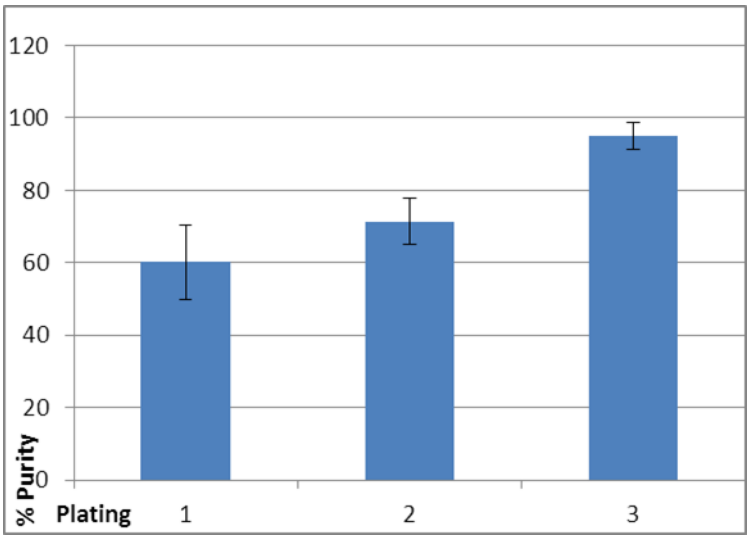


Figure 5: Indicates the relative purity of the culture starting at 60% purity at plate 1 going to 94% purity at plate 3

.

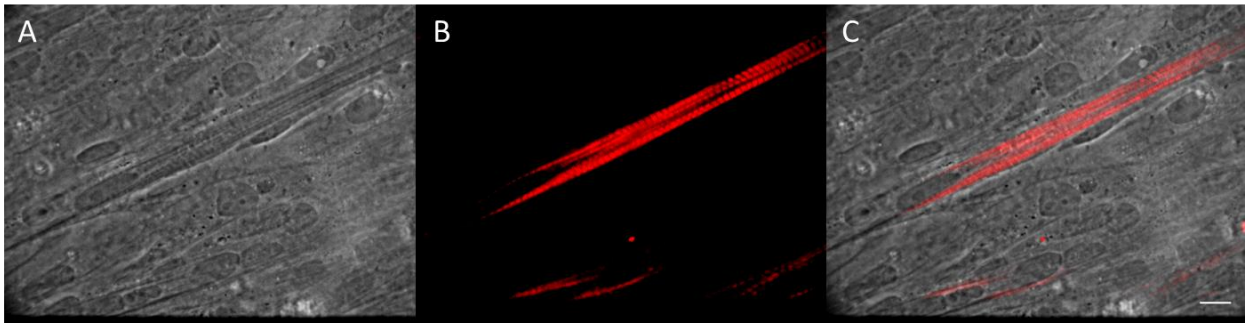


Figure 6: (A) Phase image (B) MYHC immunostaining and (C) overlay of 14 DIV myotubes. A4.1025 MyHC antibody staining showing highly striated myotubes. Striations are an indication of a highly organized contractile apparatus and mature myotubes. Scale Bar 50 µm.

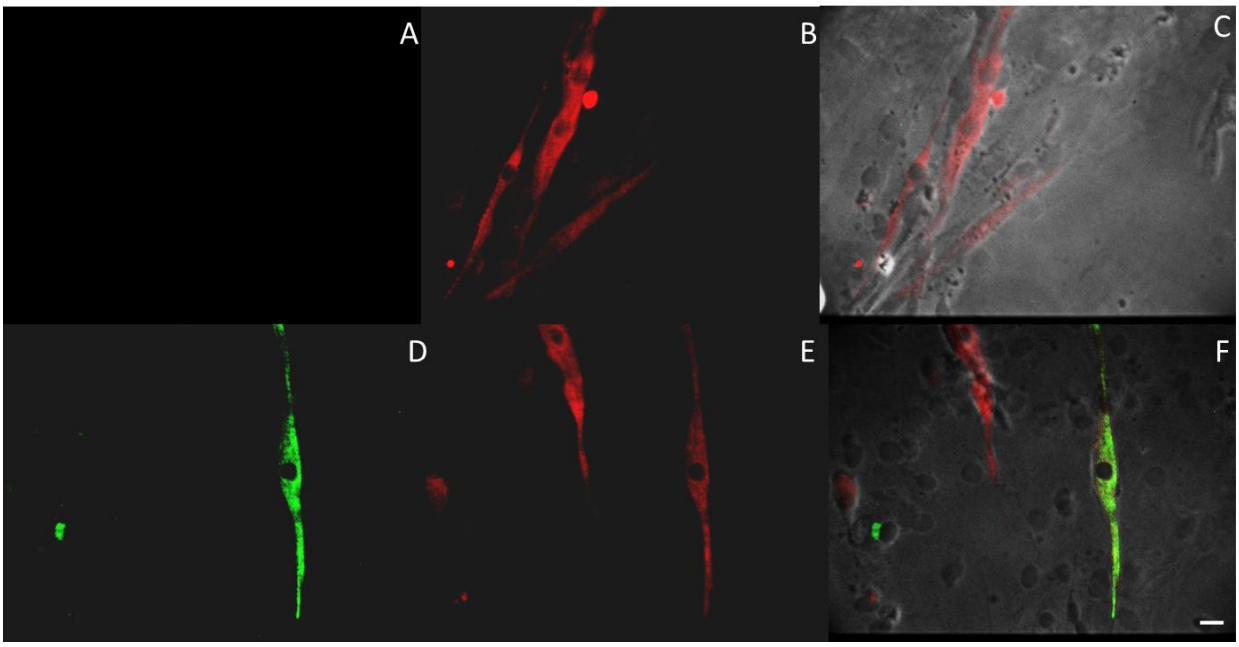
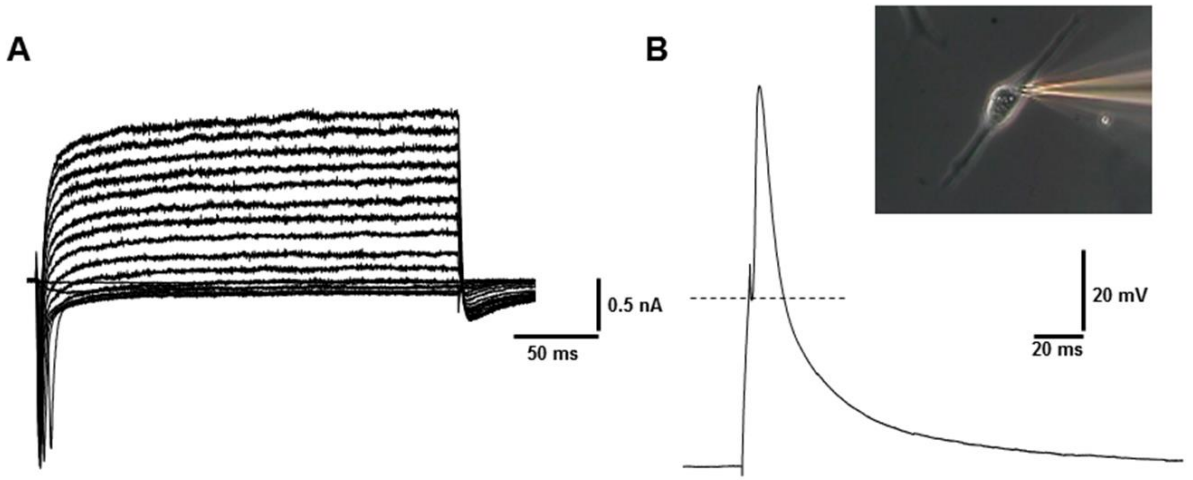


Figure 7: Immunostaining of 12 DIV myotubes for adult MyHC isoforms. Panels (A and D) show staining for adult MyHC type I isoform for two separate coverslips. Panels (B and E) show staining for adult MyHC type IIb isoforms for those same myotubes. Panels (C and F) are the composite images of those cultures. Cultures indicate at 12 DIV 73.89 ± 4.6% of myotubes stain for type IIb and approximately 10.67 ± 1.1% of those also stain for type I isoform. Scale Bar 20 µm.



C

Outward currents (pA)	Inward currents (pA)	AP amplitude (mV)	Resting Vm (mV)	Cm (pF)	Rm (mΩ)
1816.3 ± 202.2	-2998.7 ± 307.9	46.7 ± 5.8	-59.9 ± 0.8	72.7± 6.6	125.0 ± 11.5

n = 9; AP: action potential; Vm: membrane potential; Cm: Membrane capacitance ; Rm: Membrane resistance

Figure 8: Patch clamp electrophysiology of adult tissue derived myotubes. (A) Representative voltage clamp trace obtained from a 14 DIV myotube. (B) Representative current clamp trace of same myotube. Inset is a phase image of the myotube. (C) Statistics showing mean parameters derived from n = 9 myotubes. (Data acquired by Min Lin)

CHAPTER THREE: MECHANISTIC INVESTIGATION OF ADULT MYOTUBE RESPONSE TO EXERCISE AND DRUG TREATMENT IN VITRO USING A MULTIPLEXED FUNCTIONAL ASSAY SYSTEM

Introduction

The development of advanced cell culture systems, capable of accurately measuring the functional output of a variety of cell and tissue types, is one of the targets of recent efforts to engineer complex, physiologically relevant *in vitro* assay systems for drug-development and disease modeling applications. [48] Skeletal muscle is one tissue for which *in vitro* analogues have been developed which are capable of effectively recapitulating the functional maturation and performance of the native tissue, through controlled stimulation and measurement of the contractile output of embryonic [49, 50] neonatal/adult [51-53] and human cultured cells. [54] Such systems represent exciting possibilities for drug toxicity/efficacy studies, as well as for the modeling of human disease states *in vitro*. New *in vitro* systems that extend these studies to allow functional assessment of muscle maturation and recovery from injury would also be beneficial for advanced exercise physiology applications.

