# LONG TERM FUNCTIONAL AND MORPHOLOGICAL CHARACTERIZATION OF MUSCLE CELL CULTURES

by

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B.S., Mechanical Engineering
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## ABSTRACT

Skeletal muscle is the mammalian body's largest protein resevoir, serving the critical role of mobilizing proteins during acute conditions, such as trauma and starvation, and chronic conditions, such as AIDS, cancer, and muscular dystrophy. When proteins are mobilized in excess of physiological needs, muscle wasting ensues. With advances in the treatment of the primary effects of these conditions, muscle wasting, which formerly may have been of secondary concern, has become an impedement to full recovery from these conditions. With recognition of the growing importance of muscle wasting to clinical rehabilitation, the need to elucidate the mechanistic underpinnings of muscle wasting has become more acute.

Previously, whole body and organ culture have been the choice models for investigation of protein turnover in skeletal muscle. These models have been useful in showing that myofibrillar proteins are under physiological regulation by hormones and cytokines. However, these models are valid for only several hours, contain many inherent couplings and cell types, and it is difficult to separate protein synthesis from degradation, all of which make elucidation of complex proteolytic pathways difficult. Cell culture can be a very powerful system for investigation of cellular metabolic pathways, offering more precise control of the cell culture micro-environment and the ability to measure synthesis and degradation independently. However, caution must be taken to insure that the muscle specific components and degradative pathways which are regulated in adult muscle are present in the cell culture system. This study was undertaken to address these concerns and emphasize the need for thorough biochemical analysis before muscle specific protein turnover may be investigated in cell culture.

The L6 cell line was used as a model cell culture system in this study. Using creatine phosphokinase (CPK) and DNA, media conditions were optimized to produce maximal differentiation in the short-term. Next, using CPK, tropomyosin, and α-actinin, long-term studies were undertaken to evaluate the suitability of the L6 cell culture system for protein turnover experiments. Finally, static mechanical tension was applied to the cultured L6 cells as a potential method of augmenting of muscle specific function. Long-term biochemical analysis of CPK showed that differentiated muscle cells were quasi-stable in the optimized media for a period of 7 to 14 days depending on the stringency of the requirements. However, immunoflourescence staining during this period revealed that muscle specific tropomyosin degraded heterogeneously across the cultures before organizing into myofibrils. Work with short-term passive mechanical stretch showed that CPK levels increased two-fold over controls, suggesting that, after appropriate optimization, passive tension may be a viable method for maintaining muscle specific function during protein turnover studies.

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Title: Assistant Professor of Surgery

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# CHAPTER 1

## INTRODUCTION

### 1.1 Background and Motivation

Skeletal muscle is the mammalian body's largest protein reservoir and serves the critical role of mobilizing proteins in response to numerous stimuli produced during day to day nutritional variations and situations of trauma (Cuthbertson, 1930), sepsis (Baracos et al. 1983), and muscle wasting diseases (Goldberg et al. 1975). When protein mobilization occurs in excess of physiological needs, muscle wasting ensues. With advances in the treatment of the primary effects of these conditions, muscle wasting, which formerly may have been of secondary concern, has become an impediment to full recovery from these conditions. With recognition of the growing importance of muscle wasting to full clinical recovery, the need to elucidate the mechanistic underpinnings of muscle wasting has become more acute.

#### 1.1.1 Muscle Physiology

Figure 1.1 portrays the structural hierarchy of adult skeletal muscle, showing the structural hierarchy of adult skeletal muscle. Muscle fibers are the individual muscle cells, which are organized into microscopic fasicular bundles. The fascicles are interconnected by connective tissue and

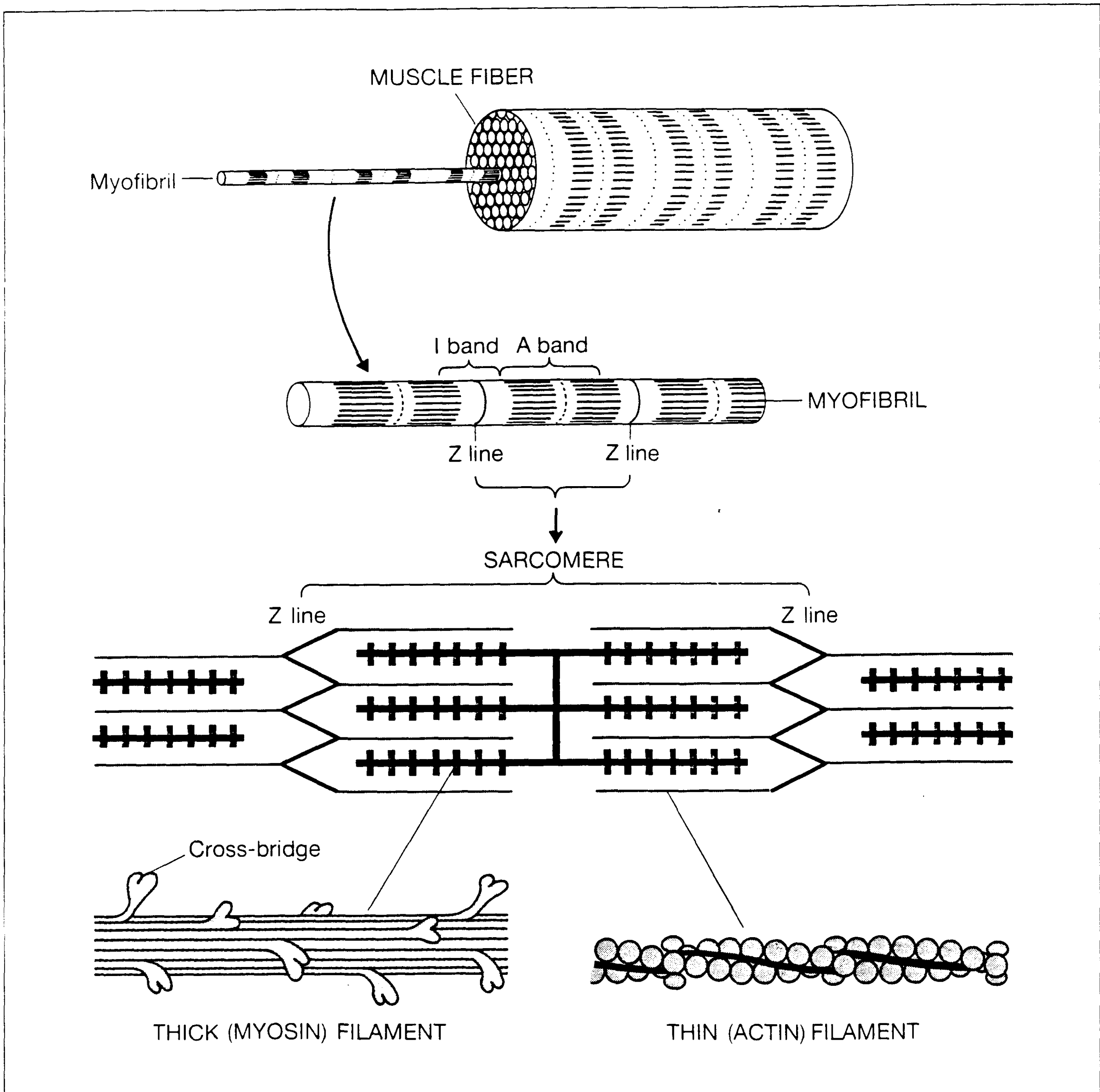


Fig. 1.1: Schematic showing the structural hierarchy of muscle.

organized into macroscopic muscle organs. The muscle fibers, also called myofibers, contain numerous myofibrils. The myofibrils are long filaments composed of sarcomeres, which are the individual contractile units of skeletal muscle. Sarcomeres contain A, I, and Z bands, each of which is characterized by its own protein (the A band is composed of myosin, the I band composed of actin, and the Z band $\alpha$-actinin). The protein isoforms which make up a sarcomere are specific for muscle tissue although many isoforms of the same proteins are found in other tissues.

The myofibril also serves as the protein store of the muscle. Its degradation is regulated by hormones (Fulks et al. 1975; Gulve et al. 1991; Vandenburgh et al. 1991b), various cytokines (Baracos et al. 1983; Flores et al. 1989), and mechanical stimulation. (Baracos and Goldberg, 1986). Specific myofibrillar proteolytic systems are associated with the myofibril and are regulated by these physiological agents. There has been extensive investigation of one component of this system, appropriately named the myofibrillar protease, which was shown to be responsive to glucocorticoids both *in vivo* and *in vitro* (Mayer et al. 1983). This protease has not been isolated to date and studies which use it rely on biochemical extracts from muscle. Further work needs to be completed before this protease extract, which may be a set of proteases and/or pathways, is fully characterized. However, it seems to be an important component of physiologic regulation of protein turnover and is one which should be present in a model culture system.

### 1.1.2 Research Models

To date, three models have been used to investigate muscle wasting: 1) whole body models, 2) organ culture, and 3) cell culture. Metabolic investigations involving whole body models are extremely difficult because of animal variability, uncontrolled influence of other organ systems and the inability to separate endogenous from exogenous metabolic insults. Whole body models are generally most useful for identifying the cytokines or hormones which may be responsible for the metabolic alterations in muscle (Flores et al. 1989), but not for elucidation of specific molecular pathways of protein degradation. Organ culture involves the removal of skeletal muscle from an animal and placing it in a culture chamber. This model has been used to investigate the molecular mechanisms of muscle protein turnover. However, a major difficulty with this model is that it is only stable for several hours (Baracos and Goldberg, 1986). Furthermore, proteolytic pathways are activated as an artifact of isolation; therefore, experiments with organ culture which investigate proteolysis in response to exogenous agents are difficult to interpret. Biochemical analysis is also confounded by protein products of the many cell types present in cultured organs. Lastly, protein synthesis and degradation are difficult to distinguish in this model.

Cell culture systems offer many advantages over the other two models. The cell type, cell density, media milieu, and extracellular matrix environment may be more precisely defined and varied to produce optimal and consistent performance of the system. Independent measurement of protein synthesis and degradation is also possible in cell culture. In addition, it is also possible to characterize the inherent limitations of cell culture models in contrast to whole body models and cultured muscle organs. One drawback of cell culture involves the fact that the myofibrils which form are generally embryonic and are not contained within their physiological environment,

which may mean that they are not be regulated as adult myofibrils are. In addition, it has been noted that muscle specific protein is almost undetectable in cell culture (Gulve et al. 1991). However, an advantage of cell culture systems, which has come from the advances in tissue engineering is that these limitations have been well-characterized and may be improved by applying methods developed for other purposes, which indirectly showed that there is potential for improving cell culture models. For example, neuromuscular researchers have found in co-culture that nerve synapses produce transition of skeletal muscle isoforms from embryonic to adult (Emerson and Beckner, 1975). Similarly, passive mechanical stretch was shown to augment many muscle specific characteristics in primary cells (Vandenburgh et al. 1991a; Kanda and Matsuda, 1994), including the induction of muscle specific proteins. Although originally not intended for such uses, stretch and co-culture systems may be used in order to optimize muscle cell cultures before protein turnover experiments are begun.

### 1.1.3 Muscle Cell Culture

Skeletal muscle cell cultures begin with a population of spindle-shaped embryonic **myoblasts**, which are the mono-nucleated, proliferative precursors of adult muscle cells. When myoblasts reach a critical density, they cease synthesizing DNA and fuse into **myotubes,** which are the multinucleated precursors of adult muscle fibers. After fusion, myotubes begin to synthesize muscle specific proteins in preparation to form adult muscle fibers. As already mentioned, adult **muscle fibers** are multinucleated and contain striated myofibrils into which the muscle specific proteins are incorporated.

Muscle cell culture systems may be characterized morphologically three ways: 1) fusion of myoblasts into myotubes with centrally located nuclei; 2) margination of nuclei to the peripheral regions of the myotubes, which is accompanied by elongation of the myotubes to hundreds of microns; 3) formation of the contractile apparatus, which is often accompanied by spontaneous beating (Konigsberg, 1965). These morphological cues have been used by previous investigators to characterize muscle cell culture systems. Biochemically, characterization of muscle cell systems is three-fold as well: 1) systems involved in ATP production; 2) systems concerned with $Ca^{2+}$ regulation; and 3) systems involved with the contraction process. Examples of muscle specific proteins associated with these systems include sarcomeric myosin, α-actin, sarcomeric tropomyosin, and creatine phosphokinase (CPK). These proteins have been used by previous investigators to characterize muscle cell cultures and show that the multinucleated cells which contain morphological similarities to muscle *in vivo* contain physiological characteristics of adult muscle biochemical systems (Yaffe D, 1970). These biochemical and morphological parameters are very critical in evaluating and optimizing muscle cell culture systems because they are the systems which are physiologically regulated during protein turnover in muscle. (Ewton et al. 1988).

