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Effects of Three-dimensional Culture Conditions on Skeletal Muscle Myoblasts

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Effects of Three-dimensional Culture Conditions on Skeletal Muscle Myoblasts

by

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Dedication

To my family and friends,

one in the same.

Non mihi, non tibi, sed nobis.

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Effects of Three-dimensional Culture Conditions on Skeletal Muscle Myoblasts

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The objectives of this research were to: 1) develop a three-dimensional *in vitro* model; and 2) subsequently, utilize this model to investigate mechanisms of myoblast adhesion, fusion, and differentiation. C2C12 cells were examined as pre-aggregated single cells and multicellular aggregates in the Rotary Cell Culture System (RCCS). At the time intervals tested, RCCS cultured cells maintained viability and did not exhibit increased apoptosis markers such as Caspase 3 (activated) and phosphatydylserine. In contrast, increases in cell death and apoptotic markers were noted in suspension culture (SC) control cells. RCCS cultured cells fused to form multinucleated syncitia and expressed sarcomeric myosin heavy chain (MHC) in significantly higher levels than SC aggregates after cultivation for 3 and 6 days. This occurred in the presence of mitogens without exogenous matrix or support structures. Myoblast fusion was inhibited by exposure to soluble anti-Neural-cadherin antibody, but this treatment increased MHC levels assessed using immunohistochemistry.

During early RCCS culture, myoblasts exhibited numerous cytoplasmic protrusions (podia). Microscopic examination of cells cultured in RCCS and SC revealed significantly more and slightly longer podia in the RCCS at 3, 6, and 9-hours. Podia were F-actin dependent as shown by exposure to an F-actin depolymerizing agent, Latrunculin A. Podia were inhibited, but recovered upon Latrunculin A removal.

Podia were postulated to play a role in cell-cell adhesion in conjunction with Neural Cadherin (N-cadherin), an adhesion molecule important in myoblast differentiation. To determine if N-cadherin was critical to cell-cell adhesion, RCCS cultured cells were examined for the presence of N-cadherin at both the podia and membrane using confocal microscopy. N-cadherin levels decreased at the podia and membrane of RCCS cultured cells but not in SC cells at 3, 6, and 9-hours.

In summary, these results revealed: 1) podia formation is F-actin dependent but N-cadherin independent; 2) N-cadherin is critical for myoblast maturation; 3) synctia formation and differentiation can occur with mitogens present, without exogenous substrates in the RCCS; 4) this novel myoblast model test system is suitable for defining muscle development/regeneration processes, identification of molecular targets for development of therapies, and potential regenerative medicine applications.

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Chapter 1: Introduction

Skeletal muscle is a mechanical organ that generates force and executes movement. The contractile function of skeletal muscle is dependent, in part, on its unique multinucleated structure and the expression of specific proteins. Skeletal muscle functions in locomotion, postural maintenance, and breathing, which are critical functions for the survival of vertebrate organisms (reviewed by Charge and Rudnick, 2004). Thus, skeletal muscle must rapidly adapt to new environmental cues and have the capability to regenerate in response to impact or injury.

The processes of development and regeneration of skeletal muscle are mechanistically linked and result from a unique progression of events. These include: recognition of other myoblasts, adhesion of individual myoblasts, alignment of cells, expression of muscle specific proteins, and fusion of mononucleated myoblasts to each other or pre-existing myotubes (Charge and Rudnick, 2004). However, despite many similarities, there are some distinct differences between these two processes that are detailed in greater depth below.

MYOGENESIS

The skeletal muscle that comprises the limbs and trunk of organisms is derived from the mesenchymal cells of the paraxial mesoderm (Buckingham et al., 2003). The majority of head muscles is derived from the paraxial and prechordal mesoderm (Hauschka, 1994; Christ and Ordahl, 1995; Lu et al., 2002). The paraxial mesoderm is located on either side of the forming neurotube and extends longitudinally along the dorasal embryo (Gilbert, 2000). The paraxial mesoderm organizes into somitomeres, and

then condenses into spherical structures, designated somites. The somites are further specialized into the dermyotome, which forms skin and muscle (Cossu et al., 1996; Miller et al., 1999) and the sclerotome, which forms the skeleton (Pirskanen et al., 2000).

Specification to myogenic lineage occurs with the upregulation of the myogenic (muscle) regulatory factors (MRF). MRFs all share a basic Helix-Loop-Helix (bHLH) domain. This domain forms a homo- or hetero-dimer with E proteins of the E2 gene family (Lassar et al., 1991) and also recognizes the E-box sequence, CANNTG, found in the promoter area of a suite of muscle-specific genes (reviewed by Parker, 2003). MRFs include MyoD, Myf5, myogenin, and Mrf4 with the latter two appearing both during and post-differentiation. The initial induction of MyoD and Myf5 in the somites is primarily under the control of a combination of signals including Shh (Sonic Hedgehog) and Wnts (1, 2, 3, 7a, and 11) from the surrounding tissues (notocord, neural tube) as well as inhibition of BMP4 by Noggin (Munsterberg and Lassar, 1995; Rawls et al., 2000). Inhibition of BMP's appears to be significant in myogenesis, since BMP2 application to isolated fetal myoblasts can induce osteogenic progression (Katagiri et al., 2006).

Myogenic cells (Myf5+ or MyoD +) migrate from the hypaxial (presumptive intercostals, abdominal, and appendicular musculature) and epaxial (presumptive back musculature) regions to form the myotome just beneath the dermyotome. In addition, proliferating cells from the central dermyotome (Pax3+/Pax7+, MRF-) migrate medially into the myotomes and continue to proliferate without expressing myogenic markers or MRFs (Relaix et al., 2005). Shortly after the formation of the myotomes, some of the cells fuse to form primary myotubes (Cossu and Borello, 1999). The cells that do not fuse continue to proliferate; however, some fuse to form secondary myotubes located around and between the primary myotubes.

As a first step toward terminal differentiation, cells withdraw from the cell cycle (Bishoff and Holtzer, 1968; Nadal-Ginard, 1978; Andres and Walsh, 1996). These cells then align (Nameroff and Manor, 1976), identify each other as myoblasts, adhere to each other (Knudsen and Horwitz, 1977) and form multinucleated myotubes by the fusion of the cell membranes (Kalderon and Guilula, 1979). Simultaneously, an array of muscle specific genes is activated.

The first fusion events result in the formation of primary myotubes. Subsequently, secondary myotubes are formed. Primary myotubes are morphologically (larger diameter, centrally localized nuclei) and temporally distinct from secondary myotubes. In addition, primary myotube formation is nearly synchronous with all myonuclei of a given myotube fusing simultaneously. This is in contrast to the sequential fusion of myoblasts that occurs during secondary myotube formation (Harris et al., 1989). Primary myotubes are believed to create a scaffold for muscle that includes the subsequent formation of secondary and tertiary myotubes (Draeger, 1987; Harris, 1989; Codon, 1990). Secondary myotubes continue to form until shortly after birth to generate the full compliment of skeletal muscle myofibers.

At the same time as the fusion process, proteins such as myosin and creatine kinase appear (Weinstock et al., 1978; Young et al., 1981). Other markers of differentiation, which are largely sarcomeric, include Myosin Heavy Chain (MHC) (Whalen 1985), two types of Myosin Light Chains (MyLC), Tropomyosin, Troponin C (Parmacek, 1990), Troponin I, and Troponin T. In addition, there is a loss of DNA polymerase and alterations in actin isoforms (see Actin below for greater detail on actin isoforms). Thus, many of these markers are useful in determining differentiation during skeletal muscle myogenesis, repair, or during cell culture.

Perhaps the most widely studied and abundant of these differentiation markers is MHC. Since MHC is the major component of the thick filaments comprising the contractile apparatus, MHC type expression and muscle fiber type determination are directly related (Gunning, 1991; Rauch and Loughna, 2004). Accordingly, MHC mRNA appears before myotube formation (Shibanauma, 2002). Contraction speed is determined by the particular MHC isoform (Whalen, 1985). Isoforms include Type I, Type II, Embryonic (emb), and Neonatal (neo). Nerve independent expression results in primary myotubes initially expressing emb and Type I MHC, while secondary myotubes express emb and neo prior to fast isoforms during myogenesis. Neural input can override this progression of isoforms and can alter adult fiber types (reviewed by Gunning and Hardeman, 1991). Although neural input is significant in studies involving human or animal models, it is not directly applicable to the cell culture methodologies described in this dissertation.

Soon after birth, a full compliment of myofibers is present (Ross et al., 1987). Additional hypertrophy (supplementary fusion or protein production) can occur throughout a vertebrate's life. Hyperplasia, the formation of new myofibers, occurs during myogenesis. It has been reported in adults following uncommon events such as certain types of overload or fiber splitting, but is not a result of neomyotube formation (Kelley, 1996). However, the issue of adult hyperplasia is controversial. Importantly, the skeletal muscle model test system developed in the studies reported here more closely resemble embryonic skeletal muscle development than adult skeletal muscle regeneration.

REGENERATION/GROWTH

The ability of skeletal muscle to regenerate is second only to bone marrow with respect to capacity (Shi and Garry, 2006). This regenerative capacity enables rapid restoration of muscle loss resulting from injury caused by trauma, disuse, disease, or exercise. To effect regeneration, skeletal muscle must replace lost nuclei and proteins. To replenish lost nuclei, satellite cells or other progenitors fuse to the damaged myofibers, which are unable to generate additional nuclei by proliferation (Konigsberg, 1963; Dienstman, 1977; Lipton and Schultz, 1979; Cossu et al., 1980; Baserga, 1981). Satellite cells are specified, quiescent skeletal muscle precursors, located between the sarcolemma (the plasma membrane of the myofiber) and the basal lamina (Mauro, 1961). Satellite cells represent only about 10% of the total nuclei of the muscle tissue (Muir, 1965). However, the percentage can range from 30 at birth to only 2-5% percent in some adult muscles. The quantity of satellite cells is dependent upon species, age and muscle fiber type (Snow, 1977; Allam, 1981; Campion et al., 1981; Bockhold, 1998; Hawke and Garry, 2001).

The mechanisms of myonuclear replacement during muscle regeneration, growth, and repair are thought to recapitulate myogenesis (Charge and Rudnick, 2004; Shi and Garry, 2006). Thus, the overall process of regeneration is identical to that described above for myogenesis with the following exceptions: 1) The source of new myonuclei are satellite cells or possibly other precursors; 2) MHC expression does not follow the same sequence as in myogenesis expression; 3) MyoD and Pax7 are required for regeneration but not for myogenesis (Parker, 2003); and 4) although hyperplasia (fusion of myoblasts to form new myotubes) and hypertrophy are common during development, hypertrophy alone is more common in adult skeletal muscle growth and regeneration.

In order for growth, repair, or regeneration to occur, additional myonuclei or satellite cells are shifted and/or activated from quiescence to a proliferative state by factors such as FGF, HGF, activated Notch, and TNF-α (Seale and Rudnicki, 2000). However, proliferation might also be modulated by additional factors such as cytokines, neurotrophic factors, growth factors, or oxygen tension. These in turn mediate the hypoxia-inducible gene program such as Hif1, Hif2, NO, and VEGF (Schmalbruch and Hellhammer, 1977; Gibson and Schultz, 1982; Wokke et al., 1989; Brown and Stickland, 1993; Gustafsson et al., 2005).

Daughters of the proliferating satellite cells fuse to existing myofibers or repopulate the satellite cell population (Moss and Leblond, 1971; Schultz and Jaryszak, 1985; Bischoff, 1994). Fusion of daughter cells typically occurs with an existing myofiber rather than to another myoblast (Yao and Kurachi, 1993), although some evidence suggests that hyperplasia can occur (reviewed by Charge and Rudnick, 2004; Rouger, 2004). The regenerative capacity of satellite cells is not unlimited as loss of proliferative capacity is observed in myopathies such as some types of muscular dystrophies (Webster and Blau, 1990; Heslop, et al., 2000). The loss of satellite cell number and proliferative capacity is also linked to other degenerative processes. For example, a decline in satellite cell number (Gibson and Schultz, 1983) and proliferative potential is seen during sarcopenia, the age-related loss of muscle mass prevalent in the elderly (Snow, 1977; Schultz and Lipton, 1982; Machida and Booth, 2004). Muscle atrophy or the progressive loss of muscle mass also occurs in certain disease processes (muscular dystrophy, amyotrophic lateral sclerosis), paralysis, denervation, inactivity, or the skeletal muscle unloading that occurs in the microgravity conditions of space or with bed rest.

The debilitating weakness caused by muscle atrophy compromises contractility force and speed, and can result in decreased strength, endurance, and agility. The resulting decrease in muscle function seriously jeopardizes the ability to perform tasks, increases the likelihood of injury, and decreases the quality of life. Loss of muscle mass occurs when 1) the nuclear number is reduced in a multinucleated myofiber (Ohira, et al., 2002) or 2) contractile proteins are decreased in the myofiber (Baldwin et al., 1990; Thomason et al., 1992; Widrick et al., 2001). In order to recover lost muscle mass, a net nuclear replacement must occur and/or net protein production increase to replace that lost.

Fusion of mononucleated myoblasts is a required step in the regeneration of skeletal muscle. Although several components have been proposed to regulate fusion, the mechanisms of syncitia formation remain undefined (reviewed by Horsley and Pavlath, 2004). For *in vitro* myoblast cell cultures, confluency is thought to be a pre-requisite for syncitia formation of myoblasts (Yaffe and Saxel, 1977), probably because it facilitates cell-cell contact and adhesion, another essential step in the fusion process.

The exact origin of satellite cells remains currently unknown. Some evidence suggests that the majority of satellite cells are derived from the dermomyotome of the somite (Gros et al., 2005; Schienda et al., 2006). Satellite cells might not be the only precursors that have the potential for myonuclei replacement. Other suggested sources include bone marrow (Ferrari et al., 1998, Gussoni et al., 1999), fibroblasts (Cosso, 1997), and mesangioblasts (blood vessel endothelial precursors) (Des Angelis et al., 1999). Interestingly, dedifferentiation of skeletal muscle tissue for muscle restoration can occur in some vertebrates such as fish and amphibians (Hay, 1959; Echeverri, 2002; Kumar, 2004). Myoseverin, a microtubule assembly inhibitor, induces myotube fission and results in mononucleated myofragments. However, cellularization alone is not

sufficient to facilitate proliferation of the mononucleated myotube fragments (Rosania, 2002). Thus, the fusion process must require the expression of proteins found in myoblasts or precursors that are reduced or lost following differentiation and/or fusion.

Overall, a basic body of knowledge has been gained regarding muscle differentiation and regeneration. Nevertheless, despite recent advances in molecular methodologies, the development of complex muscle models, and considerable research dedicated to elucidating these processes, much remains poorly understood (Charge and Rudnick, 2004). This is particularly true for the multiple regulatory pathways involved in skeletal muscle regeneration and the mechanisms of fusion (reviewed by Horsley and Pavlath, 2004). Although a variety of human and animal models have been examined, and a myriad of methodologies utilized to produce vast amounts of new information, significant gaps in our knowledge still remain.

In spite of the advances supported by current research, critical questions remain unanswered. These include the mechanisms of myoblast fusion and the full delineation of pathways during myoblast differentiation and fusion. A basic principle, form determines function, appears to have been largely ignored. The monolayer culture system has been revealing, yet the flattened morphology of cells during this type of culture bears little resemblance to the 3D nature and behavior of a cell *in vivo*. A detailed study of the cell just before and during differentiation and fusion might be revealing. Although animal and human subjects would certainly fulfill the need for *in vivo* testing, it would be extremely difficult to observe single cells in these subjects during this process. However, given a suitable model, observations of the cell-cell interaction, adhesion, and the subsequent events of differentiation and fusion would be facilitated.

In summary, it is difficult to efficiently develop or increase the efficacy of therapies for muscle atrophy, muscular trauma, and diseases when the mechanisms of

basic muscle development and regeneration remain to be elucidated. Understanding these mechanisms is an essential first step in designing novel, specifically targeted therapies as well as optimizing the efficacy of those currently in use.

SKELETAL MUSCLE MODELS

In vitro engineered muscle models mimic some of the structure and function of their *in vivo* counterparts and are essential for elucidating a variety of different mechanisms related to health, aging, disease and trauma. For example, skeletal muscle models have played a crucial role in determining the characteristics, physiology, and regulation of differentiation and fusion in skeletal muscle during myogenesis, growth, repair, and regeneration (reviewed by Charge and Rudnicki, 2004). Models have also played a role in defining the mechanisms of skeletal muscle degeneration in: 1) disease processes (i.e., cacheixia); 2) aging (sarcopenia); 3) inactivity/disuse (stroke) and unweighting (microgravity) (reviewed by Kanderian and Jackman, 2006); 4) other forms of muscle damage (trauma); and 5) pharmacological, nutritional, and exercise-based interventions. In addition, skeletal muscle models have provided insight into tissue engineering and potential regenerative medicine applications (Goodwin et al., 1993; Unsworth and Lelkes, 1998).

The ultimate goal of any model is to facilitate obtaining valid answers to experimental questions while closely approximating the functional, structural or physiological reality of the cells or tissues (Baar, 2005). However, no one model is suitable for answering every question. In order to obtain a comprehensive answer to a specific question, it is likely that several models will be required. A variety of models have been used to investigate skeletal muscle fusion and differentiation including *in vivo*

human and animal models, two-dimensional (2D) monolayer cell cultures, and three-dimensional (3D) tissue models.

Human and animal models

Human models

Ideally, humans represent the optimal test bed to study the differentiation and fusion of myoblasts for research targeting the discovery of effective treatments for human skeletal muscle problems. Clinical research is essential for translating basic research into patient care. With regard to skeletal muscle, human test subjects are generally utilized for: 1) Final clinical evaluations; 2) Gross morphology (Zange, 1999) or noninvasive data collection time course studies (LeBlanc, 1995); and 3) Muscle biopsies (Edgarton, 1995; Widrick et al., 1999) which are particularly useful for histologic examination (Behan, 2002) and gel electrophoresis analysis.

Human derived data has provided significant insight into muscle structure, function, and various diseases. However, depending on the experimental design and specific investigation, such data can also be confounding. Variations seen in skeletal muscle data can be the result of multiple factors such as: 1) limited population size; 2) population attrition (with time-course studies); 3) genetic variations among the individuals; 4) variations in the degree of pre-conditioning; and 5) differences in intake and exercise. These and other factors limit the assembly of a homogeneous test group (Turner, 2001). Although biopsies may be the most insightful of the potential analyses with regard to fusion and differentiation, they have the disadvantages of high cost,

extensive time and effort required for protocol approval, and discomfort for participating test subjects.

Animal models

The use of animal models generally provides a more homogeneous test group than human-based studies (Turner, 2001). Because there is increased latitude with animal models, the muscle as a whole may be removed and directly examined or processed for additional analyses (i.e., skinned, protein extraction, RNA isolation, etc.). In particular, strains of commercially available rodents share similarities with regard to genetics, development, physiology, and disease. In addition, the impact of specific diseases, trauma, and other conditions can be examined (NIH, National Center for Research Resources, http://www.ncrr.nih.gov/comparative_med.asp - comparative_models). The use of animal models including transgenics, mutants, inducibles, and specialized or genetically engineered strains can facilitate obtaining valid information regarding skeletal muscle regeneration.

Although human and animal models can provide the most physiologically relevant data, experiments utilizing these models are time-consuming and costly. Selection of an appropriate model is essential for obtaining relevant and applicable experimental results, but this can be difficult due to the large number of species available, as well as the inconsistencies in information, search mechanisms, terminology, and lack of support from suppliers (NIH, National Center for Research Resources, http://www.ncrr.nih.gov/comparative_med.asp - comparative_models).

Cell-Based models

Monolayer culture

Cell-based tissue models represent a compelling alternative to the more expensive, time intensive use of *in vivo* models. Cell culture can be used to investigate potentially harmful effects of radiation, toxicity, and virology (Perlman, 1968). Although these effects can also be investigated in animal models, cell-based studies allow for examination of individual cells that are more difficult to observe in human or animal models. For example, radiation survival experiments permit the assessment of doses needed to destroy the reproductive capacity of a single cell (Puck, 1960). Factors, such as genetic and precondition variability that are inherent in human test subjects, can be reduced by isolating cells displaying selected criteria and cloning these cells. Subtle cell characteristics and responses can be observed that might otherwise be obscured by heterogeneous variation in a cell, tissue, or human population.

The analysis of mechanisms using tissue culture models is relatively rapid, inexpensive, and reproducible. Sufficient repetitions and numbers of cells analyzed enable sufficient data to be obtained for rigorous statistical analyses. In addition, cell culture methods are amenable to transformations that can result in knockouts, knock-ups, silencing, and inducible/conditional expression that enable the dissection of biochemical pathways. Interventions such as drugs and other supplements (i.e., antioxidants) can be easily and rapidly screened for effectiveness in the tissue(s) of interest.

The production of a functional, differentiated cell-based model is challenging, particularly for skeletal muscle. In order to form differentiated cells, myoblasts (immature muscle cells) must proliferate, align, fuse to form multinucleated cells, and also produce new proteins required for contraction. Induction of myoblast proliferation

during *in vitro* cell cultivation is relatively easy to achieve. However, inducing myoblasts to differentiate is more problematic and requires the introduction of additional factors into an experimental procedure. A change from proliferation to specialized differentiation medium is traditionally required to induce actively growing myoblasts to fuse and produce muscle specific proteins. Currently, a number of different media are used to induce differentiation including medium without serum or medium containing various concentrations of undefined factors found in mature animal serum. The majority of studies seem to indicate that the presence of mitogens such as FGF is sufficient to positively regulate cell cycle progression and prevent differentiation (Freshney, 1994). It remains to be determined if the presence of mitogens is sufficient to prevent differentiation or if the processes of differentiation and fusion can be separated.

The cultivation of primary satellite cells and myogenic cell lines as monolayers has been used to detect participants and regulatory proteins during differentiation and fusion of skeletal muscle. Our current knowledge of the role of myoD and other myogenic regulatory factors, cell adhesion molecules (such as classical cadherins), expression of sarcomeric proteins and the assembly of contractile machinery has been greatly facilitated by the use of 2D cell culture (reviewed by Horsley, 2004; Charge and Rudnick, 2004). Compared to *in vivo* models, the 2D cell culture model system is more amenable to specifically targeted molecular methodologies, less costly, more easily controlled, and rapidly reproducible. However, despite the large body of knowledge concerning myogenesis and muscle repair generated by the "tried and true" models, the cellular and molecular sequence of events are still largely unknown. These 2D models are limited structurally, lack *in vivo*-like functionality and the 3D cell-cell interactions present in tissues (reviewed by Edelman and Keefer, 2005). Accordingly, a more *in vivo* like model test system is needed.

Three-dimensional culture

Despite advances in our understanding of the processes of differentiation and fusion of myoblasts using 2D monolayer culture, 3D modeling of tissues *in vitro* is critical to understanding the basic functions and regulation *in vivo* (Cheema et al., 2003). Three-dimensional cell culture combines the advantages of monolayer cell culture with 3D cell-cell interactions and more *in vivo*-like responses (O'Connor, 1999). Currently, 3D models are used in a wide variety of studies including adhesion, invasion, metastasis, angiogenesis, and pharmacological remediation, inhibition, and cancer biology. In addition, the potential for 3D tissue-engineered skeletal muscle suitable for regenerative medicine application is possible. However, there are certain requirements (Spaulding et al., 1993) for establishing tissue equivalency including: 1) Cell co-localization; 2) ECM secretion; and 3) interstitial fluid formation.

Since the generation and maintenance of skeletal muscle is, in part, dependent on cell:cell interactions, it seems likely that three-dimensional tissue models will play a critical role in providing additional insight into skeletal muscle differentiation and tissue organization. Three-dimensional models could be designed that allow investigation of questions that cannot be readily answered *in vivo* regarding cell-cell interactions, repair and fusion mechanisms.

Exogenous matrix and other support structures versus endogenous matrix

Advances in bioengineering have facilitated the generation of three-dimensional tissue models. Some examples of models produced include cartilage, bone, and skin tissues. All if these used an externally supplied substrate or matrix. The matrix or scaffolds provide support for the developing tissue as well as a substrate for extracellular matrix (ECM) deposition.

The ultimate goal of many of these models is to achieve tissue-equivalency for: 1) regenerative medicine applications (Edelman and Keefer, 2005); 2) analyses to answer basic science questions; or 3) testing the effects of a substance (i.e., pharmacologic or other agents) on the tissue. Many of these utilize cell seeding in a three-dimensional scaffold that has been adapted to facilitate adhesion, differentiation and/or perfusion (Radisic et al., 2003; Karande et al., 2004). Thus, these scaffolds are fabricated from a variety of materials that have been designed to support characteristics of the tissue of interest.

However, the use of a 3D scaffold to support the growth of skeletal muscle tissue has been problematic, since the choice of scaffold can have a profound impact on the characteristics of the resulting tissue. Interaction with the ECM is crucial to the development and function of cells during culture. Although numerous cell culture methodologies attempt to establish this interaction by seeding on ECM substitutes, slight but important effects on cellular behavior, growth, and differentiation can occur due to the non-native structure, composition, and proportion of ECM components (Ip and Darcy, 1996; Timmins, 2005). In addition, the gross arrangement and porosity/perfusion can influence the resulting tissue behavior (Ma et al., 2001).

Examples of commonly used support matrices

Microcarrier culture involves the growth of anchorage-dependent cells on microspheres during 3D culture (van Wezel, 1967). The use of microcarriers, which are generally 100-250 μm in size, provides a sizeable surface area for cell growth. This enables a large number of cells to be cultured within the 3D environment and allows for greater perfusion than some of the other types of support matrix. This substrate is often used to facilitate large-scale production of biological products (such as antibodies)

(Malda, 2006). Microcarriers are commonly composed of dextrans, glass, cellulose, collagen, or alginate; however, microcarriers of poly (lactic-co-glycolic acid) (PLGA) (Mercier et al., 2004; Newman and McBurney, 2004), poly-(L)-lactic acid (PLLA) (Curran et al., 2005), and hydroxyapatite (Fischer et al., 2003) have also been investigated and used with some success.

Gel-based matrix typically involves the growth of cells on the surface of the gel or embedded within the gel. Collagen, hydrogels, or a combination of these with other matrix proteins is most commonly used (Bach et al., 2004). Gels provide the following advantages: 1) a degree of structural support; 2) a mechanism to support receptor-mediated adhesion; 3) protection from mechanical insults that might otherwise result in cell dislodging; and 4) a consistency approaching that of native tissue (Edelman and Keefer, 2005). Matrigel, a commercially available commonly used gel matrix, is a mix of basement membrane components derived from the Engelbreth-Holm-Swarm mouse sarcoma. Since integrin-matrix adhesion functions in structure and signal initiation (Juliano, 2002), the undefined mix of extracellular matrix proteins and growth factors found in Matrigel may potentially result in differential behavior, morphology, and gene expression. In addition, gels in general are not entirely biodegradable and some are potentially immunogenic (Freed et al., 1994; Grande et al., 1997). Although gel embedding has been used to generate 3D skeletal muscle models, particularly in conjunction with the application of tension (Cheema, et al., 2003), these gel-embedded myoblasts and myotubes are not easily amenable to studies investigating differentiation and fusion. For example, it can be difficult to recover intact cells for essential analyses such as immunohistochemistry since the cells cannot be effectively recovered from the gel.

A scaffold is an exogenous 3D cell substrate that functions as a template for tissue formation. Initially, scaffolds were made of non-degradable materials (metals, ceramics, polymers) and remained as a permanent artificial structure within the tissue (Maquet and Jerome, 1997). Currently, scaffolds are manufactured from a number of biodegradable, biocompatible materials. These serve not only as a solid support pattern for cells and developing tissues but also provide a substrate for natural ECM production by the cells growing within. Synthetic polymers currently in use include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and PLGA, a combination of the two. Scaffolds can also be made of ECM components such as collagen and fibronectin; however, some of these natural scaffolds exhibit immunogenicity if the resulting tissue is used for regenerative medicine purposes (Liu and Ma, 2004).

Each scaffold is specifically designed (Karande, 2004) and has both advantages and disadvantages (Fuchs, 2003). Therefore, selection of the scaffold composition is partially dependent on the characteristics of the desired construct. For example, the formation of bone tissue would require a scaffold with a greater degree of stiffness than one for cardiac tissue. A number of other characteristics require consideration when selecting a scaffold. Porosity of the scaffold is also of concern since the construct typically requires a degree of perfusion (Ma et al., 2001). Connectivity of the pores and the arrangement of those pores are of interest in skeletal muscle constructs. Although these constructs are more useful than the whole organism in determining the mechanisms of differentiation and fusion, the size and organization of the forming tissue could make monitoring of single cells during the differentiation process difficult, except on the construct periphery.

Some three-dimensional skeletal muscle models have been formed without the aid of substrate attachment. “Myooids” are created using primary myoblasts in co-culture

with fibroblasts or on fibrin gel support layers (Dennis, 2001; Huang, 2005). When myotubes form, spontaneous contractions cause the tissue to detach from the monolayer substrate. Although impressive, myooids are derived from primary myoblast cultures that are, at a minimum, 88% myoblasts. Serum switches from fetal bovine to horse are used to stimulate differentiation, and alignment was only achieved when the differentiated myotubes detached from the culture vessel and were held under tension. Contractive forces measured in similar myooids constructed with C2C12 cells co-cultured with a fibroblast cell line revealed that the C2C12 derived myooids had greatly reduced contractile capacity. The use of the fibroblast cell line might not have provided sufficient support to optimize contractile capacity. Investigations of the stiffness of support layers/structures on myoblast differentiation indicate that the optimal substrate stiffness is equal to the stiffness of the myotubes (Engler, 2004). Thus, the best support layer for myoblast differentiation is a layer of the myoblasts themselves.

Anoikis/Apoptosis during culture

An area of concern when adherent cells are cultured without a substrate is the potential for induction of anoikis/apoptosis. Apoptosis, or programmed cell death, is a process regulated by the expression of specific genes (Wyllie et al., 1980) with both pro- or anti-apoptotic functions. The morphologic characteristics of apoptosis include: 1) nucleus and cytoplasmic condensation; 2) loss of specialized membrane structures (such as podia); 3) tightly packed organelles; 4) chromatin condensation; 5) loss of the nucleolus; and 6) in later stages, nuclear fragmentation and the formation of apoptotic bodies (membrane-enclosed cytoplasm, organelles and nuclear materials) (Kerr et al., 1972).

The mechanism(s) of apoptosis is highly complex and tightly regulated. Two major pathways have been described; i.e., the death receptor pathway and mitochondrial pathway. Regardless of the triggering events, typical apoptosis results in the activation by cleavage of Caspases 3, 6, and 7. It should be noted that there are some rare forms of apoptosis that do not involve the caspases (Borner and Monney, 1999; Rich et al., 2000). Caspase 3 was selected as an indicator for apoptosis in this dissertation for the following reasons. 1) Caspase 3 is involved in both death receptor and mitochondrial pathways (Lakhani, 2006) and would enable the detection of both pathways. 2) It plays a role in F-actin clevage (via gelsolin) (Kothakota et al., 1997) and the presence of F-actin in cytoplasmic protrusions (podia) is a focus of this study. 3) Caspase 3 is very commonly used to define apoptosis.

