EARLY SATELLITE CELL ACTIVATION ON ISOLATED SINGLE MUSCLE FIBERS

BY

OREST J. PILIPOWICZ

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

Masters of Science (M.Sc.)

Department of Human Anatomy and Cell Science
University of Manitoba
Winnipeg, Manitoba

(c) August, 2000.

National Library of Canada

Bibliothèque nationale du Canada

Acquisitions and Bibliographic Services

Acquisitions et services bibliographiques

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-53205-4

Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
*****
COPYRIGHT PERMISSION PAGE

Early Satellite Cell Activation on Isolated Single Muscle Fibers

BY

Orest J. Pilipowicz

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

Master of Science

OREST J. PILIPOWICZ © 2000

Permission has been granted to the Library of The University of Manitoba to lend or sell copies of this thesis/practicum, to the National Library of Canada to microfilm this thesis/practicum and to lend or sell copies of the film, and to Dissertations Abstracts International to publish an abstract of this thesis/practicum.

The author reserves other publication rights, and neither this thesis/practicum nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS 4

TABLE OF FIGURES 5

TABLE OF ABBREVIATIONS 6

1. ABSTRACT 8

2. REVIEW OF LITERATURE 10

2.1 Basic Skeletal Muscle Structure 10
Satellite Cells 11

2.2 Skeletal Muscle Regeneration 12
Techniques for Studying Muscle Regeneration 12
The Isolated Fiber Technique for Studying Regeneration 13
Key Morphological Events in Muscle Regeneration 15
Satellite Cells and the Molecular Events in Regeneration 16
a. Satellite Cell Activation 16
Hepatocyte Growth Factor 17
b. Proliferation and Differentiation 18
Markers of Proliferation - DNA Synthesis 19
Markers of Differentiation - Myogenic Regulatory Factors 19
c. Fusion of Myoblasts into Myotubes 22

2.3 Questions Still Unanswered in Muscle Regeneration 23

2.4 Nitric Oxide 23
Nitric Oxide Synthase in Muscle 24
Liver Regeneration and Nitric Oxide 25
Nitric Oxide and Muscle Regeneration 26
Nitric Oxide Donors and Inhibitors 28
Using Isolated Fibers for Nitric Oxide Studies 29

3. HYPOTHESIS AND SPECIFIC OBJECTIVES 30

3.1 Hypothesis 30

3.2 Specific Objectives 30

4. MATERIALS AND METHODS 32

4.1 Animals 32

4.2 Isolating Flexor Digitorum Brevis Muscle Fibers From Mice 32
Dissection 32
Columns 33

Plating of Fibers 34
Addition of Potential Activators 35

4.3 Fixing Isolated Fibers 35

4.4 Identifying Satellite Cells on Intact Muscle Fibers 35
FITC Fluorescence 36
Histochemistry with DAB Detection 36

4.5 Identifying the Time Course of Fiber Death Using Marcaine 37

4.6 The Effect of Marcaine on Regeneration of Muscle Fibers 37

4.7 BrdU Staining for Proliferation of Satellite Cells 38

4.8 Counting Proliferating Satellite Cells 38

4.9 Preparing Crushed Muscle Extract (CME) 39
Determining CME Concentration 39

4.10 Electron microscopy of intact fibers 40

4.11 Microscopy and Photography 40

4.12 Statistical Analysis 41

5. RESULTS 42

5.1 Characterization of fibers 42
Electron Microscopy 42
Marcaine 42

5.2 Characterization of both Free and Attached Satellite Cells 44

5.3 Measuring Activation of Satellite Cells on Fibers 45

5.4 Effect of CME on Activation 45

5.5 Effect of HGF and L-Arg on Satellite Cells on Individual Fibers 46

5.6 Effect of Marcaine-Induced Membrane Damage on Activation 47

FIGURES AND LEGENDS 48

6. DISCUSSION 68

The Fiber Isolation Process - Comparison to other Studies 68

Free vs. Attached Satellite Cells 70

HGF and L-Arg in Activation 71

Effect of Marcaine-Damaged Fibers on Satellite Cell Activation. 73

Why Do Some Satellite Cells Not Become Activated? 75

Is the Isolated Fibers Technique an Effective Method for Studying Early Activation? 76

Possible Future Experiments 79

CONCLUSIONS 81

REFERENCES **83**

ACKNOWLEDGEMENTS

I would like to thank all the many people who have made this work possible. Without their help and support, my time in the department would have been impossible.

I'd like to first and foremost like to thank my parents, who have always been solidly behind me in all the things that I've pursued, not only with my Master's degree, but in all aspects of my life.

I'd like to also thank my supervisor, Dr. Judy Anderson. I've always admired her dedication to science and felt challenged by her persistence of critical thinking and problem solving. These skills are the ones that will be the most useful for me in the future - well beyond just science. Thank you, Judy, for making this a very enjoyable and rewarding two years. I'd also like to thank the members of my advisory committee, Dr. Wayne Lautt and Dr. Elissavet Kardami for their helpful advise and in helping to keep the direction of the project going forward.

Thank you also to the other people in the lab who I've worked with - Cinthya Vargus, Dr. Jiming Kong, Mike Weber, Ryan Skrabek and Ross Baker. You made the lab an enjoyable place to work and were always available for advice, help with an experiment or just to chat. I wish you all continued success in all your endevours.

I'd also like to thank The Department of Human Anatomy and Cell Science and all the support staff who have make my life so much easier. Big thanks go out to Paul Perumal for all his last minute scanning and printing.

And a huge thank you goes out to my coffee buddies - Jason Taylor, Marla Wilson and Claire Stockwell. You guys were always there when experiments didn't work and I needed someone to talk to, as well as be the first ones there to congratulate me when things were going well. I sincerely hope that we remain good friends in the future and I wish you only the best of luck:

Orest

TABLE OF FIGURES

Figure 1	Low magnification view of a representative muscle fiber from flexor digitorum brevis muscle of C57 mice after complete dissection.
Figure 2.	Electron micrograph of an isolated fiber
Figure 3.	Staining of fiber nuclei using ethidium bromide (EtBr).
Figure 4.	Addition of Marcaine + EtBr to fibers.
Figure 5.	Addition of fluorescein diacetate (FDA) to live and hypercontracted fibers.
Figure 6.	Identification of satellite cells on muscle fibers.
Figure 7.	Assessing satellite cell activation on isolated fibers by BrdU incorporation and immunostaining.
Figure 8.	Graphical summary of a typical experiment demonstrating the effects of CME, L-Arg and HGF on satellite cell activation.
Figure 9.	A graphical summary of proliferation data for attached and free satellite cells compiled from several experiments.
Figure 10.	Summary of the experiment made to determine the effect of Marcaine on proliferation of attached and free satellite cells.

TABLE OF ABBREVIATIONS

(BrdU)	Bromodeoxyuridine
(BSA)	Bovine Serum Albumin
(CEE)	Chick Embryo extract
(cGMP)	Cyclic Guanosine Monophosphate
(CME)	Crushed Muscle Extract
(CSR-2)	Controlled Replacement Serum-2
(DAB)	Diaminobenzidine
(ddH_2O)	Double Distilled Water
(DMEM)	Dulbecco's Modified Eagle Medium
(EDRF)	Endothelial-Derived Relaxing Factor
(EtBr)	Ethidium Bromide
(bFGF)	basic Fibroblast Growth Factor
(FDA)	Fluorescein Diacetate
(FDB	Flexor Digitorum Brevis
(GTP)	Guanadine Triphosphate
(bHLH)	basic Helix-Loop-Helix
(HGF)	Hepatocyte Growth Factor
(HGF/SF)	Hepatocyte Growth Factor/Scatter Factor
(HRP)	Horseradish Peroxidase
(HS)	Horse Serum
(L-Arg)	L-Arginine
(L-NAME)	N^G-nitro-L-Arginine Methyl Ester

(LTA)	Left Tibialis Anterior
(MEM)	Minimal Essential Medium
(MRF)	Myogenic Regulatory Factor
(NADPH)	Nicotinamide Adenine Dinucleotide Phosphate
(NO)	Nitric Oxide
(NOS)	Nitric Oxide Synthase
(PBS)	Phosphate Buffered Saline
(PCNA	Proliferating Cell Nuclear Antigen
(PHx)	Partial Hepatectomy
(RSOL)	Right Soleus
(RTA)	Right Tibialis Anterior
(RT-PCR)	Reverse-Transcription polymerase chain reaction
(SNP)	Sodium Nitroprusside
([3H]Tdr)	Tritiated Thymidine
(TBS)	Tris Buffered Saline
(TEM)	Transmission Electron Microscopy

1. ABSTRACT

Satellite cells are cells of the skeletal muscle lineage located between the basal lamina and sarcolemma of muscle fibers. Upon injury, normally quiescent satellite cells become "activated" to move, proliferate and differentiate into myoblasts which fuse onto existing myofibers or form new fibers. Hepatocyte Growth Factor (HGF) and crushed muscle extract (CME) have previously been shown to activate satellite cells *in vitro*. More recently, nitric oxide (NO) release from nitric oxide synthase (NOS) was suggested to activate satellite cells *in vivo*. We used isolated muscle fibers, a system that allows detailed study of activation signals, to examine the hypothesis that HGF and NO promote satellite cell activation. Normal mouse flexor digitorum brevis (FDB) muscles were dissected, digested in collagenase, and isolated fibers were plated on vitrogen-coated dishes. Fibers were grown in basal medium plus bromodeoxyuridine (BrdU), with or without compounds that could activate satellite cells, and fixed 48 hours later. Activation was assessed by counting BrdU positive attached, as well as free proliferating satellite cells per fiber. Isolated fibers were demonstrated to be intact by electron microscopy and ethidium bromide exclusion studies. CME and L-Arginine (L-Arg), the NOS substrate, increased the proliferation of satellite cells both on and off the fibers. However, HGF only increased proliferation of satellite cells that were free of fibers. These results suggest different roles for HGF and L-Arg in satellite cell activation. HGF likely decreases the adhesion of satellite cells to fibers and increases their mobility then proliferation, whereas L-Arg also promotes satellite cells still on the fiber to become activated following an injury. These two steps are now known to be distinct in their important role in activation. Satellite cell activation was decreased by damaged membranes and restored by HGF. This

demonstrates the importance of a live fiber on satellite cell activation. These results further increase our knowledge of satellite cell activation and introduce counts of free satellite cells as a novel way of assessing activation.

2. REVIEW OF LITERATURE

2.1 Basic Skeletal Muscle Structure

Skeletal muscle is the component of the musculo-skeletal system in the body responsible for voluntary movement and locomotion. Muscle is attached to bones via tendons and always crosses at least one joint. Through the coordinated contractions and relaxations among different muscles, specific and precise movements are achieved. Skeletal muscle structure is complex and many different components such as muscle metabolism, contractions, and regeneration must act simultaneously and continuously for proper function.

One skeletal muscle is made up of several hundred to several thousand myofibers, which typically extend from one tendon to another, or may connect onto extensions of surrounding connective tissue. Myofibers are long cylindrical syncitial cells surrounded by a sarcolemma, that contain numerous peripheral nuclei, sarcoplasm, and are neighbours to a relatively small number of satellite cells. Each myofiber is composed of myofibrils placed centrally, and is rich in organelles, particularly mitochondria.

Also important in muscle structure are the connective tissues which surround and connect the different components. Connective tissue in muscle confers structural integrity when muscle contracts or relaxes, and it also transmits force from myofibers to the tendons. Each individual myofiber is surrounded by a thin delicate layer celled endomysium. Groups of myofibers, called bundles or fascicles, are bound by a thicker layer called perimysium. The perimysium provides an environment for the network of

blood vessels, nerves and lymphatics that course through and supply the muscle itself. The outside layer of connective tissue surrounding groups of fascicles is called the epimysium. This is the thick and tough layer which separates the different muscles from each other, as seen in a gross dissection (McComas, 1996).

Satellite Cells

Satellite cells, first described by Mauro in 1961 by electron microscopy, are the key elements in skeletal muscle regeneration (Carlson and Faulkner, 1983). They are found between the sarcolemma and the basal lamina (Mauro, 1961) and make up 2-5% of all the nuclei found on the periphery of the skeletal muscle fibers (Bischoff, 1986a), although the number varies with muscle and fiber type. There is a narrow and uniform gap of 15-25nm between satellite cells and the sarcolemma (White and Esser, 1989) and satellite cells sit in a concavity of the fiber. They are difficult to distinguish from myonuclei using light microscopy as they are both approximately the same size and found in similar locations. Satellite cells have an ovoid shape, have a much greater nucleus to cytoplasm ratio than most cell types and their cytoplasm is elongated along the ends (reviewed in Grounds, 1991).

In very young growing muscle, some satellite cells are actively replicating and contributing nuclei to the fiber (Moss and Leblond, 1971). However, they are typically found in a quiescent state in mature adult fibers under normal conditions and do not express myogenic regulatory proteins (Russell *et al.*, 1992). Currently, the only marker for quiescent satellite cells is c-met (Cornelison and Wold, 1997), the receptor for hepatocyte growth factor (HGF). Satellite cells are the primary source of new myoblasts during

regeneration and are recruited into active cell cycling upon injury in a process called activation. They are not found in either cardiac or smooth muscle cells, as their repair processes differ from skeletal muscle.

2.2 Skeletal Muscle Regeneration

Muscle regeneration is the sequence of events that a muscle undergoes following an injury. The sequence of repair is similar to development although the timing is quite specific to repair (Yun and Wold, 1996) and the sequences vary in their dependence on particular gene expression capacity (McIntosh *et al.*, 1998). Differences between repair and development can also be attributed to differences in the cellular environment of the respective processes. A regenerating muscle contains preexisting muscle fibers and remnants of the basal lamina, and will thus have different signals and factors than those in a developing muscle (Chambers and McDermott, 1996).

Techniques for Studying Muscle Regeneration

Muscle regeneration has been studied extensively both *in vivo* and *in vitro*. Each of these techniques has their benefits in elucidating different parts of regeneration. *In vivo* methods have been used extensively by initiating a regeneration response while maintaining the muscle in its natural environment surrounded by non-muscle cells. Starting the repair process can be achieved by either denervating and devascularizing the muscle (Zacharias and Anderson 1991), minced muscle grafts (Lawson-Smith and McGeachie, 1997), exposure to a toxin such as marcaine or notexin (Lefaucheur and Sebille, 1995) or

applying a crush injury to a muscle (Grounds and McGeachie, 1989; McIntosh *et al.*, 1994). The crush injury initiates a synchronous repair response while maintaining the orientation of the muscle fibers and the basal lamina in its normal place. While *in vivo* methods have helped define the timing and sequence of many of the components of the regeneration process, the method does not allow for the fine control of the environment. Considerations must be made for the possible influence of surrounding cells, therefore making the analysis of the results more complex.

In vitro methods, such as primary cultures of satellite cells, can be done under simpler, more carefully controlled conditions (Allen *et al.*, 1995). Specific conditions can be maintained without the concern of interference from non-muscle cells. However, because the satellite cells are not found in their natural state using this system, they would not be completely quiescent as they would under normal conditions. Another *in vitro* method, which utilizes isolated muscle fibers, maintains satellite cells in their natural position between the sarcolemma and the basal lamina and allows for studies to be done under conditions more closely resembling those in the natural environment.

The Isolated Fiber Technique for Studying Regeneration

The isolated muscle fiber technique is a powerful method to determine the mechanisms involved in muscle regeneration. The process involves dissecting muscles out of an animal, cleaning it of connective tissue, nerves and vessels then separating the fiber bundles into individual fibers. The technique was first developed in 1977 by Bekoff and Betz, who used the flexor digitorum brevis (FDB) muscle from rats (Bekoff and Betz, 1977). Recently, mouse FDB have been used to examine myogenesis by satellite cells still

attached to the parent fibers (Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1999).

The FDB muscle was chosen for its short fiber length which makes it easier to handle, as longer fibers from extensor digitorum longus or soleus are damaged much more easily. FDB also allows for many fibers to be dissected from one muscle and provides a large pool of fibers for an individual experiment. This method is ideal for studying the relationship between a satellite cell and its host fiber as both satellite cells and the membrane maintain their natural states without the interference of nerves and vessels, which are undoubtedly sources of many hormones and growth factors. The single fiber model allows for the tracking of individual satellite cells as well as populations of satellite cells over time.

Initial studies of single fiber cultures were described by Bischoff (1986a, 1986b) who grew single fibers from rat in Minimal Essential Medium (MEM) + 10% Horse Serum (HS). That media kept the satellite cells on fibers quiescent when tested for proliferation 48 hours after plating. However, Yablonka-Reuveni (1994) observed that fibers grown in MEM + 10% HS behaved as primary cultures after more time in culture (with mobilization of satellite cells to leave fibers). This is possibly due to growth factors which exist in horse serum and those released from fibers that become damaged over time. More recent studies by both groups (Yablonka-Reuveni and Rivera, 1994; Bischoff, 1990a) use a serum replacement media (Control Serum Replacement Media-2) in their basal media to maintain satellite cell quiescence instead of the horse serum. Under these conditions the spontaneous proliferation is decreased significantly from that seen in horse

serum. For studies on mouse fibers, Dulbecco's Modified Eagle Medium (DMEM) is used instead of MEM (Yablonka-Reuveni *et al.*, 1999).

