Differential regulation of satellite cell activation and quiescence by nitric oxide (NO) in normal and dystrophic muscle

BY

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Submitted to the Faculty of Graduate Studies
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Differential regulation of satellite cell activation and quiescence by nitric Oxide (NO) in normal and dystrophic muscle

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

Of

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Abstract

Satellite cells are muscle precursors cells located between the external lamina and sarcolemma of skeletal muscle fibers. The processes of satellite cell activation (withdrawal from quiescence, entry into the cell cycle and gaining the capability for motility) underlie the earliest steps in growth and regeneration of skeletal muscle. Understanding the balance between activation and quiescence should reveal regulatory mechanisms that could impact treatment of conditions where growth and repair are crucial to muscle function and quality of life. Therefore, this thesis examines the roles of nitric oxide (NO) and hepatocyte growth factor (HGF), two known mediators of satellite cell activation, in regulating activation and quiescence in normal and dystrophic muscle satellite cells. Both single muscle fiber and whole muscle culture models were employed in these experiments. Fibers or muscles were mechanically stretched using the FlexCell system in order to induce activation by a physiological mechanism, and activation was measured, by marking the later entry into S phase using bromodeoxyuridine (BrdU) uptake and counting the number of BrdU+ cells located on a single fiber. Stable levels of BrdU uptake in non-stretched cultures over the whole period under study confirmed that any intervention was an activating or inhibiting stimulus in comparison to a reproducible control condition.

Experiments on the time course of stretch-activation for normal, dystrophic (*mdx*) and NOS-1(-/-) satellite cells showed that stretching for ½ hr and 2 hrs activates normal satellite cells on single fibers, and that resting activation levels of both *mdx* and NOS-1(-/-) animals are 5-10X higher than normal. Stretching *mdx* or NOS-1(-/-) satellite cells on single fibers decreased activation levels.

Experiments were designed to reveal the roles of NO and HGF in stretch-activation by treating single fibers with BrdU and one of three treatments 1) a NOS inhibitor (L-NAME), 2) exogenous HGF or 3) a combination of 1 and 2. The experiments showed that stretch-activation is NO-dependent, but that NO dependence can be overcome by 2 hrs of stretch in the presence of HGF. They also showed that quiescence is NO-dependent. Visualization experiments to examine NO production using DAF-2, a compound that fluoresces specifically in the presence of NO, showed NO release after stretching in normal and dystrophic satellite cell cultures. Dystrophic cultures produced much less NO, and regulated NOS activity and NO release after stretching in a manner distinct from normal satellite cells.

The expression of the HGF receptor, c-met, was also demonstrated to be regulated by NO levels, and cells responded after ½ hr of stretch by upregulating the level of mRNA in normal and dystrophic animals. The number of cells that expressed c-met on a single fiber increased after ½ hr of stretch on normal single fibers, but not on *mdx* muscle fibers. Experiments on normal skeletal muscle with cycloheximide indicated that c-met was a delayed-early gene in skeletal muscle activation.

The results presented in this thesis provide direct evidence of NO release by stretch and substantial evidence that NO plays an important role in maintaining satellite cell quiescence and in initiating activation by stretch. Time course, inhibition and c-met in situ results support the hypothesis of at least 2 types of cells located in the satellite position on normal single muscle fibers, an "early activating" NO-dependent, c-met expressing population, and a "later activating" population that does not express c-met until after ½ hr

of stretching. One of these populations appears to be absent in *mdx* muscle. Data strongly implicate mechanical stimulation in conjunction with NOS manipulation as a potential treatment to normalize hyperactivation of satellite cells in Duchenne Muscular Dystrophy.

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Well the day has finally come, and my grad school career is coming to a close. The past four years have gone by so quickly that it's hard to believe that it's over. So many things have happened, both good and bad, and my life has been touched by so many people, that it seems almost impossible to keep these acknowledgments to only a couple of pages. One thing that has hit home too many times to count in my life over the last four years is that we should never take the time we have, or the people who surround us, for granted. While it may have been easier to learn this in a different fashion than I have, the lesson is one that I will never forget. So, I'm going to try to put into words the appreciation I have for those of you who have been so important in this journey.

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Ashley

But we have this treasure in jars of clay...

Abbreviations

BCIP	5-Bromo-4-chloro-3-indlyl-phosphate, 4-toluidine salt
BrdU	Bromodeoxyuridine
BGM	Basil growth medium
BSA	Bovine serum albumin
CEE	Chick embryo extract
CRS-2	Control replacement serum-2
DAB	Diaminobenzidine
DAF-2	Diaminofluorescein-2
DAF-2DA	Diaminofluorescein-2-diacetate
DAPI	4'-6-diamidino-2-phenylindole
ddH_2O	Double distilled water
DEPC	Diethyl-pyrocarbonate
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's modified eagle medium
ECC	Excitation-contraction cycle
ECM	Extracellular matrix
EDL	Extensor digitorum longus
FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FDB	Flexor digitorum brevis

FMN	Flavin mononucleotide
HGF	Hepatocyte growth factor
HS	Hindlimb suspension
HRP	Horseradish peroxidase
IGF-1	Insulin-like growth factor-1
KRP	Krebs-ringer phosphate
L-Arg	L-Arginine
L-NAME	N^{ω} -nitro-L-Arginine methyl ester
MGF	Mechano-growth factor
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium chloride
NHA	N^{ω}-hydroxy-L-Arginine
NO	Nitric oxide
NOS	Nitric oxide synthase
PBS	Phosphate buffered saline
PDZ	PSD-95 discs large/ZO-1 homology domain
PCNA	Proliferating cell nuclear antigen
PM	Proliferation medium
PSD-95	Post-synaptic density protein-95
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
TBS	Tris buffered saline

Chapter 1.
Introduction

Satellite cells are skeletal muscle precursor cells which are located on single muscle fibers and are required for the growth and repair of skeletal muscle. While satellite cells are normally inactive in adult muscle, with appropriate stimulation they will enter into the cell cycle (defined as activation) and then make the new muscle cells and larger fibers required for muscle growth. In aged muscle, satellite cells are resistant to activating stimuli. In Duchenne Muscular Dystrophy (DMD), where muscle is undergoing degeneration, satellite cells appear to require less stimulation than normal cells to become active. Both aging and DMD conditions lead to muscle wasting and abnormal muscle function. The gas nitric oxide (NO) and the growth factor hepatocyte growth factor (HGF) are thought to be essential for normal regulation of activation, with NO preceding HGF in the activation cascade. However, the experiments that previously elucidated this signaling pathway either involved satellite cells isolated away from their position adjacent to fibers and their culture *in vitro*, or involved a non-physiological (crush), or other kinds of injury to whole muscles *in vivo*. Other experiments using single muscle fibers, incorporated the addition of satellite cell activators to the culture medium, a phenomenon that is clearly not physiological.

The goal of the research presented here was to establish a more physiological model through which satellite cell activation could be studied, and to determine how NO and HGF were involved in signaling satellite cells to activate in this model. We also wanted to examine how activation differed between normal and dystrophic muscle, using the *mdx* mouse (the mouse model of DMD). Comparing normal and dystrophic satellite cell activation would allow us to determine the nature of any major variations in the process of activation in normal and dystrophic muscle. Alternate pathways to activation would suggest

that satellite cells may respond to a balance of factors or pathways that regulate activation and quiescence, and enable regeneration even in the pathological, genetic, or pharmacological absence of NO or HGF. Understanding the findings of these studies has the potential to lead us toward the development of new drug targets and therapies for the treatment of DMD and age-related muscle wasting, which are disabling to young and older Canadians and many individuals world-wide.

1. Skeletal muscle morphology

The capacity of skeletal muscle to deliver start-and-stop voluntary movement makes it unique among the organs of the human body. This function is achieved by contraction and relaxation of individual muscle fibers within the greater muscle, many of which are as long as the muscle itself. Skeletal muscle fibers are made of myofibrils connected in parallel by the cytoskeleton and held together by a membrane, the sarcolemma. Myofibrils are composed of numerous sarcomeres, the working units of muscle, linked together in series. Sarcomeres are defined as the area between 2 Z lines and contain actin and myosin, the contractile machinery of skeletal muscle. Also contained within sarcomeres are structural proteins including desmin, tropomyosin, nebulin, and titin (1). These structural proteins are involved in maintaining actin and myosin in position, both within a single sarcomere, and across multiple sarcomeres within the same fiber, during contraction and relaxation (Figure 1). This allows for all of the myofibrils within a single muscle fiber to operate collectively.

In order for action to be transmitted from one muscle fiber to another or to the

attached tendon, they must be connected in some manner. Connective tissue surrounding individual fibers (endomysium), groups of fibers (perimysium) or the entire muscle (epimysium) ensures that this occurs.

1.1. Skeletal muscle cytoskeleton

As the function of skeletal muscle inherently exposes it to large amounts of force that could potentially damage muscle fibers, the sarcolemma plays a protective role for individual muscle fibers. It is also involved in the communication between intracellular and extracellular components of muscle. This mechanical strength and communication is accomplished by the dystrophin-glycoprotein complex (DCG; (2)).

The DGC is comprised of a multitude of proteins including dystrobrevin (α and β), sarcoglycan (α, β, γ, δ), dystroglycan (α and β), syntrophin (α, $\beta 1$, $\beta 2$), sarcospan and dystrophin (reviewed in (2)). Loss of the DGC for any reason leads to increased susceptibility to mechanical injury, increased Ca^{2+} permeability, and disruption of signaling cascades (3). Associated with the DGC are proteins involved in signal transduction, enzymes and structural proteins. One of the enzymes associated with the DGC of particular interest is Nitric Oxide Synthase (NOS) which associates with dystrophin by binding $\alpha 1$-syntrophin through a Post Synaptic Density protein-95 discs large/ZO-1 homology (PDZ) domain (4-6).

1.1.1. Dystrophin

Dystrophin is a large protein (427 kDa, or 3685 amino acids) that is expressed in skeletal and cardiac muscle, as well as in the brain (7). The largest gene in the human

genome at 2.5 Mb, it is located on the X chromosome and produces mRNA that is 14 kb in size (7). Structurally, the rod-shaped protein is composed of 4 domains. The N-terminus is composed of a 240 amino acid actin-binding domain that is highly homologous to the actin-binding domain within α-actinitin and spectrin (8). Adjacent to this is a coiled-coil domain composed of 24 related triple α-helix motifs of approximately 109 amino acids each, for a total of 2849 amino acids (9). The function of this part of the protein is thought to be to give the protein a flexible structure (7). However, it is highly conserved, suggesting that it may also contain other functional domains that are as yet unidentified (7). It is also possible that this region is a physical spacer to separate the cytoskeleton and the sarcolemma (10). Between the coiled-coil domain and the C-terminus is a 280 amino acid cysteine rich region, which binds β-dystroglycan (9). Finally the C-terminus of 420 amino acids is involved in membrane association and binding of dystrobrevins and syntrophins (2;8;9). Smaller isoforms of dystrophin exist, and are expressed in various tissues including retina, central and peripheral nervous systems, liver and kidney (reviewed in (2)). Full length dystrophin expressed in skeletal muscle is concentrated at the sarcolemma, the muscle-tendon junction and the neuro-muscular junction (7). It also attaches the contractile components to the sarcolemma (7). Loss of dystrophin, such as occurs in Duchenne muscular dystrophy (DMD) and *mdx* mouse muscular dystrophy, results in the complete loss of the DGC, and extensive alterations in mechanotransduction and cellular signaling (reviewed in (2)).

1.1.2. Nitric oxide synthase

Nitric Oxide Synthase (NOS) produces NO by converting L-Arginine (L-Arg) to

citrulline (reviewed in (11)). It also produces S-nitrothiols and peroxynitrite (5). NOS was first identified in 1989 (reviewed in (11)), is approximately 300 kDa, and is quite complex (12). It is in fact 2 enzymes that are linked together (12) by a calmodulin recognition site. One enzyme is a cytochrome, located at the N-terminus and the other a cytochrome reductase, located at the C-terminus (reviewed in (11)). Biologically active NOS is a dimer of 2 NOS proteins and 2 calmodulins. The dimer is stabilized by the cofactors tetrahydrobiopterin (BH_4) and the iron protoprophyrin haem, as well as by its substrate L-Arg (11). In order to convert L-Arg to citrulline, NOS takes the substrate L-Arg along with oxygen and nicotinamide adenine dinucleotide phosphate in its reduced form (NADPH), converts L-Arg to a N^{ω}-hydroxy-L-Arginine (NHA) intermediate, and finally produces nicotinamide adenine dinucleotide phosphate (NADP), citrulline and NO. This reaction process requires 5 cofactors: flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), haem, BH_4 and calmodulin. FAD and FMN are involved in transferring electrons from NADPH to the haem domain, haem catalyzes the oxidation of L-Arg, BH_4 is thought to play a redox role and is required for NOS expression, and calmodulin increases the rate of electron transfer and triggers the transfer of electrons from the cytrochrome reductase to the haem (11).

There are 3 isoforms of NOS: neuronal, or type 1; inducible, or type 2; and endothelial, or type 3 (5;13), all named for the tissue in which they were first identified (12). The NOS enzymes are regulated dynamically and are all upregulated by hypoxia and cytokines (5). NOS-1 and NOS-2 are both upregulated during development and also with increasing concentrations of Ca^{2+} and calmodulin (5).

The NOS enzyme most prominent within skeletal muscle is NOS-1μ, an alternatively spliced isoform of NOS-1 (14), which is upregulated by activity, age and crush injuries (5) and downregulated by denervation (5;6). NOS-1μ is localized to the sarcolemma via its N-terminal PDZ domain interactions with α-1 syntrophin, as well as to the neuro-muscular junction (5), the muscle-tendon junction (6) and activated satellite cells (15). NOS-2 expression varies within normal skeletal muscle, but can be found at the sarcolemma and post-synaptically, whereas NOS-3 is associated with mitochondria (5;6).

NOS-1μ has an activity that produces 2-25 pmol NO/min/mg of muscle (5). Activity levels can be controlled by the presence or absence of calmodulin, the state of the muscle (whether it is contracting or resting), as well as by the phosphorylation state of NOS, with a decrease in activity if NOS-1μ is phosphorylated (11). Endogenous inhibitors of NOS include: PIN (protein inhibitor of NOS) which binds NOS and inhibits oxidase activity, caveolin-3, and CAPON (reviewed in (5)). Heat shock protein-90 (hsp-90) is involved in activation of NOS by incorporating haem into NOS-1 when haem is limited (11). The proper localization of NOS to the sarcolemma is also required for enzymatic activity, so loss of dystophin results in the displacement of NOS (16) and downregulation of its activity (17). Finally, NO production can also be stimulated by shear stress and pulsatile vascular flow, recalling that regulation applies to all NOS isoforms ((18); reviewed in (19))

2. Physiology and biomechanics of skeletal muscle

2.1. Energy Usage

The ability of skeletal muscle to produce force is dependent on the available energy supply, which is in the form of adenosine triphosphate (ATP). The energy in ATP is released by the breaking of a phosphate bond, resulting in inorganic phosphate (Pi) and adenosine diphosphate (ADP). The supply of ATP is, however, not unlimited, and therefore the ADP must be recycled back to ATP in order to be used again. This can occur within muscle by 3 separate mechanisms. The first of these is through direct phosphorylation of ADP by the conversion of creatine phosphate to creatine, producing 1 ATP molecule/ creatine phosphate molecule. Creatine phosphate is stored within skeletal muscle in small amounts, and therefore this mechanism of producing ATP is not sustainable for long periods of time. On the other hand, producing ATP by this mechanism is extremely fast, making it a preferred mechanism if energy is required quickly. The second way to produce ATP is by converting glucose into pyruvate through the glycolysis pathway, yielding 2-3 ATP molecules/glucose molecule. This mechanism, while not as fast as direct phosphorylation produces more ATP molecules and is sustainable for a longer period of time. Finally, glucose can be converted all the way into carbon dioxide and water if it is converted to pyruvate through glycolysis and then the pyruvate is transported into mitochondria and taken through oxidative phosphorylation. These reactions produce a total of 36 ATP molecules/glucose, and is sustainable for indefinite periods of time, however oxidative phosphorylation is a slow process relative to glycolysis and the creatine-phosphate pathway (20).

The differences in speed of these 3 reactions, and the level of their sustainability, make the processes of energy production well suited for muscles where fibers have fiber-type specific requirements. Those fibers that are required to produce large amounts of force over

a short period of time (also known as fast-twitch muscles such as gastrocnemius muscle) use direct phosphorylation and glycolysis. Those muscles that are used over extended periods of time with low force-generating requirements (also known as slow-twitch muscles such as soleus and other postural muscles) use oxidative phosphorylation for energy production. A third major fiber type is fast-twitch oxidative-glycolytic, which uses a wider range of metabolic pathways under normal conditions.

The glucose required for ATP production is transported from the blood stream into muscle by glucose transporters during periods of digestion and stored for later use as glycogen. Glucose enters into skeletal muscle by the action of insulin and the recently described hepatic insulin-sensitizing substance (HISS) (21-24).

2.2. Muscle contraction

Muscle contraction is achieved by a sliding action of the actin and myosin filaments along each other; in other words, the myosin filaments crawl along actin filaments toward the Z-line by the action of cross-bridge cycling at the myosin filament head region. This brings the Z-lines closer to each other, shortening the sarcomere. In order for myosin to move along the actin filaments and move closer to the Z-lines, a cycle of ATP binding, ATP hydrolysis and ADP release is used. This cycle is called the excitation-contraction cycle (ECC).

The ECC is initiated by the release of acetylcholine (ACh) from nerves at the neuro-muscular junction, as a result of an action potential. ACh binding to muscarinic receptors on the post-synaptic membrane causes opening of voltage-operated Ca^{2+} channels on the

sarcolemma, which induces the release of Ca^{2+} from the sarcoplasmic reticulum by ryanodine receptors. The first stage of the ECC is ATP binding to a myosin head, causing myosin to release bound actin. The active site on myosin closes around ATP, and ATP hydrolysis ensues. The energy released by hydrolysis is held within the myosin molecule for a short period of time and causes a conformational change (a high energy conformation), causing "cocking" of the myosin head. This change in configuration of the myosin head towards the Z-disc allows it to associate weakly with an actin molecule, one molecule closer to the Z disc than the previous interaction along the actin filament. Myosin releases the phosphate generated from ATP hydrolysis, causing strong binding of the myosin head to the new actin molecule, and then myosin reconverts to a low energy conformation, which moves the myosin head's C-terminus 60 angstroms to the Z-disc, and releases ADP. The cycle is now complete and the myosin has moved one actin closer to the Z-disc (20;25), shortening the sarcomere by approximately 40Å (26). The relaxation of muscle occurs when the intracellular Ca^{2+} levels decrease, due to sequestering of Ca^{2+} back into the sarcoplasmic reticulum (reviewed in(27)).

2.3. Biomechanics of skeletal muscle

The amount of force that can be generated by the contraction of muscle is governed not only by the amount of intracellular Ca^{2+}, but also by a very basic biomechanical relationship. The sarcomere length-tension relationship describes how much force the contractile components of a sarcomere can generate at a given length (Figure 2; (28)) . The optimal length for maximal force production of a sarcomere is between 2-2.5 μm. This is

the range at which there is maximal overlap of actin and myosin crossbridges. At sarcomere lengths greater than 2.5 μm, the overlap is less than optimal for a maximum number of crossbridges to act in parallel cycles. At lengths shorter than 2.5 μm, steric hindrance of the myosin crossbridges makes it difficult for actin to bind strongly to myosin, thereby decreasing the force produced (28;29).

This length-tension relationship does not, however, describe accurately what occurs within a muscle, as there is more to muscle than its contractile components. For this reason, the biomechanical relationship may be more accurately described as the amount of force that can be produced by an entire muscle at a given length. This takes both the contractile component and the parallel elastic component, formed by connective tissue and other molecules contained within a sarcomere and a fiber, into account. As can be seen by comparing Figure 2 with Figure 3, the biomechanical relationship is not as straightforward as the length tension relationship when elastic components are included in the assessment of force production. The graph essentially indicates that as length increases, so does the active tension, until at resting length, the maximum amount of active tension is produced. Past resting length, passive tension from the elastic components within muscle tissue begins to increase, until it surpasses the active tension produced by the ECC, Ca^{2+}, and actin-myosin cycling. This more complex biomechanical relationship results in 2 peaks of maximal tension, one generated by the contractile component, and one generated by the elastic component (28;29). The sum of the two functions gives the net length-tension relationship of a complex tissue.

Skeletal muscle can undergo 3 very different types of changes in length. It can be

actively shortened or alternatively, passively or actively lengthened. Active shortening of muscle is termed concentric contraction. It involves sarcomere shortening, leading to shortening of muscle and, for example, a decrease in joint angle (28). Maintaining muscle in a shortened position for some time (typically hours to days) causes sarcomeres to be held at a length shorter than optimal for maximal force production. In order to return to a resting length of the sarcomeres in a fibril that develops maximal tension, skeletal muscle tissue adapts physiologically by initiating degradation of some sarcomeres in series, to shorten the muscle fiber and fibrils within the tissue. Thus the biomechanical tissue relationship and the length tension contractile relationship are interactive.

Muscle can be lengthened in 2 different ways, either actively or passively. Active lengthening, or eccentric contraction, is caused by lengthening a contracting muscle during loading, and is typically used to decelerate joint movement (28). Active contraction of the lengthening muscle still causes activation of the ECC, subsequent increases in intracellular Ca^{2+}, and binding of actin and myosin crossbridges. However, in comparison with concentric contraction, shortening of sarcomeres is not possible, due to the load applied to the muscle; therefore the muscle lengthens while bearing a load. This lengthening mechanism could be explained simply if it was assumed that the mechanism would be the reverse movement of myosin on actin, in other words an exact reversal of the contraction mechanism detailed earlier. While we know this is not the case, the exact mechanism of lengthening accompanied by activation of the ECC is unknown. Active lengthening contractions of muscle cause the most disruption of muscle fibers, and often lead to an increase in the amount of new muscle tissue that is made, either in series at the muscle-tendon junction or

in parallel as new fibers. These features of new fiber formation from eccentric exercise are described as a characteristic mechanism of muscle damage in fibers that lack dystrophin (see Section 8). Alternatively, muscle can also be lengthened passively, with no activation of the ECC, no increase in intracellular Ca^{2+}, and no external loading. Maintaining muscle in a lengthened position for a period of time, either through active or passive stretching, leads to sarcomere elongation above a length that can deliver maximal force. As a result, muscle tissue again adapts, in this case by adding sarcomeres in series to return the muscle back to optimal resting length. The mechanisms by which skeletal muscle fibers respond to stretching through signaling to satellite cells are the focus of the research presented here.

Both passive and active stretch, applied in a chronic fashion, will result in an increase in muscle mass, due to activation of muscle precursor cells (see Section 3). However, the location at which sarcomeres are added during adaptations to muscle lengthening differs between passive and active stretch. Passive stretch leads to addition of sarcomeres in series, while active stretch leads to increased number of sarcomeres in series (lengthening) *and* in parallel (hypertrophy) (30). The amount of intracellular Ca^{2+} also differs with the type of stretch: active stretch implies that the ECC is engaged and Ca^{2+} is released, while passive stretch does not. Protein synthesis, which is required for new sarcomere addition and maintenance of the whole contractile and Ca^{2+} handling apparatus, does increase during passive stretching. Protein degradation is also increased in response to passive stretch, however to a lesser magnitude than the synthesis induced by passive stretch. Therefore passive stretch leads to an overall increase in protein content (31). The response to active stretching is not as straightforward as seen with passive stretching. The initiation of protein

synthesis and degradation is dependent on the fiber type that is experiencing stretch. If slow-twitch muscles are actively stretched, there is a decrease in protein degradation and no change in the amount of protein synthesis. For fast-twitch muscles, there is an increase in protein synthesis and no change in protein degradation (32). Regardless of the differential responses observed between fiber types, ultimately, there is an increase in protein content within a muscle undergoing active stretching, allowing for growth of the muscle by addition of sarcomeres in series and in parallel. Finally, the number of muscle precursor satellite cells recruited to add sarcomeres depends on the amount of damage that is induced by either passive or active stretch. As active stretch induces greater amounts of damage compared to passive stretch, active stretch would presumably recruit more cells than passive stretch.

Many of the elements of the following sections of the introduction have been previously published in Muscle and Nerve, 2005 (33), which is provided as an appendix.

3. Satellite cells and skeletal muscle regeneration

Satellite cells, first identified using electron microscopy by Mauro in 1961 (34), are muscle precursor cells that lie between the external lamina and sarcolemma of skeletal muscle fibers (35). In young muscle, satellite cells display organelles including ribosomes, rough endoplasmic reticulum, and Golgi complexes, indicative of cells that are metabolically active (36). In normal adult muscle, these organelles are much reduced in volume, and cells are mitotically and metabolically quiescent (36). With appropriate environmental signals,

satellite cells enter into the cell cycle to provide the precursors needed for new sarcomeres in new muscle formation, growth and repair (37). Only during periods of growth and repair from injury or in disease conditions, such as in Duchenne muscular dystrophy (DMD), do the satellite cells in adult muscle resemble those of young muscle (38).

The term "satellite cell" will be used here to refer to those myogenic precursor cells that reside in the so-called satellite position on fibers. These cells are considered distinctive from the myogenic populations that may reside in the interstitial space in a muscle, and are also distinguished from those circulating or bone marrow-resident and bone marrow-derived stem cells that may have the capability of entering the myogenic lineage. The growing field of stem cells is reviewed elsewhere (39-54). It should be noted that there may be additional non-myogenic or otherwise uncommitted or multipotent stem cells located in the satellite position on fibers (55) mixed with satellite cells that are committed to the myogenic lineage. In addition, a recent report by Collins et al (56) showed that there is no need to invoke non-satellite stem cells in order to reconcile the speed and magnitude of a muscle's regenerative capacity with the number of satellite cells on a fiber.

The capacity of satellite cells to increase muscle mass and repair muscle after damage makes them essential for the normal adaptive functions of skeletal muscle. In addition, the ability of skeletal muscle, and possibly activators from other sources, to regulate when, how many, and which satellite cells enter the cell cycle provides evidence of an extensive control system underlying this phenotypic plasticity, since muscle satellite cells rarely "run out" unless challenged by lifelong inflammatory processes or fiber damage (such as in DMD). At this time, the regulatory systems that control entry to, and departure from, the cell cycle

during growth and repair are only partially understood, although there is an expanding suite of satellite cell markers and experimental research methods for their investigation.

3.1. Satellite cell markers

Satellite cells can be identified by position, or by in situ hybridization or immunostaining for various gene transcripts and proteins. Satellite cells are positioned between the fiber membrane, outlined by dystrophin in the fiber cytoskeleton and laminin in the extracellular matrix (ECM) of normal muscle (57-59), or within the laminin surrounding dystrophin-deficient fibers (45;60). Electron microscopy, for many years the gold standard tool for identifying satellite cells, is an important, albeit technically lengthy method for experiments on satellite-cell dynamics during development and regeneration (34;36-38;61-64). Even ultrastructural studies of satellite cells can be confounded by partial sections of infiltrating macrophages or the absence of a nucleus from a cell process observed between the sarcolemma and external lamina of a given fiber, and tangential EM sections can blur the discrete ECM layer external to a putative satellite cell. By fluorescence microscopy, satellite cells are typically located by position and, in relation to their activation state, can be identified by additional immunostaining for one of the proteins that identifies a satellite-cell product, a protein that specifies the myogenic lineage of precursors, or an important receptor. Satellite cells, both quiescent and activated, are typically positioned just external to the layer of m-cadherin protein that is found in the very narrow cleft between satellite cell and muscle fiber (35;65;66), although there may be cells in the satellite cell position that do not overlie m-cadherin (65). Localization of the family of proteins expressed by the muscle regulatory

factor genes (MRFs) myf5, MyoD, myogenin, and MRF4 in nuclei of activated satellite cells, and transcripts of muscle regulatory factors such as myf5 in perinuclear cytoplasm are also used effectively to identify satellite cells in regenerating muscle, and myogenic precursor populations between fibers (54;55;65;67-72). The cytoskeletal protein desmin can be used to identify satellite cells in murine muscle as it is present in proliferating satellite cells (73). Satellite cells also deposit syndecan-3 and syndecan-4 proteins in the ECM (74;75), and pax7-expression specifies the satellite-cell population in development and persists in mature muscle satellite cells (64;76).

The expression of c-met receptor protein, the receptor for HGF, and mRNA expression provides an additional marker that can be used to identify satellite cells, since c-met is expressed in both quiescent and activated satellite cells (77). Unfortunately, immunostaining for c-met protein is not reproducible across laboratories, possibly due to differences in the immunostaining protocol. C-met protein is observed in the attenuated cytoplasm of satellite cells in normal adult muscle; in the hypertrophic cytoplasm of satellite cells in dystrophic muscle; in mononuclear, probable myogenic precursors between fibers; and in the smallest new myotubes in regenerating muscle, prior to the formation of satellite cells on those new fibers (35;77-82). Notably, there are no reports that have tested the hypothesis of a precise one-to-one correlation between satellite cells, identified by electron microscopy and by c-met immunodetection methods.

It is widely recognized that c-met protein expression is not exclusive to skeletal muscle satellite cells. Rather, c-met expression is reported in a wide variety of epithelial and mesodermal tissues, including liver, retina, smooth muscle, and lung, and additionally in

tumor and metastases of the prostate, thyroid, brain, and breast tissues (48;83-92) where it is used to identify cells of stem-like or multipotent capability. However, in studies of muscle in normal, regenerating, and disease states, c-met expression is useful as it marks both quiescent and activated satellite cells (77). It is also expressed by myogenic precursor cells outside the satellite compartment in muscle. The variety of model systems (single fiber cultures, dispersed cell cultures, muscle sections, muscle homogenates, whole mount muscles or embryos, and intact, regenerating or pathological muscle in various wild-type strains or transgenic mice on a variety of genetic backgrounds), isolation procedures (enzymatic digestion, flow cytometry, migration assays, dissection), and experimental designs (e.g., notexin or cardiotoxin injection to induce muscle degeneration, irradiation, injury, aging, and exercise) reported in characterizing satellite-cell c-met expression and localization have probably contributed to the divergent observations reported in the literature.

Each of the above proteins (the MRF's, desmin, syndecan-3 and -4, pax7 and c-met) or the respective transcripts can be employed with variable specificity to identify satellite cells, noting the important and sometimes subtle distinctions drawn from studies in various laboratories, and the specific hypotheses under examination. Indeed, any population of cells, satellite cells being one of many, would typically be characterized by a range of each property, such as protein expression or phenotype, distributed across the types and states of a normal tissue such as muscle. It should again be emphasized that satellite cells are defined differently among laboratories, to include or exclude stem-like cells in a satellite position. In view of the differences in precursor position, and changes effected in satellite cells by processes used to isolate myogenic populations, the expression, and the functional profile of

expression of each putative marker protein will also vary and require characterization for each model, design, and hypothesis. To a relatively large extent, use of the single muscle-fiber model allows a much cleaner visualization of cells in the satellite position that express c-met, mrf's or other genes. The literature lacks complete consensus on whether CD34 or muscle regulatory factor genes are expressed by quiescent satellite cells or whether their expression is restricted to activated satellite cells, stem cells, and myogenic cells in the interstitium (55;92;93). However, it is generally agreed that MRF's are only expressed by activated satellite stem cells.

3.2. Heterogeneity of muscle precursors

The ability to identify satellite cells using histological methods will remain controversial until their characteristics and activities are further elucidated. However, it is recognized that the existence of multiple populations of myogenic precursor cells (including satellite cells) allows muscle tissue to respond differentially to each particular stimulus, type of injury, or physiological demand, and thereby enable a highly controlled response. The range of characteristics displayed by muscle precursor cells is most often available experimentally from differences among cells isolated from muscle. For example, different lineages of muscle precursors can be isolated from the variety of slow- and fast-twitch muscles in typical proportions, and each can differentiate to express distinct profiles of protein isoforms typical of slow and fast muscle (94). It is also demonstrated that muscle precursor cell populations within craniofacial muscles are distinct from those of limb muscles (for example jaw muscles express super fast myosin heavy chain proteins), which

may influence their ability to regenerate after an injury (95).

Not only are myogenic and satellite cell populations observed to differ in characterized lineages and differentiation potential among various muscles of the body, there is additional evidence to suggest that more than one type of precursor cell with myogenic capacity is involved in regeneration within a single type of skeletal muscle (47;50;55;72;96-98). By identifying the differential efflux of Hoechst dye 33342 from cells using fluorescence-activated cell-sorting analyses (99), different populations of precursor cells in muscle have been separated into a "conventional" or main population of myogenic cells such as is characterized in normal muscle regeneration, and another that comprises the "side-population" cells (50). Alternative terminology has referred respectively to these two cell populations as one that is relatively more "committed" to the myogenic lineage (thought to include satellite cells) and another that is more "primitive" (100) and multipotent or stem-like which may include some cells also in a satellite position that are not committed to a myogenic lineage. However, the function or identity of these two populations is in dispute (55;92;93;101). Interestingly, similar populations can be sorted from hematopoietic and other tissues, and side-population cells can develop into different types of tissue, depending on culture conditions or the tissue environment *in vivo* after transplantation.

Heterogeneity among myogenic precursors is also examined by other methods. Three potential sub-populations of myogenic cells have been identified, based on their ability to withstand different levels of irradiation (47), and two populations have been isolated from regenerating or normal muscle based on morphology, proliferative capacity, and ability to differentiate *in vitro* (96). In the latter study, myogenic cells with these different features

were all able to differentiate and contribute to myotubes, and desmin expression increased as myogenic cells within regenerating muscle became activated and differentiated. Those distinctive populations may also differ with respect to their myogenic commitment, if the results in (96) are considered in the context of other reports (40;42;48;50;98;100;102) in which the adhesion behavior of myogenic cell populations (selected by a serial pre-plating technique) was studied in combination with myogenic potential and the expression of genes such as CD34, CD45, and Sca-1. Although reports on myogenic cell heterogeneity have not typically examined satellite cells specifically in their location on fibers, the reported differences among these populations and marker proteins probably reflect the combined contributions of satellite cells (likely to be in the large majority), interstitial and circulating myogenic precursors, and also multipotential stem or stem-like cells resident in muscle tissue. Notably, a typical preparation of single fibers isolated from muscle will include only those progenitor cells situated in a satellite positions, which certainly helps interpretation of data from a variety of experiments and methods.

3.3. Satellite cell heterogeneity

There are a few studies that directly implicate cells in the satellite position on fibers, as being heterogeneous in nature. Satellite cells in extraocular muscle, identified by position and expression, are significantly different from those in limb muscles, and display slow, continuous proliferation and ongoing fusion to fibers, reflecting ongoing fiber remodeling, even in normal animals (57). Two types of satellite cells have also been reported using immunostaining. The majority of satellite cells were defined by CD34 or myf5 expression

in combination with m-cadherin, and a minority (about 5%) were negative for both CD34 and myf5, although there is general understanding that myf5 is only expressed by activated satellite cells (65;103-105).