Anoikis, a type of apoptosis, is triggered by inadequate or inappropriate cell-substrate (integrin-ECM) interactions (Frisch, 2001). Thus, signals from the ECM are thought to be required to prevent cells from undergoing apoptosis (Gilmore, 2005). For example, in liquid overlay cultures; poly-HEMA is used to prevent anchorage-dependent cells from adhering to the substrate to form monolayers. If anchorage is prevented, many cell types will undergo apoptosis. The mechanisms of anoikis are of particular interest to cancer biologists since loss of adhesion dependence without resulting in anoikis is a defining factor of metastatic capacity. However, cell-cell adhesion, including N-cadherin-mediated adhesion, offers some degree of protection from anoikis (Glinsky and Glinsky, 1996; Makrigiannakis, 1999). Further, mesenchymal derived cells also seem to have resistance to anoikis (Benecke et al., 1978; Milasincic et al., 1996).

Since anoikis is a type of apoptosis, detection of anoikis alone has not been reported. Rather, it is the initiation of apoptosis that determines anoikis. Thus, for the experiments reported here, a second marker of apoptosis was selected. The exposure of

phosphatidylserine (PS) to the cell membrane outer leaflet, a hallmark of the dying cell (Corsten, et al., 2006)**,** is used to determine if my model system induces apoptosis. A fluorescently conjugated protein, Annexin V, which selectively binds to PS, will be utilized to determine presence of PS in the outer leaflet of the cell membrane.

METHODS OF 3D CULTURE

Suspension: liquid overlay

Many anchorage-dependent cells (primary, secondary, and cell lines) will form spheroids during static culture when attachment to the culture vessel surface is prevented. Commonly used base coatings that prevent adhesion include agar (Yuhas et al., 1977), agarose (Breul, 1995), and poly-HEMA (Folkman and Moscona, 1978). More recently, reconstituted basement membrane gels (such as Matrigel and others) have been used to generate spheroids that display morphological characteristics of the original gland or tumor (Freeman, 1994). In addition, embryoid bodies may be generated by this methodology (ATCC Protocols, 2006)

The advantages of liquid overlay culture include: 1) The proportion and maturation of cell types in the original tissue are maintained during mixed cell culture; 2) viable tissues can be maintained longer (three months) than in monolayer culture (four weeks) (Bauer et al., 1992); 3) some cell types will express features of differentiated morphology (i.e., microvilli and desmosomes) and function (i.e., secretion of cell products) (Fong et al., 1991); and 4) advances in transformation resulting in more aggressive cancer phenotypes (in Matrigel, Freeman, 1994).

The generation of spheroids using liquid overlay methodology is simple and applicable to a greater number of cell lines than the spontaneous aggregation method (O'Connor, 1999). However, the static nature of the liquid overlay method causes diffusion-based limitations as discussed in spontaneous aggregation (above). The limited mass transfer results in: 1) a viability gradient in the spheroid that limits its overall size to about 350 μm (Fong, 1991); and 2) the formation of a necrotic core with increasing size.

Liquid overlay has been extensively used in cancer biology to determine the mechanisms of tumor growth, invasion, and angiogenesis. It is also widely utilized for mechanistic studies of therapeutic drugs. However, the limited spheroid size, necrotic core, and the resulting apoptosis/anoikis seen in less transformed cells limit the usefulness of this approach when a native tissue is required for analysis.

Aggregation of cells within liquid overlay is reaction dependent, rather than diffusion dependent. During suspension culture, two steps must occur in order for aggregation to occur: i.e., the cells must come into contact (or collide) with one another and they must adhere to one another. The kinetics of cell-cell interaction during liquid overlay culture has also been modeled utilizing equations proposed by Enmon et al. (2000). These equations were originally developed by Smoluchowski (1917) but adapted to include the effects of motility, collision and adhesion, and tested empirically.

These mathematical studies showed that aggregation of cells is reaction dependent. Thus, cells may collide with one another often but will not aggregate unless adhesion occurs (Enmon et al., 2001; Enmon et al., 2002). This is particularly important in the experiments described here since Rotary Cell Culture System (RCCS) culture results in localized areas of increased cell density and, likely, an increased number of cell collisions.

The hanging drop approach, a modification of the liquid overlay method, consists of applying cells in a single drop to a substrate and inverting the culture vessel to prevent cell adhesion. The hanging drop has been used in studies investigating the effects of microgravity, N-cadherin, and to create 3D mammary constructs without the use of exogenous extracellular matrix (Timmins, 2005). However, only a limited number of cells can be cultured using this methodology and required medium changes are difficult.

In the experiments described herein, liquid overlay is useful as a control for RCCS culture. This allows for assessment of cell-cell interaction, fusion, and differentiation during static suspension culture compared to suspension in the free-fall of the RCCS culture (see below).

Spinner flasks and other methods

Although the individual spheroids generated during liquid overlay may be suitable for some experimental purposes, a greater number of spheroids can be generated by stirred bioreactors or spinner flasks (O'Connor, 1999). The impeller-driven circulation of spinner flasks not only maintains cells in suspension but also induces mixing of the medium. This mixing ameliorates the static environment and can facilitate mass transport in the spheroid. In addition, any localized microenvironments surrounding aggregates are minimized.

A disadvantage of the spinner mechanism is that it induces additional shear, turbulence, and other forces into the culture environment. This increased hydrodynamic force limits the size of the aggregate that can form. As the aggregate becomes larger, a corresponding increase in the speed of the impeller is required to keep the aggregate in suspension. This results in ever increasing shear stress and wall impacts of the aggregate

which can induce unwanted and confounding cell processes; i.e., cell death, injury, or apoptosis.

In some cases, spinner culture has resulted in the differential expression of proteins. When compared to 2D control cultures grown on supports, greater immunostaining was observed for some cell components (cytoskeletal proteins, epidermal growth factor receptor, transforming growth factor – beta, and collagen) with comparable staining for others (Clejan et al., 1996; O'Connor et al., 1997). In addition, cells in spinner flasks showed a slower growth rate due to decreased proliferation relative to 2D controls (O'Connor, 1999).

Overall, due to problems associated with impeller driven shear forces, spinner flasks are only useful in select capacities. These include the large-scale generation of embryoid bodies (Cameron et al., 2006) and for providing perfusion during cell culture with scaffolding (Mygind et al., 2006). This approach is not well suited for investigating the formation of myotubes from myoblasts.

Rotary Cell Culture System

The Rotary Cell Culture System (RCCS) provides low-shear, low-turbulence culture conditions while maintaining cells in a constant state of free-fall (Hammond and Hammond, 2001). This system consists of a power supply, rotating base, and a culture vessel, which is placed inside a traditional tissue culture incubator. Rotation of the culture vessel about the horizontal axis randomizes the gravity vector and prevents cell settling. Thus, both individual cells and cell aggregates remain in suspension. Because RCCS culture conditions favor cell co-localization and minimize sheer stress that can prevent cell aggregation, it has been used to generate 3D tissue models (Margolais et al., 1999;

Song et al., 2002; Song et al., 2004). However, few studies address the issue of single cell morphology and initial cell-cell interactions contributing to cell aggregate formation during RCCS 3D culture.

The RCCS provides a cell culture method characterized by a fluid-filled culture vessel (zero headspace) rotating about the horizontal axis. The unique features of RCCS culture include the following:

1. Shear forces and turbulence are minimized since the fluid flow within the culture vessel is near solid body. The rotation of the inner and outer walls of the culture vessel is equal, which minimizes the laminar fluid flow gradient. Shear is the product of the laminar fluid flow gradient and the viscosity of the medium. Under standard RCCS culture conditions, these forces are very low; i.e., only about 0.2 dynes/cm^2. Terminal velocity is the speed of free-fall at which no additional acceleration will occur. Terminal velocity is achieved within the RCCS by rotating the vessel in the horizontal plane so that the rate of rotation is the inverse of the terminal velocity of the cell and the medium. Shear force is caused by the sliding of one body by another either in opposite directions (such as rubbing your hands together) or within the same direction but at different rates. Shear is minimized within the RCCS vessel because the terminal velocities of the cells and the medium are nearly equal.
2. Mixing in the RCCS occurs by rotation due to secondary forces of the particle (or cell) sedimentation through a fluid. This is in contrast to spinner flasks where mixing is induced by rotation of impellers or vanes that produce substantial levels of shear stress.
3. Zero headspace prevents turbulence at the air-fluid interface. Secondary bubble formation, which may be introduced into the medium and cause additional

turbulence, is avoided. Since oxygenation and gas exchange occur by diffusion through a gas permeable silicone membrane, no bubbles are introduced. No sparging or bubbling of the culture medium for oxygenation is required.

4. Cell co-localization is facilitated via internal forces. Culture vessels are rotated at a speed that balances gravity and centrifugal force to maintain cells in free fall (see below).
5. Co-localization and subsequent aggregation may provide protection from apoptosis since aggregation directly relates to survival potential *in vitro* (Glinsky and Glinsky, 1996).

Generation of Skeletal Muscle in the RCCS

The few previous investigations of *in vitro* generation of 3D skeletal muscle tissue during RCCS culture have included the use of seeding cells on substrates (microcarriers and membrane inserts). The first two investigations (Molnar et al., 1997; Torgan et al., 2000) utilized cells seeded with microcarriers. Cells attached to beads in less than 24 hours and aggregates formed (Torgan, 2000). Bridging between beads by cells to form aggregates was observed at 48 hours (Molnar, 1997). Proliferation was reduced (Molnar, 1997), particularly after the appearance of myotubes, but did occur to some extent (Torgan, 2000).

Myotubes have been observed at 48-72 hours in the absence of a switch from growth to differentiation medium (Molnar, 1997), or at five days with a medium switch (Torgan, 2000). These differences indicate that: 1) culture conditions can alter the time in which differentiation occurs and 2) differentiation can occur within the RCCS without the change from growth to differentiating medium.

Beads were occasionally spanned by myotubes, however the majority of the myotubes were observed within multiple bead containing cell aggregates (Torgan, 2000). Since beads within the aggregate were likely to be in contact with cells from multiple directions (i.e., cells on the same bead and those from adjacent beads), myotubes might have formed and/or increased in areas where greater cell-cell contact occurred. This increased cell-cell contact might also explain how myotubes were formed to span beads

A third RCCS study of skeletal muscle myoblasts used membrane inserts seeded with myoblasts and then placed into RCCS culture (Slentz, 2001). Although the RCCS cultured cells were more densely packed with higher protein and DNA yields than controls, these conditions changed the effects of RCCS culture from free-fall conditions to one that resulted in a randomized gravity vector (similar to a Clinostat), and thus has limited relevance to the studies described herein.

Although previous investigations of skeletal muscle myoblast culture within the RCCS have provided some insight into fusion and differentiation, the studies described above remain two-dimensional in spite of the use of the RCCS. Much as the earth appears flat or round depending on perspective, so too do the beads. The beads provided a curved substrate for small areas of monolayer culture. Only in the areas of bead-bead contact did the models approach 3D interactions. The membrane inserts used by Slentz et al. (2001) also facilitated 2D attachment.

The purpose of the investigation utilizing three-dimensional tissue dictates which techniques are to be used and which characteristics are required. While the use of a complex 3D matrix may be necessary for generating models suitable for the analysis of contractility or implantation, the primary purpose of the model developed and described herein is to facilitate the elucidation of cellular and molecular mechanisms that lead to differentiation and fusion during RCCS culture.

C2C12 CELL LINE

The C2C12 mouse cell line is commonly used as a model for myoblast proliferation, differentiation, and fusion *in vitro*. C2C12 (Blau, 1985) is a subclone of the C2 cell line (Yaffe and Saxel, 1977) established from the thigh of normal C3H adult mice seventy-two hours after a crushing type injury. C2C12 cells have a doubling time of twelve (Rossi et al., 1997) to twenty hours (Blau, 1985). Typical growth medium (GM) consists of Dulbecco's modified essential (or Eagle's) medium supplemented with fetal bovine serum, which supplies necessary growth factors that stimulate proliferation. In addition, the inhibitor of differentiation (Id) proteins found in the serum of growth medium prevents induction of myogenesis by preventing dimerization of MRFs with E proteins. Studies have shown that C2C12 cells are specified, but not determined to myogenic lineage. In the presence of BMP and other factors, both C2C12 cells and primary myoblasts will covert to mesenchymally derived tissue types including the osteogenic and/or adipogenic lineage *in vitro* (Katagiri et al., 2006). Thus, the C2C12 is sensitive to medium types.

Induction of differentiation is simple and rapid. Confluent cells will begin to differentiate into myotubes in two to three days under conditions of either serum deprivation or changing from fetal bovine to horse serum. The differentiation index, which defines the amount of differentiation by the ratio of nuclei incorporated into syncitia, is 30-50% after seven days in differentiation medium (Miller, 1990). C2C12 cells retain the capacity to fuse and differentiate for up to 15 passages (ATCC). With increasing age, these cell cultures lose the ability to fuse (Marquette, personal observation). Although C2C12 myotubes were observed to actively contract in the

original strain (Blau, 1985), recent reports of spontaneously contracting C2C12 myotubes are rare.

Although transformation of anchorage-dependent to anchorage-independent cells is an identifying characteristic of tumorigenesis (Stoker et al, 1968), some anchorage-dependent cell types can be cultured in suspension without undergoing anoikis/apoptosis and remain untransformed (Sachidanandan et al., 2002; Dhawan and Helfman, 2004). When subjected to suspension without an adhesive substrate, anchorage-dependent C2C12 myoblasts become quiescent, even in the presence of mitogens. Upon reseeding to a substrate following suspension, C2C12 cells will synchronously re-enter the cell cycle or may be induced to differentiate when cultured in DM or serum-free medium (Milasincic et al., 1996). Quiescence in response to suspension culture has also been observed in other cell types of mesenchymal origin; i.e., fibroblasts (Benecke et al., 1978).

Initially, MHC expression is embryonic, and Type I expression occurs twenty-four hours after induction of differentiation via serum change. During C2C12 culture, Type II MHC was not seen until forty-eight hours post induction (Kontrogianni-Konstantopoulos et al., 2005). Interestingly, studies have induced myosin expression without fusion (cadherin coated bead-based studies) and fusion without differentiation (myoballs), although the latter did not assess myosin expression (Fischbach and Lass, 1978). In C2C12, mRNA was rapidly upregulated during *in vitro* differentiation when myotubes first appeared (Rauch and Loughna, 2004). Thus, I will use both MHC and formation of syncitia to validate my skeletal muscle model.

Accordingly, due to the ease of culture, rapid rate of proliferation, and ability to differentiate and fuse, C2C12 cells are often used in monolayer culture to study the mechanisms of cell:cell adhesion, differentiation, and fusion.

ACTIN

Actin, first purified by Straub (1942), is a highly conserved ubiquitous cytoskeletal protein. Actin functions as a structural component and in the motility of single cells (Pollard, 1976; Stossel, 1984). It is also a major contractile protein in muscle tissues and cells. Within the cell, actin is found in two distinct states; i.e., monomeric G-actin (globular) and polymerized F-actin (filamentous or fibrous) (Straub, 1943). G-actin is a 43 Kd protein with four subunits, two of which contain ATPase catalytic capabilities and an ATP binding site between subunits two and four. There are three groups of actin isoforms in humans (vertebrates), alpha, beta, and gamma (Vandekerckhove and Weber, 1979). The non-muscle or cytoplasmic isoforms include a beta (ACTB, chromosome 7) and a gamma (ACTG1, chromosome 17) type, contain a 4 amino acid difference between the two (Ng et al. 1985; Erba et al., 1988; Sonnemann et al., 2006). These isoforms are co-expressed in non-muscle cells. Interestingly, the beta isoform is resistant to cytochalasin B. The actin gamma 2 (ACTG2, chromosome 2) and alpha aortic (ACTA2, chromosome 10) are smooth muscle actin isoforms, with the former found in enteric tissues and the latter in the smooth muscle of the vasculature (Ueyama et al., 1990; Miwa et al., 1991). Alpha skeletal (ACTA1, chromosome 1) and alpha cardiac (ACTC1, chromosome 15) are found co-expressed in skeletal and cardiac muscle and one of the primary components of the contractile machinery (Gunning et al., 1984; Hanauer et al., 1984). Expression of both alpha cardiac and alpha skeletal isoforms is initiated during myogenesis around the time of fusion (Bathe et al., 2007).

Actin associated proteins serve to modulate polymerization and depolymerization within the cell. Nucleating proteins facilitate the initiation of de novo F-actin polymerization. Capping proteins typically function at the barbed end and serve to

stabilize F-actin by preventing additional polymerization and depolymerization. Cap Z is an example of a capping protein (Schafer and Cooper, 1995). G-actin binding/sequestering proteins, including profiling and thymosin-β_4, aid in the regulation of polymerization by effectively sequestering G-actin from the pool. This results in a lower F-actin to G-actin concentration and thereby stimulates F-actin depolymerization (Webber, 1999). Actin severing proteins such as gelsolin (reviewed by Kwiatkowski, 1999) actually sever and cap F-actin into fragments. These fragments can then be used to bypass the de novo nucleating step in F-actin (see below) and proceed directly to polymerization. Cross-linking proteins organize F-actin into bundles or gels and include proteins such as filamin and actinin. The sheer number and variety of these proteins can complicate defining mechanisms that involve F-actin remodeling such as the formation of cytoplasmic protrusions.

Actin remodeling is a dynamic process within the cell. F-actin is the polymerized form of G-actin and consists of two actin protofilaments arranged in a helix (Hanson and Lowy, 1963). ATP bound G-actin, in the presence of magnesium or calcium, is required for polymerization. Actin polymerization may occur on existing filaments or de novo. De novo polymerization can be divided into three steps: 1) Nucleation (lag phase) which is facilitated by a nucleating protein due to unfavorable conditions, 2) Elongation (log phase), and 3) Treadmilling (steady-state) (Oosawa and Kasai, 1962). Nucleating factors include Arp2/3, which initiates branched F-actin formation, and diaphanous (formins), which nucleate unbranched filament growth. Polymerization of existing F-actin does not require the nucleating step, but uncapping can be required. Critical concentration (C_c) is defined by the F-actin to G-actin ratio and results in F-actin treadmilling. If G-actin falls below C_c, F-actin depolymerization can occur. A rise in G-actin allows F-actin polymerization to proceed until C_c is achieved. An actin filament has polarity, with one

end (barbed) characterized by faster polymerization kinetics than the other (pointed) (Wegner, 1976).

Although this seems to be a relatively simple process, the above explanation does not take into account factors such as: 1) the state of ATP bound to G-actin; 2) the status of fragmented F-actin within the cytoplasm; and 3) the concentration and localization of F-actin-associated proteins. Changes of concentration or localization of any of these can potentially alter the ratio of F-actin to G-actin. In addition, the isoform of actin and the localization of that isoform can affect polymerization since each isoform has different association constants.

Although F-actin is ubiquitous within the cell, it is usually visibly concentrated to specific areas during monolayer culture. F-actin is arranged in stress fibers within the cytoplasm. Stress fibers, bundled F-actin filaments, will form when stable contacts (such as focal adhesions) are made with a substrate. Stress fibers are thought to play a role in cellular contraction. If cells become detached from the substrate, actin stress fibers also decrease. Stress fibers are very noticeable during 2D culture, but decrease in 3D culture (see additional information below). Another major location of F-actin is the cortical cytoskeleton, found at the periphery of the cell just under and attached to the plasma membrane. The majority of the remaining F-actin is localized to the lamellipodia, filipodia, or other cytoplasmic processes.

Actin altering agents

Remodeling of the actin cytoskeleton is necessary for proliferation, migration, and fusion of myoblasts to the myofiber (Alberts et al., 2002). Inhibition of remodeling is a technique often used to investigate the role actin plays in cellular processes. Several

pharmacological agents are currently used to manipulate F-actin. In particular, Latrunculin A (Lat A), derived from a sea sponge, affects remodeling by stochiometrically binding G-actin and effectively sequestering it from the available G-actin pool. The cell "senses" a reduction in G-actin and F-actin is depolymerized. Lat A has a high affinity for G-actin (K_d = .2-.4 nM) (Yarmola et al., 2000).

Cytochalasins have a function similar to the F-actin capping proteins. Cytochalasins bind to the barbed end of F-actin, independent of the G-actin concentration, and decrease polymerization. G-actin can still polymerize at the pointed end if conditions are favorable although at a much slower rate than at the barbed end. Thus, cytochalasin would still allow F-actin remodeling to occur. In addition, the cytoplasmic beta actin isoform is resistant to cytochalasin. I, therefore, elected to use Latrunculin A to inhibit F-actin polymerization for my experiments.

Phallotoxins are F-actin binding proteins that recognize and bind to a site between the subunits of polymerized actin (Low and Wieland, 1974). They will not bind to single actin monomers (Steinmetz et al., 1998). Phallotoxins, derived from the Death Cap mushroom, include phallacidin, phalloidin, phallisacin, phallisin and phalloin (Enjalbert et al., 1999). For my experiments, conjugated phallacidins and phalloidins were used to fluorescently label F-actin.

Effects of 3D culture on the Actin Cytoskeleton

Previous investigations of the effects of microgravity on humans have largely studied the re-arrangement of the F-actin cytoskeleton during 3D culture. Thus, the majority of these experiments have been performed in either microgravity or

microgravity analogs. Analogs include, but are not limited to: RCCS, clinostat, drop towers, and parabolic flight.

A number of cell types, including a glial cell line (C_6), human umbilical vascular endothelial cells (HUVEC), osteoblast-like cell line (ROS.SMER #14), seeded on a substrate (beads or vessel surface), gradually assumed a rounded shape in thirty minutes. Concurrently, filipodia retracted, numerous lamellipodia appeared, and the cortical actin cytoskeleton appeared disrupted (stress fibers are not mentioned). After thirty-two hours of clinorotation, a recovery of cortical actin was reported, but no recovery of the cell topography was observed (Uva et al., 2002). HUVEC-C endothelial cells cultured on beads in a rotating wall vessel bioreactor (similar to the RCCS) showed cell topography changes within four hours and these changes were maintained for 144 hours (Carlsson et al., 2003). Extended podia were noted and actin fibers were disorganized. Concentrations of disorganized actin also localized in the perinuclear area. At 144 hours, the perinuclear actin had disappeared and stress fibers were greatly reduced. The osteoblast-like cells, also cultured in a rotating wall vessel bioreactor, exhibited spherical topography with irregular edges after forty-eight hours of microgravity exposure (Rucci et al., 2002). Actin staining in these cells was intense when compared to controls.

Contrary to the above reported differences in the actin cytoskeleton, no changes in actin were noted in attached MCF-7 human breast cancer cells (Vassy et al., 2001) or suspended Jurkat T-cells (Sciola et al., 1994) during the microgravity phase of parabolic flight on the KC-135. It should be noted that these studies involve the exposure of test cells to hypergravity conditions before the brief interval (20 seconds) of microgravity. Thus, the effects of these combined conditions can make conclusions about the exclusive effects of microgravity more difficult to discern. In contrast, other studies suggest that actin polymerization can be affected by microgravity. Another parabolic flight study

showed a reduction of actin polymerization in Jurkat T-cells activated during the microgravity phase of parabolic flight (Hashemi et al., 2001). This study demonstrates that microgravity seems to affect actin in as little as the twenty seconds of microgravity exposure experienced during parabolic flight.

Cell morphology and protrusions can be changed by the presence or absence of substrate. The selective attachment of cells by shaped focal islands (small round, square or triangular adhesive areas on a non-adhesive substrate) alters cell shape, lamellipodia, and actin structure (Parker et al., 2001). Attachment of cells to substrates such as glass coverslips/slides (Huges-Fulford and Lewis, 1996; Guignandon et al., 2001; Lewis et al., 2001; Vassy et al., 2001), or beads (Carlsson et al., 2003) can mask alterations of cell shape and actin morphology changes due specifically to microgravity**.** Analysis of cell-cell aggregation in suspended cells may also influence cell shape and actin morphology (Rucci et al., 2002). Diverse treatments of cell preparation for analysis and the variation of imaging techniques (laser scanning confocal microscopy, scanning electron microscopy, epifluorescence microscopy) can also contribute to the range of results. Lastly, although some studies quantified the effects of microgravity on actin (Hashemi et al., 2001), the wide use of descriptive data can confuse an overall understanding of microgravity-induced alterations of cell topography and actin morphology. Accordingly, there is a critical need for further clarification of the effects of microgravity on the actin cytoskeleton. Such studies may provide insight into microgravity-induced muscle atrophy, an impediment to long-duration manned space exploration.

ADHESION

Molecules on the exterior of the cell play a role in adhesion and re-adhesion/aggregation following cell separation by enzymatic digestion (i.e., Trypsin)

(Moscona, 1963). The major groups of adhesion molecules that participate in myogensesis include both calcium-independent and calcium-dependent adhesion molecules. Adhesion molecules can be classified into four families depending on their biochemical and structural characteristics. These families include the selectins, the immunoglobulin supergene family, the cadherins, and the integrins (Liekins, 2001).

Selectins

Selectins are a small family of calcium-dependent cell adhesion molecules, with an N-termius lectin-like domain that functions in calcium dependent binding to sialylated (o-linked) glycans. These receptors mediate heterotypic cell-cell interactions and are most commonly linked with leukocyte adhesion, particularly in tethering (attachment) and rolling (reviewed by Juliano, 2002). However, homotypic interactions have also been reported (Alon et al., 1996). Selectins do not seem to be directly associated with skeletal muscle fusion and differentiation.

Immunoglobulin supergene family

Cell Adhesion Molecules (CAMs) are calcium-independent molecules characterized by one to several immunoglobulin folds incorporated in the external domain and a cytoplasmic tail (Aplin, 1998). Extracellular CAM binding can occur with CAM receptors or with integrins. Little is currently known of the interactions of CAM with other cytoplasmic components.

NCAM, the most intensely studied CAM, is initially expressed in somites and myotomes (Thiery et al., 1982). Later, it is expressed in myoblasts, myotubes and

myofibers until innervation (Covault and Sanes, 1985; Rieger et al., 1985). NCAM has been implicated in myogenesis (Dickson et al., 1990a; Knudson et al., 1990) and its inhibition by antibody blocking decreased the rate of fusion (Knutsen et al., 1990). In contrast, no reduction in fusion with antibody inhibition was observed by Mege (1992). These apparently discrepant results could be a result of variations in the specificity and binding strength of the antibody or differences in the concentrations of antibody utilized. In addition, Mege (1992) noted that the rate but not the extent of fusion was decreased by NCAM. Considering that over expression increases fusion rate (Dickson et al., 1990), NCAM likely modulates the rate, but not the final extent of fusion (Mege, 1992). Other CAMS including Netrins and VCAM-1 are also implicated in myogenesis but their role remains to be fully defined (Rosen et al., 1992).

Integrins

Integrins are integral glycoproteins that primarily function in cell-extracellular matrix binding, particularly with fibronectin, laminin, and collagen.

Integrins function as heterodimers of one alpha and one beta unit. Integrin-mediated adhesion of cells to ECM initiates signals that influence survival, proliferation, and expression of specific developmental phenotypes (Menko and Boettiger, 1987; Adams and Watt, 1990; Streuli et al., 1991; Chen et al., 1997). Integrin receptors undergo conformational change in response to intracellular signals that modulate binding affinity for ligands (Loftus and Liddington 1997). Antibodies to β_1 integrin block differentiation in embryonic myoblasts and cells remain in a proliferating state (Menko and Boettiger, 1987). In addition, integrin-mediated adhesion can be critical for skeletal muscle differentiation (Menko and Boettiger, 1987; von der Mark and Ocalan, 1989).

Cadherins

Cadherins are a superfamily of calcium-dependent cell-cell adhesion molecules that mediate cell-cell interactions. Members of this group have one or more cadherin domains, each consisting of approximately 110 amino acids (Wheelock and Johnson, 2003). Cadherins are translated with a protodomain that is cleaved in the cell during processing (Haussinger et al., 2004). Cadherins are a single pass adhesion protein and most members have a cytoplasmic domain that modulates adhesion to the actin cytoskeleton via the catenins.

Type I, or classical cadherins, are a subgroup of the cadherin family distinguished by five extracellular cadherin repeats and a histine-alanine-valine (HAV) sequence within the N-terminal cadherin repeat (EC1) (Nose et al., 1990). EC1 is critical to promoting the homophillic binding that typifies these adhesion molecules. Classical cadherin mediated cell-cell adhesion plays a role in tissue organization, cell-cell adhesion, sorting, and differentiation in both development and the adult. Cadherins are a major component of adherin junctions. In addition, cadherins are important regulators of apoptosis (Makrigiannakis et al., 1999). Cadherins may also play a role in the down-regulation of Integrin expression (Hodivala et al., 1994).

The extracellular cadherin repeats are capable of both cis and trans adhesion. Initial weak trans cadherin-mediated adhesion is established between cis dimmers of opposing cells (Troyanovsky, 2005). Further strengthening and formation of stable contacts occurs by: 1) recruitment of additional cadherin molecules to the site of initial contact (Mary, 2002); and 2) attachment of cadherins to the underlying actin cytoskeleton at the site of contact. Although little is known about the signaling pathways that result in cadherin recruitment, the interactions between cadherin and the cytoskeleton is better

understood. The cytoplasmic domain of a cadherin molecule can anchor to the underlying F-actin structure through the catenins (Lambert et al., 2002). The cytoplasmic domain can interact directly with either β-catenin or plakoglobin (mutually exclusive proteins) and either will bind with α-catenin, which links directly to F-actin. P120, another member of the catenin family, can bind independently of the complexes and modulate binding. Once formed, cadherin adhesion assemblies are dynamic and are continuously remodeled (Adams et al., 1998).

Although cadherins are tissue specific, each tissue can express more than one cadherin with expression temporally regulated. Myoblasts and mature muscle express two primary classical cadherins; i.e., Muscle cadherin (M-cadherin) and Neural-cadherin (N-cadherin). M-cadherin (Cdh15) is expressed in quiescent satellite cells and is up-regulated following activation (Irintchev et al., 1994; Cornelison and Wold, 1997; Beauchamp et al., 2000). M-cadherin null mice have normal muscle development and regeneration, indicating that other cadherins may have functional redundancy (Hollenagel et al., 2002).

N-CADHERIN

General description

Neural-cadherin (N-cadherin) a member of the classical cadherin subgroup, functions in a number of cellular processes during skeletal muscle development and regeneration including myoblast motility, cell sorting, differentiation, maintenance of cell-cell adhesion and, ultimately, tissue architecture (reviewed by Leckband and Prakasam, 2006). N-cadherin (Cdh2), also known as A-CAM, is a calcium dependent,

homophillic cell adhesion molecule (Takeichi, 1998). As with all classical cadherins, the external domain consists of five-tandem cadherin repeats interspersed by calcium binding sites (Shapiro et al., 1995).

N-cadherin in Development

N-cadherin is spatially and temporally regulated during development and plays a critical role in cardiogenesis (Linask, 1997), osteogenesis (Marie, 2000), chondrogenesis (Woodward and Tuan, 1999), neurogenesis (Hatta and Takeichi, 1986, Duband et al., 1988; Matsuzaki et al., 1990; Tepass et al., 2000), and myogenesis (Redfield et al., 1997; Goichberg and Geiger, 1998; Charrasse et at., 2002). Homozygous N-cad null mutation results in mouse embryonic lethality by day 10 from severe cardiac, neurulation, and somite abnormalities (Radice, 1997). N-cadherin expression and localization mediates somatogenesis. If N-cadherin is blocked at this time, irregular or multiple somites form (Linask et al., 1990). During myogenesis, N-cadherin is uniformly expressed along the surface of myoblasts and primary myotubes, but is decreased in myotubes prior to secondary myotubes formation. It is re-expressed in localized areas and seems to aid in establishing the neuromuscular junction (Cifuentes-Diaz et al., 1994). Interestingly, F-actin positive cytoplasmic projections, termed myopodia, are also seen at the time of synaptogenesis (Ritzenthaler et al., 2000).