Another study used isolated fibers as a means of attaining satellite cells directly off of fibers to use for reverse-transcription polymerase chain reaction (RT-PCR, Cornelison and Wold, 1997). They isolated muscle fibers, hypercontracted them using Marcaine, then picked off satellite cells to study the order and timing of key components involved in regeneration. Interestingly, they considered those satellite cells quiescent, even though the membrane was obviously damaged before the satellite cells were removed.

Key Morphological Events in Muscle Regeneration

Muscle regeneration following an injury eventually leads to a completely repaired muscle by 7-21 days, depending on the extent of the injury and the presence of the basal lamina (Chambers and McDermott, 1996; reviewed in Grounds, 1991). Immediately following an injury to the sarcolemma, intracellular calcium activates proteases which inhibit normal mitochondrial respiration. Membrane damage also induces the activation of the C5b-9 attack complex, causing cell lysis and the recruitment of inflammatory cells. Macrophages and leukocytes infiltrate the damaged area and remove the dead tissue through phagocytosis (reviewed in Grounds, 1991).

By 24 hours after the injury, the damaged area is sealed off to segregate the necrotic portions of the muscle from the peripheral zone surrounding the injury (Hurme and Kalimo, 1992). The remaining basal lamina may act as a scaffold for the building of new fibers and it itself affects regeneration, as contact with intact fibers decreases the amount of subsequent satellite cell proliferation (Bischoff, 1990b). Following phagocytosis

and the sealing of the damaged area, revascularization and angiogenesis begin, followed shortly by the eventual reinnervation of the muscle (Burden, 1993). During the initial morphological changes that occur, the quiescent satellite cells become activated and begin the process of building new myofibers.

Satellite Cells and the Molecular Events in Regeneration

The muscle regeneration process can be divided into several events in sequence. A satellite cell first becomes activated, it proliferates, differentiates into myoblasts, then fuses with other myoblasts to form replacement fibers called myotubes.

a. Satellite Cell Activation

Activation of satellite cells occurs when a normal quiescent satellite cell is "turned on" and begins cycling to prepare for the subsequent repair process. Activation can be brought on by several means including traumatic injury, compression, exercise, training, stretching, suspension and denervation (Darr and Schultz, 1987, 1989; Appell *et al.*, 1988; Winchester *et al.*, 1991), thus indicating that there are multiple signals capable of initiating the regeneration process.

Upon activation, satellite cells undergo several changes. The nuclei enlarge and become euchromatic, the cytoplasm expands and organelles like mitochondria and rough endoplasmic reticulum hypertrophy (Schultz *et al.*, 1978, Schultz and McCormick, 1994). To date, only one factor, hepatocyte growth factor (HGF), has been demonstrated to activate quiescent satellite cells (Tatsumi *et al.*, 1998).

Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) is a heterodimer containing both an α and β chain. The 69 kDa α-chain (440 amino acids) and the 34 kDa β-chain (233 amino acids) are linked by a single disulfide bridge (Nakamura *et al.*, 1987) showing the protein has significant homology to plasminogen (Nakamura *et al.*, 1989). HGF is translated from a single mRNA as a single chain preproHGF then later processed extracellularly by a specific serine protease which converts it into its active form (Miyazawa *et al.*, 1993). HGF has also been shown to be identical to another factor called scatter factor, so named for its ability to "scatter" tightly growing clonal epithelial cells (Gherardi *et al.*, 1989), thus hepatocyte growth factor is often referred to as hepatocyte growth factor/scatter factor (HGF/SF).

HGF has been shown to have both mitogenic and motogenic roles (Rong *et al.*, 1993). It was initially identified as a strong mitogen in liver regeneration (Matsumoto and Nakamura, 1991) and has growth regulating activities for many types of cells including epithelial, endothelial, stromal and carcinomal cells (reviewed in Matsumoto and Nakamura, 1996).

HGF was first identified in growing and regenerating muscle in 1993 by Jennische *et al.* through *in situ* hybridization and Northern Blot analysis. It was detected at 2, 4, and 10 days after birth in rats. However, HGF expression was not seen in adult muscle, thus implicating a developmental role (Jennische *et al.*, 1993). When HGF was added to primary cultures of satellite cells *in vitro*, an increase in their proliferation was seen at 24 hours after plating (Allen *et al.*, 1995). As well, the number of cells staining positive for proliferating cellular nuclear antigen (PCNA; a marker for proliferation) increased as early

as 24 hours after HGF was added to cultures, indicating that HGF caused an earlier entry into the cell cycle (Allen *et al.*, 1995).

Bischoff (1986b) identified a mitogenic saline extract from crushed muscle (CME) that was capable of activating satellite cells on isolated fibers that did not have mitogenic effects on fibroblasts. Recently, CME was found to contain HGF and had the same scattering ability on MDCK cells as HGF (Tatsumi *et al.*, 1998). Purified HGF added to primary cultures of satellite cells with a low dose of CME increased proliferation. Addition of the antibody to HGF returned the values to control levels (Tatsumi *et al.*, 1998).

In vivo immunolocalization studies showed that HGF is localized on the basal lamina surrounding the fibers, while its receptor, c-met, is found in quiescent satellite cells. However, HGF was found colocalized with c-met in the satellite cells approximately 10 minutes after a crush injury *in vivo* (Tatsumi *et al.*, 1998, Anderson, 2000). This suggests that HGF not only effects satellite cell proliferation, but may act quite early as an "activator" of satellite cells.

b. Proliferation and Differentiation

Once a satellite cell is activated, it proliferates so as to provide a large pool of available cells for new muscle formation. To date, several factors have been shown to increase the proliferation of satellite cells in primary cultures as well as on isolated fibers from rat. Using isolated fibers, Bischoff demonstrated that chick embryo extract (CEE), CME and basic fibroblast growth factor (bFGF) all induced satellite cells to proliferate over time (Bischoff, 1986a, 1986b).

Markers of Proliferation - DNA Synthesis

Several markers of the regeneration process, specifically for proliferation and differentiation have been determined. Proliferation of satellite cells, marked by DNA replication using tritiated thymidine ([3H]Tdr), has been identified to occur at 24-36 hours after crush injury (Grounds and McGeachie, 1987). As well, proliferating cell nuclear antigen (PCNA), an auxiliary protein to DNA polymerase δ which is expressed in the nuclei during S phase of the cell cycle, can also mark proliferation (Johnson and Allen, 1993). PCNA was used to identify changes in the time of satellite cell proliferation when different stimuli such as CME and FGF were added to plated satellite cells (Johnson and Allen, 1993). Proliferation on isolated fibers is also age-dependent as satellite cells from older rats (9-10 months) proliferate later than cells from younger, growing rats (3 weeks; Yablonka-Reuveni and Rivera, 1999). This reflects the latency in proliferation observed in primary satellite cell cultures of aged adult rats vs. cultures from young rats (Allen *et al.*, 1995)

Markers of Differentiation - Myogenic Regulatory Factors

Several of the regulators of muscle-specific gene expression involved in muscle cell development are also involved in proliferation and differentiation during regeneration. The myogenic regulatory factor (MRF) or MyoD family of transcription factors including MyoD, Myf-5, myogenin, and MRF4 are key regulators during muscle regeneration (Biben, 1993). MRFs are the key signals in muscle lineage development in embryogenesis and share a homologous basic helix-loop-helix (bHLH) region. The basic portion is necessary for binding to specific DNA sequences and the HLH portion is necessary for

dimerization of the MRF protein prior to it binding to the DNA (Davis *et al.*, 1990). When bound to an E-box sequence of DNA (CANNTG, where N represents any nucleotide), MRF proteins control the expression of many muscle-specific genes such as acetycholine receptor α-subunit gene (Piette *et al.*, 1990) and muscle creatine kinase (Lassar *et al.*, 1989). MRFs, when expressed either *in vivo* or *in vitro*, can induce non-determined cells like fibroblasts to enter into the myogenic program (Russo *et al.*, 1998; Weintraub, 1993). Though MRFs are not expressed in quiescent satellite cells, they are identified as the key regulators of muscle cell differentiation over the course of activation after injury (Cornelison and Wold, 1997; Yablonka-Reuveni and Rivera, 1994).

MRFs are found in 2 semi-redundant pairs: MyoD and myf5 are displayed early and are necessary for determination or commitment to the muscle lineage (Yun and Wold, 1996), whereas myogenin and MRF4 are late genes and are for differentiation (Weinthraub, 1993). MyoD is the first to be detected 6 hours after injury, and is thought to be involved in satellite cell commitment into the cell cycle and in regulatory proliferation during regeneration (Megeney *et al.*, 1996; McIntosh *et al.*, 1998). However, the absence of MyoD expression in knockout mice (myoD-/-) produces no adverse effects on development, although myf5 levels increase 4-fold in compensation (Rudnicki *et al.*, 1992). By comparison, in myf5 knockout mice, loss of myf5 expression delays development of muscle until MyoD was expressed (Braun *et al.*, 1994). Therefore, although mice lacking MyoD exhibit no obvious effect in developing muscle, the regeneration of those muscles is delayed and aberrant (Megeney *et al.*, 1996, McIntosh *et al.*, 1998).

The late genes, myogenin and MRF4, are expressed after MyoD and myf5 and are for differentiation of myogenic cells (Weintraub, 1993). Myogenin, identified as early as 6 hours after injury (Kami *et al.*, 1995; Grounds *et al.*, 1992), was demonstrated to be the most important factor for differentiation *in vivo* compared to the other members of the bHLH family (Weintraub, 1993).

The mRNAs of proto-oncogenes c-Fos and c-Jun have been detected as early as 3 hours after injury (Kami *et al.*, 1995). The proteins for these genes have been implicated in inhibiting the expression and enhancing effects of MyoD and myogenin. This suggests they are involved in regulating the regeneration process, including the transactivation of the muscle creatine kinase enhancer (Weis, 1994; Li *et al.*, 1992).

The growth factor, basic fibroblast growth factor (bFGF), has been the subject of many *in vitro* studies of cultured cell lines. bFGF suppresses muscle differentiation by repressing transcription of both myogenin (Brunetti and Goldfine, 1990) and MyoD in culture (Vaidya *et al.*, 1989). However, bFGF protein and message can be colocalized with myogenin and myoD *in vivo* in regenerating muscle (Garrett and Anderson, 1995). bFGF plays a role in proliferation during the regeneration process. On isolated fibers, more satellite cells were recruited into the cell cycle in the presence of bFGF than without it (Bischoff, 1986a; Yablonka-Reuveni and Rivera 1994) and bFGF induced a two-fold enhancement of proteins involved in muscle differentiation (i.e. myogenin and MyoD), although the general pattern of proteins expressed stayed the same both with and without bFGF (Yablonka-Reuveni and Rivera, 1994).

c. Fusion of Myoblasts into Myotubes

After myoblasts reach their peak numbers between 48-72 hours after a crush injury (McGeachie and Grounds, 1987), DNA synthesis in myogenic cells stops (Roberts *et al.*, 1989) and fusion into myotubes begins. Fusion usually is at its maximum level by 4-5 days after injury in mice (Robertson *et al.*, 1990). Fusion is facilitated by many signals including growth hormones, pH, glycoproteins and adhesion-specific receptors. In particular, four important classes of molecules are involved in this process: N-CAM, cadherins, integrins (reviewed in McDonald *et al.*, 1995) and disintegrins (Yagami-Hiromasa *et al.*, 1995).

The process of adhesion occurs in three general steps (Robert *et al.*, 1990). The first step involves the recognition of cells that are to fuse together such as two myoblasts or a myoblast to a myotube. M-cadherin also has role in the alignment and fusion of myoblasts to form and then enlarge myotubes (Donalies *et al.*, 1991). The second step has lipids from vesicles of parent cells fuse with the sarcolemma of each parent cell, thus destabilizing the cell membrane. This step is dependent on cations such as Ca^{+2} and H^{+} to neutralize the negatively charged membranes and reduce their electrostatic repulsions (Bischoff and Holtzer, 1969). In the third step, the modified membranes fuse with each other, thus forming the multi-nucleated myotube. During the fusion process, the original basal lamina persists, but after some time, the original sheath dissolves and a new layer is formed (Hansen-Smith and Carlson, 1979).

2.3 Questions Still Unanswered in Muscle Regeneration

Although muscle repair has been well studied and many of the involved factors have been elucidated, several important questions still remain, particularly concerning the activation of satellite cells. A large unsolved question in the paradigm concerns the first 10 minutes after an injury before HGF binds with c-met. For a satellite cell to be completely inactive and unresponsive for the first 10 minutes after an injury is unlikely. The results of an injury are quite traumatic, causing muscle hypercontraction and extreme shear stress on the muscle fibers. Certain enzymes, such as nitric oxide synthase (NOS), are responsive to such mechanical signals and can be activated to release nitric oxide (NO) immediately after an injury or trauma (Fleming *et al.*, 1997). In liver regeneration, where HGF also functions as an activator following an injury, NOS has been demonstrated to respond immediately after an injury (Wang and Lautt, 1998) and has recently been suggested to be an activator of satellite cells *in vivo* (Anderson, 2000).

2.4 Nitric Oxide

Nitric oxide is a free diffusible gas involved in mediating many important cellular functions. Robert Furchgott first identified a short-lived vasodilatory factor released from the endothelium associated with precontracted vascular smooth muscle cells, when acetylcholine or carbachol were added to vessels in culture (Furchgott and Zawadzki, 1980). He named the factor endothelial-derived relaxing factor (EDRF). Several years later, EDRF was identified as nitric oxide (NO). NO is involved in a vast number of processes ranging from vasoregulation to penile erectile function (Moncada and Higgs,

1993) and has recently gained notoriety as the vasodilator component in the drug Viagra (Goldstein *et al.*, 1998). It also has important roles in limb muscle as it regulates blood vessel tone (Moncada and Higgs, 1993), contractile function (Kobzik *et al.*, 1994) and increases glucose uptake into muscle cells during exercise (Roberts *et al.*, 1997). NO interacts with the heme moiety of soluble guanylyl cyclase, causing an allosteric transformation of the enzyme which and leads to the formation of cGMP from GTP (Kobzik *et al.*, 1994). It is generally believed that cGMP is a secondary messenger for NO (Reid, 1998).

Nitric Oxide Synthase in Muscle

Nitric oxide synthase (NOS) releases nitric oxide by converting L-arginine into L-citrulline, requiring NADPH, flavin adenine dinucleotide and tetrahydrobiopterin as cofactors. Nitric oxide synthase has three known isoforms in muscle: Type I (also known as neuronal constitutive NOS or nNOS), Type II (inducible or i-NOS), and Type III (endothelial constitutive or ec-NOS) (reviewed in Reid, 1998). Type I and III are constitutively expressed and Type II is regulated at the transcription level, often as part of an immunological stress response.

Neuronal NOS (nNOS) was first identified in skeletal muscle as an mRNA product in 1993 (Nakane *et al.*, 1993). An alternatively spliced form, NOS-Iμ, is found primarily in fast (Type II) muscle fibers and located just underneath the sarcolemma where it is anchored by α1-syntrophin to the dystroglycan complex (Brenman *et al.*, 1996). In the dystrophin-deficient *mdx* mouse and in patients with Duchenne muscular dystrophy, NOS-Iμ is not bound to the complex and is displaced throughout the cytoplasm and expressed

at lower than normal levels (Brenman *et al.*, 1995). NOS-I protein levels greatly increase after intense exercise (Balon, 1999), suggesting that NO helps mediate blood flow in skeletal muscle.

Liver Regeneration and Nitric Oxide

Liver regeneration and muscle regeneration share several similarities. Following an injury to the liver, normally quiescent hepatocytes begin to proliferate, undergo one or two rounds of proliferation and then return to their non-proliferative state, much like satellite cells in muscle (reviewed in Fausto *et al.*, 1995).

Studies of liver regeneration in rats have demonstrated that NO can trigger the regeneration process (Wang and Lautt, 1998). Following a partial hepatectomy (PHx; Higgins and Anderson, 1931), where two-thirds of the liver is removed forcing all portal flow through the remaining vascular bed, several reports have shown restoration of the liver mass over time. Within the first 48 hours, approximately 50% of the liver mass was restored and complete restoration of the liver was achieved within one week (reviewed in Columbano and Shinozuka, 1996). However, if a vascular shunt is constructed to divert the portal flow around the liver, so PHx does not increase portal flow, regeneration was not significant (Mann, 1940). It has been recently demonstrated that NO is released due to the shear stress resulting from the increased portal flow (Macedo and Lautt, 1998) and also released from vascular endothelial cells by shear (Dimmeler *et al.*, 1999). Proliferating factors found in the serum of liver following a PHx were decreased after addition of the NOS inhibitor L-N-nitroarginine (L-NAME) and restored with L-arginine (L-Arg), the substrate for NOS (Wang and Lautt, 1998). This suggests that NO acts as the trigger that

initiates the regeneration process in liver. Previous to the discovery of NO as that initiating factor in liver regeneration, the earliest factors known to be involved in this process were several proliferating factors found in the plasma following surgery, including HGF (reviewed in Michalopoulos and DeFrances, 1997).