Muscle cell culture can be divided into primary cultures and cell lines. Table 1.1 shows the various muscle cell culture systems used previously. With the exception of the BC3H1 cell line, these systems all form multinucleated myotubes, an irreversible process. The BC3H1 cell line is unique in that it does not fuse, but differentiates biochemically, synthesizing a number of muscle specific proteins (Schubert et al. 1974). An intriguing aspect of this biochemical differentiation is that it is reversible. The L6 and L8 cells lines were isolated by serial passage rat

primary cultures. The L8 cell line is a clone which arose spontaneously while the L6 is the transformed product of methylcholanthrene treatment of primary muscle cultures. The C2C12 is a subclone of the C2 cell line, which was isolated previously from chick embryos by serial passage (Yaffe, 1968).

Table 1.1 Summary of major muscle cell culture systems

| CELL TYPE | GENERAL INFORMATION | REFERENCE |
|---|---|---|
| **PRIMARY:** | | |
| Chick | Islolated from chick embryos | Konigsberg (1960) |
| Rat | Isolated from rat neonates | Yaffe (1969) |
| Quail | Isolated from quail embryos; less common model | Konigsberg (1971) |
| Mouse | Isolated from neonatal mice | Bowden-Essien (1972) |
| Human | Isolated from fetal tissue | Haushka (1974) |
| **CELL LINES:** | | |
| L6 | Rat primary cells transformed via methyl colanthrene | Yaffe (1969) |
| L8 | Selected by serial passaging primary rat cells without carcinogen | Yaffe (1969) |
| C2C12 | Subclone of original mouse cell line isolated from mouse embryos | Yaffe (1969) |
| BC3H1 | Isolated from mouse brain neoplasm | Schubert et. Al. (1974) |

### 1.1.4 Prior Characterization of L6 Cells

The L6 cell line was chosen for this study because previous cloning experiments showed that these myoblasts formed muscle colonies with 100% efficiency (Richler and Yaffe, 1970). Original characterization in 1968 also revealed that the L6 cells stopped synthesizing DNA and readily formed the sarcomeric bands characteristic of adult muscle (Yaffe, 1968). It is noteworthy that this original study is the only one which documented sarcomere formation. Further characterization (Shainberg et al. 1971) showed that, in parallel with fusion, CPK, myokinase, and glycogen phosphorylase, all associated with the ATP system in the muscle cell, increased. In comparison to control primary cultures, the synchrony of fusion and the increase in enzyme activity in L6 cultures, however, took place over a prolonged period of time. Early characterization did not correlate enzyme activity with other biochemical correlates of differentiation, such as α-actin or muscle specific myosin.

Nadal-Ginard (1978) was the first investigator to thoroughly characterize the L6 cell line. He used a subclone of the L6 line, L6E9, which he chose because of its ability to differentiate quickly. Furthermore, he showed that CPK and myosin heavy chain were coordinately synthesized upon myoblast fusion. A technique to quantitate morphology was also used (morphometrics), which showed that 90% of the nuclei in the cultures were in myotubes. The initial characterization was performed towards investigation of the effects of the DNA synthesis inhibitors, β-D-arabinofuranosylcytosine (AraC) and 5-Fluoro-2'-Deoxyuridine (FUdR). At high

seeding densities, it was found that these inhibitors decreased the degree of fusion and increased the observed degree of fusion to levels approaching 100%.

Further studies with L6 cells concentrated on the ability of hormones and media to modulate differentiation. The major limitations of these studies in terms of a muscle wasting model is that very few of the studies extended beyond 2 weeks. Most of the emphasis was on morphology as opposed to a dynamic biochemical analysis of muscle specific proteins, which as discussed, is the most important aspect of muscle cell culture as it pertains to protein turnover.

Mandel and Peterson (1974) were the first to report an effect of an exogenous hormone on L6 cells, showing that insulin increased the total amount of CPK, the number of nuclei per myotube, and the percentage myotube area per field, but also increased the rate of senescence. The observed effects may have been caused by an increase in general cell metabolism (i.e. more myoblast proliferation) or a specific effect on an identical number of myoblasts, causing them to fuse more rapidly and promiscuously.

Florini and Roberts (1979) characterized a serum-free media for the growth of L6 cells. While their characterization of myoblast growth was extensive, they did not analyze differentiation and fusion quantitatively, but only qualitatively in their discussion, where they indicated that their serum-free medium did not show any significance difference from controls in terms of fusion capability. Further work by Florini and Ewton (1987) more thoroughly characterized the effects of insulin and the samatomedins (now the IGF family) on the differentiation of myoblasts. They separated the mitogenic and differentiation effects of these hormones, showing that insulin and the samatomedins induce myoblast fusion which did not result from myoblast proliferation alone, but an increase in fusion of the myoblasts already present.

### 1.1.5 General Characterization of Muscle Cell Culture Systems

As outlined in Table 1.1, several cell culture systems have been used previously and some characterization was specifically directed toward extending culture life. Results with these models provide insight into methods of further stabilizing L6 cultures and also provide insight into the characterization which must be done with L6 cultures before they can be used to investigate protein turnover.

Morphological characterization has shown that rat primary cultures form sarcomeric patterns in culture, which are characteristic of the corresponding adult muscle tissue. CPK levels were also shown to increase for 50 days in culture and muscle specific myosin increased coordinately with CPK (Yaffe D, 1968). Morphological and biochemical characterization has also been repeated by different groups (Coleman and Coleman, 1972).

Manipulation of the extracellular matrix has been a widely used technique to control and stabilize different types of cell cultures, namely liver (Dunn et al. 1991) and smooth muscle cells (Kanda and Matsuda, 1994). Similarly, extracellular matrix molecules were found to increase the stability of skeletal muscle cell cultures. Early work from Konigsberg et. al. (1966) showed that surfaces coated with gelatin or collagen was preferable for primary rat myoblast differentiation. Further research by Vandenburgh et al. (1991a) found that avian skeletal muscle cells could be maintained for two weeks whereas control cultures deteriorated after 5 days. This report also showed that myosin heavy chain synthesis (MHC) increased three-fold by the final day of cultures indicating that myotube protein specifically increased but the fibroblasts did not proliferate.

Matrigel is a basement membrane preparation which has been commonly employed to enhance cell attachment and function. Muscle fibers are surrounded *in vivo* by a basement membrane and the use of Matrigel may be hypothesized to be of physiological importance. Lyles et. al. {38} investigated the effects of Matrigel vs. type I collagen to support myotube proliferation in the absence of growth factors in serum. They found that adsorbed collagen was insufficient to maintain myotubes in the absence of serum but that Matrigel in serum-free media could support and maintain myotubes for a period of three weeks.

The effect of mechanical work on skeletal muscle hypertrophy *in vivo* are well-known (Goldberg et al. 1975). Vandenburgh and Kaufman (1979) found that passive mechanical stretch of primary avian skeletal muscle cells *in vitro* resulted in the same types of changes seen *in vivo*; that is, increased transport of amino acids, increased incorporation of amino acids into total protein and myofibrillar protein during stretching. Thus, prior studies deemed passive stretch to be a valid model for investigating the mechanisms of myofibril hypertrophy. Passive stretch differs slightly from *in vivo* mechanical stretch in that the force is imposed on the myotube during passive stretch while *in vivo,* the myotube generates force biochemically while being simultaneously stretched passively. The mechanism of signal transduction in the passive model has not been elucidated to date and therefore, the physiological difference between effects of *in vitro* passive stress versus the combination of passive and active stress *in vivo* is not known.

Further work involving passive stretch of skeletal muscle cells revealed that prostaglandins are synthesized and released into the culture media (Vandenburgh et al. 1989), and glucose uptake and lactate efflux are increased (Hatfaludy et al. 1989) during passive stretch. Recently, Perrone et. Al. (1995) have shown that stretching is required to observe the effects of certain

growth factors, such as IGF-1. Upregulation of receptors, increased cell metabolism, or both may play a role in this phenomenon. From a mechanistic standpoint, this is important in that the cells must be responsive to the stimuli under investigation. Similarly, mechanical stimulation decreased the myotube atrophy seen in primary avian myoblasts exposed to the glucocorticoid dexamethasone (Vandenburgh and Chromiak, 1992). These observations point out the importance of maintaining a physiological environment when cell culture studies are undertaken.

Proteolytic pathways are another important aspect of muscle cell culture systems. The myofibrillar protease pathway found *in vivo* and discussed above has been demonstrated in primary rat myoblasts (Mayer et al. 1983) and shown to be regulated by the same agents as adult muscle. Only one study which compared specific degradative pathways found in cultured cells to those previously found *in vivo*. The conclusions from this study are critical for demonstrating the relevance of primary cultures to the *in vivo* model with respect to mature proteolytic pathways associated with sarcomere and myofibril formation.

Despite the potential advantages of muscle cell culture, very few reports have characterized protein turnover in cell culture. Gulve and Dice (1989) showed that horse serum (HS), insulin, and insulin-like growth factors (IGFs) increased protein synthesis and decreased protein degradation in L8 myotubes in a dose responsive manner. A similar study with the C2 cell line (Gulve et al. 1991) showed similar effects with HS and IGFs. The purpose of the latter study was to show that cell culture could simulate the *in vivo* effects of starvation, in which myosin, a sarcomeric protein, is the predominant protein mobilized from muscle. However, no difference was observed when myosin degradation rates in the absence of HS were compared to controls. One reason why no effect was observed was that the myosin assay was not sensitive enough to

observe differences. Alternatively, proteolytic pathways may have already been activated so that HS starvation could not properly simulate starvation *in* vivo. Or, perhaps the embryonic cell line, despite synthesizing the adult form of myosin, did not form sarcomere contractile apparatus and proteolytic pathway. Had the myosin experiments worked as planned, the C2 cell culture system could have been a powerful model to elucidate detailed mechanistic information regarding muscle wasting during starvation. This study with the C2 cell line underscores the need for thorough biochemical analysis and optimization of cell culture systems, but also emphasizes the potential these systems have as far as a model to elucidate biochemical mechanisms.

## 1.2 Scope of This Study

This study was undertaken in recognition that characterization of the differentiated phenotype, both morphological and biochemical, of skeletal muscle cells in culture is a prerequisite for the elucidation of the mechanisms behind muscle wasting. The L6 cell line was characterized as a model for this type of characterization as well as a potential model cell culture system in and of itself. Using CPK and DNA as parameters, short-term experiments were first undertaken to optimize the media for maximum, stable differentiation. Long-term experiments using sarcomeric tropomyosin, α-actinin, DNA, CPK, and total protein as biochemical markers were also undertaken. Finally, using CPK and DNA only, preliminary experiments involving the application of mechanical strain to L6 cell cultures were performed to investigate the potential of mechanical strain to stabilize L6 cell cultures.

# CHAPTER 2

## MATERIALS AND METHODS

### 2.1 Cell Culture

The L6 cell line was established by serial passage of myoblasts from rat primary skeletal muscle cells (Yaffe, 1968). L6 cells were obtained from American Tissue Culture Collection (June, 1994 passage; Rockville, Ma.). Unless otherwise noted, cells (approximately $2x10^6$/vial) were thawed immediately after purchase and divided among five Corning $75cm^2$ flasks. These cells were cultured using high glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MI) plus 10% (v/v) fetal bovine serum (FBS; Lot #3J2173, Sigma), 100 μg/ml streptomycin, and 100 μg/ml penicillin (JRH Biosciences, Lenexa, KA) in a humidified atmosphere with 10%$CO_2$/90% air and $NaHCO_3$ added to 44 mM. At approximately 80% confluence, the cells were removed with 0.125% trypsin/1mM EDTA in $Ca^{2+}$- and $Mg^{2+}$- free PBS. All experiments were performed with the cells from first passage and plated at 1000 cells/$cm^2$ in 10% FBS in six-well Corning dishes. The L6 myoblasts reached confluence by day five and after washing three times with PBS, were exposed to horse serum (HS; Lot.# 54H4656, Sigma) in accordance with the specifics of each experiment.

Primary Human Cells (lot # 2859; Clonetics Corp., San Diego, California) were thawed upon arrival and plated at 3500 cells/$cm^2$ in Corning flasks in Skeletal Muscle Basal Media (SkGM; Clonetics) in a humidified atmosphere with 5% $CO_2$/95% air. At approximately 80% confluence, cells were trypsinized with 0.125% trypsin/1mM EDTA in $Ca^{2+}$- and $Mg^{2+}$- free PBS. These cells (approximately $20x10^6$ at 80% confluence) were frozen in liquid nitrogen in SkGM + 20% FBS + 10% DMSO until use. For experiments, cells were thawed at 37°C and quickly plated at 3500 cells/$cm^2$ in SkGM in a humidified atmosphere with 5% $CO_2$/95% air. Confluency was reached approximately 5 days later and cultures were then switched to DMEM + 2% HS and placed in a humidified atmosphere with 10% $CO_2$/90% air. All other protocols with Clonetics cells were identical to those use for L6 cells.