N-cadherin expression is maintained at the cell-cell junctions of the myotomes and later, limb buds (Linask, 1997). N-cadherin also functions in cell sorting during development. Mixed non-expressing cells transfected with E-cadherin or N-cadherin segregate into distinct areas (Friedlander, 1989) and chimeric embryos with N-cadherin-deficient and wild-type cells sorted away from each other during development (Kostetskii

et al., 2001). Down-regulation of N-cadherin occurs in primary myotubes during secondary myogenesis (Durband, 1987; Hatta, 1987) and following myotubes formation in cell culture (Mege et al., 1992). This may explain why myoblasts form secondary myotubes rather than fuse with primary myotubes.

N-cadherin in myogenesis and muscle regeneration

A number of studies have examined the role of N-cadherin in aggregation and fusion. Myoblast aggregation was inhibited by polyclonal and monoclonal anti-N-cadherin antibodies (Knudsen et al., 1990; Cifuentes-Diaz et al., 1993). Cell exposure to to the Histidine, Alanine, Valine sequence of cadherins also decreased aggregation (Mege, 1992). This seems to indicate that N-cadherin plays a role in myoblast cell-cell adhesion, a first step to fusion. Anti N-cadherin antibodies and blocking peptides to the external domain also inhibited myotube formation (Knudsen, et al., 1990; George-Weinstein, 1997). Cells over expressing N-cadherin showed increased aggregation in hanging drop (3D) culture (Redfield, 1997). Interestingly, cells interacted with the external domain of N-cadherin bound to beads via small membrane protrusions (Levenberg et al., 1998) and it is possible that adhesion proteins localize to these structures. The maximum expression of N-cadherin is seen just prior to fusion and declines post fusion (Pouliot et al., 1990; MacCalman et al., 1992).

Interestingly, N-cadherin expression is increased at the time of secondary myotube formation (Pouliot et al., 1994), but is down regulated with increasing maturation of myotubes (Moore and Walsh, 1993). The inhibition of N-cadherin also inhibits fusion in myoblasts (Zeschnigk et al., 1995). N-cadherin null myoblasts cells fused to form myotubes similar to those in the wild-type. Accordingly, N-cadherin might

not be essential for myoblast fusion (Charlton et al., 1997). However, these cloned myoblasts could have developed a redundant mechanism to facilitate fusion in the absence of N-cadherin.

Differentiation

A number of investigations have suggested that N-cadherin functions to induce differentiation. Inhibition of N-cadherin by antibodies inhibited sarocomeric myosin expression (Mege, 1992). Over expression of N-cadherin in cells with low native N-cadherin expression stimulated sarcomeric myosin expression (Redfield, 1997) and upregulated myogenin (Seghatoleslami, 2000). Myogenic cells were stimulated to differentiate following exposure to N-cadherin bound to beads (Goichberg and Geiger, 1998) or plating on N-cadherin coated substrates (Gavard et al., 2004). N-cadherin binding facilitated a by-pass of growth stimulating conditions (high FBS, low cell density) in favor of differentiation when stimulated with bead bound N-cadherin or anti N-cadherin antibodies (Goichberg and Geiger, 1998). N-cadherin-mediated adhesion increases RhoA activity and activates three skeletal muscle-specific promoters (Charrasse et al., 2002).

PODIA

Cytoplasmic protrusions (podia) are observed in a number of cell types during a variety of culture conditions and are reported to serve a multiplicity of functions. The cytoplasmic protrusions observed on single cells during RCCS culture are particularly striking in both number and size. Currently, the mechanisms of podia-facilitated motility (largely lamellipodia) are the subject of intensive investigation. The development and

function of other podia, such as filopodia and microvilli, has also been the subject of a diverse array of study. Actin associated podia can extend over distances and establish contacts with neighboring cells or with matrix (Vautier, 2003).

Definition of Podia

There are many varieties of podia described. Lamellipodia are broad, flattened, veil-like protrusions, usually parallel to the substrate. Microspikes and filipodia, designations often used interchangeably, are long thin projections approximately 0.2 μm in diameter and of variable length (5-30 μm). These podia are reported to extend from the cell surface and exhibit a range of motion unless they attach to a substrate (Albrecht-Beuhler and Lancaster, 1976). Lamellipodia, microspikes, and filipodia are all thought to be involved in cell movement (Nakamura, 2001). Microvilli are morphologically cylindrical (0.1 μm in diameter, 2 μm long), and multiple microvilli can cover the surface of a cell that is not attached to a substrate (Albrecht-Beuhler and Lancaster, 1976). Microvilli are thought to be more permanent structures while lamellipodia, microspikes, and filipodia tend to be more ephemeral in nature (Nakamura, 2001). For the purposes of this work, podia are defined to include microspikes, filipodia, and microvilli.

Podia during cell culture

The most noticeable podium type during 2D culture is lamellipodia. During motility, these structures protrude in front of the cell and retract behind (Small and Resch, 2005). Adhesion with the substrate is required for lamellipodia to extend in the direction of movement by actin polymerization along the leading edge and to secure the cell to the

substrate to allow depolymerization of F-actin at the rear in a retrograde action. The 2D culture environment emphasizes and favors cell-substrate interactions. Thus, lamellipodia could be an artifact of the artificial environment. Although some studies report that cylindrical pseudopodal processes form during movement through a collagen gel (Heath and Peachey, 1989), others report that lamellipodia in this same cell type do not exist (Beningo et al., 2004). Thus, the existence of lamellipodia in 3D remains questionable. It is worth considering if these differences could be a consequence of the potential difficulty in recognizing a lamellipodium in 3D.

The spherical morphology of myoblasts cultured in either suspension or modeled microgravity is closer to the *in vivo* shape of satellite cells than the flattened cells seen during 2D culture (Sabourin et al., 1999). Inconsistencies in cell behavior and morphology during monolayer culture and *in vivo* conditions have initiated studies of cells and cell interactions during 3D culture; however, investigations of the nature and function of the podia during 3D culture are rare. Although the presence of lamellipodia in 3D culture is questionable, the presence of cytoplasmic protrusions has been reported (Huges-Fulford and Lewis, 1996; Guignandon et al., 2001)

Podia formation occurs when the degree of cell-substrate interaction decreases. Suspended cells typically exhibit more podia than their attached counterparts. For example, an electron microscopy study of 3T3 cells (mouse embryo fibroblast cell line) showed that as cells detach from a substrate, podia become more numerous (Collard and Temmink, 1976). Seher and Adam (1978) determined that podia were formed by membrane involutions as the cell detached from the substrate. The involutions were calculated, based on surface area and volume, to be a mechanism for accommodating excess membrane when transitioning from a flattened to a rounded morphology. However, this mechanism was described when investigations of F-actin in non-muscle

roles was in its infancy. The discovery of the cytoskeleton was considered a major development of the 1970's (reviewed by Davis, 1980) but the incorporation of F-actin in the role of podia formation was not yet widely accepted. However, many scientists are now examining mechanisms of F-actin involvement within cytoplasmic projections.

Several studies have reported the appearance of podia in cells exposed to microgravity and microgravity analog conditions. An osteoblast cell line (MC3T3-E1) cultured during microgravity exposure (STS-56) (Huges-Fulford and Lewis, 1996) showed morphological changes that included the appearance of numerous podia. A reduction of actin stress fibers was also noted in this study, indicating a loss of cell-substrate attachment. A second study reported the appearance of podia and noted cortical localization of a disorganized actin cytoskeleton during space flight in the ROS17/2.8 (osteoblast) cell line (Guignandon et al., 2001). Again, the disrupted stress fiber distribution seems to indicate the loss of cell-substrate attachment. It is important to note that cells, cultured in a 3D milieu, exhibit a much more rounded morphology and fewer stress fibers than seen in 2D culture

In liquid overlay cultures, single cells have been seen extending long projections toward a neighboring cell or aggregate (O'Connor et al., 2000; Enmon et al., 2001). This "tethering" mechanism was thought to be a means of establishing initial contact prior to forming closer and more permanent aggregates. However, myoblasts embedded in a collagen gel showed no sign of podia formation until eight hours in culture (Cheema, 2003). These differences might be a consequence of cell type (prostate carcinoma versus myoblast cell lines) or culture conditions (liquid overlay versus collagen embedding). Alternatively, these differences could be due to the amount of cell:cell contact present. It is far more likely that the cells in liquid overlay (suspended in a liquid medium) will

come into contact with one another than those embedded in a gel. It is possible that some culture conditions are more conducive to podia than others.

Podia Function

Cytoplasmic projections are most well known for their role in motility; however, they also serve as sensory structures and might also play a role in differentiation and cell fusion (Vautier, 2003). Specifically, filopodia and similar small actin-dependent protrusions are thought to function in cell sensory and guidance. For example, osteocyte podia serve as a sensory mechanism (You et al., 2004). Both filipodia and microvilli, mediated by hydraulic induced shear or drag forces, might regulate ion transport for several structures of the renal system (Guo et al., 2000; Liu et al., 2003; Praetorius, 2003; Du et al., 2004).

Filopodia might also play a role in myoblast differentiation and cell fusion. In an ECM study, the majority of filopodia extended from the apex of the elongated, aligned cells and were often seen in contact with the apex of the next cell. Thus, filopodia form cell-cell contact and may mediate adhesion and alignment, which are prerequisites to myoblasts fusion (Swailes, 2004). Cell projections have also been noted between myoblasts and bead-bound N-cadherin, a protein believed to play a role in differentiation in myoblasts (Goichberg and Geiger, 1998). This suggests the following question. Are podia a mechanism to facilitate cell-cell contact and adhesion, essential steps in myoblast cell fusion? A detailed examination of the role of podia in initial adhesion and subsequent maturation of myoblasts into multinucleated, MHC-expressing cells, a manifestation of skeletal muscle tissue formation *in vivo*, may provide insight into *in vivo* skeletal muscle regeneration.

THE N-CADHERIN/F-ACTIN/PODIA CONNECTION

Adhesion proteins to podia

Based on the proceeding discussion, I hypothesize that N-cadherin might localize to the podia seen in RCCS culture. Such localization has been reported for a variety of adhesion molecules in 2D culture. For example, various integrins have been shown to localize to the leading edge of lamellipodia (Choma et al., 2004), filopodia (Rabinovitz, 1997), and other membrane protrusions (Laukaitis, 2001). NCAM is localized to the tips of filopodia (Sandig, 1997) and the majority of L-selectin is localized to the microvilli of a number of (immune system) cell types (Bruehl, 1996). For the experiments reported herein, I selected N-cadherin based on its function as an adhesion molecule, the reported localization of N-cadherin to the tips of podia, and its role in fusion and myogenesis.

Engagement of adhesion proteins might induce the formation of cytoplasmic extensions. Engagement of CC151 (a tetraspanin) induces filopodial extension in several cell lines (Shigeta et al., 2003). Nectins (Kawakatsu et al., 2002) and E-cadherin show similar responses. N-cadherin binding on an N-cadherin coated substrate resulted in the formation of broad lamellipodia. Further, *de novo* N-cadherin binding at the tip of the lamellipodium appeared to be the preferred binding site (Gavard et al., 2004).

During 2D culture, N-cadherin binding results in lamellipodia formation via a PI 3-Kinase-Rac-1 dependent pathway (Gavard et al., 2004) in a similar manner to E-cadherin in epithelial cells (Kovaks and Ali, 2000). The co-localization of single cells favored by RCCS culture provides an increased opportunity for cell-cell contact. N-cad:N-cad contact is cited as the initial stage for increased N-cad recruitment to the site of contact (Mary et al., 2002). However, reports indicate increased RhoA (a small Rho GTPase) activity is associated with N-cadherin binding and may induce differentiation

(Charrasse et al., 2002). It is possible that initial contact stimulates F-actin polymerization and podia formation upon early or de novo N-cadherin contact. As the adherin complex matures, a switch to RhoA could be initiated.

Although normally diffusely localized over the entire free surface of cells (Soler and Knudsen, 1991; Mege, 1992), multiple N-cadherin molecules are recruited to the site of original contact to strengthen the adhesion, as does the interaction of the cytoplasmic tail of N-cadherin with the actin cytoskeleton. The brief contact during RCCS culture might also stimulate F-actin polymerization via an N-cadherin/ PI 3-Kinase-Rac-1 dependent pathway (Mary, 2002). Assuming this is true in 3D RCCS culture, the following is likely to occur:

1. Cell-cell collision, facilitated by RCCS co-localization, establishes ephemeral N-cadherin-N-cadherin binding on adjacent cells. This initial contact, while not strong enough to facilitate permanent adhesion, is enough to stimulate the recruitment of additional N-cadherin molecules to the site of contact and should result in an increase of N-cadherin molecules at the site of contact.
2. This ephemeral N-cadherin contact also stimulates the PI 3-kinase-Rac-1 pathway, which results in additional actin polymerization at the site of contact. Thus, podia should begin to form and extend.
3. Subsequent cell collisions are more likely to occur at the podia, which cause a localized increase in the cell radius. However, since N-cadherin may not yet have reached levels that permit permanent adhesion, steps 1 and 2 continue to re-occur with every contact. This should result in additional N-cadherin recruitment and actin polymerization seen as increasing length of podia.
4. Podia length might be limited by three primary processes: a) the amount/localization of available G-actin modulated by the amount/localization of

actin associated proteins; b) the balance of forces between the flexibility of the cell membrane and the protrusive strength of F-actin polymerization; and c) the amount of N-cadherin localized to the podia prior to permanent adhesion.

5. When N-cadherin binding reaches a critical strength, permanent adhesions form.

Chapter 2: Specific Aims

In preliminary studies, our laboratory noted two particularly important and relevant observations regarding cells cultured in the Rotary Cell Culture System (RCCS):

1. Myoblasts adhere, fuse and form syncitia (multinucleated cells) in the absence of substrate attachment and in the presence of growth (proliferation) medium. In contrast, other reports indicate that specialized differentiation medium is essential for syncitia formation and expression of muscle specific proteins (Milasincic et al., 1996). This suggests that the RCCS culture conditions enable the myoblasts to bypass the normal growth/proliferation signals/pathways and proceed to fusion and multinucleation.
2. Numerous, actin-associated cytoplasmic extensions (podia) form in unattached, single myoblast cells. In contrast, the number of podia is much lower in myoblasts in liquid overlay suspension culture compared to the RCCS. Because adhesion molecules are typically associated with podia, I propose that the cell-cell adhesion molecule N-cadherin (a protein critical to myoblast differentiation) is associated with these podia. If the podia seen in RCCS culture localize N-cadherin, this could facilitate cell-cell adhesion, a critical early step in myoblast fusion. In addition, the podia and N-cadherin would represent potential targets for cell-based treatments to combat the different forms of muscle atrophy.

Accordingly, my hypotheses are: 1) N-cadherin plays an important role in increased myoblast differentiation observed in RCCS culture in multicellular aggregates; and 2) N-

cadherin will be localized to podia in an actin-dependent manner. These hypotheses will be tested with the following Specific Aims:

Specific Aim 1: Validate that the multinucleated cells arising during culture in modeled microgravity conditions express markers specific for muscle differentiation.

Experiment 1.1 determine if differentiation occurs in myoblasts cultured in modeled microgravity by staining for skeletal muscle specific markers and evaluating the presence of syncitia in histological sections

Specific Aim 2: Inhibition of N-Cadherin decreases differentiation in modeled microgravity.

Experiment 2.1 determine if decreased differentiation occurs when N-cadherin is inhibited by antibody binding to N-cadherin

Specific Aim 3: Modeled microgravity induced podia formation in myoblasts is actin dependent.

Experiment 3.1 determine if podia are more abundant in modeled microgravity cultured myoblasts than in controls and if F-actin is associated with these podia

Experiment 3.2 examine if podia formation is dependent on F-actin by inhibiting actin polymerization with Latrunculin A

Experiment 3.3 determine if podia form when F-actin inhibition is removed

Specific Aim 4: N-cadherin is differentially localized to modeled microgravity-induced podia in an actin dependent manner.

Experiment 4.1 assess if N-cadherin is differentially localized to modeled microgravity-induced podia

The results of this proposed research will: 1) define a novel myoblast model test system that can be used to investigate and define basic regeneration processes; 2) enhance our understanding of the role of N-cadherin and podia in the adhesion, regeneration, and differentiation of myoblasts—all currently undefined but important fundamental cellular processes; and 3) identify potential mechanisms and/or biological targets for the development of molecular and cell-based treatments to improve muscle regeneration both in space and on Earth.

Chapter 3: A Three-dimensional Model for the Examination of Fusion and Differentiation in Skeletal Muscle

INTRODUCTION

Skeletal muscle models have played a crucial role in elucidating the characteristics, physiology, and regulation of differentiation and fusion of skeletal muscle (reviewed by Charge and Rudnicki, 2004). Models have also played a role in defining skeletal muscle degeneration in: 1) disease processes; (i.e., cacheixia, aging (sarcopenia), inactivity/disuse (stroke), and unweighting (microgravity) (reviewed by Kanderian and Jackman, 2006); 2) other forms of muscle damage (trauma); and 3) pharmacological, nutritional, and exercise-based interventions. In addition, skeletal muscle models may provide insight into tissue engineering and potential regenerative medicine applications (Goodwin et al., 1993; Unsworth and Lelkes, 1998). In spite of the advances supported by current skeletal muscle models, critical questions remain unanswered, including the mechanisms of fusion during myoblast differentiation and microgravity-induced muscle atrophy. It is difficult to efficiently develop and increase the efficacy of therapies when the mechanisms of basic muscle development, regeneration and muscle diseases remain poorly understood. Understanding these mechanisms is an essential step in designing novel, specifically targeted countermeasures as well as optimizing the efficacy of those currently in use.

A variety of models have been used to investigate skeletal muscle including *in vivo* human and animal models, two-dimensional (2D) monolayer cell cultures, and three-dimensional (3D) tissue models. Although human and animal models provide the most

physiologically relevant data, experiments utilizing these models can be time-consuming, costly, and are often confounded by multiple uncontrolled variables. Specific questions concerning the cellular and molecular mechanisms can be more readily addressed using cell culture based-models. Cultured cells are more amenable to specifically targeted molecular methodologies than *in vivo* models. Cell-based models are also less costly, more easily controlled, and reproducible. However, 2D cultures have limited *in vivo*-like functionality and lack the 3D cell-cell interactions present in tissues.

Despite advances in our understanding of the processes of differentiation and fusion of myoblasts facilitated by 2D monolayer culture, skeletal muscle is a three-dimensional (3D) tissue that results from 3D cell-cell and cell-tissue interactions during myogenesis. Further, regeneration of skeletal muscle is also a 3D process. The multiple regulatory pathways of myoblast fusion and differentiation remain unclear despite recent advances in molecular methodologies and considerable research dedicated to elucidating the process of skeletal muscle differentiation and regeneration (Charge and Rudnick, 2004; Horsley and Pavlath, 2004). Thus, 3D modeling of tissues *in vitro* is critical to understanding the basic functions and regulation *in vivo* (Cheema et al., 2003).

Three-dimensional tissue models will play a critical role in providing additional, more *in vivo*-like insight into differentiation and tissue organization. However, currently, very few 3D skeletal muscle models have been reported. These have included models produced both with (Molnar et al., 1997; Torgan et al., 2000; Cheema et al., 2003) and without (Dennis, 2001; Huang, 2005) an exogenous matrix. While these are suitable for measuring later events in developing skeletal muscle, such as contractility and force (Dennis et al., 2001; Cheema et al., 2003; Huang et al., 2005), the methods used to create these models make it difficult to observe the processes involved in myoblast adhesion, fusion and differentiation in 3D.

Therefore, the purpose of this study was to develop a three-dimensional skeletal muscle model from precursor myoblast cells suitable for characterizing the following: 1) initial cell binding and factors involved in this process; 2) fusion of myoblasts to form multinucleated syncitia; and 3) differentiation in the absence of externally supplied substrates or serum alterations. We developed this novel model in a unique culture environment, the Rotary Cell Culture System (RCCS). Originally designed by NASA to mimic some aspects of microgravity, the RCCS has also been recognized as an important tool for investigators in areas of tissue engineering (Dutt et al., 2003), tissue regeneration, and cancer cell biology (O'Connor, 1999; Clejan, 2001; Hammond and Hammond, 2001).

METHODOLOGY

Stock Cell Culture

C2C12 mouse myoblast cells, obtained from the American Type Culture Collection (ATCC, Rockville, Maryland), were cultured in a humidified incubator at 37^{o}C with 5% CO_2 in growth medium (GM) consisting of high glucose Dulbecco's Modified Essential Medium (DMEM, Invitrogen/Gibco, Carlsbad, California) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone, Logan,Utah) and 1% Penicillin-Streptomycin (Invitrogen/Gibco, Carlsbad, California). Cells were subcultured every 2-3 days as needed to maintain 75% confluency or less using 0.125% Trypsin/EDTA (Invitrogen/Gibco, Carlsbad, California). Culture medium was removed by aspiration, Trypsin/EDTA (Invitrogen/Gibco, Carlsbad, California) added, cultures

incubated for 2-5 minutes at 37^{o}C, fresh culture medium added, cells pipetted to facilitate removal from the culture surface, and the single cell suspension redistributed to new culture vessels. Cells in passages 6-8 were used for all experiments.

Treatment

Cells cultured as monolayers were removed from the culture surface using 0.125% Trypsin/EDTA. The resulting single cell suspensions were centrifuged, rinsed in fresh GM, and then resuspended in fresh GM. Cells were counted using an improved Neubauer hemacytometer and diluted in GM to a final concentration of 5 X 10^5 cells/ml. Cells were seeded into disposable 10 ml High Aspect Ratio Vessels (HARV) (Synthecon, Inc., Houston, TX) which were placed on a rotator base and spun at an initial velocity of 9 RPM in the horizontal plane. Rotation speed was adjusted over time to maintain cell aggregates in a constant state of free-fall. Suspension controls (SC) consisted of 10 ml of cells cultured in 60 mm tissue culture Petri dishes (Corning, Fisher Scientific, Houston, TX) pre-coated with poly-Hema (Sigma, St. Louis, MO) to prevent cell adhesion (three 2 ml coatings of 1% [poly(2-hydroxyethyl methacrylate] poly-HEMA in ETOH (W:V) (Folkman and Moscona, 1978). Petri dishes were air dried overnight in a Nuaire type II biological safety cabinet between coatings. Additional controls consisting of MG-63 osteoblast-like cells (ATCC) or MCF-7 breast cancer cells (ATCC) were also cultivated in the HARV.

At day 3 of culture, the GM containing cell aggregates was removed from the culture vessel and allowed to settle by gravity in 15 ml conical tubes. The GM was aspirated and replaced with either: 1) fresh GM and returned to culture vessels for an additional time interval; or 2) 10% neutral buffered formalin. At day 6 or 7 (6+), the

remaining cultures were fixed in 10% neutral buffered formalin. Fixed samples were processed, paraffin-embedded, and sectioned at 5 μm thicknesses. Sections were mounted on slides and stained using immunohistochemistry or with hematoxylin and eosin.

In order to determine the level of myosin heavy chain expression before culture, an aliquot of the cell suspension used to seed vessels was fixed with 3.7% formaldehyde/2mM EGTA for 30 minutes, rinsed 3 times with PBS, and stored at 4^{o} C until stained. Positive controls were obtained by seeding C2C12 cells on cover slips, which were allowed to reach confluency, and induced to differentiate by changing from GM to differentiating medium (DM) consisting of high glucose DMEM with 10% horse serum (Sigma, St. Louis, MO) and 1% Penicillin /Streptomycin. The resulting myotubes were fixed 6 days after DM application with 3.7% formaldehyde/1 mM EGTA (Sigma, St. Louis, MO) for 30 minutes, rinsed 3 times with Dulbecco's phosphate buffered saline (PBS), and stored at 4^{o} C until stained.

Aggregation Time Course

Cells were seeded in RCCS or SC as above and cultured for 0.5, 1, 2, 3, 6, and 9 hours. At the end of the culture period, cells were fixed in the culture vessel by removing 2.5 ml of medium and replacing it with 2.5 ml of 14.8% formaldehyde in 4mM EGTA (ethylenebis-(oxyethylenenitrilo)]-tetraacetic acid, Aldrich) to achieve a final concentration of 3.7% formaldehyde in 1 mM EGTA. Cells were fixed for 30 minutes and rinsed three times with PBS. Wet mounts of fixed, rinsed cells for each time point and culture condition were evaluated for aggregation by counting and classifying a minimum of 500 events as either single cells or aggregates (cells were attached to a minimum of one other cell) using a Nikon inverted microscope (Eclipse TS-100F) with

an LWD 40X/0.55 objective. The results are reported as percent single cells remaining in culture.

Quantification of Syncitia

H&E stained sections were used to quantify syncitia by manual count using a Nikon TS 100-F inverted light microscope with a PlanFluor 60X/0.5-1.25 oil immersion objective. For the purposes of this experiment, syncitia were defined as any cell containing more than three nuclei. Ten non-overlapping fields throughout the aggregate was counted, with a minimum of two sections examined per independent experiment. In the event that 10 fields could not be obtained from an individual section, a third slide was used to obtain a total of 20 different fields of view. To minimize the possibility that a syncitium would be counted more than once, sections used for enumeration were separated by no less than 150 μm.

Immunocytochemistry

Paraffin embedded, slide-mounted sections were deparafinized, rehydrated, and stored for two hours in PBS at 4^{o} C. Slides were removed from storage and washed with PBS three times for two minutes each. Excess PBS was removed and sections were permeablized/blocked with 0.1% Triton X-100 (Sigma, St. Louis, MO) and 5% goat serum (GS) (Sigma, St. Louis, MO) in PBS for 20 minutes. Excess liquid was removed and replaced with 200 μl of 1:400 MY-32 antibody (Sigma, St. Louis, MO), an antibody specific for skeletal muscle specific Myosin Heavy Chain (MHC, fast and neonatal) in 5% GS. Sections were incubated at room temperature in humidified chambers for one

hour. Excess primary antibody was removed with three PBS washes. Excess PBS was removed and 100 µl 1:500 Alexafluor 594 goat anti- mouse (Invitrogen/Molecular Probes, Carlsbad, California) in 5% GS was applied to the sections. Slides were again incubated at room temperature for 1 hour in a humidified chamber. Sections were rinsed three times with PBS to remove excess secondary and sealed with Gel Mount (Sigma, St. Louis, MO). Coverslips were permanently attached with Per Mount (Fisher Scientific, Fairlawn, NJ) or nail polish. Slides were stored in a slide box at 4^{o}C until imaged.

Images of myosin stained sections were collected for intensity analysis using a Nikon Eclipse 800 equipped with a 100W mercury vapor lamp, and a Photometrics CoolSNAP fx using a Texas Red filter set and a Nikon Plan Apo 60X oil correction objective. Exposure settings remained constant throughout image collection. Metamorph software (Molecular Devices Corporation, Sunnyvale, CA) was used to quantify both the area of myosin expression and the intensity per thresholded area. Thresholds were set to exclude background fluorescence of controls stained with the secondary antibody only. Areas were considered MHC positive if the intensity exceeded the threshold settings. One slide (one thin section) per aggregate was imaged, and a minimum of 20 fields were collected per section.

Statistics

The data is reported as the mean ± the standard error of three independent experiments. Differences were considered significant when $p<0.50$. Descriptive and statistical data were generated using SigmaStat software (Jandel Scientific) or Excel (Microsoft) software functions.

RESULTS

Histology

The three and six+ day aggregates generated in the RCCS were spherical, while SC aggregates tended to be more elongated. During early cell culture time intervals, SC cells rapidly aggregated into loosely associated cell sheets, which appeared to roll up and condense with time. Aggregates from both culture conditions occasionally appeared to be composed of multiple smaller aggregates. The RCCS aggregates showed no evidence of necrosis (Figure 3.1). In contrast, the SC aggregates contained a necrotic core as described by others (Donaldson et al., 1990; Rowley, 1992). Although proliferation had been previously noted in RCCS cultured aggregates (Molnar, 1997; Torgan, 2000), no evidence of mitotic cells were observed in either culture condition.

Aggregation

Our preliminary observations revealed that visible cell aggregates form within the first 12-24 hours of RCCS culture. Therefore, to gain a better understanding of the cellular interaction during the aggregate formation process in the RCCS, a time course study of aggregation was performed. A separate vessel was used for each time point and culture condition since aliquot removal during the study would reduce the cell density within the culture vessel and possibly affect experimental outcome. Single cells and aggregates were fixed within the culture vessel to reduce the possibility of additional adhesion or dislocation of adhering cells during cell processing and analysis. Following a 30-minute fixation, individual cells and aggregates were removed from the culture vessel and rinsed. A 100 μl aliquot was used to make a wet mount to enumerate the number of

individual cells and aggregates. A minimum of 500 events for each sample was counted and classified as either single cells or aggregates (2 or more attached cells) using light microscopy at 400X magnification. Cell aggregation was found to increase with time and resulted in a decrease in the number of single cells. Two way ANOVA analysis of the data revealed a significant interaction between treatment type (RCCS vs SC) and time of culture ($p<0.001$). Multiple Pairwise Comparisons (Holm-Sidak method) indicated significantly more aggregates in SC than in RCCS at all time points up to and including 3 hours ($p<0.001$ at all time points). Time in culture appears to have had a greater effect on aggregation during SC than in RCCS culture. When compared to the 0 hour pre-seeding control (96.81 ± 0.538), SC cultures at 0.5, 1, 2, and 3 hours are all significantly more aggregated ($p<0.001$ at all time points) with means of 79.08 (±2.90), 60.02 (± 4.16), 48.84 (± 2.59), and 45.43 (± 2.57), respectively. RCCS cultures do not significantly differ from the 0 hour controls until 2 and 3 hours (82.52 ± 0.516, 76.09 ± 2.22) ($p=0.006$, <0.001, respectively) (Figure 3.2). In addition, the number of cells per aggregate increased over time. SC aliquots at the 6 and 9-hour time points consisted of very large aggregates (sheets of cells). The remaining single cells at these time points were few in comparison to the number of cells comprising the aggregated sheets; however, since a single massive aggregate was counted as a single event, single cells were disproportionately in greater in number and resulted in skewed data. Therefore, the 6 and 9-hour time points were excluded from statistical evaluation.

Syncitia formation

The fusion of mononucleated cells to form multinucleated syncitia is a critical event in the maturation of skeletal muscle. We previously noted the appearance of some

visibly larger cells attached to the periphery of RCCS aggregates cultured for 8+ days. Further, examination of H&E stained thin sections revealed that these enlarged cells contained multiple nuclei (Sognier et al., 2004). Since the RCCS culture conditions used did not include cell attachment to an artificial substrate, were these synicita the result of RCCS culture or of suspension culture conditions alone? To test this, cells were prevented from attaching to the substrate in the control cultures. In addition, an approximate time course for syncitia formation was determined at two time intervals. Cells used in seeding the culture vessels were examined prior to exposure for the presence of syncitia. Although we noted a few bi-nucleated cells, cells with multiple nuclei were not apparent. A 3-day time interval was selected for analysis since this is the commonly accepted time required for the appearance of multinucleated cells using other methods (such as serum replacement or removal). A 6 or 7-day period was also selected for analysis because of our previous observations of the appearance of large attached cells during RCCS culture. Cells in both RCCS and SC were cultured in GM, which was replaced with fresh GM on day three of culture. RCCS rotation speed was increased as needed to maintain the cell aggregates in a constant state of free-fall suspension.