Nitric Oxide and Muscle Regeneration

Present studies from our lab suggest that NO may be the messenger that activates satellite cells to enter the cell cycle (Anderson, 2000). In an injury, there is significant movement and shearing between the sarcolemma and the basement membrane. Because NOS is responsive to physical stress of a cell (Fleming *et al.*, 1997) an injury could activate NOS to release massive amounts of NO. Because satellite cells are located below the basement membrane and are in close contact with the sarcolemma, they are ideally situated to be responsive to a large influx of NO from NOS-Iμ.

In vivo studies in our lab examined the role of NO in the activation of satellite cells (Anderson, 2000). Mice were injected with either L-NAME, L-Arg or saline and given a crush injury to the right tibialis anterior (RTA). The RTA, left tibialis anterior (LTA) and right soleus (RSOL) were dissected at various times afterwards and the number of satellite cells which had loosened from the fibers were counted. Immediately following the crush injury to a normal mouse, twice as many satellite cells were isolated from crushed RTA than the contralateral left tibialis anterior (LTA). Ten minutes after the injury, the RTA cell yield decreased and the LTA increased from the basal level. Injecting L-NAME (the inhibitor for NOS) into the mouse before the injury delayed the increase of satellite cells coming off the RTA for 10 minutes. Injecting the NOS substrate, L-Arg, into the mouse

increased the number of satellite cells coming off both the RTA and LTA and maintained the high level for 30 minutes. These results show that modifying the function of NOS (and thus NO release) following an injury changes both how and when satellite cells come off the fiber. Specifically, enhancing NOS activity increased activation and inhibited NOS decreased activation.

Histological and immunostaining studies have demonstrated that differences in the size and position of satellite cells in the RTA compared to the LTA immediately following an injury were delayed by NOS inhibition by L-NAME (Anderson, 2000). Immediately following an injury in saline injected mice, the RTA showed many larger satellite cells, had a higher ratio of cytoplasm to nucleus and had a higher proportion of cells with c-met and HGF colocalization than the contralateral LTA. However, in L-NAME injected mice, the RTA and LTA both had thin attenuated cells at time 0. Large satellite cells and colocalization of c-met with HGF was not seen until 10 minutes after the injury in the RTA. The inhibition of NOS in normal mice through L-NAME delayed the onset of changes to a satellite cell that typically occur within the few minutes following an injury.

The cell yields from the saline-injected dystrophin-deficient *mdx* mouse were quite different to those of normal mice and resemble those of NOS-inhibited normal mice (Anderson, 2000). The normal LTA basal levels were 30% higher that those in normal mice. The RTA cell yield immediately after the injury did not increase and was similar to the LTA. The rise in cell yield did not occur until 10 minutes after the injury and dropped somewhat at 30 minutes. NOS-I knockout mice showed very similar cell yields to *mdx* mice (Anderson, 2000). Because *mdx* mice have low levels of NOS distributed throughout

the cytoplasm they would not have a large, concentrated release of NO upon injury due to the shear stress.

Nitric Oxide Donors and Inhibitors

Several molecules have been used experimentally to mimic an endogenous release of NO. Because NO is so unstable and cannot be directly added to a system, molecules that either release NO into the system or enhance the activity of NOS must be used. Even bubbling of NO gas in media is not sufficient, as the NO reacts with oxygen dissolved in the buffer and is rarely effective (Feelisch, 1998). NO donors, also known pharmacologically "nitrovasodilators", have been used therapeutically for over a century. Nitroglycerin and isoamyl nitrite were commonly given for relief of angina pectoris in the late 1800's (Feelisch, 1998) and are known as the classic NO donors. These compounds are transformed by the body, releasing NO into the system. The NO activates soluble guanylate cyclase thus producing vasorelaxation (Kishnani and Fung, 1996).

Several classes of donors have been discovered, each releasing NO into the system by chemical, enzymatic or a combination of both mechanisms (reviewed in Feelisch, 1998). There are five types of NO donors: organic nitrates, organic nitrites, inorganic nitroso compounds, sydnonimines and s-nitrosothiols (Tullett and Rees, 1999). Each of these families vary in their potency, mode of delivery and mechanism of NO release. NO is toxic at too high a concentration and causes cellular damage. Therefore, the type of donor is an extremely important consideration when NO release is produced experimentally.

The most commonly used inhibitor of NOS is N^G-nitro-L-arginine methyl ester (L-NAME). It is a competitive inhibitor of the enzyme and can be administered either orally

(Miller *et al.*, 1996), via injection (Anderson, 2000) or added to culture media (Schott *et al.*, 1995)

Using Isolated Fibers for Nitric Oxide Studies

The purpose of our experiments is to further characterize the initiation process of muscle regeneration using an *in vitro* isolated fiber technique. This technique has been used extensively to characterize the regeneration process during proliferation and differentiation (Bischoff 1986a, 1986b, 1990a, 1990b; Yablonka-Reuveni and Rivera, 1994; Cornelison and Wold, 1997), but has never been applied as a model to study the immediate effects after an injury.

The isolated fiber technique may be ideal for studying the activation of satellite cells as it is the only *in vitro* method which maintains satellite cells situated in their normal position. Therefore, it must be demonstrated that both the basal lamina and the membrane are intact and not damaged during the isolation process. Any disruption to either of them would hinder any studies on early activation as NO release may be dependent on shear stress - possibly between the basal lamina and the sarcolemma. It must therefore be established that the fiber is intact.

Many different methods have been used for assaying satellite cell activation and myogenesis on isolated fibers. Each method involves measuring the downstream markers after activation. These include proliferation through measurements of PCNA (Yablonka-Reuveni and Rivera, 1994) and tritiatied thymidine ([^{3}H]TdR; Bischoff, 1986a, 1986b, 1990a, 1990b) as well as MRFs such as MyoD and myogenin (Yablonka-Reuveni and Rivera, 1994). The thymidine analogue, 5-bromo-2-deoxyuridine (BrdU), has also been used for measuring proliferation of satellite cells in culture (Allen *et al.*, 1995).

3. HYPOTHESIS AND SPECIFIC OBJECTIVES

While many of the molecules involved in muscle regeneration have been characterized, the relationship between fibers and satellite cells has not been well examined during the regeneration process, particularly at the earliest stages which concern activation of satellite cells. To this date, studies on the effect of the two most early activators, HGF and NO, have been done exclusively on cultured satellite cells or *in vivo*, which either do not maintain satellite cells in their natural position or are overly complex to enable detailed study. Better *in vitro* studies are needed to ensure quiescent satellite cells become activated when situated in their normal position and help in defining any relationship between HGF and NO.

3.1 Hypothesis

It is hypothesized that nitric oxide release following injury activates quiescent satellite cells on normal single intact fibers, initiating the cascade of events that lead to DNA synthesis, and proceeding onto muscle regeneration *in vivo*.

3.2 Specific Objectives

1. Isolate and characterize individual fibers from the flexor digitorum brevis muscle from normal mice and test the viability of single fiber culture as a technique for studying satellite cell quiescence and activation. Fibers will be examined using electron microscopy on single fibers to ensure that the basal lamina is still intact and characterized as intact using the exclusion of ethidium bromide (EtBr) from myonuclei.

2 Test the effect of potential activators or compounds on satellite cell activation (proliferation) by counting the number of satellite cells that have incorporated BrdU into DNA at 48 hours after treatment of intact fibers in culture.

4. MATERIALS AND METHODS

4.1 Animals

The C57-BL/6 mice used for these experiments were maintained and housed according to the guidelines of the Canadian Council of Animal Care at the University of Manitoba Animal Care Facility. All mice were male between 8 and 10 weeks of age and were provided with access to food and water 24 hours per day. Each experiment used fibers from 1-3 mice which were pooled together after the dissection.

4.2 Isolating Flexor Digitorum Brevis Muscle Fibers From Mice

Dissection

Flexor digitorum brevis (FDB), the muscle which flexes the toes, was isolated from C57-BL/6 mice. All instruments were autoclaved before use and periodically rinsed in 70% ethanol to maintain sterility at all times. The mice were anaesthetized by ether inhalation then sacrificed by cervical dislocation. The mouse was skinned and the FDB muscle, found on the plantar surface of the foot, was exposed. FDB was carefully removed by grasping and lifting the proximal tendon and cutting away the connective tissue found on either side of it. When the muscle was completely exposed, the four distal tendons were cut and the muscle was placed into a 60mm culture dish with approximately 5ml of Dulbecco's Modified Eagle Medium (DMEM; Gibco) + 10% Horse Serum (HS; HyClone). If more than one mouse was used for an experiment, the muscles were combined at this time.

The muscles were individually transferred to separate dishes with DMEM-HS Fat, nerves and vessels were then pulled off the muscle using sharp forceps under a dissecting microscope. Connective tissue surrounding the muscle was also removed by holding the proximal tendon with one pair of forceps then using the other to pull off the connective tissue. The muscle connective tissue was then digested with 0.2% collagenase (Type I, Sigma) in DMEM for 2½ hours at 37°C in an atmosphere of 5% CO_2 with occasional mixing.

The muscles were then transferred into another dish using a flamed wide-bore glass pipette filed and flame polished and rinsed in 5ml DMEM-HS. The individual bundles were then transferred into fresh DMEM-HS, separated, and any remaining connective tissue removed using the forceps. This dissection lasted approximately 45 minutes for 6 FDBs. The fibers were then gently triturated 30-50 times using the wide-bore pipette to dislodge individual fibers from the bundles. The remaining bundles were then transferred into another dish with fresh DMEM-HS and fibers were further removed by using a brushing motion with forceps moved along tendon remnants. The bundles were again triturated 30-50 times and remaining bundles were transferred to a fresh dish of medium. This was repeated 2-3 more times until a sufficient number of fibers had been teased off the tendons. Any remaining fiber bundles were then removed from the dish using forceps and discarded.

Columns

To separate the fibers from any remaining connective tissue and bundles, the liquid from the dishes containing the free fibers was pooled and placed into a 15ml glass column

containing approximately 10ml of DMEM-HS After 10-15 minutes, the fibers sedimented to the bottom, and most connective tissue and bundles floated After the fibers had settled to the bottom, media was removed from the top of the column until 2ml remained This was then gently mixed and placed into a new column containing 10ml of media and again allowed to settle. This procedure was repeated 2 more times. After the final column, the media was transferred into a 60mm dish and any remaining bundles and connective tissue was removed by visual inspection under a microscope, leaving a clean preparation containing only individual fibers.

Plating of Fibers

Excess media was removed from the dish until the appropriate volume needed for an individual experiment remained, according to the desired number of dishes per treatment. The fiber suspension was then plated in 35-40µl aliquots onto 35mm dishes. Dishes were precoated with 120µl of a 1:6 mixture of vitrogen in 7×DMEM, by using 200µl pipette tips which had been cut and flamed smooth to make a larger bore. The fibers were then placed at 37°C with 5% CO_2 for 20-30 minutes to allow the vitrogen to dry and for fibers to adhere to the bottom of the dish. The plates were then removed from the incubator and 1.0ml of basal growth media was added. Each dish contained between 100-400 fibers and at least 3 dishes were used and averaged for each compound tested.

Basal growth media consisted of DMEM + 20% Controlled Replacement Serum-2 (CSR-2; Sigma) + 1% HS + 1% antibiotic/antimycotic (Gibco) + 0.1% gentamycin (Gibco) + 0.002% BrdU (Sigma). Various supplements were added depending on the experiment. Fibers were incubated at 37°C with 5% CO_2.

Addition of Potential Activators

Potential activators of satellite cells were added at the time of plating and replenished in fresh media at 24 hours. All compounds were added in the basal media as follows. L-Arginine (L-Arg; Sigma) was made up as a 10mM stock solution in sterile double distilled water (ddH_2O) and added to the media to its final concentration. Hepatocyte Growth Factor (HGF; R&D Systems) was added from frozen aliquots of 100ng/uL. Crushed muscle extract was prepared as noted below (4.9).

4.3 Fixing Isolated Fibers

To fix fibers for immunohistochemistry, dishes were removed from the incubator and rinsed twice with 1.5ml warm DMEM. One ml of an alcohol-acid mixture (90% ethanol, 5% glacial acetic acid) was then added to the dish for 20 minutes, removed and dishes were allowed to air-dry in a laminar flowhood for 10-15 minutes. The dishes were stored in tris buffered saline +1% HS (TBS-HS) at 4°C until needed.

4.4 Identifying Satellite Cells on Intact Muscle Fibers

To positively identify satellite cells attached and free from fibers, an antibody against c-met, the receptor for HGF, was used. Two different methods were used, one using FITC and the other using histochemistry and diaminobenzidine (DAB).

FITC Fluorescence

Following a minimum incubation of 24 hours in TBS-HS at 4°C, the fibers were rinsed three times with TBS + 0.05% Tween20 (TBS-Tween20). The fibers were incubated in anti-c-met primary antibody made in rabbit (1:75 dilution in TBS-HS, Santa Cruz) at room temperature for 1 hour, then overnight at 4°C. Negative controls lacked the primary antibody. The plates were then rinsed 3 times with TBS-Tween20 and incubated with secondary anti-rabbit FITC (1:200 dilution in TBS-HS) for 2 hours at room temperature. Bis-benzimide (Hoescht 33258), a stain for nuclei, was added to plates for 30 seconds, then plates were rinsed 5 times with TBS-Tween20 and mounted with aqueous mounting medium.

Histochemistry with DAB Detection

Fiber dishes were blocked for a minimum of two hours in primary diluent (1% HS, 0.1% Bovine Serum Albumin (BSA) and 0.3% Triton in phosphate buffered saline (PBS)), then rinsed 3 times in PBS. Anti-c-met antibody was diluted 1:300 in primary diluent and placed on fibers to incubate at room temperature for 1 hour, then overnight at 4°C. The dishes were next rinsed in PBS and blocked overnight in secondary antibody diluent (1% BSA in 0.3% Triton in PBS). Secondary antibody (anti-rabbit, HRP-linked whole antibody) was diluted 1:200 in secondary diluent, placed on fibers to incubate for 2 hours then rinsed well in PBS. DAB solution was prepared by added 25mg DAB solution, 100µl Ni/Co colour intensifier and 200µl for 15 minutes at room temperature until sufficient colour had developed and were then coverslipped.

4.5 Identifying the Time Course of Fiber Death Using Marcaine

Marcaine, a myotoxic anaesthetic, was used to kill live fibers. Ethidium bromide (EtBr) is a fluorescent dye which intercalates into the DNA of dead cells but is not taken up into live fibers. In order to examine whether the fiber membrane was important for activation, the concentration of Marcaine which caused fiber damage, but did not hypercontract the fibers immediately was needed. Media was mostly decanted from freshly plated fibers and a number of fibers were located using a microscope at 40× magnification. Two or three drops of either EtBr (2.5μg/ml) in DMEM or several different concentrations of Marcaine (0.005% to 0.05%, bupivicaine, Sigma) dissolved in DMEM + EtBr (2.5μg/ml) were added to live, unfixed fibers under continual observation. Fiber hypercontraction was observed using a phase lens and the time course of EtBr nuclear staining was observed under UV light using a red filter.

4.6 The Effect of Marcaine on Regeneration of Muscle Fibers

Activation of satellite cells was examined after briefly exposing fibers to a low dose of Marcaine, permeabilizing them (allow entry of EtBr) without hypercontraction. Freshly plated fibers were incubated in either Marcaine (0.025%) in DMEM-HS or just DMEM-HS for 5 minutes at room temperature. The plates were rinsed once with DMEM then grown in basal media with or without HGF (20ng/ml) at 37°C for 48 hours. Fibers were fixed, stained and counted for satellite cell activation.

4.7 BrdU Staining for Proliferation of Satellite Cells

Fixed plates of fibers were rinsed with PBS then incubated in 2N HCl at 37°C for 1 hour to denature the DNA. The plates were rinsed and incubated with a 1:1000 dilution of primary antibody (anti-BrdU; Amersham) in primary diluent (90% 0.01M PBS, 10%HS and 1% BSA) for 2 hours at room temperature with gentle agitation. The dishes were rinsed and secondary antibody, anti-mouse HRP-linked with secondary diluent (1:200 Amersham, 1:300 Sigma in1% BSA in PBS), was added for 1 hour at room temperature. The dishes were rinsed well with PBS then DAB solution was added for 15-20 minutes until sufficient colour had developed.