## 2.2 Preparation of Aliquots for Biochemical Assays

Aliquots for biochemical assays were obtained by washing cell layers three times with 1ml PBS chilled to 4°C and scraping the cell layer into 250 μl PBS with a rubber policeman. The cell suspension was quickly frozen at -80°C until analysis. For assay preparation, the cell suspension was thawed for five minutes at 37°C and homogenized by sonicating for 5-10 seconds (Branson Sonicator Model 450, power level 1; Branson Corp., Danbury, CT ). For each experimental time point, aliquots for all three assays were taken from the same homogenate and each biochemical assay run in triplicate, with the average value used for comparison to respective standard curves. The amount contained in a given aliquot volume was scaled by the appropriate factor to obtain

the amount in the 250 μl of cell homogenate, or the amount per well, which is the value presented in the results.

## 2.3 DNA Assay

DNA was assayed flourimetrically using a method originally developed by MacDonald et. al. (1991) and modified for use with the L6 cell culture system and creatine phosphokinase (CPK) assays. Hoechst 33258 dye (Molecular Probes, Eugene, OR) was diluted to a 1.6 μM concentration in DNA buffer (TNE; 10mM Tris-HCL, 2M NaCl, pH 7.4, 1 mM EDTA). Twenty μl aliquots from the cell homogenate were mixed with 200 μl of Hoechst-TNE buffer and placed in 96 well plates. Plates were covered with aluminum foil and incubated for 30 minutes at ambient temperature on a plate shaker. Flourescence intensity was measured with a Cytofluor 2350 Flourimeter (Millipore Corp., Marlborough, MA), using an excitation filter 360 nm, an emission filter of 460nm, and a sensitivity level set to 5. The DNA content of each sample was determined using purified calf thymus DNA (Sigma) as a standard. Standards (6-150 μg) were prepared in PBS and one stock used for all experiments, which was stored in PBS at 4°C.

## 2.4 CPK Assay

CPK catalyzes the formation of creatine from creatine phosphate. The phosphate is transferred to ADP to form ATP and then the formation of ATP which can be monitored by linking the ATP formation to the reduction of Nicotinimide Adenine Dinucleotide (NAD). The

reduction of NAD is then measured by the rate of absorbance increase at 340 nm (Oliver, 1955). Clinical diagnostic kits, rabbit CPK standards, and controls for the kit were purchased from Sigma and adapted for the L6 cell culture system. Fifty µl of cell homogenate was spun down for 2 minutes in an eppendorf centrifuge at 14,000 rpm and 4°C. In a cold room, 10 µl of the supernatant was mixed with 200 µl of Sigma substrate in a 96 well plate. After 5 minutes, samples and standards were transferred to a plate reader (Molecular Devices, Millipore) and the rate of NAD conversion monitored at 340 nm and ambient temperature. The assay was performed in triplicate for each sample and the average compared to a rabbit CPK standard curve. The standard was prepared by mixing 1000 units of rabbit CPK with 1 L of 10 mM glycine buffer with 5 mg/ml BSA at pH 8.0 and freezing 1 ml aliquots at -80°C. One batch of aliquots was used for all experiments. Aliquots were thawed at 37°C for 5 minutes and then diluted with PBS to yield a standard curve ranging from 0.3 mU to 10 mU. The storage buffer did not affect the linearity of the assay. After thawing, standards and samples were kept on ice. To maintain consistency, standard and samples were thawed in parallel.

### 2.5 Total Protein Assay

A protein assay kit based on the Bradford (Bradford, 1976) method was purchased from Biorad (Thousand Oaks, CA). Aliquots of cell homogenate were solubilized by diluting 1:1 with 1 M NaOH and incubating for 30 minutes at 37°C. Samples were then maintained at 4°C for the duration of the assay, typically not more than 3 hours. Depending on the cell culture density, some samples had to be further diluted with 0.5 M NaOH/0.5X PBS solution in order to fall

within the linear range of the assay. Five μl samples were then mixed with 200 μl of Biorad reagent in a 96 well plate, incubated at ambient temperature for 15 minutes, and the absorbance at 570nm read on a plate reader (Molecular Devices). The amount of protein in samples was determined using Bovine Serum Albumin (BSA; Sigma) as a standard. Standards (50 - 300 μg/ml), were prepared by dissolving BSA in PBS. Standards were prepared once for all experiments and stored at 4°C.

### 2.6 Immunoflourescence

A protocol was developed to triple stain cell cultures in order to localize nuclei, F-actin and either sarcomeric tropomyosin or α-actinin in the same petri dish. All procedures were done at ambient temperature unless otherwise indicated. Cells growing on polystyrene were washed three times with PBS and fixed for 20 minutes with 4% paraformaldehyde in PBS (pH 7.4) solution. After washing with PBS for 10 min, cells were permeabilized for 5 min in 0.1% Triton X-100 and held in PBS until staining.

For immunolocalization of F-actin, cells were incubated for 60 minutes with rhodamine conjugated phalloidin (Molecular Probes) and diluted 1:100 (to 3.3μM) in PBS. Phalloidin is a fungal protein which binds to all polymerized forms of actin (F-actin). After incubation, cells were washed in PBS for 15 min. Following F-actin staining, nuclei were localized by staining DNA. Cells were incubated for 20 min with 1.6μM Hoechst dye in PBS. After washing in PBS for 10 min, immunolocalization of α-actinin and sarcomeric tropomyosin was begun by incubating cells for 1 h in blocking solution (3% BSA in PBS, pH 7.4). Cells were then stained for 1 h at

37°C with a mouse monoclonal antibody to rabbit skeletal muscle α-actinin (diluted 1:200 in PBS and spun down for 2 min @ 14,000 RPM in an Eppendorf Centrifuge; Sigma) or a mouse monoclonal antibody to chicken skeletal muscle tropomyosin (diluted 1:50 in PBS and spun down; Sigma). Following four washes in PBS (15 min each), cells were incubated at 37°C for 1 h with flourescein-conjugated anti-mouse IgG (Sigma; diluted 1:100 in PBS). Finally, dishes were covered and incubated overnight on a shaker.

For objective lenses smaller than 20X, flourescent micrographs were taken through the bottom of the polystyrene dishes. Objective lenses greater than 20X, however, required an additional protocol to visualize cells. PBS was aspirated from dishes and a drop of n-propyl-gallate (preparation described below; Sigma) placed in the center. A glass cover slip was then placed over the n-propyl-galete and fixed to the polystyrene dish with nail polish. The walls of the dish were then trimmed off with pliers with care taken not to crack the dish. Images were obtained by placing the dish on the microscope stage with the plastic side up and the cover slip down. With this technique, flourescence could be visualized with objectives up to 100X magnification.

n-Propyl Gallate solution preserves flourescence by scavenging oxygen free radicals in solution. It was prepared by mixing 4.5 ml $H_20$, 5 mls Tris, 0.5 ml NaCl, 35 ml glycerol, and .5 g n-Propyl Gallate in a beaker while stirring. After the solution was thoroughly mixed, glycerol was added to obtain a final volume of 50 ml. Stocks were stored at 4°C.

### 2.7 Morphometry

Morphometry was used to obtain a numerical average for number of myotube nuclei per field, percentage of each field covered by myotubes, and percentage of nuclei contained within myotubes per field. This method was only feasible with the 1% HS cultures because the 10% HS cultures were too dense to reliably distinguish myotube nuclei from myoblast nuclei. After the triple staining procedure described above, random digital pictures of 11 fields were taken with a 20X objective lens and analyzed using metamorph software (Universal Imaging, Westchester, PA). At this magnification, a field of view represented 1/3500 of a 9.6 $cm^2$ petri dish. Total nuclei per field was obtained from Hoechst staining. Myotube area was obtained by outlining myotubes stained with tropomyosin and calculating the percentage area contained within these outlines. During the initial stages of myotube formation, all myotubes stained with tropomyosin; however, as the cultures deteriorated and myotubes degraded, bright field images had to be used to outline myotubes. The percentage of nuclei within myotubes was determined by transferring the myotube outline to the accompanying Hoechst image and counting the number of nuclei within the outlines. The nuclei were considered myotube nuclei only when there were three or more present within an outlined area.

### 2.8 CPK Control Experiments

A large number of control experiments were performed in order to substantiate the biochemical assays. The majority of controls, however, focused on the CPK, because its stability, both in the cell homogenate and when frozen, is of major importance in evaluating cell culture experiments. A batch of cell homogenate was aliquoted, frozen, and assayed on successive days

for enzyme activity to test the stability at -80°C. Storage of CPK standards was similarly tested this way. Enzyme levels were also measured on successive freeze-thaws and before and after sonication to check stability during these processing steps. Sample stability was also evaluated while the sample was maintained on ice. In addition, because the proportion of myotubes and myoblasts changes throughout an experiment, a number of controls were performed to insure that the heterogeneous population of cells did not affect enzymatic determination. Enzyme standard was spiked into PBS, which was then used to homogenize cell cultures. This procedure was repeated a number of times throughout the time course of the experiments to insure that the proteolysis seen during the deterioration of the cell cultures did not taint data. Homogenate from myoblast cultures was also mixed with homogenate from myotube cultures to insure that myoblasts did not contain an inhibitor of CPK which could affect observed enzyme levels during the experiments.

### 2.9 Passive Stretch

The Flexercell Strain Unit (rented from Flexcell International Corporation, McKeesport, Pa.) is a device to apply mechanical strain to cells on petri dishes with flexible silicon bottoms. Petri dishes with laminin coated, flexible silicon membrane bottoms (Flex I) were also purchased from Flexcell. The laminin coated membranes were used because they induced the greatest amount of myotube formation, as observed visually during initial testing. The Flexercell Unit was connected to the in-house vacuum system through output ports in the incubators.

Several minor changes were made to the above protocols to account for slower growth rates and smaller well sizes (now 4.9 $cm^2$ instead of 9.8 $cm^2$). Cells were plated at a seeding density of 30,000/$cm^2$ so that confluence was attained in four days. The media in the plates was gently swirled every five minutes to prevent cell pooling in the center of the wells. Biochemical aliquots were obtained by scraping cells into 100 μl rather than 250 μl. All other procedures were identical.

Experiments in this study investigated passive stretch only. That is, the membrane was stretched once and remained stretched throughout the experiments except when media was changed and biochemical aliquots were prepared. The strain level of the unit was set by adjusting pressure control valves on the computer which accompanied the unit. The maximum strain used was 25% ± 3% for all experiments. This parameter is the unit's variable parameter and represents the maximum surface strain on the membrane. The strain profile across the membrane for this maximum strain and a discrete set of other maximum strains were characterized previously (Gilbert et al. 1989).

## 2.10 Data Analysis and Statistics

With the exception of the passive stretching experiment, each experiment was repeated at least twice with different batches of reagents and cell stocks. Each experimental time point is the average of three identically treated wells, and all biochemical assays were performed in triplicate. The error bars in all figures with the exception of the morphometric figures represent the standard deviation from the three wells. The error bars in the morphometric figure and represent the

standard error of the mean. A one-sided Student's t-test with p values smaller than 0.05 was used to assess statistical significance where indicated. In the morphometric curves, all points were compared to the maximum point in the graphs.

# CHAPTER 3

## RESULTS

### 3.1 Short-Term Effects Of Horse Serum On L6 Cell Cultures

L6 cells were grown in DMEM + 10% FBS until confluence (5 days), at which time the media was switched to DMEM with different concentrations of HS. After 6 days in the HS media (11 days total), cultures in 10% HS contained distinctly fewer myotubes and dense regions of myoblasts whereas 1% HS cultures contained fewer myoblasts and what appeared to be a greater density of myotubes. Fig. 1 shows representative fields from L6 cell cultures on day 6 after media switch. These cultures contained three distinct morphological characteristics associated with myotubes (Yaffe D, 1970): 1) numerous marginated nuclei; 2) diameters ranging from 10 to 50 μm; and 3) lengths on the order of hundreds of microns. Cultures in 10% HS contained distinctly fewer myotubes and dense regions of myoblasts whereas 1% HS cultures contained fewer myoblasts and what appeared to be a greater density of myotubes.