H&E stained thin sections of 3, and 6 or 7-day (6+) aggregates from RCCS and SC cultures were examined for the presence of syncitia (Figure 3.3). Random fields were assessed (40X magnification). The number of syncitia per field was counted prior to advancing to the next, non-overlapping field. RCCS generated aggregates are spherical, thus when examining the periphery of the aggregate only a portion of the field of view was occupied (Figure 3.4). In these cases, the portion of the field occupied by the aggregate was estimated (such as 0.75 field of view) and the syncitia number was adjusted to reflect an entire field to accommodate for this difference in field of view. Thus, although a minimum of 20 fields was counted, a minimum of 15 fields is reported.

Few syncitia were observed at 3 days of culture in the RCCS or SC (Figure 3.5).The mean number of syncitia per field in the RCCS at 3 days was 1.092 ± 0.226 and in SC was 0.397±0.126. However, by 6+ days, the RCCS cultures had a significantly greater number of syncitia per field (7.867 ±0.507) than the SC at the same time point (1.990 ±0.327). Two way ANOVA revealed a significant interaction between the culture type (RCCS vs SC) and time (3 and 6+ days) ($p<0.001$). Multiple Pairwise Comparisons (Holm-Sidak method) revealed that there were significantly more syncitia at 6+ days than at 3 days in both RCCS ($p<0.001$) and SC culture ($p=0.001$). However, at 6+ days of culture, the number of syncitia in RCCS culture is significantly higher than in SC ($p<0.001$). Interestingly, the majority of syncitia were at the periphery of the cell aggregates for all treatments. Peripheral localization and increased numbers of syncitia have also been observed in human skeletal muscle myoblasts (Cambrex/Clonetics, East Rutherford, NJ) in RCCS cultures (unpublished data).

Myosin expression

To determine if RCCS culture also induced differentiation of myoblasts, an early marker of myoblast differentiation was used. Thin sections of 3 and 6+ day aggregates from RCCS and SC culture were stained using an antibody to skeletal muscle specific myosin heavy chain (MHC) (Figure 3.6). As with the syncitia data above, a correction for sections occupying partial fields was applied prior to statistical analysis. The threshold was set to exclude background staining using a secondary only stained sample, which was maintained during all data collection. Images were collected and analyzed for the 1) thresholded area and 2) intensity within the thresholded area.

Two way ANOVA (RCCS vs SC, Time) of thresholded areas revealed a statistically significant interaction between the Treatment and Time ($p = 0.009$) (Figure 3.7). Multiple Pairwise comparisons (Holm-Sidak method) revealed 1) a significant increase in RCCS culture compared to SC at 6+ days ($p<0.001$) and 2) increases at day 6 when compared to day 3 in RCCS culture ($p<0.001$). The graph represents the mean number of pixels per field of view and error bars represent standard error. A minimum of 31 fields was counted per treatment for three independent experiments. Average Intensity within the thresholded areas did not significantly differ between treatments or time.

In addition to SC, several additional controls were evaluated for myosin expression and syncitia formation. Although syncitia are apparent, secondary only stained controls revealed no non-specific staining. Aggregates of other cell types cultured for the same time period show that RCCS culture does not result in myosin expression or syncitia formation in cell lines such as breast cancer (C) or bone (D) cell lines. Images on the left are light microscopy, while those on the right represent fluorescent images of the same area. Increased areas of myosin expression have also been noted in human skeletal muscle myoblasts (Cambrex/Clonetics, East Rutherford, NJ) in RCCS cultures (unpublished data).

DISCUSSION

The purpose of this study was to develop a tissue culture based model that 1) fuses to form syncitia, 2) differentiates without exogenous substrates or serum alterations, and 3) encourages a more *in vivo*-like 3D interaction. Our unique approach produces a 3D model that fuses to form syncitia and expresses skeletal muscle specific myosin heavy chain without the use of growth stimulants, serum deprivation or exogenous substrates.

This skeletal muscle model was generated in the RCCS which has been previously utilized for basic research as well as 3D tissue engineering (McGuckin et al., 2005). In addition, RCCS culture has been used in cancer research (Moon et al., 2001; Clejan, 2001) pathology (Nickerson, 2003; Honer, 2006), signaling (Clejan, 2001; Vincent, 2005) and reproductive medicine research (Di Agostino, 2004). RCCS culture is not limited to single cells. Tissue explants (Margolas, 1999), fertilized eggs, and embryos (Shimada, 2005) have been successfully cultured and studies in the *in vivo*-like environment of the RCCS. During RCCS culture many cells types were found to assume a more normal cellular architecture and exhibit gene expression profiles that were reflective of an authentic differentiated phenotype (Kleinman, 1987; Stoker, 1990). Three-dimensional *in vitro* systems have been shown to recapitulate the drug sensitivity patterns of tumor cells *in vivo* (Ohmori, 1998). Multicellular tumor spheroids have a well organized spherical symmetry of morphological and physiological features including complex cell–cell and cell–matrix interactions resembling avascular tumor sites and or micrometastatic regions *in vivo* and thus are a very useful model in tumor biology (Kim, 2004).

Myoblast cells are typically cultured in medium supplemented with serum containing high quantities of growth factors, nutrients, minerals, and hormones that serve to encourage proliferation. Altering the amount and type of serum used to supplement medium is often used to induce myoblast differentiation. Myoblasts respond to this serum alteration by switching from a proliferating to a differentiating state (Freshney, 1994).

Aggregation

Cell-cell adhesion is a necessary step that precedes fusion of mononucleated myoblasts to form multinucleated muscle cells. We show here that RCCS culture promotes myoblast fusion and differentiation as defined my MyHC expression. Rapid cell-cell adhesion/aggregation during RCCS culture might explain the earlier and greater degree of syncitia in RCCS culture when compared to SC culture. To determine rate of aggregation during time points preceding the appearance of visible aggregates in culture, a time course study was performed. Although visible aggregates were noted by 12 hours in this study as well as others, (Infanger et al., 2004), a greater single cell to aggregate ratio was found during RCCS than in SC culture. In a similar study by Manley and Lelkes (2006), >70% of HepG2 were composed of 4 cells or less at 3 hours of RCCS culture. Although we use percent single cells to aggregate ratios to define degree of aggregation over time rather than the small aggregates of the Manley and Lelkes study, our results of 76.09 ± 2.22 single cells at 3 hours are similar. Aside from the differences in methodology, differences between our study and that of Manley and Lelkes include the rate of RCCS rotation (9 versus 12 rpm), seeding density (5 X 10^5 versus 2.5 X 10^5 cells/ml) and adhesive characteristics of C2C12 cells and HepG2 cell lines all may contribute to the differences (Enmon et al., 2002) observed between the studies.

In a kinetics study during suspension culture of three human prostate cell lines, Enmon et al., (2002) show that adhesive properties and not diffusion is the rate-limiting factor for cell aggregation. Since the adhesive properties of the cells in our study are initially a constant for the two culture conditions, and co-localization (and thus a localized increase in density) is increased within the RCCS, aggregates would be expected to occur more readily in RCCS culture than in the suspension controls.

Interestingly, our suspension control cells exhibited a greater degree of aggregation. Thus, some other mechanism may be limiting the aggregation of cells within the RCCS.

The delayed cell-cell adhesion observed in RCCS compared to SC culture might be explained by the mechanics of RCCS culture. While it might seem logical to assume that the co-localization favored by RCCS culture would encourage cell-cell adhesion, bodies within the RCCS are also subjected to shear forces estimated 0.2 to 0.5 dynes/cm^2 (Synthecon, Inc.; Unsworth and Lelkes, 1998). Although this sheer is minimal when compared to the physiological range of 10-100 dynes/cm^2, which in turn is much less than arterial pressure (force of gravity is approx. 1 dyne/cm^2) (Nerem, 1991), it may be sufficient to delay permanent cell-cell adhesion during early culture.

Beads have been used to mimic cells in RCCS culture in order to determine the kinetics of cells in RCCS culture in several studies. Time-lapse photography of hollow microspheres, designed specifically as a support mechanism during RCCS culture, illustrated the trajectory that cells might take during RCCS culture. Images show that "cells" should follow an inward spiraling trajectory until colocalized near the center of the RCCS (Qui et al., 1999). Recently, the trajectory and colocalization was validated using real-time monitoring of cell cultures (Manley and Llekes, 2006). As a body (or cell) rotates in the RCCS, a given point upon the body will rotate 360^o with every rotation of the RCCS culture vessel. This rotation or tumbling adds additional movement to the individual cell. Tumbling, in combination with the low sheer and the collisions which are likely to occur between cells during RCCS culture, might inhibit cell-cell adhesion. Adhesion might then occur when 1) adhesion proteins are recruited and/or 2) matrix secretion increases to sufficient levels at the site of contact to counter the effects of these forces and facilitate adhesion.

Interestingly, we noted a large number of cytoplasmic protrusions, particularly in the RCCS culture. These projections may prolong the time required for adhesion by reducing the amount of surface area available for contact with adjacent cells. Conversely, since a number of adhesive molecules have been localized to similar structures in monolayer culture (Rabinovitz, 1997; Sandig, 1997; Choma et al., 2004), these projections may be involved in the cell-cell adhesion process.

Syncitia Formation

Cell fusion of mononucleated myoblasts into multinucleated cells is a necessary step in the maturation of skeletal muscle. Although several components have been proposed as regulatory components of fusion, the mechanisms of syncitia formation remain undefined (reviewed by Horsley and Pavlath, 2004). Confluency is thought to be a pre-requisite for syncitia formation of myoblasts *in vitro*. Prevention of confluency is suggested as a means for maintaining proliferating myoblast cultures (ATTC) and, upon achieving a high degree of confluency, myoblasts in cell culture will differentiate and fuse (Baldwin and Kayalar, 1986). Confluency facilitates a degree of cell-cell contact and adhesion, a necessary step for fusion. However, during 2D culture, cell-cell contact is limited to small areas at the cell periphery due to the flattened morphology of the cell during monolayer culture. In contrast, substrate-free RCCS culture forces cells with a more spherical morphology into localized areas of 3D high-density within the culture vessel (Qui et al., 1999; Manley and Lelkes, 2006). Since a more rounded shape of the actin cytoskeleton may play a role in differentiation (Solursh, 1989), the cells in this model may be using mechanical or cytoskeletal signaling mechanisms to switch from a proliferating to a differentiating program (Gibson & Perrimon, 2003; Ingber, 2005).

The very large cells noted at the periphery of the aggregates during RCCS culture of this model were identified as syncitia containing multiple nuclei. Since cells seeded into the RCCS culture vessel were largely single mononucleated cells with very few bi-nucleated cells, these syncitia were formed during RCCS culture. It is likely that co-localization of cells in the RCCS was at least in part responsible for syncitia formation. High cell density seeding is commonly used methodology during 3D tissue engineering to increase the tissue equivalency of skeletal muscle (Okano and Matsuda, 1998), myocardium, (Radisic et al., 2003) and bone tissues (Takagi et al., 2007). High density is also used in regenerative medicine such as myoblast transplantation (Blat et al., 2003; Skuk, 2004).

Although it is possible that mitosis may have occurred without cytokinesis to create the syncitia seen here, reduced proliferation has been reported for myoblasts (Molnar et al., 1997; Torgan et al., 2000) and other cell types (i.e., Cogli et al., 1984; Coinu et al., 2006) during RCCS culture. In addition, no mitotic cells were observed within the syncitia at 3 or 6 + days, and little BrdU uptake was observed during the first 24 hours of RCCS culture (unpublished data). It seems likely that syncitia occurred as a result of cell fusion.

Interestingly, syncitia were seen only on or near the periphery of the aggregate. Down-regulation of cells surface proteins such as N-cadherin occurs in primary myotubes during secondary myogenesis (Durband, 1987; Hatta, 1987) and following myotube formation in cell culture (Mege et al. 1992). It is possible that the peripheral location of syncitia was due to cell sorting.

Skeletal muscle MHC expression

In addition to syncitia formation, maturation of myoblasts into skeletal muscle requires the expression of muscle specific proteins. For this study, the expression of skeletal muscle specific MHC was used to determine whether differentiation was occurring. MHC mRNA expression is thought to occur shortly after fusion (Gunning et al., 1987) and commonly used as an early marker of muscle differentiation.

One of the advantages of RCCS culture is that it facilitates co-localization and cell-cell interactions. Increased cell-cell contact would seem to increase signal pathways initiated by this contact. Cell:cell interactions are implicated in myogenic control (Cossu et al., 1995; reviewed by Krauss et al., 2005). Although cell-matrix interactions are critical to myoblast differentiation (Melo et al., 1996; Osses, 2002), since our model lacks an exogenous matrix, and adhesion begins to occur shortly after cell seeding, it is more likely that adhesion occurs as a result of cell-cell and not cell matrix adhesion. Mammalian myoblasts typically express two classical cadherin family members; M- and N-cadherin (Knudsen et al., 1990; Hahn and Covault, 1992; Pouliot et al., 1994) that facilitates cell-cell interactions, and both have been linked to differentiation (Zeschnigk, et al., 1995; Redfield, 1997; Seghatoleslami, 2000). It seems possible that one or both may facilitate the increased differentiation seen in our model.

In summary, we present a novel 3D non-necrotic skeletal muscle model that differentiates and fuses to forms multiple multinucleated cells that may be may be suitable for studying the following: 1) initial cell binding and factors involved in this process; 2) fusion of myoblasts to form multinucleated syncitia; and 3) differentiation in the absence of externally supplied substrates or serum alterations. This model is also suitable for examination of the effects of early (pre-aggregation) 3D culture on individual

cells. In addition, we show for the first time *in vitro* that myoblasts will fuse to form syncitia and express markers of differentiation in the presence of growth medium and without the use of exogenous substrates or supports.

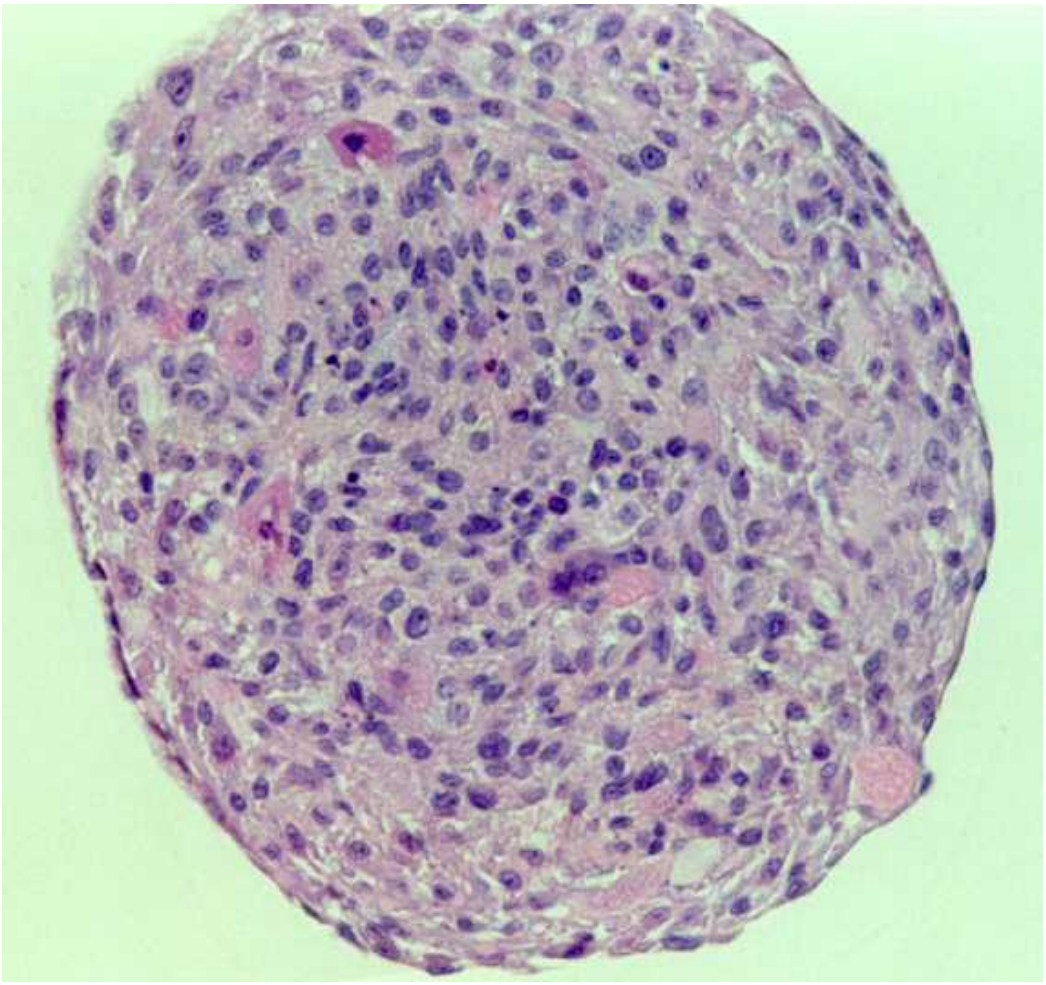


Figure 3.1: Example of whole aggregate

Example of an H and E stained thin section (5 μm) of an aggregate cross section. Note that the core of the aggregate shows no sign of necrosis. (DIC, original magnification 10X).

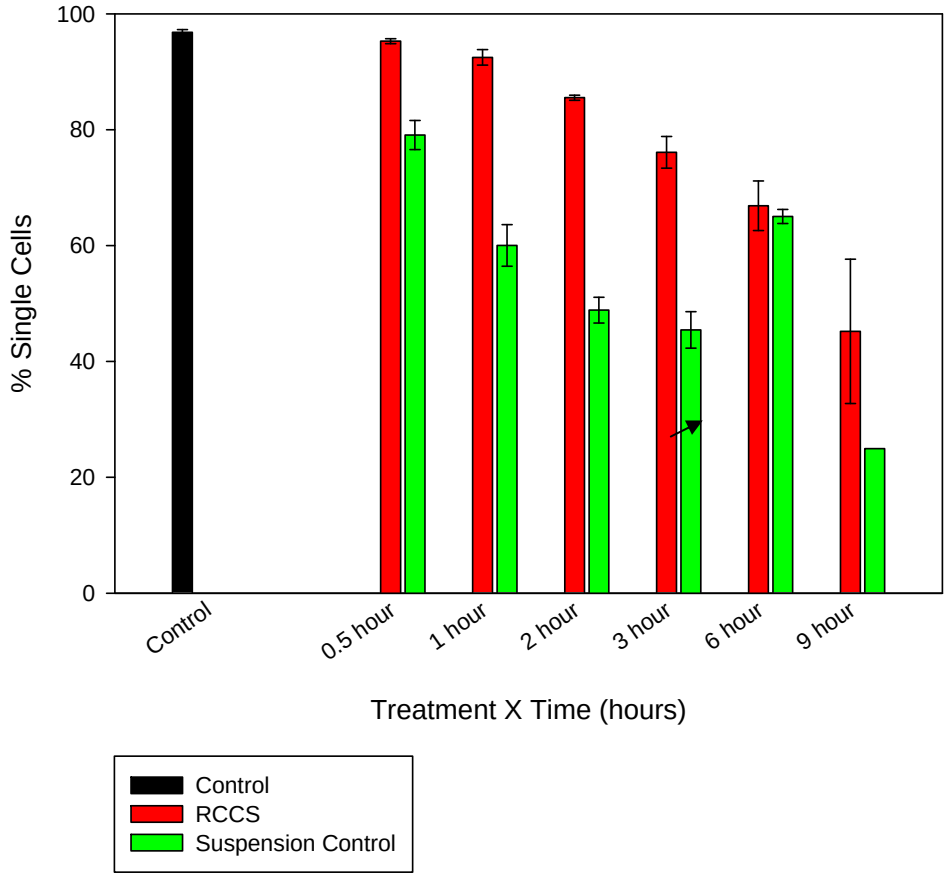


Figure 3.2: Aggregation time course

Aggregation was measured by manual count of 500 events. The mean percentage of single cells remaining is reported. Error bars represent the standard error of 3 replicates. The few aggregates seen in the SC at 6 and 9 hours were very large and counts of single cells were difficult to obtain. The results above at SC 6 and 9 hours represent 2 and 1 replicate, respectively, and are not included in the statistical analyses.

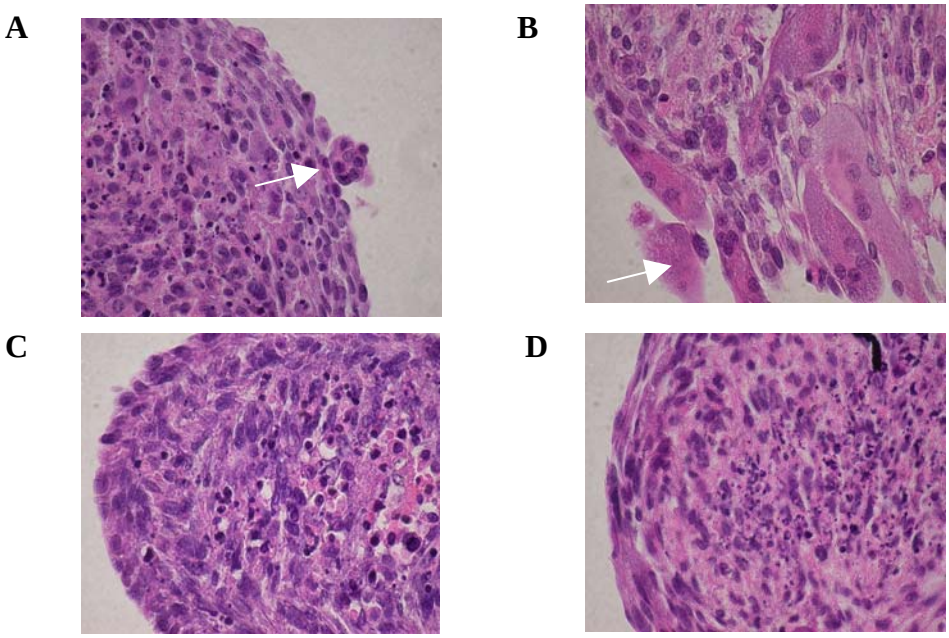


Figure 3.3: Example of aggregate thin sections, H&E stained

A) 3 day and B) 7 day aggregate cultured in RCCS. Note the syncitia at the periphery of both RCCS images. White arrows indicate examples of syncitia. C) 3 day and D) 7 day aggregate from suspension control. All DIC images were collected at 20X. Image 3B has been cropped and enlarged to enable visualization of syncitia.

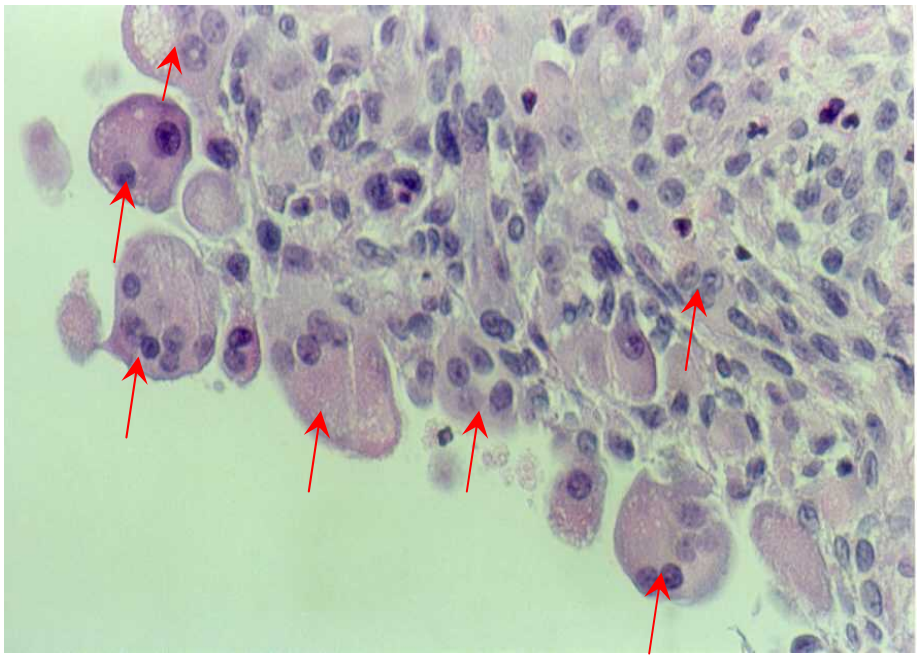


Figure 3.4: Example of syncitia in 7 day aggregate

H and E stained 5 μM thin section of a seven day aggregate. Arrows indicate the presence of syncitia (Original magnification 40X)

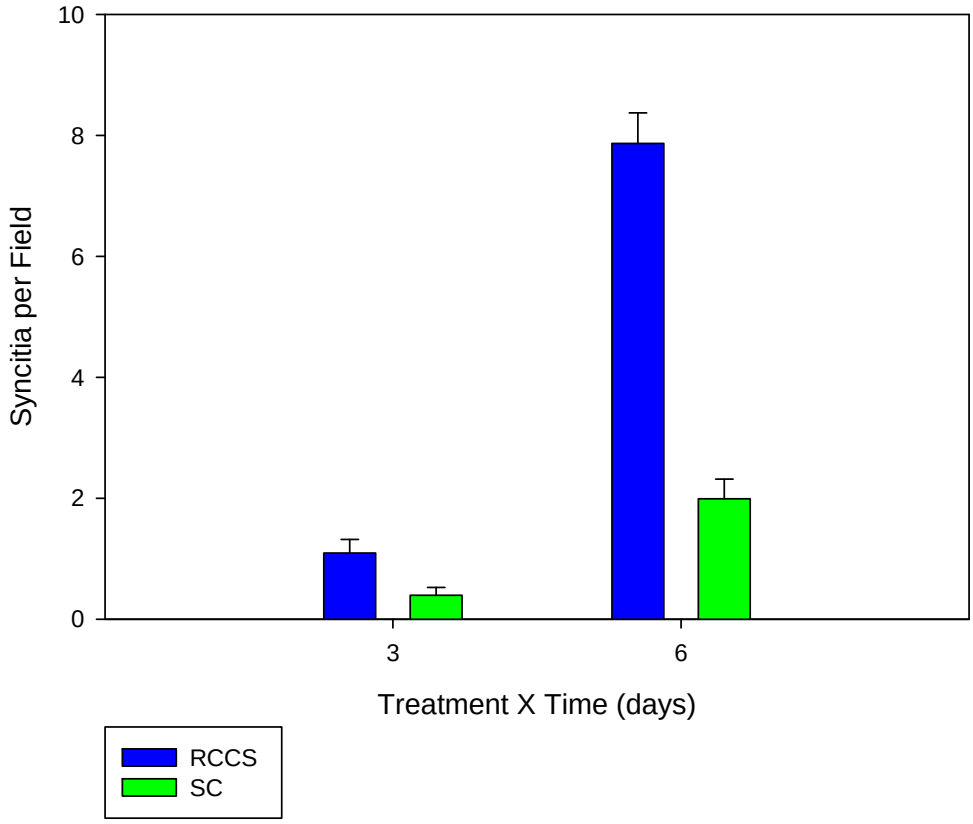


Figure 3.5: Syncitia formation

H and E stained thin sections were used to count syncitia. Results are mean number of syncitia per field for 3 independent experiments. Error bars represent standard error.

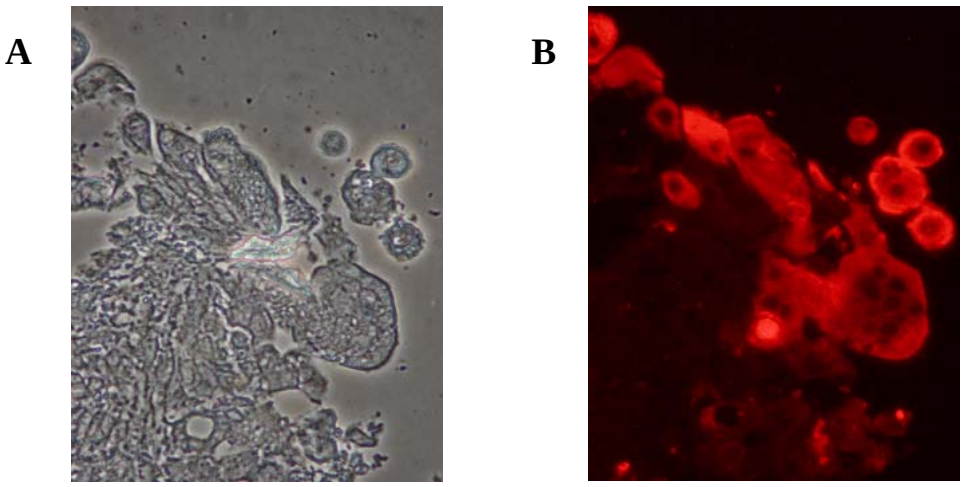


Figure 3.6: Myosin in 6+ day cultures

A) DIC image of an unstained thin section. Compare to the same section in B) showing areas of differentiation indicated sarcomeric myosin heavy chain staining. Original magnification of all images is 60X.

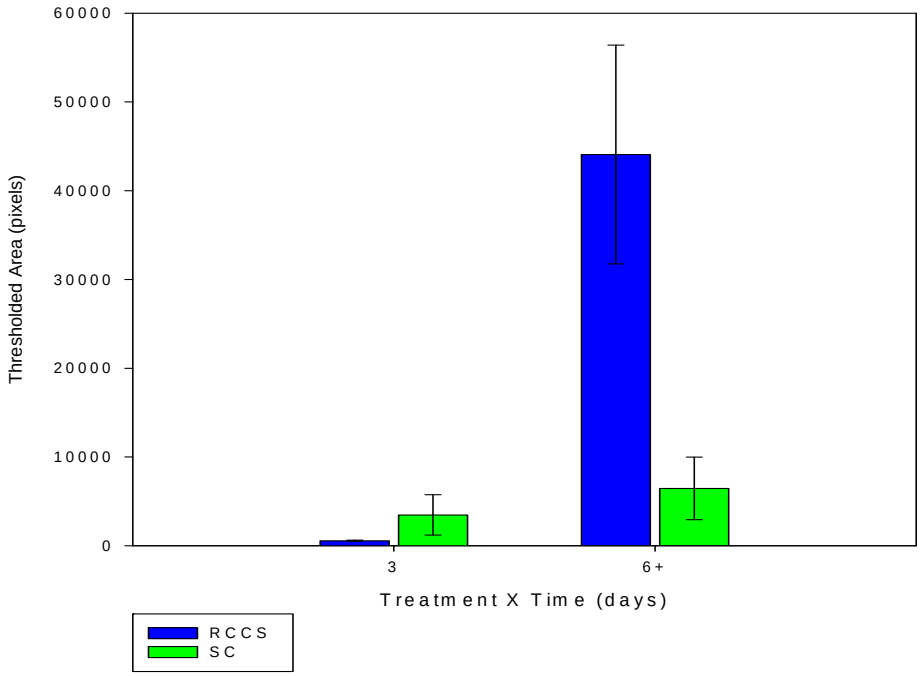


Figure 3.7: Myosin Expression

Deparaffinized thin sections were stained for skeletal muscle specific myosin heavy chain, imaged, and analyzed for areas expressing MHC. Secondary only controls were used to set lower limits of the threshold. Stained thin sections were used to count syncitia. The results represent the mean number of syncitia per field for 3 independent experiments. Error bars represent standard error.