Importantly, the integration of these functional skeletal muscle models with established long-term stimulation parameters and/ or hypertrophic drug treatments such as creatine will allow investigators to effectively simulate and assess muscle building and exercise *in vitro* [10, 11, 53] Application of such technology could have important implications for the study of the training regimes used by elite athletes, as well as for developing appropriate therapies for patients with muscle wasting conditions such as muscular dystrophy and sarcopenia. Previous work in this field has focused on assessment of the molecular responses and metabolic output of skeletal muscle cells subjected to bouts of *in vitro* exercise and/or drug treatment. [20, 55-57] In

creatine studies, increased transcript expression for contractile machinery proteins, such as Myosin Heavy Chain (MyHC), have been observed in treated cultures. [56, 58] Similarly, an increase in gene expression for markers of mitochondrial biogenesis and measurements suggesting increased glucose metabolism in cells subjected to *in vitro* exercise protocols has been reported. [10, 11, 59] However, to date very little work has been done on directly measuring the cell's functional response to such *in vitro* stimulation regimes. Hence, there remains some ambiguity as to whether the molecular and metabolic changes observed in response to *in vitro* exercise correspond to a measureable change in the contractile behavior of cultured cells. Moreover, no published work has yet studied the effect of *in vitro* exercise on muscle fiber (myotube) fatigue, meaning little is known about the fatigability of cultured muscle, or how it compares to the *in vivo* tissue. Verifying an improvement in cultured skeletal muscle myotube contractility in response to exercise, as well as establishing a physiologically accurate model of fatigue, would be an important step in the development of a functional systems to model exercise physiology *in vitro*.

In this study, a previously reported [60, 61] serum-free, multiplexed assay system utilizing silicon cantilevers was used to record the contractile output of adult rat skeletal muscle cells subjected to long-term stimulation protocols and creatine treatment. Both long-term stimulation and creatine treatment produced improved peak force (PF) and time to fatigue (TTF) data, as well as increased transcript expression for MyHC and mitochondrial biogenesis markers in line with previously published reports. These data highlight not only the functional improvement of muscle cells subjected to bouts of *in vitro* exercise, but also their biologically correct hypertrophic response to treatment with a known muscle building compound. The use of

multiplexed micro-scale cantilevers facilitates the interrogation of individual myotubes [21, 56, 58, 62], allowing analysis of the effects of exercise and/ or drug treatment at the single cell level but since each cantilever is a separate experiment, with a high statistical power. Mathematical scaling of measured outputs is possible using this model, enabling data obtained at the single cell level to be used in predictions of whole tissue responses to physical and chemical challenges.

Materials and Methods

Cantilever Fabrication and Surface Preparation

Cantilever chips were fabricated from silicon wafers, using previously published methods. [33, 50] Briefly, 100 mm Silicon-On-Insulator (SOI) wafers had a 4 μm thick device layer and buried oxide layer of 1 μm. In the device layer, the cantilevers were patterned using S1818 photoresist and etched using deep reactive ion etching (DRIE). To protect the cantilevers during processing, a 1 μm layer of silicon dioxide was deposited on the top surface. The bottom of the wafer was similarly patterned and etched so that the silicon beneath the cantilevers was removed, producing a large window underneath. The oxide layers were removed using a buffered oxide etch solution. The resulting structures were silicon cantilevers that could be imaged from above and interrogated with a laser from below.

These cantilevers, as well as the coverslip controls used in this study, were coated with an amine-terminated alkylsilane, (3-Trimethoxysilyl propyl) diethylenetriamine (DETA) (United Chemical Technologies, Bristol, PA) using methods previously described. [5, 35, 36, 50] DETA is an analog of spermidine, which is known to promote long-term survival of cells *in vitro*, and

has been previously validated in the culture of various cell types. [31, 32] Thus it was used in this study as a surface coating, to promote cell adhesion and survival.

The silanization of the cantilever surfaces with DETA was performed using a solution of 0.1% (v/v) DETA-silane in toluene, heated to 70°C for 30 minutes, followed by a series of toluene rinses and reheating to 70°C for 30 minutes in fresh toluene to remove any unreacted silane. The surfaces were oven cured at 110°C for 2 hours and stored in a desiccator until use. The surface coating composition was verified using X-ray photoelectron spectroscopy and contact angle goniometry.

Dissection and Cell Culture

Adult rats were euthanized using excess CO_2 in accordance with IACUC standards. The *Tibialis Anterior* muscles were isolated from the left and right hind limbs. The muscles were placed in a dish containing phosphate buffered saline (PBS), and scalpels were used to remove excess skin and hair. The muscles were then transferred to a Petri dish containing an enzyme solution consisting of 20 mL Dulbecco's Modified Eagle's Medium (DMEM), 25 mg collagenase type I (Worthington, Lakewood, NJ) and 1 mg neutral protease (Worthington, Lakewood, NJ). The tissue was minced in this dish, and the resulting fragments were transferred to a 50 mL conical tube. This tube was placed horizontally in a 37°C water bath and left for 1 hour at 100 rpm. Following this enzymatic digestion, the tube was removed from the water bath and its contents were mechanically triturated and filtered through a 100 μm mesh to remove undigested tissue fragments. The tube was then centrifuged at 400 G for 4 minutes. The cell pellet was re-suspended in 20 mL of DMEM, and then separated into 2 100 mm Petri dishes. These dishes were placed in an incubator for 45 minutes to allow adhesion of fibroblasts to the

surfaces, and so enrich the non-adherent population for myogenic cells. The dishes' contents were then collected in a 50 mL tube and centrifuged again at 400 G for 4 minutes. The cell pellet was re-suspended in 4 mL of proliferation medium (Table 3) and plated onto 4 DETA coverslips in a 12 well plate and was left to incubate overnight. The following day, the medium was collected into a 50 mL tube and centrifuged at 400 G for 4 minutes. The supernatant was removed and the cell pellet was again re-suspended in proliferation medium. The cells were plated onto 7 coverslips and 6 cantilever chips and left overnight. The following day, the medium on these plates was aspirated and 1 mL fresh proliferation medium was added to each well. Once the plated cells achieved confluency and myotubes began to form (at about 6 days after the first plating), 500 µL of differentiation medium (50:50 Neurobasal:L15 medium plus 10 ng/ml insulin-like growth factor) was added to each well. Every 4 days subsequently, half the culture medium on each well was replaced with fresh differentiation medium. Cultures were maintained for 7-9 days after addition of differentiation medium prior to analysis.

Table 3: Composition of serum-free growth medium components in 50:50 neurobasal:L15 medium

Component	Company	Catalogue #	Quantity
Antibiotic-antimycotic	Invitrogen	15240062	5 ml
aFGF	Invitrogen	13241-013	20ng/mL
Calcium chloride	Fisher	10035-04-8	500ng/mL
VEGF	RND systems	293-ve-010	20ng/mL
bFGF	RND Systems	3339-FB-025	40ng/mL
CNTF	Cell Sciences	CRC 401B	40ng/mL
NT-3	Cell Sciences	CRN 500B	20ng/mL
NT-4	Cell Sciences	CRN 501B	20ng/mL
GDNF	Cell Sciences	CRG 400B	20ng/mL
BDNF	Cell Sciences	CRB 600B	20ng/mL
CT-1	Cell Sciences	CRC 700B	20ng/mL
LIF	Sigma	L5158	20ng/mL
Vitronectin	Sigma	V0132	100ng/mL

Chronic Low-Frequency Stimulation (CLFS) Protocol and Creatine Treatment

After 7-9 days in differentiation medium, 2 cantilevers and 1 coverslip were placed into a chronic low-frequency stimulation apparatus. The remaining cantilevers and coverslips were used as unstimulated controls. The apparatus consisted of carbon electrodes housed in a 6 well plate and connected to a pulse stimulator. Electrical pulses had a magnitude of 3 V, with a pulse width of 1.5 ms; 5 pulses were delivered to the cultures at 20 Hz every 4 seconds. This stimulation protocol was devised based on previously published work [63, 64]. The cultures were incubated at 37°C within this apparatus for 4-7 days, and the medium was replaced every other day. Similarly, cultures were fed with medium supplemented with 40 mM creatine (Sigma) following 7-9 days in differentiation medium. Cultures were maintained in creatine containing medium for a further 4-7 days before analysis and the medium was replaced every other day.