4.8 Counting Proliferating Satellite Cells

As the index of satellite cell activation and proliferation, BrdU-stained satellite cells, both attached and free from fibers, were counted under the microscope using magnification of 100 or 200. All dishes were given a code that was not broken until after all the dishes were counted. The average number of proliferating satellite cells on all live fibers were first scored on each dish. The same dish was then re-examined to count the number of live and hypercontracted fibers, and the number of free satellite cells. The number of free satellite cells per fiber was calculated from those observations. Free cells were confirmed by c-met staining to be satellite cell derived myogenic cells.

4.9 Preparing Crushed Muscle Extract (CME)

CME was prepared as described by Chen and Quinn (1992). C57-BL/6 mice were sacrificed by cervical dislocation then the quadricep, triceps surae tibialis anterior, triceps brachii and pectoralis major muscles were dissected from the mice by cutting carefully at both tendons. The muscles were given a gentle squeeze by depressing the muscles approximately 1mm with a dull pair of forceps for 1 second at 6-7 sites along the larger thigh muscles or 5 times along the smaller ones. The muscles were then incubated on ice in PBS (approximately 1g muscle/1.5ml PBS) for 90 minutes with gentle stirring. Approximately 2.5g of muscle was obtained from each mouse. Following the incubation, the slightly pink soluble extract was separated by centrifugation at 1000g for 5 minutes at 4°C, decanted then sterilized by filtering through a 0.2μm filter. The CME was distributed into 100μl aliquots, immediately frozen and stored at -20°C until needed. The CME was never thawed more than once and typically was made fresh every 6-8 weeks. Deterioration of CME was only noted in two experiments when stored longer than 8 weeks and resulted in a much greater number of hypercontracted fibers over time when added to live fibers.

Determining CME Concentration

The protein concentration in the CME was assayed by the method of Bradford (1976) where unknown concentrations of samples were compared to a standard curve of known protein concentrations. An immunoglobulin standard (1.5μg/μl) was diluted 1:9 in ddH_2O (100μl:900μl). It was distributed into 5 tubes so their final concentrations ranged from 0.0038μg/μl to 0.019μg/μl when diluted up to 1ml with ddH_2O. The blank contained none of the standard, but was otherwise treated the same. The CME sample was prepared

by diluting 10μl of the sample into 390μl ddH_2O, mixing, then adding 100μl of that solution to 900μl H_2O in replicate. All standards and samples were mixed well, and 400ml of a 2:1 dilution of BioRad Protein Assay in ddH_2O was added to each tube and mixed. The reaction was allowed to proceed for a minimum of 5 minutes. The absorbencies were read at 595nm and the samples were averaged to get the final result. Concentrations of CME extracts were approximately 6.0mg/ml (7.0mg/g muscle).

4.10 Electron microscopy of intact fibers

Fibers were visualized under electron microscopy to confirm the presence of the basal lamina after the dissection and isolation of individual fibers. Freshly isolated fibers were fixed in 2% glutaraldehyde in PBS and rinsed with fibers settling to the bottom of an eppendorf tube between steps. The fiber pellet was embedded in warm agarose, post-fixed in OsO_4 and processed for routine TEM in methylate. Sections were stained in uranyl acetate and lead citrate and viewed (Phillips 201) to visualize the satellite cell relationship to external lamina and the sarcolemma.

4.11 Microscopy and Photography

Fibers were observed using an Olympus BHT-2 RFCA light microscope equipped with a bright field, phase contrast and epifluorescence optics. Photographs were made with an Olympus C-35AD-4 camera using Fuji Professional Colour Film (ASA400) and scanned using an Olympus ES-20 scanner. Images were transferred to Corel Draw and Adobe Photoshop for enlargement and contrast enhancement. Images were printed using an Epson 700 ink-jet printer on high quality glossy paper.

4.12 Statistical Analysis

Appropriate statistical analysis were made using NWA Statpak (Northwest Digital Inc) and Excel (Microsoft) software according to standard criteria (Hassard, 1991) Single sample t-tests were used to investigate whether there was significant difference between ratios of pooled means of potential activators to controls from individual experiments ($p \leq 0.05$).

5. RESULTS

5.1 Characterization of fibers

FDB fibers isolated from C57 mice were approximately 50µm in width and 500µm in length (Fig. 1a). Fibers that had hypercontracted were about 1/5th of their original size and were approximately 100µm in width and 120µm in length (Fig 1b). Each dish contained between 100-400 fibers and approximately 95-98% of the fibers were live (not hypercontracted) upon plating. Within 48 hours, between 10-20% of the fibers showed hypercontraction, most of which occurred after the first 24 hours in culture.

To ensure that the fibers were still "intact" following isolation, both electron microscopy and studies using ethidium bromide incorporation were carried out.

Electron Microscopy

Electron microscopy studies showed that the fiber basal lamina was still present. The sarcolemma was smooth around the fibrils and sarcoplasm, without any obvious lesions or tears along the outside of the fiber (Fig. 2). Satellite cells were localized just below the basal lamina and had a cytoplasm that ran along the edge of the fiber. Myonuclei were also identified and were easily distinguishable from satellite cells as they did not have a distinct cytoplasm delineated by a plasma membrane.

Marcaine

Studies were done to test the integrity of the membrane of freshly plated fibers by assessing the permeability of EtBr into the fiber. EtBr only stains the DNA of dead cells,

but not live ones where the membrane can exclude the dye Positive controls, where EtBr was added to fixed fibers, showed nuclei within both fibers and satellite cells stained red (Fig 3a,b). Live fibers, either freshly plated or after 24 hours, did not have nuclei stained when EtBr was added to the dish (Fig 3c,d). The nuclei of fibers that had hypercontracted during the isolation process or which hypercontracted over time in culture stained positive within one minute of EtBr being added to the dish.

When Marcaine was added at a high dose (0.10%), a vast majority of the fibers hypercontracted almost immediately (Fig. 4a,b). Hypercontraction usually occurred within 10 seconds as the fibers bunched in from the ends, making them much smaller and square shaped. The nuclei of hypercontracted fibers stained positive within one minute after the addition of EtBr or when a high dose of Marcaine was added with the EtBr (Fig 4c). Marcaine added at a lower dose (0.025%), did not cause fiber hypercontraction for at least 10 minutes, but EtBr did enter the fiber and nuclear staining was visible within 90 seconds after the Marcaine was added (Fig 4d,e).

When fluorescein diacetate (FDA), a vital stain, was added to live fibers, the entire fiber stained green immediately (Fig 5a,b). When fibers were already hypercontracted or were hypercontracted by a high dose of Marcaine, only satellite cells remained viable, and the cytoplasm no longer stained positive (Fig 5c,d). If FDA was added just after fibers were hypercontracted with Marcaine, fibers were stained positive. If FDA was added 15 minutes later, only the satellite cells cytoplasm was stained, and fibers were not.

5.2 Characterization of both Free and Attached Satellite Cells.

Cells attached to and free from fibers were stained with c-met to confirm their identification as satellite cells since it was important not to include contaminant fibroblasts or other non-muscle cells in later cell counts. By light microscopy, attached satellite cells were usually very difficult to distinguish from myonuclei both on live and fixed fibers (Fig 6a). Staining fixed fibers with bisbenzimide stains all nuclei - both satellite cells and myonuclei (Fig 6b). However, only satellite cells stain with the c-met immunostain (Fig 6c). Satellite cells were only found on the periphery of fibers and looked slightly larger than myonuclei. There were between 1-3 c-met+ cells per fiber. Fibers fixed anytime within the first 24 hours after plating and stained for BrdU incorporation only occasionally had cells that stained positive for new DNA synthesis. However, after 48 hours, fibers cultured in the presence of BrdU had between 0 and 6 very dark, concentrated cells on and off the fibers (Fig 7a). Cells on the fibers had a cytoplasm that did not stain (Fig 7b).

Cells that were free from fibers were stained positive for c-met. Nearly all those free cells had cytoplasm that stained very dark, whereas nuclei remained unstained for c-met (Fig 7c). Their cytoplasm was elongated on the bottom of the dish. Mitotic figures were often seen among free cells (and also on attached cells) and always stained positive for BrdU (Fig 7d). With further experience, it was possible to distinguish by shape and colour between satellite cells and fibroblasts by using a light microscope after staining for BrdU. Satellite cells stained for BrdU had nuclei that were dark black, their nuclei appeared quite compact and the cytoplasm did not stain. Fibroblasts nuclei stained for BrdU incorporation were a lighter brown color and not as compact in appearance.

5.3 Measuring Activation of Satellite Cells on Fibers

The addition of potential activators to dishes generally increased the amount of proliferation of satellite cells 48 hours after plating The number of proliferating attached and free satellite cells was counted by counting dark cells with concentrated black nuclei. The lighter stained, more flat fibroblasts, were not counted. Control dishes, containing only basal media, typically contained 0.4-0.6 proliferating attached satellite cells per live fiber and approximately 0.1-0.15 free proliferating satellite cells per fiber. To account for variations between the individual experiments, all values were compared to the mean value of proliferation by satellite cells in the basal media, and are shown in figures and tables as ratios of potential activators/control.

5.4 Effect of CME on Activation

CME, previously demonstrated to be a strong activator of satellite cells *in vitro*, activated satellite cells on intact fibers. Relative to basal media, CME greatly increased the amount of activation of proliferating satellite cells both attached and free from fibers. However, a much greater increase in activation of free cells was seen than for attached cells, though both showed the impact of the activator. Treatment with CME resulted in a 61% increase in the number of proliferating attached cells per fiber and a 460% increase in the number of proliferating free cells per fiber when compared to controls (n=4; where n = average values of 3 dishes for each of 3 individual experiments; summarized in Fig 9a,b). Although the CME was filter sterilized before use, dishes containing CME had some brown cloudy material covering the bottom, presumably components of the extract that

precipitated while frozen or when added to the dish. CME was the most potent activator tested and was therefore used as the positive control to ensure the experimental conditions and staining had worked properly in subsequent experiments.

5.5 Effect of HGF and L-Arg on Satellite Cells on Individual Fibers

HGF and L-Arg both increased the activation of satellite cells on isolated fibers, though there were differences in the type and extent of activation that was present for each. Fig 8a shows the results of adding HGF and L-Arg along with the negative and positive controls in a typical experiment. Results for this experiment show that for attached cells, there was 49% increase in proliferation in the presence of CME, a 10% increase in the presence of HGF and a 12% increase in the presence of L-Arg relative to basal media (Fig 8a). Only the CME value is considered significant in this experiment. However, when these values are pooled together with other experiments, counts of attached cells show that HGF (5ng/ml and 20ng/ml; n=2 and n=3 respectively) did not significantly increase activation over control levels (summarized in Fig 9a). However, L-Arginine did show a 30% increase in the number of proliferating attached cells per fiber relative to basal media. Dishes with HGF or L-Arg added did not have the brown precipitate that was evident in CME.

In contrast to the different effects of HGF and L-Arg on attached cells, HGF and L-Arg both increased the number of proliferating free cells per fiber. Fig 8b, from a typical experiment and the same dissection as Fig 8a, shows the number of proliferating free cells per fiber for each treatment group. For this particular experiment, HGF increased the

number of proliferating free cells 4-fold and the L-Arg increased it 2-fold over basal media. When results from several experiments were pooled together, counts of proliferating free cells after addition of HGF showed 260% (20ng/ml; n=3) and 200% (5ng/ml; n=2) increases over control. L-Arg (500μM; n=3) increased the number proliferating free cells 41% over control values (Fig 9b).

5.6 Effect of Marcaine-Induced Membrane Damage on Activation

Fibers that were exposed to 5 minutes of Marcaine and then allowed to grow for 48 hours showed a decrease in activation. When the results of several experiments were pooled together, mean proliferation levels were 62% for attached and 67% for free cells (Fig 10). Adding HGF (20ng/ml) to the Marcaine injured dishes with basal media elevated the number of proliferating free fibers 290% compared to Marcaine alone but only 72% of HGF alone. The addition of HGF to Marcaine-damages fibers did not have any significant effect on the number of attached cells compared to Marcaine alone.

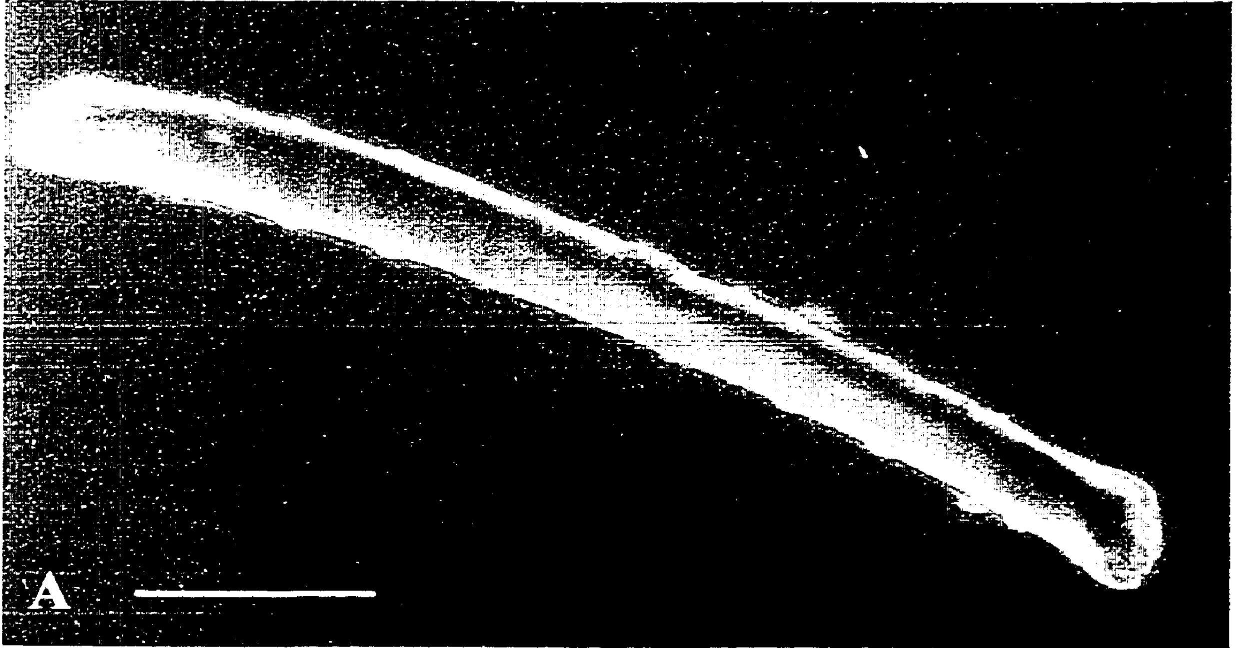
FIGURES and LEGENDS

Figure 1.

Low magnification view of a representative muscle fiber from flexor digitorum brevis muscle of C57 mice after complete dissection. (×300; bar=100μm) Connective tissue, nerves and vessels are absent.

A) Live fiber - nuclei are observed in and on the fiber, and some appear bulging from fiber contour.

B) Live and hypercontracted fibers. The hypercontracted fiber was damaged during the isolation procedure and is approximately 1/5th as long as the live fiber. These figures also show that satellite cells cannot be identified with certainty by using phase contrast alone.


A

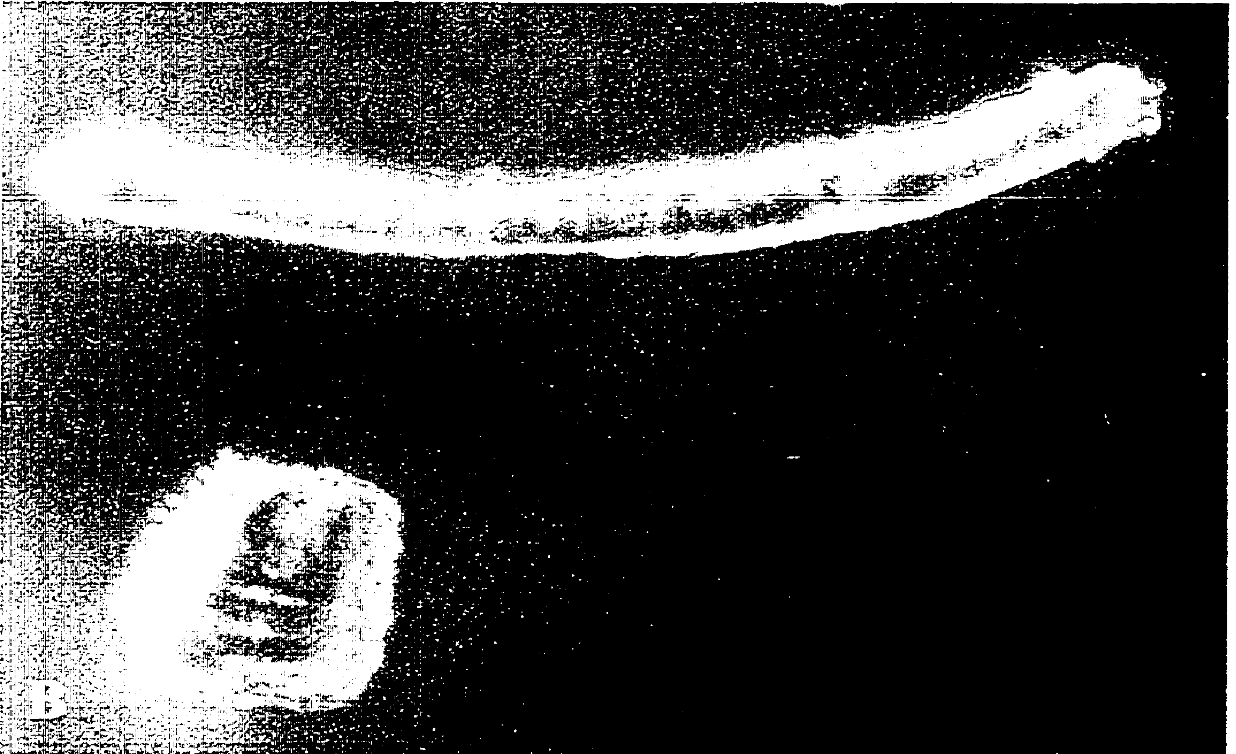

B

Figure 2.