Chapter 4: Analysis of Podia in Three-dimensional Cell Cultures

INTRODUCTION

Under two-dimensional (2D) culture conditions, the attachment of cells to the substrate influences their shape, cytoskeletal arrangement (Huang and Ingber, 2000), and the morphology of cytoplasmic projections (Beningo et al., 2004). When detached from a substrate, cells become more spherical in shape and cytoplasmic projections become both more apparent and numerous (Collard and Temmink, 1976). Cytoplasmic projections have been noted on both single cells and on the periphery of aggregated cells during suspension culture (Gershman, 1978). Cytoplasmic protrusions have also been observed in cells grown under microgravity conditions (Huges-Fulford and Lewis, 1996; Guignandon, 2001) and microgravity analogs (Uva, 2002), including the NASA-designed bioreactor or Rotary Cell Culture System (RCCS).

In RCCS culture, cells are maintained in a constant state of free-fall in low-shear, low-turbulence conditions (Hammond and Hammond, 2001). The rotation of the culture vessel about the horizontal axis randomizes the gravity vector and cells remain in suspension. Originally designed by NASA as a means to protect living cells from hypergravity effects experienced during launch, the RCCS has more recently been recognized as an important tool for tissue engineering applications (Dutt, 2003), the study of tissue regeneration, and cancer cell biology (O'Connor, 1999; Clejan et al., 2001; Hammond and Hammond, 2001). Because culture conditions favor cell co-localization and minimizes the sheer stress that can prevent cell aggregation in other cell culture systems, the RCCS has been used to generate 3D tissue models (Goodwin et al., 1993; Hammond and Hammond, 2001; Manley and Lelkes, 2006). However, in spite of the

diverse uses of the RCCS, few studies have examined single cell morphology during 3D culture conditions. In particular, skeletal muscle myoblasts have not been previously investigated in detail with regard to adhesion, aggregation, and differentiation in the RCCS.

Previously, we have shown that C2C12 myoblasts will begin to differentiate and fuse in the RCCS 3D environment at 3 days (Marquette et al., 2007). Cells in this 3D skeletal muscle model form syncitia and express the skeletal muscle specific marker, MyHC (sarcomeric myosin heavy chain), without externally supplied substrates or changes in culture medium serum types or levels typically used to induce differentiation. Interestingly, numerous cytoplasmic projections have been observed: 1) on single cells, prior to cell-cell adhesion; and 2) extending between cells at early time intervals preceding differentiation and fusion. These podia appeared to be more abundant in the RCCS compared to liquid overlay culture (Marquette, personal observations).

Cytoplasmic protrusions have been described using a number of terminologies including lamellipodia, filipodia, and microvilli (Abercrombie et al., 1970; Albrecht-Beuhler and Lancaster, 1976; Nakamura, 2001). Microvilli are thought to be more permanent structures while lamellipodia and filipodia tend to be more ephemeral in nature (Nakamura, 2001). Some of these descriptions were generated based on characteristics observed in a 2D milieu (such as lamellipodia). However, in a 3D environment, some of these 2D defined characteristics and terminologies may no longer be as applicable. Accordingly, for the purposes of this study, the term “podia” is used as a general descriptor of all cytoplasmic projections seen during 3D culture.

Although podia are perhaps best known for their role in motility, filopodia can form cell-cell contacts and adhere to adjacent cells. Such contacts are prerequisites to myoblast fusion (Swailes, 2004). Cytoplasmic projections have also been reported

between cells and N-cadherin coated beads. N-cadherin is a protein believed to play a role in differentiation in myoblasts (Goichberg and Geiger, 1998). These findings suggest that podia may be an important mechanism to facilitate cell-cell contact, an essential step in fusion. The investigation of the role of podia in the adhesion, fusion, and differentiation of myoblasts into multinucleated, MHC-expressing cells in our RCCS model may reveal new information regarding *in vivo* skeletal muscle regeneration.

Regardless of the specific function, lamellipodia, filopodia, and microvilli depend upon F-actin polymerization for both structure and function. These experiments will determine if the podia noted during RCCS culture are homologous to F-actin dependent cytoplasmic protrusions originally defined in 2D culture conditions.

The overall goal of these studies is to utilize our previously described RCCS model test system (Marquette et al., 2007) and skeletal muscle myoblasts to investigate the following: 1) Are the podia more abundant in the RCCS when compared to liquid overlay culture? 2) Do podia contain F-actin? 3) Are podia F-actin dependent? Cytoplasmic projections participate in critical events such as cell-cell adhesion, differentiation (Goichberg and Geiger, 1998), and fusion in 2D culture (Huang et al., 1978). Since 3D cell-cultures exhibit more *in vivo*-like responses (O'Connor, 1999), it is critical to gain an understanding of the structure of podia and the role that these podia might play in adhesion, fusion, and differentiation during RCCS culture.

METHODOLOGY

Cell Culture

C2C12 cells, a murine myoblast cell line (Blau, 1985), obtained from the American Type Culture Collection (ATCC, Manassas, VA) were cultured in a humidified, constant temperature incubator at 37^{o}C with 5% CO_2/95% air. Cells were cultivated in growth medium (GM) consisting of high glucose Dulbecco's Modified Essential Medium (DMEM, Gibco-BRL, Carlsbad, California) supplemented with 10% Fetal Bovine Serum (FBS, HyClone, Logan, UT) and 1% Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, MO). Cells were subcultured as needed to prevent confluency (approximately every 2-3 days) using 0.125% Trypsin/0.05 EDTA (Gibco-BRL, Carlsbad, CA). GM was aspirated, Trypsin/EDTA added and cells incubated for 3-5 minutes until detached. Fresh medium was added and cells vigorously pipetted (triturated) to produce a single cell suspension, centrifuged 5 minutes at 1000 RPM, resuspended in fresh GM and placed in culture vessels. Cells in passages 6-8 were used for experiments.

Sample Preparation

Cells were removed from monolayer culture using Trypsin/EDTA (as described above). Following resuspension in GM, cells were counted using an improved Neubauer hemacytometer and volumes were adjusted to a final concentration of 5 X 10^5 cells/ml by adding GM. For some experiments, GM was supplemented with Latrunculin A (Lat A, Invitrogen/Molecular Probes, Carlsbad, California) to achieve a final concentration of 2 μM. An equivalent volume of the DMSO carrier (Sigma-Aldrich, St. Louis, MO) was

added to controls. Cells subjected to RCCS culture were seeded into 10 ml High Aspect Ratio Vessel (HARV, Synthecon, Inc., Houston, TX) disposable culture vessels and rotated at 9.0 RPM. Suspension controls (SC) were seeded in previously prepared poly(2-hydroxyethyl methacrylate (poly-HEMA, Sigma-Aldrich, St. Louis, MO) coated Petri dishes to prevent adhesion as described by Folkman and Moscona (1978). Briefly, a 10X stock was made by dissolving 4.8 g of poly-HEMA in 40 ml of 95% ethanol (AAPER, Shelbyville, KY) with continual rocking overnight at room temperature until completely dissolved, and then stored at 4^{o}C. To prepare dishes, the stock was diluted 1:10 in 95% ethanol, 2 ml were added to a 60 mm tissue culture Petri dish, and the dish was air dried overnight in a Nuaire Biological Safety Cabinet. Three coats of poly-HEMA were required to prevent attachment of C2C12 cells. Coated Petri dishes were rinsed with sterile PBS and allowed to air dry as above, then stored in an airtight container at 4^{o}C until use. For some experiments, cells were cultured for 3, 6, and 9 hours. Additional time points were added for viability and Caspase 3 expression. For Lat A experiments, cells were either fixed at 3 hours of culture or subjected to Lat A washout with 37^{o}C GM and re-seeded into fresh culture vessels with or without 2 μM Lat A for an additional 3 hours of incubation. At the end of the treatment period, a 2.5 ml aliquot of cells in GM was removed from the culture vessel and used to assess viability. The remaining cells in the culture vessel were fixed by slowly adding 2.5 ml of 14.8% formaldehyde (Sigma-Aldrich, St. Louis, MO) in 4 mM EGTA (ethylenebis-(oxyethylenenitrilo)]-tetraacetic acid, Sigma-Aldrich, St. Louis, MO) to achieve a final concentration of 3.7% formaldehyde/1 mM EGTA. Cells were fixed for 20 minutes, rinsed 3X with Dulbecco's Phosphate buffered saline (PBS) and held at 4^{o}C for analysis or further processing. All experiments were repeated a minimum of three times.

Confocal Microscopy

To quantify the number of podia, 3, 6, and 9 hour time points were chosen for analysis to precede the formation of visible aggregates (Marquette, 2007). Membrane projections were detected using a lipophillic stain and F-actin visualized using a phallacidin counterstain. Since it was unknown whether all podia were a result of cytoskeletal projections or merely the presence of excess membrane (Seher and Adam, 1978), the membrane portions of the cell were stained with a lipophillic stain in order to enumerate and measure podia.

To quantify the number of podia per single cell and to determine whether F-actin was present within the podium, an aliquot of fixed, rinsed cells was prepared for confocal imaging by the application of 500 µl of 1 µg/ml CM DiI (chloromethylbenzamido, Invitrogen/Molecular Probes, Carlsbad, California) for 5 minutes at 37^{o}C followed by 10 minutes at 4^{o}C from a reconstituted stock solution prepared according to manufacturer's directions (50 µg in 50 µl DMSO). CM DiI is a lipophillic carbocyanine derivative targeting cell membranes. Cells were rinsed with PBS and incubated with 250 µl of 1 unit/200 µl BoDipy FL Phallacidin (Invitrogen/Molecular Probes, Carlsbad, California) in 5% goat serum (Sigma-Aldrich, St. Louis, MO) and 0.1% saponin (Sigma-Aldrich, St. Louis, MO) in PBS for 30 minutes at room temperature to stain F-Actin. Cells were rinsed twice with 0.1% Saponin in PBS, once with PBS, and stored at 4^{o}C until imaging or analysis. Two hundred µl of stained cells suspended in PBS placed on a coverslip were used to collect three-dimensional, two channel images using a Zeiss LSM 510 with a C-Apochromat 40X /1.2W objective. Focal planes were 0.5 µm and encompassed the entire cell. A minimum of thirty 3D images was collected per replicate per treatment.

Images were analyzed using LSM 510 Image Browser Software. To quantify podia, a cell image was viewed and the depth (z vector) of the cell was determined by using an orthogonal view. Cell diameter, defined by the CM DiI membrane stain, was determined by calculation based on the number of Z planes comprising the interior of the cell (Figure 4.1A) (# of focal planes ∗ 0.5 μm per plane) and empirically by measuring the interior of the x and y directions of the center focal plane of the cell (Figure 4.1B) .All measurements were performed using the overlay ruler function.

The total number of z planes was used to calculate the range of planes comprising the center ¼ of the cell, which were utilized to count and measure podia. Beginning with the uppermost focal plane, projections beyond the cell membrane were marked and measured from the interior of the cell to the longest portion of the projection using a straight line from the overlay menu (Figure 4.1C). This process was continued for all focal planes in the center ¼ of the cell. These lines were lengthened as necessary to measure the apex of the projection. Podia were defined as projections greater than 2 μm (red channel). Although an individual podium may have appeared in a number of planes during the analysis, it was only counted as a single podium.

F-actin intensity

To quantify the effects of Lat A application on F-actin during RCCS culture, aliquots of fixed, rinsed cells were incubated with Alexafluor 532 phalloidin (Invitrogen/Molecular Probes, Carlsbad, California) 5% goat serum/0.1% saponin in PBS for 30 minutes at room temperature. Cells were rinsed twice with 0.1% saponin in PBS, once with PBS and analyzed for F-actin intensity using a Guava Personal Cell Analysis (PCA) system. The PCA is a modified micro capillary flow cytometer.

Microscopy

To quantify the number of cells that contained podia, a wet mount of fixed cells was examined using a Nikon Inverted Microsope (Eclipse TS-100F) with an LWD 40X/0.55 objective. A minimum of 500 cells was analyzed per treatment per replicate to determine if cells were podia positive or negative. Cells were scored as podia positive when cytoplasmic projections could be seen extending beyond the cell periphery.

Viability

Trypan blue dye exclusion was used to determine cell viability. Two hundred fifty microliters of cells, from the 2.5 ml aliquot removed from culture vessels prior to fixation, was placed in a microcentrifuge tube with an equal volume of 0.4% trypan blue (Sigma-Aldrich, St. Louis, MO) and was allowed to incubate for 5 minutes at room temperature. A drop of this solution was used to make a wet mount. A minimum of 500 cells was counted per treatment per replicate and classified as living (colorless) or dead (blue or partially blue). Viability was reported as the percent of live cells.

Viability was also determined using a Guava personal flow cytometer and ViaCount reagent (Guava Technologies, Hayward, CA) according to the manufacturer's instructions. Viability is determined by differential permeability of the DNA-specific dyes in the reagent. Briefly, 450 µl of the ViaCount reagent was added to a 50 µl aliquot of cells that had been removed from culture vessels prior to fixation. The sample was incubated at room temperature for 5 minutes. An unstained 50 µl aliquot of cells, diluted with 450 µl of PBS, was used to set thresholds. Samples with reagent were gently mixed and analyzed using the Guava PCA a minimum of two times with 1000 cells analyzed per data set. Data is reported as the percent viable cells.

Apoptosis determination

A Guava Nexin kit (Guava Technologies, Hayward, CA) was used to determine the external expression of phosphatidyl serine (PS), an early indicator of apoptosis. An aliquot of cells, removed from culture vessels prior to fixation, was washed with cold 1X Nexin Buffer, centrifuged, re-suspended in 40 µl 1X Nexin Buffer supplemented with 5 µl of Annexin V-PE (specific for phosphatidyl serine) and 5 µl 7-ADD, and incubated on ice in the absence of light for 20 minutes. At the end of the incubation period, an additional 450 µl of 1X Annexin buffer was added, cells were centrifuged, and analyzed using the Guava PCA. Thresholds were set using an unstained sample. The results are expressed as the percentage of Annexin positive cells.

Imunohistochemistry was also used as an additional means of Apoptosis assessment. An aliquot of fixed, rinsed cells was centrifuged, rinsed, and incubated with 0.25 ml of 1:1000 rabbit anti-Caspase 3 (activated, Sigma-Aldrich, St. Louis, MO) in 5% goat serum/0.1% saponin for 1 hour at room temperature. Then, cells were rinsed 2 times with 0.1% saponin in PBS. Subsequently, cells were incubated with 0.25 ml of 1:500 Alexafluor 532 Goat anti-rabbit (Invitrogen/Molecular Probes, Carlsbad, CA) in 5% goat serum/0.1% saponin in PBS for 1 hour at room temperature. Cells were rinsed 2X with 0.1% saponin/PBS and once with PBS. Cells were held at 4^{o}C and were analyzed within 24 hours using the Guava PCA. In order to prevent aggregates from being recorded, forward scatter was set using fixed, unstained cells before treatment. Cells receiving secondary antibody staining only were used to set the upper limits of background staining. Caspase 3 positive cells are reported as the percent of cells above background.

Statistics

The data reported are the mean ± the standard error of three independent experiments. Descriptive and statistical data were generated using SigmaStat (trial version) software (Jandel Scientific) or Excel (Microsoft) spreadsheet functions.

RESULTS

Assessment of the number of podia

To determine whether the cytoplasmic projections were the result of RCCS culture conditions or suspension culture without adhesive support, the number and length of podia from cells cultured in the RCCS were compared with those found on cells grown in suspension in poly-HEMA coated Petri dishes. Because cells removed from RCCS culture are roughly spherical when examined by light microscopy, confocal images were used to determine diameters in two directions. The mean diameter based on the number of focal planes in the z axis was 17.6 ± 0.26 μm. The measured diameter was similar at 16.93 ± 0.26 μm (the mean of the x and y measured diameters) illustrating the spherical nature of the cells. The membrane thickness was 0.85 ± 0.02 μm.

For the purposes of this study, all membrane projections that were greater than 2 μm (red channel) in length were considered podia. To ensure accuracy and consistency, these measurements also accounted for the general thickness of the cell membrane plus any localized membrane thickening by utilizing a 2 μm limitation for inclusion of podia (1 μm for average membrane thickness and 1 μm for podia). Thus, to be classified as a podium, a projection must extend 2 μm beyond the cell membrane thickness. The number of focal planes per cell ranged from 10-14. All experiments were repeated 3 times.

In order to alleviate any confounding factors resulting from shape changes as the apex of the cell is approached in the z-plane of a hollow sphere, only the center ¼ of the 3D confocal images was used to measure and count podia. Further, since confocal images are a series of 2D sections, resolution of spherical structures are lost as the z plane apices of the cell are approached. We previously determined that cells are oriented randomly with respect to F-actin (Marquette, unpublished data). Accordingly, it was concluded that the center ¼ of the cell was representative of the whole image.

Confocal image analysis revealed that the 3-hour RCCS cultured cells contained the highest number of podia (7.47 ± 0.63) as compared to 4.33 ± 0.62 in the SC as defined by the lipophillic staining. The number of RCCS podia decreased over time with mean podia numbers of 7.19 ± 0.82 and 5.52 ± 0.66 at 6 and 9 hours, respectively. Over this same time interval, the number of podia in SC cultures remained constant (4.81± 0.84 and 4.38 ± 0.84) (Figure 4.2). The mean number of podia (per the center ¼ of the cell) between RCCS and SC culture treatment is significantly different (two way ANOVA, p= <0.001). In contrast, the decreases noted over time were not significant (p=0.300).

Determination of podia length

Podia were also significantly longer as a result of RCCS culture (two way ANOVA, p= 0.003). The mean podia length was 3.18 ± 0.093 at 3 hours and mean length increased slightly over time (3.48 ± 0.12, 3.60 ± 0.12 at 6 and 9 hours, respectively) (Figure 4.3A). At 3 hours, SC podia were shorter (3.07 ± 0.14) and did not show the same increases in length over time (3.16 ± 0.13, 3.09 ± 0.12 at 6 and 9 hours). The longest podia, occasionally exceeding 10 μm in length, were only observed during RCCS culture

at 9 hours. The majority of moderate length podia (7-10 μm) were seen in RCCS culture at 6 and 9 hours, with only one podium in this range noted in the SC 9 hour culture.

Assessment of podia for presence of F-actin

In order to determine whether podia were F-actin positive, podia that had been previously counted were re-examined for the presence of F-actin. The red channel (CM DiI) membrane stain was eliminated from the two color confocal images, and each podia mark was examined for the presence of F-actin (green channel) (Figure 4.3B and C). F-actin was noted in a minimum of 97 ± 0.2% of the podia in the SC at 3 hours to a maximum of 100% of the podia in the RCCS at 6 and 9 hours of culture as determined by the presence of green fluorescence. For the remainder of this study, all podia were considered as F-actin positive.

Viability during culture

Cell viability and apoptosis are potential areas of concern when culturing adherent cells without substrate attachment. In order to determine the effects of our culture methods on these parameters, cells were analyzed for viability and apoptosis at 0.5, 1, 2, 3, 6, and 9-hour time points. An aliquot of cells, removed from the culture vessels to accommodate fixative volume, was used to determine viability using the Trypan Blue Dye Exclusion method. The results indicate that there is a significant interaction between time and treatment (two way ANOVA, $p<0.001$). A Holm-Sidak Multiple Comparison versus Control (0 Treatment, 0 Time) indicates no significant differences within the RCCS over time. In contrast, there are significant differences at 6 (60.78 ± 7.68) and 9

hours (62.32 ± 7.52) within the SC treatment when compared to the 0 Hour, 0 Treatment control (93.20 ± 2.04, p <0.001 and <0.001, respectively). Further, viability is significantly higher in the RCCS at 6 (90.49 ± 0.89) and 9 (81.25 ± 2.93) hours when compared to SC at the same time points (p<0.001 and <0.001, respectively) (Figure 4.4).

An aliquot of cells, removed from the culture vessels prior to fixation, was used to determine viability via Guava PCA methodologies. The results indicate that there is not a significant interaction (two way ANOVA on Ranks, p=0.604) between time and treatment. However, the viability of RCCS cultured cells was significantly greater than those cultured in SC (p<0.001) (Figure 4.5).

Caspase 3 (activated) expression

To assess apoptosis, an aliquot of fixed cells for each treatment was stained with an antibody to the activated (cleaved) form of Caspase 3, secondary, and analyzed on a Guava PCA. Data are reported as the percent cells positive for Caspase 3 (activated) above the background and represent the mean of three experiments with a minimum of two replicates (1000 cells each) per experiment analyzed. Two way ANOVA on ranks indicate that there is a significant difference between RCCS and SC culture (p<0.001). Although there is a general decrease of Caspase 3 in RCCS culture and an increase in SC culture over time, these differences are not statistically significant (Figure 4.6). The positive controls shown in Figure 4.6 consisted of SC cells exposed to 500 uM hydrogen peroxide (H_2O_2) to induce activated Caspase 3 expression.

Annexin V staining

An aliquot of cells removed prior to fixation was used to determine whether cells were expressing a second marker of apoptosis, phosphatadyl serine (PS), which appears on the outer leaflet of the cell membrane during apoptosis. The presence of PS on the cell membrane is determined by fluorescently conjugated Annexin V, which binds to cell-surface expressed PS and cannot penetrate an intact cell membrane. A second indicator, 7-AAD (7-amino-actinomycin D), can also penetrate permeable cell membranes and stain the nucleus. Thus, cells staining positive for Annexin V will be apoptotic. Cells in late apoptosis are both Annexin V and 7-AAD positive, while earlier stages of apoptosis are only positive for Annexin V. Positive staining for 7-AAD indicates the presence of permeable cells while those cells that are 7-AAD positive and Annexin V negative are necrotic, but not apoptotic. Cells with no staining of either indicator are considered viable. Less than 2 % of the cells were Annexin V positive at the 3, 6, and 9-hour time points (not shown).

The effects of Lat A induced F-actin depolymerization on podia

To determine if RCCS induced podia were F-actin dependent; cells were cultured in the presence of Lat A, an actin-depolymerizing reagent. Cells were cultured for 3 hours in the RCCS with (Lat A+) or without (Lat A-) 2 μM Lat A. The results shown in Figure 4.7 reveal that cells with podia are significantly reduced by Lat A application (12.00 ± 1.10) when compared to Lat A- cells (84.37 ± 2.65) (T-test, $p<0.001$). A minimum of 500 cells was classified per treatment for each of three replicates (Figure 4.7).

The effects of Lat A application on F-actin during RCCS culture was determined by staining aliquots of fixed cells with AlexaFluor 532 Phalloidin. Stained cells were

analyzed using the Guava PCA to determine relative F-actin intensity via flow cytometry (Figure 4.8). Gates were set using unstained cells (described above). The intensity of F-actin was significantly reduced in Lat A+ cells (1415.86 ± 45.82) when compared to the control (Lat A-) (1659 ± 81.94) (T-test p=0.020).

The effects of Lat A removal on F-actin polymerization and podia formation

To ascertain if podia recovery occurs with Lat A removal, cells were cultured with or without Lat A as previously described (above). At the end of the 3-hour incubation period, cells were removed from the culture vessel and Lat A was removed using the washout procedure. Cells were returned to new culture vessels with or without 2 μM Lat A in fresh GM for an additional 3 hours of culture. Fixed cells were examined using light microscopy and classified as podia positive or podia negative. Recovery of the number cells with podia (86.72 ± 0.76) was observed with Lat A (+/-) washout when compared to control cells (Lat A-/-) (80.62 ±0.98) and cells continually exposed to Lat A (10.10 ±1.55) (Figure 4.9). The differences between the test groups are statistically significant (ANOVA, $p < 0.001$). A Holm-Sidak Multiple Pairwise Comparison revealed that cells with no Lat A treatment (Lat A -/-) have significantly more podia than those receiving Lat A +/+ (p=0.025) exposure. Lat A +/- cells also have significantly more podia than the positive controls (Lat A +/+) (p=0.017). Interestingly, in the Lat A +/- treatment, the number of cells with podia are more numerous than in the Lat A -/- cells (p=0.050).

The recovery of F-actin intensity following Lat A washout via flow cytometry is somewhat less apparent, but the same general trends seen in the data above are present (Figure 4.10A). Although the F-actin intensity of the experimental cells (Lat A+/-,

1762.55 ± 80.14) exceeds that of the negative control (Lat A-/-, 1741 ± 54.59) and of the positive control (Lat A +/+, 1624.85 ± 66.78), the differences between the three groups are not significant (ANOVA on ranked data, p=0.314). Large, highly fluorescent clusters of F-actin could be seen in association with the cortical cytoskeleton in the Lat A -/- cells. This is likely to be responsible for the increased F-actin intensity in this group (Figure 4.10B, C).

Viability, Caspase 3 (activated) expression during Lat A and Lat A washout

Cell viability was evaluated using Trypan Blue Dye Exclusion during Latrunculin A application and washout as describe above. The viability ranged from 92.83 ± 1.81% (Lat A +/+) to 95.33 ± 1.18% (Lat A + group) with no significant difference between the groups (not pictured).

Apoptosis was analyzed by assessing Caspase 3 (activated) expression during Latrunculin A treated and washout as previously described. The expression of activated Caspase 3 was minimal in both Lat A application and Lat A washout groups. The differences between Lat A – (2.37% ± 0.16%) and Lat A + (0.631 ± 0.21) was not significant (t-test, p=0.108). The greatest expression was seen in the Lat A -/- (washout negative control) with 7.57% ± 1.12% of the cells positive for Caspase 3 expression. This is slightly higher than the 6 hour Caspase 3 expression seen above without washout. This might be attributed to the washout process which involved centrifugation. Interestingly, the Caspase 3 expression in the experimental group (Lat A +/-, 4.84% ± 0.55%) and the positive control (Lat A+/+, 2.87% ± 0.29) was significantly lower than the negative control (ANOVA on ranks, p=0.002). This seems to indicate that Lat A offers some small

degree of protection from Caspase 3 expression during RCCS culture (graphed data not shown).

DISCUSSION

The RCCS provides a unique culture environment to examine cell-cell interactions that are essential for skeletal muscle tissue assembly and regeneration. During RCCS culture, cells co-localize, adhere, and aggregate (Qiu et al., 1998). This facilitates self-assembly of functional tissue including bone (Granet et al., 1998), cartilage (Freed et al., 1993), pancreatic islets (Rutzky et al., 2001; Tobin et al., 2001) and tumors from single cells (Clejan et al., 2001). Previous studies of skeletal muscle in RCCS culture have utilized microcarriers (Molnar et al., 1997; Torgan et al., 2000) or membranes (Slentz, 2001) as a support mechanism for skeletal muscle myoblasts. We have previously shown that C2C12 myoblasts can fuse and differentiate in the RCCS in GM without serum alterations or the use of an artificial matrix (i.e., scaffolding or beads) in 3D aggregates. Since the use of exogenous support can influence cell structure and function (Huang and Ingber, 2000; Mao and Schwarzbauer, 2005), our model provides unique advantages to observe 3D cell-cell contact and aggregation without these confounding factors.

It seems surprising that the unique structure of single cells during 3D culture has rarely been examined, particularly in the absence of some form of exogenous matrix. This is may be due to the possibility that cells undergo anoikis, a specific type of apoptosis, when cultured in isolation (Reddig and Juliano, 2005). However, this phenomenon may be more characteristic of some cell types (i.e., epithelial cells, Frisch and Francis, 1994) than others (such as cells of mesenchymal origin). Regardless of the

triggering events, typical apoptosis results in the activation (by cleavage) of Caspases 3, 6, and 7. It should be noted that there are some rare forms of apoptosis that do not involve the caspases (Borner & Monney, 1999; Rich et al., 2000). Caspase 3 was selected as an indicator for apoptosis in this study due to the vital role it plays in both the death receptor and the mitochondrial pathways of apoptosis (Lakhani, 2006), its role in F-actin cleavage (via gelsolin) (Kothakota et al., 1997), and the well-characterized use of this method in defining apoptosis. Translocation of PS to the outer leaflet of the cell membrane, which occurs downstream of mitochondrial alterations and Caspase 3 activation, is also considered to be an indicator of apoptosis. Annexin V binds to phosphatidylserine (PS) in the outer leaflet and is often used as an indicator of PS translocation. Therefore, we tested for Annexin V staining in order to detect the level of apoptosis in our system. The data shows that these indicators of anoikis/apoptosis are not expressed by C2C12 myoblasts during the first 9 hours of RCCS culture. This may be due, in part, to the mesenchymal origin of C2C12 cells. The C2C12 cell line (Blau, 1985) is derived from activated skeletal muscle satellite cells, which are believed to be mesenchymally derived (Gros et al., 2005; Schienda et al., 2006). Some mesenchymal cell types typically become quiescent rather than apoptotic when cultured in isolation (Milasincic, 1996; Sachidanandan et al., 2002; Dhawan and Helfman, 2004). Further, C2C12 cells are thought to be more resistant to apoptosis than other satellite cell lines (Wittstock et al., 2003). Interestingly, cell-cell interaction may offer protection against anoikis/apoptosis (Glinsky and Glinsky, 1996; Makrigiannakis, 1999). The co-localization and potential for cell-cell contact (and, ultimately, aggregation) favored by RCCS culture may also protect cells from anoikis/apoptosis. Coupled with our viability data, it is unlikely that RCCS culture results in an increase in cell death as a result of anoikis/apoptosis in C2C12 cells

within the first 9 hours of culture. These data suggest that RCCS may offer protection from cell apoptosis and necrosis when compared to static liquid overlay culture.

The appearance of podia on single cells during RCCS culture is striking. Podia like structures have been reported on the free surface of cells after they detach from a substrate (Collard and Temmink, 1976) or establish contact with other cells (Enmon et al., 2001; Enmon, 2002). In addition, previous studies have shown that adherent cells, grown in suspension culture (liquid overlay) without an exogenously supplied substrate, typically exhibit podia (Ukena and Karnovksy, 1976). However, if the appearance of podia were solely a consequence of the lack of substrate adhesion then increases would not be noted during RCCS culture.

Although we assessed cells at earlier times for the presence of podia (0.5, 1, and 2 hours), the 3 hour time interval was selected as our initial time point for two reasons: 1) a great number of the podia observed before 3 hours were shorter than our proposed definition; and 2) complete spreading of C2C12 cells on a culture substrate occurs in approximately 3 hours. This time is in agreement with the analysis of cell spreading by Bereiter-Hahn et al. (1990). Therefore, it was reasoned that this might be an optimum time to assess podia formation.

We have shown here that podia are more numerous in RCCS culture, may increase in length with time in culture and are F-actin positive. The use of the actin depolymerizing agent, Lat A, affects remodeling by stochiometrically binding G-actin and effectively sequestering it from the available G-actin pool. The cell "senses" a reduction in G-actin and F-actin is depolymerized (Yarmola et al., 2000). The application of Lat A resulted in a decrease in the number of cells with podia and Lat A washout resulted in a recovery of these structures. Similar reductions of cytoplasmic projections in 2D and 3D have been previously reported (Keller et al., 2002; Campi, 2005). Thus, the

Lat A experiments not only indicate that podia formation is dependent on F-actin polymerization, it also indicates that RCCS podia may be similar to or modified versions of the above mentioned 2D cytoplasmic projections.