The literature therefore describes a wide range of muscle-derived muscle precursor cells, satellite cells, more stem-like cells, and non-satellite myogenic cells, which have varying profiles of gene expression and behavior (41;45;48;50;92;99;100;106;107). Differences in experimental design, staining protocols, and models at least partly preclude comparisons of data from the various reports. Such a variable combination of approaches and hypotheses in the literature suggests that an international collaboration would fruitfully cross-reference the character of such cell populations by each of the commonly applied methods. As yet it is not known how many functionally distinct satellite cell populations are contained within skeletal muscle and have functionally significant capacity to contribute to muscle growth and regeneration. There are reports that provide evidence for heterogeneity among satellite cells and among myogenic precursors, based on generally well characterized markers of function (e.g capacity for self-renewal, ability to give rise to multiple tissue lineages, higher proliferative capacity and possibly slower or faster rates of cycling), activation state, position and gene expression. However, integrating the functional distinctions between satellite and stem cells is very challenging: to date, the latter are mostly isolated from muscle prior to their characterization, and observations of cell behavior can vary according to markers of satellite-cell identification, their unknown origins, modeling, and potential differences related to muscle disease and regeneration.

3.4. Skeletal muscle regeneration

The complexity of the skeletal muscle regeneration process in repairing muscle tissue damage and forming new fibers has been explored carefully for many years (63). Regeneration begins simultaneously with key events, such as satellite cell activation and the inflammatory response to damaged fibers (108). As the removal of damaged tissue begins, muscle fiber regeneration ensues under extensive controls, by the state of both the external lamina and sarcolemma (109), growth factors (110), and mechanical events affecting fibers in the ECM-wrapped complex with satellite cells (108) as well as reperfusion. Muscle regeneration can be usefully considered in four stages, apart from events in non-myogenic tissues in muscle that also contribute to tissue repair: (1) satellite cell activation, (2) myoblast or precursor proliferation, (3) differentiation, and (4) return to quiescence, as reviewed briefly in the following paragraphs.

3.4.1. Stage 1: Satellite cell activation

Activation is defined as the process by which satellite cells leave quiescence and become mitotically active and mobile (reviewed in (33;111)). Once activated, the proliferating cells generate precursors for new muscle formation. For our purposes, the focus will be on satellite cell activation from fiber-derived signals until the time HGF binds to the c-met receptor, defining activation as satellite cell entry into the cell cycle and mobilization *from a quiescent state* (G0). Cell cycle entry, or withdrawal from G0, begins the process of preparing for DNA synthesis. The process includes hypertrophy of the satellite cell (reviewed in (35)), as well as an increase in motility, resulting in migration away from the

parent fiber (112;113). This explicit definition of activation necessitates experiments that are designed to examine the very early stages of activation from quiescence. There are however, various definitions for activation within the field. For this reason it is essential that markers of satellite cells be considered within the context of the definition of activation applied in a particular experimental context.

Activation can be measured using a wide variety of methods. Changes in morphology and contents have been extensively used to develop structural criteria for the activation state of satellite cells. A quiescent satellite cell is a spindle shaped cell with little cytoplasm and few organelles (37) whereas an activated satellite cell has hypertrophied organelles and an expanded cytoplasm (35). Another method that is used widely to mark activation is the incorporation of tritiated-thymidine or BrdU into new DNA (37). Although this is a very useful method, it requires that cells enter into S phase, and therefore does not mark activation as it begins, but rather uses DNA synthesis as an effective marker of earlier activation. Application of this technique requires sufficient controls to maintain a baseline quiescence among a non-stimulated population and necessitates catching the appropriate interval when DNA synthesis will start or progress in one cycle, but not enter a second cell cycle. Proliferating nuclear cell antigen (PCNA), which appears in cells during DNA synthesis (114) can also be used as a marker for prior activation, as can the onset of muscle regulatory factor gene and protein expression (104). Further investigations are needed to distinguish among specific markers of activation in satellite cells, and those, such as c-met, which are accepted as marking satellite cells independent of their activation state. At present, the earliest known marker of activation in progress in satellite cells is the co-localization of

c-met with its ligand, HGF (35;81). Finally the expression of immediate early genes, c-fos and c-jun can also be used to mark prior activation, as the proteins are present in satellite cells 3 to 6 hours after injury (115). Interestingly, c-fos expression is prominent in normal diaphragm muscle satellite cells *in vivo*, which appear to be more activated without injury than satellite cells in limb muscle, likely by respiratory movements and fiber shear. Interestingly, in liver tissue, c-fos mRNA expression is increased as soon as 15 minutes after activation of hepatocytes (116).

3.4.2. Stage 2: Satellite cell proliferation

The second stage in skeletal muscle regeneration is the proliferation of satellite cells and myogenic precursor cells (113;117). Daughter cells (myoblasts) then commit to enter the myogenic lineage by the expression of myf5 and MyoD, approximately 6 hours after activation *in vivo* (104). Committed myoblasts continue to proliferate and express muscle regulatory genes, including myogenin, until the balance of protein expression pushes the cells toward differentiation (70;71;71). $p27Kip^1$, a cyclin-dependent kinase inhibitor which negatively regulates the cell cycle regulator, negatively regulates satellite cell proliferation (118), such that the inhibition of progression in cycling effectively decreases satellite-cell proliferation. Two growth factors, insulin-like growth factor-1 (IGF-1) and mechano-growth factor (MGF; an alternatively spliced version of IGF-1) are involved in increasing proliferation of activated satellite cells (119;120). MGF mRNA peaks 1 day after a stretch-stimulation protocol (meant to mimic active stretch) and IGF-1 mRNA levels peak 5-7 days after the same protocol (121). Passive stretching also increases MGF and IGF-1 levels,

however the level of response is lower (119;120).

A major limitation in studies of muscle regeneration is that expression of myogenic marker genes and proteins cannot be used alone to determine whether all satellite cells that are initially stimulated to activate will ultimately progress to proliferation. For this reason, in studying satellite cell and muscle precursor behavior, it would be very effective to combine nucleotide incorporation into new DNA with techniques to localize protein or gene expression as tandem markers of proliferation in myogenic cells (70;122). These experiments may reveal a non-uniform distribution of proliferative progenitor cells (67).

3.4.3. Stage 3: Precursor differentiation

Differentiation is the process whereby proliferating myoblasts derived from activated satellite cells and other myogenic precursors withdraw from the cell cycle and either fuse to existing fibers in repair of damaged segments or fuse to each other to form new fibers (123). Fusion events occur after cells execute mitosis and enter into G1 (124). The onset of differentiation in cell or fiber cultures can be marked by the expression of the myogenic regulatory genes myogenin and MRF4 (54;71;72;125), although myogenin can be expressed in the late proliferation stages of myoblasts during regeneration *in vivo* (70). Early differentiation can also be measured by an increase in creatine kinase activity in muscle tissue (123), particularly the isoform creatine kinase-BB (126;127), and later, by the shift in expression of contractile protein isoforms from immature or developmental isoforms to adult isoforms (128;129). The requirements for, and initiators of, myoblast differentiation and fusion as new fibers grow and subsequently become innervated, have been the focus of

extensive research (71;72;105;123;130-133). Since satellite cells are self-renewing and satellite-derived myoblasts do not uniformly fuse into fibers, at least some myoblasts likely maintain, resume, or take up the satellite cell position, as evidenced by retention of markers of earlier DNA synthesis (62;134;135), and may ultimately return to quiescence (136;137).

3.4.4. Stage 4: Return to quiescence

The ability of satellite cells to return to quiescence has been less thoroughly investigated than activation, proliferation, and regeneration. Under normal conditions the proportion of satellite cells that actually reside in G0 rather than in a long lag phase in G1 is not known, as are many important aspects of satellite cell quiescence. For example, it is uncertain whether the same satellite cells that originally respond to an activating stimulus will return to quiescence, or whether the satellite cell compartment is repopulated only by daughter cells. It is also not established whether the requirements for maintaining quiescence in the absence of an activating stimulus are the same as those required for a possible return to quiescence after activation and cell division. The details of these events likely have impact on the extent and decline of life-long regenerative capacity and relate to regulatory signals during development that are recapitulated during regeneration. Furthermore, these signals may be affected by the transgenic expression strategies often used for study of muscle regeneration. It is likely, however, that the expression of a repertoire of proteins must be upregulated during late differentiation in order to mediate satellite cell quiescence.

Myostatin is acknowledged as a key regulator of quiescence because its overexpression blocks regeneration (138). Satellite cell release from the effects of myostatin

expression in the myostatin knockout mouse enables significant increases in muscle growth and regeneration (139;140). The precise identity and characterization of all the quiescence factors, beyond myostatin and other members of the transforming growth factor-β superfamily, and their interaction with genes that are upregulated during early activation, have yet to be determined.

4. Models used to study satellite cells

Models used to examine satellite cell activation from quiescence include isolated satellite cell cultures (141;142), single muscle fibers (82;112;143;144) and *in vivo* studies in various experimental regeneration protocols (35;65;81). Each model is capable of studying satellite cells to a variable degree of complexity. However, the maintenance of satellite cell quiescence during isolation procedures is essential to address the timing or nature of satellite cell withdrawal from G0 and entry to cycling during activation. This is accomplished in the cell culture model by isolating satellite cells from older animals, since those cells have a longer latent period of quiescence that is maintained after an activating stimulus, before they make DNA, compared to satellite cells from younger animals (141;142). The single fiber culture model involves isolating myofibers, usually from the flexor digitorum brevis muscle (FDB) of mice or rats (144;145), although fibers can be prepared from other muscles. This process of isolating fibers should maintain satellite cells in the normal, quiescent state, and in their characteristic position between the basal lamina and sarcolemma of skeletal muscle fibers. However, protocols for methods of isolation,

digestion, plating, and culture vary among laboratories, and variations may cause inadvertent activation of satellite cells that may not be revealed by the markers of function, expression, or position that are selected for study. The basic method was pioneered by Bekoff and Betz in 1977 (146) and later established by Bischoff (144). It allows for the isolation of intact single whole muscle fibers with their satellite cells still attached and quiescent. Muscles are isolated and digested in collagenase-I, and then fibers are separated from one another using gentle trituration with a wide bore pipette. Fibers are then separated from debris and dead fibers through gravity sedimentation. The clean fiber suspension is then plated on dishes using a variety of adhesives (Vitrogen, Matrigel etc.). Over time, variations of this basic method have arisen. One variation used extensively within the literature is that established by Rosenblatt et al (147). This isolation is not as concerned with keeping satellite cells quiescent as it employs collagenase digestion in a shaker. This allows for a shorter length of time needed for digestion in collagenase, however, the constant motion of the liquid may activate satellite cells. In this method fibers are not subjected to gravity sedimentation; rather they are transferred individually to a fresh dish. This is required as the longer fibers (from EDL muscle for example) which are used by many investigators who use this variation, become tangled in the columns, and this leads to fiber death.

Finally, *in vivo* studies can provide the most comprehensive picture of satellite cells during activation in situ in muscle (78;78;81). However the effects of treatments directed to satellite cells require careful interpretation, due to the complex contributions from non-muscle tissues, perfusion-dependent changes, constraints of tissue sampling, animal activity, and systemic physiology that each may introduce significant variability to indices under

study.

5. Satellite cell activators

5.1. Nitric oxide

Nitric oxide (NO) is a low molecular weight, hydrophobic gas (30 Da) that is highly reactive (12;13). NO was first implicated in cellular processes in 1988 (148-150) when it was identified as the endothelium relaxation factor which caused vascular relaxation. It is now known to be involved in liver regeneration (116), inhibition of platelet aggregation (19), control of vasomotor tone and tissue respiration (5).

NO is unique as a messenger molecule, in that it interacts chemically (covalently) with its target molecules and has an extremely short half-life (151). This short half life is a direct result of its highly reactive nature; however, NO has been found to be highly diffusible (6) and is able to move between 150- 300μm during its estimated half-life of 4-15 seconds (151). These characteristics of NO ensure that the effects of NO signaling happen very close to the site of NO production, leaving no opportunity or requirement for a storage mechanism for the reactive gas (6). The features of transience and volatility suggest the NO signal is potent, and the recent wealth of literature on NO bears out this notion. NO reacts with oxygen (O_2) or peroxide (O_2^-) to make free radicals like peroxynitrite (ONOO-), which are involved in mediating cellular oxidative damage (6). NO can also react with transition metals including haem iron and iron-sulfur centres to make metal-NO addition products, or with reduced protein -thiol groups (RSH, RS^-) which results in formation of RS-NO proteins,

influencing protein function (6).

The role of NO in muscle (smooth, cardiac and skeletal) is pleiotropic. Briefly, in smooth muscle, NO produces vascular relaxation (reviewed in (5)) and inhibits proliferation of vascular smooth muscle cells (12). In cardiac muscle, NO is involved in Ca^{2+} release in cardiomyocytes (152), leading to increases in force production. The release of Ca^{2+} occurs due to stretch, after which NO is released, and in turn causes the opening of ryanodine receptors due to ryanodine S-nitrosylation and subsequent increases in intracellular Ca^{2+} concentration.

NO also influences the ECC in skeletal muscle; however, its impact appears to depend on the mechanism of NO delivery, including the timing and form of NO delivery, and the source (location) of NO (5), as well as O_2 levels and the local pH (6). In skeletal muscle, NO attenuates Ca^{2+} release from ryanodine receptors (5;6), however, it also increases Ca^{2+} release from the sarcoplasmic reticulum, which in turn increases the open probability of the ryanodine receptor (5;6).

Not only is NO involved in modifying the ECC, but it also influences many aspects of muscle growth and repair (35) as well as glucose uptake, gene expression (153) and mitochondrial respiration (5) in skeletal muscle. NO is involved in modifying pre-synaptic function at the neuro-muscular junction by acting as a retrograde signal that inhibits acetylcholine release (6) and acts in general as an anti-inflammatory molecule (154). NOS-1 is anchored in location in skeletal muscle via the DGC (see Section 1.1.2) and the neuronal NOS-1 synaptic protein, CAPON was recently described in skeletal muscle (60).

Relevant to muscle regeneration, Anderson (35) reported that NO played an important

role in mediating activation and repair in skeletal muscle. A bolus release of NO by a skeletal muscle fibers, which occurs in circumstances of injury, was postulated to diffuse through the fiber to the satellite cells that are adherent to the fiber, and to activate them. This NO release was proposed to be the first signal for satellite cells to activate (112;141).

In muscle *in vivo*, the systemic exposure to a non-specific NOS inhibitor, N^{ω}-nitro, L-Arginine methyl ester (L-NAME), just prior to a crush injury inhibited the rapid component of satellite-cell activation by NO (35). L-NAME treatment also prevented the hypertrophy of satellite cells and co-localization of HGF and c-met proteins in satellite cells, one of the earliest signs that activation was inhibited by NOS inhibition. Satellite cell hypertrophy, co-localization of HGF with c-met, and satellite cell activation were observed in the injured muscle of saline-treated mice at 0 and 10 min, but were delayed 10 min or more by the L-NAME treatment (35). Without NOS inhibition, satellite cells showed rapid, NO-dependent cell and organellar hypertrophy, co-localization of HGF and c-met proteins, and reduced adhesion to fibers (35).

In vivo studies have also shown that short-term treatment with L-Arginine resulted in an increase in activation, as marked by increased DNA synthesis in *mdx* animals (111). Further studies of satellite cells resident on single fibers have confirmed the involvement of NO (112). Although activation of individual cells is considered an all-or-none process when defined by BrdU staining (positive or negative), the satellite cell population showed a dose-related response to the activating stimulus of L-Arginine (112). NO is also known to regulate the release of HGF from satellite cell cultures after mechanical stretching (141), and increase the number of sarcomeres in series during muscle growth (155).

5.2. Growth factors

Hepatocyte growth factor (HGF), also known as scatter factor due to its ability to initiate cell motility, was first detected in the plasma of rats which had undergone partial hepatectomy, and was shown to be a potent mitogen for hepatocytes in culture (156). HGF has since been isolated from liver, kidneys, human lung and skin fibroblasts, and human plasma (157-161). HGF first identified in skeletal muscle in 1993 when it was shown that growing and regenerating muscle expressed HGF (162). Bischoff (143) showed that satellite cells on single fibers become activated by an extract of crushed muscle (CME). According to studies of proliferation, measured using BrdU or tritiated-thymidine incorporation into DNA, the HGF contained in CME (81) is released upon injury or gentle compression from skeletal muscle, and is responsible for inducing activation of quiescent satellite cells in cell cultures (141;142;163;164), in fiber cultures (112) and *in vivo* (81).

HGF is synthesized and secreted as a biologically inactive precursor, pro-HGF, which is a single chain protein (165) of 728 amino acids (157). Active HGF is a heterodimer of α and β subunits, which are both products of the proteolytic cleavage of pro-HGF (157;165;166) and are linked together by a disulfide bond (165). The α subunit, which has a size of 60 kDa, is composed of a N-terminal leader sequence that is hydrophobic, contains a hairpin loop and has 4 kringle loops. The β subunit is smaller at 32-36 kDa, and has a sequence with high homology to the catalytic domain of serine proteases; however it does not exhibit enzymatic activity due to the replacement of 2 amino acids within the active site of the enzymatic sequence (165).

Activation by HGF occurs through the HGF receptor, c-met (84), a tyrosine kinase

expressed by satellite cells (77). The binding of HGF to c-met triggers the enzymatic activity inherent to the tyrosine kinase. This causes rapid autophosphorylation of the receptor (80;84) and initiates multiple signaling cascades including the mitogen-activated protein kinase (MAPK) and phosphatidyl inositol-3 kinase pathways (167), that are involved in driving the transcription of genes required for growth and cell division (168). HGF is expressed in, and enhances proliferation of, satellite cells and inhibits differentiation of myogenic precursor cells (169).

Other growth factors, including fibroblast growth factor-1, and -2, platelet-derived growth factor, and IGF are not demonstrated to induce satellite cell activation *de novo* from a quiescent state (170). Rather these growth factors appear to be more specifically involved in promoting the proliferation and differentiation of satellite cells that were already activated at the time of exposure to those proteins, than in activation *per se*. In the absence of an effective marker for quiescence, it is important to demonstrate that under control conditions, the maintenance of satellite cells in a quiescent, stable, non-proliferative state during the same interval in which growth factor or other stimulation induces proliferation. Fibroblast growth factor, although a potent mitogen for muscle, does not reproducibly activate satellite cells from quiescence (163). Given that activation also triggers cell cycling, the distinction is often difficult to address without specific attention to experimental design. This consideration had particular implications for the present thesis. Fibroblast growth factor does stimulate an increase in the proliferation of already-activated satellite cells, and represses the ability of activated satellite cells to differentiate. IGF, by comparison, is known to promote both processes (110), and is involved in the hypertrophy response to stretch (reviewed in

(155)).

5.3. Physiological stimulation from exercise

Muscles exposed to enough physical activity and exercise are reported to experience traumatic micro-tears. The trauma can be either direct or indirect (108), and results in activation of normally quiescent satellite cells upon sufficient disruption of the sarcolemma (108;109). This was demonstrated by a variety of indices. In rat muscle after resistance exercise induced by motor nerve stimulation, satellite cell activity increased; by 5 hours after a single bout of exercise, intracellular signaling via phosphorylation of extracellular signal-regulated kinase-2 (ERK2) and p70 S6 kinase-1 (S6K1) had increased; by 10-24 hours after exercise, there was higher mRNA expression of IGF-1 and the variant isoform, MGF, cyclin D1, and myogenic regulatory genes (myogenin) (171).

Different types of activities will evoke different magnitudes of response by satellite cells (as discussed in Section 2), as will different frequencies and magnitudes of one type of activity. *In vivo*, satellite cells are known to proliferate in response to exercise, stretching, and weight loading, and their proliferation is reduced by loss of weight-bearing in the hindlimb suspension model of microgravity (37;172;173). It is not known, however, whether exercise is able to induce widespread activation of satellite cells or whether it is a more localized signal that might induce a response near the neuro-muscular or muscle-tendon junctions or in a region within the muscle architecture defined by the highest strain on muscle fibers or segments (174;175).

Mechanisms by which the mechanical stimuli of exercise are able to induce satellite

cell activation are not well understood. It has been proposed that decreased pH, reduced calcium cycling, glycogen depletion, and ATP depletion may be involved in the transduction of the exercise signal into a response (176). It is also known that one bout of exercise is sufficient to stimulate a more than 20-fold increase in c-fos mRNA within 4 minutes (174). Since c-fos expression can be used as a marker of activation that occurred a short time earlier, this information suggests that satellite cells have a rapid response to exercise *in vivo*, similar to the rapid response by satellite cells to injury of muscle (35), consistent with experiments on single cells, muscle fibers and muscles that are stretched in culture.

6. Potential quiescence factors

Examination of the literature reveals considerable debate over the gene expression profile during satellite cell quiescence, which is by definition a cell residing in G0 of the cell cycle. One example of this debate concerns the expression of the myf5 gene, a marker of myogenic commitment in skeletal muscle satellite cells (as reviewed in (55) and Section 3.3 of this thesis). It is possible that this controversy may be explained by the number of satellite cells in G0 of the cell cycle, as opposed to a G1 lag phase in individual experiments. It is important to note however, that the majority of gene expression profiles examined up to this point have been of genes that are involved in the *activation* of satellite cells, and not those involved in the return to, or maintenance of satellite cell *quiescence*.

Published data indicate that a key feature of activation is the loss of adhesion between satellite cells and the muscle fibers to which they attach (35;112). This implies that adhesion

is a primary requirement for quiescence, suggesting that endogenous or exogenous expression or secretion of adhesion factors, for example integrins or other components of the extracellular matrix, may aid in the return to an inactive state. Another possible participant in the return to quiescence is the growth factor myostatin, which has been shown to inhibit both satellite cell proliferation (139) and differentiation (177). Finally Q6, a quiescence-induced gene which is upregulated in lung fibroblasts returning to quiescence (178), and reportedly expressed in skeletal muscle (179), may play a vital role in stabilizing and/or restoring the quiescence of satellite cells.

6.1. Integrins

Integrins are a family of cell-surface receptors involved in adhesion. They are comprised of α and β subunits (as reviewed in (180)). While there are at least 24 different combinations of α and β subunits, those important in muscle differentiation are αv, α5β1, α6β1 and α7β1; α5β1 being the receptor for fibronectin, and α6β1, the receptor for laminin (180). Sastry and colleagues suggested (181;182) that α5β1 and α6β1 integrins strike a balance in determining the cell cycle fate of primary myoblasts isolated from nine day old Japanese quail embryos. In that model, ectopic overexpression of α5β1 integrin retained myoblasts in a proliferative stage, allowing for fusion only in low to no-serum conditions. On the other hand, ectopic expression of the α6β1 integrin in the same myoblasts resulted in an inhibition of myoblast proliferation and enhancement of differentiation (181;182). For this reason, α6β1 may be involved in myoblast cell cycle withdrawal.

6.2. Myostatin

Myostatin was first identified and placed within the TGF-β superfamily in 1997 by McPherron and colleagues (139). Loss of myostatin gene expression is the cause of the double-muscled phenomenon in cattle (183), and loss of the myostatin gene in mice results in a "large and widespread increase in skeletal muscle mass" (139). Myostatin protein is present in satellite cells of pectoralis major and biceps femoris in chickens (184), and is ubiquitous in muscles of both mice and humans (reviewed in (185)). It was further demonstrated that myostatin inhibits proliferation (186-188) and differentiation (177;188) in C_2C_{12} cells, a mouse myoblast cell line used to study satellite cell proliferation and differentiation. The ability of myostatin to halt proliferation is due to its ability to downregulate cdk2 (186), the cdk that binds cyclin E (25). This cyclin E-cdk2 complex regulates the transition from G1 to S, suggesting that downregulation of the complex blocks C_2C_{12} cells from entering into S phase (DNA synthesis), thereby inhibiting proliferation (186). The eventual inhibitory effect that myostatin has on differentiation is also due to downregulation of the proteins required for differentiation in skeletal muscle, MyoD and myogenin (188). The capacity of myostatin to halt cells from entering S phase suggests that myostatin upregulation would stop satellite cells from proliferating, which could possibly initiate quiescence. Furthermore, since myostatin inhibits differentiation, it may keep proliferating satellite cells from fusing into myotubes to contribute muscle nuclei. This fusion process would have the potential to deplete the satellite cell contingent of skeletal muscle tissue if prolonged. These observations imply that myostatin plays a role in the induction and the maintenance of satellite cell quiescence.

6.3. Quiescence-induced genes

Quiescence-induced genes, or quiescins, are 10 genes that were found to be upregulated in lung fibroblasts returning to quiescence (178). The majority of the quiescins code for previously identified proteins, including the extracellular matrix components, collagen α1(I, III and VI), collagen α2(I) and decorin (178). Two of the genes that were upregulated in the return to quiescence were not previously identified, and were named Q6 and Q10. Q10 is expressed at low levels in cycling and senescent fibroblasts, and at higher levels in quiescent fibroblasts (179). Q6 on the other hand is only expressed in quiescent cells, and has been identified in skeletal muscle extracts (179). Since muscle fiber nuclei are post-mitotic, and do not enter the cell cycle, it is unlikely that they would be the nuclei expressing Q6, as they are terminally post-mitotic rather than quiescent. This notion suggests that it is the satellite cells in particular that express Q6 during quiescence.

There are data to suggest that induction of quiescin gene expression is initiated by cell-cell contact in the growth cycle (178), suggesting that Q6 may work in combination with integrin α6β1 (described above as binding laminin and involved in cell-cell adhesion). It has also been suggested that since integrins influence gene expression through the regulation of ERK pathways - via focal adhesion kinases (FAK) (189) - that the same process may help regulate expression of the quiescins (179). In other words, when integrin α6β1 downregulates FAK activity (181), it may induce Q6 expression. Coppock and colleagues also showed that the addition of TGF-β to cultures of rapidly growing fibroblasts induces Q6 expression in those cells (179). Since myostatin is a member of the TGF-β superfamily (139), myostatin may induce Q6 expression in a similar manner. Finally, it has been shown

that integrin signaling is not independent from growth factor signaling and therefore, α6β1 and myostatin may work together in order to withdraw a satellite cell from the cell cycle (182).

7. Aging

As individuals age, the ability of skeletal muscle to function decreases. This is caused by a progressive decrease in musculoskeletal activity and loading, and leads to a loss of muscle mass (atrophy; (190)), and an increase in connective tissue content. In fact, by the age of 70, up to 30% of muscle mass has been lost (reviewed in (191)). This atrophy leads to disruptions in gait and an increased tendency to fall (192), along with a decrease in the ability to live independently, a decreased quality of life of older individuals, and an increase in mortality (193).

The mechanism by which aged muscle undergoes sarcopenia is under a great deal of investigation, as the social costs of this disease within an aging population are climbing. In 2001, 1 out of every 8 Canadians was over the age of 65. By 2006, this percentage is expected to rise to 1 in every 5. In terms of the burden on the health care system, in the late 1990s, falls accounted for 65% of injuries, 84% of injury-related admissions to the hospital and 58% of deaths related to injuries in the senior population. In 2002, the Canadian government spent 27 billion dollars on issues related to the senior-citizen population (194).

7.1. Molecular and cellular changes due to aging

Many discoveries related to the mechanism by which muscle atrophies and becomes weaker were revealed by limb immobilization studies, where entire limbs have been casted for varying lengths of time, or through hindlimb suspension (HS) experiments. Changes in gene and protein expression, morphology as well as function that accompany muscle loss can then be measured. There has also been extensive research using the aging population to examine ways to combat atrophy. As a result of such studies, it is now thought that sarcopenia, or muscle wasting, is a secondary disease process that is caused by a combination of muscle atrophy and motor unit loss (195). Sarcopenia can be induced by neurological changes, alterations in metabolic, hormonal or nutritional factors (195;196), or after a reduction of physical activity ((197-200); reviewed in (201)).

While HS and disuse models can be used to mimic the loss of muscle mass that accompany and result from the aging process, they are unable to account for multiple changes that occur within aging skeletal muscle. For this reason, it is important to consider age-related changes in the response by satellite cells to stimulation in the context of changes within whole skeletal muscle (i.e., as a tissue) due to aging, including the accumulation of free radicals (202;203), loss of vascular perfusion and peripheral neuropathy (204), in addition to the altered biomechanics that result from decreased use and can be effectively modeled using HS and disuse models.

One of the major contributors to muscle wasting is increased protein catabolism (205;206), and a decrease in protein synthesis (206-208). Loss of muscle mass is evidenced by a decrease in the number of fibers within a muscle, as well as a decrease in the numb of sarcomeres, in parallel and in series ((155;209); See Section 2.3). Satellite cell number

also decreases (as much as 10-fold) as aging progresses (210;211), limiting the magnitude of a regenerative response after an activating stimulus such as exercise. In immobilized muscle, after 2 weeks of HS, there is an absence of c-met, m-cad, myf-5 and syndecan-4 expression, all genes required for myogenic lineage specification. However, in the same muscles, there is no change in pax7 expression (212) after 2 weeks of immobilization, meaning that the *number* of satellite cells did not decrease over time in young muscle with HS, but that the expression of myogenic specification genes was lost. This loss of myogenic gene expression associated with HS may explain the increased chance of satellite cells becoming adipogenic instead of myogenic with increasing age (213). HS in young animals did not however explain the general loss of satellite cell as identified morphologically in old muscle (211).

The functional phenotype of satellite cells as precursors decreases with age, in addition to the decrease in the satellite cell number. This decrease in function is evidenced by changes in proliferation and differentiation of young muscle that was previously atrophied by HS (212). By the same token, there is a lack of satellite cell response in old muscle to intermittent mechanical reloading during HS, which is a common characteristic of reloading in young animals (191). Experiments have shown that satellite cells that are isolated from young muscle following HS-induced atrophy and cultured for 24 hrs, show reduced MyoD expression (a measure of cells in the proliferative stage of myogenesis) when compared to MyoD expression in cells from young muscle that did not experience HS (212). The ability of satellite cells from atrophic muscle to form myotubes in culture is delayed by 2 days compared to control (non-atrophic) muscle, or to muscle that has undergone reloading during

HS. There is also a decrease in the formation of large myotubes in cell cultures from atrophic young muscle. Notably, these changes are reversible after 2-3 weeks of reloading (212). In aged rats, however, reloading after 2 weeks HS induces less satellite cell activation than in young rats (reviewed in (191)). This means that there is an increase in the amount of mechanical activity required to counteract the tendency to progress into atrophy with age, since activity induces significantly lower responses from stress- and damage-responsive or inflammatory genes. These observations suggest that processes that regulate muscle growth and the hypertrophy response to exercise also adapt to the consequences of aging (214).

Cellular changes within muscle fibers that accompany aging include a shift within the muscle fiber itself to smaller amounts of cytoplasm within each nuclear domain in a fiber. However there is no change in either the number of myonuclei or the number of nuclear domains (191). The cellular environment in aged muscle is also quite different than in young muscle. It is believed that this is primarily due to changes in levels of growth factors that either increase or decrease regenerative capacity (reviewed in (196)). For example, there is a decrease in IGF-1 (215) and MGF (216;217) in aged muscle, limiting proliferative signals to satellite cells. The expression of myostatin, which is a cell cycle inhibitor, increases with age (218), reducing the general tendency for muscle precursor cells to enter the cell cycle and make new muscle even when required. This reduction in myogenic cell proliferation is thought to be responsible for the replacement of muscle with connective tissue in aged muscle (155;209). NOS-1 expression is decreased by 43% when muscles are immobilized for 10 days (219), and it is likely that this would be observed in aged muscle. Since NO is

known to be involved in the first stages of satellite cell activation, this reduction could account for altered activation kinetics after stimulation.

7.2. Functional changes due to aging

Atrophy also has significant effects that result in decreased function. The decrease in muscle mass leads to a decrease in the amount of force produced per unit cross sectional area (220-224), a decrease in the rate of relaxation after contraction (225) and an increase in the passive stiffness of muscle (possibly due to the increased collagen content; (226)). There is also a decrease in Ca^{2+} levels after an active stretch compared to control weight-bearing muscles. It is believed that Ca^{2+} levels change due to changes in the membrane, and an increase in the amount of reactive oxygen species (ROS) present in aged muscle. Both Ca^{2+} and ROS could potentially damage (227;228), change the control mechanism of (27;225;229), or uncouple (230) ryanodine channels on the sarcoplasmic reticulum, or the voltage-dependent channels located on the sarcolemma. The ability of muscle to establish a working ECC mechanism in a timely fashion is diminished with age, meaning that any new muscle fibers formed in the muscle of an older individual would not be functional. This was shown in studies of myotubes in culture. When myotubes were cultured from muscle cells from young patients (up to 21 years-of-age), the coupling mechanism appears within 6 days in culture (231). This time-frame increases with age, since myotubes grown from muscle of 48 year-Olds establish a coupling mechanism only after 9 days in culture. The latency prior to establishing the ECC mechanism is due to the delayed appearance of ryanodine receptors and voltage-operated Ca^{2+} channels. Cells isolated from 76 year-Olds failed even to

differentiate into myotubes in culture after 9 days, and no coupling mechanism was evident, as there was a complete absence of voltage-operated Ca^{2+} channels (231). The same study indicated that the changes seen in old myoblasts in culture were not due to cell senescence or reduced mobility. For this reason, it seems reasonable to conclude that the cellular environment influences the ability of myoblasts and satellite cells to respond to signals that induce repair. This is supported by studies that indicate that when satellite cells from young muscle are transplanted into old muscle, they are not able to regenerate new muscle fibers properly. By contrast, when satellite cells from old animals transplanted into young muscle form normal muscle fibers in a timely fashion (232), and when a young animal was joined by parabiosis to an old animal, the ability of the older muscle to repair after injury was restored (233). However, it is important to note that there are reports which argue for the occurrence of intrinsic changes within satellite cells with age. One of these studies showed that if satellite cells are removed from either environment (i.e. from muscle in young or older animals) and placed in culture, they respond the same way they do *in vivo* (212).

These changes can also occur quite quickly after immobilization, with a 30% decrease in muscle mass after 6 days (190) and decreases in force production after 9 days immobilization (234). Immobilization studies in young animals, which are used to model age-related atrophy, show that satellite cells from atrophic muscle are not able to proliferate or differentiate as well as satellite cells from non-immobilized muscle of the same age. Myotubes formed by satellite cells that are isolated from atrophic muscle are also smaller than normal (212). However when immobilizing casts are removed from limbs and animals are allowed to resume normal weight-bearing for 2-3 weeks, these satellite cells respond

identically to satellite cells from non-immobilized muscles. This is additional evidence that the satellite cell environment, either atrophic or normal, contributes to the functional phenotype of satellite cells.

Studies involved in finding ways to alleviate muscle atrophy have shown that not all muscle loss can be attributed to decreased activity levels, as endurance activity on its own was not able to stop decreases in muscle strength (235). In fact, resistance exercise is thought to be better for maintaining muscle size during otherwise atrophy-inducing conditions (236), possibly because resistance exercise induces upregulation of MGF and IGF-I, whereas endurance activity only upegulates IGF-I (237). It is possible to increase muscle mass even in the very elderly with resistance exercise. However the use of additional supplements along with resistance exercise increases the effectiveness of the recovery from atrophy as increases in muscle mass, muscle size, gait speed and balance in elderly patients rise further than in those receiving exercise alone (reviewed in (236)). From these data it becomes clear that while atrophy is a debilitating condition that affects the aging population, there are ways to stimulate satellite cells to be responsive to activating stimuli in a more "normal" fashion. Understanding this mechanism of restoring the normal muscle phenotype of younger individuals will be important in decreasing the financial and human impact of atrophy and increasing the quality of life of the senior population.

8. Duchenne muscular dystrophy and mdx mice

Dystrophin, the product of the DMD locus, (238), is absent from the sarcolemma of

muscle fibers in DMD and the mouse homologue, the *mdx* mouse. Lack of dystrophin causes disruption of the DGC (reviewed in (2)), resulting in displacement of NOS-1 to the cytosol and its downregulation (16;17). This change may be the cause of the abnormal activation of satellite cells in *mdx* mice, and possibly also in DMD patients.