Peak Force and Time-to-Fatigue Calculation

A laser and photo-detector system, adapted from Atomic Force Microscopy technology, was utilized to calculate peak force (PF) of individual myotube contractions, as well as the myotubes' time-to-fatigue (TTF) (Figure 10). Application of this system for similar studies has been described in detail elsewhere[60, 61]. Each cantilever chip was transferred to a heated stage housed within a modified electrophysiology rig. The culture dish on this heated stage was filled with Differentiation Medium (+10 mM HEPES) to maintain the cells during analysis.

Briefly, the automated system consisted of a class 2 photodiode laser positioned below the stage so that the beam was focused on the cantilever tip and reflected into the center of a 4-quadrant photo-detector. [49, 65]. A pulse stimulator was used to generate pulses capable of

eliciting contraction in the cultured myotubes. The biphasic pulses were 3V in magnitude and had a pulse width of 40 msecs with a frequency of 1 Hz. The resulting cantilever deflection was measured in terms of laser displacement (in V). Software was written in labVIEW (National Instruments) to control laser and photo-detector positioning to allow scanning across all cantilevers on each chip for contractile activity. The acquired raw data was converted into a direct measurement of force using a modified Stoney's equation. Previous work[60] had demonstrated that measurements analyzed using the Stoney equation with a similar cantilever system exhibited strong correlation with data generated using finite element analysis, which takes into account a more detailed set of cell dimensions when calculating force production. The assumptions made for analysis using Stoney equation were therefore deemed acceptable for determining changes in force production per cell in response to creatine treatment and long-term stimulation.

To determine which cantilevers had contracting myotubes, the chip in the culture dish was first subjected to broad field electrical stimulation using the stimulation parameters listed above. The cantilevers were scanned for 5 seconds each to elicit and record contractile responses. The active cantilevers were then scanned, with continuous electrical stimulation, until their peak force was reduced by half. Thus, the time-to-fatigue was calculated by measuring the time elapsed in which a 50% reduction in peak force was observed. Subsequent bright-field images were collected of each contracting cantilever and those supporting multiple myotubes were discounted from further analysis to ensure the calculation of force generated per myotube was accurate. Preliminary studies were performed to ensure that this 50% reduction in PF was indicative of myotube fatigue and not physical damage to the myotubes such as tearing of the

sarcolemma induced by stimulation. After a 7 minute rest and recovery period approximately 88% of original PF was observed, suggesting force reduction was indeed due to cellular exhaustion (Data not shown).

Immunocytochemistry

Cells were fixed for 10 minutes in 4% paraformaldehyde in PBS. These cultures were then permeabilized with 0.1% Triton-100 in PBS for 20 minutes followed by a 20 minute incubation in 5% donkey serum with 0.1% Bovine Serum Albumin (BSA) in PBS (Blocking solution). The blocking solution was then removed and the cells were further incubated overnight at 4°C in a primary antibody solution consisting of a mouse monoclonal antibody against MyHC (DSHB A4.1025) at a 1:10 dilution in blocking solution. The cells were washed 3 times with PBS and then incubated in the dark for 2 hours at room temperature with a secondary antibody solution consisting of a donkey anti-mouse Alexafluor-594 conjugate diluted 1 in 200 in blocking solution. The cells were washed 3 times in PBS before being mounted on microscope slides using a hard-set DAPI containing mounting medium (Vectashield) for visualization.

Quantitative Polymerase Chain Reaction (qPCR)

Samples designated for PCR analysis were homogenized in TRIzol reagent and the RNA isolated according to the manufacturer's protocol. Purified RNA was converted to cDNA and the qPCR reactions run using the BIO-RAD iScript™ One-Step RT-PCR kit with SYBR® Green (BIO-RAD Laboratories, Hercules, CA - catalog #170-8892) according to the manufacturer's protocol. Briefly, the PCR reaction mixture (12.5 µL 2x SYBR® Green RT-PCR reaction mix, 0.5 µL iScript reverse transcriptase, 0.75 µL forward primer, 0.75 µL reverse primer, 10 ng RNA

template and nuclease-free water to a total reaction volume of 25 µL) was prepared for each gene to be analyzed from each sample in triplicate wells of a 48 well plate. PCR primers used in this study were taken from those previously tested and verified (Table 4). [66] The reaction plate was then transferred to an MJ Mini thermal cycler (BIO-RAD) and incubated at 50°C for 10 minutes and 95°C for 5 minutes before being cycled 40 times at 95°C for 10 seconds, followed by 60°C for 30 seconds. The qPCR protocol and fluorescent output from the reactions were implemented and recorded using Opticon Monitor software (BIO-RAD).

Table 4: PCR primer sequences

Gene	Sequence (5' to 3')
MyHC 1 FWD	GCCAACTATGCTGGAGCTGATGCCC
MyHC 1 REV	GGTGCGTGGAGCGCAAGTTTGTCATAAG
MyHC 2A FWD	GGCACAAAACTGCTGAAGCAGAGGC
MyHC 2A REV	GGTGCTCCTGAGGTTGGTCATCAGC
MyHC 2B FWD	GCAGCTACTGGATGCCAGTGAGCGC
MyHC 2B REV	CTGGACGATGTCTTCCATCTCTCC
MyHC 2X FWD	GGCAGCAGCAGCTGCGGAAGCAGAGTCTGG
MyHC 2X REV	GAGTGCTCCTCAGATTGGTCATTAGC
PGC-1 α FWD	GTGCAGCCAAGACTCTGTATGG
PGC-1 α REV	GTCCAGGTCATTCACATCAAGTTC
ERR-γ FWD	TGACTTGGCTGATCGAG
ERR-γ REV	CCCAGGATCAAGATTTCC

The threshold cycle (C_T) was defined as the fractional cycle at which the fluorescence generated by the binding of SYBR® Green molecules to double-stranded DNA exceeded a fixed threshold above the baseline. The amount of each target gene present in the sample was quantitated using the comparative C_T method.[67]. Mean C_T values from triplicate reactions for

each sample were determined and normalized to that of an endogenous housekeeping gene (β-actin) run in parallel. The amount of target amplification relative to the endogenous control was calculated using the formula $2^{-\Delta\Delta C}{}_{T}$.

Image Analysis

The width of myotubes cultured on cantilevers was measured as a means to estimate cross-sectional area (CSA). Phase-contrast images were collected using a Zeiss Axiovert 200 inverted microscope and analyzed using ImageJ software. Lines were drawn vertically across each image at points 1/3 from each edge of the field of view. The width of each myotube that crossed either of these lines was measured at that point to eliminate selection bias.

Statistical Analysis

All experiments were repeated at least 3 times using cultures prepared on different days, with tissue from different animals. Differences in force and time-to-fatigue measurements among the three conditions ("Untreated", "CLFS", "Creatine-Treated") were each evaluated statistically using one-way repeated measures ANOVA ($\alpha = 0.05$). Following the repeated measures ANOVA with a statistically significant F-statistic, means were compared using Tukey's HSD test for multiple comparisons ($\alpha = 0.05$). QPCR expression data for CLFS and creatine-treated samples were expressed relative to untreated controls; as such, control values were always 1. For these experiments, a one-sample T-Test was used to calculate whether or not mean expression levels were statistically different from 1. All values noted in the text are expressed as mean ± standard error.

Results

Primary adult rat skeletal muscle myotubes were maintained on silicon cantilevers for 14 days in differentiation medium before being analyzed for contractile function. Immunocytochemical analysis at this time-point demonstrated the development of a distinct striated morphology, indicating the formation of mature and functionally capable sarcomeres within these cells (Figure 9). This end-point enabled maximal levels of myotube differentiation and maturation (approximately 70% of cultured myotubes responded to broadfield stimulation), while minimizing myotube loss due to detachment and thereby maximizing data collection.

Functional Assessment of Cultured Myotubes

Using the described protocol, contractile activity was recorded from the cultured myotubes, verifying the ability for this system to measure the functional contraction (Figure 10A). Continuous recording of stimulated cantilever cultures indicated substantial reductions in PF measurements over extended periods that enabled the assessment of skeletal muscle fatigue in real-time (Figure 10B). Analysis of functional data from the experimental conditions demonstrated that both creatine treatment and long-term stimulation produced significant increases in PF compared with untreated controls (Figure 11A, $p<0.02$). No significant differences were observed between creatine treated and CLFS cultures over 4 or 7 day applications ($p>0.5$). Similarly, creatine and CLFS regimens were found to statistically increase the TTF capacities of cultured myotubes roughly 5 fold, when compared with untreated controls (Figure 11B, $p<0.001$). No significant differences in TTF were observed between any of the treatment regimens examined ($p>0.6$). These combined data suggest that each treatment regime

examined was capable of promoting the same magnitude of functional improvement in myotube performance over the time-course investigated in this study.

Assessment of Hypertrophy in Cultured Myotubes

During the culture of the primary adult skeletal muscle cells, it was observed that creatine treated cultures developed substantially thicker myotubes than all other conditions examined (Figure 10). Image analysis confirmed that 4 and 7 day creatine treated cultures promoted a significant increase in myotube CSA compared with both CLFS and untreated controls ($n = 3$, $p < 0.0001$). Both creatine treatment and CLFS were found to improve functional performance but only creatine treatment produced a significant hypertrophic response in the cultured myotubes. The collected data therefore implied that the observed differences in functional performance between treated and untreated cultures were due to the adaptation of different molecular mechanisms activated through the two methods of myotube stimulation employed.