The biochemical response of L6 cultures to different doses of HS was assessed in order to maximize muscle specific protein and minimize myoblast proliferation before long-term experiments were begun. Biochemical quantitation (Fig. 2B) revealed that DNA synthesis rate per well decreased from 5 μg/day to 0 μg/day as the dose decreased from 10% HS to 1% HS. By day 13, the 10% HS cultures contained 10-fold more DNA than the 1% cultures. The DNA levels in the 0% HS cultures were stable, but morphologically, few myotubes formed and they

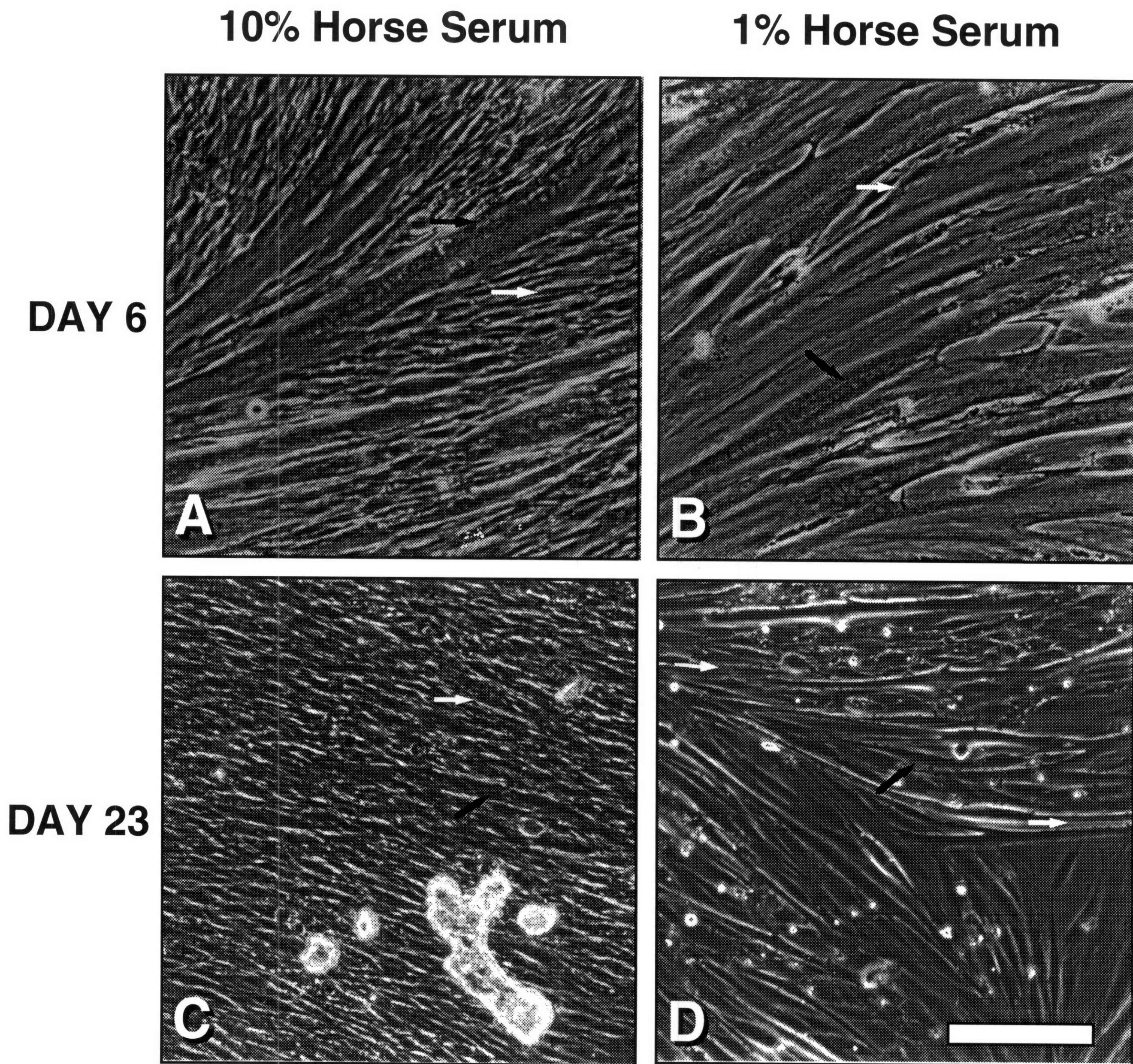


Figure 1: Effect of horse serum on L6 muscle cell cultures. The dark arrows depict myotubes with numerous marginated nuclei and white arrows depict myoblasts. Scale bar is 100μm.

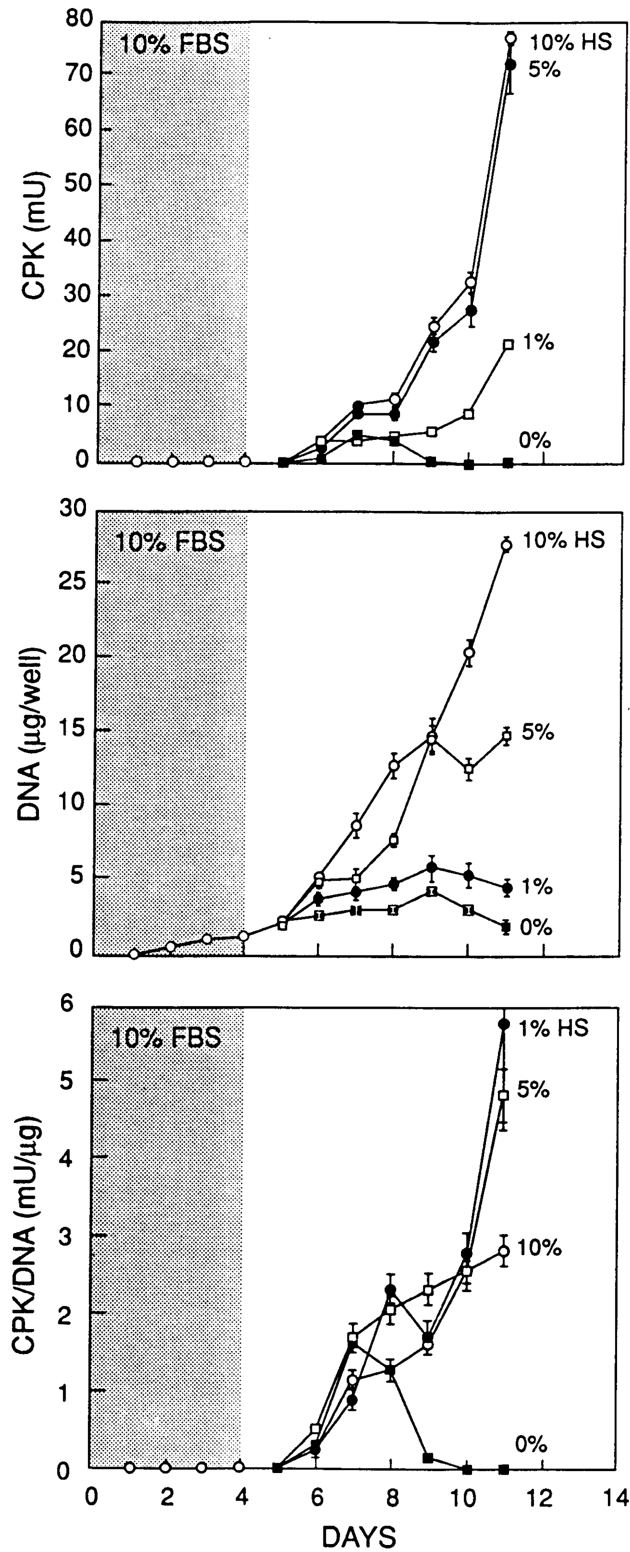


Fig. 2: Short-term effect of HS on L6 cell cultures. Cells were grown to confluence in 10% FBS (dotted regions) and then switched to the indicated concentrations of HS. Data points represent average and standard deviation of 3 experiments.

deteriorated rapidly. These results indicate that as the dose of HS decreased, myoblast growth was progressively inhibited.

CPK was used as a marker of biochemical differentiation in L6 cell cultures in order to investigate the effect of HS on a marker of differentiated muscle function (Yaffe D, 1970). Absolute levels of CPK in the 10% HS cultures increased with time from undetectable levels on day 5 to over 70 mU per well on day 11 (Fig. 2A). This increase in CPK was at least 50-fold greater than the CPK before fusion. The increase in CPK also occurred in a somewhat dose responsive manner, as the 10%, 1%, and 0% HS cultures showed distinctly different rates of increase. The CPK in the 0% HS culture increased and then decreased rapidly to 0 after day 8. CPK normalized to DNA showed that the maximum amount of CPK relative to DNA occurred in the 1% HS cultures (Fig. 2C) and also that the rapid rise in CPK could not be explained by myoblast proliferation. Normalized CPK also decreased in a dose responsive manner. This decrease could be due to either an increase in the number of myotubes or an increase in CPK per myotube. These dose response experiments were repeated at different times with different reagents and cell batches and biochemical trends were identical in both experiments.

Thus, the short-term culture experiments showed that: 1) biochemical information corroborated the short-term morphological information in that DNA in the 10% HS cultures increased in accordance with myoblast proliferation, the 1% culture DNA reached a plateau in accordance with a cessation of myoblast growth, and the CPK in culture increased in tandem with the morphological formation of myotubes; 2) 1% HS was the superior media for long-term experiments, given the requirements of decreased myoblast proliferation and increased expression

of CPK. For the remainder of this study, 1% HS was used and 10% HS was used for comparison.

## 3.2 Long-term Effects of Horse Serum on L6 Cultures

### 3.2.1 Morphology

To evaluate the suitability of L6 cultures for long-term experiments, myoblasts were grown to confluence in DMEM with 10% FBS, at which time (5 days), cultures were switched to 1% HS or 10% HS with DMEM. Cultures were subsequently analyzed over a 35 day period. In 1% cultures (Fig. 1D), although the myoblasts proliferated to some extent, the cultures consisted primarily of myotubes. There was a marked difference in myotube morphology between day 6 and day 20, with the myotubes in day 20 cultures appearing thinner and shorter. The myotubes in 10% HS deteriorated by day 23 (Fig. 1C) and the cultures became dominated by myoblasts.

To obtain a quantitative assessment of the apparent degradation in long-term cultures and substantiate the observed morphology, morphometric analysis of 1% HS cultures was undertaken. The percentage of the microscopic field of view covered by myotubes (Fig. 3A) showed that myotube area increased 5 fold over a 9 day period, peaked at day 20, and then declined 5 fold 9 days after the peak. The decline in myotube area corroborates the morphological differences seen between day 6 and day 23 cultures in 1% HS. The number of myotube nuclei increased 10-fold over the six-day period from day 12 to day 18 (Fig. 3B), indicating that the majority of myotubes

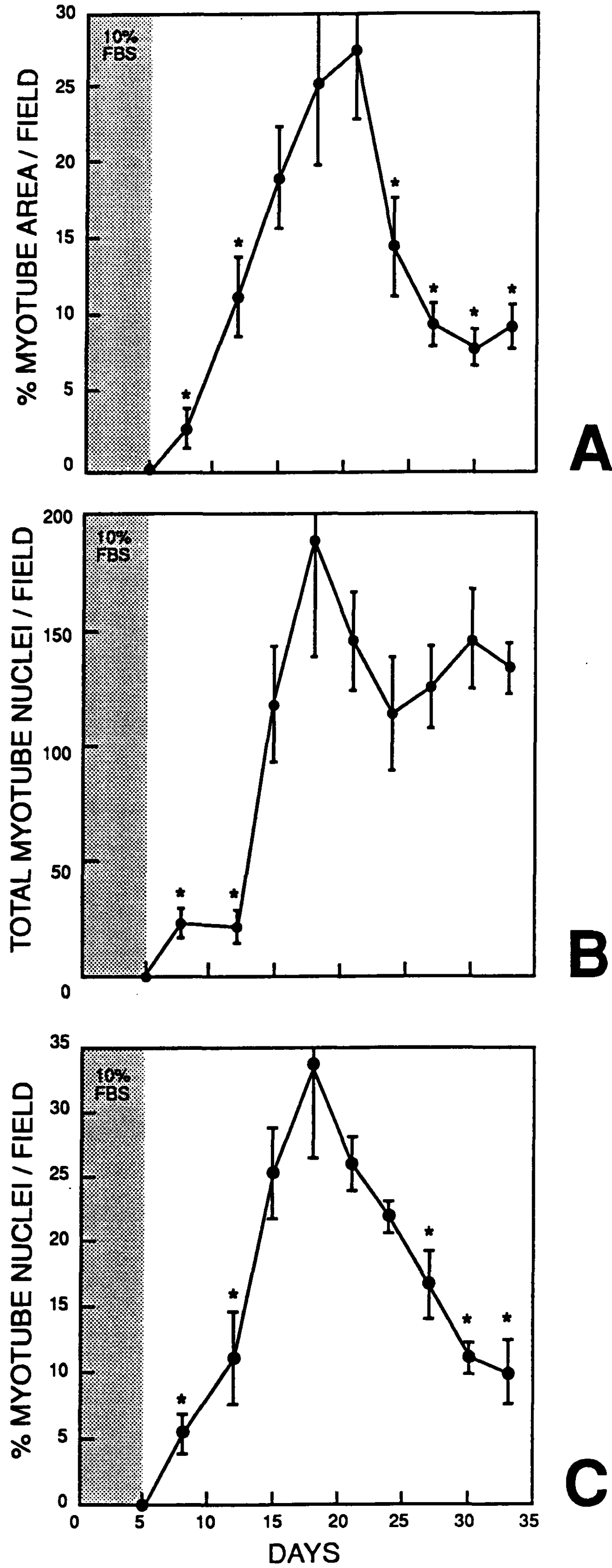


Fig. 3: Cells were grown to confluence in 10% FBS and then switched to 1% HS at day 4 (confluence). Data points represent the average of 11 fields from on petri dish. Error bars represent standard error. All values were compared to the maximum and * denotes $p<.05$.

were formed during this period. The number of myotube nuclei then reached a constant value toward the end of the culture. Along with the data which showed a declining percentage myotube area, this result suggests that most of the myotubes remained intact in the cultures, or identically, that the myotube density remained constant and the myotubes atrophied. Finally, the percentage of myotube nuclei per field in long-term cultures reached a maximum and continued to decline toward zero (Fig. 3C), which along with the constant number of myotube nuclei, indicates that after day 20, myoblasts proliferated in excess of myotubes.