Clinical signs of DMD include hypertrophied calf muscles, accompanied by delayed walking and an awkward gait (reviewed in (239)). Creatine kinase levels are elevated in the serum, (reviewed in (239;240)) and other developmental delays are common (reviewed in (239;241)). Over time, patients exhibit progressive muscle weakening, loss of ambulation, contractures, respiratory and cardiac insufficiency, cardiomyopathy, and eventually death.

The *mdx* mouse was first identified by Bulfield (242) as a potential model for DMD, and has provided extensive insight into the pathology and pathophysiology of DMD. Initially there are repeated cycles of muscle degeneration and regeneration, similar to that seen in muscle of DMD patients. However, unlike the conditions in DMD, the *mdx* mouse is able to recover significantly (but not completely) from this process and live to a relatively old age before the disease process causes death (243). It appears that this ability of *mdx* mouse muscle to withstand multiple rounds of damage to a greater extent that muscle in DMD patients is due not only to a slower disease process than that seen in DMD (62), but also due to a sustained compensatory hypertrophy mechanism (244) and upregulation of utrophin (a protein highly homologous to dystrophin) (245) which can at least partially take the place of dystrophin at the sarcolemma.

While the dystrophic phenotype in *mdx* mice is milder than that seen in DMD, the mouse strain is still a very relevant model for studying disease, treatment, pathogenesis and

muscle regeneration. The dystrophic muscles exhibit contraction-induced injury (246), especially during eccentric exercise, identical to that seen in DMD. As well, the diaphragm muscle of the *mdx* mouse exhibits a dystrophic phenotype which is substantially closer to that seen in DMD muscle than shown by limb muscles (247;248). Both *mdx* mouse muscle and muscle from young DMD boys initially show hypertrophic responses to the degeneration-regeneration cycle, which is thought to be a compensatory mechanism to help maintain strength. In DMD patients, this response decreases over time, as the capacity of skeletal muscles to replace damaged fibers decreases, and the muscle is replaced with connective tissue and fat. In the *mdx* mouse, the hypertrophy mechanism does increase the size of the muscle over the life of the animal until quite late in life. However, the muscles are each weaker in terms of force output per muscle cross-sectional area compared to normal muscle (249), indicating that the hypertrophied muscle is not fully functional, healthy, skeletal muscle.

8.1. Initiation of the dystrophic phenotype

One of the original hypotheses used to explain the initiation of the dystrophic phenotype was that damage to muscle was due to disruption of the weakened sarcolemma. However, it has been shown that in very young *mdx* animals, muscles are resistant to injury from a mechanical stimulus (250). Muscles from young *mdx* animals also have similar levels of stiffness compared to normals, suggesting that at an early age the dystrophin-deficient membrane complex can accommodate mechanical stress, possibly through elements that would stabilize either the sarcolemma and/or the muscle tendon junction (suggested by

(250)). Alternatively the small body weight, immature muscle phenotype attachment to cartilaginous bone elements, and reduced activity pattern may each contribute to alleviating the onset of fiber damage. These data seem to suggest that the initiation of the disease process is not due to mechanical perturbation (250). Another mechanism that may initiate the dystrophic process is the presence of abnormally high concentrations of Ca^{2+} in muscle fibers, which have been shown to be linked to increased levels of protein degradation (251). It has been shown that there is an enhanced open probability of mechanosensitive Ca^{2+} channels, located at the sarcolemma of *mdx* muscle (252), leading to increased influx of Ca^{2+} and increased intracellular Ca^{2+} levels under resting conditions. When subjected to stretch, these channels close in the membrane of *mdx* muscle (251), which is the opposite response to that in normal muscle, where mechanosensitive channels remain closed until stretching opens them (ibid). It has also been suggested that the loss of NO signaling may cause induction of the disease process from an ischemia-related mechanism (250). The normal vascular relaxation (dilation) that occurs at times of high demand (e.g. exercise) is diminished in contracting *mdx* muscle. This appears to result in lower perfusion at times of high demand compared to normal. High levels of ischemia are then thought to lead to muscle damage. This idea of an ischemic onset to dystrophy is supported by studies of *mdx* mice which overexpress NOS-1 and show less severe dystrophy (154). However, if ischemia were a major contributor to the disease process, widespread degradation of muscle would be expected along the vascular tree, and this is not the case. In fact, dystrophy is characterized by focal damage throughout the muscle bundle. Also, it may be the case that reduced perfusion serves to provide some additional regenerative capacity, since in tissue culture the

muscle stem cell-like phenotype is enhanced in 6% oxygen culture conditions (202).

8.2. Skeletal muscle changes due to dystrophy

While the mechanism that initiates the disease process is still under contention, the progression of the disease, once initiated, is known to involve mechanical damage to the sarcolemma. As the muscle matures, there is a decrease in the number of folds of membrane at the muscle-tendon junction, leading to a decrease in the surface area of membrane that is available to bear stress and tension (253). As well, when normal muscle fibers are stripped of the sarcolemma and stretched in experimental conditions (254), the amount of damage is the same as that observed in *mdx* muscle fibers. This suggests that the DGC stabilizes the membrane and protects the fiber from damage (255). Further support for there being a change in response to mechanical stimuli during maturation is supported by data from Petrof (256) which show that mechanical stress-induced injury is greater in muscle of older *mdx* mice than that produced in muscles of young *mdx* animals.

Sarcolemmal damage may not, in and of itself, be enough to generate the phenotype of massive cycles of degeneration-regeneration, and the accompanying decreases in muscle function, that are so evident in DMD and *mdx* muscle. For this reason, a "2-hit hypothesis" was proposed outlining a mechanical and then a metabolic progression of disease (257). The first "hit" proposed in the pathophysiology of dystrophy is the disruption of the membrane due to mechanical damage. This is believed to become exacerbated by the loss of NOS-1 from the sarcolemma (secondary to dystrophin deficiency), which leads to ischemic damage. This process does not, however, necessarily prevent recovery of muscle fibers, since fibers

are able to repair small tears in the sarcolemma without inducing necrosis. However, there is a second "hit"proposed; the disruption of signaling pathways due to the loss of the DGC and its associated signaling proteins, which may cause an increased susceptibility to metabolic stress. These two processes together are proposed to push fibers down a necrotic rather than a repair pathway in DMD and *mdx* dystrophy. As well, MGF is absent from *mdx* and DMD muscle (258). As previously noted, MGF is involved in inducing proliferation, and is followed in this induction pathway by expression of IGF-1 which increases proliferation (See Section 3.4.2). The complete absence of MGF may therefore cause the atrophy of muscle by abrogating the ability of dystrophic muscle to sustain high levels of proliferation. In turn, the decrease in muscle mass could lead to an increase in connective tissue deposition. Fibrosis also tends to increase when regeneration processes are imposed on an ischemic tissue. By comparison, other authors report that although repair capacity does decline with age in muscle in DMD and *mdx* dystrophy, these muscles are still able to regenerate a significant amount of new contractile tissue fairly late in the disease (259;260). Others suggest that it is the dysregulation of fibrosis and production of collagen that are the primary reason for eventual failure to regenerate (261-264).

Damage to muscle fibers recruits satellite cells into the cell cycle. Ultimately, satellite cell proliferation will replace damaged muscle fibers or fiber segments in normal muscle. However, due to the loss of NO release from the sarcolemmal region of dystrophic fiber (from the downregulation of NOS-1), satellite cells within *mdx* muscle are thought to respond to a greater degree than necessary, and/or to respond to stimuli that would not normally activate satellite cells. In other words, the satellite cells may be "hyperactive"(35).

It is hypothesized that this hyperactivity is due to the loss of pulsatile NO that is normally released from muscle fibers under resting conditions (219), which is thought to maintain satellite cell quiescence (35). This constitutive or uncontrolled hyperactivity, along with the real need for satellite cell activation to replace damaged or necrotic fibers, results in rapid senescence of the satellite cell population within *mdx* and DMD muscle as dystrophy and aging progress (244;264;265). The ability of *mdx* muscle to regenerate after injury therefore decreases with age (135), and becomes significantly replaced by connective tissue, effectively modeling the overall pathophysiology of DMD (266;267).

During the course of the dystrophin-deficient muscular dystrophy, force production by dystrophic muscle decreases. This is certainly due to a decrease in the number of fibers as the dystrophy progresses, and eventually to the decreased cross-sectional area of the muscle. However, the decrease may also be due to a disturbance of the normal excitation-contraction process as a result of membrane disruption from contraction-mediated damage (3). It is also possible that the gradual decrease in force generating capacity is due to greater susceptibility (and therefore the greater loss) of fast twitch fibers to damage in dystrophy, probably due to their greater force production and speed of contraction compared to slow twitch muscle fibers (268). The shift in fiber-type distribution to a slower phenotype may however, be a mechanism by which the muscle decreases energy requirements, promotes fiber survival, and increases muscle endurance (256). Interestingly, the fiber-type shift may be limited by the apparent ischemia that accompanies perfusion of dystrophic muscle. Another possible reason for reduced force production is a change in the myosin motor in *mdx* compared to normal muscle (269). This causes a decrease in the number of crossbridges

made in *mdx* diaphragm, as well as a decrease in the amount of force generated per crossbridge (269). These data led to the hypothesis that there is a myosin motor dysfunction in the *mdx* diaphragm muscle. However, there is no difference in physical tensile strength between normal and *mdx* muscle sarcolemmal membranes, which is more of a property of the lipid bilayer than the cytoskeleton (270;271). This observation has led to the idea that there are important distinctions between strength and stability (271) that would reflect the differences in function between the membrane and the cytoskeleton. It also suggests that changes to either the membrane or the cytoskeleton would have different consequences to structure and function adaptations to the primary dystrophic process and to disease progression.

9. Significance

While information exists on NO and HGF signaling in skeletal muscle and gives an idea about how these 2 molecules interact with each other, models used to elucidate these roles did not adequately capture physiologically relevant events. The work presented in this thesis was designed to examine the roles of NO and HGF in satellite cell activation using a more physiological appropriate model. This was accomplished by using the single fiber model, where satellite cells are maintained in their native position adjacent to muscle fibers, and passive stretching as the activating stimulus, a phenomenon which occurs often to skeletal muscle *in vivo*. Also, how the response to stretch, and the signaling due to this stimulation, changes in dystrophy was examined, as the control of satellite cell activation in dystrophy is largely overlooked.

Elucidating the requirements for NO and HGF, and their interactions or independence in satellite cell activation in normal and diseased muscle will further our understanding of the repair mechanism and open avenues for the potential to promote muscle tissue repair. Possible deficiencies in the signaling pathways that are responsible for the hyperactivity and early senescence in dystrophic *mdx* muscles will be revealed by such experimental approaches to these requirements and interactions. Alternate pathways to satellite cell activation, in either normal or diseased muscle would suggest the possibility of therapeutically driving satellite cells down these pathways to effect muscle growth, repair or stabilization. Coupled with the information on the roles of NO and HGF, these new data could also lead to the identification of new drug targets, with the potential for developing

new therapies for muscle diseases in which abnormal activation, such as in DMD and age-related atrophy, contributes to a decrease in function.

10. Hypothesis and specific aims

The overall hypothesis underlying this thesis is that both NO and HGF/c-met signals are required for <u>normal</u> stretch-activation of satellite cells on single fibers. As well, we hypothesized that alternate pathways to satellite cell activation are present in disease conditions.

Specific Aims:

1. Determine the time course of stretch-activation of normal, *mdx* and NOS-1(-/-) satellite cells.
2. Characterize the interactions or independence of NO and HGF signaling events in satellite cell activation in normal and *mdx* muscle.
3. Visualize NO release from cultures of isolated muscle cells and determine how stretch influences this release.
4. Determine the gene expression profile of quiescent and activated normal satellite cells and determine if c-met is an immediate early gene.
5. Determine whether there are alternate pathways to satellite cell activation, apart from an NO-HGF mediated pathway.

The experiments described here were conducted using the single fiber model which allows for the maintenance of satellite cell quiescence under normal conditions without vascular,

nerve and other non-muscle influence (82), and also using short term whole-muscle cultures. Fibers and muscles from normal mice and from *mdx* and NOS-1(-/-) mice were used to investigate differences in the regulation of satellite cell activation by stretch, NO and HGF/c-met in normal and diseased muscle.

Figure 1. Molecular structure of a sarcomere (adapted from (1))

The contractile and structural components of a sarcomere. Actin and myosin are the contractile machinery of skeletal muscle. Structural proteins help maintain actin and myosin in position within a single sarcomere, and across multiple sarcomeres within a muscle fiber, ensuring that sarcomeres within a muscle fiber act as one functional unit.

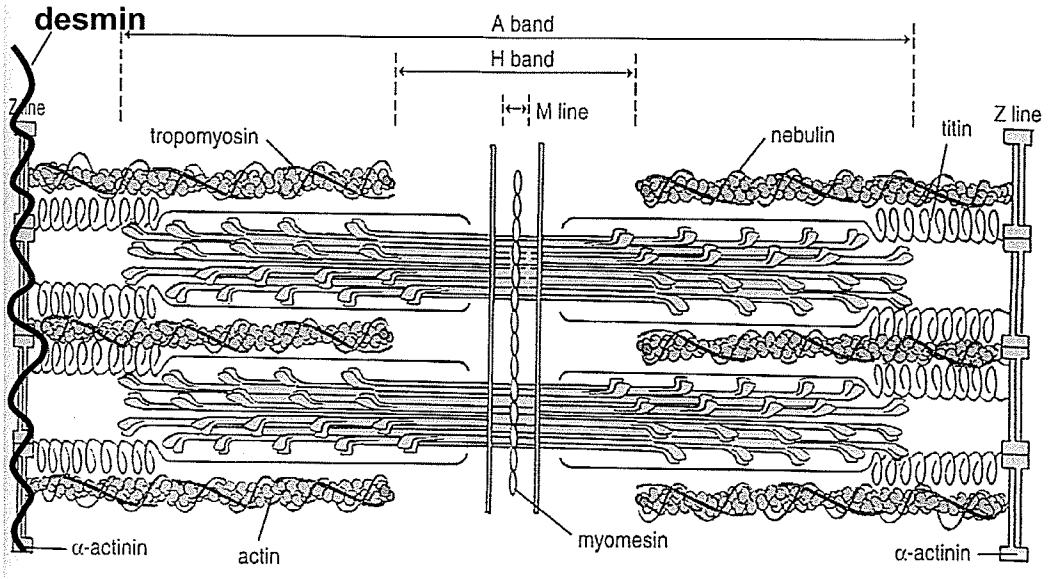
desmin
A band
H band
M line
Z line
tropomyosin
nebulin
titin
Z line
α-actinin
actin
myomesin
α-actinin

Figure 1.

Figure 2. Skeletal muscle sarcomere length-tension curve (28)

A simplistic interpretation of the ability of a sarcomere to develop maximal force by the amount of overlap of the actin and myosin crossbridges. At resting length, actin and myosin overlap maximally. At longer lengths, there is a decreased overlap of crossbridges, resulting in decreased force production. At shorter lengths, steric hinderance results in sub-optimal binding, and decreased force production.

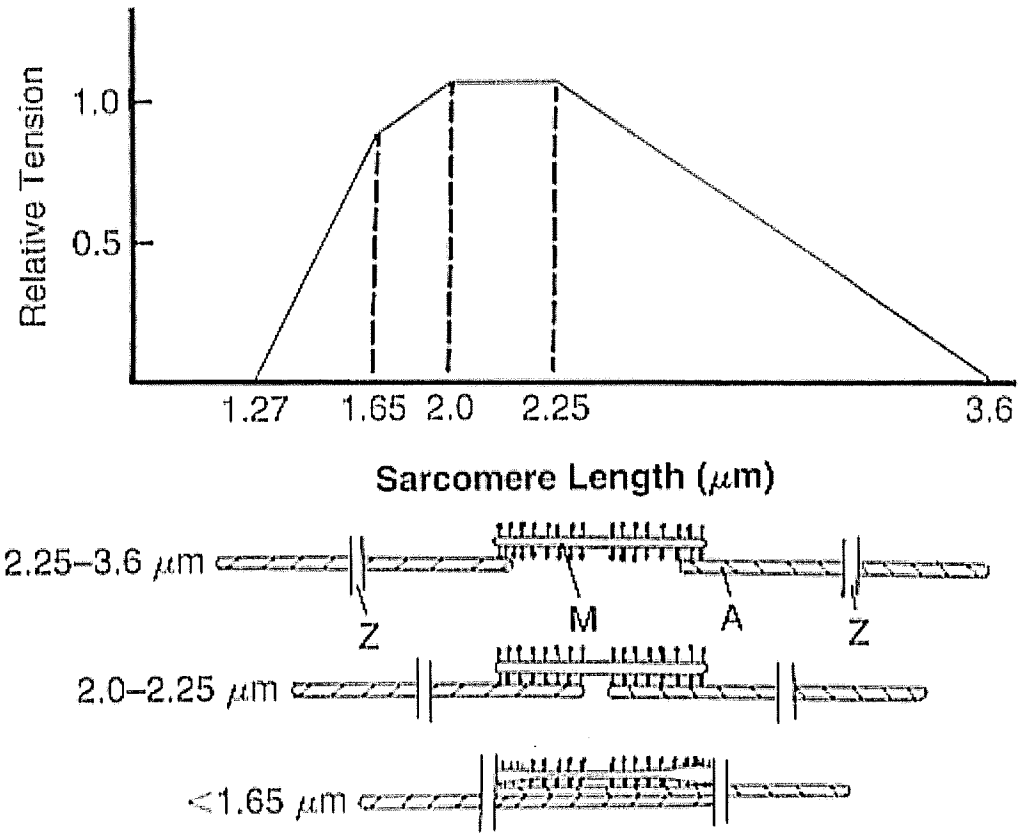
Relative Tension
1.0
0.5
1.27
1.65
2.0
2.25
3.6
Sarcomere Length (μm)
2.25–3.6 μm
Z
M
A
Z
2.0–2.25 μm
<1.65 μm

Figure 2.

Figure 3. True biomechanical length-tension relationship of skeletal muscle (28)

Force production is not only dependent on the contractile component. It is also determined by the elastic component of a sarcomere. At resting length, optimal overlap of the contractile component occurs, resulting in maximal active force production. At shorter lengths, the curve appears much like that seen in Figure 2, with low active and passive forces. At longer lengths however, the elastic component of the sarcomere becomes stretched, leading to an increase in passive force, while active force (produced by actin and myosin) decreases. The resulting curve does not show a complete loss of force at longer lengths, as seen in Figure 2. Instead is shows a small dip as active force decreases and before passive force, generated by the elastic component, surpasses the active force and leads to an overall increase in force

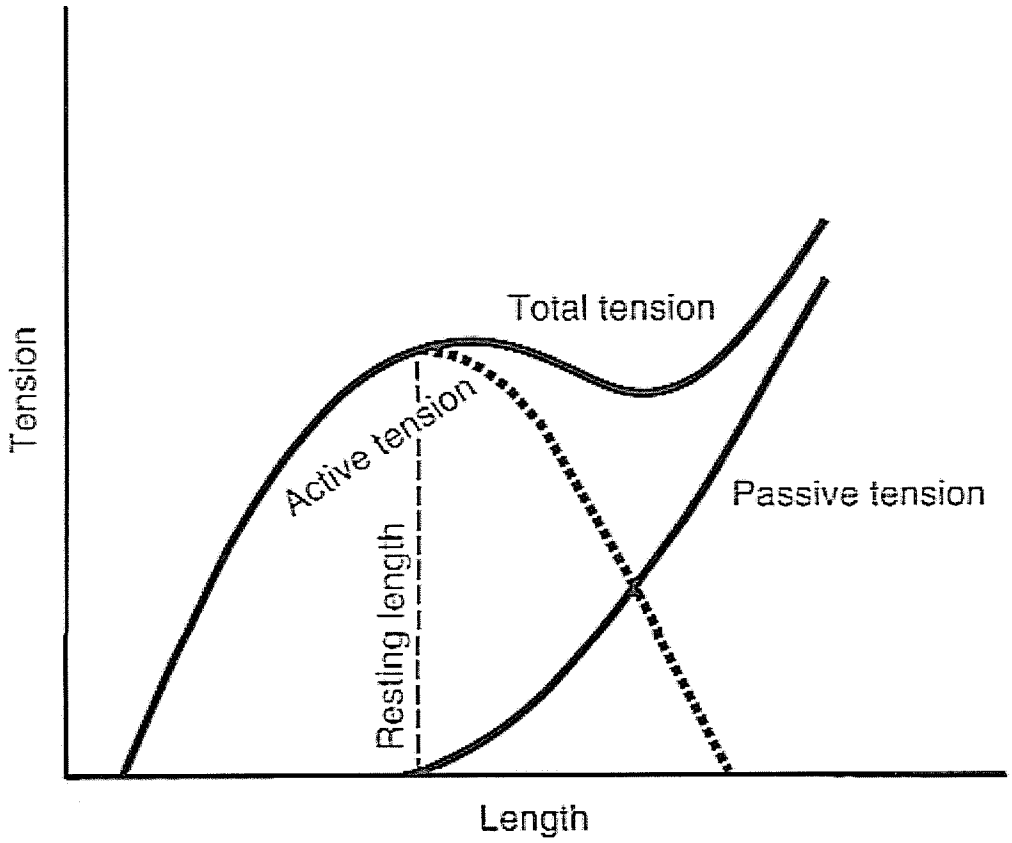
Tension
Total tension
Active tension
Passive tension
Resting length
Length

Figure 3.

Chapter 2.
C-met expression and mechanical activation of satellite cells on cultured muscle fibers

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ABSTRACT

Single fiber cultures can be used to model satellite cell activation *in vivo*. While, technical deficiencies previously prevented study of stretch-induced events, here a method was developed for studying satellite cell gene expression by in situ hybridization using protocol modifications for fiber adhesion and fixation. The hypothesis that mechanical stretching activates satellite cells was tested. Fiber cultures were established from normal flexor digitorum brevis muscles, and plated on FlexCell dishes with a layer of Vitrogen. After 2 hours of stretch in the presence of BrdU, satellite cells on fibers attached to Vitrogen were activated above control levels. In the absence of activating treatments or mechanical stretch, in situ hybridization studies showed 0-6 c-met+ satellite cells per fiber. Time course experiments demonstrated stable quiescence in the absence of stretch and significant peaks in activation after 30 min and 2 hours of stretch. Frequency distributions for unstretched fiber cultures showed a significantly higher number of quiescent c-met+ satellite cells than were activated by stretching, suggesting that typical activation stimuli did not trigger cycling in the entire c-met+ population of satellite cells. These methods have a strong potential to further dissect the nature of stretch-induced activation and gene expression among characterized populations of individual quiescent and activated satellite cells.

INTRODUCTION

Satellite cells are muscle precursor cells that lie between the external lamina and sarcolemma of skeletal muscle fibers. In normal adult muscle, these cells are mitotically and metabolically quiescent (36). Once stimulated to leave quiescence, they become activated, a state defined by satellite cell entry into the cell cycle and preparation for DNA synthesis. This initiates the process whereby precursors are provided for new muscle formation in growth and repair (37). For this reason, satellite cells are essential to normal functions of skeletal muscle. *In vivo* satellite cells are known to proliferate in response to exercise, stretching and weight overload, and their proliferation is reduced by loss of weight-bearing in the suspension model of microgravity (172;272). However, the mechanism that integrates mechanical stimuli into satellite cell dynamics is not well understood.

According to studies of proliferation, measured using BrdU or tritiated-thymidine incorporation into DNA, hepatocyte growth factor (HGF) and nitric oxide (NO) activate satellite cells from quiescence. HGF is contained in an extract of crushed muscle (CME) and, similar to NO, is demonstrated to activate satellite cells *in vivo* and *in vitro* (35;81;112;141-143;163;273). Much, however, is still not known about the normal activation pathway or how the population of quiescent satellite cells is distributed on individual normal fibers. In part, this is due to the technical complexity of sampling, and studying satellite cells that form such a small constituent of the mass or volume of skeletal muscle tissue. Determining the proportion of satellite cells that is available for activation by a given stimulus would help to interpret the effectiveness of regeneration or growth that follows. C-met protein, the receptor for HGF, is expressed by both quiescent and activated satellite cells (46;77;81) although

other gene markers have also been used to study satellite cell populations and responses (e.g., (55;92;104)).

Isolated cultures of satellite cells have been used for many years to study the dynamics of satellite cell proliferation, gene expression and myogenic differentiation that ensues after fusion of the precursors. As such, isolated cell cultures are useful for simulating some aspects of muscle regeneration *in vivo*. However, cell cultures are recognized to be somewhat limited for modelling activation *per se*, since their isolation disrupts the intercellular relationship between a fiber and quiescent satellite cells. Cell cultures are most useful in exploring activation when they are isolated from older animals as they exhibit a long latent period prior to activation (DNA synthesis) and can be maintained for control purposes, in a quiescent state for almost 48 hours in culture. The initial entry of those satellite-derived cells into cycling activity can then address the process of activation from quiescence (e.g. (141;142)). Otherwise, dispersed cultures more aptly model tissue responses and new myotube formation after injury (274).

The use of single muscle fibers in culture is an alternative approach to study activation and subsequent events in early regeneration. The method of single fiber isolation was pioneered by Bekoff and Betz (146) and firmly established by Bischoff (143;144). The method allows for the isolation of single intact fibers with satellite cells in their characteristic position, still in a quiescent state beneath the external lamina, and not incorporating labelled nucleotides into new DNA under control conditions. This method therefore usefully models many *in vivo* conditions of the satellite cell micro-environment while maintaining interactions between fibers and satellite cells (112;143;144;275;276). To date, the application

of single fiber cultures to study mechanical signal transduction is not reported.

The FlexCell culture system delivers a stimulus of cyclical stretching to cells in culture. Using this system, stretch was shown to activate isolated quiescent satellite cells in culture within 2 hours, as judged by significant increases in BrdU incorporation (142). Cells of other origins are also known to proliferate and enter tissue-specific processes as a result of stretching (277;278). Stretch-induced activation has not been examined using the single fiber model, since the conditions required for the experiments have not been developed. While previous experiments without stretching, reported a low level of spontaneous activation by satellite cells (112), the frequency distribution, denoting the population response by satellite cells, was not reported. We hypothesized that mechanical stimulation via stretch would activate quiescent satellite cells on single fibers, once appropriate conditions for maintaining the cultures were identified. We also applied the in situ hybridization protocol to single fiber cultures to enable observations of molecular events at the level of individual satellite cells. In situ hybridization for c-met mRNA was used to determine the distribution of quiescent satellite cells on single fibers after modifications to the procedure.

METHODS

REAGENTS

Vitrogen 100 was supplied by Cohesion Technologies (Palo Alto, CA). Dulbeccos Modified Eagle Medium (DMEM), antibiotic/antimycotic, chick embryo extract, fetal bovine serum (FBS), trypsin, and gentamycin were supplied by InVitrogen (Carlsbad, CA). Araldite resin was supplied by Cedarlane (Hornby, Canada). All other electron microscopy materials were obtained from Electron Microscopy Sciences (Fort Washington, PA). BrdU, Serum Replacement-2 (S-9388; different from the serum replacement used in earlier studies by Yablonka-Reuveni and colleagues, and which is currently not available), HGF, diaminobenzidine, anti-BrdU antibody, HRP-linked anti-mouse antibodies, and diethyl-pyrocarbonate (DEPC) were obtained from Sigma (Oakville, Canada). The c-met plasmid template was a gift from Dr. Carola Ponzetto, University of Turin, Italy. FlexCell plates were obtained from FlexCell International (Hillsborough, NC), and Falcon 35 mm plates were obtained from Becton Dickinson (Franklin Lakes, NJ).

PLATING FIBERS WITH VITROGEN

Fibers from the flexor digitorum brevis muscle (FDB) of mice were isolated according to published methods, as established by Bischoff (143;144) and modified by others (72;112;274). Once fibers were isolated, they were plated in 6-well FlexCell plates with either a rigid or flexible surface substrate that was pre-cooled and coated with 80 ìl pure Vitrogen100. The rigid plastic layer under the elastomer substrate provided a control, unstretched condition. In situ hybridization experiments had fibers plated on 35 mm culture

plates (Falcon). Fibers were allowed to adhere for 20 minutes at 37°C and 5% CO_2, after which basal growth medium was added to each well (2 ml/well) as reported (112) using DMEM, 20% Serum Replacement-2, 1% FBS, 1% antibiotic-antimycotic and 0.1% gentamycin. Fibers were allowed to attach overnight at 37°C and 5% CO_2 prior to stretching. Fiber preparations that contained tissue debris were discarded and not included in experiments.

RESIN SECTIONING

Fibers attached to culture plates were fixed for 20 minutes in acid alcohol (90% Absolute ethanol, 5% glacial acetic acid, 5% H_2O), rinsed in phosphate buffer (pH 7.4), post-fixed in 1% osmium tetroxide and rinsed in double distilled water. After staining en bloc in 8% uranyl acetate for 60 min, fibers were rinsed in water (1hr), and dehydrated in ethanols and methanol. After infiltration steps using propylene oxide, fibers were embedded in araldite resin. The flexible substrate was removed from the hardened resin which was further embedded in methacrylate to identify and protect the Vitrogen layer. Discs of hardened resin containing fibers were removed from petri dishes, and fibers were identified and marked under a dissecting microscope. Blocks of a few fibers were cut from the resin, trimmed and sectioned (½ μm thick). Sections were transferred to slides, stained with toluidine blue, viewed under a light microscope and photographed with Fujichrome professional slide film (ASA 400). Measurements of the Vitrogen layer thickness were made from 9 regions adjacent to each of 9 different fibers, and calculated as mean ± SEM.

IN SITU HYBRIDIZATION

Fixations using paraformaldehyde and a variation of acid-alcohol fixation were tested. The former is known to preserve RNA and for this reason is often used for in situ hybridization (e.g., (103)). Acid-alcohol fixation is also used for fixation of fibers in Vitrogen (112) and was modified as follows for these experiments. Fibers were rinsed with RNAse-free 1X PBS (treated with DEPC) and then fixed in acid-alcohol for 10 minutes. Fixative was removed and fixed fibers were allowed to air-dry in a laminar flow hood for 10-15 minutes and rinsed with three times RNAse-free 1X PBS and stored in the same solution at 4°C until ready to use. The remaining steps of the in situ protocol were carried out as reported in order to detect c-met transcripts in individual satellite cells on fibers (103;122). Templates for c-met receptor were obtained by transforming bacteria with a pBluescript SK+ plasmid containing bases 300-1572 of the met proto-oncogene cDNA (279;280), generously supplied by Dr. C. Ponzetto (281). Antisense, digoxigenin-labelled riboprobes were synthesized following Boehringer Mannheim protocols, as reported in detail (15). Labelled probes were run on formaldehyde-agarose gels, transferred to a nylon membrane, and visualized using anti-digoxigenin antibodies and alkaline phosphatase colour detection to confirm the probe size of 1.27 kb. The target mRNA was 9 kb. Hybridized transcripts were localized in satellite cells resident on fibers. In other experiments, a sense riboprobe for c-met showed no signal, similar to other procedural controls that omitted probe, anti-digoxigenin antibody or colour detection steps.

STRETCHING SINGLE FIBERS

After fiber attachment overnight on Vitrogen-coated FlexCell plates, 0.02% BrdU was added to medium and fibers were placed immediately in a FlexCell system. Fiber cultures were subjected to mechanical stretching at 4 cycles/minute (8 seconds on, 7 seconds off) with 20 kPa stretch. This applies approximately a 10% stretch (282), which is within the physiological range for muscle contraction. Control plates were also placed in the FlexCell system but did not stretch. In initial experiments, fibers were stretched for 0 or 2 hrs. In addition, time course experiments included fibers stretched for 0 hr, 15 min., 0.5, 1, 1.5, 2, and 3 hr periods. After a given period of cyclical stretch, fibers were maintained at 37°C and 5% CO_2, still in the presence of BrdU, until 24 hours had elapsed from the initiation of stretching, and then fixed using acid-alcohol. Fibers were immunostained to detect the incorporation of BrdU using anti-BrdU (1:1000) and secondary HRP-linked anti-mouse antibodies (1:300) visualised with diaminobenzidine (25 mg/ml) (112). The number of satellite cells that accumulated BrdU per fiber was counted blind, without knowledge of source, by systematically scanning the entire area under each coverslip. Only small numbers of mononuclear cells not adherent to fibers were observed, and they were not counted in this population study as they could not be attributed to a particular fiber of origin. Myonuclei were not positive.

STATISTICAL ANALYSIS

Data were compared by analysis of variance (ANOVA) with repeated measures where appropriate (Statpak, NorthWest Analytical Inc.), with pair-wise comparisons between groups using Least Significant Difference (LSD) tests. Frequency distributions were

compared using Chi-square statistics applied to raw data (not percentage data). Significance was determined at the $p < 0.05$ level.

RESULTS

While mechanical stretching is known to increase activation of quiescent satellite cells in dispersed cultures and *in vivo* ((141;142); Tatsumi et al. personal communication), it is not known whether cultures of isolated muscle fibers can be used to model mechanical activation of satellite cells. Use of fiber cultures would also enable the study of satellite cell gene expression, since these cells are easily accessible to staining procedures. To our knowledge this is the first report in which satellite cells on single fibers were prepared successfully for in situ hybridization studies of c-met mRNA expression, and displayed stretch-induced activation.

IN SITU HYBRIDIZATION FIXATION AND STAINING

Fixation is typically required during immunostaining and in situ hybridization protocols, and the latter often employs paraformaldehyde. However, fibers plated on Vitrogen showed a marked and problematic loss of adhesion after brief paraformaldehyde fixation. Here the in situ hybridization protocol used to identify c-met-expressing satellite cells was adapted to incorporate fixation with acid-alcohol which is used during BrdU immuno-detection (112). Fixation included the typical in situ hybridization wash procedures. This acid-alcohol fixation retained the adhesive properties of Vitrogen, without degrading the c-met mRNA signal supplied by in situ hybridization.

In situ hybridization procedures produced intense, specific staining for c-met transcripts. The cells were identified in a satellite position outside the sarcolemma, as observed by focusing through the fiber thickness. C-met-positive cells had a narrow rim of

darkly-stained cytoplasm located around pale nuclei (Figure 1A). The number of c-met-positive cells identified in fiber cultures 18 hours post-plating and without stretching was 0-6 cells per fiber, with a mean (± standard error) of 1.38 ± 0.07. The frequency distribution of c-met-expressing satellite cells is shown in Figure 1B.

STRETCH-INDUCED ACTIVATION AND PROLIFERATION

Initial stretching experiments indicated that a Vitrogen substrate was essential for stable fiber adhesion in FlexCell plates. Fibers were observed embedded at various depths in the Vitrogen layer, as visualized with resin sections, from a position anchored on top of the Vitrogen to one completely surrounded by Vitrogen. The Vitrogen layer itself varied from 40-200 μm in thickness when examined in ½ μm thick resin sections (Figure 2). Although fibers initially adhered in the absence of Vitrogen coating, many fibers rapidly detached from the collagen-coated elastomer layer when the plates were subjected to stretch.

Once fiber attachment was stabilized by Vitrogen, the fiber model was used to identify mechanical stretch as an activating stimulus. Satellite cells on stretched fibers were significantly activated after 2 hours ($p<0.05$, Figure 3), as previously reported for dispersed cultures of satellite cells (141;142). In the absence of stretch (on rigid substrate) very few satellite cells stained positive for BrdU, indicating that satellite cells were quiescent. To confirm that attachment was required for transduction of the mechanical signal, fibers plated directly on collagen-coated FlexCell plates without Vitrogen, did not show activation of satellite cells after 2 hours of stretch (Figure 3).