F-actin intensity was also reduced by Lat A application, indicating that the podia seen during RCCS culture are dependent on F-actin polymerization. However, comparisons between the 3 and 6-hour intensities of our RCCS controls (LatA- and Lat A-/-) may indicate that there is a slight increase of F-actin with time in culture as reported by others (Shimada et al., 2005). Nevertheless, RCCS induced upregulation does not explain the lack of significant differences between the washout groups. Lat A stimulated upregulation of actin is unlikely (Lyubimova et al., 1997). Although we cannot exclude the effects of short term gravitational loading via centrifugation during Lat A washout as a possible stimulus for increased F-actin, it is likely that these effects would be temporary and F-actin would be depolymerized upon return to RCCS culture. The intensely fluorescing F-actin clusters localized under the membrane of Lat A treated cells likely contribute to the fluorescence seen in the positive controls (Lat A -/-). This F-actin cluster arrangement may be a result of an increased expression of actin-associated proteins, which can overcome Lat A effects. Although actin stress fibers and cytoplasmic extensions may be rapidly depolymerized at low (1 μM) concentrations of Lat A, immature actin myofibrils are more resistant to depolymerization up to a 5 mM concentration. Furthermore, established actin microfibrils, which are seen in myotubes, are resistant to Lat A concentrations up to 50 μM (Wang et al., 2005). Although we attempted to eliminate these F-actin clusters by increasing Lat A concentrations, utilizing cytochalsin D, and a combination of cytochalisn D and Lat A, the clusters would not disassemble (Marquette, unpublished data). Thus, it is possible that these clusters are protected by some unknown mechanism. One possibility is the actin capping proteins

(such as CapZ), which would confer protection from Lat A induced depolymerization during our test period.

Classification of the 3D cytoplasmic projections in terms of 2D derived definitions is difficult. Although the lengths of 3D RCCS podia ranged up to 12+ μm in 9 hour culture and seem to fall within the definition of filopodia, the diameters of the RCCS podia varied widely. Some of these podia extended beyond ¼ of the cell (unpublished data) and thus do not fit the 0.2 μm diameter that defines filopodia (Albrecht-Beuhler and Lancaster, 1976; Nakamura, 2001). RCCS podia might be considered lamellipodia if the diameter were used as a defining measurement; however, these podia do not resemble the broad, flattened, veil-like protrusions described by Abercrombie et al. (1970). With the increased use of 3D culture for analyses, additional defining factors may be needed to further classify cytoplasmic projections. Since the single cell morphology of suspension and RCCS cultures are similar to that of lymphocytes, application of the terms microvilli and ruffles might be applicable.

Although our investigations demonstrate that RCCS culture results in an increase of F-actin dependent podia, it does not explain the mechanism of podia formation, the function of these structures, or the increases in quantity and length seen during RCCS culture.

Mechanism

Several signaling pathways have been proposed to drive podia protrusion (Faix and Rottner, 2006). However, the exact sequence of events remains undefined. The majority of these proposed pathways consist of a basic sequence of events including: 1) engagement of a cell adhesion protein (such as cadherins or integrins); 2) coupling of the

adhesion molecules to the actin cytoskeleton; 3) recruitment of additional adhesion proteins to the site of attachment for strengthening of the adhesion complex; and 4) stimulation of additional F-actin polymerization via Rho family GTPases. The increase in podia length seen during these studies may indicate that a similar mechanism is occurring in and facilitated by RCCS culture.

Increases seen in RCCS culture

We previously showed that cell-cell adhesion and aggregation occurs more rapidly in SC culture compared to RCCS cells. The decreased adhesion and aggregation as well as the greater number and increased length of podia observed might be explained by the RCCS culture environment. RCCS culture favors 3D cell co-localization (Qui et al., 1999); however, this co-localization occur in the presence of other forces including shear (0.2 dynes/cm^2, Synthecon, Inc., personal communication; Unsworth and Lelkes, 1998) induced by freefall through the medium and also impact and rotating/tumbling forces. These forces, which are not present in SC, may be sufficient to prevent permanent cell-cell adhesion between the adhesion molecules of co-localized cells. It is possible, however, that this temporary contact is enough to recruit additional adhesion molecules and stimulate F-actin polymerization. These temporary adhesion events would continue until the level of adhesion proteins could counter the destabilizing forces and facilitate permanent cell-cell adhesion. Interestingly, this same temporary contact may protect cells from apoptosis. This may explain why RCCS cells have decreased expression of activated Caspase 3 when compared to SC. Potential future investigations could include an examination of cell adhesion proteins that function in cell-cell and cell-aggregate formation.

Function

Although best known for their role in motility, cytoplasmic projections have also been reported to serve as sensory structures and may also play a role in differentiation and cell fusion. Filopodia and similar small actin-dependent protrusions are thought to function in a sensory capacity in bone (You et al., 2004). If mediated by hydraulic induced shear or drag forces, both filipodia and microvilli may regulate ion transport in several structures of the renal system (Guo et al., 2000; Liu et al., 2003; Praetorius, 2003; Du et al., 2004). Filopodia may also play a role in myoblast differentiation and cell fusion (Vautier, 2003).

CONCLUSIONS

An improved understanding of cell morphology during 3D culture is important for: 1) elucidating the mechanisms of myoblast fusion and differentiation; and 2) generating tissue-equivalent skeletal muscle tissue constructs suitable for regenerative medicine. Using a novel approach for analyzing podia, we show, for the first time, that the quantity of podia is increased during RCCS culture when compared to liquid overlay suspension culture. We also show that these podia are F-actin dependent and decrease with time but may increase in length with time in culture. In addition, RCCS culture appears to offer protection from cell death and apoptosis compared to liquid overlay suspension culture. Finally, RCCS culture facilitates further examination of the role of cell-cell contact in skeletal muscle fusion and differentiation.

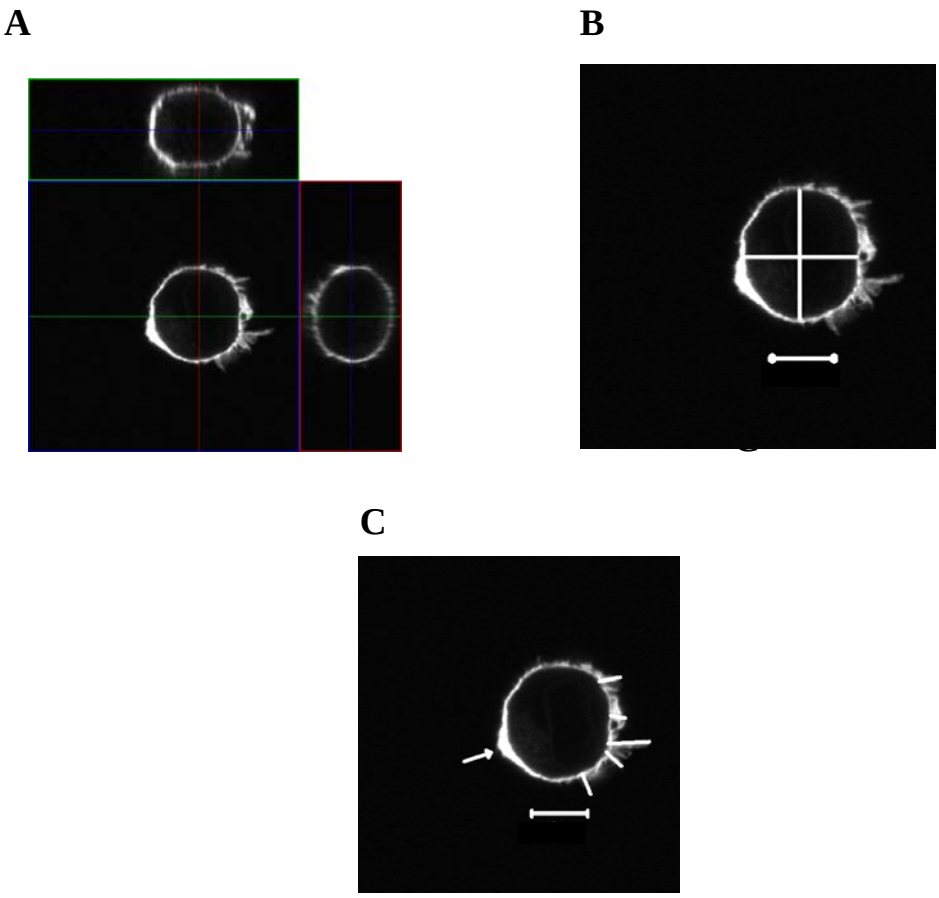


Figure 4.1: Methodology used to enumerate and measure podia

A) Example confocal microscopy orthogonal view used to determine number of focal planes, calculate the cell center and ascertain calculated diameter. B) Center focal plane used for empirical determination of diameter. White lines indicate the locations where diameters were collected. C) Example of the process used for podia quantification and length. White lines indicate the method used to count and measure podia. The arrow indicates membrane thickening that was not counted as a podium. All images shown are from a 9 hour RCCS culture. The cell membrane has been stained with CM DiI. Bar represents 10 microns.

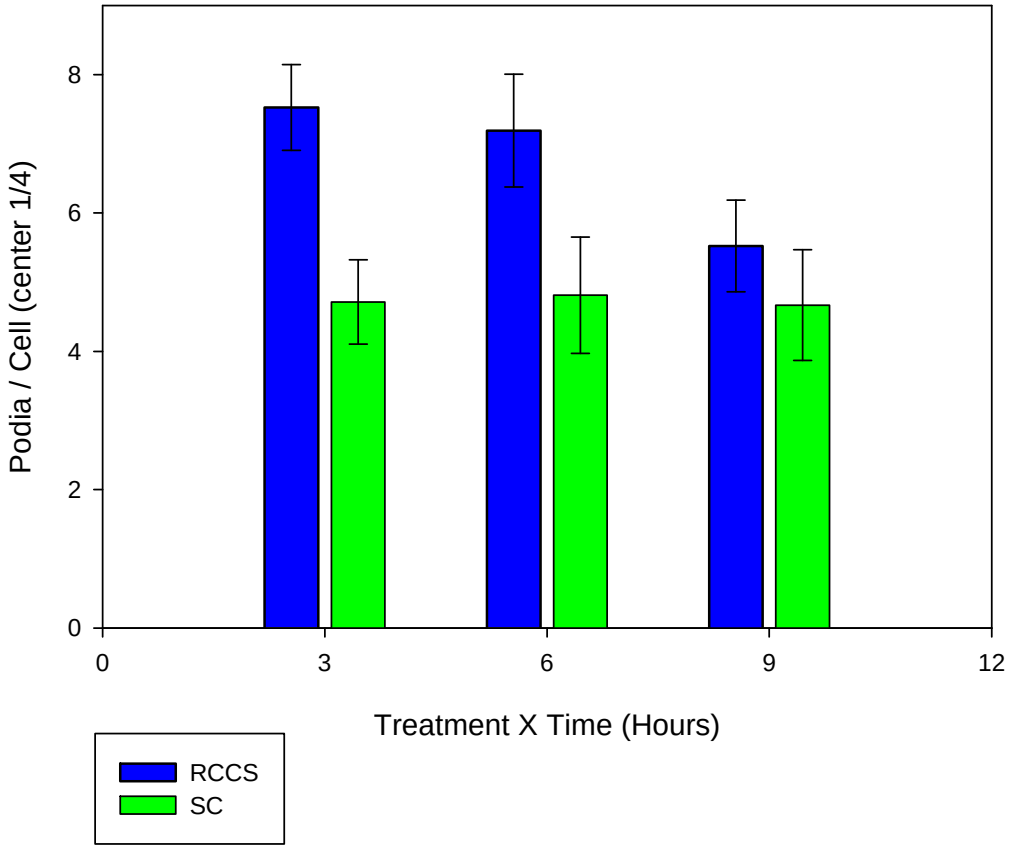


Figure 4.2: Podia enumeration

Podia are significantly more numerous during RCCS culture than in SC culture ($p<0.001$). There is also a decrease in podia number with time in RCCS culture while no decrease is noted in SC culture. Bars represent standard error.

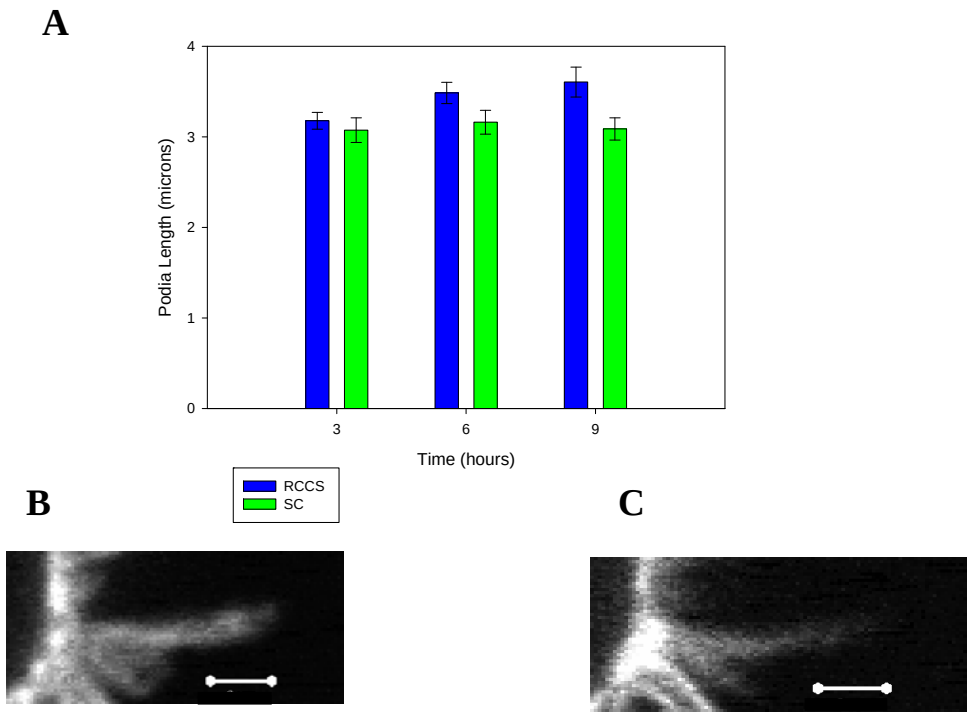


Figure 4.3: Podia measurements and determination of the presence of F-actin

A) Podia length increases with time during RCCS culture; however this increase is not significant. Bars represent standard error. B) Confocal microscopy view of podia highlighted by CM DiI staining (lipophillic membrane stain, red channel only) used to determine podia number, length, and as a marker for the presence of F-actin. C) F-actin staining in podia highlighted by BoDipy FL Phalacidin. To determine the presence of podia, podia were marked using the red channel. The red channel was then removed and green channel turned on. If F-actin (green channel) staining was present in the same area, podia were considered F-actin positive. Bar represents 10 microns

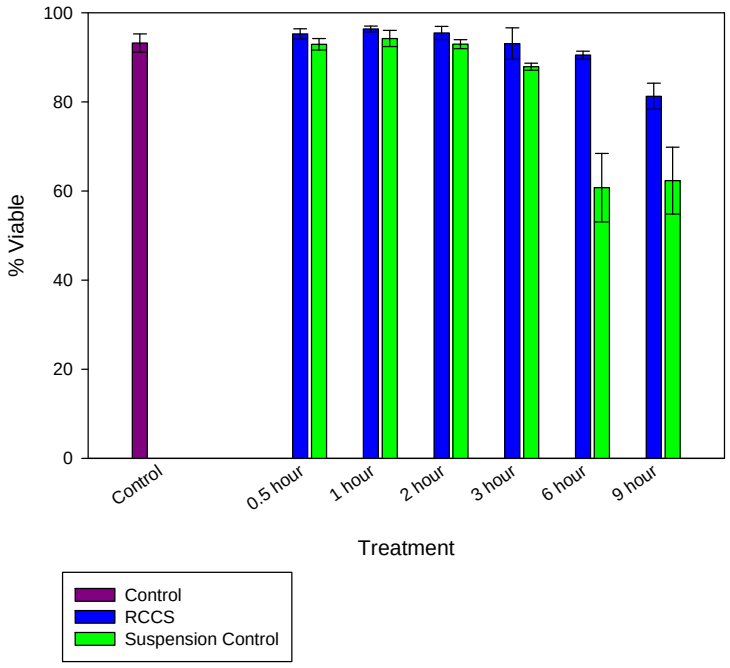


Figure 4.4: Viability via Trypan Blue Exclusion

An aliquot of cells was removed from culture and exposed to Trypan Blue. A minimum of 500 cells was counted and classified as either viable (no blue staining) or dead/dying (blue staining in any part of the cell). There are no significant differences within the RCCS over time; however, there are significant differences at 6 hours (60.78 ± 7.68) and 9 hours (62.32 ± 7.52) in the SC treatment when compared to the 0 hour, 0 treatment control (93.20 ± 2.04, p <0.001 and <0.001, respectively). Further, viability is significantly higher in the RCCS at 6 hours (90.49 ± 0.89) and 9 hours (81.25 ± 2.93) compared to SC at the same time points (p<0.001 and <0.001, respectively). Graph represents the means of 3 replicate experiments. Bars represent standard error.

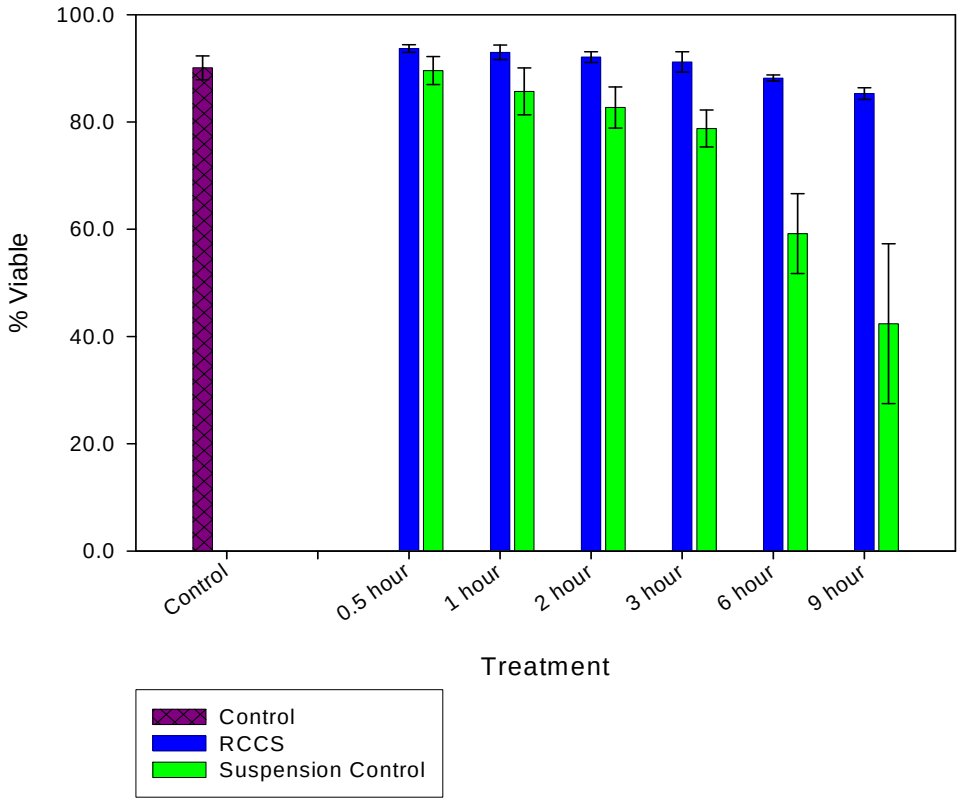


Figure 4.5: The Effects of Culture on Viability -Flow Cytometry

An aliquot of cells was removed from culture and subjected to Guava ViaCount analysis to determine viability. The viability of cells in the RCCS is significantly greater than those cultured in SC ($p<0.001$). Graph represents the means of 3 replicates. Bars represent standard error.

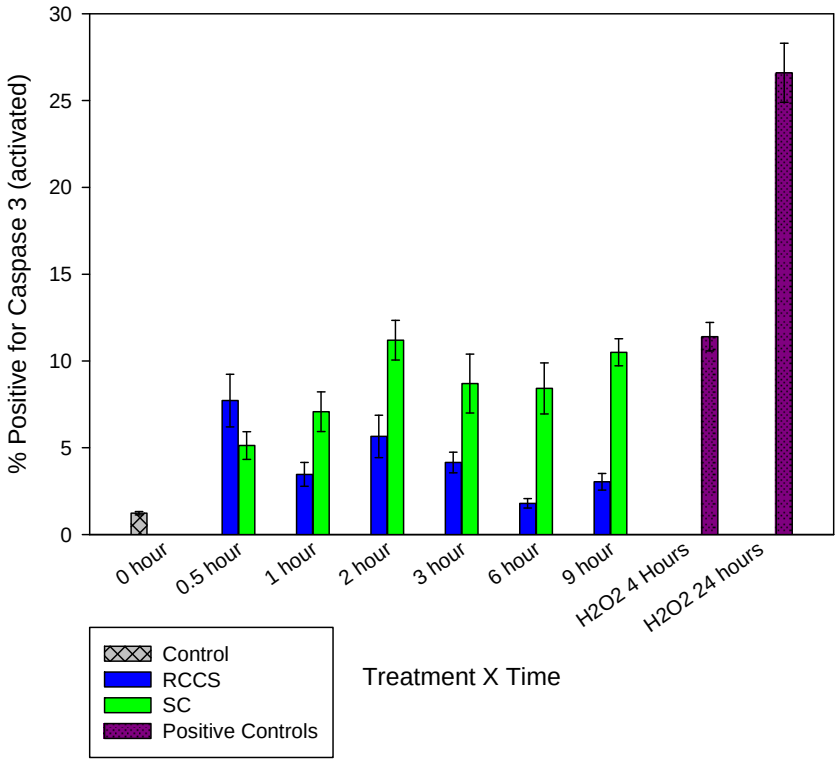


Figure 4.6: Markers of apoptosis - Caspase 3 (activated) expression

Caspase 3 (activated) was determined using a Guava PCA. Fixed rinsed cells were stained with an antibody to Caspase 2 (activated) and fluorescently labeled with a secondary antibody. The results represent the means of 3 independent experiments. Two way ANOVA on ranks indicate that there is a significant difference between RCCS and SC culture ($p<0.001$). The decreases in RCCS culture and the increases in SC culture are not significant. Positive controls included C2C12 cells subjected to 500 μM of H2O2 for 4 and 24 hours. Bars represent standard error.

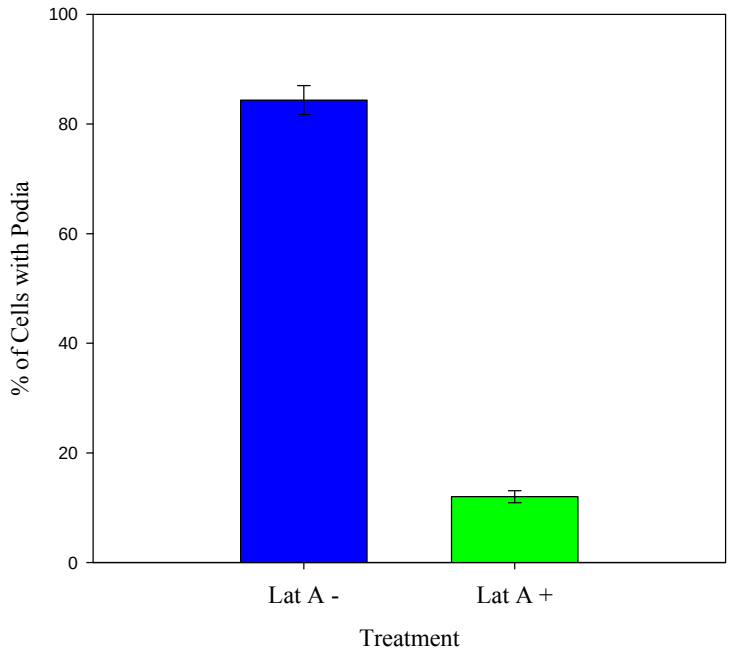


Figure 4.7: Effects of Latrunculin A on the number of podia positive cells

Cells were subjected to 2 μM Latrunculin A during RCCS culture for 3 hours. Cells were examined under light microscopy and a minimum of 500 cells classified as podia positive or podia negative. Cells subjected to Latrunculin A treatment show a significant reduction in the number of cells with podia (T-test, $p<0.001$). Graph represents the means of 3 independent experiments. Bar represents standard error.

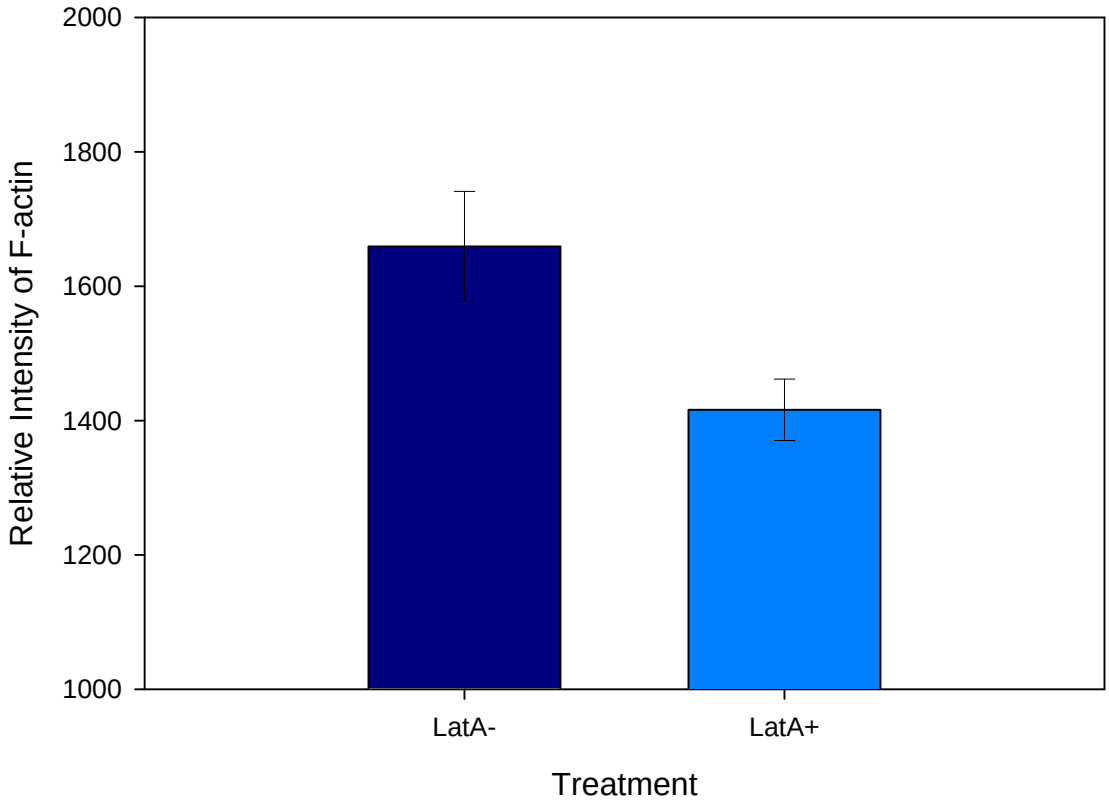


Figure 4.8: The Effects of Latrunculin A on F-Actin during RCCS Culture

Cells were subjected to 2 μM Latrunculin A during RCCS culture for 3 hours as above. Cells were stained with AlexaFluor 532 Phalloidin and analyzed using Guava PCA flow cytometry methodology. Cells subjected to Latrunculin A treatment show a significant reduction in F-actin fluorescence (T-test, p=0.020). Graph represents the means of 3 independent experiments. Bar represents standard error.

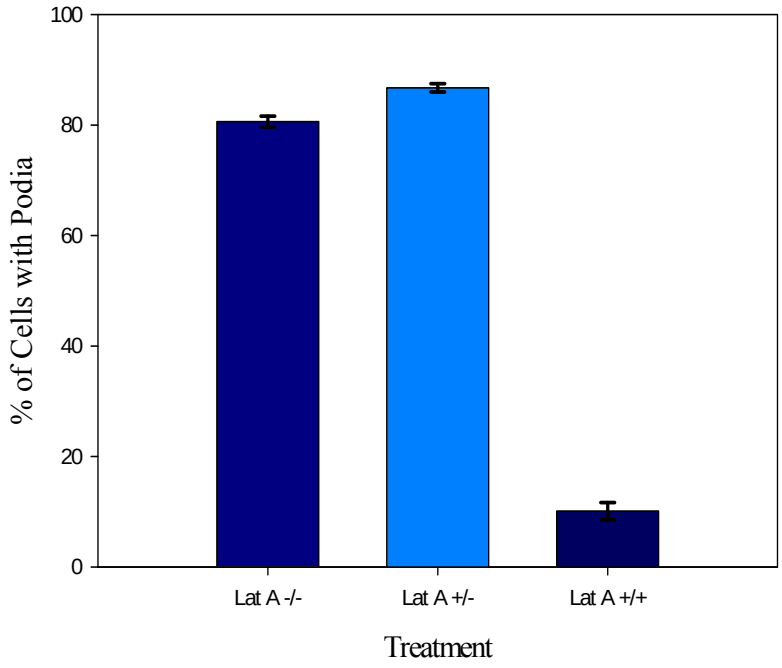


Figure 4.9: The Effects of Latrunculin A Washout on the Number of Cells with Podia

Cells were subjected to 2 μM Latrunculin A during RCCS culture for 3 hours as above. At the three hour time point, cells were washed to remove Latrunculin A, and then replaced in culture vessels with or without 2 μM Latrunculin A. At the end of the test period, fixed cells were examined under light microscopy. A minimum of 500 cells were classified as podia positive or podia negative. Positive controls (Lat A +/+) showed a significant reduction in the number of cells with podia, whereas the Lat A washout group (Lat A +/-) showed recovery of cells with podia in slightly higher levels than cells never treated with Lat A (Lat A -/-). There is a significant difference between the 3 groups (ANOVA, $p < 0.001$). Graph represents the means of 3 independent experiments. Bar represents standard error.