Gene Expression Analysis of Cultured Myotubes

Data collected from qPCR experiments highlight that both creatine treatment and CLFS have a significant effect on transcription profiles within primary adult myotubes *in vitro*. Application of creatine to cultured cells for 4 days produced a 2 to 4 fold increase in all MyHC transcripts examined ($n = 4$, $p < 0.002$, Figure 13), whereas four day CLFS protocols were found to promote an upregulation in estrogen related receptor (ERR) γ transcripts ($n = 3$, $p < 0.03$). The differences observed in upregulated genes within these cultures highlight the alternative pathways activated by the treatment regimens examined. The collected data confirmed the physiological differences in myotube CSA discussed earlier, and is in line with previously

published work assessing the hypertrophic effect of creatine on skeletal muscle and the cellular response to chronic exercise regimes *in vivo* and *in vitro.* [21, 62, 68]

Discussion

The development and widespread application of an *in vitro* model of exercise and fatigue capable of modelling skeletal muscle functional response would be of substantial benefit to exercise physiology studies and investigations of muscle building and wasting. While three-dimensional cultures have obvious advantages for modeling whole tissue behavior, multiplexed, two-dimensional systems are more easily adapted for application in high-throughput drug efficacy/toxicity studies. Furthermore, the ability to record contractile data from individual myotubes is useful for examining alterations in individual muscle fiber phenotype in healthy and diseased states. Multiplexed cantilever assays provide information on maturation and performance variability between myotubes, and provide multiple data points for the generation of more statistically relevant observations. Analysis of 3D myotube bundles either relies on integration of invasive force transducers or lower resolution optical mapping. The non-invasive nature of the cantilever analysis system means cultures can be reanalyzed at multiple time-points for continued assessment of individual cells. The high resolution data acquisition system, utilizing laser and photo-detector hardware, enables higher sampling rates (1000 Hz) than optical techniques (5 – 500 Hz), facilitating more in depth analysis of changes in functional contractile waveforms. Finally, 2D systems such as the one described here are likely to be more readily integrated with complex, multi-cell-type platforms for body-on-a-chip applications [48, 69, 70] and for developing phenotypic assays for drug evaluation.

Previous work in this lab has developed and optimized this cantilever system for assessing embryonic myotube development. [61, 65] While such a culture model is beneficial as a proof of principle, application of this technology to investigate age-related myopathies and exercise regimens in aged tissue would benefit from integration of the cantilever platform with cells derived from adult tissue. This study builds on the published data to utilize a cell population derived from adult tissue within a functional *in vitro* assay system designed to quantify contractile responses in real-time and over extended periods in response to both drug and exercise treatments.

The collected data demonstrate that this multiplexed cantilever array can be used to effectively monitor rates of muscle fatigue *in vitro*. While previous work from a number of groups [59, 63, 64, 71] has used long-term stimulation and creatine treatment to evaluate molecular changes during cellular development *in vitro*, this study is the first to assess the effect of such treatment applications on long-term functional performance. Such data is important for confirming that the molecular changes observed *in vitro* correlate to alterations in functional output, thereby validating such studies for producing predictions of whole tissue responses *in vivo*.

Analysis of the cultured cells highlights that both peak force generation and rates of fatigue improve significantly in response to either creatine or long-term stimulation protocols. The observed improvements to myotube output and endurance correlate to established data evaluating the effects of similar treatments *in vivo*. [22, 72-75] Assessment of gene transcription profiles in treated cells suggests that the improvements to functional output induced by these treatments are facilitated by activation of different molecular mechanisms. Creatine application

promoted a hypertrophic response in the cultured cells, leading to the upregulation of contractile protein apparatus and the maturation of sarcomeric structures, resulting in myotubes with significantly larger cross-sectional areas compared with untreated controls (Figures 12, $p < .0001$). Long-term stimulation is known to initiate alterations in transcription of genes relating to mitochondrial biogenesis, such as ERRγ. [55, 68, 76] Upregulation of these genes likely induces increased mitochondrial presence within these cells, facilitating a greater endurance capacity. [55, 57] This understanding of the effects of long-term stimulation on skeletal muscle is supported by the data presented in this study (Figure 13).

Improvements to peak force observed in CLFS cultures suggest the repeated stimulation likely also had an effect on sarcomeric development in these cultures. No significant difference was observed in PF production between creatine treated cultures and those subjected to CLFS. However, significant differences in myotube CSA were observed between these culture conditions. Together, this data implies that creatine treatment promoted a hypertrophic response, leading to greater myotube volumes and therefore more cytoplasmic space for sarcomere formation resulting in greater PF production. Since no hypertrophic response or upregulation of MyHC transcripts was observed in CLFS cultures, the observed improvements in force likely result from improved development of the sarcomeres already present within these cells. Previously reported results indicate evidence for the upregulation of myosin synthesis in electrically stimulated *in vitro* cultures utilizing chick [19] and C_2C_{12} cell lines. [67] On the surface, these data seem contradictory to those presented in this manuscript. However, there are significant differences between this work and those published previously that inhibit direct comparison. Our protocol provides a defined, serum-free medium formulation which eliminates

many unknown factors contributed to the culture system by fetal bovine serum addition. The use of serum in the cited work may have led to the generation of significantly altered responses to electrical stimulation, due to the activation of different pathways by unknown factors in the medium. The use of our defined culture system obviates this issue, thereby providing a more controlled environment for investigating the direct effects of electrical stimulation on culture maturation and development. Furthermore, our functional assay system utilizes cells derived from adult rat tissue, which may demonstrate significantly different physiological responses to long term electrical stimulation than alternate cell sources.

This study provides baseline parameters for measuring endurance of single fibers *in vitro*. While previous studies have characterized fatigue parameters *in vivo*, this study is the first to do so for *in vitro* muscle fibers. As such, it was necessary to determine an appropriate level of peak force decline which could be taken as evidence of muscle fatigue. Using the described functional assay, it is not possible to characterize a 100% force reduction since the baseline fluctuations make such small recordings unreliable. Muscle failure, as is measured *in vivo,* is not possible to investigate in this system since failure does not necessarily confirm that the muscle fibers are no longer contracting, but rather that they are no longer able to generate sufficient force to overcome the mass of the animal. It is not clear at what point this occurs and how much variability exists between different muscle types and between individuals. The parameters assessed in this study provide the means to compare between experimental repeats and facilitate normalization of measurements to a consistent evaluation of cellular exhaustion to potentially produce a more reliable assay of muscle function than some *in vivo* models. Measurement of a 50% reduction in peak force is sufficient to reliably compare fatiguability between experimental

samples while restraining experimental lengths to a time-frame which would facilitate investigation of multiple cellular phenotypes and/or treatments on a given day.

This system demonstrates, for the first time, the capacity to directly measure both absolute peak force and rates of fatigue in individual cultured myotubes. The developed system thereby allows full functional assessment of skeletal muscle responses to both small molecule treatment and exercise regimes controlled via broad field electrical stimulation. The successful application of cells derived from adult tissue and maintained in serum-free defined culture conditions makes this model appropriate for investigations of adult myopathies and/or drug efficacy and toxicity studies by utilizing a phenotypic assay system. The model is also appropriate to combine with molecular biological techniques for advanced mechanistic studies, as well as target identification in drug evaluations. Further development of this model to improve its high-throughput nature will likely be beneficial to future pre-clinical screening technologies and exercise physiology protocols.

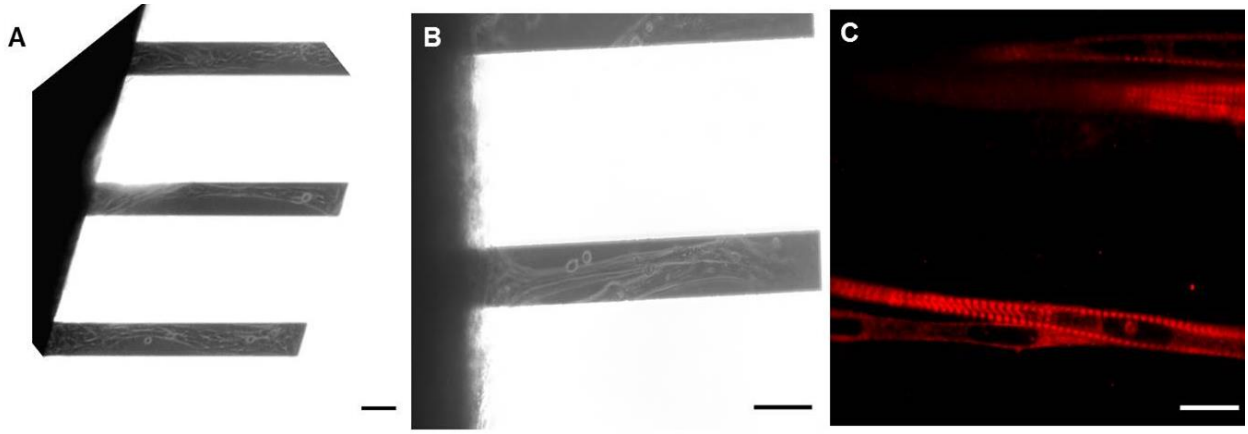


Figure 9: Images of adult derived skeletal muscle myotubes. Phase contrast images of myotubes on silicon cantilevers at A) 10x and B) 20x. C) Immunostaining of cultures using an antibody against MyHC all classes (A4.1025, DSHB) indicating highly striated and mature muscle fibers. Scale Bar = 100 µm.