A comparison of the distributions of 2 hr stretched and unstretched fibers (Table 1,

Figure 4) show a significant increase ($p=0.01$, $\chi^2 = 9.21$, $df=2$) in the number of BrdU+ cells/fiber in the stretched compared to unstretched fiber cultures. The frequency distribution of c-met + cells per fiber compared to that of BrdU+ satellite cells per fiber on the 2 hr stretched fibers (Table 1, Figure 4), shows a significantly greater number of quiescent satellite cells positive for c-met (1.38 ± 0.07) than BrdU+ cells (0.4 ± 0.03) after fibers were stretched (Table 1, $p<0.001$, $\chi^2 = 147.83$, $df=2$).

After determining that a period of stretching stimulated satellite cell activation on single fibers, the time course of activation was examined (Figure 5). In all of the 6 repeated experiments, satellite cells in unstretched cultures showed a very low level of activation, which was maintained for the duration of the experiment. Again, satellite cells attached to single fibers were activated by stretching (2 way ANOVA, $p<0.0001$; $df = 6, 1$). The increase in activation occurred rapidly, as soon as 30 minutes after initiation of stretch ($p<0.05$). Activation peaked at 30 minutes of stretch, declined and then rose to a second peak above control levels at 2 hours ($p<0.05$).

DISCUSSION

These experiments established the number and distribution of quiescent c-met+ satellite cells on a population of normal single fibers. Stretch was also determined to stimulate activation of satellite cells on cultured single fibers, while quiescence was maintained in the absence of stretch. A time course study was conducted to elucidate the nature of stretch-induced activation, and showed 2 peaks of activation within 2 hours of stretching. The demonstration of a time-dependent relationship between stretching and activation of satellite cells has direct relevance to the response to exercise and the repair capacity of skeletal muscle.

Vitrogen was essential for adhesion of single fibers to FlexCell plates. In contrast, reports on dispersed cell cultures of skeletal muscle and other cell types that were plated on collagen-coated FlexCell plates did not require Vitrogen (141;142;282). Without a Vitrogen coating, in the present experiments, there was no increase in activation observed after stretching fibers, likely a result of poor adhesion to the collagen-coated substrate, and shear between long, contractile fibers and the underlying substrate. The Vitrogen substrate did not activate satellite cells, as indicated by the low levels of spontaneous activation seen in unstretched cultures. These findings indicate that secure adhesion of fibers is required for the stretch-stimulus to be transduced to satellite cells through fibers.

The modification of the fixation protocol for in situ hybridization was necessary since the typical paraformaldehyde fixation for in situ hybridization was problematic and allowed fibers to detach from the Vitrogen substrate (Rivera and Yablonka-Reuveni unpublished observations; Anderson unpublished observations). Previous reports of single fiber cultures

examined by immunostaining used methanol fixation and were able to maintain the adhesive properties of Vitrogen (e.g. (72;145)). However, methanol fixation has not been investigated in connection with in situ hybridization. Acid-alcohol fixation, typically used for anti-BrdU immunostaining, maintained the adhesive properties of Vitrogen during in situ protocols. While c-met is also expressed by non-satellite cells in other tissues including pericytes (283-286), c-met expression is reported as a marker of satellite cells as viewed on fibers in sections of skeletal muscle (35;78;81), and in single fiber cultures (112), any fibroblast that theoretically might remain attached to a fiber would not be positive for c-met expression.

The number of c-met + cells/fiber, here identified using in situ hybridization, was taken to represent the number of satellite cells expressing c-met under quiescent conditions. This assumption was based on the low levels of spontaneous activation under basal conditions, measured by BrdU incorporation, in the parallel experiments presented here. Since c-met is expressed by satellite cells regardless of the activation state (77;81), the size of the satellite cell population on normal mouse fibers is now available for detailed comparisons among different muscles, transgenic strains, environments and treatments.

The average number of quiescent c-met+ cells per fiber was 1.38 ± 0.07. The number apparently under-represents the population with the potential to become activated according to earlier studies. Bischoff (144;273) reported 2.1 to 3 cells per rat FDB were activated after 48 hours in proliferation medium. Yablonka-Reuveni and colleagues reported 4-5 cells per rat fiber were positive for proliferating cell nuclear antigen after 36 hours in basal medium containing 1% horse serum and controlled processed serum replacement (a product named CPSR2 by Sigma, and somewhat different from controlled serum replacement used here and

by (112)) (72).

Four explanations may account for the apparent differences between the satellite cell populations identified to date. First, different methods of isolation and culture likely contribute to populations defined by expression of particular gene products (48;55;92). Second, FDB fibers are longer in rats than mice and would show more satellite cells if the density per unit length was the same. Third, the method using Vitrogen to anchor fibers to dishes may affect penetration of the riboprobe used to define c-met+ satellite cells. Although a thick layer of Vitrogen substrate could potentially impede penetration of a 1.2 kb riboprobe, causing some satellite cells to be unidentified by this protocol, this explanation is unlikely since satellite cells were observed in all positions relative to the fiber and the Vitrogen layer, and showed intense staining for c-met mRNA regardless of location. Finally, c-met expression may be restricted to a subset of satellite cells when probed in cultures under conditions that promote satellite cell quiescence. This possibility is under investigation, as it suggests that some activating stimuli may induce significant de novo expression of c-met in a second population of satellite cells, in addition to activating those cells that already express c-met mRNA (and presumably c-met protein) during quiescence.

The number of c-met+ cells per fiber apparently over-represents the number of satellite cells that were activated by stretch in these experiments or by various stimuli reported previously (112). The low numbers of satellite cells positive for either c-met or BrdU on a single fiber under quiescent conditions demonstrate that the basal conditions maintained quiescence and allowed only a small number of satellite cells to become activated during the approximate 40 hrs in culture after plating (Table 1). In the presence of an

activating stimulus (HGF, CME or 2 hr stretch), there was a contingent of c-met + cells that were not activated (i.e. not BrdU+). These data imply that activating conditions do not uniformly engage the entire population of satellite cells that is presumably available for activation. We are currently exploring the idea that there is a subset of satellite cells that reproducibly remain quiescent (i.e., BrdU-negative) even in the presence of the activators known to date.

Satellite cells on single fibers were shown here to be activated by a stretch stimulus. The activation is likely accompanied by an increase in metabolic activity as suggested by experiments on dispersed cultures of rabbit muscle cells which showed a doubling ($p<0.05$) of calcium-phosphatidylserine-dependent protein kinase C activity after1 hour of stretching (McComb, Greenway, Scott and Anderson, unpublished results). This observation is consistent with the reported increase in metabolic activity in other cell types subjected to stretch (277;278;287).

Two peaks of activation were observed in the time course study of skeletal muscle satellite cells on single fibers. Both peaks occurred within the 2 hour time frame examined in a previous report on dispersed cells in culture, and occurred to the same extent (approximately 1.5-1.8X increase) (142). The presence of 2 peaks of activation can be interpreted in two ways. It is possible that the same satellite cells enter proliferation after 30 min and 2 hours of stretch and each cell requires more that one stimuli to become activated. This would imply that activation is not an all or none process since satellite cells were not activated 1 hour after stretching, but does not account for a mechanism that turns off activation processes in the intermediate period. The other possibility is that two (or more)

populations of satellite cells enter proliferation after stretch stimuli of different periods (30 min and 2 hours) due to differences in cell-dependent activation characteristics, and that the first requires different stimuli to be maintained. This latter possibility suggests potential for a differential regulation of activation among various satellite cell populations. This would have an impact on anticipating the level of regeneration or growth resulting from different stimuli. However, in either case, it is difficult to explain the decrease observed in BrdU incorporation when fibers are stretched for 1 and 1.5 hrs. It would be expected that the amount of BrdU observed in cells would be cumulative, since BrdU in present in the medium from the beginning of stretch until fixation 24 hours later. However, if satellite cells on fibers stretched for 1 and 1.5 hrs do not exit from G1, or are prevented from making the G1 to S transition, due to an unfavourable environment to initiate DNA synthesis, then BrdU may not accumulate in these cells. However, it is also likely that once cells are activated by stretch-induced HGF release (141), those cells become mobilized and migrate from fibers (112), and would not be included in the counts between 1 and 1.5 hours reported here. Analysis of the number of satellite cells that express an earlier marker of activation (i.e. PCNA; (114) or c-fos (15)) would address the potential for satellite cells to withdraw from activation under particular circumstances depending on different thresholds of responsiveness to HGF, stretch and other activators including NO.

To our knowledge this is the first report using the single fiber model for studies of stretch-induced satellite cell activation. Control conditions of these experiments maintained satellite cells in the quiescent state in their normal position, and accounted for the potential regulation of activation by subjacent fibers once mechanical stretch was applied. This

mimicked *in vivo* conditions more closely than experiments on stretch-induced activation of dispersed satellite cell cultures. Fiber attachment was required for the study of satellite cells during mechanical stretching, which induced satellite cell activation after 30 minutes and 2 hours. The entry to cycling was visualized with anti-BrdU immunostaining and was maintained at very low levels in quiescent satellite cells on unstretched fibers. In addition, acid-alcohol fixation was used to enable in situ hybridization studies of gene expression in satellite cells attached to the fibers. The size and distribution of the satellite cell population that expressed c-met was characterized in detail in non-stretched cultures. Population studies of activation using frequency distribution were consistent with the idea of heterogeneity among satellite cells (97;145) as prominently suggested by two peaks of stretch-induced activation in the present experiments. With the new information that a mechanical stimulus effects the transition from quiescence to activation and proliferation by resident satellite cells, interactions between stretch and activating treatments can now be explored. This will allow us to fully characterize the signals transduced by fibers and/or inherent to satellite cells, that are required for growth and repair.

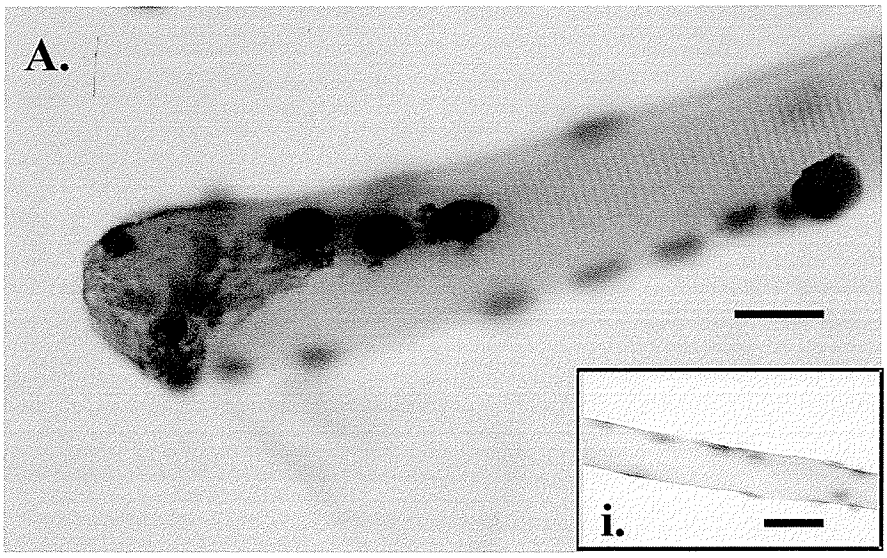
Tables

	CONDITIONS	0	1	2	3	>4	total
1	c-met in situ hybridization Unstretched	154 (24.9)	223 (36.1)	143 (23.1)	64 (10.3)	34 (5.5)	618 (100)
2	Control rigid well 2hr stretched	236 (81.4)	39 (13.4)	14 (4.8)	1 (0.3)	0	290 (100)
	Control flexible well 2hr stretched	142 (70.3)	48 (23.8)	10 (4.95)	2 (0.99)	0	202 (100)
3	Control unstretched	3799 (65.7)	1357 (23.5)	476 (8.2)	107 (1.8)	46 (0.8)	5785 (100)
	HGF unstretched	1701 (58.8)	740 (25.6)	327 (11.3)	79 (2.7)	45 (1.6)	2892 (100)
	CME unstretched	1330 (43.0)	959 (31.0)	513 (16.6)	195 (6.3)	96 (3.1)	3093 (100)

Table 1. Tabulation of raw data and percentage distribution (in brackets) of the number of c-met positive (1) and BrdU positive (2 and 3) satellite cells per fiber from 3 sets of experiments. Data for 3 are provided for comparison and were derived from (112).

Figure 1. A. Satellite cells were identified on single fibers using in situ hybridization and riboprobes to detect c-met mRNA. Four cells appear on this fiber, as detected by a dark rim of cytoplasm surrounding paler nuclei, lightly counterstained with hematoxylin. Fiber cytoplasm did not stain for transcripts of c-met. Inset: A negative control for the in situ procedure omitting the riboprobe for c-met shows no signal for mRNA.

B. Frequency distribution (proportion (%) of total fibers counted) of quiescent satellite cells per fiber that were positive for c-met mRNA in a population of 618 normal mouse FDB fibers, fixed 18 hours after isolation and without stretching. From 0-6 cells per fiber expressed c-met transcripts, with a mean (± standard error) of 1.38 ± 0.07 c-met+ cells per fiber.


A.
i.

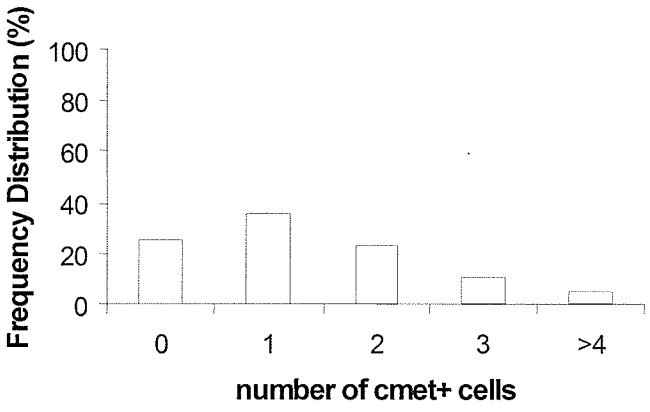

B.
Frequency Distribution (%)
100
80
60
40
20
0
0
1
2
3
>4
number of cmet+ cells

Figure 1.

Figure 2. A light micrograph of an isolated fiber in transverse section partially embedded in the Vitrogen layer. Sections through all layers were made perpendicular to the original culture plate (½ μm in thickness) and stained with toluidine blue to show the fiber (dark) and the paler Vitrogen layer (delimited by arrows) above the region of resin that replaced the flexible substrate (lower grey region). Note the variation in the thickness of the Vitrogen layer. Bar = 20 μm (original magnification 620X).

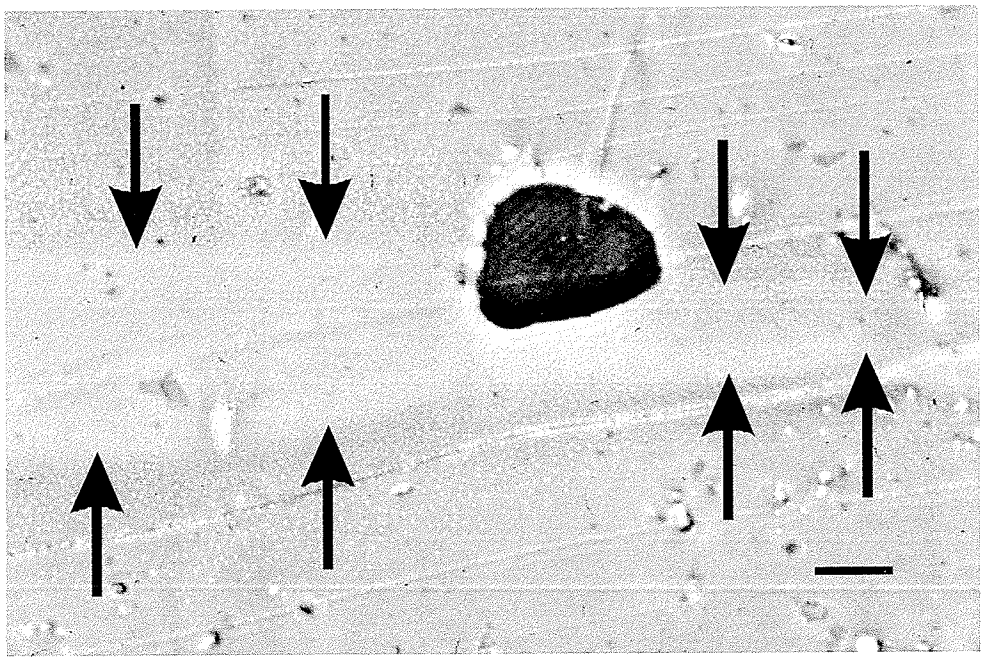


Figure 2.

Figure 3. A graph showing the number of activated (BrdU+) satellite cells per fiber plated either in collagen-coated FlexCell dishes (as supplied) or the same dishes with an additional layer of Vitrogen gel. Fibers were plated on flexible or rigid wells and allowed to adhere overnight (16 hours). BrdU was added, and cultures were subject to stretching for 2 hours (plates with rigid-bottomed wells were placed in vacuum mats but do not stretch). There was no difference in activation between fibers plated on collagen-coated or Vitrogen-covered dishes in the absence of stretch for either flexible- or rigid-bottomed wells. However, stretching for 2 hours induced a significant increase in activation (BrdU+ cells/fiber) when fibers were plated into Vitrogen-covered dishes (*, $p<0.05$). Note that there was no change in activation induced by stretching fibers that were plated onto collagen-coated wells.

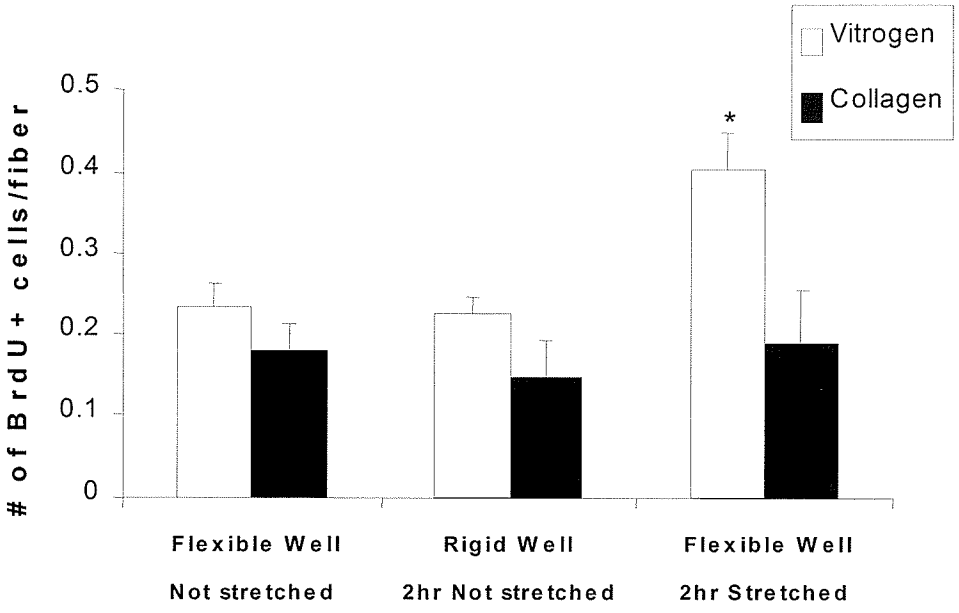

Vitrogen
Collagen
# of BrdU+ cells/fiber
0.5
0.4
0.3
0.2
0.1
0
*
Flexible Well
Not stretched
Rigid Well
2hr Not stretched
Flexible Well
2hr Stretched

Figure 3.

Figure 4. Frequency distributions (proportion (%) of total fibers counted) of the number of activated satellite cells on isolated stretched or unstretched fibers. Activation was determined according to immunostaining for the incorporation of BrdU into DNA. The distribution of fibers was shifted to significantly higher numbers of activated satellite cells per fiber by 2 hours of stretching compared to unstretched conditions ($p<0.05$). Both distributions were significantly lower than the distribution of c-met+ quiescent satellite cells per fiber shown in Figure 2.

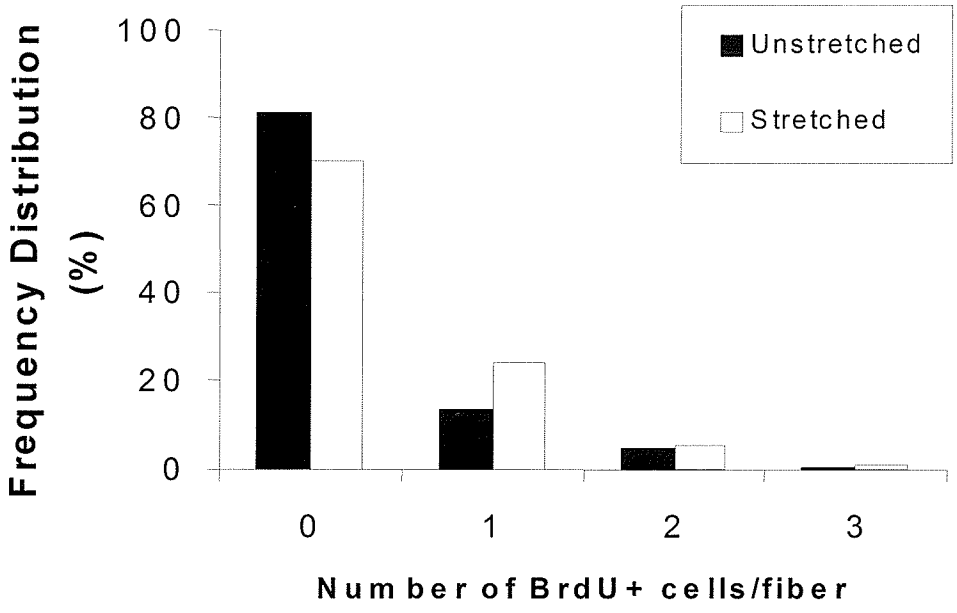

Frequency Distribution (%)
100
80
60
40
20
0
0
1
2
3
Number of BrdU+ cells/fiber
Unstretched
Stretched

Figure 4.

Figure 5. A graph showing a time course of the number of activated satellite cells per fiber achieved by stretched and unstretched cultures. There was a significant difference as early as 30 minutes after the start of stretch and a second peak of activation after 2 hours of stretch (* $p<0.05$). Data (mean ± SEM) were collected from 4 repeated experiments, n=5-14 wells, with 10-233 fibers each, per time point.

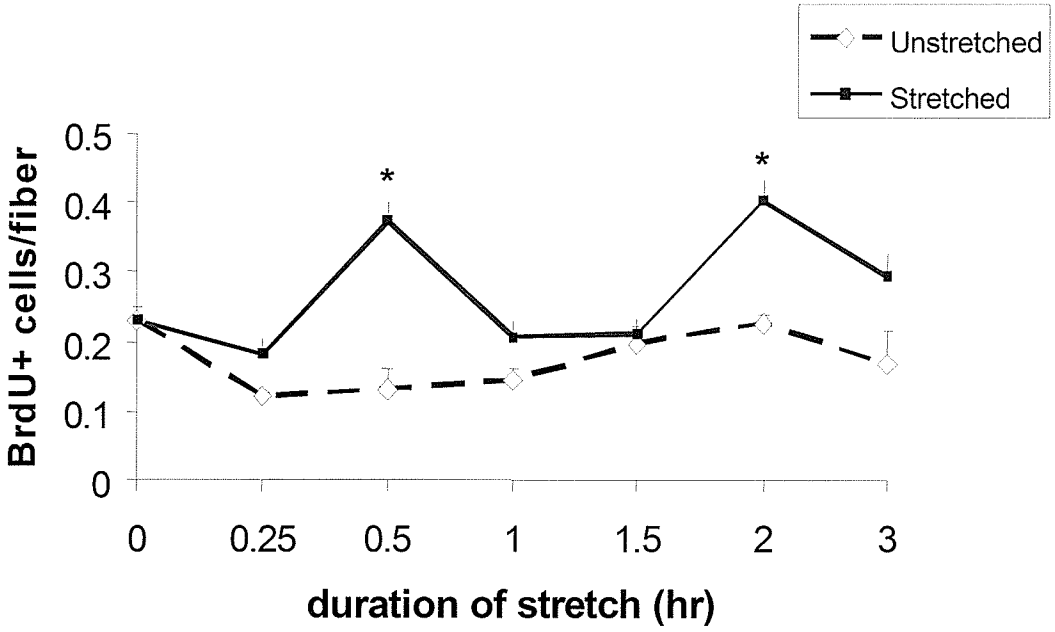

Unstretched
Stretched
BrdU+ cells/fiber
0.5
0.4
0.3
0.2
0.1
0
*
*
0
0.25
0.5
1
1.5
2
3
duration of stretch (hr)

Figure 5.

Chapter 3.
Single fiber isolation and maintaining satellite cell quiescence

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ABSTRACT

The activity of satellite cells during myogenesis, development or skeletal muscle regeneration, is strongly modelled using cultures of single muscle fibers. However, there are variations in reported features of gene or protein expression, as examined with single-fiber cultures. Here, we examined the potential differences in activation of satellite cells on normal mouse muscle fibers produced during a standard isolation protocol, with or without agitation during collagenase digestion. Activation was detected in satellite cells on fibers after 24 and 48 hours of culture in basal growth medium, using immunodetection of the incorporation of bromodeoxyuridine (BrdU) into DNA and quantification of the number of BrdU-positive cells per fiber. At 24 and 48 hours in culture under nonactivating conditions, the number of activated (BrdU+) satellite cells on fibers was greater on fibers that had received gentle agitation during collagenase digestion, than those that were subject to digestion without agitation during isolation. The findings are interpreted to identify that at least some of the variation among published reports may derive from the application of various methods of fiber isolation. The information should be useful for maintaining satellite cell quiescence during studies of the regulatory steps that lead to satellite cell activation.

INTRODUCTION

Single fiber muscle cultures have been used to examine satellite cell gene expression, myogenesis on fibers, regenerative capacity and activation processes. This model is very powerful in that it allows for the maintenance of the satellite cell-fiber interaction while eliminating non-muscle influences and maintaining satellite cell quiescence. While powerful the use of the single fiber model, first pioneered by Bekoff and Betz (146) and expanded by Bischoff (144) is sensitive to activating stimuli. This makes it technically difficult to maintain satellite cell quiescence state which is essential for studying gene expression in quiescence and activation, current areas of importance and interest. However there are contradictions within the literature regarding the state and expression of satellite cells on fibers at isolation. Published reports disagree on gene expression patterns in quiescent satellite cells ((55;65;92) and reviewed in(33)), as well as on the requirements for activating satellite cells from quiescence (reviewed in (33;111)). For example, the expression of muscle regulatory genes such as myf5 by satellite cells is generally reported only in activated satellite cells (77), and continues *in vivo* during myogenic regeneration through precursor fusion into myotubes up to a diameter of roughly 75 μm (103). Most reports confirm these findings (92), although there is at least one report that satellite cells on fibers immediately after isolation, and presumably quiescent according to other markers, also express myf5 transcripts (55). The basis of these differences has not been established.

Since the single fiber model was validated, modifications to the method have been used in various laboratories. The two most widely used isolation methods are those that use gentle shaking during collagenase digestion (ex. (147)) or omit that step (82;112;144). A

comparison of these two techniques for isolating single fibers was carried out in order determine if differences in isolation technique and therefore the activation state of satellite cells at the time of plating could explain some of the discrepancies within the literature.

METHODS

Single fibers were isolated from the flexor digitorum brevis (FDB) of C57BL/6 animals according to (82). Briefly, the FDBs were removed from the animals, and cleaned of connective tissue under a dissecting microscope in proliferation medium (PM; 1X DMEM with 10% fetal bovine serum, 2% chick embryo extract, 1% antibiotic/antimycotic and 0.1% gentamycin). After the muscles were clean, they were placed in a dish with 1X DMEM + 0.2% collagenase and placed in the incubator (37°C, 5% CO_2) for 2 ½ hours. At this point muscles were either gently shaken (as described by (147); i.e. no wave front of liquid was visible on a rotary shaker speed 30, Slow Speed Rotomix, Thermolyne, Dubucq, Iowa), or not (as described by (82;112;144)). After 2 ½ hours, the muscles were removed from the collagenase using a flamed, wide-bore pasteur pipette, and put in fresh dishes of PM. Muscle bellies of the FDBs were then separated from any remaining connective tissue and tendon and then triturated gently using a wide bore pipette. The fiber solution was then cleaned four times using gravity sedimentation. Fibers were then plated according to (82), in basal growth medium, which uses serum replacement rather than high concentrations of fetal bovine serum, in order to limit activation of satellite cells due to culture conditions (1X DMEM with 20% controlled replacement serum-2, 1% fetal bovine serum, 1% antibiotic/antimycotic and 0.1% gentamycin). Vitrogen was used to adhere fibers to culture dishes. BrdU was added to the medium to mark satellite cell activation that was due to isolation. Fibers for both groups (shaken or not) were isolated from the same animals at the same time, and were kept separate from each other throughout the experiment. At either 24 or 48 hours fiber cultures were fixed using acid-alcohol and stained for BrdU incorporation. BrdU+ satellite

cells were counted blind without knowledge of source by scanning the entire area under the coverslip. Only those cells that were BrdU+ and adherent to a live muscle fiber were counted as activated satellite cells, as reported previously (82;112). A typical culture is illustrated in Figure 1. Myonuclei were not positive. The number of activated satellite cells per fiber due to time and isolation procedure were analyzed using 2 way ANOVA. Significance was determined between groups using least significant difference post hoc tests.

RESULTS AND DISCUSSION

Satellite cells on fibers that were isolated without shaking exhibited low levels of BrdU+ (activated) satellite cell at 24 hours and no significant increase by 48 hours (Figure 2). Fibers prepared from FDBs exposed to gentle shaking during digestion showed significantly higher activation after 24 hours in culture, and activation was further increased by 48 hours. Results indicated that gentle shaking during collagenase digestion activates satellite cells from quiescence and suggest that satellite cells are highly sensitive to isolation procedures. This finding may account for variations among reported expression profiles for satellite cells in the quiescent and activated state.

Figure 1. A typical single fiber culture stained for BrdU incorporation.

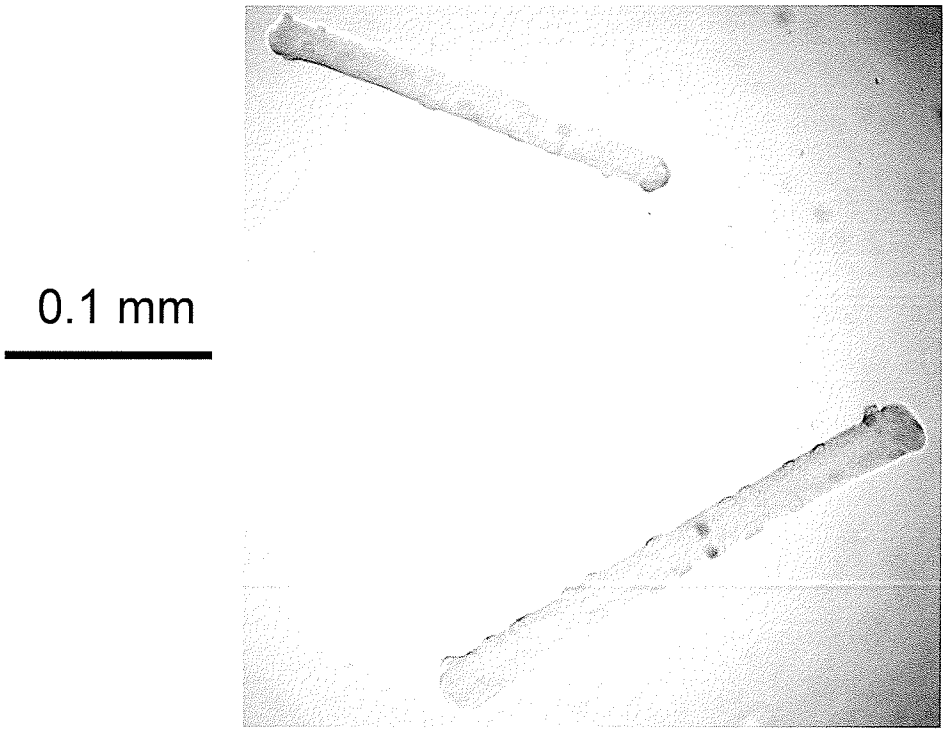

Figure 1.

Figure 2. Single fibers were isolated with or without gentle agitation during collagenase digestion. Fibers were cultured until 24 or 48 hrs had elapsed from time of addition of BrdU, fixed and immunostained for BrdU. The number of fibers visible under a coverslip varied between culture dishes, and there were 2-6 cultures per group (total fibers counted per treatment group: n=306 not agitated, 24hrs; 58 not agitated, 48hrs; 392 agitated, 24hrs; 81 agitated, 48hrs). Data indicate that gentle shaking during isolation activates satellite cells above quiescence (ANOVA2 $p<0.0003$, LSD $p<0.05$*).

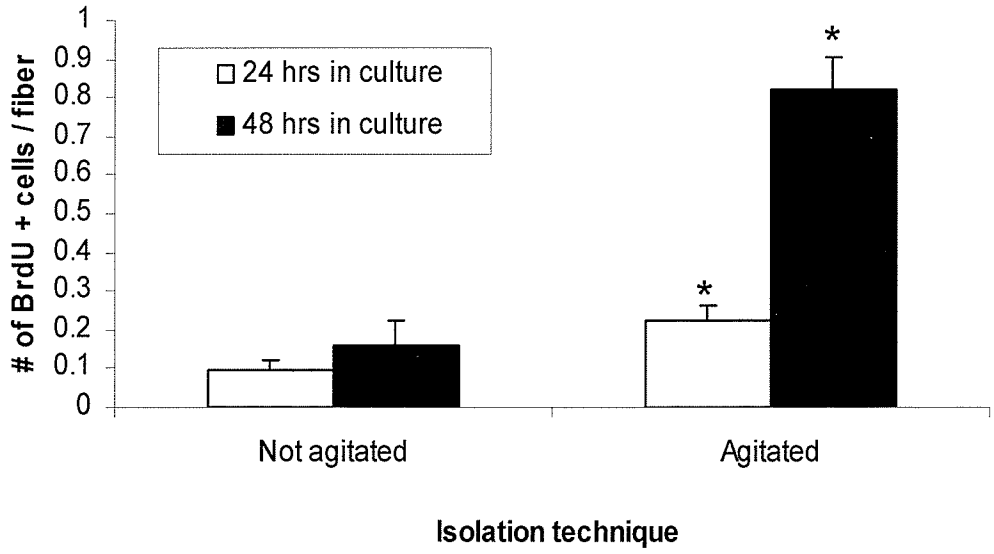
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
# of BrdU + cells / fiber
24 hrs in culture
48 hrs in culture
*
*
Not agitated
Agitated
Isolation technique

Figure 2.

Chapter 4.
Nitric oxide (NO)-dependence of satellite cell stretch-activation and quiescence on fibers.