### 3.2.2 Biochemical Analysis of Long-Term L6 Cultures

Since morphometric data showed myotube degradation and myoblast proliferation, it is important to see how these were reflected in biochemical functions that are more pertinent to skeletal muscle cultures. To this end, cells were grown to confluence for five days in 10% FBS and then stimulated with 1% HS for a period of 33 days. CPK and DNA were used as biochemical parameters and 1% HS results compared to a condition that promotes proliferation (10% HS). The DNA in 1% HS cultures appeared stable through day 15 (Fig. 4), at which point the DNA slowly increased, indicating that the myoblasts began to proliferate. In comparison, DNA in 10% HS cultures increased until day 20, when the cultures appeared to reach a steady state of approximately 35 μg/well. Between day 10 and day 25, the 10% HS cultures increased 7-fold whereas the DNA in the 1% HS cultures increased only 2-fold, indicating that the myoblasts proliferated at a slower rate. Thus, long-term analysis of DNA revealed that 1% HS maintained myotube cultures for approximately 7 days but was not sufficient to keep myoblasts quiescent for

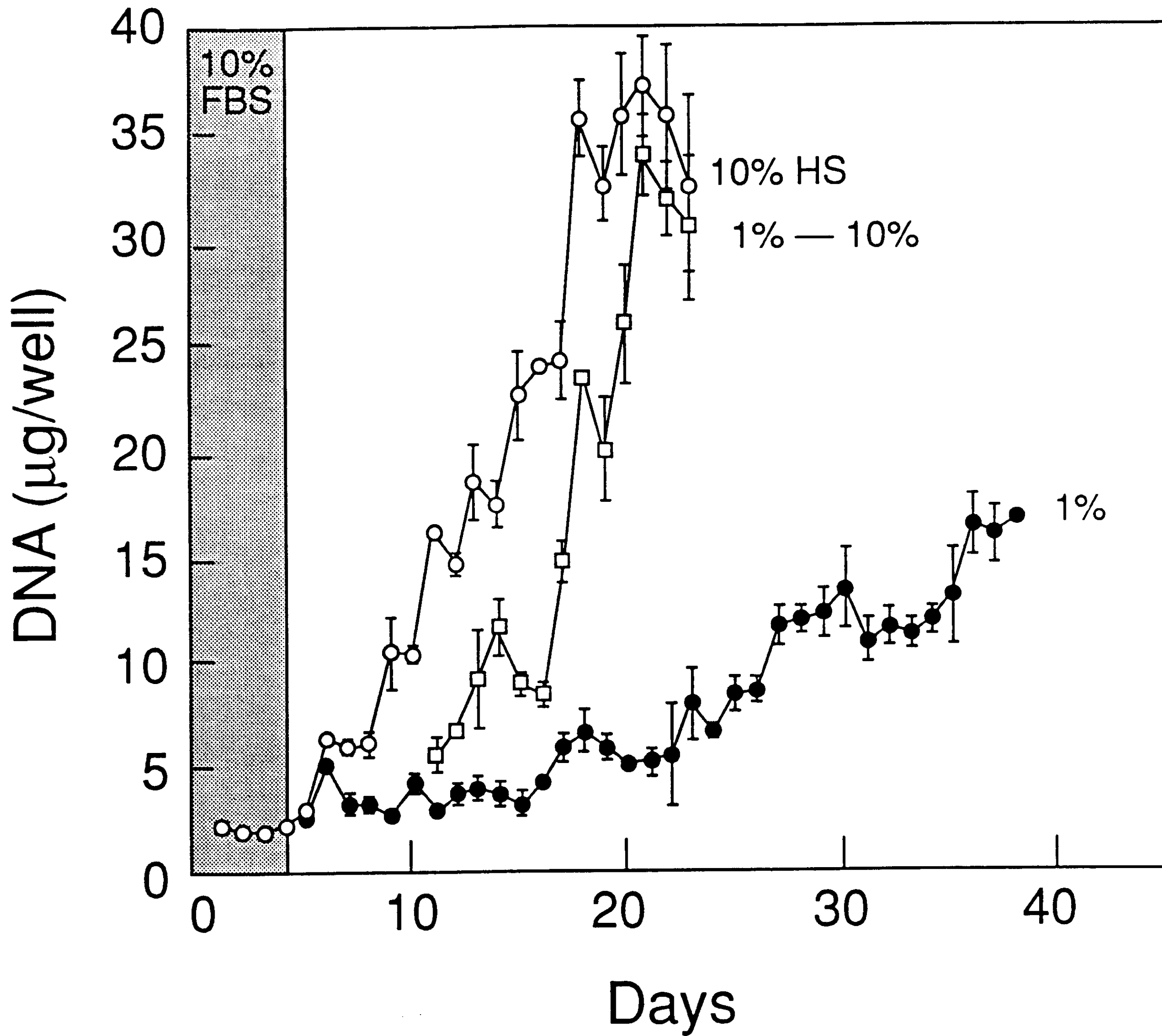


Fig. 4: Long-term effect of HS on L6 cell cultures. Cells were grown in 10% FBS (dotted regions) to confluence at which time media was switched to the indicated concentrations of HS at day 4 (confluence). On day 10, some cultures were switched back to 10% HS (squares). Data points and error bars represent the average and standard deviation of three experiments.

longer periods of time. Experiments with the 1% HS cultures were continued until day 38, at which point, the multinucleated cells were mere remnants of the myotubes which were once present. By day 23 in 10% HS cultures, there was excessive cell turnover and experiments were stopped.

To investigate the responsiveness of the culture system, 1% HS cultures were stimulated on day 10 with 10% HS (open squares, Fig. 4). The DNA resumed its increase, with a slope similar to the 10% HS cultures. By day 20, the DNA levels in the 1% HS and 10% HS cultures were comparable, indicating that the myoblasts present in the 1% HS cultures remained competent to rapidly proliferate. This result indicates that the myoblasts were held in a non-proliferating state when the concentration of HS was decreased to 1%. Absolute and normalized CPK data during this experiments showed results similar to the DNA (data not shown), where the levels began close to the 1% HS levels and then reached the levels of the 10% HS curves. This result may be explained by 1) the proliferating myoblasts fused into myotubes or 2) the myotubes already present began to produce more CPK.

CPK was used to assess long-term function of the L6 myotubes. The absolute level of CPK in long-term experiments was detectable two days after media was switched (Fig. 5A) and increased through day 18, when a peak was reached in both the 10% HS and 1% HS cultures. In 1% HS, the level of CPK reached a peak on day 18 and thereafter, began to decline. After day 21, the cultures attained a quasi-steady state for the remainder of the long-term experiments, with the rate of decrease approximately 1 mU/day. In comparison, after day 20, the level of CPK in 10% HS cultures declined at a rate of 25 mU/day. However, the maximum level attained in the 10% HS cultures was approximately 3 times higher than the level attained in the 1% HS cultures.

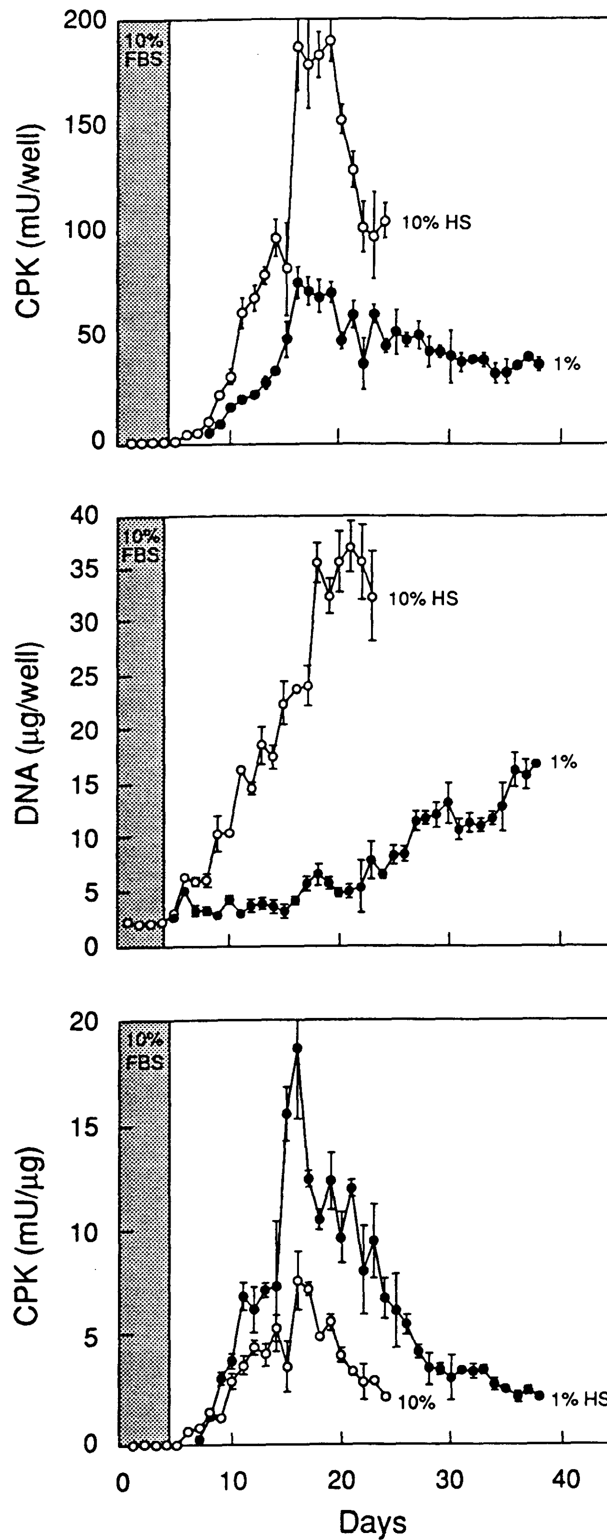


Fig. 5: Effect of 1% HS and 10% HS on CPK and DNA levels in L6 muscle cell cultures. Dotted regions depict the time before confluence when cells were grown in 10% FBS. Each data point represents the average of 3 petri dishes and error bars represent standard deviations.

The morphological appearance of the 10% HS cultures paralleled the enzyme degradation and proliferation of myoblasts (Fig. 1C); on day 23, 10% HS cultures were stopped. The higher level of CPK in the 10% HS cultures can be explained either by an increase in CPK per myotube or an increase in the total number of myotubes. These results indicate that myotube function deteriorated rapidly in 10% HS cultures and more slowly in 1% HS cultures. The fact that the enzyme degradation slopes of 10% and 1% cultures are different suggests that the myoblasts may have a deleterious effect on the myotubes. Normalized CPK levels accounted for the myoblast proliferation in the cultures (Fig.5C) and showed that normalized CPK increased and then decreased rapidly. This result shows that CPK per DNA also increased and decreased with similar slopes in both 1% and 10% HS cultures, which again suggests that the myoblasts exert a deleterious effect on the myotubes. In addition, there was a sharp increase in DNA and a sharp decrease in normalized CPK, which coincided with the peak in absolute CPK levels. This result may be explained by media conditioning by the myotubes secreting growth factors, such as IGF-1 (Tollefsen et al. 1989). Conversely, a fast growing myoblast clone may have been selected for, which could have resulted in an increase in myotubes and a decrease in normalized CPK as well. The decline in absolute CPK levels correlates with the conclusion from area percentage of myotubes in the morphometric data, both of which indicate a decline in myotube function. Similarly, the decrease in normalized enzyme levels correlates with the DNA levels and the percentage myotube nuclei from the morphometric data, both of which indicate increased myoblast proliferation. The long-term experiments were repeated twice and trends were identical.