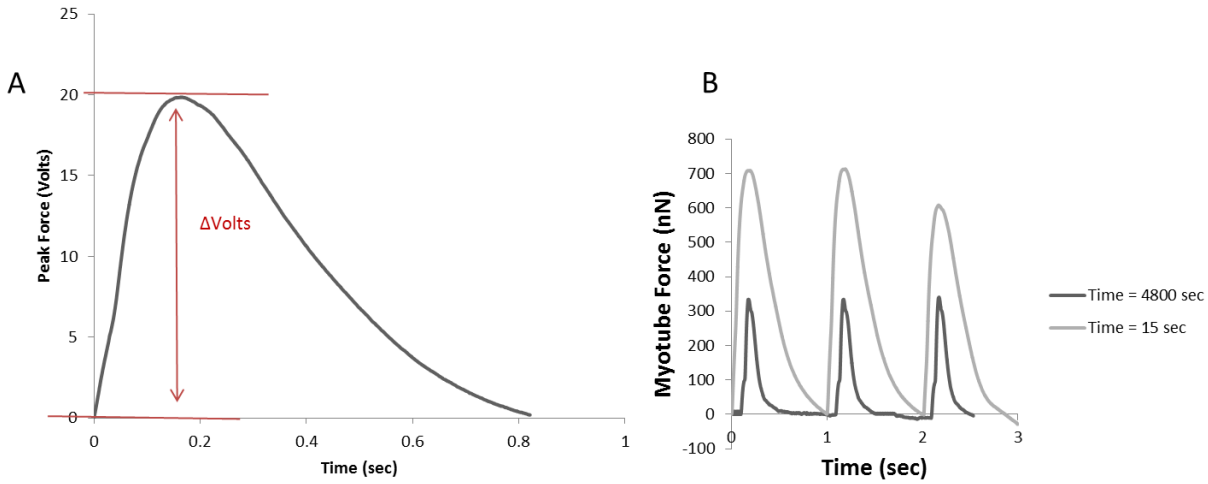


Figure 10: Measurement of force generation and fatigue. A) A representative peak force trace indicating how peak force is measured as a change in voltage by the photo-detector. This change in voltage can be converted using a modified version of Stoney's equation to force in nN. B) Representative traces of peak force at initial time (15s) and when peak force has reached a 50% reduction in force (4800s). Recordings were measured in Volts then converted to force using Stoney's equations and re-plotted.

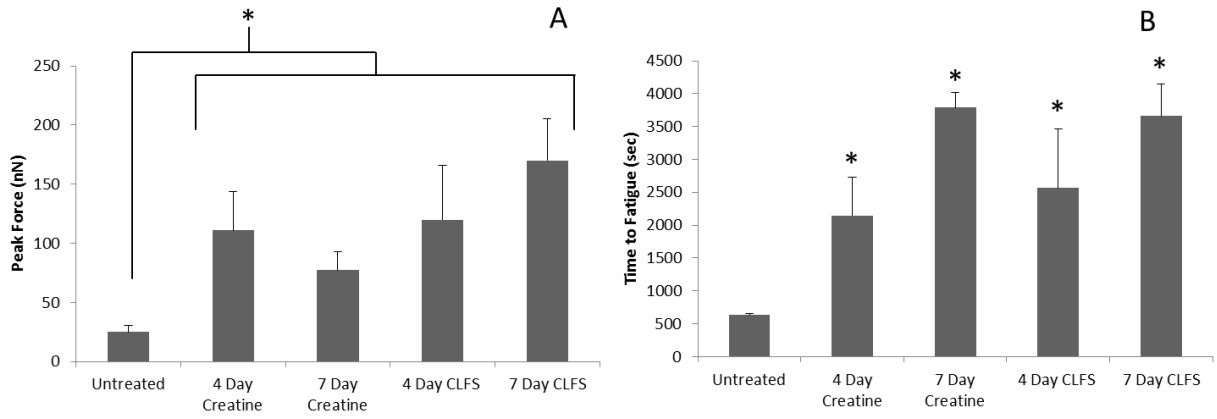


Figure 11: Comparison of peak force and fatigue. A) Graphical representation of peak force changes of four and seven day creatine and CLFS treatment regimens $p<0.02$. B) Graphical representation of time to fatigue changes of four and seven day creatine and CLFS treatment regimens $p<0.001$.

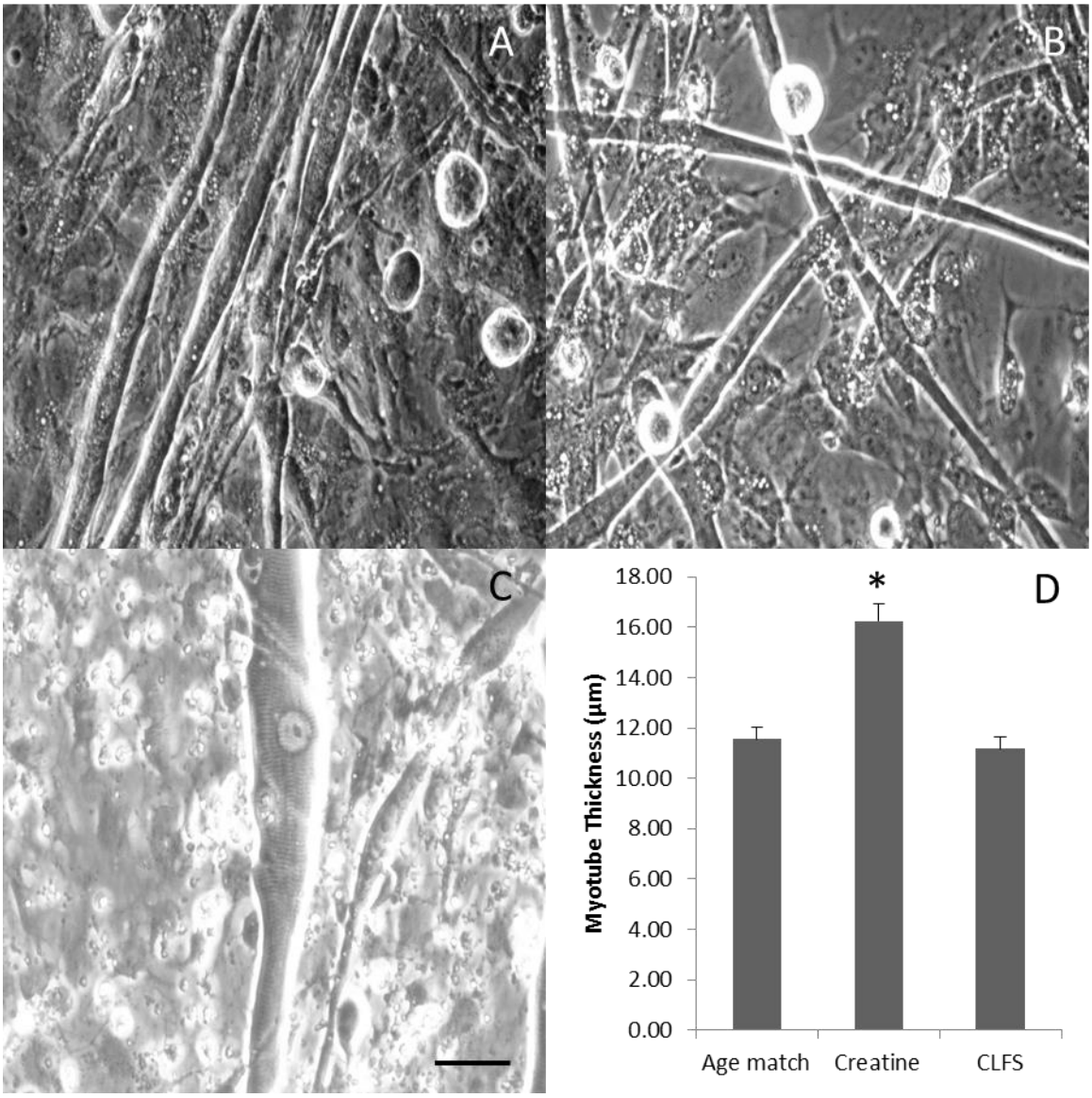


Figure 12: Assessment of hypertrophy in cultured myotubes. Phase images of multinucleated myotubes in A) control, B) CLFS, and C) creatine treated cultures. Scale bar = 50 µm. D) Graphical comparison of myotube thickness for comparison of hypertrophy $p<0.001$.