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ABSTRACT

While nitric oxide (NO) and hepatocyte growth factor (HGF) are involved in satellite cell activation, absolute requirements for either have not been established. In human and *mdx* mouse muscular dystrophy, satellite cells appear "hyperactive", possibly due to lower or dysregulated NO Synthase-1 (NOS-1) activity in fibers and satellite cells. Experiments were designed to test whether NO and HGF both mediate stretch-activation of normal satellite cells on fibers by employing NOS inhibition (L-NAME) and/or HGF treatments in normal fiber cultures. The response to stretch by satellite cells on normal muscle fibers was compared to the response displayed by two independent genetic models of NOS-1 deficiency, *mdx* and NOS-1(-/-) mutants. In normal fiber cultures, NOS inhibition increased activation and reduced the response to stretching. HGF compensated for NOS inhibition at 2 hr of stretching. In normal fiber cultures, exposure to HGF during stretch resulted in decreased activation, consistent with stretch-related changes in the dose-response to HGF. Activation in *mdx* and NOS-1(-/-) fiber cultures was 3-4X higher than normal, and was reduced by stretching. NO is therefore involved in satellite cell quiescence and activation, and responses to NO and HGF are modulated by mechanical stretch. Findings implicate stretch and NO manipulation as potential treatment for normalizing satellite cell activation in human muscular dystrophy.

INTRODUCTION

Skeletal muscle satellite cells are essential for the growth of normal muscle and the progression of repair in injured and diseased muscle (37;46;63;65;67;78;204;288-290). However, the mechanism of satellite cell activation from quiescence (G0) and entry into the cell cycle (G1 and eventually S phase), (reviewed by (33)), and the regulation of stretch-induced activation are not well-established.

One molecule and one growth factor have been identified as activators of satellite cells in studies on cell cultures, single fiber cultures and *in vivo*. Nitric oxide (NO) activates satellite cells from quiescence (35;112;141), and precedes HGF in the activation cascade in isolated cultures (141) and in unstretched single fibers (112).

NO is produced by Nitric Oxide Synthase (NOS; reviewed in (11)), of which NOS-1μ is the skeletal muscle-specific isoform (14). NOS-1μ is localized to the fiber sarcolemma by association with the dystrophin-glycoprotein complex (DGC) via α-1 syntrophin (4) and possibly other members of the complex (suggested by (60;257)). NO is normally produced in a pulsatile manner under quiescent conditions (219). Low levels of pulsatile NO are thought to maintain satellite cell quiescence (35), while activating conditions, such as shear forces, upregulate NOS-1μ expression and activity (291). This upregulation of NOS-1μ is proposed to release a bolus of NO that activates satellite cells (35). Hepatocyte growth factor (HGF) is made by fibers and stored in the extracellular matrix. Studies involving skeletal muscle *in vivo*, single cell cultures, and the single fiber culture model show that HGF binding to its receptor, c-met (located on muscle satellite cells), initiates satellite cell activation (81;112;141;163). However, the details of satellite cell responses to NO and HGF have not

been tested in a model system that allows cell by cell study of satellite cells on fibers.

Recent work in this laboratory demonstrated rapid mechanical stretch-activation of normal satellite cells on single fibers (82). In the absence of stretch, fibers maintained in presence of bromodeoxyuridine (BrdU) showed very low incorporation into DNA, demonstrating the stable baseline of quiescence. By observing cultures at 24 hours after BrdU addition, the stability of quiescence in control cultures can be confirmed, and acts as a baseline for comparison with responses to putative activating signals. Stretching for only ½ hr, using a cyclical 10% length change at 4 cycles per minute, stimulated satellite cell activation. Work by others on stretched cultures of normal satellite cells has shown that activation results from NO release, which facilitates HGF binding to the c-met receptor (141). However, since fibers mediate the balance between quiescence and activation and transmit the signals that activate satellite cells (reviewed in (33;111)), the details of activation signaling are best investigated using the single fiber culture model, which retains satellite cells in quiescence until stimulated to activate.

In dystrophin-deficient myopathy (Duchenne muscular dystrophy, DMD, and *mdx* mouse muscular dystrophy), muscle fibers are susceptible to damage from mechanical stress (292) due to the loss of the DGC from the sarcolemma. This DGC deficiency also displaces NOS-1μ from the sarcolemma and down-regulates NOS-1 activity. As a result, satellite cells in dystrophic muscle do not receive the fiber-derived NO signals from NOS-1μ that are thought to maintain quiescence. Together with the environment of fiber damage and the secondary inflammatory response, satellite cells are more susceptible to activation (35). While experiments *in vivo*, on NOS-1-overexpressing *mdx* mice and the *mdx* mutant strain

have shown that additional NO can alleviate the progression of dystrophy and improve the related regenerative response (15;154;293), the impact of NOS-1 downregulation in *mdx* or NOS-1 knockout muscle on satellite cell activation is not known.

We hypothesized that stretch-induced satellite cell activation of normal fibers in culture depends on both NO and HGF signaling, and that NOS-1 downregulation, such as in *mdx* fibers, would alter the satellite cell response to stretch. We also hypothesized that satellite cells on *mdx* single fibers would not demonstrate a normal response to stretch, given the down-regulation of NOS-1μ in dystrophic fibers. Experiments were designed to examine the roles of NO and HGF in stretch-induced activation of normal satellite cells, using single fiber cultures, and how *mdx* satellite cells would respond to stretch. Since NOS-1 downregulation in *mdx* muscle is not fully understood and may involve variable upregulation of NOS-2 and/or NOS-3, responses of satellite cells on NOS-1(-/-) fibers to stretching served as a genetic negative-control condition for comparison to satellite cells on normal single fibers examined during non-specific NOS inhibition. Results demonstrated an important role for NO in early activation signaling and in the regulation of satellite cell quiescence in normal fiber cultures, stretch-related responses to HGF, and hyperactivation of satellite cells on *mdx* and NOS-1(-/-) fibers that was partly offset by mechanical stretch.

METHODS

REAGENTS

Vitrogen 100 was supplied by Cohesion Technologies (Palo Alto, CA). Dulbecco's Modified Eagle Medium (DMEM), antibiotic/antimycotic, chick embryo extract, fetal bovine serum (FBS), trypsin, and gentamycin were supplied by InVitrogen (Carlsbad, CA). BrdU, Serum Replacement-2 (S-9388; different from the serum replacement used in earlier studies by Yablonka-Reuveni and colleagues, and which is currently not available), N^{ω} -nitro-L-Arginine Methyl Ester (L-NAME), HGF, diaminobenzidine, anti-BrdU antibody, HRP-linked anti-mouse antibodies were obtained from Sigma Chemical Co. (Oakville, Canada). FlexCell plates were obtained from FlexCell International (Hillsborough, NC)

.

FIBER ISOLATION

Fibers were isolated as reported earlier (82) from flexor digitorum brevis muscles of normal, *mdx* and NOS-1 (-/-) mice (5-8 weeks old). Briefly, muscles were gently isolated, cleaned of connective tissue in proliferation medium (1X DMEM with 10% fetal bovine serum, 2% chick embryo extract, 1% antibiotic/antimycotic and 0.1% gentamycin) and placed in a 0.2% collagenase solution at 37°C and 5% CO_2 for 2 ½ hrs. Muscles were transferred to fresh proliferation medium without collagenase, cleaned of any remaining connective tissue and tendon remnants, and gently triturated to release single fibers. Fibers were further cleaned using gravity sedimentation and plated in 6-well FlexCell plates with either a rigid or flexible surface substrate which was pre-cooled and coated with 80 µl pure Vitrogen100. Fibers were allowed to adhere for 20 minutes at 37°C and 5% CO_2, after

which basal growth medium (DMEM, 20% Serum Replacement-2, 1% FBS, 1% antibiotic-antimycotic and 0.1% gentamycin) was added (2 ml/well), as reported (82). Fibers were allowed to attach overnight at 37°C and 5% CO_2 prior to stretching. Fiber preparations that contained tissue debris, or broken or hypercontracted fibers were discarded and not included in experiments. At the start of experiments (time 0hr), bromodeoxyuridine (BrdU; 0.002% wt/vol) was added to the medium and cultures were maintained for 24 hrs prior to fixation.

ROLES OF NO AND HGF IN STRETCH-ACTIVATION

Normal fiber cultures received one of three treatments at the beginning of the experiment (time 0 hr): a non-specific NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co., 0.2 μg/ml), HGF (20 ng/ml; the concentration known to stimulate quiescent satellite cells on single fibers to activate (112)) or a combination of L-NAME and HGF. For the HGF dose-response experiments, between 0-30 ng/ml of HGF was added to medium (time 0 hr).

Plates were placed in a FlexCell system and stretched for ½ or 2 hours (82) at 4 cycles per minute (one cycle being 8 seconds in a stretched position and 7 seconds in an unstretched position). The degree of stretch, applied along the radius of each culture well, was 10%, similar to the physiologic range of length changes during a contraction, and was provided by a vacuum of 20 kPa applied to the culture plates (282). Following the period of cyclical stretching, the vacuum line was disconnected and cultures maintained (without stretch) for 24 hours and fixed in acid-alcohol.

Fibers were immunostained to detect the incorporation of BrdU using anti-BrdU antibody (1:1000; Sigma Chemical Co.) and secondary HRP-linked anti-mouse antibody (1:300; Sigma Chemical Co.) and visualised with diaminobenzidine (25 mg/ml) (82;112). Fiber cultures were coded and viewed at 200X magnification to clearly visualize the satellite cell nuclei that were stained positive for BrdU incorporation. The total number of satellite cells is not available for counting; nuclei in quiescent satellite cells are indistinguishable from myonuclei in normal muscle fibers as both are not positive for BrdU incorporation. The number of BrdU-positive (BrdU+) satellite cells per fiber was counted without knowledge of source, by systematically scanning the entire area under each coverslip. Data were compiled from 8 independent experiments.

STRETCH-ACTIVATION OF *MDX* AND NOS-1(-/-) SATELLITE CELLS

Experiments with *mdx* and NOS-1(-/-) fiber cultures were prepared using identical protocols to normal fiber cultures. BrdU was added to fiber cultures at time 0 hr. Fibers were subjected to stretching (or not) for 0-3 hours (as in (82)). Data were obtained from 3 repeat experiments for each mouse strain.

STATISTICAL ANALYSIS

Data were compared by analysis of variance (ANOVA) with repeated measures, where appropriate (Statpak; NorthWest Analytical Inc.). Differences between groups were assessed using Least Significant Difference tests. Significance was determined at the $p < 0.05$ level. Only significant changes are reported in the text.

RESULTS

RELATIVE ROLES OF NO AND HGF

Satellite cells on normal fibers showed a low level of activation under control conditions, without stretching or additional treatment, measured as the number of BrdU+ satellite cells per fiber in each culture. Stretching for either ½ hr or 2 hrs increased the number of BrdU+ satellite cells per fiber ($p<0.05$), as described earlier (82).

Fiber cultures treated with L-NAME or HGF for 24 hr in culture showed 2-3X higher levels of satellite cell activation compared to control conditions without treatment ($p<0.05$). By comparison, when cultures of normal fibers were treated with either L-NAME or HGF during stretching, the number of BrdU+ satellite cells per fiber was reduced ($p<0.05$), and was not different from the level observed for unstretched, control (untreated) cultures (Figure 1).

Fiber cultures treated with both L-NAME and HGF together showed no activation above unstretched control fiber cultures, either without stretch or with ½ hr stretch. More prolonged stretching (2 hr) in the presence of L-NAME and HGF resulted in a very high level of activation, above the level observed for cultures that were stretched for 2 hrs with either L-NAME or HGF alone.

HGF DOSE RESPONSE

HGF was added to the medium of stretched and unstretched fiber cultures in increasing concentrations (0 ng/ml to 30 ng/ml), and the level of satellite cell activation (the number of BrdU+ satellite cells per fiber) was compared between stretched and unstretched

cultures. Activation increased as a function of increasing HGF concentrations in cultures without stretching ($p<0.05$; Figure 2), similar to the relationship reported for satellite cells in culture (142). By contrast, for those fibers cultured under cyclical stretched conditions, the level of activation peaked at a lower concentration of HGF (10 ng/ml) than for fibers maintained in unstretched conditions ($p<0.05$), and showed decreased activation at higher HGF concentrations (Figure 2).

SATELLITE CELL RESPONSE TO STRETCH ON *MDX* AND NOS-1(-/-) FIBERS

These experiments investigated the role of NO signaling in satellite cell activation by examining the cultures of fibers with NOS-1 deficiency, isolated from muscle of *mdx* and NOS-1(-/-) mice. The two types of fibers, respectively, formed natural and transgenic comparisons to the studies of normal fibers during NOS inhibition using exposure to L-NAME.

Satellite cells on *mdx* fibers showed high levels of activation compared to normal fibers. In the absence of stretching, there was an approximately 4-fold increase in the number of BrdU+ satellite cells per fiber than observed for satellite cells on normal fibers (Figure 3A). The level of activation was plotted as a function of stretch and normalized against the level of activation in unstretched *mdx* fiber cultures (at 0 hr). Activation on stretched *mdx* fibers decreased after ½ hour of stretch ($p<0.05$), and returned to the level observed on unstretched fibers after 1 hr of stretching.

Satellite cells on NOS-1(-/-) fibers showed a pattern similar to *mdx* fiber cultures in the level of activation without stretching, and in the stretch-response of activation. There

were high numbers of satellite cells per fiber stained positive for BrdU in the absence of stretching (Figure 3B). Again, stretching NOS-1(-/-) fibers in culture resulted in a decreased level of activation after ½ hr of stretch, to lower levels than observed for satellite cells on *mdx* fiber cultures after the same interval of stretching. This decrease in activation was maintained for a longer duration of stretch in NOS-1(-/-) fiber cultures compared to *mdx* fiber cultures (1 hr vs. ½ hr). After stretching for 1½ hr, satellite cell activation on NOS-1(-/-) fibers had returned to the level observed for unstretched fibers. Satellite cell activation on NOS-1(-/-) fibers decreased again after 3 hours of stretching compared to unstretched NOS-1(-/-) fibers.

DISCUSSION

Results of these experiments demonstrated for the first time, that NO as well as HGF play a role in satellite cell activation that was stimulated by mechanical stretching in the physiological range. Previous studies had shown that L-Arginine, a NOS substrate, induced a dose-dependent satellite cell activation on fibers cultured in the absence of stretching (112), and that stretching induced an increase in activation after ½ hr and 2 hrs of stretch (82). The current experiments integrated those earlier reports by demonstrating that HGF stimulated satellite cell activation on fibers, and that stretch-induced satellite cell activation on normal fibers in culture was NO-dependent, since it was prevented by exposure to a NOS inhibitor (L-NAME). Interestingly, exposure to HGF during stretching also reduced the observed level of satellite cell activation relative to unstretched cultures treated with HGF. Results of dose-response experiments demonstrated that the activation stimulated by HGF was potentiated by stretching, since the response to HGF peaked at lower levels in the presence of stretching. Furthermore, experiments on fiber cultures from two genetic models of NOS-1 down-regulation, *mdx* and NOS-1 knockout mice, strongly suggested that NO is required to maintain satellite cell quiescence. Consistent with the observed increase in satellite cell activation on normal fibers treated with the NOS inhibitor, satellite cell activation on unstretched *mdx* and NOS-1(-/-) fibers was 3-4-fold higher than observed for normal fibers. Together, these results demonstrate a dynamic interaction between a mechanical stimulus and satellite cell activation, and extend our understanding of the regulation of activation. In these experiments, satellite cell activation from a relatively quiescent state (with a very low level of cell cycling) was measured as a change in the number of BrdU-positive satellite cells per

fiber. This model provides a binary read-out of cycling behaviour as a result of a stretching stimulus for the first few hours (½ to 3 hrs) of each experiment. Following stretch, cultures were maintained for a total of 24 hrs in the presence of BrdU. The "baseline" level of quiescence, established by observations of a stable low level of cycling by satellite cells on control cultures of normal muscle fibers, is thereby available for observation. A stable baseline in control cultures confirms that any changes in activation observed at 24 hrs in the experimental cultures have resulted specifically from the interventions (e.g., stretching, L-NAME and HGF) rather than as an artifact of culture conditions. The model therefore examines entry into S-phase subsequent to a stimulus, and the counts of BrdU+ satellite cells per fiber are not confounded by cytokinesis, since cultures were fixed within one cell cycle period (24 hrs). Other indices of activation, such as immunostaining for the expression of a muscle regulatory gene such as myf5 or proliferating cell nuclear antigen, would also reveal activation from a prior stimulus. While they may provide a read-out of activation that is temporally closer to a "real-time" measure, their use prevents confirmation of stable quiescence over at least one cell cycle, and interpretation of such observations may be subject to various definitions of the quiescent state (55;92). Experiments using BrdU incorporation into DNA of satellite cells on fibers in culture over 24 hours were therefore sensitive to changes in cycling behaviour due to earlier activation, and were established as specific to the interventions under examination.

These experiments established for the first time that NO, released from muscle fibers underlying satellite cells, is a putative factor in maintaining satellite cell quiescence observed in normal fibers in culture. Studies using 3 different model systems were consistent in

demonstrating that down-regulation of NOS activity or expression was associated with a significant increase in satellite cell activation. Exposure of satellite cells on normal fibers to a NOS inhibitor, L-NAME, resulted in greater satellite cell activation. Satellite cells on muscle fibers isolated from NOS-1 knockout mice had a significantly higher level of activation. Finally, satellite cells on *mdx* mouse muscle fibers, which have down-regulated NOS-1 expression secondary to dystrophin deficiency also showed 3-4-fold greater activation than on normal fibers in culture. To our knowledge, this is the first demonstration that NO plays a role in maintaining satellite cell quiescence. Together with results showing NOS inhibition prevented stretch-induced activation on fibers, these experiments provide strong support for the model of NO as a mediator of satellite cell quiescence (35).

Previous studies using dispersed cultures of satellite cells (without underlying fibers) showed that L-NAME prevented satellite cell activation by stretching (141). Moreover, L-NAME was reported to prevent activation induced by muscle injury *in vivo* (35), and current results extend that observation to include activation induced by stretching normal fibers in culture. Therefore, we interpret these results as showing that the molecular signal, NO, has a novel role in normal muscle fibers in regulating the equilibrium between satellite cell quiescence and activation. The environment of satellite cells, as defined in part by the anatomical relationship to normal muscle fibers, has direct impact on the activation state of satellite cells. Apparently opposite roles for single molecules are not unknown. There are other signaling pathways, such as those for gonadotropin releasing hormone and growth hormone, where different patterns of delivery (pulsatile vs. sustained, or even changes in periodicity) have distinctive effects on a target tissue (294;295). In muscle, ryanodine

receptor activity is both stimulated and blocked by NO and related compounds, and the pattern of delivery is important in determining the direction of the effect (reviewed in (5)). The present experiments reinforce the notion of bi-directional signaling by the NO molecule in skeletal muscle and myogenesis.

Similar to the bi-directional regulation of satellite cell activation by NO signaling, results of the present experiments on stretched fibers in culture also demonstrated that HGF plays a complex role in mediating the activation state of satellite cells. HGF was shown previously to activate satellite cells on fibers without a stretch stimulus (112). HGF is also known to stimulate activation in satellite cells in culture, and to be released from the extracellular matrix surrounding satellite cells into the medium by the stretch-related release of NO (141). Here, satellite cells were activated by HGF in the absence of stretch, consistent with earlier results, and additionally, HGF prevented stretch-activation of satellite cells on fibers. Results of the dose-response experiments were consistent with these observations. Without stretch, increasing concentrations of HGF resulted in greater satellite cell activation on fibers, while the same exposures to increasing concentrations of HGF gradually reduced activation in stretched cultures. These observations are interpreted to suggest that the c-met receptor may be desensitized at some threshold concentration of HGF, resulting in a "*de*activation" of satellite cells. It is likely that continuous or high exposure to HGF induces the desensitization. HGF is sequestered in the matrix of muscle fibers (78;81), and stretch-induced NO release from fibers likely releases that HGF, as it does in satellite cell cultures. That would augment the level of HGF acting on satellite cells in stretched fiber cultures. Activated primary satellite cell cultures also show decreased activation when HGF

concentrations exceed optimal levels (169). A very high level of HGF was also observed to decrease satellite cell activation in cell cultures (500 ng/mL, R. Tatsumi, personal communications) compared to a lower inhibitory concentration for single fibers in culture (20 ng/mL) which was observed here. Evidence of c-met receptor desensitization is found in other systems including HeLa cells (296) and whole-animal clearance studies in liver (297). Therefore, the addition of HGF to the culture medium by treatment and through stretching could desensitize c-met and result in the decreased activation observed in HGF-treated stretched fibers in culture.

It is apparent from other reports that the process of satellite cell activation *in vivo* can bypass NO signaling from NOS-1, since muscle regeneration occurs in dystrophic *mdx* and NOS-1(-/-) mice. Two approaches were used here in an attempt to determine whether NO signaling was required during satellite cell activation in normal muscle. In the study of L-NAME and HGF treatments, added singly or combined to culture media, only those fibers that were exposed to both L-NAME and HGF in addition to 2 hrs of stretching showed a significant rise in satellite cell activation over control cultures. Activation under the same conditions and with ½ hr of stretching showed no increase. Therefore, NO appears to be required for activation early in the course of the mechanical stretching, while HGF treatment and a longer period of stretching was able to bypass the requirement for NO release. This possibility would be consistent with the suggestion that there are 2 distinct satellite cell populations on normal fibers, each with distinctive activation characteristics (82).

A second approach was utilized to reveal the possible impact of down-regulated NOS-1 and activation without NO. Experiments were designed to study stretch-activation

in *mdx* and NOS-1(-/-) fiber cultures, where NO would not be released from NOS-1. Results of these experiments showed high levels of activation in the absence of stretching, while activation was reduced by stretching, rather than increased as in normal fiber cultures. One explanation is that there are more satellite cells which populate fibers from *mdx* or NOS-1 (-/-) muscle compared to normal fibers. However, such a difference would not account for the observed decrease in activation by stretching. We have not previously observed strain-related difference in the numbers of satellite cells resident on *mdx* and normal fibers (298), although one report shows approximately twice the number of satellite cell progeny (per mm^2 of membrane) are produced from single fiber explants after 90 hours in culture (264). A second explanation for the stretch-related decrease in activation on *mdx* and NOS-1(-/-) fibers is that NO may be produced from alternate isoforms or locations. For example, NOS-1μ is expressed in activated *mdx* satellite cells, and there is NOS activity present (from NOS-2 or 3) in NOS-1(-/-) satellite cells (15). Since stretch generally up-regulates NOS activity (219;291), these alternate sources may release NO during stretch, and shift satellite cells toward quiescence. The duration of this response to stretch was longer in NOS-1(-/-) than *mdx* fibers, and may also be related to the alternate sources of NO. These experiments provide evidence that there may be one population of satellite cells absent from *mdx* fibers as suggested elsewhere (47), while the NOS-1(-/-) fibers may retain at least 2 different populations with distinct time-dependent responses to stretching, similar to those on normal fibers in culture (82).

In summary, we propose a model for the role of NO in satellite cell activation, where the level of activation is a function of NO concentration surrounding satellite cells on fibers

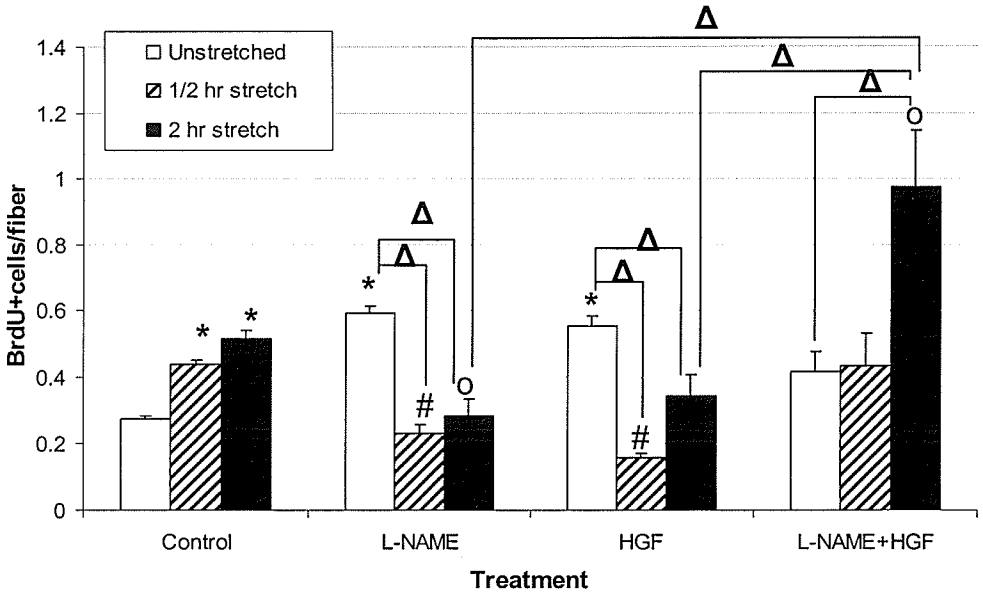
(Figure 4). Experiments demonstrate that satellite cell activation is differentially regulated over time during stretch, and provide evidence consistent with a requirement for NO in early stretch-activation in normal muscle. Experiments on *mdx* and NOS-1(-/-) fiber cultures extended earlier *in vivo* studies by demonstrating that mechanical activity partly normalized the hyper-activated satellite cells on fibers where NOS-1is down-regulated. Together with these results, the novel observation of a dual role for NO in mediating quiescence and activation, and the apparent desensitization of c-met receptor activity in satellite cells suggest new treatment possibilities for promoting skeletal muscle growth and regeneration.

Figure 1. Relative Roles of NO and HGF in stretch-activation

A. Satellite cells on fibers were subjected to stretch (or not) for ½ hr or 2 hrs in the presence of BrdU and either 1) no additional treatment, 2) L-NAME, a NOS inhibitor (0.2 μg/mL), 3) HGF (20 ng/mL) or 4) a combination of 2) and 3). Data are plotted as mean ± standard error of the mean. Significant increases in activation were evident in unstretched cultures treated with either L-NAME or HGF, whereas stretching in the presence of these treatments significantly decreased activation levels. Co-treatment of L-NAME and HGF with 2 hrs of stretch overcame NOS inhibition, indicating that satellite cells activated by 2 hrs of stretch may have different activation requirements from those activated by ½ hr of stretch; N= 72-1121 fibers per group (3-6 experiments). * indicates a difference from unstretched controls; # indicates a difference from ½ hr stretch control; o indicates a difference from 2 hr stretch control; Δ indicates a difference between 2 treatment groups connected by a line.

B. A summary of **A.** indicating overall changes in activation due to stretch and/or treatment.

A.



B.

Treatment	0hr	½ hr	2 hr
Control		↑	↑
L-NAME	↑	↓	↓
HGF	↑	↓	↓
L-NAME+HGF	no Δ	no Δ	↑ ↑

Figure 1.

Figure 2. HGF dose response with and without stretch

Satellite cells on single fibers were treated with increasing concentrations of HGF (0-30 ng/ml) with or without ½ hr of stretch, and the level of activation was measured as the number of BrdU+ satellite cells per fiber (mean ± SEM, n= 8-78 fibers per group). Increasing concentrations of HGF resulted in increased activation in unstretched cultures. Stretching in the presence of HGF (at increasing concentrations) decreased activation. At higher HGF concentrations, stretch significantly decreased activation in comparison to fibers cultured without stretch. * indicates a difference from the unstretched control culture at 0 ng/ml HGF; Δ indicates a difference between stretched and unstretched cultures exposed to the same concentration of HGF.

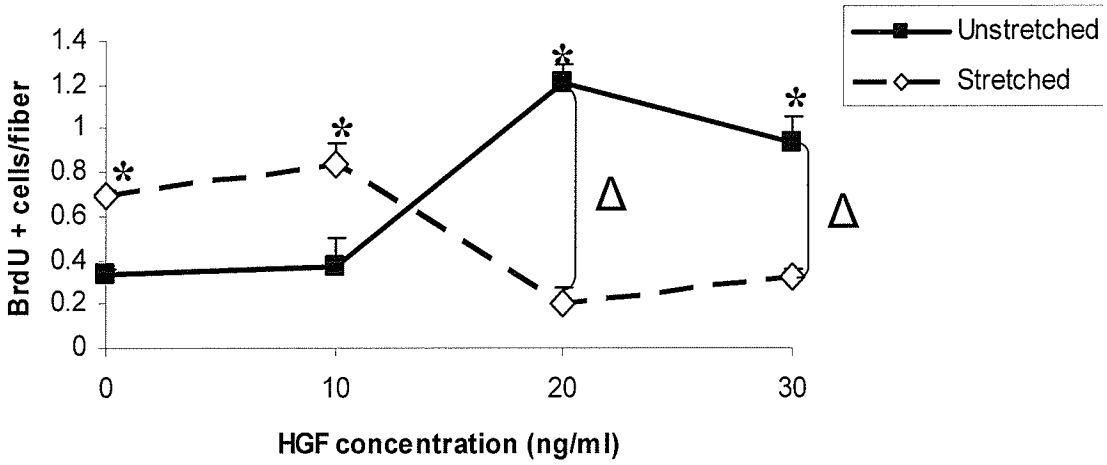

Unstretched
Stretched
BrdU + cells/fiber
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
HGF concentration (ng/ml)
*
*
*
Δ
Δ

Figure 2.

Figure 3. The time course of *mdx* and NOS-1(-/-) stretch-activation

Single fibers from *mdx* (A) and NOS-1(-/-) (B) muscles were stretched for 0-3 hours and activation was measured (number of BrdU+ satellite cells per fiber), here plotted as a function of the duration of stretching and relative to the level of activation at time=0 (mean ± SEM). In both *mdx* and NOS(-/-) fiber cultures, satellite cell activation in unstretched cultures was higher than observed for normal fiber cultures. A short period of stretching for ½ hr, resulted in significant reductions in activation in both *mdx* and NOS-1(-/-) fibers ($p<0.05$), which was transient in *mdx* cultures (N=30-432 fibers per group) and more persistent in NOS-1(-/-) fiber cultures (N=8-156 fibers per group).

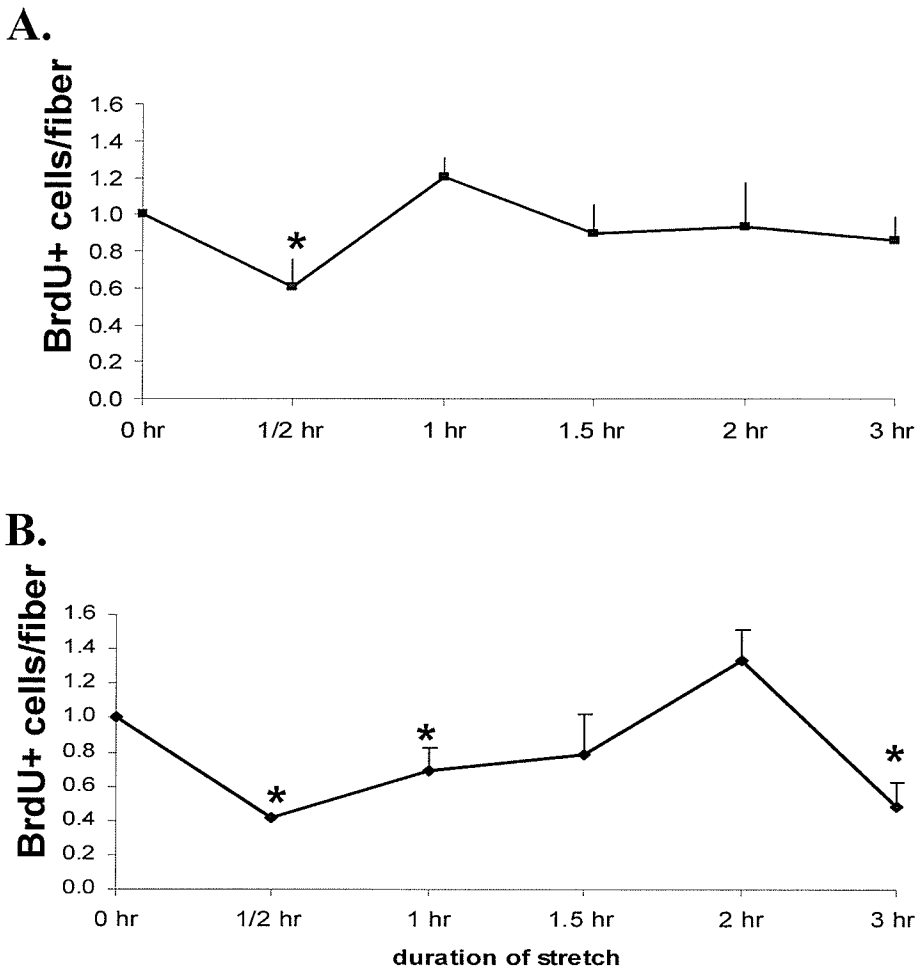

A.
BrdU+ cells/fiber
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
0 hr
1/2 hr
1 hr
1.5 hr
2 hr
3 hr
B.
BrdU+ cells/fiber
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
0 hr
1/2 hr
1 hr
1.5 hr
2 hr
3 hr
duration of stretch

Figure 3.

Figure 4. A model of activation as a function of NO concentration.

The model proposes that the level of activation is presented as a function of NO concentration acting on satellite cells that are resident on fibers. At 'X' concentration of NO in normal muscle, satellite cells are maintained in a quiescent state through NOS-1 activity localized to the sub-sarcolemmal cytoskeleton. For normal muscle, stretch induces NO release which activates satellite cells, while a lower NO concentration resulting from NOS inhibition (e.g., L-NAME treatment in normal muscle) or NOS-1 down-regulation (in *mdx* and NOS-1(-/-) muscle), also allows activation. Stretch may stimulate NOS activity from NOS-2 or NOS-3 isoforms (and possibly NOS-1 in *mdx* satellite cells) and shift satellite cells toward a decreased level of activation. In satellite cell cultures, this fiber-dependent NO signaling would be absent.

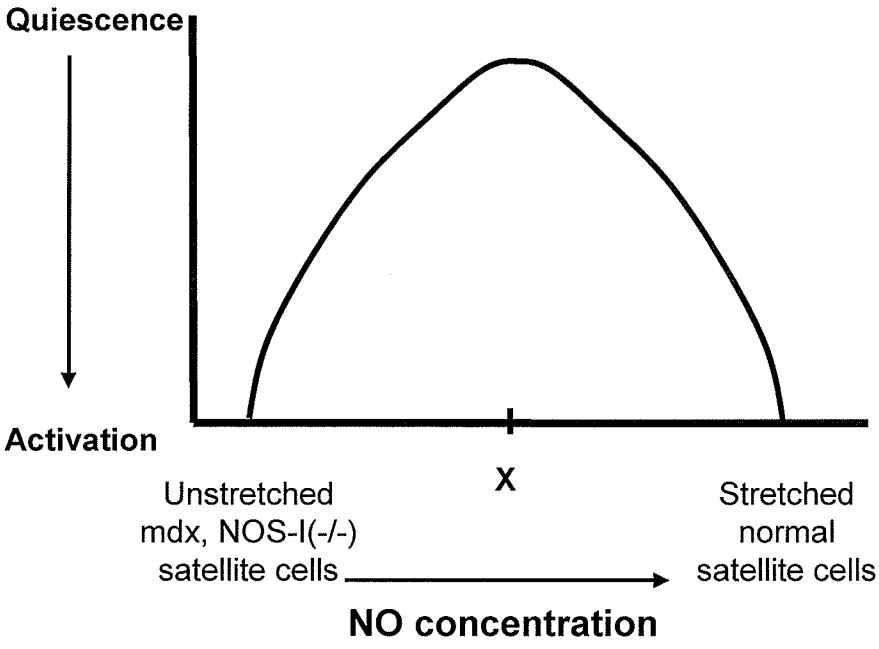

Quiescence
Activation
X
Unstretched
mdx, NOS-I(-/-)
satellite cells
Stretched
normal
satellite cells
NO concentration

Figure 4.