Electron micrograph of an isolated fiber. (×30,000; bar=500nm).

The basal lamina (arrow) and sarcolemma persist and remain smooth with the fiber contour after the isolation without any obvious lesions or tears. A satellite cell remains attached on the fiber between the basal lamina and sarcolemma.

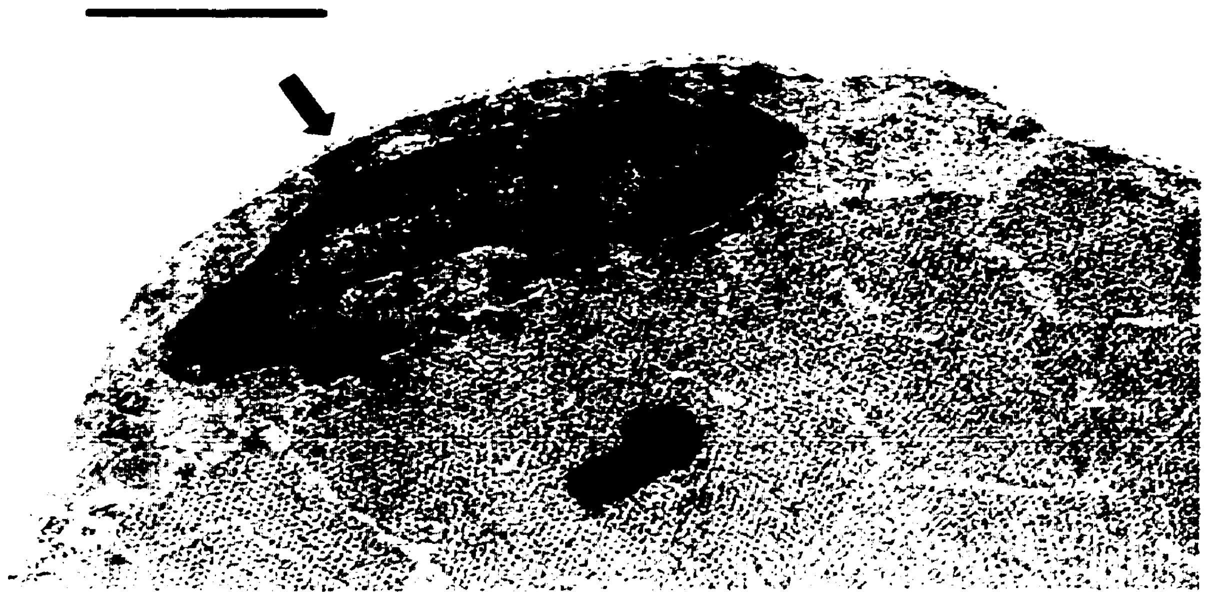


Figure 3.

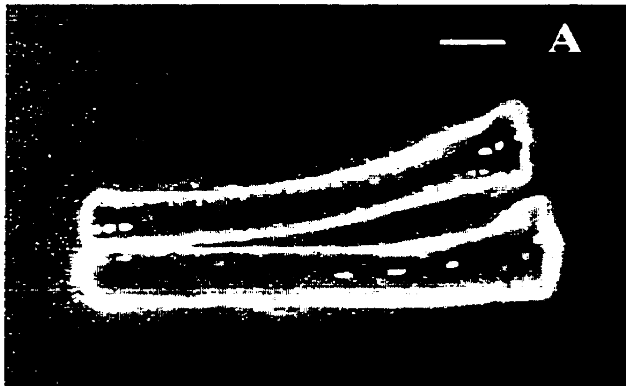
Staining of fiber nuclei using ethidium bromide (EtBr).

A) Two fibers fixed immediately after plating (×160; bar=50μm). Membrane blebs appear on and close to the fiber due to hypotonic conditions during incubation in this experiment.

B) The nuclei in the same two fixed fibers as in A were stained immediately after the addition of EtBr (2.5 μg/ml; positive control).

C) Unfixed live and hypercontracted fibers (×150; bar=50μm).

D) Nuclei in the same fiber shown in C. Nuclei in the hypercontracted fiber stained red immediately after EtBr was added to the dish, but fiber nuclei on the live fiber did not stain, even after this 30 minute incubation.


A

B

C

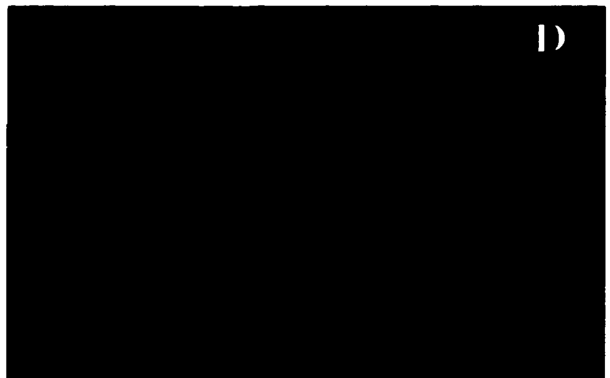

D

Figure 4.

Addition of Marcaine + EtBr to fibers. (×150; bar=50μm).

A) Phase contrast picture of an unfixed live fiber.

B) Same fiber as A one minute after Marcaine (0.01%) + EtBr were added to the dish. The fiber has hypercontracted.

C) Nuclei in the hypercontracted fiber are stained red.

D) Phase contrast picture of an unfixed live fiber.

E) Live fiber 90 seconds after the addition of Marcaine (0.025%) and EtBr. The fiber has yet to hypercontract but the nuclei are stained positive, indicating that Marcaine allows EtBr to permeate into the fiber.

A

B

D

Figure 5.

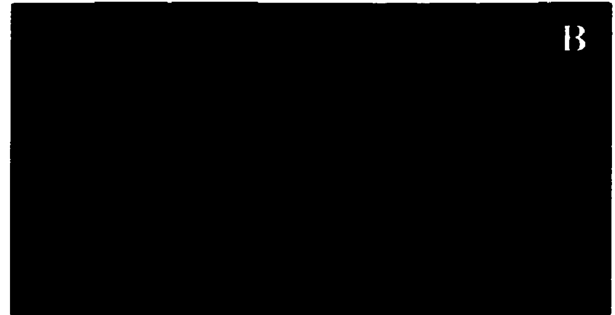
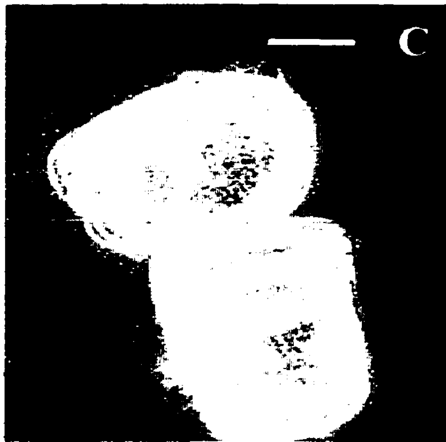
Addition of fluorescein diacetate (FDA) to live and hypercontracted fibers.

A) Live fiber 24 hours after plating (×150; bar=50μm).

B) Live fiber 60 seconds after the addition of FDA. The entire fiber cytoplasm stained green, indicating that the fiber is alive.

C) Two fibers hypercontracted with 0.1% Marcaine (×220; bar=50μm).

D) FDA added to the fibers, one hour after hypercontraction with Marcaine. Only the satellite cells stain green, demonstrating that the fiber has been killed with this high dose of Marcaine, but satellite cells remain alive.

A

B

C

D

Figure 6.

Identification of satellite cells on muscle fibers. (×300; bar=50μm)

A) Fixed muscle fiber.

B) Bis-benzimide stain under UV illumination and using epifluorescence optics showing that both myonuclei and satellite cell nuclei are stained.

C) C-met immunostaining of the culture distinguishes between satellite cells (stained positive) and myonuclei. The cytoplasm of satellite cells is stained for c-met protein, but satellite cell nuclei or myonuclei within the fiber and fiber sarcoplasm are unstained. Negative control fibers stained with the same procedure but without primary antibody did not show c-met positive satellite cells.

A

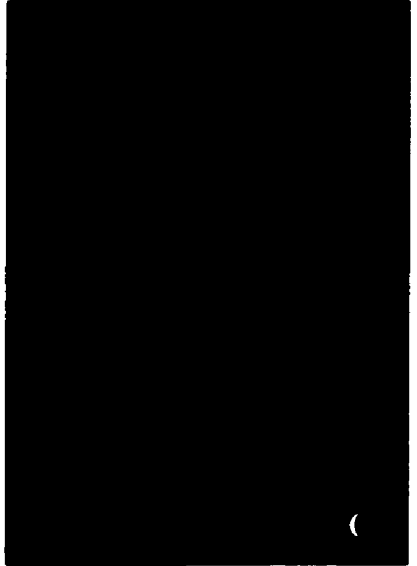
B

C

Figure 7.

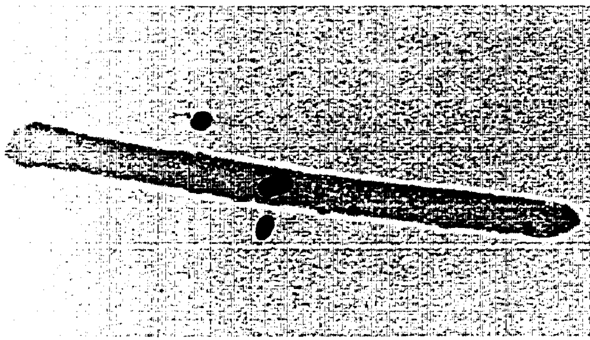
Assessing satellite cell activation on isolated fibers by BrdU incorporation and immunostaining.

A) A fiber fixed 48 hours after plating demonstrates proliferating satellite cells (×185; bar=50μm). Satellite cells that were activated have nuclei that stain black for incorporation of BrdU, whereas nuclei in quiescent satellite cells and myonuclei do not stain for BrdU. Counts were made of both the number of proliferating satellite cells per live fiber (attached) and the number of proliferating free satellite cells as a ratio with the total number of fibers in the dish (free).

B) Proliferating satellite cell clearly shows a stained nucleus and unstained cytoplasm (×300; bar=50μm).

C) Free satellite cell stained for the identification marker, c-met (the receptor for HGF) at 48 hours, showing that the free cells are satellite cells and not fibroblasts or contaminants (×280; bar=50μm).

D) Mitotic figure of proliferating satellite cell beside a fiber 48 hours after plating (×350; bar=50μm).



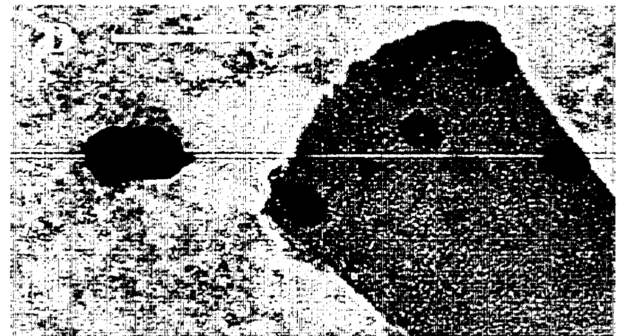
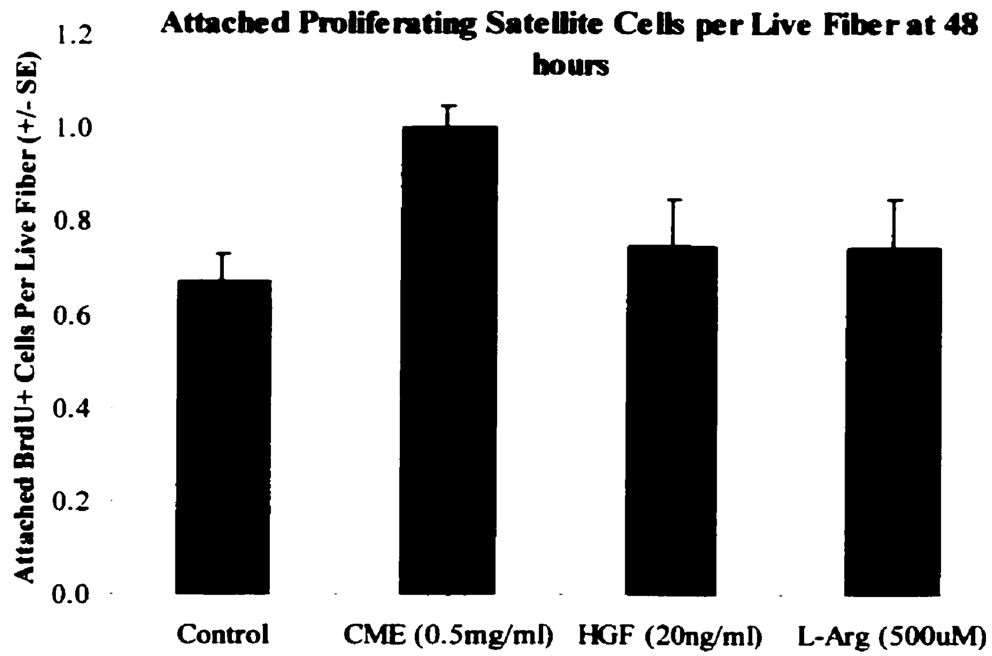


Figure 8.

Graphical summary of a typical experiment demonstrating the effects of CME, L-Arg and HGF on satellite cell activation.

A) Isolated fibers were grown for 48 hours, fixed, and the number of cells staining positive for BrdU+ incorporation on the fibers (attached) were counted per live fiber. Each bar represents counts for BrdU and attached cells per live fiber, from 3 dishes (+/- standard error). In this experiment only the CME significantly stimulated proliferation above control levels.

B) In the same experiment as above, the number of free proliferating satellite cells (free) were counted as a ratio to total fibers in the dish. For free satellite cell proliferation, CME, HGF and L-Arg all showed a significant increase over control levels.