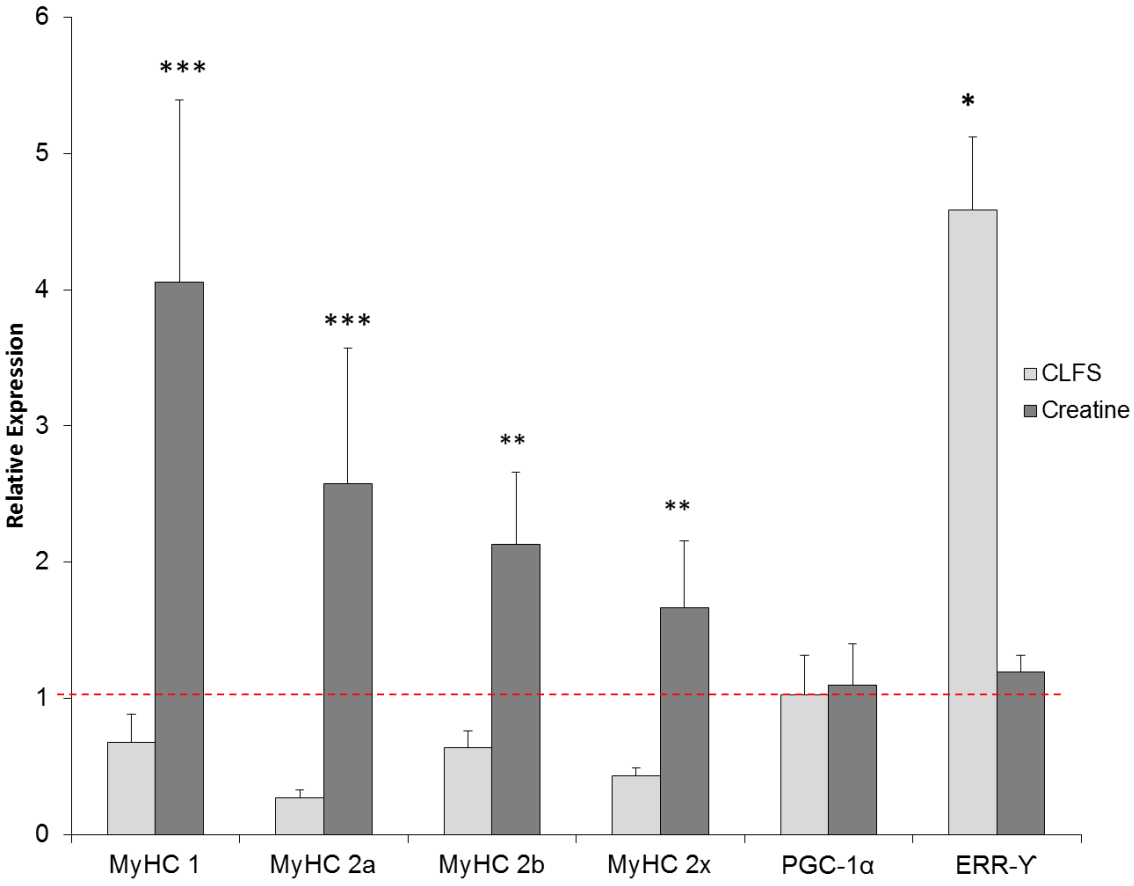


Figure 13: Gene expression analysis of cultured myotubes. The gene expression profiles for myotubes undergoing a four day treatment of either CLFS or creatine were assessed using real-time quantitative PCR analysis. All MyHC genes were significantly upregulated under creatine treatment ** $p<0.002$, *** $p<0.0001$. CLFS treatment resulted in a greater than fourfold upregulation in ERR-γ transcripts, * $p<0.03$. This PCR analysis suggests different mechanisms of action for equivalent changes in functional outputs.

CHAPTER FOUR: A MULTIPLEXED HIGH-CONTENT FUNCTIONAL IN VITRO ASSAY TO ASSESS THE EFFECTS OF PGC-1α MISREGULATION ON MOUSE MYOTUBE ENDURANCE

Introduction

The development of *in vitro* assay systems capable of accurately and reliably measuring the functional output of a multitude of cell types is important in the advancement of drug discovery and toxicity studies. Skeletal muscle is one cell type in which *in vitro* systems adept at recapitulating *in vivo* functional output has been well characterized. Tissue engineering of skeletal muscle has made considerable progress over recent years in the ability to integrate these cells into functional devices which are capable of measuring force output of single myotubes as well as myofibers in 2D and 3D *in vitro* environments[50, 54, 61, 65]. Current advances in these *in vitro* systems have made great strides in scaling up content recovery while scaling down the amount of tissue needed for functionality. These systems represent exciting avenues for the modeling of human disease and drug efficacy studies.

In vitro muscle culture systems have been successful in recapitulating the *in vivo* transcriptional and translational effects that occur as a result of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) mis-regulation. Specifically, the alterations in downstream proteins responsible for muscle and fat development as well as mitochondrial biogenesis have been interrogated[77, 78]. Simultaneously, in vivo/in situ studies have also shown that deviations in PGC-1α expression effect mitochondrial biogenesis and subsequently the endurance of skeletal muscle. The modeling of disease states has benefitted greatly from the ability to culture cells derived from protein deficient, "knockout", tissue. *In vitro* systems have also shown the ability to measure functional force output of disease state and healthy skeletal

muscle tissue[79]. However, these systems are limited in their ability to accurately measure the endurance profile of the muscle utilized. An *in vitro* system capable of measuring muscle functional parameters such as force output and fatigue of healthy, disease state, and drug treated tissue, especially in a high-content manner, could have important implications for the study of the exercise regimes used by highly trained athletes and soldiers, drug efficacy studies, as well as give overall insight into the exercise physiology of muscle.

In this study, a serum-free, high-content assay system utilizing silicon cantilevers was used to investigate the functional force output and endurance of mouse skeletal muscle whereby PGC-1α has been mis-regulated. The down-regulation of PGC-1α caused a significant decrease in endurance while in contrast an up-regulation of PGC-1α exhibited a significant increase in myotube endurance profiles. Neither up-regulation nor down-regulation of PGC-1α caused changes in the initial peak force (PF) output of the skeletal muscle myotubes. These data are in line with previously published in vivo phenomenon related to alterations in PGC-1α expression[80, 81]. We have established a high-content monitoring system for the recapitulation of *in vivo* phenomenon on an individual cell scale

Material and Methods

Dissection, Cell Culture, and Drug Incubation

PGC-1α deficient mice were derived from a previously established protocol[82]. Adult mice, both WT and KO, were euthanized using excess CO_2, in accordance with IACUC standards. The *Gastrocnemius* was removed from both the left and right hind limbs. The muscle was then processed according to previously established protocols[83, 84]. Briefly, muscle was

minced and incubated in a digestion solution consisting of 20 mL Dulbecco's Modified Eagle's Medium (DMEM), 25 mg collagenase type I (Worthington, Lakewood, NJ), and 1 mg neutral protease (Worthington, Lakewood, NJ). The cells were then triturated, spun down, and resuspended in DMEM in a 90mm dish for 45 minutes to remove fibroblasts. This solution was transferred to a 50 mL conical tube and spun down to pellet. The resulting pellet was then resuspended in a previously defined growth medium and serially plated every 24 hours to enhance the percentage of myogenic cells. Once the cells had reached confluency, 500 µL of differentiation medium was added to each well. Every 4 days subsequently, half the culture medium on each well was replaced with fresh differentiation medium. Cultures were maintained for 13-14 days after addition of differentiation medium prior to analysis. For the drug treated muscle, WT myotubes were incubated for 24 hours in 10 ng/ml of SBI-477 drug solution (courtesy of the Sanford-Burnham medical research institute) prior to testing.

Cantilever fabrication and surface preparation

Cantilever chips were fabricated from silicon wafers, using previously published methods[29, 65, 85]. Briefly, 100 mm Silicon-On-Insulator (SOI) wafers had a 4 µm thick device layer and buried oxide layer of 1 µm. In the device layer, the cantilevers were patterned using S1818 photoresist and etched using deep reactive ion etching (DRIE). To protect the cantilevers during processing, a 1 µm layer of silicon dioxide was deposited on the top surface. The bottom of the wafer was similarly patterned and etched so that the silicon beneath the cantilevers was removed, producing a large window underneath. The oxide layers were removed using a buffered oxide etching solution. The resulting structures were silicon cantilevers that could be imaged from above and interrogated with a laser from below.

These cantilevers, as well as the coverslip controls used in this study, were coated with an amine-terminated alkylsilane, (3-Trimethoxysilyl propyl) diethylenetriamine (DETA) (United Chemical Technologies, Bristol, PA) using methods previously described[29, 65, 85]. DETA is an analog of spermidine, which is known to promote long-term survival of cells *in vitro*, and has been previously validated in the culture of various cell types[31, 32]. Thus it was used in this study as a surface coating, to promote cell adhesion and survival.

The silanization of the cantilever surfaces with DETA was performed using a solution of 0.1% (v/v) DETA-silane in toluene, heated to 70°C for 30 minutes, followed by a series of toluene rinses and reheating to 70°C for 30 minutes in fresh toluene to remove any unreacted silane. The surfaces were oven cured at 110°C for 2 hours and stored in a desiccator until use. The surface coatings were verified using X-ray photoelectron spectroscopy and contact angle goniometry.

Multiplexed Assay Recording System to Measure Peak Force and Time-to-Fatigue

A novel laser and photo-detector system, adapted from Atomic Force Microscopy technology, was utilized to calculate peak force (PF) of individual myotube contractions, as well as the myotubes' time-to-fatigue (TTF). Application of this system for similar studies has been described in detail previously [60, 86]. Each cantilever chip was transferred to a heated stage housed within a modified electrophysiology rig. The culture dish on this heated stage was filled with Differentiation Medium (+ 10 mM HEPES) to maintain the cells during analysis.