Chapter 5.
A cellular nitric oxide aurora: Video-capture microscopy experiments reveal stretch-induced release of nitric oxide is deficient in cultured dystrophic muscle cells.

Submitted to Journal of Muscle Research and Cell Motility

ABSTRACT

Potent nitric oxide (NO) signals are described for many forms of cell-cell communication. However, there is no direct evidence for a proposed NO-transient signal released from skeletal muscle during stretch. Differentiated muscle cell cultures from normal and dystrophic *mdx* mice were stretched and intracellular NO release was examined using fluorescence video-microscopy. Stretching released NO from normal myogenic cultures with a time-dependent, pulsatile decay. Dystrophic muscle cell cultures showed only minimal, irregular pulses of NO release at a level much less than normal cultures. Direct visualization of mechanotransduction through NO release will enable exploration of the regulatory balance of satellite cell activation and quiescence, and possible application of NO-based therapies targeted to skeletal muscle.

INTRODUCTION

Nitric oxide (NO) is a low molecular weight gas (30 Da) that is produced in mammals by the enzyme Nitric Oxide Synthase (NOS; reviewed in (11)). The 3 isoforms of NOS, neuronal (type 1), inducible (type 2), and endothelial (type 3) (5;13) are named for the tissue in which they were first identified (12).

The first evidence of NO as a biological messenger (148-150) identified endothelium-derived relaxation factor as NO. Since 1988, NO involvement in many biological processes has been demonstrated, including liver regeneration (116), blood flow and tissue respiration (reviewed in (5)), as well as myoblast differentiation (299;300). NO signaling also modifies pre-synaptic function at the neuromuscular junction by acting as a retrograde signal that inhibits acetylcholine release (6) and NO acts in general as an anti-inflammatory molecule (154). Of late, this laboratory demonstrated roles for NO in regulating the activation of skeletal muscle precursor or satellite cells from their normal quiescent state in adult tissue, in modulating muscle regeneration ((35;111;112); reviewed in (33)), and more recently in maintaining satellite cell quiescence (Wozniak and Anderson, submitted).

The muscle-specific NOS-1μ isoform (14;35) is the most prevalent isoform expressed in skeletal muscle fibers. It is localized to the sarcolemma in conjunction with members of the dystrophin glycoprotein complex (DGC (5)) and to the neuromuscular (5) and muscle-tendon junctions (6). NOS-1μ is also expressed by activated satellite cells (15). Sarcolemmal localization is required for normal enzyme expression and enzymatic activity. This is illustrated by the condition in muscles of Duchenne muscular dystrophy (DMD) patients and *mdx* mice (a mouse homologue of DMD), where loss of dystrophin causes

displacement of NOS-1 protein (16) and down-regulation of NOS-1 activity and gene expression (17). This down-regulation of NO production at a dystrophin-deficient cytoskeleton underlying the sarcolemma is hypothesized to account for hyperactivation of satellite cells and the dysregulation of muscle regeneration in dystrophic muscle, as a consequence of satellite cells not receiving normal levels of the NO signal that modulates the balance between quiescence and activation (35).

Stretch or shear stress is proposed as a potent stimulus to skeletal muscle that results in an increase in NOS-1μ activity and the release of a bolus of NO (35), similar to the role of shear stress and pulsatile blood flow that up-regulate NOS-3 activity in vascular tissues ((18); reviewed in (19)). However, there is no direct evidence of NO release from mechanically stimulated skeletal muscle, despite recent demonstration that activation of isolated satellite cells in culture, on single fibers in culture and *in vivo* is NO-dependent (35;141)(Wozniak and Anderson, submitted).

Experiments were designed to: 1) visualize the extent and time-course of NO release stimulated by stretching differentiated primary cultures of normal and *mdx* muscle, containing myoblasts and multinucleated myotubes formed by myoblast fusion; and 2) to determine by direct observation, whether that NO release varies between myotubes with and without normal NOS-1 localization, in normal and *mdx* cultures respectively. NO release was visualized using diaminofluorescein-2 (DAF-2), a dye that fluoresces in the presence of NO (301) by binding to the short-lived intermediate NO• free radical without changing either the downstream effects of NO or the activity of the NO signal.

METHODS

Satellite cells were isolated from muscle of normal (C57BL/6) and mutant (*mdx* dystrophic) mice (276;302). Cells were plated on a removable sylastic membrane on the bottom of StageFlexer™ FlexCell 100 mm culture plates (FlexCell International Corp., Hillsborough, NC) and cultured for 10 days in proliferation medium (1X DMEM containing 15% fetal bovine serum, 2% chick embryo extract, 1% antibiotic/antimycotic and 0.05 mg/mL gentamycin). Twenty-four hours before an experiment, cultures were switched to basal-growth medium containing a low serum concentration (82) to stimulate cell fusion and myogenic differentiation into myotubes. At the start of an experiment, cultures were washed with Krebs-Ringer Phosphate (KRP) solution, and the cells in culture were pre-loaded with the dye DAF-2DA by incubation in 10 μM DAF-2DA in KRP for 1 hr at 37°C. When incorporated into cells, the diaceate of DAF-2DA is cleaved, forming the active dye, DAF-2. Cultures were protected from light after pre-loading. Negative control cultures were incubated in KRP without DAF-2DA. Cultures were rinsed with KRP containing the vital nuclear stain, 4'-6-diamidino-2-phenylindole (DAPI; 5 μg/mL), rinsed with fresh KRP, and incubated at 37°C until use.

For each experiment, conducted in near darkness, one sylastic membrane was removed from a culture dish and mounted in the StageFlexer apparatus which was attached to the FlexCell system vacuum line. The unit, assembled with a mounted cell-culture membrane, was placed securely on the stage of an Olympus BH2-RFCA microscope equipped with epifluorescence optics (490 nm excitation filter, and 515 nm emission filter to detect DAF fluorescence). The microscope was also fitted with a ToUCam Pro™ Webcam

(Philips Electronics) attached to the photographic aperture for real-time collection of the fluorescence signal, (CCD detection to < 1 lux illumination, with a still image resolution of 1280-H X 960-V) as employed for astro-video-photography (303;304). The FlexCell vacuum system was set to produce a stretch of approximately 10% (282), and the microscope was focused on DAPI + cells in the plane of an unstretched culture membrane. Cultures were subjected to mechanical stretching as follows: 2 sec of 10% stretch (out of focus), followed by either 20 or 60 sec, in the unstretched position (in the original plane of focus). Data of the fluorescence signal were recorded at 30 frames per second using the Philips VRecord program of the ToUCam software package. For analysis, the real-time digital video of the fluorescence signal was broken into individual frames using Videomach v.2.6.3 software (Gromada.com), sampled at 1 frame per second at the start of each second (t=0 established by the FlexCell system, with second-by-second sampling beginning when the myotube regained focus), and analyzed using ImageJ freeware (National Institutes of Health). Fluorescence intensity was calculated as the mean-grey value (minus the mean-grey value of the respective negative control culture) divided by the area of fluorescence. The calculated fluorescence intensity at each time point for a given culture (n=2-6) was also normalized to the initial fluorescence value and plotted as a function of time after the end of stretching. For detailed study of the pulsatility of NO release, fluorescence was sampled from smaller areas of interest within myotubes, and analyzed for fluorescence intensity as described above.

RESULTS

We were able to observe the first direct demonstration of differences in NO release between normal and dystrophic muscle in culture. While DAF fluorescence was observed in normal and *mdx* cultures after stretch (Figure 1A and 2A, respectively), four key features distinguish the observations from the two types of muscle in culture. First, the level of fluorescence that appeared in *mdx* cells and myotubes without stretching was markedly less than observed in normal cultures under the same conditions. This comparison is presented graphically in Figure 3A. Second, a time-dependent decrease in this NO-specific fluorescence (301) in normal cultures was not observed in *mdx* cell cultures. The low initial fluorescence intensity in *mdx* cultures did not change over the same time frame after stretch. These data are shown in Figure 3B. Third, the time-dependence of the decay function in normal myotubes in culture displayed a pulsatile character representing the signal of NO-related fluorescence, as presented in Figure 3C. The pulses of NO release in normal myotubes were identical in frequency and amplitude over the entire fluorescent area of a myotube, independent of the local magnitude of fluorescence. Additionally, regions of sarcoplasm toward the myotube periphery or between myonuclei showed the most intense fluorescence. Fourth, although the low level of NO released by *mdx* myotubes was also pulsatile in character, the pulse frequency differed from that in normal muscle cultures, varied irregularly over the 58-second interval following a stretch, and gradually decayed in frequency (Figure 3C).

DISCUSSION

The direct visualization of stretch-induced NO release from differentiated, multinucleated muscle cells in culture demonstrated features of a "NO transient" signal. The experimental protocol provides the basis for further study of mechanisms that transduce mechanical activity and regulate muscle functions, such as the adaptation to exercise and the balance between satellite cell activation and quiescence. Mechanisms of disease pathophysiology that are proposed to be related to mechanical signal transduction via NO, and effects of potential NO-based treatments of neuromuscular and other disease conditions will also be available for investigation.

The present findings are direct evidence for the original working hypothesis that mechanical shear or stretch causes a bolus release of NO (35). Observations are also consistent with the idea that the NO concentration is a key determinant of the level of activation, in that satellite cells are hyperactivated on muscle fibers from dystrophic *mdx* or NOS-1(-/-) mutant mice and are made more quiescent by stretching (Wozniak and Anderson, submitted). The observation of pulsatile NO release also confirms earlier work (219), and recalls the potency of a signaling mechanism that operates through a physiologic "transient" signal, such as an electrical signal through ionic channel transients (as discussed in (305)). While current experiments were not conducted to observe the detail of baseline and signal changes in NO concentration, the time-course of the NO signal appears too long to be a "spark" such as observed in Ca^{2+} signaling (306). However, somewhat similar to transients in Ca^{2+} ion concentration that serve to synchronize cells in renal tissue (307;308), NO concentration oscillated internally within multinucleated myotubes. Strict regulation of NOS

activity is required in mitochondria, since NO has a critical role in vital functions including cellular respiration (309). A parallel role for NO in skeletal muscle would suggest that the NO-release pattern detected here by DAF-2 fluorescence implies a very high level of regulation in the metabolic sequelae to mechanical stretch. Results further suggest the feasibility of this methodology for testing whether NOS-1 up-regulation may effectively reduce the progression of muscular dystrophy by restoring or partly normalizing control over the dysregulated, hyperactivated satellite cells in dystrophic muscle. Further applications of this stretch-culture model of exercise will extend our understanding of stretch-dependent NOS activity in cells from muscle and non-muscle tissues, and enable the visual, real-time verification of NO delivery during testing of potential new NO-based therapies for DMD and other conditions.

CONCLUSIONS

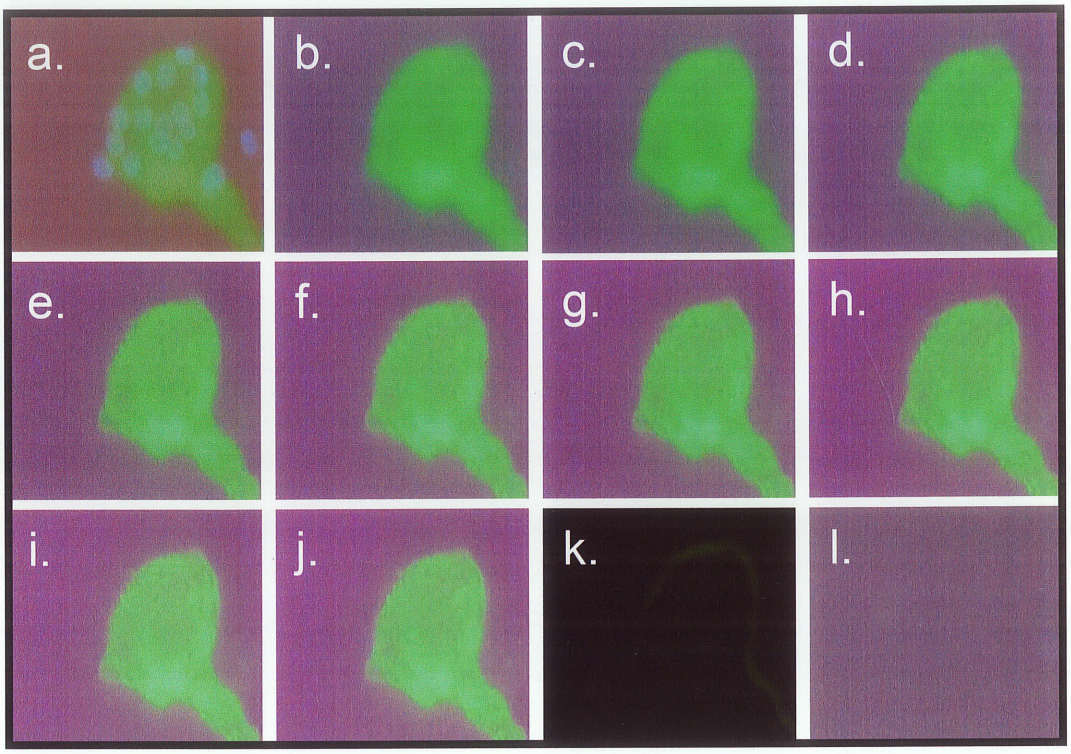
Results presented here indicate that NO is released by stretching in differentiated primary cultures of normal and *mdx* muscle. The release of NO varies between myotubes with and without normal NOS-1 localization, in normal and *mdx* cultures respectively. Changes in the activity of NOS-1 over time after stretch and the pulsatile delivery of NO are also affected in *mdx* cultures, and should be considered during the development of NO-based therapies for the treatment of DMD.

Figure 1. Nitric oxide release due to stretch in normal muscle satellite cells

A. Cultures from muscle of normal C57BL/6 mice were pre-loaded with DAF-2 and stretched for 2 seconds. A time-series of micrographs (panels a-l) shows representative images of NO fluorescence, demonstrated by the presence of a green fluorescent signal. The first micrograph (panel a) in the series shows nuclei (blue from DAPI staining of DNA) within the myotube producing the fluorescence signal was captured just prior to the 2-second stretch. NO release was highest immediately after stretching (panel b) and decreased over a 58 sec period after the end of stretch (panels c to j). Panel k shows an image produced by subtraction, pixel-by-pixel, of the last frame (panel j) from the first image-frame (panel b) of the sequence after stretch. The final panel (l) shows the negative control micrograph, from a myotube not pre-loaded with DAF-2.

B. The NO-fluorescence signal produced by a 2-second period of stretch followed by release from stretching (different post-stretch intervals, 20 sec (n=5) or 60 sec (n=1), were captured for the same myotube) in the presence of DAF was analyzed using NIH ImageJ freeware. Fluorescence-intensity values are plotted as the mean-grey values normalized against the initial level of fluorescence recorded immediately after the end of stretch. An increase in mean-grey value indicates a decrease in fluorescence. NO concentration (produced by NOS) decreased over time after stretch in normal muscle satellite cell cultures. The initial level of fluorescence and the time-dependent decrease in fluorescence were consistently reproduced over multiple (n=6) stretch cycles.

A.


a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
k.
l.

B.

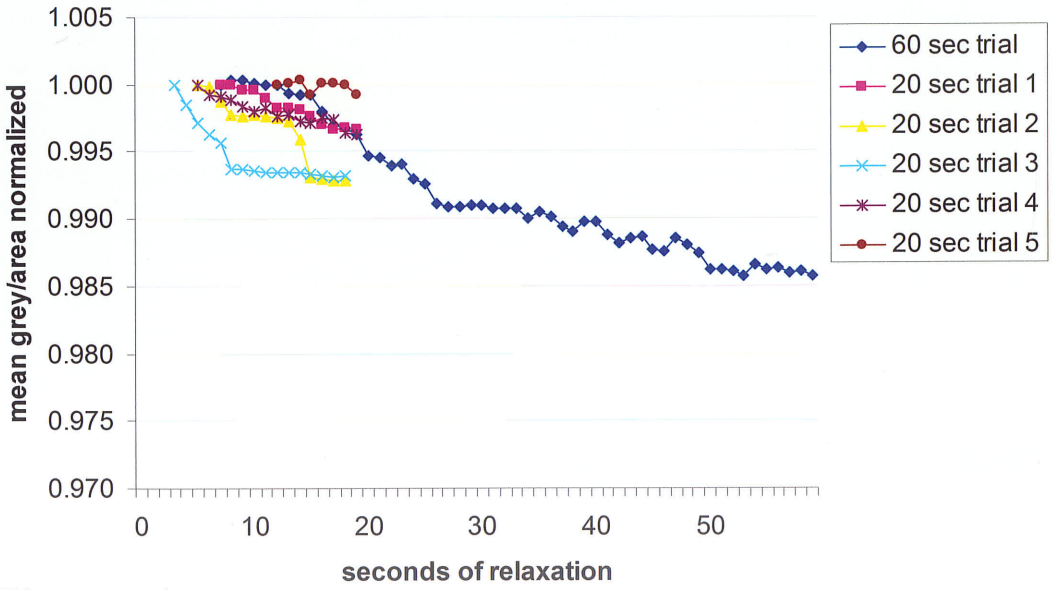

1.005
1.000
0.995
0.990
0.985
0.980
0.975
0.970
mean grey/area normalized
0
10
20
30
40
50
seconds of relaxation
60 sec trial
20 sec trial 1
20 sec trial 2
20 sec trial 3
20 sec trial 4
20 sec trial 5

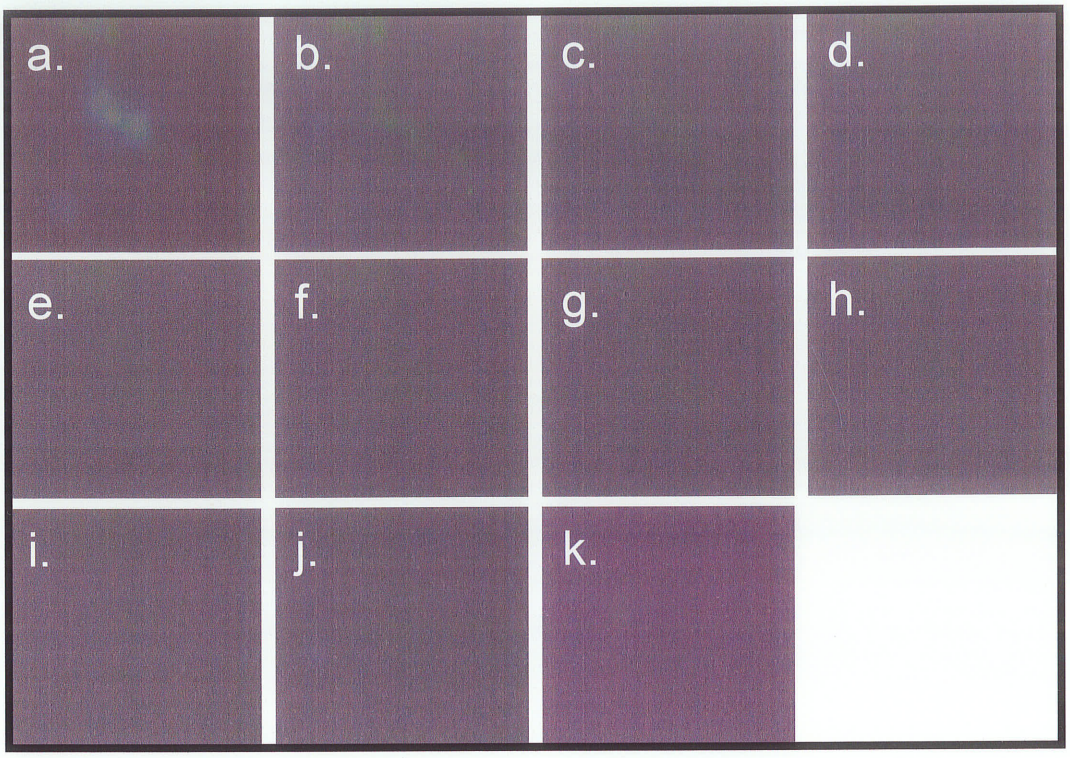
Figure 1.

Figure 2. Nitric oxide release due to stretch in *mdx* satellite cells

A. Satellite cell cultures from skeletal muscle of *mdx* mice were loaded with DAF-2 and stretched for 2 seconds, then released from stretch. Again, the presence of a green fluorescent signal indicates the presence of NO. The first panel (a) in the series shows the nuclei in blue (DAPI staining) that are surrounded by the sarcoplasm that gave rise to the fluorescence signal. NO release was much lower in *mdx* cultures immediately after stretching (panel b) compared to normal cultures, likely due to the decreased activity of NOS-1μ, and stayed at a very low level (panels c to j). The final panel (k) shows the negative control image from a myotube that was not pre-loaded with DAF-2.

B. The NO-specific fluorescence signal recorded from *mdx* myotubes was plotted over time after the end of stretch, after being collected in an identical manner to the experiments on normal myotubes in culture, represented by Figure 1. NO-related fluorescence in *mdx* muscle cultures did not show the changes over time after stretch in cells or myotubes that were observed in normal muscle cells and myotubes.

A.


a.
b.
c.
d.
e.
f.
g.
h.
i.
j.
k.

B.

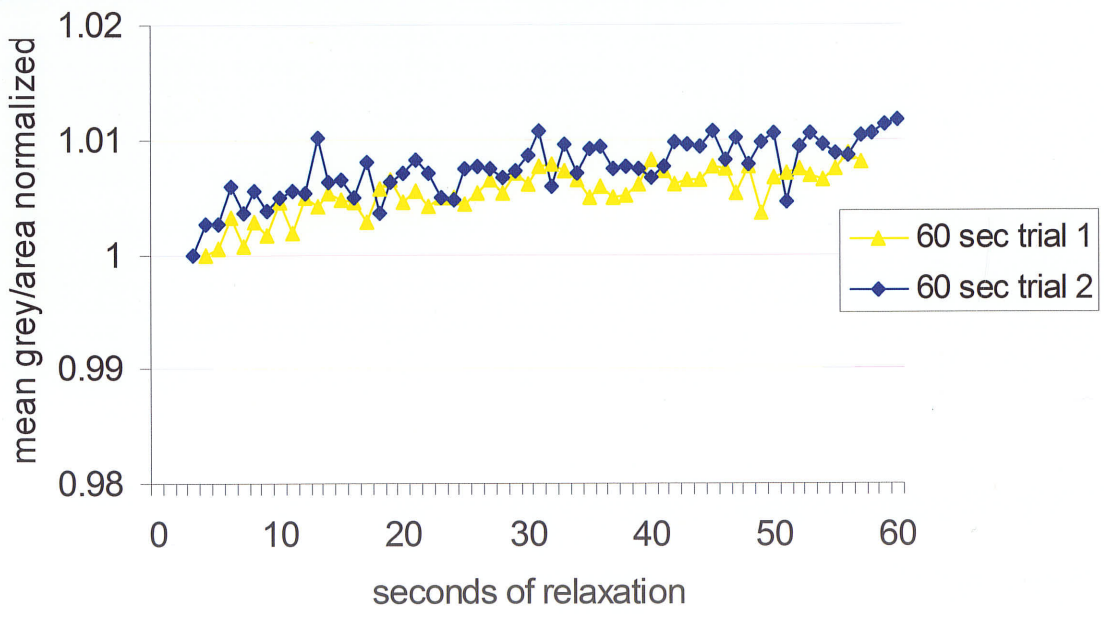

mean grey/area normalized
1.02
1.01
1
0.99
0.98
0
10
20
30
40
50
60
seconds of relaxation
60 sec trial 1
60 sec trial 2

Figure 2.

Figure 3. Studies of the time-course of fluorescence

A. NO-related fluorescence was recorded from normal and *mdx* myotubes, formed by fusion of muscle cells maintained in culture, and plotted on the same scale for comparison. Here, a decrease in the value of mean-grey/area indicates a rise in fluorescence. There was a dramatic differences in the areal intensity of fluorescence between control and *mdx* myotubes, a finding that is interpreted to indicate a lower level of NO release from *mdx* myotubes compared to that in normal myotubes.

B. Fluorescence intensity in this graph is plotted as intensity normalized to the initial fluorescence value recorded immediately after a stretch. The decrease in fluorescence over time demonstrated by normal myotubes after a 2-second stretch was not observed in cultures of *mdx* myotubes. This observation suggests that in addition to a lower NOS-1μ enzyme activity in *mdx* cells (shown in panel **A**), there is also a dysregulation of NO produced by *mdx* myotubes. Both factors may contribute to the hyperactivation of satellite cells in dystrophic muscles.

C. This panel shows the time-course of NO-specific fluorescence signals sampled from 3 identical areas, two sampled from a normal myotube (blue and pink curves) and from one sampled from a comparable *mdx* myotube (yellow curve). Data are presented as mean-grey value divided by the area of fluorescence and normalized to the level of initial intensity of fluorescence recorded from exactly the same region and area immediately after the end of a stretch. The two curves from a representative normal myotube show an identical and stable

frequency of pulsatile NO release. The curve representing the fluorescence sampled from the identically-sized area of a representative *mdx* myotube suggests that there is a time-dependent decay in the pulse frequency that occurs after stretch, while only small changes in fluorescence intensity occured over time. Insets show the normal (i.) and *mdx* dystrophic (ii.) myotubes with the sampled regions indicated by areas circled in the same colour as the curves plotted in panel C.

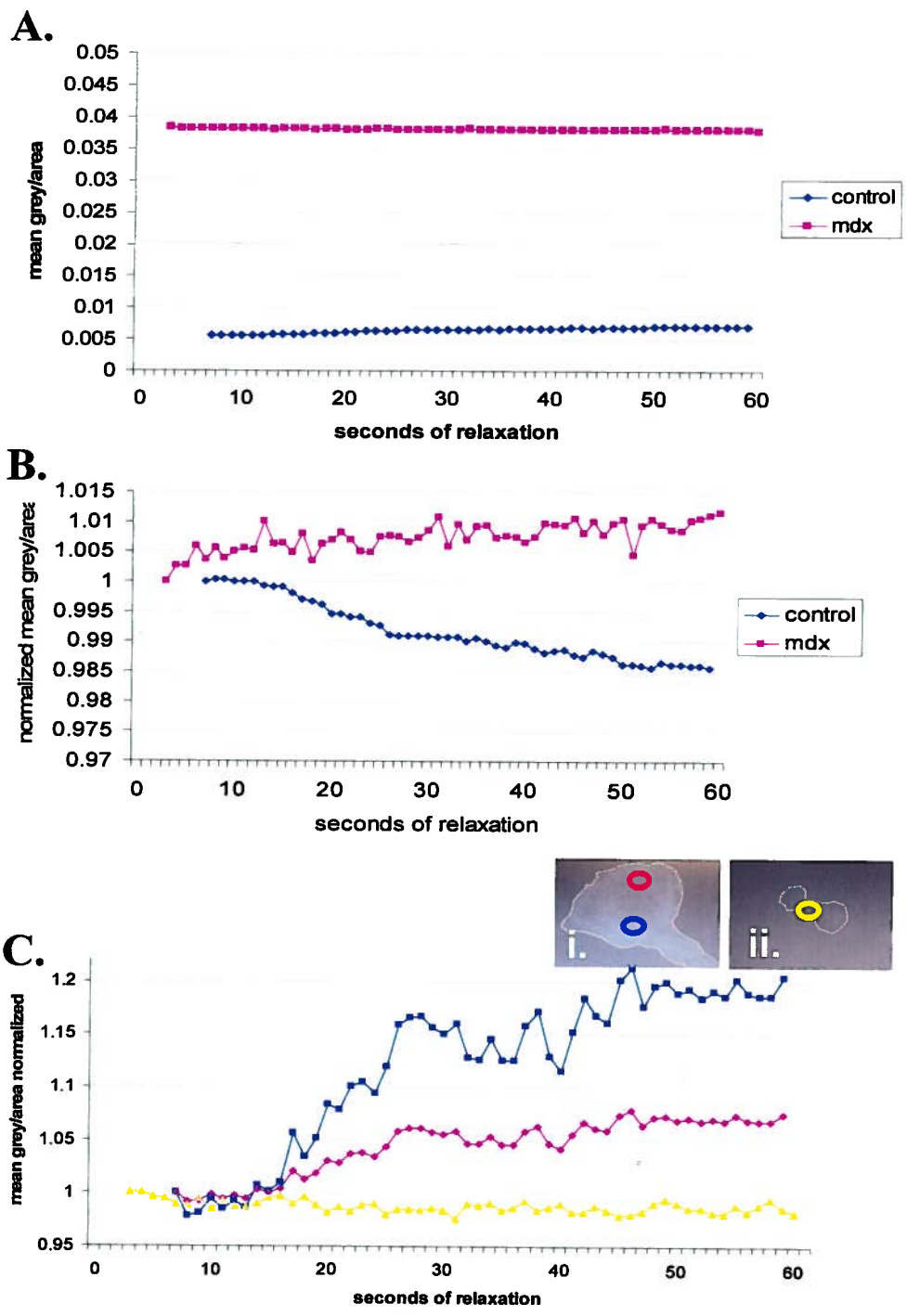

A.
mean grey/area
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
0
10
20
30
40
50
60
seconds of relaxation
control
mdx
B.
normalized mean grey/area
1.015
1.01
1.005
1
0.995
0.99
0.985
0.98
0.975
0.97
0
10
20
30
40
50
60
seconds of relaxation
control
mdx
C.
i.
ii.
mean grey/area normalized
1.2
1.15
1.1
1.05
1
0.95
0
10
20
30
40
50
60
seconds of relaxation

Figure 3.

Faint DAF fluorescence was observed briefly just prior to a 2-second stretch interval, when the membrane is pulled downward, out of the plane of focus. The myotube then returns at the end of the stretch interval to a view that is in focus for 59 of the 60 seconds in the unstretched interval. However, only faint fluorescence is observed from the *mdx* myotube at the end of stretch, and the level of fluorescence does not show any marked changes over 59 sec. At the end of 59 seconds viewing the DAF fluorescence signal, the microscope view is once again adjusted (by changing the filters through a brief red illumination) to show the same DAPI+ nuclei in the myotube that was viewed at the start of the video sequence.

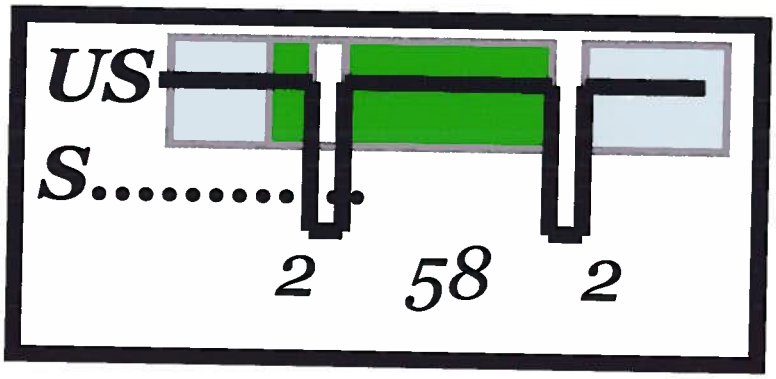
Supplemental figure 1. A schematic diagram of the experimental design, captured by video-microscopy of normal and *mdx* myotubes in culture.

A. The video of control cultures begins by showing the microscope focused on DAPI-stained nuclei within a myotube. The view then changes from blue fluorescence in DAPI+ nuclei to red illumination (very briefly) followed by green fluorescence, as the filter cubes of the microscope were changed to visualize the DAF fluorescence. Within a few seconds, the video picture appears out of focus for 2 seconds, during which time the 2 sec of stretching pulls the substrate of the culture plate and the attached myotube downward from the objective and out of the plane of focus. After 2 seconds, the stretch period ends and the membrane returns to the plane of focus. The DAF fluorescence produced as a result of NO release from the stretched myotube is then observed in focus over a 58 sec time frame (the total time in the unstretched position was 60 seconds). The fluorescence can be observed to decay gradually in intensity, and to show a pulsatile character throughout the sarcoplasm of the myotube. After 58 seconds, the membrane was again stretched; this second stretch is observed as the myotube again moving out of focus for 2 sec, during which the microscope was switched back to viewing through the UV filter for to observe fluorescence from DAPI. The same myotube was observed throughout the video sequence.

B. This video was recorded from a representative culture of *mdx* myotubes. This sequence begins by focusing on the myotube nuclei while viewing DAPI staining. The view of the myotube then changes from blue DAPI+ nuclei to red illumination (very briefly), and then to green fluorescence, in order to observe the NO-specific DAF fluorescence signal.

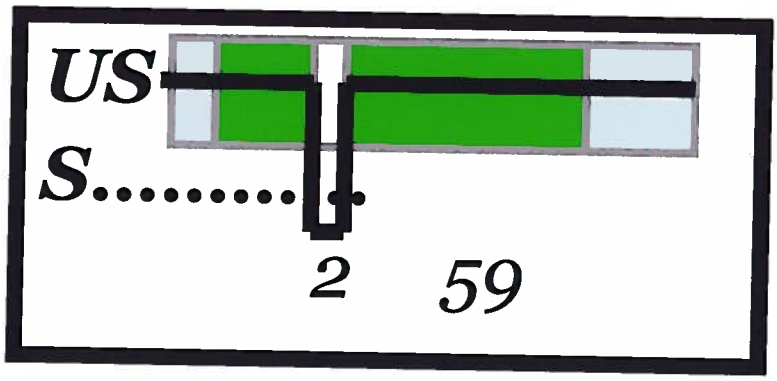
Faint DAF fluorescence was observed briefly just prior to a 2-second stretch interval, when the membrane is pulled downward, out of the plane of focus. The myotube then returns at the end of the stretch interval to a view that is in focus for 59 of the 60 seconds in the unstretched interval. However, only faint fluorescence is observed from the *mdx* myotube at the end of stretch, and the level of fluorescence does not show any marked changes over 59 sec. At the end of 59 seconds viewing the DAF fluorescence signal, the microscope view is once again adjusted (by changing the filters through a brief red illumination) to show the same DAPI+ nuclei in the myotube that was viewed at the start of the video sequence.

A.



Control

B.



mdx

Supplemental Figure 1.

Real-time video capture of NO release by visualization with DAF

The two digital video sequences recorded from **A**. a normal myotube, and **B.** a dystrophic myotube in culture. Cells were pre-loaded with the dye DAF-2DA by incubation in 10 ìM DAF-2DA in KRP for 1 hr at 37°C. (When incorporated into cells, the diaceate side chain of DAF-2DA is cleaved, leaving the active fluorochrome, DAF-2.) Cultures were protected from light after pre-loading. Negative control cultures were incubated in KRP without DAF-2DA. Cultures were rinsed with KRP containing the vital nuclear stain, DAPI (5 µg/mL), rinsed with fresh KRP, and incubated at 37°C until use. The sylastic membrane was removed from a culture dish and mounted in the StageFlexerJ apparatus which was attached to the vacuum line of the FlexCell™ culture system. The unit was placed on the stage of an Olympus BH2-RFCA microscope equipped with epifluorescence optics (490 nm excitation filter, and 515 nm emission filter to detect DAF fluorescence). The FlexCell™ vacuum system was set to produce a stretch of approximately 10% in the radial direction in each culture well (282), and the microscope was focused on DAPI+ nuclei in myotubes on the plane of an unstretched culture membrane. Cultures were subjected to 2 sec of 10% stretch, when myotubes moved out of the plane of focus; the stretch was followed by a 60 sec interval when the sylastic membrane and myotubes returned to the "unstretched position" in the original focal plane setting. Data for the fluorescence signal were recorded at 30 frames per second using the Philips VRecord feature of the ToUCam™ software package.