Initial characterization of protein turnover focused on total protein (Fig. 6). After stimulation of the cultures with 1% HS, protein content increased during the first five days. From

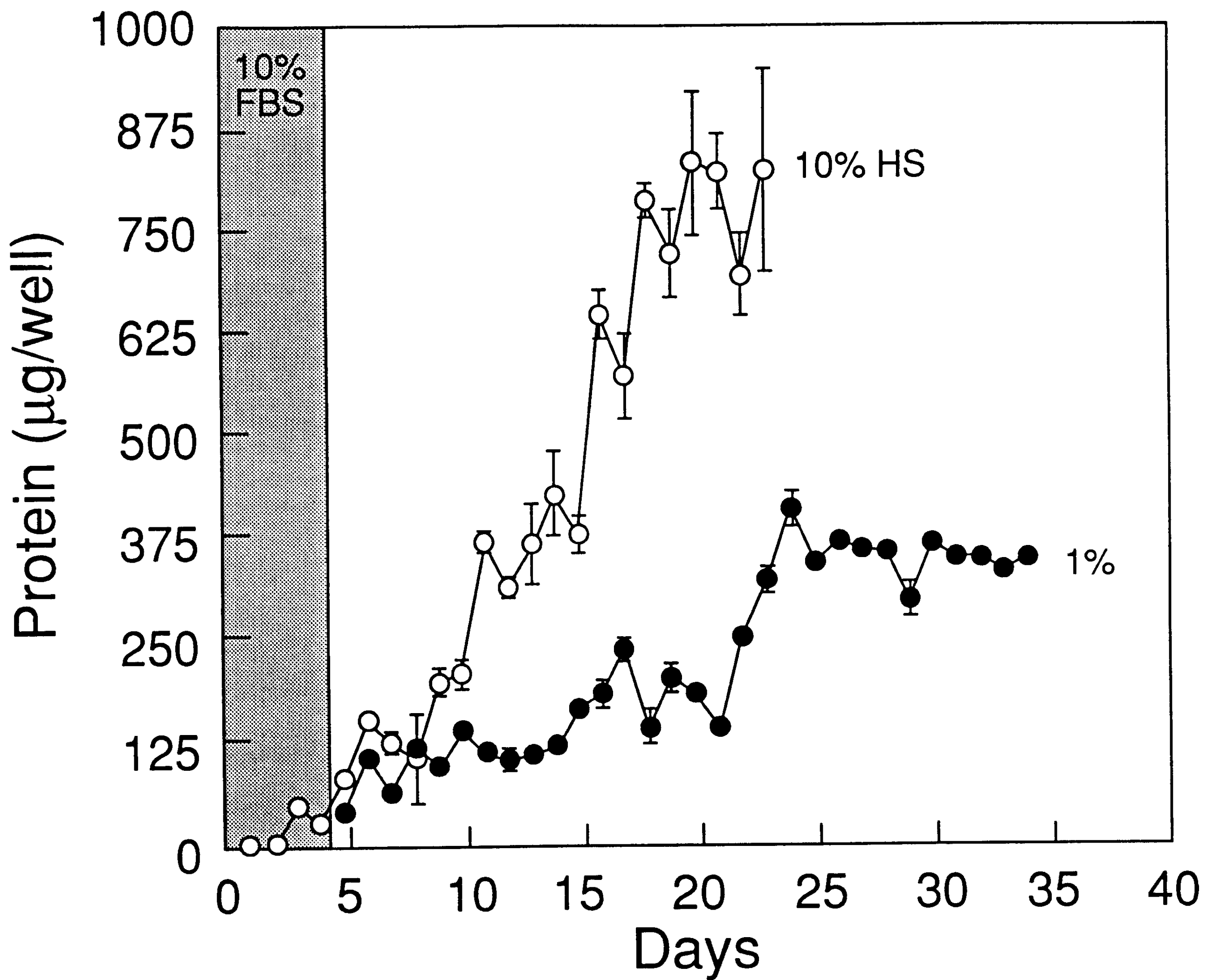


Fig. 6: Long-term effect of HS on L6 cell culture total protein. Cells were grown in 10% FBS (dotted regions) to confluence at which time media was switched to indicated concentrations of HS. Data points and error bars represent the average and standard deviation of three experiments.

day 10 to day 20, protein stabilized in the 1% culture, but continued to increase in the 10% HS cultures. After day 20, total protein increased in a manner similar to DNA in both 1% HS and 10% HS cultures. DNA analysis showed that myoblasts proliferated after day 20 as well; therefore, total protein in the cultures after day 20 appeared to depend on myoblast proliferation.

Long-term quantitative biochemical studies suggest that myotube function is quasi-stable from day 22 until day 38 in cultures maintained in 1% HS over the 38 day period. The overall biochemical state of the culture system, however, is unstable because myoblasts begin to proliferate after their apparently quiescent state immediately after stimulation with 1% HS. However, based solely on the stable levels of CPK, this muscle culture system may be useful for protein turnover studies if muscle specific biochemical markers as long as markers are used.

### 3.2.3 Immunoflourescence Staining and Birefringence Microscopy of L6 Cultures

To further document muscle specific protein degradation and myotube deterioration *in situ* in culture, immunoflourescence staining was undertaken using monoclonal antibodies for muscle specific tropomyosin and α-actinin, markers of differentiation. The specificity of these antibodies has been shown previously (Lin et al. 1985; Lin and Lin, 1986). Tropomyosin staining after 3 days in 10% FBS showed that myoblasts contained little tropomyosin (Fig. 7A,B). However, after six days in 1% HS, myotubes showed significantly brighter flourescence (Fig. 7C,D) than the myoblasts. By day 28, tropomyosin degraded in a large number of myotubes, but the degree of degradation (Fig. 7E,F) was heterogeneous throughout the cultures. While the staining in a number of myotubes was bright and diffuse (Fig. 7E), many myotubes contained

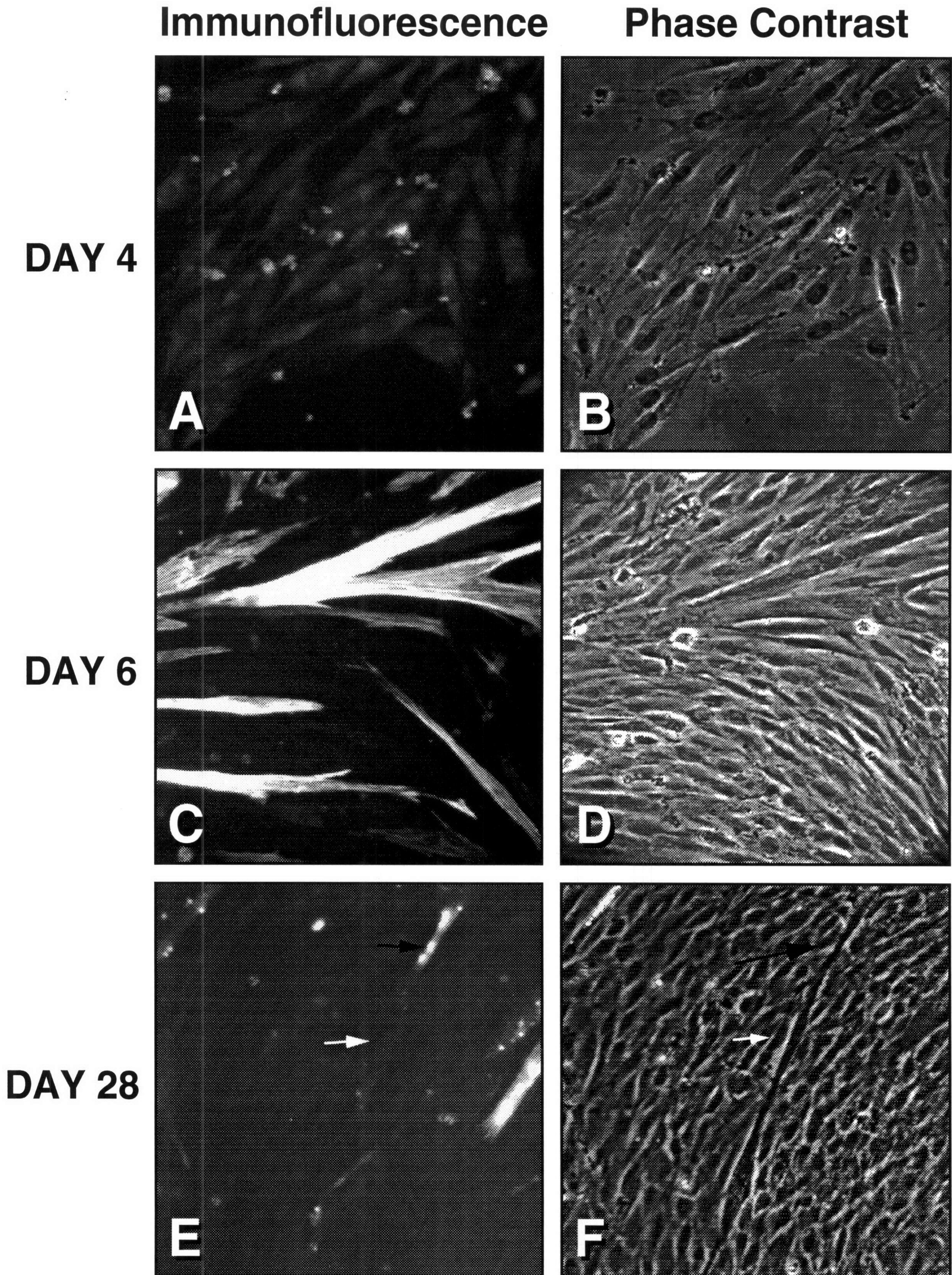


Figure 7: Effect of 1% HS on tropomyosin expression in L6 cell cultures. Cells were grown in 10% FBS and stained on day 4 (A&B). On day 5, media was switched to 1% HS and stained on days 6 and 28. Dark arrows depict same myotube with regions of no staining. Scale bars represent 100 µm.

stained and unstained regions in different areas of the same myotube. The flourescent micrographs in all six panels were taken at identical camera settings to ensure that the intensity levels were comparable. In addition, controls with secondary antibody did not show any staining. Thus, these results indicate a two-fold conclusion: 1) the antibody stained specifically for sarcomeric tropomyosin in the L6 myotubes; 2) degradation of skeletal muscle tropomyosin correlated with the degradation of CPK, supporting the use of CPK as a marker of muscle specific function and as an indicator of the state of the contractile proteins.