Briefly, the automated system consisted of a class 2 photodiode laser positioned below the stage so that the beam was focused on the cantilever tip and reflected into the center of a 4-

quadrant photo-detector[49, 65]. A pulse stimulator was used to elicit contraction in the cultured myotubes, and the resulting cantilever deflection was measured in terms of laser displacement (in V). The acquired raw data was converted into a direct measurement of force using a modified Stoney's equation as detailed previously[60]. Software was written in labVIEW (National Instruments) to control laser and photo-detector positioning to allow scanning across all cantilevers on each chip for contractile activity.

To determine which cantilevers had contracting myotubes, the chip in the culture dish was first subjected to broad field electrical stimulation, with pulses of 3 V and 40 ms, delivered at a frequency of 1 Hz. The cantilevers were scanned for 5 seconds each to elicit and record contractile responses. The active cantilevers were then scanned, with continuous electrical stimulation, until their peak force was reduced by half. Thus, the time-to-fatigue was calculated by measuring the time elapsed in which a 50% reduction in peak force was observed.

Immunocytochemistry

Cells were fixed for 10 minutes in 4% paraformaldehyde solution in PBS. These cells were then subjected to a permeabilization solution consisting of 0.2% Triton-100 in PBS for 30 minutes followed by incubation in 5% donkey serum plus 0.5% Bovine Serum Albumin in PBS (Blocking solution). The blocking solution was aspirated and replaced with a 1:200 dilution of primary antibody against Complex IV (Life technologies 439500) solution in blocking solution and was incubated overnight at 4 ^{o}C.

Statistical analysis

All experiments were repeated at least 3 times using tissue from different animals on cultures from different days. Differences in force and time-to-fatigue measurements among the three conditions ("WT", "SBI-477-Treated", "PGC-1α KO") were each evaluated statistically using a two-tailed students T-test ($\alpha = 0.05$). All values are expressed as mean ± standard error.

Results

The laser-photodetector system utilized in this study makes use of modified atomic force microscopy (AFM) technology, using a laser to measure substrate deflection in response to cellular contractile activity. Through application of modified Stoney's equations, used to calculate stress in a thin film, this measurement of deflection can be converted into a direct read-out of force production in the contracting cells. The high sampling rate of the photodetector module (300 – 1000 points per second) far supersedes other published models, which rely on video analysis of contractile activity (20 – 30 frames per second), and provides high resolution information on contractile waveform for in depth analysis of changes in functional performance. The non-invasive nature of this assay provides superiority to the system over and above alternative platforms which rely on integration with sensitive force transducers, and enables repeat analysis of single cultures over multiple time points *in situ*. Furthermore, the non-invasive nature of this model makes it well suited to incorporation into multi-organ models currently under development, where fast access to a particular cell type may be limited by the complexity of the platform.

Although this system possesses substantial inherent benefit for the reliable and reproducible evaluation of cultured contractile cell types, the software and hardware configurations installed on this system enable scanning of multiple cantilevers across multiplexed arrays. This functionality enables multiple data points to be collected from single culture surfaces, providing improved statistical power to observed changes in functional performance. Given that most current platforms for investigating skeletal muscle activity *in vitro* rely on multiple myotubes incased in hydrogels, the ability to interrogate single myotubes in such systems is lacking. This 2D assay therefore provides a read-out of inter fiber variability for more accurate assessment of culture maturation over time, and provides multiple data points per condition for more statistically relevant functional analyses.

The scanning functionality developed by this group is also of substantial importance for the continued development of this technology toward higher-throughput applications. The experiments detailed in this study utilize cantilever arrays maintained in a single culture well in consistent conditions. However, the ability to scan across multiple cantilevers successfully provides proof of concept data for the adaptation of this system to support cantilever arrays in multiple wells, enabling concurrent investigation of multiple experimental conditions. Integration of this technology with a 96 well format or greater would facilitate high-throughput investigations of novel drug compound efficacy and toxicity, as well as more informative mechanistic studies of pathological conditions and basic scientific research into muscular physiology. Adaptation of the chips used in this study to incorporate larger cantilever arrays would be relatively simple, and the proof of concept data collected here suggest integration of

the system with a multiwell environment for high-throughput screening would likewise be straight-forward.

Force measurement

The displacement of the laser position on the photodetector is software converted to a "Peak" that is reflection of a change in position of the cantilever tip indicating the maximum deflection and maximal stress changes (Figure 15A). PGC-1α is a regulator of mitochondria content and should not affect muscle morphology or the initial peak force output of the myotubes. There was no discernable difference in myotube size or morphology between WT (Figure 14A), SBI-477 treated (Figure 14B), and PGC-1α KO (Figure 14C). Consequently, there was no significant difference in initial peak force between WT and experimental groups (Figure 15C, $p>0.31$). The change in peak voltage is evaluated by clampfit software as a measure from initiation of the peak to maximum deflection of the cantilever (Figure 15B). Maximal peak force was evaluated for WT, PGC-1α knockout and SBI-477 drug treatment. The peak force was measured as 412 +/- 32 nN, 464 +/- 38 nN, and 397 +/- 44 nN respectively (Figure 15C).

Endurance of Myotubes

The endurance of myotubes, or time-to-fatigue, has been previously defined and established as the time course necessary to observe a 50% reduction in maximum peak force output[84] (Figure 16A). The TTF was calculated as 1134 +/- 36 seconds for WT, 462 +/- 91 seconds for PGC-1α knockout myotubes and 2610 +/- 300 seconds for SBI-477 drug treated cultures. A significant decline in endurance was observed between WT and PGC-1α knockout myotubes ($p< .005$). However, when WT myotubes were treated with SBI-477 to upregulate

PGC-1 α activity there was observed an over two-fold increase in endurance profiles (Figure 16B, $p < .0002$).

Discussion

Skeletal muscle is one of many organ systems of importance in drug efficacy and toxicity studies. The scientific community has developed a multitude of assay systems for force interrogation in vitro[54, 61, 87]. However, these systems are either limited in their capacity to measure endurance, or in their capacity to interrogate single myotube force measurements in real time. Functionally based in vitro systems for the query of skeletal muscle performance provide a unique tool in the drug discovery process. To date, a fully functional in vitro system for the real time acquisition of muscle force and fatigue has not been developed. Through this study we have provided a system capable of interrogating the endurance profiles of individual myotubes in real time.

As previously described the analysis of skeletal muscle functionality utilizes a modified AFM based multiplexed scanning device and a novel laser scanning system. At ideal capacity this system provides 32 individual points of data collection per cantilever chip. Each 15" by 15" cantilever chip fits into a single well of a standard 12 well plate. With 32 points of data collection per well this provides 384 individual sources of data acquisition. The novel scanning system allows for each cantilever board of a chip to be individually assessed in real time in an automated fashion. These 384 sources of data acquisition per plate provide a 4-fold increase in single cell analysis per plate over a standard 96-well assay system.

PGC-1α is a co-activator of Peroxisome Proliferator of Activated Receptor-gamma (PPAR-γ). These two in conjunction regulate a multitude of biological pathways including mitochondrial content and biogenesis. In the myotubes derived from PGC-1α KO mice there was a significant decrease in mitochondrial content which corresponded to the observed decrease in time to fatigue. This parallels what is found *in vivo* and *in situ*[82, 88, 89]. SBI-477 is a novel and patent pending agonist for PGC-1α, graciously donated by the Sanford-Burnham medical research institute. After a 24-hour incubation with this compound, myotubes showed an over 2-fold increase in their fatigability (Figure 16) which is supported by the significant increase in mitochondria content compared to the wild-type age matched controls.

Due to the limited viability of PGC-1α deficient offspring, it was not possible for the scope of this study to treat PGC-1α KO myotubes with an agonist to downstream proteins regulated by PGC-1α to investigate the recapitulation of WT activity.

This multiplexed system demonstrates, for the first time, the ability to measure force and endurance of protein deficient myotubes in a relatively high throughput manner. These functional endpoints can be manipulated by small molecule application which makes this assay system ideal for pre-clinical drug testing/efficacy studies.

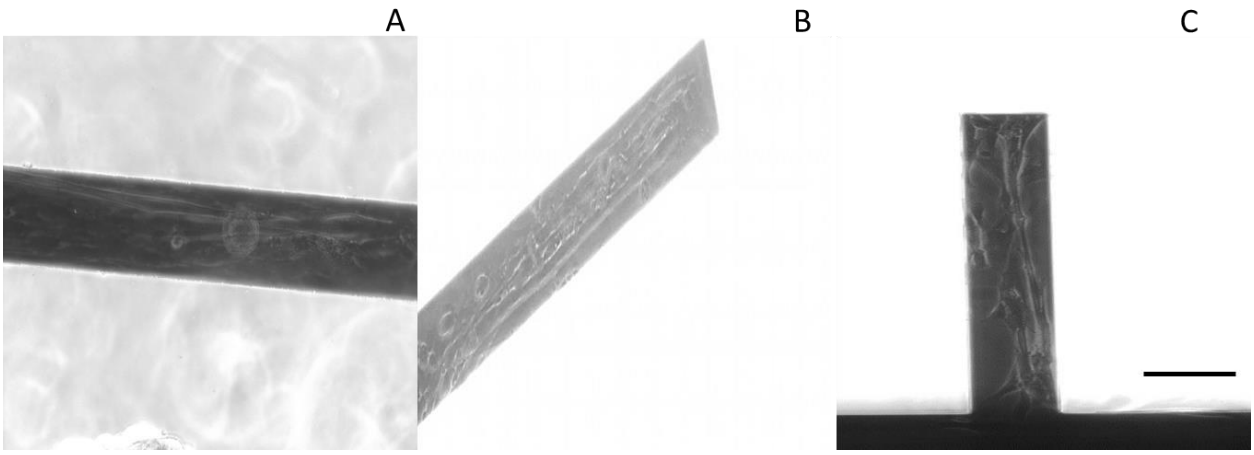


Figure 14: Phase contrast images of myotubes on cantilevers. WT (A), SBI-477 treated (B), and PGC-1α (C) myotubes at 12 DIV on cantilevers. Scale bar =50 μm.