Chapter 6.
C-met: a delayed-early gene in skeletal muscle satellite cells

ABSTRACT

C-met, the tyrosine kinase receptor for hepatocyte growth factor (HGF), is expressed in skeletal muscle satellite cells. This receptor is essential for development, as c-met knockouts are embryonic lethal. Activation of skeletal muscle satellite cells also appears to be mediated in part by HGF binding to c-met, which then initiates multiple signaling cascades involved in cell proliferation, migration and survival. Recent evidence has indicated that there may be more than one population of satellite cells resident on single fibers and that c-met expression is increased after ½ hr of stretching. These results suggested the value of re-evaluating the expression profile of c-met in satellite cells on single fibers during quiescence and activation. Single muscle fibers from normal and *mdx* mouse flexor digitorum brevis muscles were cultured with or without stretch for ½ hr, and immediately processed by in situ hybridization to detect the number of c-met+ cells per fiber. The number of c-met+ cells on normal single fibers increased with stretch, but did not change for *mdx* single fibers after stretch. Whole muscles treated with cycloheximide showed increased c-met expression after ½ hr and 4 hrs of exposure. A time course experiment to examine c-met expression with cycloheximide indicated that the peak of c-met expression was reached after 1 ½ hrs of exposure, slightly after the peak of expression of c-fos, a known immediate early gene. We conclude that c-met acts as a delayed-early gene in skeletal muscle satellite cells.

INTRODUCTION

Normally quiescent in adult muscle, skeletal muscle satellite cells have been shown to activate after ½ hr and 2 hrs of mechanical stretch in a single muscle fiber model (82). More recently it has been shown that NO and HGF are involved in the stretch-activation cascade, and that the 2 peaks of activation have different characteristics and signaling requirements (Wozniak and Anderson, submitted). One of the possible explanations for the different peaks is the presence of more than one population of satellite cells on single isolated fibers. This idea of satellite cell heterogenity in skeletal muscle is not new (reviewed in (33)). Indeed, in *mdx* mouse muscular dystrophy, it has been suggested that there is a population of satellite cells that is absent from single muscle fibers ((47), Wozniak and Anderson, submitted).

C-met, the receptor for hepatocyte growth factor (HGF) is thought to be expressed by satellite cells under both quiescent and activated conditions (77), and in unstimulated, or unstretched cultures is expressed in 1.38 satellite cells per fiber in the flexor digitorum muscle (FDB) of normal mice (82). C-met activity is intimately linked to signaling pathways involving migration (35), cell cycling and cell survival (83-91). These traits suggest that c-met is required for satellite cell activation, and that all satellite cells on a single fiber should express c-met during activation. However, if there are multiple populations of satellite cells (based on their activation characteristics), it seems possible that c-met expression in satellite cells on normal fibers could also be heterogenous. As well, recent data have shown that the method of isolating single muscle fibers is important in maintaining satellite cell quiescence (310), and that total c-met expression increases after ½ hr of stretching (111). These

findings strongly encourage a re-examination of the detailed character of c-met expression in satellite cells on single fibers during quiescence and activation, and have led us to hypothesize that the distribution of satellite cells expressing c-met would be regulated by stretch.

Experiments were designed to examine the level of expression (using whole muscles) and the pattern of distribution (using single muscle fibers) of c-met in normal and dystrophic muscle with or without stretch. Comparisons of changes in the distribution of c-met + cells on single fibers between normal and dystrophic muscle were conducted in an attempt to determine whether the satellite cell heterogeneity observed in normal muscle is indeed lost in *mdx* muscle as measured by this indicator. Whole muscle and single cell cultures were also treated with cycloheximide to determine whether c-met acts as an immediate early gene, which would account for the rapid upregulation of c-met expression after limited stimulation by stretch.

METHODS

IN SITU HYBRIDIZATION

Normal and *mdx* fibers, isolated according to (82) were stretched (or not) for ½ hr and then taken through in situ hybridization as reported (82). Satellite cells on fibers from normal and *mdx* muscle were assessed for c-met expression. Antisense, digoxigenin-labelled riboprobes were synthesized following Boehringer Mannheim protocols, as reported in detail (15). Labelled probes were run on formaldehyde-agarose gels, transferred to a nylon membrane, and visualized using anti-digoxigenin antibodies and alkaline phosphatase colour detection to confirm the probe size of 1.27 kb. The target mRNA was 9 kb. Hybridized transcripts were localized in satellite cells resident on fibers. In other experiments, a sense riboprobe for c-met showed no signal, similar to other procedural controls that omitted probe, anti-digoxigenin antibody or colour detection steps.

C-MET AS AN IMMEDIATE EARLY GENE

Whole extensor digitorum longus (EDL) muscles were isolated and pinned according to (33), and primary satellite cell cultures were grown for 1 ½ weeks before initiation of experiments. Cycloheximide (10 μg/ml) was added to muscle cultures and incubated for 4 hr according to (311), after which they were immediately frozen in liquid nitrogen. Other muscle cultures were either stretched for ½ hr or not in the presence or absence of cycloheximide, after which they were immediately frozen in liquid nitrogen. Cells in culture received cycloheximide in the medium (10 μg/ml) for 0 hr, 15 min, 30 min, 1 or 1 ½ hrs after which RNA was isolated immediately. RNA was isolated from muscles and cells by phenol

chloroform extraction (312), and c-met and c-fos mRNA were detected using RNAse protection assays.

RESULTS

STRETCH INFLUENCES C-MET EXPRESSION IN NORMAL BUT NOT *MDX* SATELLITE CELLS

This experiment was conducted to test the influence of stretching on the distribution of c-met + cells located on normal and *mdx* single muscle fibers. Stretching normal single fibers resulted in a significant shift in the distribution of c-met + satellite cells to higher numbers (Figure 1; $p<0.005$). There was no significant change in the distribution of c-met+ satellite cells on *mdx* fibers after ½ hr of stretching (Figure 1). There was also no significant difference in the distribution of c-met+ satellite cells between normal and *mdx* unstretched fibers.

C-MET RESPONSE TO CYCLOHEXIMIDE TREATMENT

To test whether c-met acts as an immediate- or delayed- early gene, whole muscle cultures were treated with the protein synthesis inhibitor, cycloheximide. Treatment of whole muscle cultures with cycloheximide for ½ hr without stretch resulted in a 1.2 fold increase in c-met expression over untreated controls using RNAse protection assays (Figure 2). Treatment with cycloheximide for 4 hours without stretch resulted in a 1.3 fold increase over untreated controls. Stretching for ½ hr in the presence of cycloheximide did not increase c-met expression over untreated, stretched controls, possibly due to the short period of time of cycloheximide treatment (see below).

C-MET AS A DELAYED EARLY GENE

To determine whether c-met acts as an immediate or delayed early gene, a time-course of cycloheximide exposure was carried out on isolated satellite cells from normal animals. Expression of transcripts for c-fos, a known immediate early gene, was used for comparison, as it would be expected to rise rapidly upon exposure to cycloheximide. As anticipated, c-fos expression increased dramatically within 15 minutes of exposure and reached a plateau after 30 minutes (Figure 3). The level of c-met increased after 15 minutes of exposure, to a level somewhat less than c-fos expression. After this initial rise, c-met expression decreased by 30 minutes of cycloximide treatment. C-met expression increased again at 1 hr of exposure, and the increase was almost 3X greater after 1½ hrs of exposure (Figure 3). Since there was a dramatic increase in c-met expression on a time frame distinguishable from the significant increases in c-fos expression, we conclude that c-met acts as a delayed early gene in skeletal muscle satellite cells.

DISCUSSION

C-met expression was previously demonstrated to increase after ½ hr of stretch in whole muscle cultures from normal and dystrophic mice (111). Another report showed 2 peaks of satellite cell activation were induced by stretch in normal muscle fibers (82). Here we extend those observations and show that the distribution of satellite cells that are expressing c-met, shifted to significantly higher numbers after ½ hr of stretch. By contrast, the distribution of c-met+ satellite cells on *mdx* fibers subjected to stretch over the same interval did not change. Results are interpreted to suggest that the number of c-met + cells increases after stretch in normal muscle. Together with an earlier report (111), these results suggest further that the level of c-met expression within each satellite cell also increases during activation. The differential patterns of changes in c-met gene expression in satellite cells between normal and *mdx* muscle following stretch was interpreted to indicate that in dystrophic fiber cultures, the level of c-met expression per satellite cell increases with stretch, while the number of cells expressing c-met does not change.

C-met expression was explored in these experiments since previous reports demonstrated a change in satellite cell activation after stretching (82;142), and the effect of a potent growth factor, HGF, to activate satellite cells from quiescence (81;164). Thus, c-met appears to be the primary receptor responsible for initiating the early stages of activation, with HGF binding c-met rapidly, in about 10 minutes after stimulation (35;81). Since 2 peaks of activation are demonstrated in stretched single fiber cultures from normal muscle (82), it seems possible that the distribution of c-met expressing cells on single fibers would change during stretch to accommodate, or reflect, these 2 apparently distinct sets of cells.

Results presented here indicate that stretching for ½ hr increased the distribution of c-met expressing cells on normal single fibers. This increase towards higher numbers of c-met+ satellite cells per fiber was not observed for c-met expressing cells on *mdx* single fibers. There was also no difference between normal and *mdx* single fibers in terms of the number of c-met+ cells on unstretched fibers. These observations are consistent with those made by Reimann et al. (313) that showed no difference in the number of m-cadherin+ satellite cells in normal and *mdx* muscle. The difference in the response to stretching by satellite cells on normal and *mdx* single fibers may be due to alterations in activation levels of normal vs. *mdx* satellite cells when subjected to ½ hr of stretch. In normal single fiber cultures, satellite cell activation increases after ½ hr of stretch (82). On the other hand, ½ hr of stretch decreases satellite cell activation levels in single fiber cultures of *mdx* muscle (Wozniak and Anderson, submitted). Since satellite cells are highly activated in *mdx* muscle, it is also possible that all satellite cells in *mdx* muscle express c-met under typical conditions, and that there are no other c-met negative satellite cells to respond during stretch by initiating synthesis of c-met gene transcripts. Finally, it is possible that there is a satellite cell population that is lost in *mdx* muscle, as suggested elsewhere ((47)(Wozniak and Anderson, submitted)).

The observations that c-met expression had increased within ½ hr of stretch-stimulation suggests that c-met acts as an immediate, or at least delayed, early gene. Immediate early genes are those genes that are able to respond to growth factors within minutes, regardless of the presence of protein synthesis inhibitors (reviewed in (314)). In fact, it is often the case that treatment with a protein synthesis inhibitor such as cycloheximide will superinduce gene expression of immediate early genes (e.g., (315)). Two

of the most notable immediate early genes are c-fos and c-jun, which when combined become the activating protein AP-1 (316). Delayed early genes are those that are activated by immediate early transcription factors (317).

Here we show that c-met expression was induced by cycloheximide treatment of normal muscles in culture. C-met expression in whole muscle cultures increased in the absence of stretching after ½ hr or 4 hrs of cycloheximide treatment. Stretching muscles in the presence of cycloheximide for ½ hr, however, did not increase the levels of c-met expression in the tissue. This may be due to maximal stimulation by cycloheximide alone, which would prevent any further increase by stretching. The increase in c-met expression was not as large as would be expected, according to other reports showing dramatic increases in expression of immediate early genes. The lower level may be due to the time points that were selected for cycloheximide exposure. C-met expression would be expected to rise and then begin to decrease over time, even in the presence of cycloheximide, such that the current experiments may have missed the peak in c-met expression.

In order to determine the timing of peak c-met expression due to cycloheximide exposure, c-met expression was measured as a function of time in isolated single satellite cell cultures. The time course of c-fos expression was used to compare upregulation of c-met with that of c-fos, a known immediate early gene (318) that is expressed in satellite cells (15). Expression of c-fos increased after only 15 min of cycloheximide exposure, and peaked after ½ hr. C-met expression on the other hand increased at 15 minutes, declined at 30 min and then increased after 1 hr and 1.5 hrs of cycloheximide exposure. This finding of a dramatic increase in c-met expression somewhat later than the peak in c-fos expression

suggests that c-met is a delayed-early gene in skeletal muscle.

The data presented here on c-met as a delayed early gene are consistent with work done by Boccaccio and colleagues (311), where epithelial cells were shown to induce transcriptional expression of c-met after stimulation with HGF, serum or a protein kinase C stimulator (TPA). By comparison, the level of c-met mRNA was low in unstimulated cells. C-met mRNA levels were increased after increases in c-fos and c-jun, but before uPA (311). As well, increases in c-met mRNA were observed using the protein synthesis inhibitor cycloheximide in epithelial cells. Increased expression of c-met in response to stimulation in hepatocytes (HepG2 cells) has also been demonstrated (319).

This is the first report to show that the distribution of satellite cells that express c-met on normal single fibers increases after ½ hr of stretch, and that the c-met receptor gene acts as a delayed-early gene in skeletal muscle satellite cells. It is important to note that this upregulation can only be observed if satellite cells are quiescent upon isolation. For this reason, the method by which single fibers or whole muscles are isolated is pivotal in conducting experiments involving the early stages of activation (reviewed in (33)). The time course of the response to cycloheximide exposure suggested that c-met acts as a delayed-early gene in skeletal muscle satellite cells, since expression reached a peak shortly after the peak in expression of the known immediate early gene, c-fos. Satellite cells from *mdx* muscle showed the same distribution of c-met+ cells per fiber as that seen on normal single muscle fibers. However, the number of c-met expressing satellite cells per *mdx* fiber did not increase after stretching. If anything, there was a tendency to shift the distribution of c-met+ satellite cells per fiber toward lower numbers in stretched *mdx* fiber cultures. This may be

due to the loss of a satellite cell population as a consequence of the dystrophic process (47) and/or changes in NOS-1 expression and activity that appears to regulate activation and quiescence. Further experiments measuring the activation of the c-met receptor in skeletal muscle by examining autophosphorylation will clarify the time required after stimulation and NO release ((35), Wozniak and Anderson submitted) to activate the c-met receptor for initiation of cellular signaling pathways.

Figure 1. Response of distribution of c-met+ satellite cells to stretch

The number of c-met+ cells per fiber were counted on plates that had either been stretched or not. Frequency distributions were then complied for both normal (A) and *mdx* (B) single fibers and χ^2 was used to determine if the distribution changed between normal and *mdx* fibers, and between stretched and unstretched fibers of the same mouse strain. There is no significant difference between the number of c-met+ cells on normal single fibers compared to *mdx* single fibers under unstretched conditions. Stretching shifts the distribution of c-met+ cells to higher numbers in normal fiber cultures ($p<0.005$), but not in *mdx* fiber cultures. The frequency distribution of c-met+ cells on stretched normal single fibers is also significantly higher than the frequency distribution on stretched *mdx* single fibers ($p<0.05$).

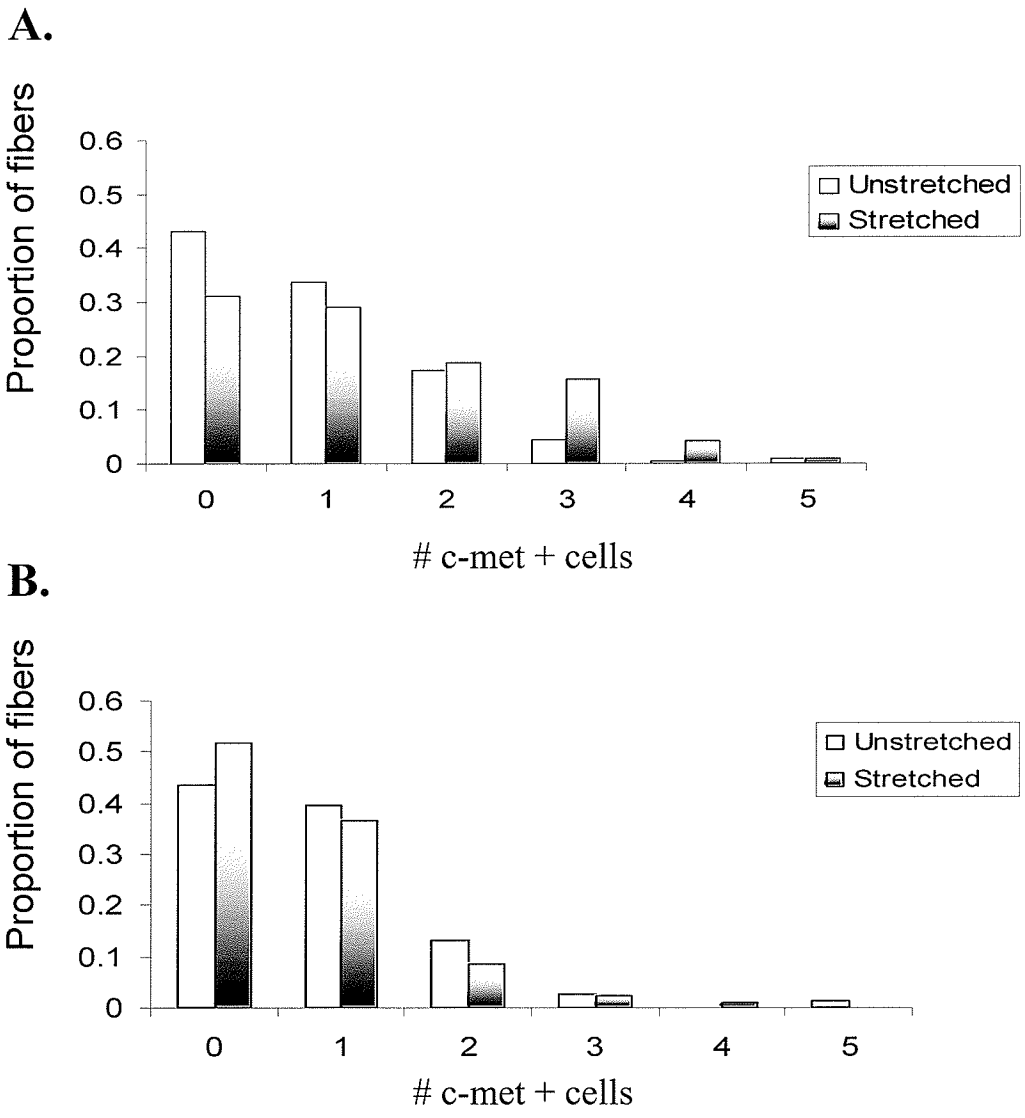

A.
Proportion of fibers
0.6
0.5
0.4
0.3
0.2
0.1
0
Unstretched
Stretched
0
1
2
3
4
5
c-met + cells
B.
Proportion of fibers
0.6
0.5
0.4
0.3
0.2
0.1
0
Unstretched
Stretched
0
1
2
3
4
5
c-met + cells

Figure 1.

Figure 2. Response of c-met expression to ½ hr or 4 hr cycloheximide treatment

Whole muscle cultures of normal EDL's were stretched or not with or without cycloheximide for ½ hr. Whole muscle cultures were also treated with cycloheximide (or not) for 4 hours according to (311). C-met expression was measured using RNAse dot blots, and data were plotted as normalized to unstretched controls. Cycloheximide treatment for ½ hr without stretching resulted in an increase in c-met expression to the same degree as treatment for 4 hours. Stretching in the presence of cycloheximide did not increase c-met expression, possibly due to overstimluation.

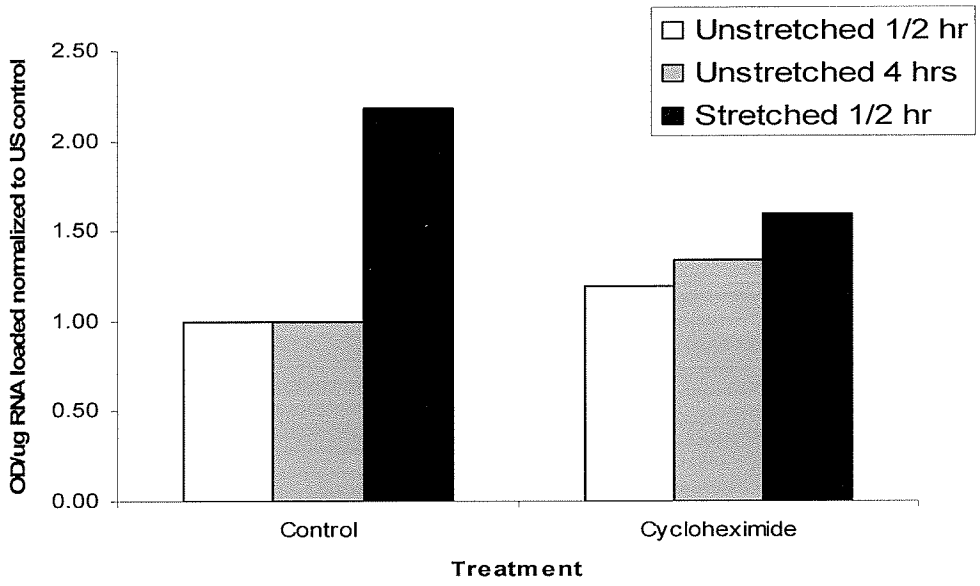

Unstretched 1/2 hr
Unstretched 4 hrs
Stretched 1/2 hr
OD/ug RNA loaded normalized to US control
2.50
2.00
1.50
1.00
0.50
0.00
Control
Cycloheximide
Treatment

Figure 2.

Figure 3. C-met as a delayed-early gene

Single satellite cell cultures isolated from normal mice were grown for 1 ½ weeks, and then treated with cycloheximide for 0-90 minutes. Cells were isolated and RNA isolated according to (312). C-met expression was visualized using RNAse protection dot blots (i.). In order to determine if c-met was acting as an immediate- or delayed-early gene, c-fos expression over the same time period was measured from the same samples. C-fos expression increases after only 15 minutes of exposure to cycloheximide and continues to increase until 30 minutes, after which it declines. C-met expression on the other hand increases slightly after 15 minutes of exposure, decreases after 30 minutes, and then increases until reaching a maximum at 90 minutes of exposure to cycloheximide. Due to the delay in the increase in c-met expression compared to c-fos expression (a known immediate-early gene), these data suggest that c-met acts a delayed-early gene in skeletal muscle satellite cells.

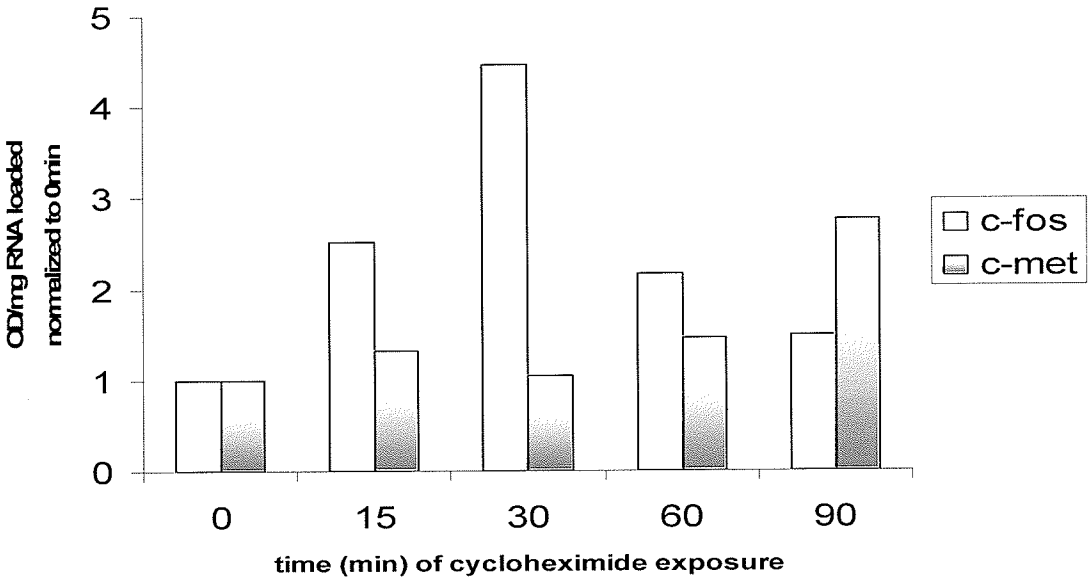

5
4
3
2
1
0
OD/mg RNA loaded
normalized to 0min
0
15
30
60
90
time (min) of cycloheximide exposure
c-fos
c-met

Figure 3.

Chapter 7.
Additional Data

INTRODUCTION

The earlier work presented in this thesis raised the following questions related to signaling in the regulation of quiescence and activation: 1) Are NO and HGF involved in stretch-inactivation in *mdx* muscle?, 2) Does the loss of NOS-1μ from *mdx* muscle result in high levels of satellite cell activation before the onset of dystrophy? 3) What is the expression profile in quiescent and activated satellite cells of genes (such as myostatin) that may be involved in maintaining quiescence, and those involved in myogenic determination (e.g. myf-5 and MyoD)?, and finally 4) Are there alternate pathways to activation, as suggested by results obtained from NOS-1(-/-) animals?

The role of NO and HGF in normal stretch-activation has now been established. The role that these 2 compounds play in stretch-induced decreases in activation of satellite cells on *mdx* single fibers (after ½ hr of stretch) remains unknown. This is especially important for potential treatment design for DMD. This information would indicate whether short-term exercise or stretching protocols (e.g. Peeler and Anderson, unpublished data) paired with a drug targeted to enhance NO production or increase HGF concentrations would further normalize satellite cell activation in dystrophic muscle.

NOS-1 activity plays a pivotal role in activating satellite cells from quiescence. The absence of NOS-1 leads to hyperactivation of satellite cells (seen in *mdx* and NOS-1(-/-) muscle; see Chapter 4). However, the impact of a NOS-1 deficiency before the onset of dystrophy, i.e. during growth, has not been established. Since muscle growth and muscle regeneration are 2 distinct processes, the role of NOS-1 would be expected to change, depending on the stage of growth or disease, in a muscle under investigation.

The identification of genes expressed during quiescence and early activation of satellite cells has been debated within the literature (for example (55;65)). One possible explanation for the reported inconsistencies is likely due to the different experimental protocols between laboratories ((310) and reviewed in (33)). In this thesis project, isolated single fibers were maintained with satellite cells in quiescence (82;310), such that ½ hr of stretch induced activation (82). The model of single fibers in culture is well suited for determining the gene expression profile of key myogenic genes during quiescence and activation. Another model that can be useful is the whole muscle culture model, as satellite cells respond by DNA synthesis in the same pattern after stretch in the 2 models (33). Using these 2 models we investigated the changes in gene expression from quiescence to activation to reveal potential mechanisms that regulate satellite cell activation. Those mechanisms could provide new treatment possibilities to reduce the activity of satellite cells in DMD and *mdx* muscle (to conserve regenerative capacity), or stimulate activation in conditions where satellite cells are resistant to activating stimuli (as is the case for satellite cells in aging muscle).

To date, the main pathway identified for satellite cell activation from quiescence involves the release of NO after a stimulus, leading to HGF release and subsequent binding to the c-met tyrosine kinase receptor (112;141). However, the capacity for muscle in dystrophic and NOS-1(-/-) strains to regenerate at all (albeit somewhat more rapid or delayed respectively in comparison to normal (35)), is strong evidence to support the hypothesis that there is at least one alternate pathway for satellite cell activation that does not involve NO release from NOS-1.

G proteins may play an extensive role in the activation of satellite cells. G proteins are involved in initiating DNA synthesis in fibroblasts cultured from human skin (320) and fetal lung ((320) and reviewed in (321)) via activation of the adenylate cyclase pathway, and in cardiomyocytes through unknown mechanisms (reviewed in (322)). Activation of the adenylate cyclase pathway was induced in skin and lung fibroblasts by cholera toxin (320) and produced an increase in HGF mRNA and HGF secretion after 24 hours. Adenylate cyclase is also modulated by NO signals through G-protein-coupled receptors (323). If similar steps were involved in regulating satellite cell activation, this increase in HGF would result in enhanced binding of HGF to c-met, and lead to increased satellite cell activation (324). The involvement of G proteins in the activation of protein kinase C (PKC) via the PIP_2 pathway is also suggested by data from studies on cultured human alveolar cells (325), human skin fibroblasts (158) and epithelial cells (90). In addition, however, these signaling pathways may be activated by tyrosine kinases that are extensively involved in activation of DNA synthesis in many systems ((90) and reviewed in (321)).

While the roles of the two types of receptors involved in PKC activation (G-protein or tyrosine kinase) were not distinguished in these studies, stimulation induced notable increases in HGF (158;325) and in c-met mRNA (311). The increase in HGF was reported to induce up-regulation of c-met through stimulation of the c-fos and c-jun (172;326). The mechanism by which HGF increases c-met gene expression may account for signal amplification. Such amplification could recruit more than one satellite cell after a single stimulus such as brief stretching, and could explain some of the apparent heterogeneity in the satellite cell population (82).

Stretch-activated ion channels may also be involved in activating satellite cells, since ion channel activity can induce PKC activation via the PIP2 pathway in vascular smooth muscle cells (321). The regulation of NOS activity by calcium-calmodulin, for example in endothelial cells (327-329), further suggests there may be a similar pathway in skeletal muscle fibers and possibly satellite cells themselves, since NOS is expressed in satellite cells upon activation *in vitro* (141) and *in vivo*, even in the absence of the stable localization of NOS-1 normally afforded by dystrophin (15). The Ca^{2+} influx that occurs with fiber damage also leads to hypercontraction and generates shear forces which are potent signals in many tissues. Shear acting on endothelial cells increases activity by Akt and NOS (327). In skeletal muscle, hypercontraction shear results in NO-dependent satellite cell activation *in vivo* (35), and in liver after partial hepatectomy, shear increases hepatocyte c-fos expression and cell proliferation (116;330). Calcium-calmodulin also reverses caveolin-3 dependent inhibition of NOS activity. L-type calcium channel opening is stimulated by S-nitrosylation via nitroso-thiols, while NO itself inhibits channel activity (331). Therefore the movement and concentration gradients of Ca^{2+} ions may play a substantial role in activation, acting via shear or directly on NOS, to release NO and activate satellite cells in skeletal muscle.

While not an exhaustive list of possibilities, some of the pathways active in other tissue systems may ultimately be shown to play a significant role in satellite cell activation. Together the capacity for one stimulus to recruit more than one satellite cell per fiber into activation, to recruit an apparently heterogeneous satellite cell population with distinct courses in time (82), and the tight control of activation and quiescence shown by satellite cells are suggestive that multiple pathways engage various positive and negative regulatory

loops in that control. The following experiments were designed to test some of these possible pathways involved in satellite cell activation.

METHODS

SINGLE FIBER AND WHOLE MUSCLE ISOLATION

Single fibers were isolated from the flexor digitorum brevis (FDB) of normal (C57BL/6) and *mdx* mice according to established methods (82;112;144). Fibers were plated in 6-well FlexCell plates (FlexCell International) which had either a flexible or rigid surface substrate, pre-coated with 80 µl of Vitrogen100. After 20 min at 37°C and 5% CO_2, 2 ml of basal growth medium (112) was added to each well and fibers cultured overnight. Whole EDL muscles were removed from normal animals, cleaned and pinned at each tendon, arranged radially in flexible or rigid FlexCell plates. Each experiment used a balanced design where control and stretched fibers (or muscles) were collected from the same animal preparations.

ROLE OF NO AND HGF IN STRETCH-DEACTIVATION

Bromodeoxyuridine (BrdU; 0.002%) alone or in conjunction with either L-Arg (500µM), L-NAME (0.2µg/ml), HGF (10ng/ml) or anti-HGF (2µg/ml) were added the next morning (time 0 hr) to fiber cultures. Plates were placed in a FlexCell system and stretched for ½ hr at 4 cycles per minute with 20 kPa of vacuum, maintained until 24 hours and fixed in acid-alcohol (82).

MEASURING ACTIVATION

Activation was measured as DNA synthesis, visualized by BrdU immunostaining (82), using anti-BrdU (1:1000; Sigma) and secondary HRP-linked anti-mouse antibodies (1:300; Sigma) visualized with diamino-benzidine (DAB, 25 mg/ml; Sigma). The number of BrdU-positive

satellite cells per fiber was counted without knowledge of source, by systematically scanning the entire area under the coverslip. Myonuclei were not positive.

ROLE OF NOS IN SATELLITE CELL ACTIVATION OF YOUNG ANIMALS

Young (1 ½ week old; n=3 (C57) and 2 (*mdx*)) and mature (3 ½ - 4 ½ months old; n=1 C57 and *mdx*) normal and *mdx* mice were injected with triated thymidine (^{3}H thymidine; [200µl/mL]) and euthanized under anesthesia after 2 hrs. Hindlimb muscle was collected from all animals; all muscles were pooled for the young animals, while the right and left muscles were kept separate for the mature animals. Muscle samples were frozen at -80°C. DNA was isolated according to (332). Scintillation counts were obtained for all DNA samples, and total DNA measured according to (332).

GENE EXPRESSION IN QUIESCENT AND ACTIVATED SATELLITE CELLS

Whole muscles isolated from normal and *mdx* animals were stretched for ½ hr, after which they were immediately flash frozen in liquid nitrogen. RNA was isolated from whole muscles according to (312), and mRNA detected by RNAse protection assays (15) or northern blots as appropriate to the size of the transcript. N = 1 group per treatment.

ALTERNATE PATHWAYS TO ACTIVATION

Whole muscles from normal animals, isolated and pinned in culture as described above, were treated with one of 6 compounds: Aluminum fluoride (formed from a mixture of [30µM] $AlCl_3$ and [10mM] NaF, a G-protein activator); Wortmannin ([0.75µM], a G-protein

inhibitor); Genestein ([5μM], a non-specific ttyrosine kinase inhibitor); Geldanamycin ([0.25μM], a tyrosine kinase inhibitor more specific for c-met); the Ca^{2+} ionophore A23187 ([5μM], a Ca^{2+} channel agonist); or Nifedipine ([5μM]; an L-type Ca^{2+} channel inhibitor). Whole muscles were then stretched for ½ hr, removed from the FlexCell system and incubated with ^{3}H-thymidine [2μl/mL] for 24hrs, and then frozen at -80°C. DNA was isolated according to (332). Scintillation counts were obtained for all DNA samples, and total DNA measured according to (332) using a Hoescht dye assay on an ELISA plate reader against a standard curve. N=1 muscle per group.

RESULTS

ROLE OF NO AND HGF IN STRETCH-DEACTIVATION

Stretching in the presence of L-Arg significantly decreased satellite cell activation on *mdx* single fibers ($p<0.05$; Figure 1). Treatment with anti-HGF alone also decreased activation of satellite cells on unstretched *mdx* single fibers (Figure 1). No other treatments (L-NAME or HGF) were able to influence satellite cell activation, with or without stretching. The amount of stretch was quantified once again to corroborate results published earlier (282). It was determined that the protocol employed throughout this thesis applies an elongating stretch, ranging from 7.5-10% (tendon to tendon, or end-to-end; Table 1) through a change of 20 kPa applied as a negative pressure to the bottom of a FlexCell plate.