To further document myotube atrophy and the degradation of tropomyosin, high magnification images were obtained (Fig. 8). Cultures were stained simultaneously with F-actin and tropomyosin. Day 11 myotubes showed that the two structural molecules co-localized along peripheral stress fibers (Fig. 8A). The multinucleation can also be seen clearly in this micrograph. Later cultures (Fig. 8B) showed that tropomyosin staining was punctate. Parts of the myotube stained diffusely while other parts of the same myotube did not stain at all, although the stress fibers remained intact as visualized with F-actin. Thus, high magnification staining suggests a number of conclusions: 1) early cultures show the beginnings of myofibril formation, which has been shown previously to begin with stress fiber localization of muscle specific proteins (Wang et al. 1988); 2) two different proteins (F-actin and tropomyosin) within the the same myotube may degrade at different rates; 3) these images confirm that tropomyosin degradation seen in Figure 7E is in fact specifically located within the myotubes, a conclusion which could not be drawn from the low magnification images; 4) these results showed that tropomyosin, a muscle specific protein, degraded while F-actin did not appear to degrade and the myotube remained intact. This correlates with the morphometric result which suggested that myotubes atrophied, as indicated by

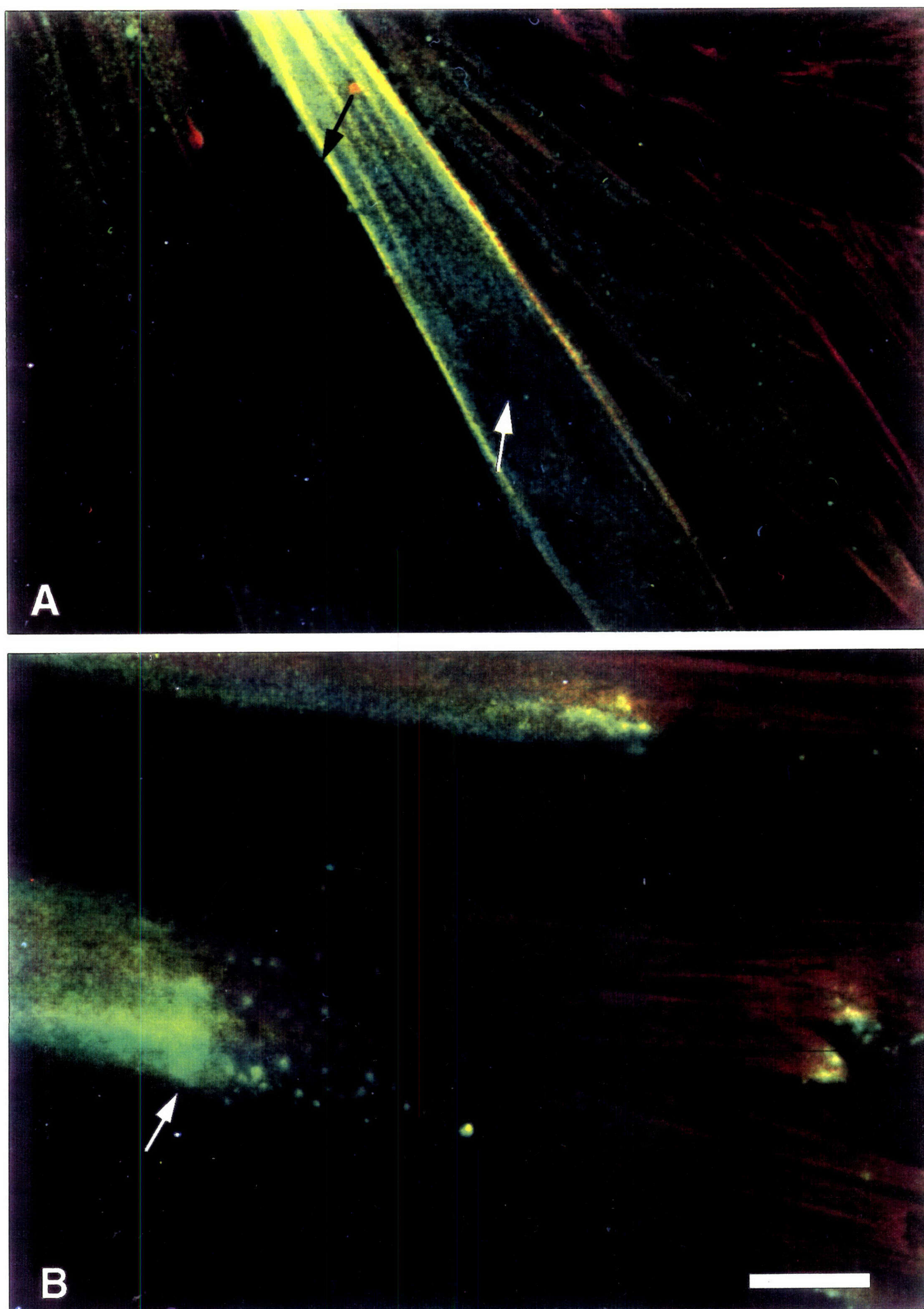


Fig.8 Confocal images of 1% HS L6 cultures stained for F-actin (red) and tropomyosin (green) White arrow in A depicts multinucleation and dark arrow depicts tropomyosin localized to peripheral stress fibers. In B, the white arrow points to degrading tropomyosin and the dark arrow points to intact stress fibers.

a decrease in myotube area per field in combination with a constant total number of myotube nuclei.

Immunoflourescence staining for α-actinin (Fig. 9) and birefringence microscopy (Fig. 10) were also used in order to assess the level of structural differentiation of L6 myotubes. The well-known characteristics of adult muscle are the well-defined sarcomere patterns. Although tropomyosin, myosin, α-actin, or α-actinin can be used to visualize sarcomeric patterns, α-actinin offers the greatest resolution because it incorporates into the Z-bands (see Fig. 1.1). Birefringent microscopy depicted the striated pattern as well, but only with the resolution to separate the I-bands and A-bands. The myotube in Fig. 9A shows diffuse staining with α-actinin, but there is some indication that sarcomeres are forming. Most myotubes, however, stained even more diffusely than the one shown in Fig. 9A. In contrast, Fig. 9B shows a primary human myotube with distinct sarcomeric patterns. The majority of myotubes in the primary human myotube cultures showed this level of structural differentiation. Fig. 10 shows a birefringent myotube indicating that this cell contained sarcomeric patterns. Again, very few birefringent myotubes were seen in the L6 cell cultures. These results suggest that little structural differentiation is attained by the L6 cell myotubes. However, including α-actinin staining, at least two muscle specific proteins are expressed by the L6 myotubes. The specific staining shown in the human primary cultures indicates that the the antibody is specific and the microscopy techniques allow for the visualization of sarcomeres if they are present in the myotubes.

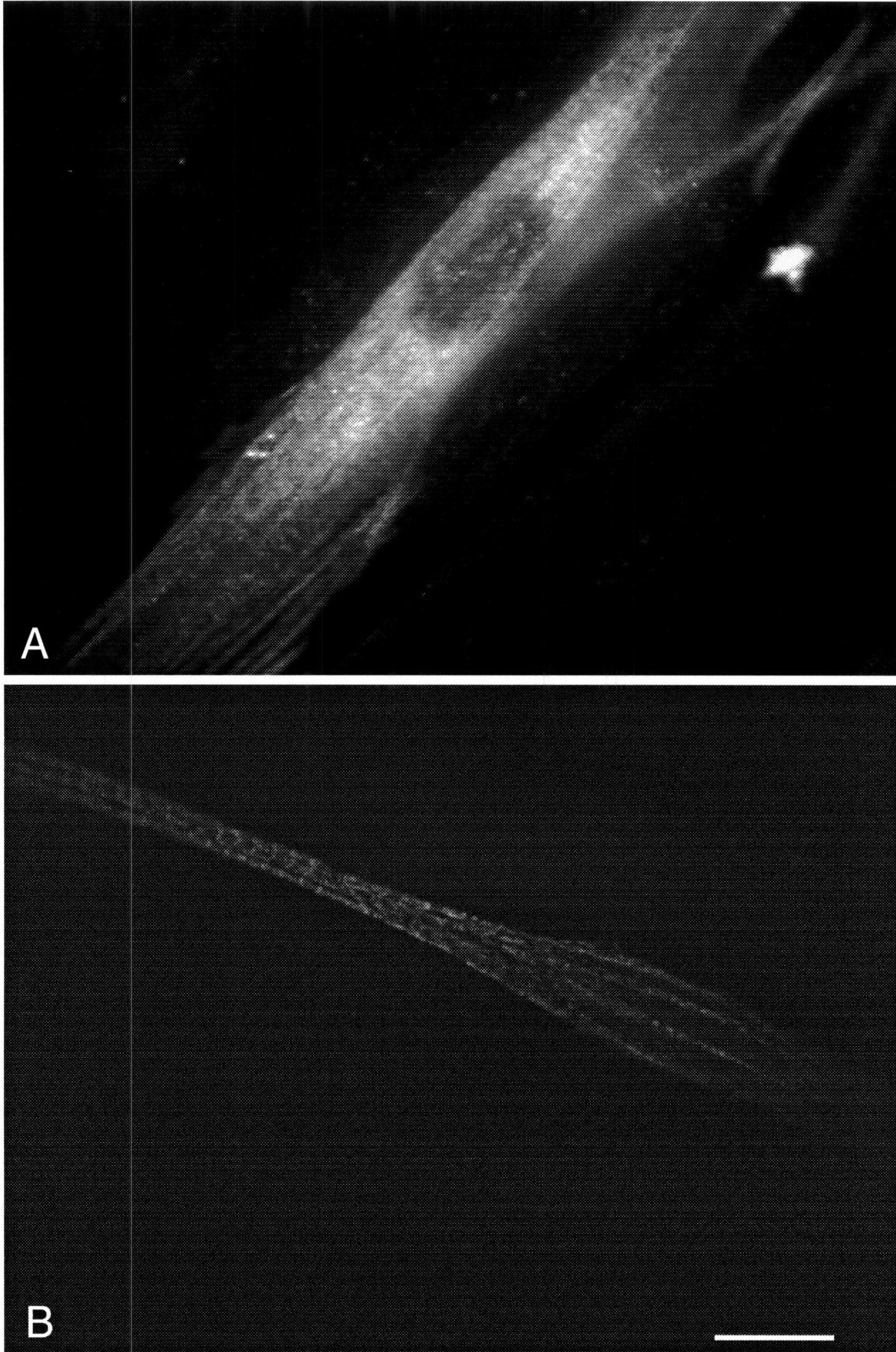


Fig. 9: $\alpha$–actinin staining of an L6 myotube (A) and a primary human myotube (B). Scale bar represents 10 $\mu$m.

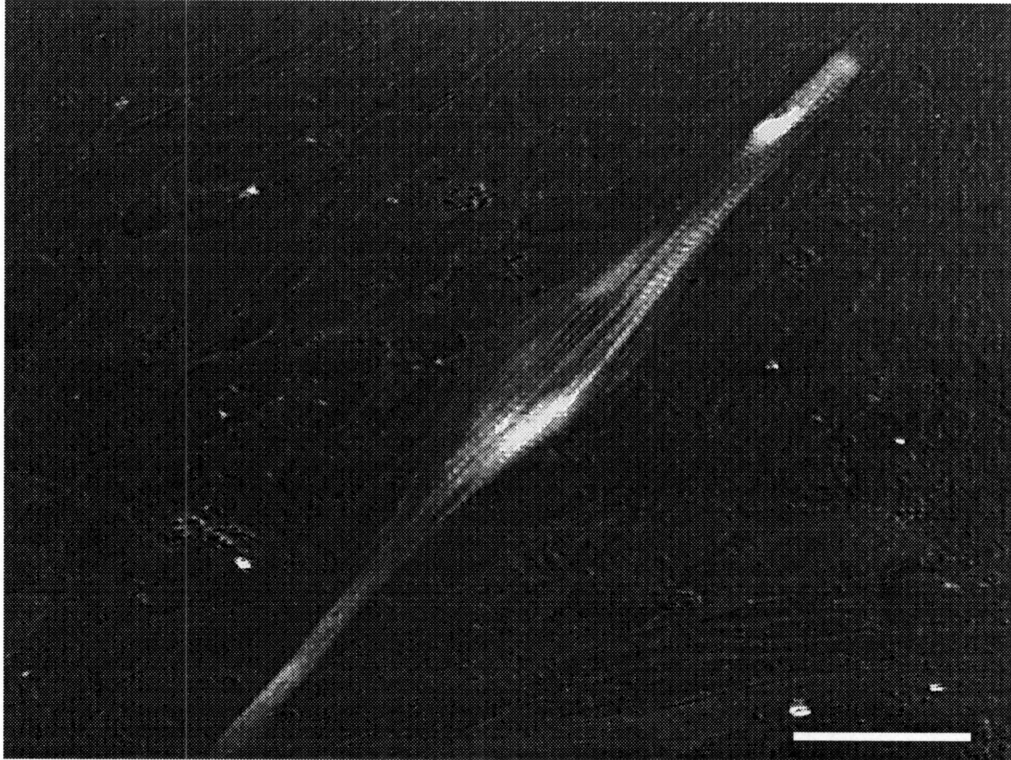

Figure 11: Birefringent L6 myotube. Scale bar represents 10 μm

## 3.3 Passive Stretch Experiments

Passive stretch experiments were performed as a preliminary test of the hypothesis that myotube function can be augmented and potentially stabilized when cells are maintained under tension. L6 cells were grown on flexible laminin coated silicon membranes in DMEM + 10% FBS. After 5 days, the cells became confluent and the media was switched to 1% HS. Following 7 days of incubation, plates were either stretched statistically at 25% maximum strain or left unstretched (controls). Table 1.1 shows that both DNA and CPK showed a statistically significant increase over controls, but the normalized DNA remained constant. The numbers in parentheses represent the standard deviation across the 3 wells. These preliminary results indicate that passive stretch influences the metabolic state of the culture system either by directly signaling the cells, changing the substratum configuration, or sufficiently separating the cells so as to avoid contact inhibition and promote growth.