Attached Proliferating Satellite Cells per Live Fiber at 48 hours
Attached BrdU+ Cells Per Live Fiber (+/- SE)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
CME (0.5mg/ml)
HGF (20ng/ml)
L-Arg (500uM)

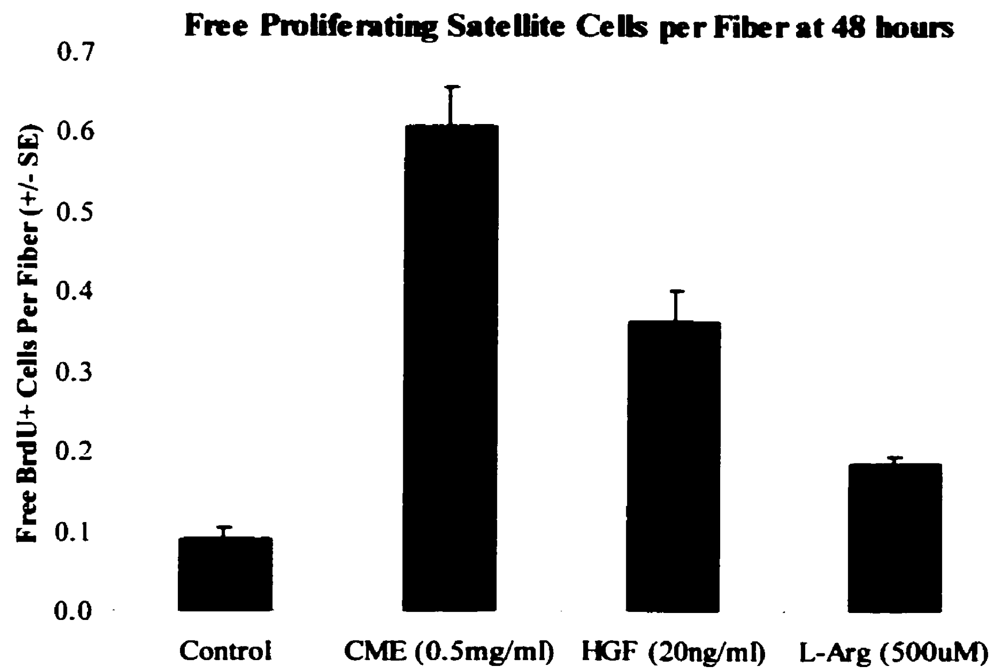
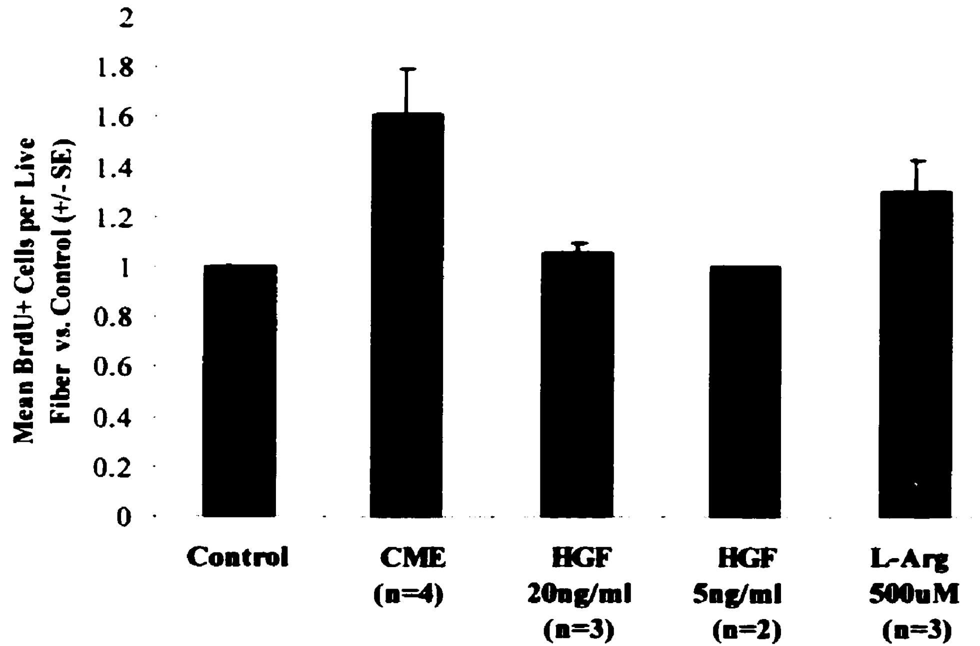

Free Proliferating Satellite Cells per Fiber at 48 hours
Free BrdU+ Cells Per Fiber (+/- SE)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
CME (0.5mg/ml)
HGF (20ng/ml)
L-Arg (500uM)

Figure 9.

A graphical summary of proliferation data for attached and free satellite cells compiled from several experiments. Each bar represents the average +/- SEM of all mean values from several experiments for the designated potential activator compound standardized as a ratio to CSR control in the same experiment (n=number of experiments from which results were pooled together from).

A) Summary of data for attached proliferating satellite cells. CME and L-Arg both significantly increased the amount of satellite cell activation (proliferating cells/live fiber) over control levels, whereas two different concentrations of HGF (20ng/ml and 5ng/ml) did not affect the activation of attached satellite cells.

B) Summary of data for free proliferating satellite cells. CME, HGF and L-Arg all significantly increased the number of free proliferating satellite cells per fiber at 48 hours. The number of experiments compiled for this figure differs among conditions and is indicated in brackets (n) below the bars


Summary of Attached Satellite Cell Proliferation
Mean BrdU+ Cells per Live Fiber vs. Control (+/- SE)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
CME (n=4)
HGF 20ng/ml (n=3)
HGF 5ng/ml (n=2)
L-Arg 500uM (n=3)

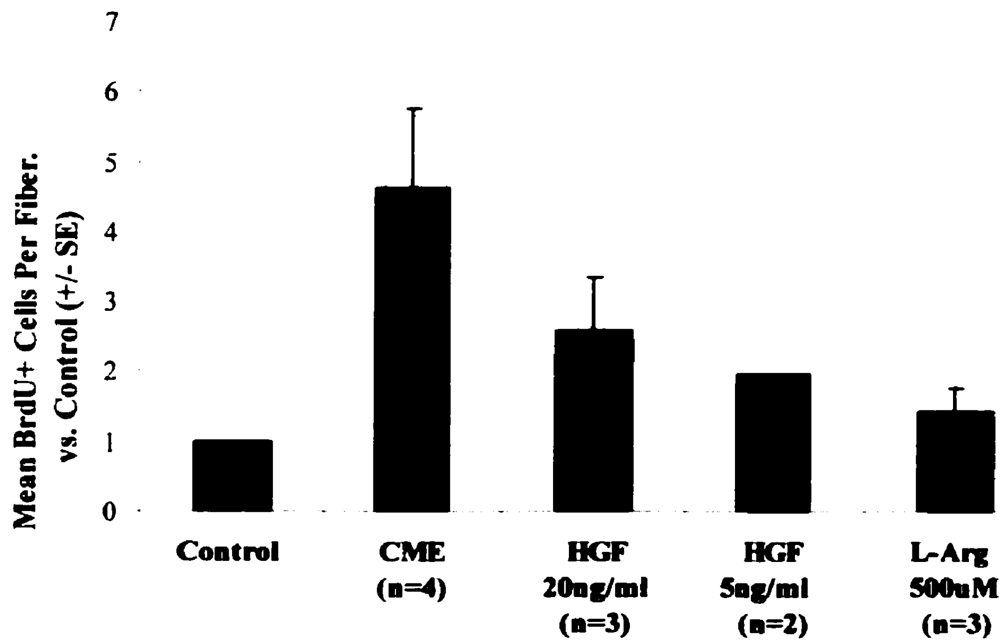
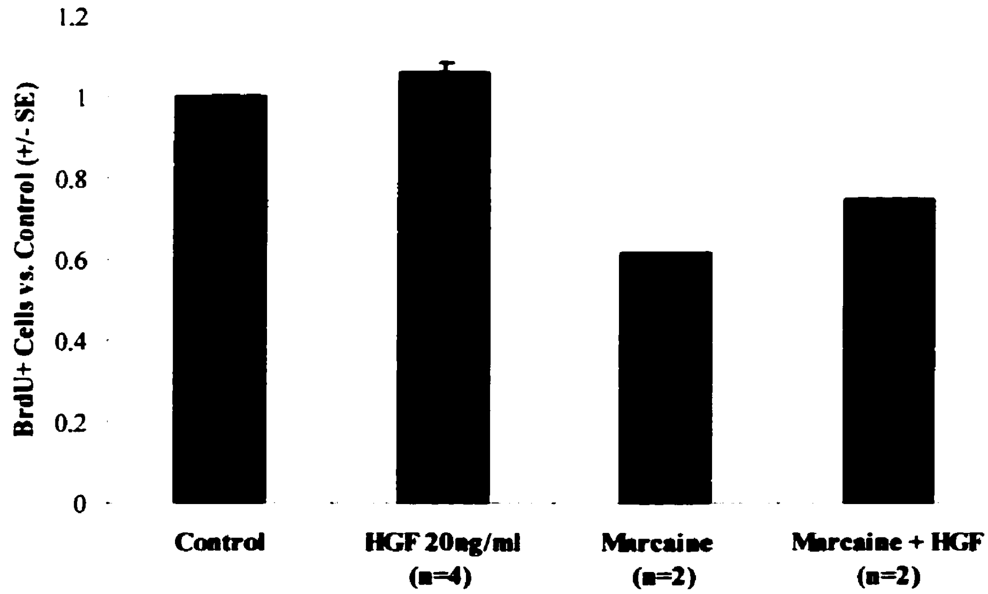

Summary of Free Satellite Cell Proliferation
Mean BrdU+ Cells Per Fiber. vs. Control (+/- SE)
7
6
5
4
3
2
1
0
Control
CME (n=4)
HGF 20ng/ml (n=3)
HGF 5ng/ml (n=2)
L-Arg 500uM (n=3)

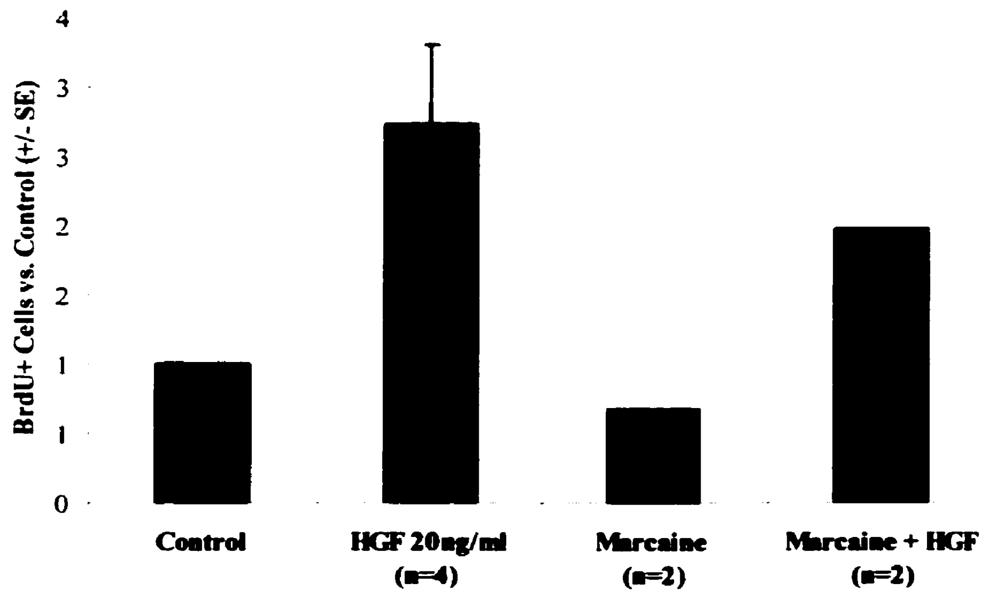
Figure 10.

Summary of the experiment made to determine the effect of Marcaine on proliferation of attached and free satellite cells.

A) Summary of data for attached proliferating satellite cells per live fiber. Addition of Marcaine to the dishes for five minutes prior to culture for 48 hours in media without Marcaine produced a decrease in the number of activated attached cells per fiber. Addition of HGF to Marcaine-damaged fibers elevated the number of attached cells above Marcaine levels, but activation was still lower than on undamaged fibers.

B) Summary of data for proliferating free satellite cells per fiber. Marcaine-damaged fiber cultures show a decrease in the number of proliferating free cells per fiber. That proliferation is partly restored toward control levels with the addition of HGF.


Summary of Attached Satellite Cell Proliferation
BrdU+ Cells vs. Control (+/- SE)
1.2
1
0.8
0.6
0.4
0.2
0
Control
HGF 20ng/ml
(n=4)
Marcaine
(n=2)
Marcaine + HGF
(n=2)


Summary of Free Satellite Cell Proliferation
BrdU+ Cells vs. Control (+/- SE)
4
3
3
2
2
1
1
0
Control
HGF 20ng/ml
(n=4)
Marcaine
(n=2)
Marcaine + HGF
(n=2)

6. DISCUSSION

Satellite cell activation occurs when a satellite cell leaves its normal quiescent state. It is the first step after a muscle injury which triggers the cascade of events including entry to the G_1 state of the cell cycle that eventually leads to the making of new muscle fibers. Using proliferation of satellite cells as the marker for activation, we have demonstrated that both nitric oxide (NO) and hepatocyte growth factor (HGF) activate quiescent satellite cells on intact fibers and have suggested that activation is decreased when the fiber membrane is partially damaged with Marcaine.

The Fiber Isolation Process - Comparison to other Studies

The isolation of FDB fibers for purposes of studying satellite cell activation and proliferation has been done primarily by three groups: Bischoff (1986a, 1986b and 1990), Yablonka-Reuveni and Rivera (1994, 1999) and Cornelison and Wold (1997), though each group uses the technique for slightly different purposes. Many similarities exist between our dissections and theirs, including the method and general characterization of the fibers. As described previously (Bischoff, 1986a), early dissections which had many dead fibers caused a large proportion of satellite cell proliferation on fibers, even in the control dishes, likely due to release of mitogens like bFGF from the fibers upon hypercontraction. Once the technique was better established and adjustments were made, dissections contained much cleaner preparations (fewer dead fibers) for experiments.

There were several important differences between observations by other groups and ours Bischoff (1986a) noted that when a fiber hypercontracted with Marcaine, the basal lamina persisted in its normal position while the inner components enclosed by the sarcolemma retracted within it. Upon hypercontraction, satellite cells were found intimately attached to the loose basal lamina which was larger than the rest of the contracted fiber. This differs from our finding that the entire fiber, including the outside basal lamina, was hypercontracted after exposure to Marcaine or when damaged during the dissection process. The Bischoff and Yablonka-Reuveni groups also did not observe satellite cells that became dissociated from the fiber ("free" cells) as were noted in our studies. Because of the persistent basal lamina and the absence of free cells in previous studies, the counts of proliferating satellite cells at various times after plating were significantly higher than we observed (Yablonka-Reuveni, personal correspondence). Bischoff and Yablonka-Reuveni papers recorded up to 20-30 proliferating satellite cells attached onto a rat fiber 48 hours after plating. Only rarely were more than 5-7 proliferating satellite cells found on any one fiber in our studies. Electron microscopy studies verified that the basal lamina was intact on our fibers, and looked identical to those found *in vivo* (Carpenter and Karpati, 1984). This excludes the possibility that satellite cells were allowed to leave the fiber due to the absence of the ensheathing basal lamina. The differences could be attributed to differences in the species used (rats vs. mice), since rat and chicken myoblasts respond differently to HGF (Gal-Levi *et al.*, 1998; Tatsumi *et al.*, 1998). Alternatively, other groups may have presumed the free cells to be fibroblasts, since at the time of those earlier studies, no marker for quiescent satellite cells (c-met) was available.

Free vs. Attached Satellite Cells

This work looked at activation and proliferation of satellite cells both attached to and free from fibers. In previous studies, only the number of attached cells was studied (Bischoff, 1986a, 1986b, 1990a, 1990b; Yablonka-Reuveni and Rivera, 1994) and no mention is made of the presence of satellite cells coming loose from the fiber. *In vivo*, satellite cells do travel through the basal lamina and attach onto other fibers (Maltin *et al.*, 1983, Anderson *et al.*, 1987). This phenomenon has been seen in our studies where c-met+ satellite cells were released from fibers and migrated onto the dish, since the basal lamina was intact.

The presence of both attached and free satellite cells could demonstrate the different events that must occur following an injury. Proliferation and mobility must both occur for new fibers to be made, as satellite cells must divide so as to provide a large number of cells available for new fibers, as well as move to where the damage has taken place. Our results suggest that these are two separate events, each with their own signals and pathways controlling the unique processes, since proliferation by the free and attached cells was affected differently by different potential mitogens. The attached proliferating satellite cells could be responsible for replicating and providing the pool of progeny cells for repaired fibers, likely for repairing the same fiber from which they were originally found. Attached cells could also be acting as stem cells, with a self-renewal function that would "bank" myogenic cells in the satellite cell position for future activation upon injury.

The free cells could demonstrate the presence of satellite cells that are released and move to the damaged area of the muscle, though not necessarily on or toward the same parent fiber. If damage is extensive, and many cells are necessary, then satellite cells from greater distances could be recruited to contribute to an injury further away from the initial damage. We know this occurs as new fibers are seen between the surviving ends of fibers at a distance from the segment where injury occurred (McIntosh *et al.*, 1994; McIntosh *et al.*, 1995; Pernitsky *et al.*, 1996). Because some non-proliferating satellite cells were observed but not counted, it is not known how many satellite cells had left the fiber and not yet proliferated. Although a double stain for BrdU/c-met would be necessary to confirm the identity the cells found off the fibers, or use of a transgenic that expressed (for example) green fluorescent protein on the c-met gene, the existence of the cells does again indicate two aspects of activation. Whether these constitute completely separate or independent pathways of the activation cascade is not known.

HGF and L-Arg in Activation

HGF and L-Arg both increased the amount of satellite cell activation at 48 hours after plating, as measured by counts of proliferating satellite cells per fiber. HGF only affected the number of free cells (260% increase) whereas L-Arg increased both the number of attached (30%) and free cells (41%) over control levels. This suggests a different role for each of these as activators. Since HGF only affects proliferation by the cells that come off the fibers, it could function to loosen the cells and induce them to move to the required destination. This is similar to what is seen in postnatal development, as

previous studies have demonstrated that HGF and c-met have a role in myoblast migration to the limb bud during development (Blandt *et al.*, 1995) and HGF has an effect on the migration of satellite cells in culture (Bischoff, 1997) As well, during a myotoxic injury, satellite cells detach from the fiber and migrate within the muscle (Maltin *et al.*, 1983). Given that the c-met receptor is known as motogenic (as well as mitogenic; Rong *et al.*, 1993), our results agree with these findings and demonstrate the important role of HGF in satellite cell migration during regeneration.