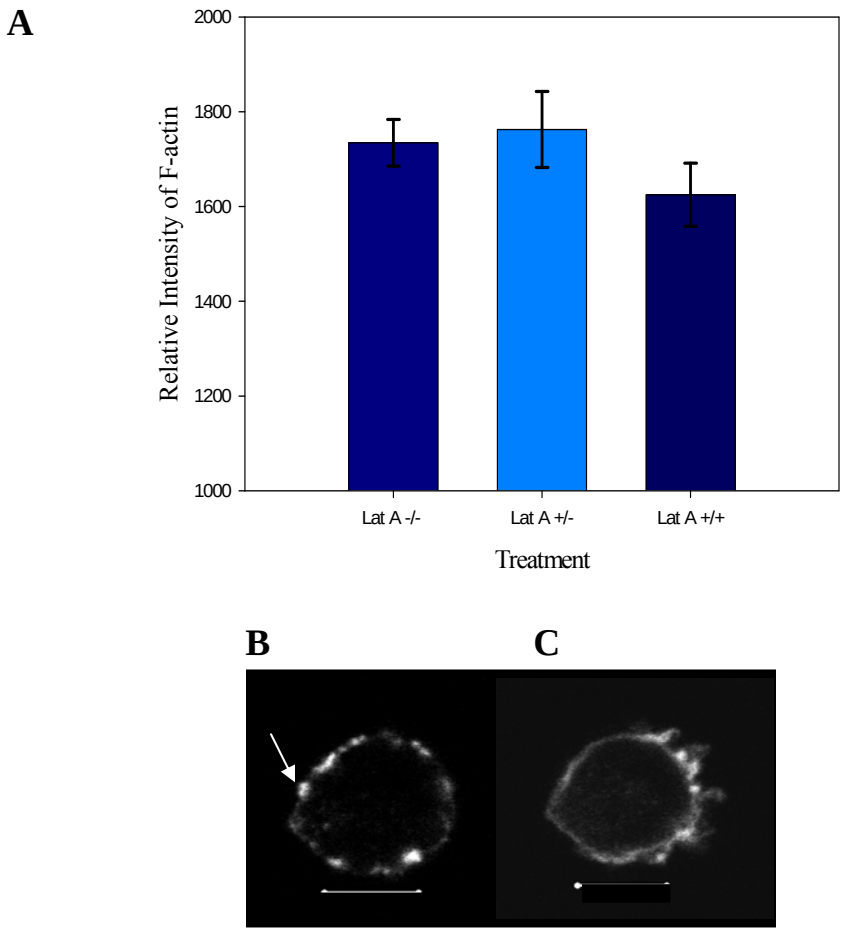


Figure 4.10: The Effects of Latrunculin A Washout on F-Actin

A) Cells subjected to Latrunculin A washout during RCCS culture as above, stained with AlexaFluor 532 Phalloidin, and analyzed using Guava PCA. F-actin intensity of the experimental cells (Lat A+/-) exceeds the negative controls (Lat A-/-) and the positive control (Lat A +/+), the differences between the three groups are not significant (ANOVA on ranked data, p=0.314). B) Large, highly fluorescent clusters of F-actin (arrow) are seen using confocal microscopy in association with the cortical cytoskeleton in the Lat A +/+ cells. C) F-actin arrangement in untreated cells.

Chapter 5: Cadherins: Effects on myoblast morphology, fusion and differentiation in the Rotary Cell Culture System

INTRODUCTION

Cadherins are a superfamily of cell-cell adhesion molecules that play a major role in cell-cell interactions. Classical cadherins, a subgroup of the cadherin family, are calcium-dependent single-pass transmembrane cell-cell receptors that typically function in homophillic binding. Classical cadherin mediates tissue organization, cell-cell adhesion, and differentiation. N-cadherin, a member of the classical cadherin subgroup, functions in a number of cellular processes during skeletal muscle development and regeneration. These functions include myoblast motility, cell sorting, differentiation, as well as the maintenance of cell-cell adhesion and tissue architecture (reviewed by Leckband and Prakasam, 2006).

The role of N-cadherin during skeletal muscle differentiation and fusion has been extensively studied. Culturing myogenic cells on N-cadherin coated substrates or in contact with N-cadherin coated beads induced differentiation (Gavard et al., 2004) even in the presence of growth stimulating conditions; i.e., a high percentage of Fetal Bovine Serum in the culture medium and low cell density (Goichberg and Geiger, 1998). Over expression of N-cadherin in cells with low native N-cadherin expression stimulated sarcomeric myosin expression (Redfield, 1997). Similarly, inhibition of N-cadherin by antibodies inhibited sarcomeric myosin expression (Mege, 1992). Fusion of myoblast cells, an essential step in differentiation, was inhibited by exposure of cells to cadherin

blocking peptides to the external domains (George-Weinstein, 1997). These studies indicate that N-cadherin likely plays a vital role in fusion and differentiation.

We have previously shown that single myoblasts fuse and differentiate into 3D skeletal muscle aggregates during RCCS culture (Marquette et al., 2007). Moreover, in the RCCS differentiation occurs without the withdrawal of growth factors. A similar observation was reported by Goichberg and Geigers (1998); however, in their study, an externally supplied matrix was used in a 2D culture environment. In contrast, the cells in RCCS culture adhere, aggregate and finally differentiate/fuse to form a maturing skeletal muscle model in a 3D culture environment. Cell adhesion molecules such as N-cadherin may mediate these processes. We have also reported the appearance of a significant number of F-actin positive cytoplasmic extensions on pre-aggregate single cells, which we designated "podia" (Marquette et al., 2007). These podia may also be a result of N-cadherin adhesion.

It has long been recognized that molecules on the exterior of the cell may play a role in adhesion and aggregation (Moscona, 1963). Blocking peptides to the Histidine, Alanine, Valine sequence of cadherins decreased aggregation (Mege, 1992) and cells over expressing N-cadherin showed increased aggregation in hanging drop (3D) culture (Redfield, 1997). Interestingly, cells interacted with the external domain of N-cadherin bound to beads via small membrane protrusions (Levenberg et al., 1998) and it is likely that adhesion proteins localize to these structures. Indeed, a number of adhesion molecules are localized to cytoplasmic projections. Examples include: 1) various integrin combinations localize to the leading edge of lamellipodia (Choma et al., 2004), filopodia (Rabinovitz, 1997), and other membrane protrusions (Laukaitis, 2001); 2) NCAM is localized to the tips of filopodia (Sandig, 1997); and 3) the majority of L-selectin is localized to the microvilli of a number of (immune system) cell types (Bruehl, 1996). In

addition to localization, adhesion molecules stimulate podia formation. Binding of CC151 (a tetraspanin) induces filopodial extension in several cell lines (Shigeta et al., 2003). Nectins (Kawakatsu et al., 2002) and E-cadherin also show filopodial extension (Kovacs et al., 2002). N-cadherin binding on an N-cadherin coated substrate resulted in the formation of broad lamellipodia. Further, de novo N-cadherin binding at the tip of the lamellipodium appeared to be the preferred binding site (Gavard et al., 2004).

The cell co-localization facilitated during RCCS culture provides an increased opportunity for N-cad:N-cad contact between single cells. N-cad:N-cad contact is cited as the initial stage for increased N-cad recruitment to the site of contact. Over time, the recruitment of multiple cadherin molecules serves to strengthen this adhesion. The brief contact during RCCS culture might also stimulate F-actin polymerization (Gavard, 2002) and stimulate the increases in podia number and length observed. However, shear and other forces within the RCCS create an environment that may prevent permanent binding upon initial contact requiring a greater recruitment of N-cadherin to the area before stable contacts can be established. This may also increase the number and length of podia and seen during RCCS culture. We believe this may explain the more rapid aggregation of cells cultured in suspension when compared to RCCS cultured cells. During SC culture, no shear forces are induced, and cell tumbling, if present, is minimal compared to that which occurs in the RCCS. Therefore, N-cadherin facilitated binding occurs more rapidly in SC and less N-cadherin is required to create stable contacts. If forces in the RCCS prevent initial binding and additional N-cadherin molecules are required for binding, then N-cadherin should increase at the podia over time in single myoblasts in the RCCS.

Therefore, the purpose of this study is to determine if: 1) podia in single myoblasts exhibit increased N-cadherin localization over time during RCCS culture prior

to permanent cell adhesion; and 2) N-cadherin inhibition will decrease fusion and differentiation in myoblasts in RCCS culture.

METHODS

Cell Culture

C2C12 cells, obtained from the American Type Culture Collection (Rockville, Maryland), were cultured in a humidified incubator at 37^{o}C with 5% CO_2/95% air in growth medium (GM) consisting of high glucose Dulbecco's Modified Essential Medium (DMEM, Gibco-BRL, Carlsbad, California) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone, Logan, UT) and 1% Penicillin-Streptomycin (Gibco-BRL, Carlsbad, California). Cells were subcultured every 2-3 days as needed to maintain less than 75% confluency using 0.125% Trypsin/EDTA (Gibco-BRL, Carlsbad, California). Culture medium was removed by aspiration, Trypsin/EDTA added, cultures incubated for 2-5 minutes at 37^{o}C, fresh culture medium added, cells strongly pipetted for complete removal from the culture surface, and the single cell suspension redistributed to new culture vessels. Cells in passages 6-8 were used for all experiments.

Monolayer cell cultures were subcultured using Trypsin/EDTA (as above). Although Trypsin promotes cell detachment by digestion of the adhesion proteins, including cadherin in the presence of EGTA, some recovery of adhesion molecules is likely by the 3-hour time point (Charrasse et al, 2002). Cells were collected from culture flasks by aspiration, centrifuged, rinsed, and resuspended in GM. Cells were counted using an improved Neubauer hemacytometer and volumes were adjusted to a final concentration of 5 X 10^5 cells/ml with GM. For some experiments, GM was

supplemented with anti-N-cadherin (GC-4 clone, Sigma-Aldrich, St. Louis, MO) to achieve a final concentration of 100 μg/ml. Cells subjected to RCCS culture were seeded into 10 ml High Aspect Ratio Vessel (HARV, Synthecon, Inc., Houston, TX) disposable culture vessels and rotated at 9.0 RPM. During 4-day culture periods, rotation speed was adjusted empirically to maintain the cell aggregates in freefall. Suspension controls (SC) for N-cadherin localization experiments were seeded in previously prepared poly(2-hydroxyethyl methacrylate) (poly-HEMA, Sigma-Aldrich, St. Louis, MO) coated Petri dishes to prevent adhesion as described by Folkman and Moscona (1978). Briefly, a 10X stock was made by dissolving 4.8 g of poly-HEMA in 40 ml of 95% ethanol (Folkman and Moscona (1978) and rocking overnight at room temperature, then stored at 4^{o}C. To prepare dishes, the stock was diluted 1:10 in 95% ethanol, two ml was added to a 60 mm tissue culture Petri dish, and the dish was air dried overnight in a Nuaire biological safety cabinet. Three coats of poly-HEMA were required to prevent attachment of C2C12 cells. Coated Petri dishes were rinsed with sterile Dulbecco's Phosphate buffered saline (PBS) and allowed to air dry as above, then stored in an airtight container at 4^{o}C until use. RCCS cultured cells without anti-N-cad served as controls for anti-N-cadherin inhibition studies. For N-cadherin localization experiments, cells were cultured for 3, 6, and 9 hours. Cells were cultured for 4 days in the N-cadherin inhibition experiments.

Preparation for Syncitia Counts and Immunohistochemistry

At day 4 of culture, the GM containing cell aggregates was removed from the culture vessel and allowed to settle by gravity in 15 ml conical tubes. The GM was aspirated and replaced with 10% neutral buffered formalin. Fixed samples were

processed, paraffin-embedded, and sectioned at 5 μm thicknesses. Sections were mounted on slides and stained using immunohistochemistry or with hematoxylin and eosin (H&E).

Quantification of Syncitia

H&E stained sections were used to quantify syncitia by manual count using a Nikon TS 100-F inverted light microscope with a PlanFluor 60X/0.5-1.25 oil immersion objective. For the purposes of this experiment, syncitia were defined as any cell containing more than three nuclei. A maximum of eight non-overlapping fields along the periphery of the aggregate were counted, with a minimum of three sections examined per independent experiment. To minimize the possibility that a syncitium would be counted more than once, sections used for enumeration were separated by no less than 60 μm.

Immunohistochemistry

Paraffin embedded, slide-mounted sections were de-parafinized, rehydrated, and stored for two hours in PBS at 4^{o} C. Slides were removed from storage and washed with PBS three times for two minutes each. Excess PBS was removed and sections were permeablized/blocked with 0.1% Triton X-100 and 5% goat serum (GS) in PBS for 20 minutes. Excess liquid was removed and replaced with 200 μl of 1:400 MY-32 antibody (Sigma-Aldrich, St. Louis, MO), an antibody specific for skeletal muscle specific Myosin Heavy Chain (MHC, fast and neonatal) in 5% GS. Sections were incubated at room temperature in humidified chambers for one hour. Excess primary antibody was removed with three PBS washes. Excess PBS was removed and 100 μl 1:500 Alexafluor 594 goat anti-mouse secondary antibody (Invitrogen/Molecular Probes, Carlsbad, California) in

5% GS was applied to the sections. Slides were again incubated at room temperature for 1 hour in a humidified chamber. Sections were rinsed three times with PBS to remove excess secondary antibody and sealed with Gel Mount (Sigma-Aldrich, St. Louis, MO). Coverslips were permanently attached with Per Mount (Fisher Scientific, Fairlawn, NJ) or nail polish. Slides were stored in a slide box at 4^{o}C until imaged.

Images of myosin stained sections were collected using a Nikon Eclipse 800 microscope equipped with a 100W mercury vapor lamp, a Photometrics CoolSNAP fx, a Nikon Plan Apo 60X oil correction objective and a Texas Red filter set. Exposure settings remained constant throughout image collection. Metamorph software (Molecular Devices Corporation, Sunnyvale, CA) was used to quantify both the area of myosin expression and the intensity per thresholded area. Thresholds were set to exclude background fluorescence of controls stained with the secondary antibody only. Areas were assessed as myosin positive if the intensity exceeded the threshold settings. Five slides (thin section) per aggregate were imaged, and a minimum of 60 fields about the periphery of the aggregate(s) was collected.

Confocal Microscopy

To determine whether N-cadherin was localized to podia, an aliquot of fixed, rinsed cells was prepared for confocal imaging by application of 250 µl 1:200 anti-N-cadherin (H-63 clone, Santa Cruz Biotechnologies, Santa Cruz, California) in 5% goat serum (Sigma-Aldrich, St. Louis, MO) and 0.1% saponin (Sigma-Aldrich, St. Louis, MO) in PBS for 1 hour at room temperature. Counterstaining was accomplished with 250 µl of 1:500 AlexaFluor 594 goat anti-rabbit IgG (Invitrogen/Molecular Probes, Carlsbad, California)**.** F-actin was labeled by subsequent application of 250 µl BoDipy FL

Phallacin (5 units/250 µl of 5% goat serum and 0.1% saponin in PBS for 30 minutes. Cells were rinsed and stored in PBS at 4^{o}C until imaging. To image, 200 µl of stained cells suspended in PBS were placed on a coverslip and used to collect three-dimensional, two-channel images using a Zeiss LSM 510 with a C-Apochromat 40X /1.2W objective. Focal planes were 0.5 µm and were collected to encompass the entire cell.

Confocal Image analysis

Images were prepared for analysis using LSM 510 Image Browser Software. The center ¼ of the cell was calculated as previously described (Marquette et al., 2007b). Briefly, the number of z-planes comprising the depth (z vector) of the cell was determined. The total number of z planes was then used to calculate the range of planes comprising the center ¼ of the cell. The average intensity of N-cadherin was calculated from this center ¼ of the cell (range of planes).

The F-actin channel was used to delineate the cell interior from the exterior periphery (Figure 5.1A). To create a cortex only image, a 12 point closed freeform circle was used to delineate the interior limits of the cortical cytoskeleton. Once the circle was adjusted to maximize exclusion of the cortex while including the cell interior within the circle, the cut function was used to remove all interior staining. This process was repeated plane-by-plane for the entire center ¼ of the cell (Figure 5.1B). To create the podia only image, the 12 point closed freeform circle was then used to include the cortical region but exclude the podia. The cut function removed the cortical region, and the remaining image included only podia (Figure 5.1C). This process was repeated for all focal planes of the center ¼ of the cell.

In order to analyze for increased localization to the podia, all three images of a given cell (whole cell, cortex, and podia) were opened in Metamorph. The F-actin channel was separated and removed. The N-cad channel was thresholded to exclude background and maintain podia using the whole cell image. Voids created by LSM deletion and those caused by the nucleus in the whole cell were excluded by the threshold settings. This threshold was then applied to the remaining images of the cell. N-cadherin average intensity per thresholded area was collected for each image.

N-cadherin intensity

Cells were fluorescently labeled using the procedure described above with the exception that 250 μl of 1:500 AlexaFluor532 goat anti-rabbit antibody (Invitrogen/Molecular Probes, Carlsbad, California) was used. N-cadherin intensity was then determined using a Guava Personal Cytometer Analyzer (PCA, Guava Technologies, Hayward, CA) and the Protein Express settings according to the manufacturer's instructions. An aliquot of unstained untreated cells collected at 0 hour was used to set the forward light scatter gates. Cells were analyzed using the Guava PCA a minimum of two times with 1000 cells analyzed per data set. Data is reported as a ratio of the intensity of each sample to the isotype in order to standardize between replicates.

Statistics

The data reported are the mean ± the standard error of three independent experiments. Descriptive and statistical data were generated using SigmaStat software (Jandel Scientific) or Excel (Microsoft) software functions.

RESULTS

N-cadherin inhibition

To investigate the role of N-cadherin in myoblast differentiation, the well-characterized monoclonal antibody to N-cadherin, GC-4, was used to inhibit N-cadherin binding. Although the use of siRNA to N-cadherin was considered, the GC-4 clone was selected due to its binding strength and widespread use in previous N-cad inhibition studies. In addition, there is evidence that cadherins associate with various catenin molecules during transport to the membrane via vesicles prior to localization to the plasma membrane (Hinck et al., 1994; Kojima et al., 2003)**.** Therefore, siRNA may result in cellular changes in the localization of catenins. Since increases in beta-catenin levels suppress adherin junction formation and arrest of myogenic progression, cadherin silencing could potentially alter cell behavior (Goichberg et al., 2001). However, the use of the GC-4 clone inhibits N-cadherin homophillic binding at the membrane where cell-cell interaction occurs. Although these antibodies would seemingly be incorporated into the cell with N-cadherin during recycling, no untoward effects in cell function have been reported.

Cells were cultured in the RCCS with or without anti-N-cadherin for four days. We previously demonstrated that myoblasts in the RCCS begin to fuse and differentiate by day three as indicated by the appearance of syncitia and MHC (II) expression. Accordingly, the four-day culture period in these studies was used because it provided sufficient time to establish any differences that might occur due to N-cadherin inhibition. In addition, we previously determined that the majority of syncitia and myosin staining occurred near the periphery of the aggregate and no syncitia were found in the interior of

the aggregate. Thus, for these studies, syncitia were counted and imaged for MHC only near the periphery of the aggregate.

H&E stained sections were used to count syncitia. A minimum of seven peripheral fields from a minimum of three sections was analyzed and data was expressed as number of syncitia per field. A Mann-Whitney Rank Sum Test shows that the mean number of syncitia per field was significantly reduced in the N-cadherin inhibited cells (2.28 ± 0.25) compared to cells cultured without the antibody (3.88 ± 0.25, $p<0.001$) (Figure 5.2). These results indicate that inhibition of anti-N-cadherin binding inhibits fusion in 3D culture.

To determine if anti-N-cadherin also inhibited differentiation of myoblasts in RCCS culture, an early marker of myoblast differentiation was used. Thin sections of 3 and 6+ day aggregates from RCCS and SC culture were stained using an antibody to skeletal muscle specific myosin heavy chain (MHC). As with the syncitia data above, a correction for sections occupying partial fields was applied prior to statistical analysis. The threshold was set to exclude background staining using a secondary only stained sample, which was maintained during all data collection. Images were collected and analyzed for the 1) thresholded area and 2) intensity within the thresholded area.

T test analysis of thresholded areas revealed a statistically significant increase between anti-N-cadherin treated aggregates ($5.7 \text{ X}10^5 \pm 3.2 \text{ X } 10^4$) compared to the untreated control aggregates ($1.4 \text{ X } 10^5 \pm 1.4 \text{ X } 10^4$) ($p<0.001$) (Figure 5.3). However, the differences in average intensity between anti-N-cadherin treated aggregates (506 ± 5.4) and untreated control aggregates (544 ± 22.0) were not significant, indicating that although there are smaller areas expressing N-cadherin within the RCCS controls, the degree of expression within those areas is comparable to the degree of expression in the anti-N-cadherin treated aggregates (not shown). Thus, it appears that anti-N-cadherin

prevents myoblast fusion but promotes the expression of MHC, a marker of differentiation during 3D RCCS culture.

N-cadherin localization

We previously showed that RCCS culture resulted in increased fusion and differentiation compared to suspension control cells. Since N-cadherin is reported to function in differentiation and fusion, we wished to determine if RCCS culture resulted in increased N-cadherin expression over time. To test this possibility, cells were cultured for 3, 6, and 9 hours in the RCCS, fixed, and then stained for N-cadherin. These time points were selected based on our observations that podia were of minimal length at the two-hour time point and few single cells remained by the 12 hour time point. Further, application of trypsin in the presence of a chelating agent results in cadherin cleavage, but recovery is reported by 4 hours (E-cadherin**,** Stehbens et al., 2006)**.** Cells were then analyzed for N-cadherin intensity using Guava PCA analysis (Figure 5.4). The suspension control cells have an intensity almost twice that of RCCS cells at the 3 hour (2.45 ± 0.18 vs 1.27 ± 0.13), 6 hour (2.78 ± 0.18 vs. 1.22 ± 0.088), and 9 hour (2.59 ± 0.22 vs. 0.98 ± 0.13) time points. Two way ANOVA on ranks reveal that N-cadherin intensity is significantly greater than that seen in the RCCS cultured cells ($p<0.001$). There is a slight decrease over time observed in the RCCS cells, but the changes over time are not significant.

Although N-cadherin expression did not increase over time, it was possible that a redistribution of cytoplasmic N-cadherin to the podia from the cytoplasmic pools might occur. In order to determine if N-cadherin localized to the podia seen in RCCS culture, we subjected cells to RCCS culture for 3, 6, and 9 hours, and then analyzed if there was

increased N-cadherin expression at the podia. To define if the differences that occur in RCCS culture are not simply a consequence of culture without an externally supplied substrate, cells grown in suspension were used as controls.

A novel method was utilized to describe the differential localization of N-cadherin to the podia over time. A subset of each confocal image consisting of the center ¼ of the cell was used to sequentially delete cell compartments defined by the F-actin cortical cytoskeleton. Average N-cadherin intensity data was gathered on: 1) whole cell (WC, in which no portion of the cell removed), 2) cortical region (roughly equivalent to the membrane region, M, in which all fluorescence interior to the cortical cytoskeleton was removed), and 3) podia only (P, where the cortical cytoskeleton and interior of cell was removed). The average intensity was gathered for each of the three compartments for each cell analyzed via Metamorph software. Since confocal adjustments were necessary to obtain optimal images, and thresholds were adjusted for each cell, localization is reported as a ratio of the average intensities to allow for comparisons between cells and time points.

Comparisons of the average N-cadherin intensity ratios of P:WC during the 3, 6, and 9 hour time points show that there is a general decline of N-cad at the podia over time in during RCCS culture (Figure 5.5). A two way ANOVA on ranks revealed a statistically significant interaction between Treatment and the Time in culture ($p=0.016$). To determine the times and treatments where differences were significant, a Holm Sidak Multiple Pairwise comparison was used and revealed that there is significantly more N-cadherin localized to podia in the SC than in the RCCS at 6 hours (0.51 ± 0.057 vs 0.28 ± 0.022) and 9 hours (0.42 ± 0.036 vs. 0.28 ± 0.025) of culture ($p<0.001$, $p=0.002$ respectively). The decreased localization of N-cadherin to SC podia over time is not

significant. However, the reduction of N-cadherin localized to the podia is significantly reduced at 6 and 9 hours when compared to the 3 hour time point ($p<0.001$, $p<0.001$).

Since it is possible that the decreased intensity localized to podia might be caused by a decrease to the membrane and not to the podia, two additional compartmental comparisons were performed. P:M intensities over time reveal a similar trend to the P:WC if not to the same degree (Figure 5.6). Two way ANOVA on ranks reveal that the interaction between treatment and time is significant ($p=0.006$). Holm-Sidak pairwise multiple comparisons once again showed that there is significantly more N-cadherin localized to podia in the SC than in the RCCS at 6 hours (0.76 ± 0.063 vs. 0.63 ± 0.073) and 9 hours (0.69 ± 0.032 vs. 0.60 ± 0.027) of culture ($p=0.004$, $p=0.026$, respectively). The decreased localization of N-cadherin to podia over time in SC cells is not significant. However, the reduction of N-cadherin localized to the podia is significantly reduced at 6 and 9 hours when compared to the 3 hour time interval ($p=0.004$, $p=0.011$). Thus N-cadherin does not appear to increase localization to the podia nor to the membrane over time during RCCS or SC 3D culture and may decreases over time.

DISCUSSION

N-cadherin in 3D fusion and differentiation

The role of N-cadherin in cell-cell adhesion, fusion, and differentiation has been studied extensively. However, these investigations have largely used 2D monolayer culture methodologies. Although studies have indicated that cells with myogenic potential will differentiate only during hanging drop culture (Edwards et al., 1983; Slager

et al, 1993; Redding et al., 1997), few studies have addressed the role of N-cadherin during 3D culture of myoblasts.

Although originally used to model some aspects of a microgravity environment in cell culture, the RCCS is more recently utilized for 3D tissue engineering, regenerative medicine applications (Goodwin et al., 1993; Rutzky et al., 2001; Unsworth and Lelkes, 1998), and generating 3D tissue models (Margolais et al., 1999; Song et al., 2002; Song et al., 2004). Zero headspace, a minimal laminar gradient, and solid body rotation about the horizontal axis reduces shear stress and turbulence as well as co-localizes particles (cells and aggregates) of varying sedimentation rates in a 3D milieu (reviewed by Hammond and Hammond, 2001). Gas exchange occurs via a gas permeable silicone membrane at the rear of the RCCS culture vessel. The RCCS facilitates aggregation by effectively concentrating cells in the center, a small portion of the overall vessel volume (Infanger et al., 2004; Manley and Lelkes, 2006). This area of high density co-localizes the cells into a much smaller area and increases the opportunity for N-cadherin mediated cell-cell contact and adhesion, critical events in myoblast fusion and differentiation.

We show for the first time that inhibition of N-cadherin mediated adhesion results in decreased cell fusion and increased expression of skeletal muscle specific MHC during RCCS 3D culture. Similar decreases in fusion (Mege et al., 1992) and increases in expression of differentiation markers have been reported in monolayer culture methods by N-cadherin blockade/activation using antibodies, peptides, or N-cadherin ectodomains (Goichberg and Geiger, 1998; Gavard, 2004). Furthermore, some inhibition of differentiation has also been noted (Mege et al., 1992; Charrasse et al., 2002). These differences are likely due to the anti-N-cadherin protein type and the method of delivery used.

The GC-4 anti-N-cadherin antibody, used in N-cadherin inhibition, is reported to interact with an epitope located between the first and second of five cadherin repeats in the external domain (Harrison et al., 2005). The first cadherin repeat (ECD1) is thought to play a critical role in cadherin specificity (Sakisaka and Takai, 2005). The area between ECD 1 and 2 contains a calcium binding pocket that is required for N-cadherin mediated adhesion (Kitagawa et al., 2000) and is critical for both cis and trans dimerization (Ozawa, 2002). Thus, the soluble GC-4 clone binds to N-cadherin molecules at the surface and inhibits cis and trans binding of other N-cadherin molecules. This action prevents adhesion via N-cadherin. The binding to this location may also serve to activate the differentiation program. Thus, fusion can be inhibited while intensifying differentiation by GC4 clone binding. The ability to activate differentiation while inhibiting cell-cell interaction and fusion will differ depending on the epitope of the antibody, peptide, or ectodomain being used.

Although N-cadherin binding is sufficient for differentiation (Goichberg and Geiger, 1998), it is not required. N-cadherin null satellite cells differentiate (as determined by spontaneous contraction) and fuse to form multinucleated myotubes both *in vitro* and *in vivo* (Charlton et al., 1997). Thus, other cadherins have been suggested as the protein necessary for fusion and differentiation. A leading candidate is M-cadherin. M-cadherin knockdown by siRNA results in decreased fusion, but the expression of MHC, myogenin and Tropin T is not affected compared to differentiating myotube controls (Charrasse et al., 2006). Further, M-cadherin null mice have normal musculature (Hollnagel et al., 2002). These results seem to indicate the potential for functional redundancy among cadherins and the importance of downstream events in the induction of fusion and differentiation.

The role of N-cadherin in Podia Formation

Since the binding strength of a single N-cadherin unit (dimer) is relatively small, multiple units are required to facilitate a permanent bond (Kusumi et al., 1999). According to the findings and model proposed by Mary et al. (2002), N-cadherin contact between cells recruits additional N-cad to the site of contact to strengthen the bonds between N-cadherin molecules. This additional N-cadherin recruitment facilitates a stronger, more permanent binding. If N-cadherin contact occurs between the suspended cells during RCCS culture, additional N-cadherin might be localized to these areas.

Accordingly, this investigation assessed if increased levels of N-cadherin would be observed on the structures we defined as podia. These F-actin dependent structures are more numerous in RCCS culture when compared to cells in SC. The longest podia were measured on those cells following 9 hours of RCCS culture. Interestingly, the formation of cytoplasmic extensions between cells and bead bound N-cadherin chimera has been noted (Goichber and Geiger, 1998; Lambert et al., 2000) as have large lamellipodia after binding to N-cadherin coated Petri dishes (Gavard et al., 2003). N-cadherin binding subsequently induced differentiation in single cell myoblasts. Thus, it is possible that podia form as a result of N-cadherin contact. In addition, N-cadherin activation via binding also activates the small GTPases including Rac1 (Gavard et al., 2003) and RhoA (Charrasse et al., 2002). Rac1, associated with N-cadherin mediated lamellipodia formation (Gavard et al., 2003), inhibits differentiation when constitutively expressed (Gallo et al., 1999). RhoA, which positively regulates myogenesis, is more closely associated with the formation of stress fibers during 2D culture (Wei et al., 1998). Thus, cell-cell contact could result in temporary N-cadherin binding. Such binding would

stimulate increased recruitment to the site of contact and could also stimulate podia formation via Rac1.

While the RCCS environment favors 3D cell co-localization (Qui et al., 1999), it occurs in the presence of shear (0.2 dynes/cm^2, Synthecon, Inc., personal communication; Unsworth and Lelkes, 1998) and other forces. These forces, which are not present in liquid overlay (SC), may be sufficient to initially prevent stable cell-cell adhesion between N-cadherin proteins located on adjacent cells. Indeed, we previously showed that cell-cell adhesion and aggregation occurs more slowly during RCCS culture when compared to static SC culture. In addition, we showed that podia increase in length during these tested time points. We hypothesized these ephemeral cell-cell contacts would be sufficient to induce recruitment of additional N-cadherin to the site of contact and would also stimulate F-actin polymerization via a Rac1 pathway. This would result in increasing podia length and increased localization of N-cadherin to the podia apices.

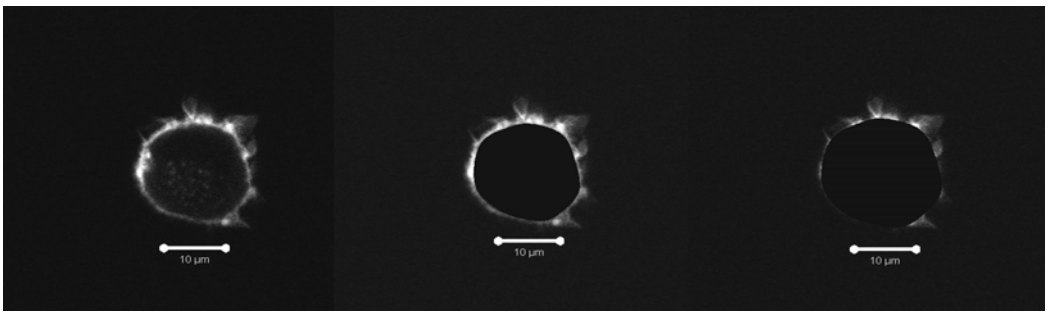
To test this hypothesis, we utilized a novel method for quantifying N-cadherin (or other fluorescently labeled proteins) to the podia and the cortical regions of a 3D cell. Areas of the multifocal plane confocal images of cells were sequentially deleted to remove areas of non-interest and then reported as a proportion of intensity localized to these areas with respect to the whole cell. Using this approach, our preliminary studies revealed that there was increased localization of cadherins to the podia over the 9-hour test period using Pan-cadherin (unpublished data). The Pan-cadherin antibody used in this preliminary study recognizes a number of cadherins including E, N, P, R, K, C, and OB-cadherins (SIB BLAST Network Service, 2006).