Table 3.1 Effect of passive mechanical stretch on L6 muscle cell cultures

| DAYS | CPK (mU) (control) | CPK (mU) (stretched) | DNA (μg) (control) | DNA (μg) (stretched) | Norm.CPK (control) | Norm. CPK (stretched) |
|---|---|---|---|---|---|---|
| 5 | 3.2 (.7) | -- | 1.02 (.27) | -- | 3.2 (.11) | -- |
| 14 | 5.84 (0) | -- | 1.63 (.44) | -- | 3.6 (.29) | -- |
| 16 | 6.7 (.1) | 7.8 (.57) | 1.55 (.07) | 2.45 (.3) | 4.32 (.21) | 3.2 (.08) |
| 18 | 6.9 (.02) | 9.6 (.18)* | 1.46 (.03) | 2.49 (.03)* | 4.73 (.38) | 3.9 (.15) |
| 20 | 8.6 (.04) | 15.2 (.02)* | 1.78 (.14) | 3.18 (.75)* | 4.8 (.19) | 4.8 (.09) |

$p<0.05$; n=3

## 3.4 CPK Controls

When CPK standard was spiked into PBS and used to homogenize cells at different time points during a 40 day cultures, the concentration of enzyme recovered was identical to the original concentration, indicating that proteases were not a problem during the extraction or assay steps at any time. In the experiments in which myoblasts and myotubes were mixed together, the resulting concentration of CPK was additive, indicating that the myoblasts did not contain an inhibitor of CPK. The cell homogenate and CPK standards were stable for over 50 days at -80°C and were also stable for at least 12 hours when kept on ice after thawing. Sonication did not result in any decrease in enzyme. The reliability of the biochemical assays was demonstrated by the fact that dilution and standard curves for all three biochemical assays were linear and slopes were within 15% of one another.

# CHAPTER 4

## DISCUSSION

Previous studies on muscle cell cultures, both primary and cell lines, have primarily focused on myotube formation and expression of muscle specific genes. However, the level of differentiation attained as well as the long-term stability of muscle specific characteristics has not been thoroughly characterized. In this study, we quantitatively studied the functioning of L6 cells in both static and mechanically stimulated culture conditions. The major finding of this study is that differentiated L6 myoblasts, maintained under static culture conditions, are stable for a period of 7 to 14 days depending on the stringency of the steady state requirements. However, the muscle specific markers undergo a slow rate of degradation in longer term cultures. Results with static passive stretch of L6 cells after 10 days in static culture showed that mechanical stimulation of muscle cells may prolong the culture time and decrease the rate of degradation.

Results from long-term studies showed that L6 cells in 1% HS differentiated into a population of myotubes which contained a maximum of 30% of the nuclei per field. The total number of myotube nuclei remained constant after 15 days, suggesting that most of the myotubes were formed by 15 days and the number remained relatively constant. Alternatively, but less likely given the net degradation seen at later times, a steady state of myotube formation and disappearance may existed. In the original characterization of the L6 cell line (Yaffe, 1968), it was noted that "100% of the myoblast clones formed muscle forming colonies." It was also noted

that as the line was passed, the stringency of the culture media requirements to maintain myoblast clones was reduced. It is not clear exactly what the muscle forming colony index referred to and thus it could not be compared to current studies. Nadal-Ginard (1978) quantitated the percentage nuclei per field contained within L6E9 myotubes in a manner identical to current studies; he reported an index of 80%-90% in 10% HS depending on the initial seeding density. He also reported that in 20% fetal calf serum, the index was 0%. The L6E9 cell line is an L6 subclone specifically selected by Nadal-Ginard for its ability to differentiate quickly. Thus, there is considerable variability in the degree of differentiation seen in different studies, which emphasizes the need for characterization of media and cell lines in order to understand the limitations prior to use of muscle cell cultures for various physiological studies.

It is possible that genetic drift occurs with continued propagation of the L6 cells and with time, its ability to differentiate declines. In primary cultures, cells lose their ability to differentiate after a finite number of cell divisions, the value of which is sensitive to the exact culture conditions employed (Yaffe, 1968). A similar effect was seen in the current studies: with continued passage, the ability of the cells to differentiate rapidly declined, which was the reason why we carefully controlled passage number. It may be that muscle cell lines must be recloned to maintain differentiation, which is a difficult task given that when the cells differentiate, they stop proliferating. Nonetheless, care must be taken to standardize and characterize culture procedures and media in order to obtain reproducible results.

Initial short-term experiments were undertaken in order to maximize muscle specific markers and minimize myoblast growth. Experiments with HS doses ranging from 0% to 10% over a period of 7 days showed that DNA per well remained reasonably constant in the 1% HS

cultures, while the levels of CPK increased, indicating that the 1% HS cultures had the largest proportion of muscle specific protein. The 10% HS cultures had the highest absolute levels of CPK but increasing DNA levels suggested that the undifferentiated myoblasts continued to proliferate. This point was reflected in the normalized CPK, which showed that 1% HS cultures attained the highest levels of specific CPK activity. CPK has been used as a marker of myogenic differentiation in previous studies with primary and L6 muscle cell cultures (Coleman and Coleman, 1972; Yaffe D, 1994), and is considered a marker of contractile protein synthesis, which is easier to quantitate than the protein levels themselves (Konigsberg, 1965; Devlin and Emerson, 1978). In at least two studies with L6 cells, initiation of CPK and myosin synthesis were were observed to be coordinated at the time of fusion and increase with similar slopes afterward (Nadal-Ginard, 1978; Coleman and Coleman, 1972). Furthermore, in the present study, tropomyosin expression was shown to begin coordinately with fusion and CPK synthesis. Thus, there is strong evidence from past literature and present work that CPK correlates with contractile protein synthesis in myotubes. With 1% HS producing reasonably high levels of CPK and limiting myoblast growth, long-term studies using 1% HS were undertaken in order to investigate the magnitude and stability of muscle specific protein in L6 cell cultures.

Long-term DNA data from 1% HS cultures showed a proliferation of myoblasts after day 20. From day 20 to day 38, myoblasts proliferated slowly, which indicates that protein turnover measurements may be obscured by non-muscle cells; thus, measurements of protein turnover should make use of a muscle specific marker such as myosin, tropomyosin, or $\alpha$-actinin. Alternatively, DNA inhibitors such as cytosine arabinoside (C-arA) (Gulve and Dice, 1989) and 5-fluorodeoxiuridine (FudR) (Coleman and Coleman, 1972; Nadal-Ginard, 1978), which have been

used in previous work to either augment differentiation and/or eliminate myoblast proliferation, may be employed to eliminate non-differentiated muscle overgrowth. However, there are discrepancies in past studies regarding the toxic effect of these inhibitors on myotubes. Zalin et. al. (1974) showed that FudR and C-arA decreased the degree of fusion in cultures, but Nadal-Ginard (1978) and Coleman and Coleman (1972) showed a less detrimental effect, indicating that further characterization is required before use of these inhibitors in muscle cell culture.

Data from 1% HS cultures showed that CPK levels continued to increase until day 20, and thereafter, declined slowly. The decline in protein levels of approximately 1 mU/day indicates a deteriorating population of myotubes. However, the period of time 7 to 14 days after the peak (i.e. days 27-34 of culture) was a relatively stable region and is therefore the most reasonable for experimentation. CPK normalized to total DNA showed a peak at day 20 with a subsequent sharp drop-off correlating with myoblast proliferation. It is possible that the myoblasts proliferated as a result of media conditioning by the L6 myotubes. Primary myotubes have been shown to secrete at least one growth factor, IGF-1, upon fusion (Tollefsen et al. 1989). An alternative explanation is that a faster growing myoblast clone was selected for as the myoblasts slowly proliferated in 1% HS. Previous work (Yaffe, 1970) which characterized the L6 cultures for periods of time longer than two weeks showed that CPK normalized to protein rose steadily until day 41 and peaked at day 50, with the next and last time point showing a 25% decline. This study is difficult to compare with current work because: 1) only one data point after the peak was shown and statistical significance not assessed; 2) corresponding morphology was not shown; and 3) the enzyme levels were normalized to total protein and the absolute levels not given, i.e. the decline could potentially be attributed to myoblast proliferation.

Total protein is an important parameter in the characterization of protein turnover. In this study, total protein increased while DNA was stable during the 5 days after media was switched. After day 10, total protein remained constant for approximately ten days and then began to increase in parallel with DNA after day 20. The sudden increase in total protein after day 20 while muscle specific proteins degraded (tropomyosin and CPK) can be explained by the increased myoblast proliferation. The increase in total protein from day 5 through day 10 and subsequent stabilization correlates with previous previous work (Coleman and Coleman, 1972), which reported a similar increase and stabilization of total protein in chick primary cultures in the absence of DNA synthesis. It is not clear why there is an increase in total protein. It would be simple to attribute it to an increase in muscle specific protein; however, even after total protein stabilization, CPK continues to increase in our study, and both CPK and myosin increased in the study by Coleman and Coleman (1972) while total protein remained at best constant. This indicates that the total protein level is not strongly correlated with muscle specific proteins. Indeed Gulve et. al (1991) discussed the relative scarcity of myofibrillar protein in muscle cell cultures, which again points to the need to study a muscle specific protein during turnover experiments, as opposed to total protein levels.

Immunoflourescence staining for sarcomeric tropomyosin proved to be a powerful and relatively simple *in situ* technique to investigate temporal and spatial changes in muscle specific protein expression in L6 cultures. In the mixed population of myoblasts and myotubes, a monoclonal antibody to sarcomeric tropomyosin was used to support CPK data, to visualize myotubes at low magnification and to analyze structural differentiation at high magnification. Fluorescent micrographs revealed that sarcomeric tropomyosin was expressed after myoblast

differentiation, and in parallel with CPK and morphological analyses, tropomyosin degraded toward the end of the culture period.

Analysis of tropomyosin over the time course of these studies showed that tropomyosin degraded at different rates within each myotube and among different myotubes. Parallel immunoflourescence with F-actin showed that the myotubes remained intact while tropomyosin degraded. This observation may lead to the additional hypothesis that myotube proteins are specifically degraded during the deterioration process, which would suggest that specific and different degradation mechanisms occur within the cell as the deterioration process progresses. Additional data would be required to prove this hypothesis, but if proven, the present culture system may be used as a model to investigate these differing proteolytic mechanisms.

Preliminary results with primary human cells showed that a large number of the myotubes formed mature sarcomeric patterns. To date, very little work has been done with primary human skeletal muscle cells, and specifically, there are no published reports documenting culture of the skeletal muscle cells from Clonetics Corp. Used in the present study. However, human cells may represent a more physiologically relevant model than L6 cell for studying muscle wasting, and thus, recent work in our lab has focused on additional characterization of primary human muscle cells, using phase microscopy and tropomyosin staining. Results to date showed that these cells formed abundant myotubes (>80%) with very little myoblast overgrowth and well-developed sarcomeric banding patterns. However, myotube morphology appeared to degrade with time constants similar to the L6 cell cultures. Work is underway to characterize the human cultures to evaluate their potential as a model system.

It is well established both *in vivo* and *in vitro,* that passive mechanical stretching increases the level of muscle specific function (Vandenburgh and Kaufman, 1979; Goldberg, 1975). Passive stretching alters many different aspects of muscle cell function, including IGF-1 secretion (Perrone et al. 1995), amino acid uptake (Hatfaludy et al. 1989), prostaglandin release (Vandenburgh et al. 1989), and most importantly, myofibrillar protein turnover (Vandenburgh and Kaufman, 1979). These studies were all performed with primary cell cultures and no work has been done with cell lines. In particular, no work has been done with CPK regulation by mechanical stretching. Results with passive stretching indicated that total CPK, and hence, muscle specific activity can be increased in L6 cell cultures. These preliminary results indicate that CPK might also be regulated by stretching. If CPK can be shown to increase coordinately with the contractile proteins under stretching conditions, future studies would benefit because CPK rather than contractile proteins could be used as a measure of muscle specific function in order to optimize the cultures.

Future work with passive stretching requires that a more detailed biochemical study be completed before the true effect of stretching on muscle specific function can be assessed. In addition, dynamic stretching needs to be investigated where a number of parameters such as amplitude, frequency, and pulse width can be modulated in order to optimize cultures. Additionally, controls have to be run as well to investigate the causes of the increases in CPK. As it stands, the observed increase in CPK during mechanical stretching may have been due to myoblast growth, possibly caused by a disruption of contact inhibition after the substratum was stretched or by an increase in synthesis of growth factors by myotubes after they are stretched.

Thus, although preliminary results obtained using mechanical stretching appear promising, further biochemical characterization of this culture system is needed.

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