L-Arg affects proliferation of both the free and attached satellite cells, though the increased proliferation of free cells was less than for HGF. This indicates a different role for L-Arg and therefore NO in regeneration, in that NO does affect both the proliferation and migration of satellite cells. Alternatively, it could mean that NO is an earlier signal which initiates the activation of attached satellite cells (and nearby free satellite cells). However, the downstream factors needed from the media to carry out activation to a sufficient degree to result in a large proliferation (which was the marker we used for complete activation) were not present. This would suggest that NO acts earlier than HGF, since if NO were further downstream, the results on proliferation would be expected to be greater by amplification of earlier signals. As well, given that NO is a freely diffusible gas and does not persist very long after its release, it would likely be the first and possibly the most specific in the activation pathway. However, longer term inhibition of NOS does reduce the effectiveness of muscle repair *in vivo* (Anderson, 2000) and NO is reported necessary for myoblast fusion *in vitro* (Lee *et al.*, 1994). Therefore, the initial NO release to activate satellite cells is not the only effect of NO during muscle repair.

Previous studies have demonstrated that NO immediately loosens satellite cells to come off fibers following a crush injury to the TA, since loss of adhesion is inhibited by injecting an NO inhibitor before the injury (Anderson, 2000). Our results are in agreement with these findings, as an increase in free satellite cells was seen after 48 hours.

Effect of Marcaine-Damaged Fibers on Satellite Cell Activation.

Fibers which were exposed to a high dose of Marcaine (0.10%) hypercontracted almost immediately, as was previously demonstrated (Bischoff, 1990b; Cornelison and Wold, 1997). Our results showed that EtBr permeated into the fibers upon exposure to Marcaine and was seen within one minute after hypercontraction. To induce an injury to the isolated fibers without hypercontracting them, as well as test the effect of slight membrane damage to fibers, Marcaine was added to fibers at a lower dose (0.025%). EtBr was able to permeate through the slightly damaged membranes on these fibers, but hypercontraction was not seen for at least 15 minutes. This suggests that Marcaine causes sufficient damage and makes holes in the fiber membrane large enough to allow EtBr into the fiber and stain the nuclei. Unpublished data have shown that Marcaine, even in low doses, poisons the mitochondrial production of energy (Bernardi, University of Milan, personal communications). It is not possible to visually distinguish between a normal intact fiber and one that has been exposed to a low dose of Marcaine and then recovered an intact membrane. However, the number of proliferating satellite cells 48 hours after plating are lower than in control dishes (62% lower for attached cells and 67% lower for free cells). This indicates that the membrane or a metabolically active fiber is important and

necessary for activation. Because NOS is found just under the membrane linked to syntrophin within the dystrophin cytoskeleton, membrane damage could disrupt the NOS complex and therefore affect activation. The addition of HGF elevated the levels of satellite cell proliferation almost back to control levels with only HGF. This indicates that the HGF step in activation is likely found following the release of NO. This would also indicate that NO may not always be absolutely necessary for activation, but instead NO release is a step that may help trigger the downstream factors, or that there are alternate pathways for activation, one of which occurs via NO release.

Previous studies have demonstrated that contact with the fiber reduces the amount of satellite cell proliferation and that hypercontracted fibers had greater numbers of proliferating satellite cells than live ones (Bischoff, 1990b). As well, fibers killed by Marcaine only needed 8 hours of exposure to muscle extract to commit to proliferation to the same extent as live fibers that were exposed to muscle extract for the entire 48 hours (Bischoff, 1990a). Interestingly, our results show that satellite cells on fibers damaged, but not completely killed by Marcaine, actually lowered levels of activation and that although HGF elevated the levels of satellite cell proliferation on those fibers after Marcaine, activation never reached the same levels as after HGF alone. This rationale further supports the idea that an intact fiber is important for normal activation, especially via NO and HGF mechanisms.

Why Do Some Satellite Cells Not Become Activated?

Prior studies by Bischoff (1986a, 1986b) have shown that on isolated fibers, all satellite cells are activated and proliferating 48 hours after plating fibers with CME at 0.5mg/ml. However, in our experiments, there were never more than 70% of the fibers with satellite cells that had activated after 48 hours with extract. An important factor in accounting for the variance could be species differences, as Bischoff used rats and our experiments used mice. It could also mean that not all satellite cells are responsive to a stimulus to the same degree and that some may require a higher dose or different conditions to become activated. That would suggest that different subsets of satellite cells exist, and may be responsive to different factors, or to different concentrations and combinations of stimuli. The unactivated cells in our experiments could serve as reserve cells that may be used to respond to future injuries or needs. Some satellite cells may also have co-extracted off the fibers during the dissection. However, a limitation of this technique is that we do not know how many cells are not activated under a given condition.

Our experiments used a CME concentration of 0.5mg/ml as the positive control because that was previously shown to be the lowest concentration of CME that activated all satellite cells (Bischoff, 1986b). To verify this, a concentration curve of CME was done early in the project. However, at that time the technique and proper CME storage were not well refined in our lab. As a result, early results were inconclusive, as the CME killed the majority of the fibers on some dishes by 48 hours, likely an effect of autolytic enzymes released from excessive crushing of muscle extract preparation. Although the curve was not performed again and 70% was regarded as sufficient activation for studying potential

increases and decreases, it may be worthwhile to pursue to help answer the question of non-activated satellite cells.

Because satellite cells that came off the fibers were counted, it could be possible that some of the free proliferating satellite cells had originated as attached satellite cells, then migrated off by 48 hours. However, free satellite cells were most commonly found quite near the fiber and there were often fibers grown in CME that had no proliferating attached satellite cells and also had no free ones nearby. Additionally, in looking at the live and dead stain studies, when FDA was added to hypercontracted fibers, most fibers had between 1-3 satellite cells. However, there were occasionally (5-10%) hypercontracted fibers that had no live satellite cells on them. Although further testing was not done and there was no way of controlling the results using this FDA/EtBr method, the variable number of satellite cells per fiber could also have some effect on overall results. It would be interesting to pursue whether fibers either did not have any satellite cells on them *in vivo*, or that some satellite cells may have died or been mobilized and lost during the dissection or Marcaine hypercontraction.

Is the Isolated Fibers Technique an Effective Method for Studying Early Activation?

This work consisted of learning, refining and evaluating the isolated fiber technique to determine if it can serve as a good model for studying early satellite cell activation. The technique allows for the study of satellite cells that are still intimately attached to the fiber and lie between the sarcolemma and basal lamina, a state which most closely resembles *in*

vivo conditions. Unlike other *in vitro* methods, it allows for fibers to be intact, as demonstrated through the EM, EtBr, and Marcaine studies

However, several questions and concerns become apparent when evaluating this technique. One of the most obvious is the long learning curve for the method, which can hinder the number of people who are able to perform it, as well as the relatively few people who can be used as a resource for questions or problems encountered. Another concern is that even under optimal conditions and dissection that yield very few dead fibers, a small percentage of satellite cells may still proliferate by 48 hours under basal (CSR) conditions. This can be partly explained by the few fibers that do hypercontract and release activators into the media, as well as potential activators that still exist in the minimal serum replacement (CSR-2) or the low dose of horse serum. We never tested the effect of removing horse serum, as that was maintained in all the other studies using isolated fibers, presumably because it had other components that were needed in maintaining fibers viable. There could be a trade off between having healthy intact fibers, and having a small percentage of activated satellite cells. As well, because we used young mice (7-9 weeks), the satellite cells would still be very responsive to any manipulation and to ongoing growth demands, and would be expected to be more responsive to any stimuli than satellite cells on fibers from older mice. For these reasons, proliferation from potential activators was always expressed as a ratio to CSR-2 controls when evaluating the effect on activation.

Another consideration is the choice of marker for activation. We chose to use proliferation as our marker as that was used by Bischoff, who used tritiated thymidine. We used BrdU incorporation because it was much quicker to assay than using

autoradiography, and was also very reliable and quite easy to study. One could use some of the early markers such as MyoD and myogenin staining as used by others (Yablonka-Reuveni and Rivera, 1994). However, those markers would also detect cells that had started to enter the cycle but did not become completely activated to achieve proliferation within 48 hours. This could be an important consideration in comparing our results and previous studies, since the marker that is employed will affect the specificity of results. As well, since NO is a very short-lived gas, its effects may only be on initial steps in activation, whereas other factors may be needed for activation to continue into DNA synthesis. If a marker can be identified which is part of the NO signaling pathway in activation, it could be used to help identify the exact role and timing of NO, especially the sequence of factors (i.e. HGF) in the activation cascade.

The intact fiber technique has been used in several different ways. Yablonka-Reuveni (1994, 1999) used the technique as a way of monitoring and tracking the timing of MRF expression and proliferation. Cornelison and Wold (1997) used the technique as a method of collecting "quiescent" satellite cells off the hypercontracted fibers, then testing them for the expression of MRFs over time using RT-PCR. However, Bischoff's reported technique and purpose of experiments most closely resembles what we tried to accomplish. He added various compounds to the fibers, then counted the number of proliferating satellite cells over time, as an investigation of satellite cell activation.

The isolated fiber technique may not actually be the most conducive or appropriate for the studies of myogenesis undertaken by Yablonka-Reuveni. Because isolated fibers should not undergo any myogenesis (new muscle formation) in a dish without any stimuli, tracking gene expression over time could be misleading, as it does not imitate the *in vivo*

situation in repair. Conversely, using the technique to test the effects of compounds on early activation allows for a more realistic representation of what takes place *in vivo*, as activation should occur if these compounds are released into a damaged area following injury and should allow for a comparison of the timing and degree of activation that results in proliferation in earlier or later events. Since the muscles were still young and therefore quite responsive to the addition of a putative activator, the method allowed for subtle effects of the activators while maintaining the satellite cells situated in their normal milieu, at least at the start of the experiment. Therefore, since we expected the satellite cells to become somewhat activated following the trauma of a dissection, we accepted a small baseline level of activation (proliferation) in CSR-2 medium and then tested for effects of an activator or Marcaine and compared stimulated to baseline levels of proliferation.

Possible Future Experiments

Because the isolated fiber technique and details of satellite cells activation are relatively new, many experiments can be devised to help answer the questions that have been spawned from this study:

- Because L-Arg has many different roles in the body, experiments should be devised using different NO donors. We attempted sodium nitroprusside (SNP), but it caused extensive fiber hypercontraction. Other, more gentle donors such as SIN-1 should be tested to seen if they have an effect on activation. These may be preferred to L-Arg as they act directly, and do not need NOS to act, although extant NO may not be the same signal as a large stimulative increase in NO from NOS activity.

- NO likely acts very briefly during activation, but our experiments added L-Arg for the entire 48 hours. To more closely mimic the hypothesized model of NO activation, studies should be undertaken to determine if the NO donor is needed for only a few hours or minutes. It will also be interesting to determine the degree to which different lengths of NO exposure will affect proliferation.
- It would be of interest to see if activation decreased after inhibiting NOS using L-NAME exposure. This would be very useful testing the role of NO.
- Experiments combining HGF and L-Arg would determine whether the two share the same signaling pathway to activation and if they have synergistic, additive or overlapping affects.
- Interesting studies by Allen have demonstrated that plated satellite cells from older mice that receive a mechanical stretch in culture showed much more proliferation than controls (Allen, personal communication). Stretching studies on isolated fibers would be an effective way to cause satellite cell activation, hopefully without injuring or hypercontracting the fibers. This is similar to the rationale that was initially proposed for the Marcaine studies. Even adding a NOS inhibitor, L-NAME, while doing the stretching studies either in fibers or with cells (as done by Allen), may determine the role of NO in satellite cell activation.
- The isolated fiber technique would make an ideal model for visualizing NO release using fluorescent dyes (Sarti *et al.*, 1999). It would be very interesting to follow the effects of a mechanical stimulus that would mimic shear, such as stretching or hypercontraction of the fibers. Other signals that cause activation (cold, pressure, denervation) could also be studies for effects on activation. One could also determine

whether continued shearing causes the NO release to persist, or whether release is just an initial event following injury, succeeded by other steps in activation.

- The isolated fiber technique can also be used to isolate fibers for measuring the conversion of H^3-L-arginine into H^3-L-citrulline. This could be used to quantify directly the extent of NO release following an injury, as L-citrulline is a stable by-product of the reaction and can be measured directly.

CONCLUSIONS

1. Fibers from flexor digitorum brevis (FDB) muscle were isolated from mice. Single fibers maintained their basal lamina after the dissection by electron microscopy and were defined as intact by EtBr exclusion studies.
2. Hepatocyte growth factor (HGF), a component of CME, increased the number of free proliferating satellite cells per fiber 260% over controls 48 hours after plating. HGF did not increase the number of attached proliferating satellite cells per live fiber over control levels.
3. Nitric oxide increased satellite cell activation. L-Arg increased both the number of attached (30%) and free (41%) satellite cells 48 hours after plating over control levels.
4. Fibers need to be intact for satellite cell activation to take place. Damaging the fiber membranes with Marcaine decreased the number of attached proliferating cells to 62% of control levels and free cells to 67% of control levels. This

indicates that the fiber and an intact fiber are important and necessary for activation.

5. Satellite cell activation is likely a multi-step cascade during which activators (and suppressors) have distinctly different effects on attached and free satellite cells.

REFERENCES

Allen, R.E., Sheehan, S.M., Tayler, R.G., Kendall, T.L., Rice, G.M. (1995). Hepatocyte growth factor activates quiescent skeletal muscle satellite cells in vitro. *J. Cell. Phys.* **165**: 307-312

Anderson, J.E. (2000). A Role for Nitric Oxide in Muscle Repair: Nitric Oxide-Mediated Activation of Muscle Satellite Cells. *Mol. Bio. Cell.* **11**:1859-1874.

Anderson, J.E., Ovalle, W.K., Bressler, B.H. (1987). Electron microscopic and autoradiographic characterization of hindlimb muscle regeneration in the mdx mouse. *Anat. Rec.* **219**: 243-57.

Appell, H.J., Forsberg, S., Hollmann, W. (1988). Satellite cell activation in human skeletal muscle after training: evidence for muscle fiber neoformation. *Int. J. Sports. Med.* **9**(4): 297-9.

Balon, T.W. (1999). Integrative biology of nitric oxide and exercise. *Exerc. Sport. Sci. Rev.* **27**: 219-53.

Bekoff, A. and Betz,W. (1977). Properties of isolated adult rat muscle fibres maintained in tissue culture. *J. Physiol. Lond.* **271**(2): 537-47.

Biben, C. (1993). Myogenic factors: What might their role be? *Bull. Inst. Pasteur.* **91**: 161-171.

Bischoff, R. (1986a). Proliferation of muscle satellite cells on intact myofibers in culture. *Dev. Biol.* **115**: 129-139.

Bischoff, R. (1986b). A satellite cell mitogen from crushed adult muscle. *Dev. Biol.* **115**: 140-147.

Bischoff, R (1990a). Cell cycle commitment of rat muscle satellite cells. *J. Cell. Biol.* **111**(1): 201-7.

Bischoff, R. (1990b). Interaction between satellite cells and skeletal muscle fibers. *Development.* **109**: 943-952.

Bischoff, R. (1997). Chemotaxis of skeletal muscle satellite cells. *Dev. Dyn.* **208**(4): 505-15.

Bischoff, R., Holtzer, H. (1969). Mitosis and the processes of differentiation of myogenic cells in vitro. *J. Cell. Biol.* **41**(1): 188-200.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A., Birchmeier, C (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature*. **376**(6543): 768-71.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-54

Braun, T., Bober, E., Rudnicki, M.A., Jaenisch, R., Arnold, H.H. (1994). MyoD expression marks the onset of skeletal myogenesis in Myf-5 mutant mice *Development*. **120**(11): 3083-92.

Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., Froehner, S.C., Bredt, D.S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell*. **84**(5): 757-67.

Brenman, J.E., Chao, D.S., Xia, H., Aldape, K., Bredt, D.S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. *Cell*. **82**(5): 743-52.

Brunetti, A., and Goldfine, I.D. (1990). Role of myogenin in myoblast differentiation and its regulation by fibroblast growth factor. *J. Biol. Chem.* **265**: 5960-5963.

Burden, S.J. (1993). Synapse-specific gene expression. *Trends. Genet.* **9**(1): 12-6.

Carlson, B.M., and Faulkner, J.A. (1983). The regeneration of skeletal muscle fibers following injury: A review. *Med. Sci. Sports. Exerc.* **15**: 187-198.

Carpentor, S., and Karpati, G. (1984). Pathology of Skeletal Muscle. Churchill Livingstone Publishing Inc.