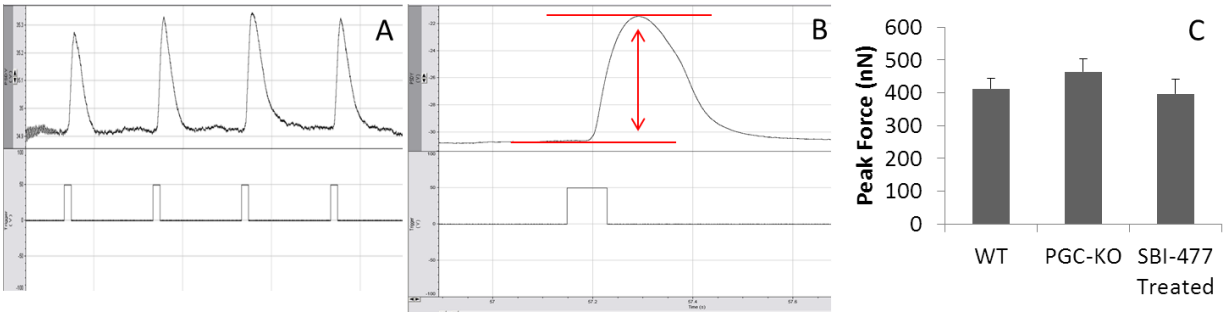


Figure 15: Measurement of skeletal muscle force output utilizing AFM based detection system. Graph of raw data acquired by the axoscope program in response to cantilever detection from myotube response (A) and a rendering of how peak deflection is measured (B). Initial Peak force measurements between WT, PGC-1α KO, and SBI-477 treated myotubes indicating no significant difference in original peak force (P>.31).

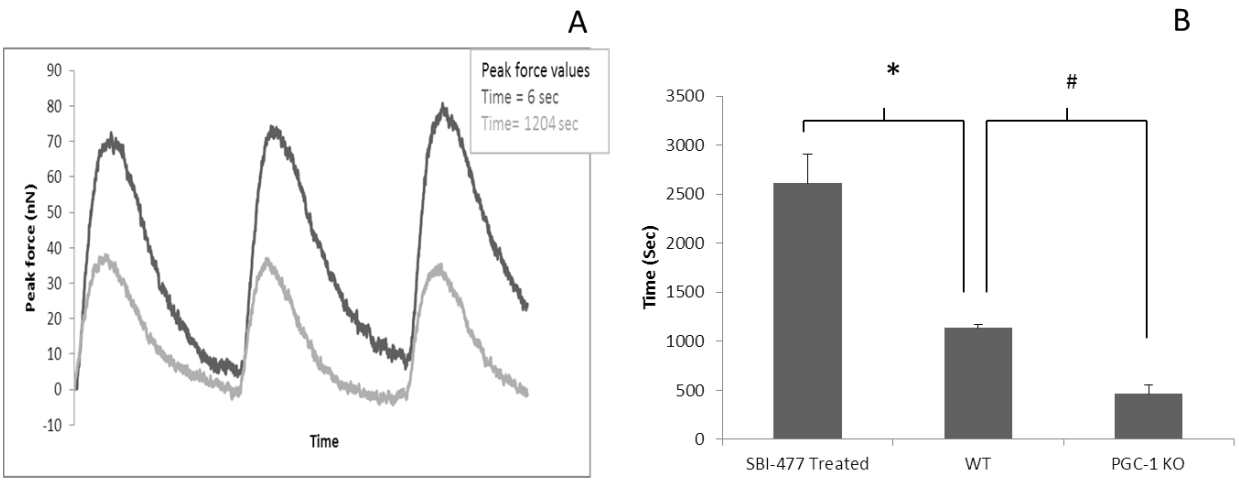


Figure 16: Endurance profiles of skeletal muscle. Representative initial peak force traces at time=6 sec and at 50% in peak force at time=1204 indicating myotube fatigue. Graphical representation of time to fatigue data of SBI-477 treated, WT, and PGC-1α KO myotubes. A significant upregulation in endurance was seen between SBI-477 treated and WT ($p<.005$) while a significant decrease was observed from WT with PGC-1α KO myotubes ($p<.0002$).

CHAPTER FIVE: GENERAL DISCUSSION

One of the limiting factors involved in translating tissue engineering and regenerative medicine research to clinical purposes is the poor correlation between basic research and application in human subjects. Some of these limiting factors are due to the cells involved for testing and the use of poorly defined animal sera in medium formulations. Also, the endpoint of most in vitro research studies don't look at actual functionality of tissue. They are typically limited to:

1. Morphological changes
2. Alterations in gene transcription or protein expression
3. Ability for cell survival

While these endpoints have their value, an up-regulation in certain proteins isn't necessarily commensurate with a functional change. However, the ability to measure both parameters simultaneously helps to establish causality and may provide for a more accurate *in vitro / in vivo* correlation and further application in clinical medicine.

This dissertation research utilizes basic cell biology and physiology knowledge, combined with a previously developed cantilever array system, to further enhance our knowledge about basic functional properties of skeletal muscle. Specifically, the development of a new serum-free culture system to derive myotubes from adult satellite cells and investigate force and fatigue changes of this muscle in response to chemical and physical stimuli.

Skeletal muscle tissue engineering constructs are important due to their potential to be used in grafts, prosthetic devices, and other bio-hybrid devices for drug discovery.[65, 90] There

are multiple limitations to these grafts, one being the human immune responsible to sera containing medium and the incompatability of fetal tissue. Therefore the development of a serum-free culture system of adult based cells eliminates two of these potential pitfalls. To that end, Chapter 2 discusses the formulation of a serum-free medium and the development of a culture technique by which functional muscle on the single cell level can be formed from adult derived skeletal muscle myoblasts. The addition of any new culture system and medium formulation to the system requires a comparison to previously developed and currently utilized methodologies. The muscle was interrogated for MyHC expression, highly striated sarcomere formation, function of ion channels by electrophysiology and shown to be more developed than its embryonic or adult sera containing counterparts. This is a promising advancement in the tissue engineering field. It also creates further excitement in that this formulation is not species specific as it is functional for rat (Chapters 2 and 3), mouse (Chapter 4), and primary human biopsies (data not shown).

The initial goal for the experiments performed in chapter 3 was to utilize the skeletal muscle to alter myotube endurance by an exercise regime and to increase force output by the addition of creatine. It has been discovered that subjecting the whole body *in vivo* to different exercise regimes causes a change in the MyHC isoform expression. Specifically, a chronic low frequency regime such as marathon running or long distance walking causes upregulation of type I myosin expression. While conversely, a sprinting protocol drives an upregulation of type II myosin expression.[63, 91, 92] Creatine has been shown to help in the upregulation of force output and creatine phosphate levels. We wanted to test these *in vivo* phenomena in our *in vitro* muscle system. Incorporating a previously developed AFM based cantilever system developed

by this lab [50, 65] it was possible to measure the effects of chronic low frequency stimulation and 40mm creatine treatment on muscle endurance and more importantly fatigue. To our knowledge this is the first in vitro platform to ever measure muscle endurance profiles of WT or treated skeletal muscle. The results were intriguing that both force and endurance were upregulated in both treatment regimes. Further investigation pointed to a hypertrophic response and upregulation of MyHC expression resulting in increased force and likely an upregulation of creatine phosphate was responsible for increased endurance. CLFS resulted in increased ERR-γ levels which are responsible for mitochondrial biogenesis. Likely the increased force was due to a maturation in the overall contractile machinery within the myotube.

Lastly, this medium system was used to study the endurance changes that occur as a result of a misregulation of a particular protein. Tissue was derived from the hind limbs of adult mice deficient in PGC-1α (mice were used for ease and cost purposes). WT and WT muscle treated with an agonist for downstream protein also responsible for mitochondria biogenesis were tested as well. It was demonstrated that we can increase and decrease muscle fatigue utilizing small molecule application and protein deficient tissue. Another novel point is this new medium and plating protocol were not limited to WT tissue alone or to specific sources of cells.

The development of a new culture protocol utilizing serum-free medium and the ability to culture these cells on a bio-MEMs cantilever device for the measurement the force and fatigue responses to external stimuli provides a new platform in which to study drug efficacy and in discoveries related to exercise physiology.

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