Our results also indicate that N-cadherin decreases to the podia during the time period tested. Interestingly, these significant decreases were not noted in SC controls. In addition, N-cadherin was more abundant in SC than in the RCCS. M-cadherin, another

classical cadherin protein that plays a role in differentiation and fusion (Zeschnigk et al., 1995), showed a similar pattern to that of the N-cadherin data (unpublished results, Marquette). It is possible that one or more other cadherins recognized by the Pan-cadherin antibody is localized to the podia. In addition to cadherins, integrins have been reported to facilitate aggregate formation in 3D hanging drop culture, and thus may also function in initial cell-cell adhesion (Robinson et al., 2002). Since our experimental cells aggregated and differentiated, it is likely that adhesion molecules other than N-cadherin facilitate cell-cell adhesion during RCCS culture. It is also possible that N-cadherin may be recruited to the site of adhesion at time points not examined in these studies to facilitate differentiation and fusion.

CONCLUSIONS

The co-localization favored during RCCS culture provides increased opportunity for N-cadherin: N-cadherin contact between single cells. An improved understanding of the events of cell-cell adhesion and fusion/differentiation during 3D culture is important for: 1) increased understanding of differentiation and fusion; 2) the development of models to elucidate the mechanisms of myoblast fusion and differentiation *in vivo*; and 3) generating tissue-equivalent skeletal muscle tissue constructs suitable for regenerative medicine. We show for the first time during RCCS 3D culture that an antibody to N-cadherin, inhibits cell fusion but increases expression of MHC. Utilizing a novel technique of sequential deletion, we show that N-cadherin significantly decreases during the initial 9 hours of RCCS culture and these differences are not seen in the suspension control cells. Preliminary studies indicate that other cadherins may increase localization over time. These are currently under further investigation.



A **B** **C**

Figure 5.1: Sequential deletion of images to isolate membrane and podia compartments

A) Previously collected confocal images were opened using LSM 510 software. The red channel (N-cadherin) was removed, leaving only F-actin (green channel). B) The closed freeform circle was used to trace the interior limits of the cortical cytoskeleton and the cut function was used to remove all interior staining. C) To create the podia only image, the closed freeform circle was enlarged to include the cortical cytoskeleton but exclude the podia. The cut function removed the cortical region and the remaining image included only podia. These processes were repeated plane-by-plane for the entire center ¼ of the cell.

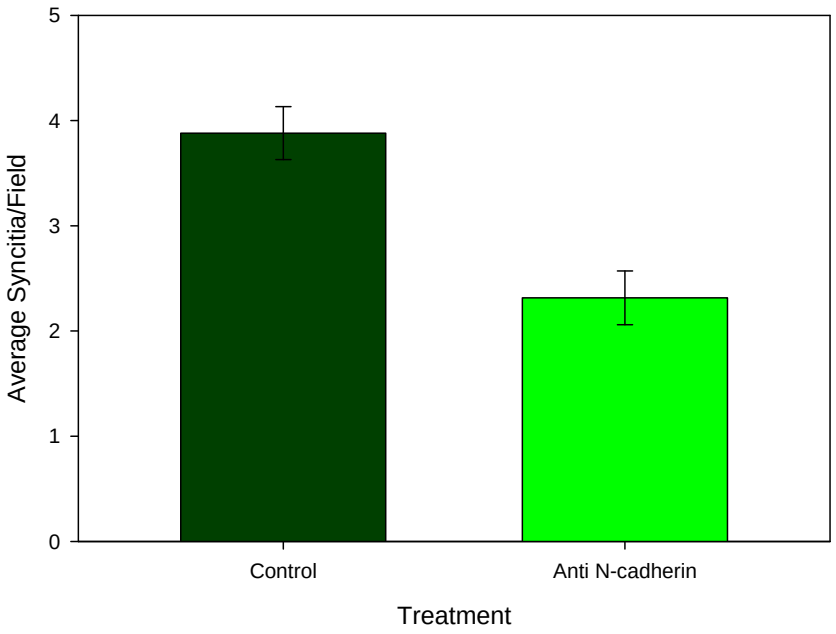


Figure 5.2: The effects of anti N-cadherin on syncitia formation during RCCS culture

Cells were culture in the RCCS for 4 days with or without 100 μg/ml anti-N-cadherin antibody (GC-4 clone). H&E stained sections were used to count syncitia per field of view using a 60X objective. Syncitia (more than three nuclei) were counted in eight non-overlapping fields along the periphery of the aggregate with a minimum of three sections examined per independent experiment. The number of syncitia were significantly reduced by anti-N-cadherin application (Mann-Whitney Rank Sum Test, $p<0.001$).

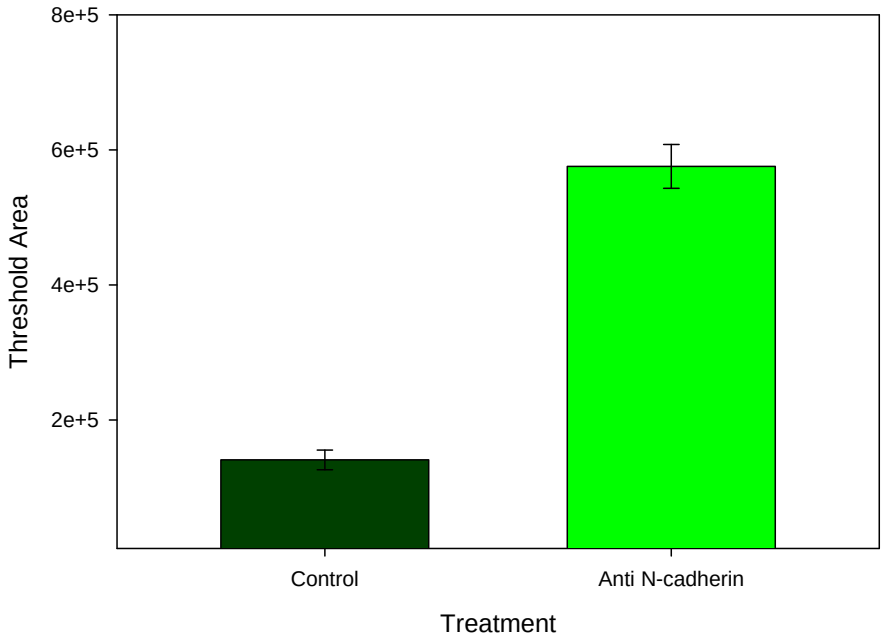


Figure 5.3: The effects of anti N-cadherin on MHC expression during RCCS culture

Cells were culture in the RCCS for 4 days with or without 100μg/ml anti-N-cadherin antibody (GC-4 clone). De-parafinized sections were stained with antibodies (MY32, Alexafluor 594 goat anti-mouse secondary antibody) to determine differentiation as determined by the presence of sarcomeric myosin heavy chain. Images of the sections were analyzed using Metamorph software. Threshold settings were set so that secondary only stained sections showed no area expressing MHC. Anti-N-cadherin significantly increased the areas of MHC expression (T test, $p<0.001$).

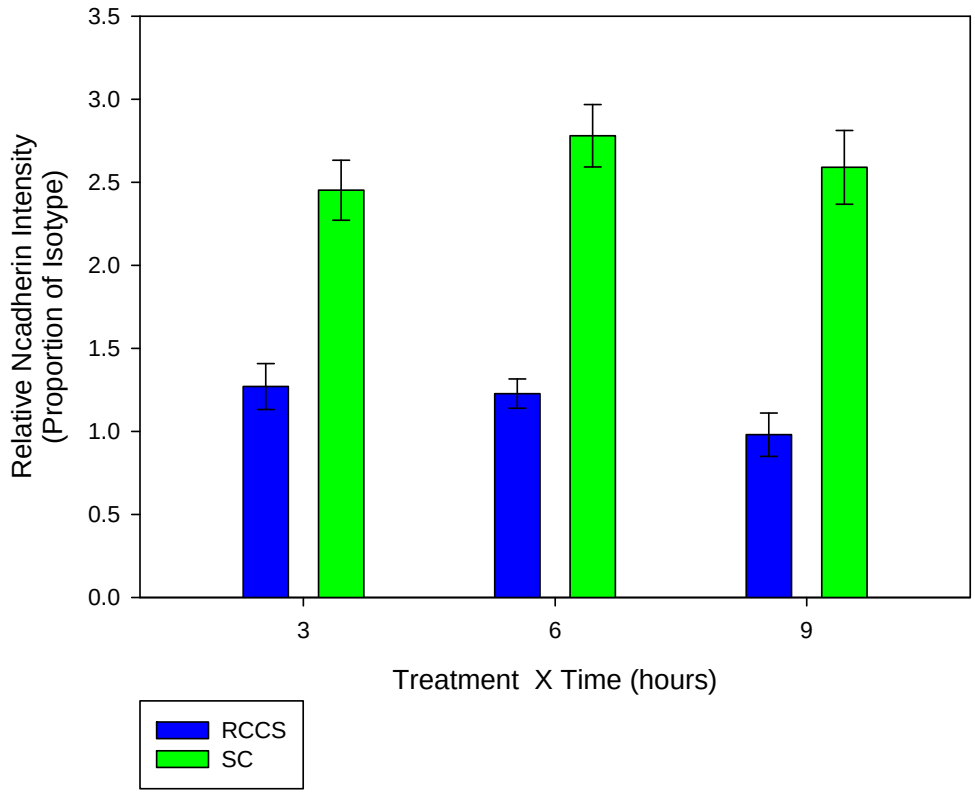


Figure 5.4: The effects of RCCS culture time on N-cadherin

Comparisons of the average N-cadherin intensity ratios of P:WC during the 3, 6, and 9 hour time points show that there is a general decline of N-cad at the podia over time in during RCCS culture. There is a statistically significant interaction between Treatment and the Time in culture (two way ANOVA on ranks, $p=0.016$). To determine the times and treatments where differences were significant, a Holm Sidak Multiple Pairwise comparison revealed that there is significantly more N-cadherin localized to podia in the SC than in the RCCS at 6 hours and 9 hours of culture ($p<0.001$, $p=0.002$ respectively).

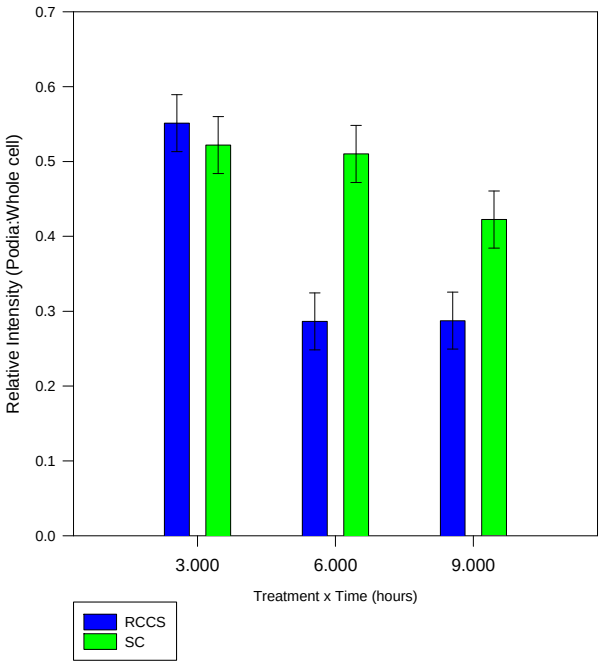


Figure 5.5: The effects of RCCS culture time on N-cadherin Podia/Whole Cell

Two way ANOVA on ranks reveal that the interaction between treatment and time is significant (p=0.006). Holm-Sidak pairwise multiple comparisons showed that there is significantly more N-cadherin localized to podia in the SC than in the RCCS at 6 hours (0.765 ± 0.0632 vs 0.638 ± 0.0731) and 9 hours (0.692 ± 0.0328 vs 0.607 ± 0.0273) of culture (p=0.004, p=0.026, respectively). The decreased localization of N-cadherin to podia over time in SC cells is not significant. However, the reduction of N-cadherin localized to the podia is significantly reduced at 6 and 9 hours when compared to the 3 hour time interval (p=0.004, p=0.011).

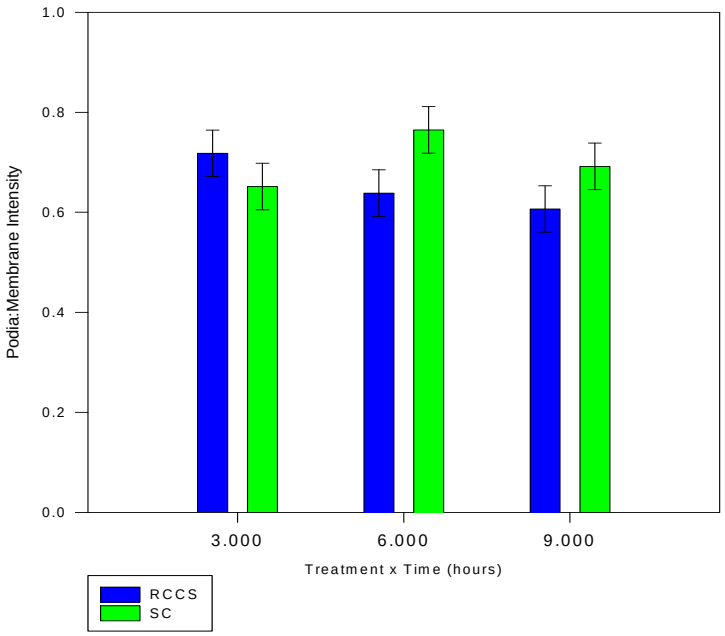


Figure 5.6: The effects of RCCS culture time on N-cadherin Podia/Membrane

Comparison of podia/membrane localization over time showed that there is significantly more N-cadherin localized to podia in the SC than in the RCCS at 6 hours and 9 hours of culture ($p=0.004$, $p=0.026$, respectively). The decreased localization of N-cadherin to podia over time in SC cells is not significant. However, the reduction of N-cadherin localized to the podia is significantly reduced at 6 and 9 hours when compared to the 3 hour time interval ($p=0.004$, $p=0.011$).

Chapter 6: Summary

Currently, the mechanisms of skeletal muscle myogenesis and regeneration remain largely undefined. During skeletal muscle organogenesis or regeneration, two processes are involved in differentiation. Skeletal muscle precursor cells must: 1) change from either a quiescent or proliferating state to a differentiating program; and 2) fuse to form multinucleated muscle cells. Elucidation of the mechanisms of myogenesis and skeletal muscle regeneration *in vivo* is complicated by: 1) the concurrence of the processes of fusion and differentiation; 2) inherent difficulties of developmental research; and 3) the paucity of skeletal muscle precursors (satellite cells) in adult skeletal muscle. Utilizing an *in vitro* skeletal muscle model to examine myoblasts undergoing the transition from single cell progenitors to functional synctial muscle has provided a more effective means for defining these mechanisms.

In vitro monolayer cultures are routinely and successfully used to investigate a myriad of cellular mechanisms and overcome some difficulties encountered with *in vivo* studies. However, the two-dimensionality of monolayer cell culture can severely limit the cell-cell interactions required for skeletal muscle fusion and differentiation. Further, the artificial 2D substrate modulates signals that are initiated by cell-substrate attachments. These alterations in cell behavior are observed during comparisons of 2D versus 3D cell culture (O'Connor, 1999).

For myoblasts cultivated in 2D culture, confluence is considered to be a pre-requisite for syncitia formation (Freshney, 1994). In fact, avoiding confluency is the only means of maintaining proliferating myoblast cultures (ATCC). Upon achieving a high degree of confluence, myoblasts in cell culture differentiate and fuse (Baldwin and Kayalar, 1986). Confluence produces extensive cell-cell contact resulting in adhesion, a

necessary step for fusion. However, during 2D monolayer culture, cell-cell contact is limited to small areas at the cell periphery due to the flattened morphology of the cells. In contrast, substrate-free RCCS culture results in more spherical cells centrally localized in 3D high-density areas within the culture vessel (Qui et al., 1999; Manley and Lelkes, 2006). The resulting cytoskeletal alterations, coupled with the cell co-localization, could induce a change from the proliferating to a differentiating cell stage via mechanical or cytoskeletal signaling mechanisms (Farge, 2003; Gibson & Perrimon, 2005; Ingber, 2005).

In our RCCS generated cell aggregates, the exceptionally large cells observed at the periphery contained multiple nuclei. These syncitia were significantly more numerous in RCCS compared to SC culture. Since cells seeded into the RCCS culture vessel were almost exclusively single mononucleated cells, RCCS culture conditions likely induced cell fusion. Interestingly, we saw syncitia only on or near the periphery of the aggregate. Since down-regulation of cell surface proteins, such as N-cadherin, occurs in primary myotubes during secondary myogenesis (Durband, 1987; Hatta, 1987) and following myotube formation in cell culture (Mege et al., 1992), the peripheral location of syncitia is probably a result of cell sorting.

In addition to syncitia formation, maturation of myoblasts into skeletal muscle requires the expression of muscle specific proteins (reviewed by Charge and Rudnick, 2004). For this investigation, the expression of skeletal muscle specific MHC was used to determine whether differentiation was occurring. MHC mRNA synthesis is thought to occur shortly after fusion (Gunning et al., 1987) and is commonly used as an early marker of muscle differentiation. We found that MHC expression was significantly greater in the RCCS generated aggregates when compared to controls. Thus, RCCS cell

culture induces a switch from the proliferating to a differentiating stage in spite of the presence of mitogens in the culture medium.

Since one of the advantages of RCCS culture is that it facilitates co-localization and cell-cell interactions in the culture vessel, increased cell-cell contact would be expected to activate signal pathways by this contact. Increased cell-cell adhesion/aggregation during RCCS culture might explain the earlier appearance of syncitia and greater degree of myosin expression observed compared to SC culture. However, the results of our aggregation experiments in Chapter 3 have shown that the single cell to aggregate ratio was higher for RCCS versus SC culture. An analogous study by Manley and Lelkes (2006) reported a similar degree of aggregation in the RCCS. Our decreased cell aggregation in the RCCS might be explained by the mechanics of RCCS culture. While the RCCS favors cell co-localization that would encourage cell-cell adhesion, a number of forces, including shear, are present in the RCCS culture vessel. Although these forces are minimal when compared to those present in conventional spinner flasks, they might be sufficient to delay stable cell-cell adhesion during early culture time intervals. Adhesion might only be able to occur when: 1) adhesion proteins are recruited and/or 2) matrix secretion increases to sufficient levels at the site of contact to counter the effects of these forces thereby facilitating adhesion. Results of aggregation, fusion, and differentiation are further discussed in Chapter 3.

Interestingly, we noted a large number of cytoplasmic projections on single cells grown in both RCCS and suspension controls. These projections seemed particularly abundant in the RCCS culture. Since a number of adhesive molecules have been localized to similar structures in monolayer culture (Rabinovitz, 1997; Sandig, 1997; Choma et al., 2004), these projections could be involved in the cell-cell adhesion process. Although

these structures have been reported in numerous 3D studies, few detailed investigations of these structures have been attempted.

Surprisingly, the unique morphology of single cells during 3D culture has rarely been examined, particularly in the absence of some form of exogenous matrix. One possible explanation for the lack of this type of study is that cells undergo anoikis, a specific type of apoptosis, when cultured without adhesion (Reddig and Juliano, 2005). However, this fate could be more characteristic of some cell types (i.e., epithelial cells, Frisch and Francis, 1994) than others (such as cells of mesenchymal origin). Our investigation found expression of the activated Caspase 3 and cell surface expression of phosphatidylserine, indicators of anoikis/apoptosis, in few C2C12 myoblasts during the first 9 hours of RCCS culture. This sparcity revealed that C2C12 cells were not undergoing apoptosis during the time intervals tested. This could be due, in part, to the mesenchymal origin of C2C12 cells, which are more likely to become quiescent than apoptotic when cultured without substrate adhesion (Milasincic, 1996; Sachidanandan et al., 2002; Dhawan and Helfman, 2004). Coupled with our viability data, it is unlikely that RCCS culture results in an increase in cell death as a result of anoikis/apoptosis in C2C12 cells within the first 9 hours of culture.

The podia we observed on single cells during RCCS culture were numerous and lengthy. Podia like structures have been reported on the free surface of different types of cells after they detach from a substrate (Collard and Temmink, 1976), establish contact with other cells (O'Connor et al., 2000; Enmon, 2002) or when grown in suspension (Ukena and Karnovksy, 1976). Since the RCCS cells had a significantly greater number of podia than suspension controls, some mechanism in RCCS culture is likely inducing the podia. Further, these podia were F-actin dependent since application of Lat A, an actin depolymerizing agent, resulted in loss of podia that were recovered with Lat A

washout. Interestingly, the F-actin content of the LatA +/+ positive control cells (as detected by flow cytometry) remained high during washout experiments. The intensely fluorescing F-actin clusters localized under the membrane of Lat A treated cells likely account for the high F-actin content in the cells. As discussed in Chapter 4, the clusters were resistant to increased concentrations of Lat A,cytochalasin D, and a combination of both reagents. These clusters might form in response to increased expression of one or more of the actin-associated proteins that provide protection for polymerized F-actin (such as Cap Z).

Although our investigation demonstrated that RCCS culture results in an increased number of F-actin dependent podia and an increase in podia length compared to suspension controls, it does not explain the mechanism of podia formation, the function of these structures, or the decrease in quantity and increase in length seen during longer RCCS culture times. Although the exact sequence of events remains undefined, collectively adhesion-mediated pathways consist of the following basic sequence of events including: 1) engagement (binding) of the extracellular region of the cell adhesion protein (such as cadherins or integrins); 2) coupling of the adhesion molecules to the actin cytoskeleton; 3) recruitment of additional adhesion proteins to the site of attachment for strengthening of the adhesion complex; and 4) stimulation of additional F-actin polymerization via Rho family GTPases. The increase in podia number and length described in Chapter 4 could indicate that a similar engagement of adhesion proteins might be facilitated by conditions in the RCCS.

The reduced cell-cell adhesion and aggregation results reported in Chapter 3 might be a consequence of small forces in the RCCS environment, not present in liquid overlay. Forces, such as those generated during cell collision within the RCCS, could be sufficient to prevent stable cell-cell adhesion between the adhesion molecules of co-

localized cells. It is also possible that this temporary contact is sufficient to recruit additional adhesion molecules and stimulate F-actin polymerization. These temporary adhesion events would continue until the level of adhesion proteins could counter the destabilizing forces and facilitate stable cell-cell adhesion.

The role of N-cadherin in cell-cell adhesion, fusion, and differentiation has been studied extensively (reviewed by Leckband and Prakasam, 2006). However, investigations of N-cadherin during 3D culture are rare. The RCCS facilitates N-cadherin contact by effectively concentrating cells in the center, a small portion of the overall vessel volume (Infanger et al., 2004; Manley and Lelkes, 2006). This area of high density increases the opportunity for N-cadherin mediated cell-cell contact and adhesion, critical events in myoblast fusion and differentiation.

We showed for the first time that inhibition of N-cadherin mediated adhesion resulted in decreased cell fusion and increased expression of skeletal muscle specific MHC during RCCS 3D culture. The dissociation of fusion and differentiated was mediated by the use of the soluble anti N-cadherin antibody (GC-4 clone). The GC-4 antibody used in this study binds to N-cadherin molecules at the surface and inhibits cis and trans binding of other N-cadherin molecules, thus preventing homophillic N-cadherin interactions. Binding of this antibody between the first and second cadherin repeat domains may also serve to activate the differentiation program. Thus, fusion can be inhibited while intensifying differentiation by GC4 clone binding. The similarities between our 3D studies and those previously performed provide further evidence of the validity of our skeletal muscle model and are discussed in Chapter 5 (Mege et al., 1992; Goichberg and Geiger, 1998; Gavard, 2004).

We hypothesized that RCCS induced co-localization would result in ephemeral N-cadherin mediated cell-cell contacts, which would be sufficient to induce recruitment

of additional N-cadherin to the site of contact. According to a model proposed by Mary et al. (2002), N-cadherin would also stimulate F-actin polymerization via a Rac1 pathway. This should result in the increasing podia length described in Chapter 4 and increased localization of N-cadherin to the podia apices as discussed in Chapter 5. Our preliminary studies revealed that there was increased localization of cadherins to the podia over the 9-hour test period using Pan-cadherin (unpublished data), an antibody that recognizes an epitope at the N-cadherin at the podia decreased with time during RCCS culture but not in SC culture. M-cadherin, another classical cadherin protein that plays a role in differentiation and fusion (Zeschnigk et al., 1995), showed a similar response. The Pan-cadherin antibody used in this preliminary study recognizes a number of cadherins including E, N, P, R, K, C, and OB-cadherins (SIB BLAST Network Service, 2006). Since our preliminary studies using Pan-cadherin showed increase localization to podia, it is possible that one or more of the cadherins recognized by this antibody is localized to the podia and plays a role in the podia mediated cell binding process.

FUTURE STUDIES

The results of the research described in this dissertation suggest a number of interesting possibilities for future investigation. Although others had demonstrated the importance of N-cadherin during myogenesis and muscle repair as well as an increased localization of N-cadherin following cell-cell contact (reviewed by Juliano, 2002, Charge and Rudnick, 2004)), the degree of contact required to initiate recruitment of N-cadherin and stimulate podia formation remains undefined. To simulate effects of collision and separation that likely occur during RCCS culture, repeated tapping of bead bound N-cadherin on single cells by Atomic Force Microscopy (AFM) could be used to determine

whether recurring ephemeral N-cadherin contact might stimulate podia formation. These results would provide evidence that N-cadherin mediated temporary adhesion could result in the podia formation occurring during RCCS culture.

The preliminary results indicating that Pan-cadherin levels increased in the podia are intriguing. When considered together with the observed decreases of N-cadherin (and possibly M-cadherin) at the podia, this suggests that another cadherin localizes to the podia during 3D RCCS cell culture. Several cadherins identified by the Pan-Cadherin antibody might be excluded based on the likelihood of expression of these cadherins by C2C12 or other skeletal muscle myoblasts. The novel methodology described in this project might then be utilized to investigate the remaining cadherins. This knowledge could be of particular interest to the field of cancer research since a cadherin switch has been implicated in invasion and metastasis (Kim et al., 2004).

Another potential area for future investigation centers on the mechanisms driving the formation and lengthening of podia during 3D RCCS culture. Localization and quantification of Rac1 would reveal whether podia in 3D RCCS culture respond in a similar manner as 2D lamellipodia. Further, since Rac1 inhibits differentiation, this small GTPase might be down regulated at some point during RCCS culture. In addition, RhoA is implicated in differentiation and is likely upregulated during or just prior to the initiation of differentiation. This sequence of events could also be examined utilizing the experimental approaches and techniques described in this dissertation.

CONCLUSIONS

In summary, we present a novel 3D skeletal muscle model that exhibits differentiation and fusion to form multinucleated cells suitable for studying the

following: 1) initial cell binding and factors involved in this process; 2) fusion of myoblasts to form multinucleated syncitia; and 3) differentiation in the absence of externally supplied substrates or serum alterations. This model is also useful for the examination of the effects of early (pre-aggregation) 3D culture on individual cells. In addition, *in vitro* myoblasts were found for the first time to fuse and form syncitia as well as to express differentiation markers in the presence of growth medium and without the use of exogenous substrates or supports.

Using this 3D skeletal muscle model and a novel approach for analyzing cytoplasmic projections (podia), we have demonstrated for the first time that the number of podia per cell increased during RCCS culture when compared to liquid overlay suspension culture. We also determined that these podia are F-actin dependent and decrease with time but may increase in length with time in culture. In addition, RCCS culture appeared to offer some protection from necrosis and apoptosis compared to liquid overlay suspension culture. Finally, RCCS culture was found to facilitate further examination of the role of cell-cell contact in skeletal muscle fusion and differentiation.

Additional studies demonstrated for the first time that an antibody to N-cadherin inhibits cell fusion but increases the expression of MHC during RCCS 3D culture. Utilizing a novel technique of sequential deletion, N-cadherin levels were shown to significantly decrease during the initial nine hours of RCCS culture. These changes are not seen in the suspension control cells. Preliminary studies indicate that other cadherins increase localization to the podia over time during RCCS culture and may play an important role in myoblast adhesion. These are currently under investigation.

The results of this dissertation project contribute to our knowledge in several areas. 1) A novel myoblast model test system has been characterized that can be used to define muscle development/regeneration processes including cell-cell adhesion,

differentiation, and fusion; 2) An enhanced understanding was obtained for the important fundamental role of Neural Cadherin during the muscle development/regeneration processes; and 3) Cytoplasmic projections (podia) observed during 3D culture were identified and characterized. This project also yielded an innovative methodology to assess alterations in single cells cultivated in a 3D environment. The novel model test system presented and characterized here has a number of applications including: 1) increasing our understanding of the mechanisms of differentiation and fusion; 2) identifying targets for cell or molecular-based treatments for increasing muscle regeneration; and 3) facilitating the generation of tissue-equivalent skeletal muscle tissue constructs suitable for regenerative medicine applications.

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VITA

Michele Lynn Marquette was born on May 24, 1957 to Zoe Oneta Griffin Marquette and Gerald Charles Marquette. In 1975, she began undergraduate work at the University of Louisiana - Lafayette in Lafayette, Louisiana, where she attained a Bachelor of General Studies in Natural Sciences the fall of 1985. From 1980 through 1984, Michele was employed as an Emergency Medical Technician – Basic, - Intermediate, and –Paramedic with Acadian Ambulance service in Lafayette, Louisiana and with Baton Rouge Emergency Medical Services in Baton Rouge, Louisiana. Michele also worked as a Technical Supervisor in an Environmental Laboratory. In the summer of 1994 she began work on Alternate Teachers Certification at the University of Texas at Brownsville in Brownsville, Texas. From the fall of 1994 to the spring of 2002, Michele was employed as a high school educator at Harlingen High School in Harlingen, Texas. She matriculated into the Educational Administration Program at the University of Texas Brownsville and received a Masters of Education in December 1997. In the fall of 2002, Michele matriculated into the Cell Biology Program within the Graduate School of Biomedical Sciences at the University of Texas Medical Branch at Galveston, Texas and began her doctoral research under the guidance of Dr. Marguerite Sognier. Michele was awarded NASA Graduate Student Research Program fellowships at NASA – Johnson Space Center, Who's Who in American Universities and Colleges, South Texas Academics Rising Scholars scholarship, and the UTMB Retirees' Award Scholarship. She has also recently received a NASA Johnson Space Center Postdoctoral Fellowship.

Michele has significant teaching experience. As a Nationally Registered EMT – Paramedic, she instructed numerous courses including Cardiopulmonary Resuscitation

(CPR), basic first aid, Advanced Cardiac Life Support (ACLS), EMT – Basic, -Intermediate, and –Paramedic. She served as a Teaching Assistant laboratory instructor for the basic biology classes at UL – Lafayette, and later instructed Pre AP and AP Biology classes at the high school level. She has participated in UTMB Educational Outreach activities including co-instructing several sessions of Educational Outreach Saturday Biomedical Academy and Expanding Your Horizons in Math and Science Career Conferences for 6-8th grade girls.

Michele has accepted a postdoctoral research position in the laboratory of Dr. Diane Byerly at NASA Johnson Space Center in Houston, Texas.

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