Chambers, R.L., McDermott, J.C. (1996). Molecular basis of skeletal muscle regeneration. *Can. J. Appl. Physiol.* **21**(3): 155-84.

Chen G, and Quinn, LS. (1992). Partial Characterization of Skeletal Myoblast Mitogens in Mouse Crushed Muscle Extract. *J. Cell. Physiol.* **153**: 563-574.

Columbano, A., Shinozuka, H. (1996). Liver regeneration versus direct hyperplasia. *FASEB. J.* **10**(10): 1118-28.

Cornelison, D.D.W. and Wold, B.J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Dev. Biol.* **191**: 270-283

Darr, K.C., Schultz, E. (1987). Exercise-induced satellite cell activation in growing and mature skeletal muscle. *J. Appl. Physiol.* **63**(5): 1816-21.

Darr, K.C., Schultz, E. (1989). Hindlimb suspension suppresses muscle growth and satellite cell proliferation. *J. Appl. Physiol.* **67**(5): 1827-34

Davis, R.L., Cheng, P.F., Lassar, A.B., Weintraub, H. (1990). The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. *Cell.* **60**(5): 733-46

Dimmeler, S., Hermann, C., Galle, J., Zeiher, A.M. (1999). Upregulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **19**(3): 656-64.

Donalies, M., Cramer, M., Ringwald, M., Starzinski-Powitz, A. (1991). Expression of M-cadherin, a member of the cadherin multigene family, correlates with differentiation of skeletal muscle cells. *Proc. Natl. Acad. Sci. USA.* **88**(18): 8024-8.

Fausto, N., Laird, A.D., Webber, E.M. (1995). Liver regeneration. 2. Role of growth factors and cytokines in hepatic regeneration. *FASEB. J.* **9**(15): 1527-36.

Feelisch, M. (1998). The use of nitric oxide donors in pharmacological studies. *Naunyn. Schmiedebergs. Arch. Pharmacol.* **358**(1): 113-22.

Fleming, I., Bauersachs, J., Busse, R. (1997). Calcium-dependent and calcium-independent activation of the endothelial NO synthase. *J. Vasc. Res.* **34**(3): 165-74.

Furchgott, R.F., Zawadzki, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature.* **288**(5789): 373-6.

Gal-Levi, R., Leshem, Y., Aoki, S., Nakamura, T., Halevy, O. (1998). Hepatocyte growth factor plays a dual role in regulating skeletal muscle satellite cell proliferation and differentiation. *Biochim. Biophys. Acta.* **1402**(1): 39-51.

Garrett, K.L., and Anderson, J.E. (1995). Colocalization of bFGF and the myogenic regulatory gene myogenin in Dystrophic mdx muscle precursors and young myotubes *in vivo. Dev. Biol.* **169**: 596-608.

Gherardi, E., Gray, J., Stoker, M., Perryman, M., Furlong, R. (1989). Purification of scatter factor, a fibroblast-derived basic protein that modulates epithelial interactions and movement. *Proc. Natl. Acad. Sci. USA.* **86**(15): 5844-8.

Goldstein, I., Lue, T.F., Padma-Nathan, H., Rosen, R.C., Steers, W.D., Wicker, P.A. (1998). Oral sildenafil in the treatment of erectile dysfunction. Sildenafil Study Group *N. Engl. J. Med.* **338**(20): 1397-404.

Grounds, M.D. (1991). Towards understanding skeletal muscle regeneration. *Pathol. Res. Pract.* **187**(1): 1-22.

Grounds, M.D., Garrett, K.L., Lai, M.C., Wright, W.E., Beilharz, M.W. (1992) Identification of skeletal muscle precursor cells in vivo by use of MyoD1 and myogenin probes. *Cell. Tissue. Res.* **267**(1): 99-104.

Grounds, M.D. and McGeachie, J.K. (1989). A comparison of muscle precursor replication in crush-injured skeletal muscle of Swiss and BALBc mice. *Cell. Tissue. Res.* **255**(2): 385-91.

Grounds, M.D., McGeachie, J.K. (1987). Reutilisation of tritiated thymidine in studies of regenerating skeletal muscle. *Cell. Tissue. Res.* **250**: 141-148.

Hansen-Smith, F.M., Carlson, B.M. (1979). Cellular responses to free grafting of the extensor digitorum longus muscle of the rat. *J. Neurol. Sci.* **41**(2): 149-73.

Hassard, T.H. (1991). Understanding Biostatistics. Mosby-Year Book, St. Louis, Missouri.

Higgins, G.M., and Anderson, R.M. (1931). *Arch. Pathol.* **12**: 186.

Hurme, T. and Kalimo, H. (1992). Adhesion in skeletal muscle during regeneration. *Muscle. Nerve.* **15**(4): 482-9.

Jennische, E., Ekberg, S., Matejka, G.L. (1993). Expression of hepatocyte growth factor in growing and regenerating rat skeletal muscle. *Am. J. Physiol. Cell Physiol.* **265**: C122-C128.

Johnson, S.E., Allen, R.E. (1995). Activation of skeletal muscle satellite cells and the role of fibroblast growth factor receptors. *Exp. Cell. Res.* **219**(2): 449-53.

Kami, K., Noguchi, K., Senba, E. (1995). Localization of myogenin, c-fos, c-jun, and muscle-specific gene mRNAs in regenerating rat skeletal muscle. *Cell. Tissue. Res.* **280**: 11-19.

Kishnani, N.S., Fung, H.L. (1996). Nitric oxide generation from pharmacological nitric oxide donors. *Methods. Enzymol.* **268**: 259-65.

Kobzik, L., Reid, M.B., Bredt, D.S., Stamler, J.S. (1994). Nitric oxide in skeletal muscle. *Nature.* **372**(6506): 546-8.

Lassar, A.B., Buskin, J.N., Lockshon, D., Davis, R.L., Apone, S., Hauschka, S.D., Weintraub, H. (1989). MyoD is a sequence-specific DNA binding protein requiring a

region of myc homology to bind to the muscle creatine kinase enhancer. *Cell.* **58**(5): 823-31

Lawson-Smith, M.J., McGeachie, J.K. (1997). Experimental skeletal muscle grafts as a model of regeneration. *Aust. N. Z. J. Surg.* **67**(1): 35-9.

Li, L., Chambard, J.C., Karin, M., Olson, E.N. (1992). Fos and Jun repress transcriptional activation by myogenin and MyoD: the amino terminus of Jun can mediate repression. *Genes. Dev.* **6**(4): 676-89.

Lee, K.H., Baek, M.Y., Moon, K.Y., Song, W.K., Chung, C.H., Ha, D.B., Kang, M.S. (1994). Nitric oxide as a messenger molecule for myoblast fusion. *J. Biol. Chem.* **269**(20): 14371-4.

Lefaucheur, J.P., Sebille,A. (1995). The cellular events of injured muscle regeneration depend on the nature of the injury. *Neuromuscul. Disord.* **5**(6): 501-9.

Macedo, M.P., Lautt, W.W. (1998). Shear-induced modulation of vasoconstriction in the hepatic artery and portal vein by nitric oxide. *Am. J. Physiol.* **274**(2 Pt 1): G253-60.

Maltin, C.A., Harris, J.B., Cullen, M.J. (1983). Regeneration of mammalian skeletal muscle following the injection of the snake-venom toxin, taipoxin. *Cell. Tissue. Res.* **232**(3): 565-77.

Mann, F.C. (1940). The portal circulation and restoration of the liver after partial removal. *Surgery.* **8**: 225-238.

Matsumoto, K., Nakamura, T. (1991). Hepatocyte growth factor: molecular structure and implications for a central role in liver regeneration. *J. Gastroenterol. Hepatol.* **6**(5): 509-19.

Matsumoto, K., Nakamura, T. (1996). Emerging multipotent aspects of hepatocyte growth factor. *J. Biochem. Tokyo.* **119**(4): 591-600.

Mauro, A. (1961). Satellite cells of skeletal muscle fibers. *J. Biophys. Biochem. Cytol.* **9**: 493-495.

McComas, A.J. (1996). Skeletal Muscle: Form and Function. Human Kinetics Publishing.

McDonald, K.A., Lakonishok, M., Horwitz, A.F. (1995). Alpha v and alpha 3 integrin subunits are associated with myofibrils during myofibrillogenesis. *J. Cell. Sci.* **108**(7): 2573-81.

McGeachie, J.K., Grounds, M.D. (1987). Initiation and duration of muscle precursor replication after mild and severe injury to skeletal muscle of mice. An autoradiographic study. *Cell. Tissue. Res.* **248**(1): 125-30.

McIntosh, L.M., Pernitsky, A.N., Anderson, J.E. (1994). The effects of altered metabolism (hypothyroidism) on muscle repair in the mdx dystrophic mouse. *Muscle. Nerve.* **17**(4): 444-53.

McIntosh, L.M. and Anderson, J.E. (1995). Hypothyroidism prolongs and increases mdx muscle precursor proliferation and delays myotube formation in normal and dystrophic limb muscle. *Biochem. Cell. Biol.* **73**(3-4): 181-90.

McIntosh, L.M., Garrett, K.L., Megeney, L., Rudnicki, M.A., Anderson, J.E. (1998). Regeneration and myogenic cell proliferation correlate with taurine levels in Dystrophin and MyoD-Deficient Muscles. *Anat. Rec.* **252**: 311-324.

Megeney, L.A., Kablar, B., Garrett, K., Anderson, J.E., Rudnicki, M.A. (1996). MyoD is required for myogenic stem cell function in adult skeletal muscle. *Genes Dev.* **10**: 1173-1183.

Michalopoulos, G.K., and DeFrances, M.C. (1997). Liver regeneration. *Science.* **276**(5309): 60-6.

Miller, M.J., Thompson, J.H., Liu, X., Eloby-Childress, S., Sadowska-Krowicka, H., Zhang, X.J., Clark, D.A. (1996). Failure of L-NAME to cause inhibition of nitric oxide synthesis: role of inducible nitric oxide synthase. *Inflamm. Res.* **45**(6): 272-6.

Miyazawa, K., Shimomura, T., Kitamura, A., Kondo, J., Morimoto, Y., Kitamura, N.(1993). Molecular cloning and sequence analysis of the cDNA for a human serine protease reponsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. *J. Biol. Chem.* **268**(14): 10024-8.

Moncada, S., Higgs, A. (1993). The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329**(27): 2002-12.

Moss, F.P., and Leblond, C.P. (1971). Satellite cells as the source of nuclei in muscles of growing rats. *Anat. Rec.* **170**(4): 421-35.

Nakamura, T., Nawa, K., Ichihara, A., Kaise, N., Nishino, T. (1987). Purification and subunit structure of hepatocyte growth factor from rat platelets. *FEBS. Lett.* **224**(2): 311-6.

Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., Shimizu, S. (1989). Molecular cloning and expression of human hepatocyte growth factor. *Nature.* **342**(6248): 440-3.

Nakane, M., Schmidt, H.H., Pollock, J.S., Forstermann, U., Murad, F. (1993). Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS. Lett.* **316**(2): 175-80.

Pernitsky, A.N., McIntosh, L.M., Anderson, J.E. (1996). Hyperthyroidism impairs early repair in normal but not dystrophic mdx mouse tibialis anterior muscle. An in vivo study. *Biochem. Cell. Biol.* **74**(3): 315-24.

Piette, J., Bessereau, J.L., Huchet, M., Changeux, J.P. (1990). Two adjacent MyoD1-binding sites regulate expression of the acetylcholine receptor alpha-subunit gene. *Nature.* **345**(6273): 353-5.

Reid, M.B. (1998). Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta. Physiol. Scand.* **162**(3): 401-9.

Robert, A.M., Groult, N., Six, C., Robert, L. (1990). The effect of procyanidolic oligomers on mesenchymal cells in culture. II--Attachment of elastic fibers to the cells. *Pathol. Biol. Paris.* **38**(6): 601-7.

Roberts, C.K., Barnard, R.J., Scheck, S.H., Balon, T.W. (1997). Exercise-stimulated glucose transport in skeletal muscle is nitric oxide dependent. *Am. J. Physiol.* **273**(1 Pt 1): E220-5.

Robertson, T.A., Grounds, M.D., Mitchell, C.A., Papadimitriou, J.M. (1990). Fusion between myogenic cells in vivo: an ultrastructural study in regenerating murine skeletal muscle. *J. Struct. Biol.* **105**(1-3): 170-82.

Rong, S., Oskarsson, M., Faletto, D., Tsarfaty, I., Resau, J.H., Nakamura, T., Rosen, E., Hopkins, R.F., Vande-Woude, G.F. (1993). Tumorigenesis induced by coexpression of human hepatocyte growth factor and the human met protooncogene leads to high levels of expression of the ligand and receptor. *Cell. Growth. Differ.* **4**(7): 563-9.

Rudnicki, M.A., Braun, T., Hinuma, S., Jaenisch, R. (1992). Inactivation of MyoD in mice leads to up-regulation of the myogenic HLH gene Myf-5 and results in apparently normal muscle development. *Cell.* **71**: 383-390.

Russell,B., Dix, D.J., Haller, D.L., Jacobs, E.J. (1992). Repair of injured skeletal muscle: a molecular approach. *Med. Sci. Sports. Exerc.* **24**(2): 189-96.

Russo, S., Tomatis, D., Collo, G., Tarone, G., Tato, F. (1998). Myogenic conversion of NIH3T3 cells by exogenous MyoD family members: dissociation of terminal differentiation from myotube formation. *J. Cell. Sci.* **111**(6): 691-700.

Sarti, P., Lendaro, E., Ippoliti, R., Bellelli, A., Benedetti, P.A., Brunori, M. (1999). Modulation of mitochondrial respiration by nitric oxide. investigation by single cell fluorescence microscopy. *FASEB. J.* **13**(1): 191-7.

Schott, C.A., Bogen, C.M., Vetrovsky, P., Berton, C.C., Stoclet, J.C. (1994). Exogenous NG-hydroxyl-L-arginine causes nitrite production in vascular smooth muscle cells in the absence of nitric oxide synthase activity. *FEBS. Lett.* **341**(2-3): 203-7.

Schultz, E., McCormick, K.M. (1994). Skeletal muscle satellite cells. *Rev. Physiol. Biochem. Pharmacol.* **123**: 213-57.

Schultz, E., Gibson, M.C., Champion, T. (1978). Satellite cells are mitotically quiescent in mature mouse muscle: an EM and radioautographic study. *J. Exp. Zool.* **206**(3): 451-6.

Tatsumi, R., Anderson, J.E., Nevoret, C.J., Halevy, O., Allen, R.E. (1998). HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. *Dev. Biol.* **194**: 114-128.

Tullett, J.M., Rees, D.D. (1999). Use of NO donors in biological systems. *Mol. Biotechnol.* **11**(1): 93-100.

Vaidya, T.B., Rhodes, S.J., Taparowsky, E.J., Konieczny, S.F. (1989). Fibroblast growth factor and transforming growth factor beta repress transcription of the myogenic regulatory gene MyoD1. *Mol. Cell. Biol.* **9**: 3576-3579.

Wang, H.H., Lautt, W.W. (1998). Evidence of nitric oxide, a flow-dependent factor, being a trigger of liver regeneration in rats. *Can. J. Physiol. Pharmacol.* **76**(12): 1072-9.

Weintraub, H. (1993). The MyoD family and myogenesis: redundancy, networks and thresholds. *Cell.* **75**: 1241-1244.

Weis, J. (1994). Jun, Fos, MyoD1, and myogenin proteins are increased in skeletal muscle fiber nuclei after denervation. *Acta. Neuropathol. Berl.* **87**(1): 63-70.

White, T.P. and Esser, K.A. (1989). Satellite cell and growth factor involvement in skeletal muscle growth. *Med. Sci. Sports. Exerc.* **21**(5 Supp): S158-63.

Winchester, P.K., Davis, M.E., Always, S.E., Gonyea, W.J. (1991). Satellite cell activation in the stretch-enlarged anterior latissimus dorsi muscle of the adult quail. *Am. J. Physiol.* **260**(2 Pt 1): C206-12.

Yablonka-Reuveni, Z., Rivera, A. (1994). Temporal changes of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult rat fibers. *Dev. Biol.* **164**: 588-603.

Yablonka-Reuveni, Z., Rudnicki, M.A., Rivera, A.J., Primig, M., Anderson, J.E., Natanson, P. (1999). The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. *Dev. Biol.* **210**(2): 440-55.

Yagami-Hiromasa, T., Sato, T., Kurisaki, T., Kamijo, K., Nabeshima, Y., Fujisawa-Sehara, A. (1995). A metalloprotease-disintegrin participating in myoblast fusion. *Nature.* **377**(6550): 652-6.

Yun, K. and Wold, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. *Curr. Opin. Cell. Biol.* **8**(6): 877-89.

Zacharias, J.M., Anderson, J.E. (1991). Muscle regeneration after imposed injury is better in younger than older mdx dystrophic mice. *J. Neurol. Sci.* **104**(2): 190-6.