DIFFERENCE IN MUSCLE CELL PROLIFERATION BETWEEN YOUNG NORMAL AND DYSTROPHIC ANIMALS; *IN VIVO* EXPERIMENTS

There was no significant difference in muscle cell proliferation between normal and *mdx* muscle as assayed using ^{3}H-thymidine uptake into muscle DNA at 1 ½ -2 weeks of age (3123.6 ± 630.41 CPM/ug total DNA (normal, n=3) vs. 1811.6 ± 453.02 (*mdx*, n=2); Figure 2). There was also no strain difference (between normal and *mdx*) in muscle cell proliferation at 3-4 months of age (3896.2 ± 573.61 (control) vs. 4501.9 ± 437.35 (*mdx*)) or between normal muscle cells regardless of the age of normal mice. However, there was a significant increase ($p<0.05$) in muscle cell proliferation in older compared to younger *mdx* mouse muscle (Figure 2).

GENE EXPRESSION IN QUIESCENT AND ACTIVATED SATELLITE CELLS

The expression of some of the genes involved in responding to satellite cell activation and quiescence was assessed using whole muscle cultures assayed by northern blotting or RNAse protection assay; results are summarized in Table 2. Resting levels of c-met expression were quite low (Figure 3; data published in (111)) in normal muscle extracts, indicating a general quiescence of satellite cells in this model. Resting levels of c-met were substantially higher in extracts of *mdx* muscle under identical conditions.

The levels of c-met expression increased rapidly after only ½ hr of stretching in both normal and *mdx* muscle. Furthermore, the expression of c-met was NO-dependent, as shown by experiments with L-NAME. Treatment of normal and *mdx* muscle cultures with L-NAME in the absence of stretch induced an increase in c-met expression as shown for satellite cell activation by L-NAME in fiber culture experiments (Chapter 4). Stretching muscle cultures in the presence of L-NAME reduced c-met expression. Again, this was similar to effects of L-NAME on stretched fiber cultures (Chapter 4). By comparison, mRNA transcripts for myf-5 (Fig. 4A), MyoD (Fig. 4B) and myostatin (Fig. 4C) were slightly reduced by stretching at ½ hr after the stimulus. This is consistent with previous reports on the timing of MRF gene expression during activation.

ALTERNATE PATHWAYS TO ACTIVATION

Data suggest a trend toward increased activation after treatment with either a G-protein activator (AlF) or a G-protein inhibitor (Wortmannin). By comparison, stretching for ½ hr in the presence of either compound tended to decrease activation (Figure 5A).

Treatment with tyrosine kinase inhibitors did not appear to affect satellite cell activation in whole muscles in the absence of stretch. However, when cyclical stretching was applied to whole muscle cultures, there was a large increase in satellite cell activation after treatment with Genistein, and there was a smaller increase observed after Geldanamycin treatment of muscle cultures (Figure 5B). Both tyrosine kinase inhibitor treatments resulted in fibers in culture that appeared to have all nuclei incorporating BrdU to a small degree (See inset Figure 5), including myonuclei. Treatment with the L-type Ca^{2+} channel blocker, Nifedipine, did not change activation in unstretched muscle cultures, while treatment with the Ca^{2+} ionophore, A23187 appeared to increase activation. Stretching appeared to have induced an increase in activation in Nifedipine-treated cultures, and did not appear to change activation levels in the A23187 treated muscles (Figure 5C).

DISCUSSION

The experiments in this chapter were conducted to address the specific aims related to exploring the possibility of alternate pathways in satellite cell activation and the role of NO and HGF in *mdx* satellite cell activation, as well as additional questions raised by the work presented earlier in this thesis. While not all the experiments provided conclusive findings, the current results give some insight into the regulation of satellite cell activation and quiescence by gene expression and by disease.

The observation that anti-HGF treatment resulted in a significant reduction in activation of satellite cells on *mdx* single fibers indicates that HGF-c-met signaling is maintained in *mdx* satellite cells. The results also indicated that HGF-c-met signaling is required for activation on *mdx* fibers, regardless of the changes in satellite cell activation kinetics due to the loss of NOS-1 signaling evident in *mdx* mouse muscle.

L-Arg treatment decreased satellite cell activation after ½ hr of stretching. This experiment indicates that the stretch-*de*activation of satellite cells on *mdx* single fibers (Chapter 4) is NO dependent. Findings lend support for the notion that we could develop therapies for DMD that use both NOS manipulation and short-term exercise protocols.

Possible developmental changes in NO-regulated satellite cell activation have not been fully clarified. Originally, we hypothesized that young *mdx* animals would show elevated satellite cell activation even before the initiation of the dystrophic phenotype. This was thought to be the definitive proof that NO or NOS-1 deficiency from fibers (secondary to dystrophin deficiency) was already contributing to hyperactivate satellite cells even prior to dystrophy. However, the hypothesis was not supported by the data presented here. There

was no significant difference between normal and *mdx* satellite cell activation at a young age, or at an older age, although there was a significant increase in muscle cell activation in old compared to young *mdx* muscle. While it is possible that small sample sizes and large variation among animals obscured a difference between young and old *mdx* muscle cell proliferation, data suggest that development and regeneration are distinct processes with different signaling requirements.

During development, satellite cells are constitutively active, as they are required for large amounts of muscle growth during a relatively short period of time. Therefore, they would not be expected to withdraw into quiescence, and then exit that state, before entry into the next cell cycle; they would generally be under continuous stimulation to continue proliferating. By comparison, once a muscle is fully grown, satellite cells would generally enter into quiescence, and remain in that state until they are recruited to repair damaged muscle or support further growth. During regeneration in adult muscle, satellite cells are recruited from the quiescent state, and the environment around the cells must provide the proper signals for repair. For this reason the signaling molecules and pathways leading to activation would not be expected to be identical during growth and regeneration. As such, the absence of NOS-1 during development does not appear to significantly affect the capacity for muscle growth in *mdx* muscle, although a) NO is the initial signal for the exit from quiescence ((35); Chapter 4), and b) NO is a signal for maintaining quiescence (Chapter 4), as satellite cells do not reside in a quiescent state in growing muscle. It is only when satellite cells are required to exit quiescence, due to damage, or to re-enter quiescence after repair, that the loss of NOS-1 appears to becomes detrimental, and the dystrophic process

exacerbated. These ideas could be developed in further investigations

Studies of gene expression in satellite cells during quiescence, and the changes after mechanical stimulation that activates satellite cells, may help to determine the genes involved in the first stages of satellite cell activation. Although these preliminary data encourage further gene profiling studies, they provide initial evidence for corroborating the distribution studies of satellite cells expressing c-met. Those experiments showed a shift to higher numbers of c-met+ satellite cells per fiber as a result of stretch in normal single fiber cultures (Chapter 6). However, *mdx* single fibers in culture did not show this increase in the number of satellite cells expressing c-met (Chapter 6). The difference may be due to the loss of a stem-like satellite cell population in *mdx* muscle (47), proposed to be due to ongoing damage and depletion of the precursor pool for continuous regeneration. By comparison, the level of c-met expression increased after stretching in studies of whole muscle RNA extracts of whole muscle from normal and *mdx* mice (111). Therefore the levels of c-met expression by a single satellite cell may increase in both normal and *mdx* activated satellite cells, while the number of c-met+ activated satellite cells appears to increase with stretch only in normal fiber and muscle cultures, and not in *mdx* cultures. These findings were consistent and highly supportive of the notion that one population of satellite cells is lost from *mdx* muscle fibers. The present experiments also show that c-met acts as a delayed-early gene in skeletal muscle, as found in whole muscle and isolated single satellite cells in culture (Chapter 6).

Despite numerous reports on myogenic gene expression following satellite cell activation, the typical response to stretch-activation in satellite cells was not known. Here we showed that the expression of both myf5 and MyoD decreased slightly after stretch, as

determined from studies of RNA extracts of whole muscles. This was a surprise, given that activation *in vivo* (65;67;104) increases myf5 and MyoD expression. However, the RNA was collected after only ½ hr of stretch in cultures (using the same samples as for the c-met expression studies), and it is unlikely that myf5 and MyoD would have been upregulated within ½ hr of stretch as they are not immediate early genes. Myogenic genes have been shown previously to upregulate no sooner than 3-6 hours after an activating stimulus (77). Myostatin levels also did not change significantly after ½ hr of stretch. Since satellite cells may not yet have entered the cell cycle at this time point after stimulation, it would not be expected that the level of myostatin expression would change substantially. It remains to be determined how the distribution of satellite cells expressing myf5, MyoD and myostatin changes after a stretch stimulus, and how the genes that are expressed during the myogenic differentiation program (myf-5 and MyoD) change in relation to those which are suspected to play a role in maintaining quiescence (myostatin), over the first 24 hours after an activating stimulus.

The presence of pathways to satellite cell activation, other than those requiring NO or HGF, was suggested earlier by observations from muscle regeneration studies in NOS-1(-/-) and *mdx* mice. However, the identity of the alternate pathways has not been elucidated. In (33) we proposed a variety of pathways that are known to play a role in activation of cycling in other tissue systems, and whose signaling molecules are present in skeletal muscle. Here we chose to focus on the G-protein, tyrosine kinase and Ca^{2+} channel pathways.

The pattern observed for activation with and without stretching after treatment with a G-protein inhibitor, Wortmannin, is very reminiscent of the activation pattern with L-

NAME treatment (Chapter 4), suggesting that G-proteins may be acting through a NO-dependent pathway. Treatment with the G-protein activator, AlF, resulted in an increase in activation without stretching, and decreased activation during stretch. This pattern resembles the one observed for single fibers treated with HGF (Chapter 4). The experiment was not designed to distinguish whether signaling was occurring through NO or was bypassing NO to signal through HGF. Both of these scenarios are engaged by G-protein signaling pathways in other systems (33). Further experiments to examine the effects of NOS inhibition during treatment with Wortmannin and AlF would distinguish between the 2 scenarios.

Based on the literature that established c-met as a tyrosine kinase receptor, treatment with tyrosine kinase inhibitors was expected to inhibit activation completely, with or without stretching. However, this does not appear to be the case, as activation increased with stretching and after treatment with either a general tyrosine kinase inhibitor, Genistein, or with a more specific c-met inhibitor, Geldanamycin. These findings are interpreted to be due to small amounts of DNA synthesis that occur before cells enter into the apoptosis pathway (333). This hypothesis is supported by experiments on single fibers, which show that the morphology of single fibers treated with Genistein appears very abnormal, with diffuse BrdU staining in every nucleus on the fiber. The observation remains without explanation.

Treatment with the compound A23187 induced an increase in activation without stretching. This finding is consistent with data on single satellite cells in culture (Tatsumi and Allen, personal communication). However, stretching A23187-exposed cell cultures did not increase activation over stretched control cultures (untreated). This observation suggests that Ca^{2+} channels can be used to activate satellite cells from quiescence, as in unstretched

cultures, although stretch-activation is not increased further by additional opening of these channels. As well, Nifedipine treatment only induced an increase in activation under conditions of stretch. The reasons for these findings are not clear, although it is possible that by decreasing the amount of HGF that is released from the extracellular matrix through stretching, or by Ca^{2+} channel opening without stretch, that the c-met receptor is being hyper-sensitized over this short time frame. From previous results, one might anticipate that adding HGF to these Nifedipine treated cultures would prevent activation.

While these preliminary observations from experiments on the alternate pathways to activation were not conclusive, due to a small number of samples and few repeat experiments, and pose more questions than answers, they hint at the role of other signaling molecules in satellite cell activation. Confirmation that these alternate pathways may be present and employed in activating satellite cells in *mdx* and NOS-1(-/-) muscle (where the concentration of NO is low) would be definitive proof that early activation signaling can operate outside or around the NO-HGF pathway that has been demonstrated here to be operationally required in normal muscle after an activating stimulus. These pathways could offer new avenues for development of therapies for patients with DMD, and are acknowledged to require much more investigation than provided in these preliminary experiments.

Tables

	Unstretched Length	Stretched Length	% change
Whole muscle	8.22 mm	8.83 mm	7.5 %
Line	8.26 mm	9.07 mm	9.8 %

Table 1. Changes in length due to 20 kPa applied pressure.

Gene	**Ratio (Stretch/Unstretch; O.D./ ug RNA loaded)**
c-met (C57)	2.17
c-met (C57 + L-NAME)	0.42
c-met (*mdx*)	1.4
c-met (*mdx* + L-NAME)	0.62
myf5 (C57)	0.81
MyoD (C57)	0.67
myostatin (C57)	0.95

Table 2. Ratio of ½ hr stretched to unstretched mRNA levels of c-met, myf, MyoD and myostatin in normal and *mdx* EDL. Animal strain and treatment are listed in brackets.

Figure 1. The role of NO and HGF in *mdx* satellite cell activation

Fibers were stretched for ½ hr in the presence of BrdU and one of L-NAME, L-Arg, HGF or anti-HGF. Treatment with a NOS inhibitor (L-NAME) or a NOS substrate (L-Arg) had no effect under unstretched conditions, suggesting that NOS is unable to impact activation without a mechanical stimulation. This is supported by the observation that L-Arg treatment decreases activation under cyclical stretching. Treatment of HGF in the presence of stretch did not change activation levels, contrary to what is observed in normal fiber cultures. Treatment with anti-HGF resulted in decreased activation without stretch, suggesting that the HGF-c-met signaling cascade is present and working in *mdx* satellite cells. * indicates a significant difference from unstretched untreated controls.

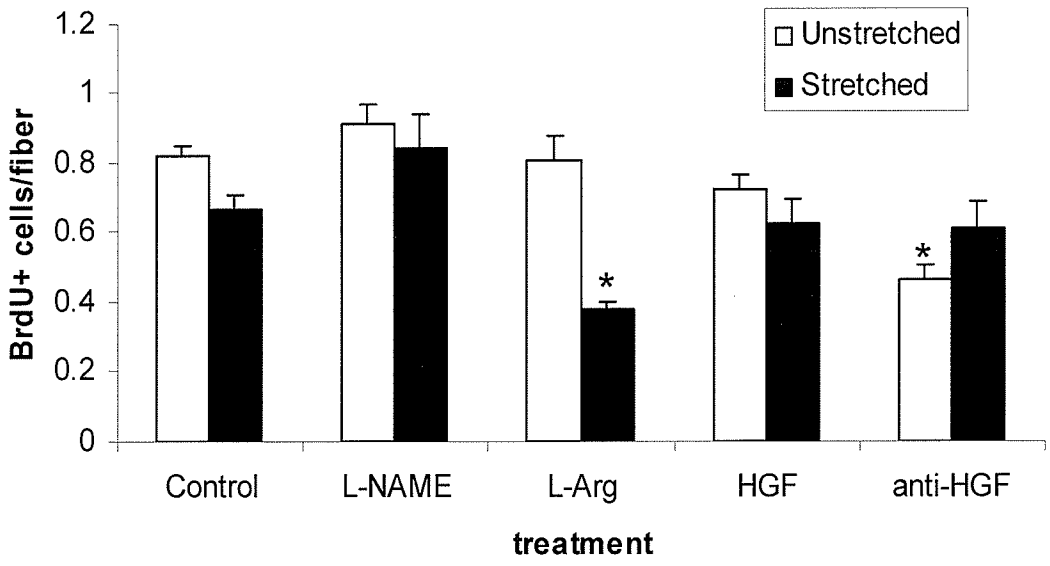

1.2
1
0.8
0.6
0.4
0.2
0
BrdU+ cells/fiber
Unstretched
Stretched
*
*
Control
L-NAME
L-Arg
HGF
anti-HGF
treatment

Figure 1.

Figure 2. Satellite cell activation in young and mature normal and *mdx* animals

Young (1½ -2 wks) and mature (3½ - 4½ months) animals were injected with ^{3}H and hindlimb muscle harvested for analysis of resting DNA synthesis. A significant difference was observed between young and mature *mdx* animals ($p<0.05$), suggesting that the growth process is distinct from the regeneration process, and that the loss of NOS-1μ is only detrimental when satellite cells are required to enter into and exit from quiescence. N= 2-3 samples per group from one experiment.

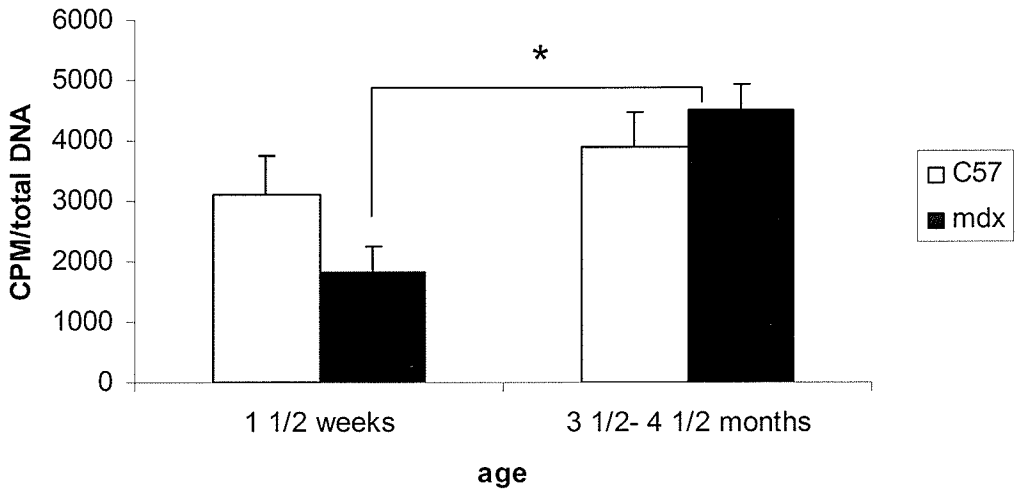

6000
5000
4000
3000
2000
1000
0
CPM/total DNA
*
C57
mdx
1 1/2 weeks
3 1/2- 4 1/2 months
age

Figure 2.

Figure 3. C-met expression in normal animal and dystrophic muscle (taken from (111))

EDLs were isolated and pinned, after which they were either stretched or not in the presence or absence of L-NAME. RNA was isolated from muscles immediately after the 30-min treatment period, and the relative expression of c-met was quantified by RNAse protection assay. C-met gene expression was higher in *mdx* muscle compared to normal muscle. C-met expression increased with stretch or with L-NAME treatment in both types of muscle. C-met levels were reduced by stretching in the presence of L-NAME, indicating that the stretch-induced increase in c-met expression was NO-dependent.

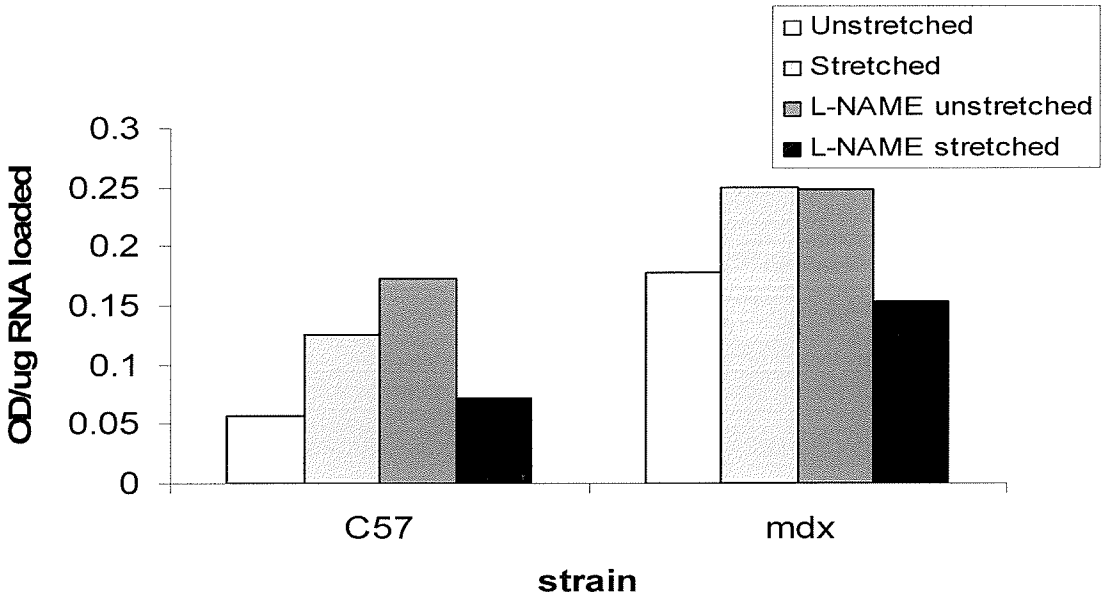

Unstretched
Stretched
L-NAME unstretched
L-NAME stretched
OD/ug RNA loaded
0.3
0.25
0.2
0.15
0.1
0.05
0
C57
mdx
strain

Figure 3.

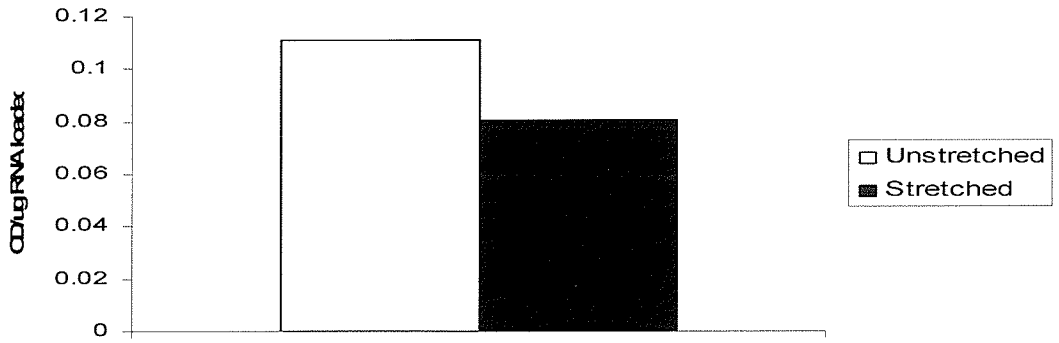
Figure 4. Whole muscle gene expression in normal animals

EDL's were isolated and pinned, after which they were stretched for ½ hr and then immediately flash frozen in liquid nitrogen. RNA was extracted according to (312) and mRNA was detected by Northern blotting. N=1 sample per group.

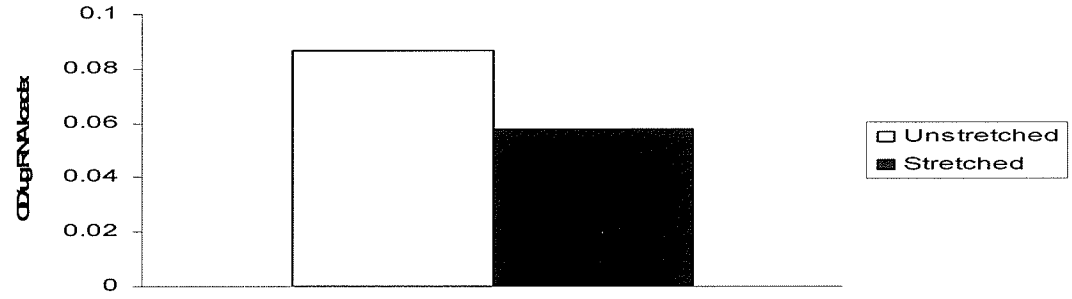
Expression of 2 myogenic genes myf5 (**A.**) and MyoD (**B.**) decreased after ½ hr of stretch. It is not expected that expression would increase after ½ hr, as neither gene is an immediate early gene. The response over time after stretch still needs to be determined.

Expression of the potential quiescence factor myostatin was reduced slightly after ½ hr of stretching (**C.**). Further decreases would be anticipated to occur over time after the stretch stimulus, however these experiments have not been completed.

A.


0.12
0.1
0.08
0.06
0.04
0.02
0
Unstretched
Stretched

B.


0.1
0.08
0.06
0.04
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0
Unstretched
Stretched

C.

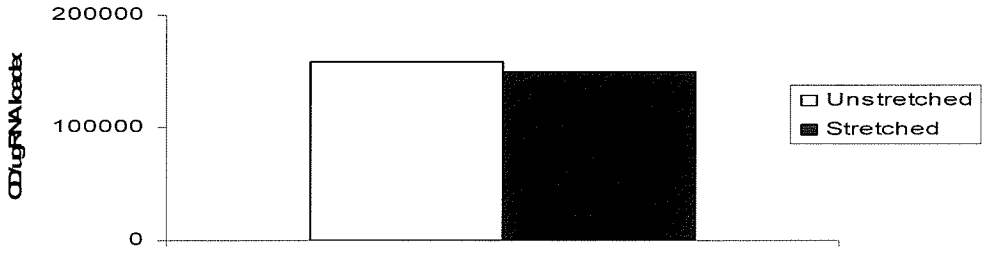
200000
100000
0
Unstretched
Stretched

Figure 4.

Figure 5. Alternate pathways to satellite cell activation

Whole EDL cultures were either untreated (controls) or treated with one of 6 treatments: AlF, Wortmannin, Genistein, Geldanamycin, A231887 or Nifedipine. Muscle cultures were then stretched or not and then treated with ^{3}H thymidine and cultured for 24 hrs. Muscles were frozen, and then DNA isolated for scintilllation counts and total DNA counts. Results are plotted as CPM/ug of DNA. Treatment with either AlF or Wortmannin (**A.**) increases activation without stretching, and decreases activation with stretching compared to unstretched values. Genistein and Geldanamycin treatment do not appear to influence activation without stimluation (**B.**). Increased activation with stretching, especially with Genistein treated muscles, may be due to initiation of apoptosis, which would explain the presence of "crazy" fibers (inset, i.). The Ca^{2+} ionophore A23187 increases activation without stretch, while the L-type Ca^{2+} blocker, Nifedipine, increased activation with stretching (**C.**). N=1 muscle per treatment, all from 1 experiment.

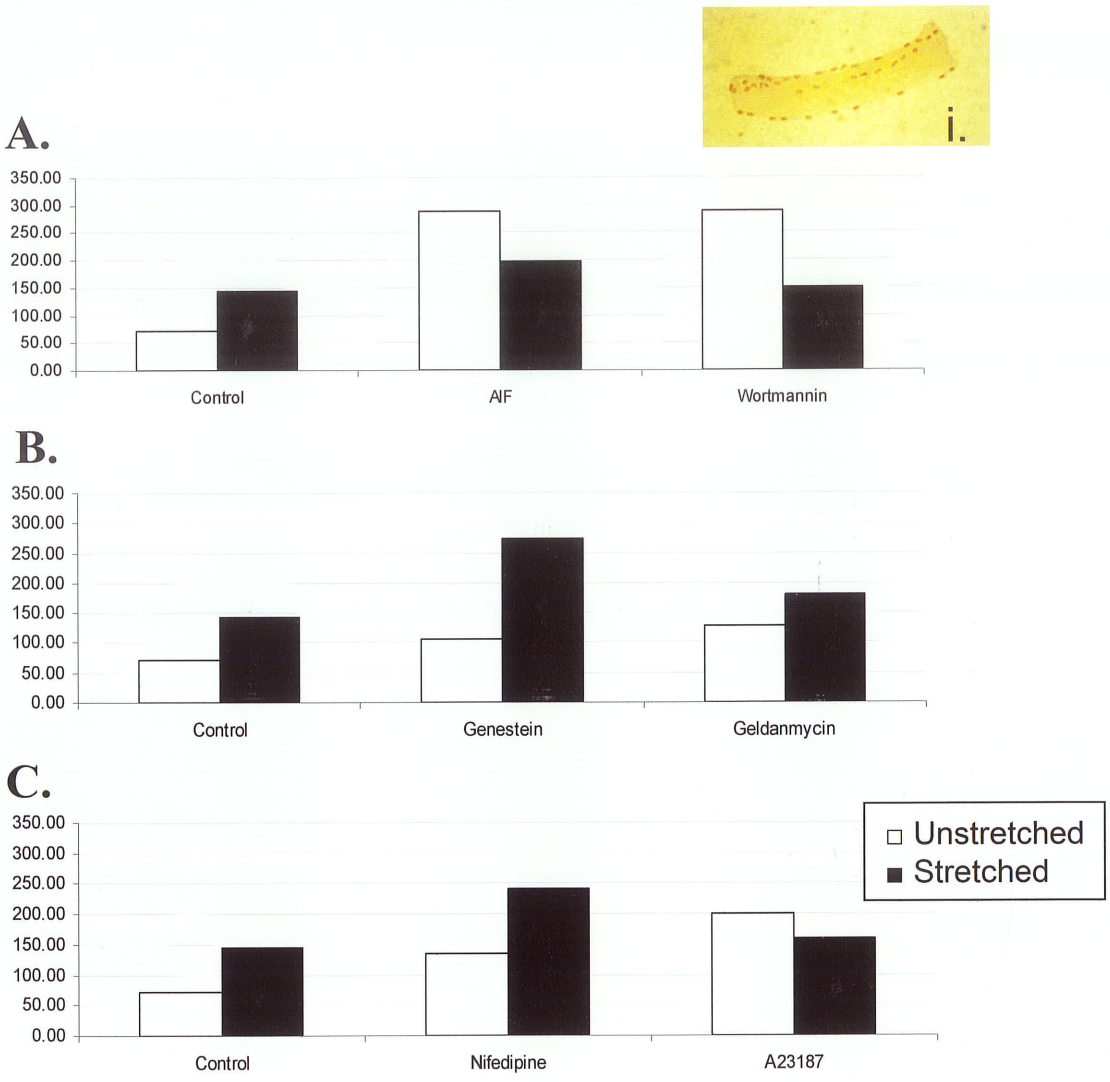
i.
A.
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Control
AIF
Wortmannin
B.
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Control
Genestein
Geldanmycin
C.
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Control
Nifedipine
A23187
Unstretched
Stretched

Figure 5.

Chapter 8.
Discussion

The results presented in this thesis will be discussed as follows. First a general overview of the data will be presented, followed by an evaluation of the weaknesses and strengths of the work. The results will then be discussed in the context of already published literature, and the implications of the research will be outlined. Finally, future directions for this research will be discussed.

1. General Summary

The work presented in this thesis was undertaken to determine the roles of NO and HGF in the activation of skeletal muscle satellite cells. The single fiber model was chosen for its ability to mimic *in vivo* conditions by maintaining satellite cells in their characteristic anatomic position in a quiescent state. We also developed a whole muscle culture model (33), which is one step closer to *in vivo* conditions in order to study RNA expression in the context of the whole tissue.

Chapter 2 experiments addressed the need for a model through which we would be able to examine the relative roles of NO and HGF in satellite cell activation under conditions that could be considered physiological. In other words, a model that was able to activate satellite cells without exogenous treatment. Mechanical stimulation is known to activate satellite cells (see Chapter 1 Section 5.3.), but prior to these experiments it was not known whether stretch could activate satellite cells on single fibers, or what duration of stretch was required for satellite cell activation. Using the FlexCell system we were able to show that satellite cells on single fibers are activated by ½ hr and 2 hrs of stretching. We hypothesized

that these 2 peaks in activation were due to the presence of 2 populations of satellite cells on single muscle fibers. Findings were interpreted to indicate that cyclical stretching of single fibers was a relevant physiological model through which early stages of satellite cell activation could be studied.

Chapter 3 experiments were designed to address questions within the literature about the genes that are expressed in satellite cells on single fibers during activation and/or quiescence. We examined the methods by which single fibers are typically isolated, to investigate whether different procedures had the potential to affect the gene expression in satellite cells that were understood to be quiescent upon fiber isolation. Two basic variations of the method are used by researchers: one involves gentle shaking during collagenase digestion, the other does not. Results from experiments comparing the 2 methods showed that even gentle shaking during collagenase digestion resulted in satellite cell activation. These results are interpreted to mean that appropriate measures are required during fiber isolation in order to maintain satellite cell quiescence for experiments where the early stages of activation are to be studied.

Chapter 4 addressed the central objective of the thesis: to characterize the roles of NO and HGF in stretch-activation. Results indicated that NO is required for satellite cell activation and for quiescence, and that during NOS inhibition the requirement for NO can be overcome after 2 hrs of stretch with exogenous HGF. We also showed that stretching in the presence of HGF decreases satellite cell activation at ½ hr and 2 hrs, likely due to desensitization of the c-met receptor. Results from *mdx* and NOS-1(-/-) time course experiments led us to hypothesize that the presence of even low levels of NOS-1μ do not

result in sufficient upregulation of other NOS isoforms to compensate for the altered balance between activation and quiescence in those muscles, and that the complete absence of NOS-1 is in fact better for muscle satellite cells than low levels of NOS-1. Time course data also suggested that there is a population of satellite cells that is absent from *mdx* single fibers, in agreement with the previous literature (47).

Chapter 5 is the first report of the visualization of an NO release sequence produced by stretching skeletal muscle. Experiments were designed to observe differences between normal and dystrophic satellite cell cultures. Results indicated that there are 3 aspects of NO production that are aberrant in dystrophic (*mdx*) satellite cells after stretching: the amount of NO produced, the regulation of the enzyme activity over time in releasing NO after stretch, and the character of the pulsatile delivery of NO in the sarcoplasm.

In Chapter 6, experiments were designed to re-examine the expression profile of c-met during activation. Data from our laboratory showed that c-met expression increased after ½ hr of stretch in normal and *mdx* whole muscle cultures (111). This, along with evidence for multiple satellite cell populations on single muscle fibers (reviewed in (33) and interpreted from the published and submitted reports from this thesis work (82), led us to believe that c-met was not expressed uniformly and homogeneously in all satellite cells under quiescent conditions. For this reason, studies of the distribution of c-met-expressing satellite cells on normal and *mdx* single fibers were undertaken to see if the distribution of c-met expressing cells changed with stretch. Here we showed that the number of cells that express c-met on normal single fibers increased with stretching, while it did not change on *mdx* single fibers. In view of the previous report (111) that c-met is upregulated by stretching in

both normal and *mdx* muscle tissue, these data are interpreted to mean that the level of c-met expression per satellite cell increases during activation in both normal and *mdx* muscle during stretching, and also that the number of c-met-expressing cells increases in normal muscle during stretching. Results of experiments in which muscles were exposed to cycloheximide (with and without stretching) also indicated that the increase in c-met expression after ½ hr of stretch was due to c-met acting as a delayed-early gene in skeletal muscle satellite cells.

Finally, in Chapter 7, results from experiments on young and older *mdx* animals showed that the role of NOS in development was different than its role in regeneration. Results from studies on *mdx* mouse single muscle fibers in culture suggested that the level of physical activity in an individual with muscular dystrophy should be considered when designing drug therapies for dystrophy. Here, L-Arg was effective in reducing satellite cell activation when single fibers were stretched, but this result was not found in fibers that were left unstretched. Anti-HGF on the other hand, was able to reduce activation under unstretched conditions as anticipated from the literature, and did not affect activation when fibers are stretched. Alternate pathways to activation are likely to exist, based on evidence of relatively normal regeneration in NOS-1(-/-) animals, however experiments presented in Chapter 7 were not significant, likely due to the experimental design.

2. Limitations and strengths

2.1. Limitations

One limitation of the studies presented here is that measurements of BrdU and ^{3}H-thymidine incorporation are not measures of activation *per se*, but rather of DNA synthesis subsequent to satellite cell entry into G1, and prior to cell proliferation. If only some of the satellite cells that are activated in an experiment actually enter into S phase during the period of incubation (on fibers or muscles in culture), then the method will miss some of the satellite cells that are indeed being activated and which exit G0. To address this issue, it would be essential, although technically more difficult, to use an earlier marker of activation, such as increases in c-met expression (RNA or protein) or co-localization of HGF with c-met. However, given the requirement to process a very large number of experiments and culture plates for activation, and the perspective that entry to S-phase is indeed activation to cycling (with a very long G1 phase required to push satellite cells resistant to activation outside the 24 hour period of exposure to the labelling nucleotides), we conclude that the BrdU and ^{3}H-thymidine incorporations are useful as data that represents most of the satellite cells that are activated during the culture period.

Another issue that arose from these experiments is that an increase in c-met mRNA production may not be indicative (or rigorously correlated) of an increase in the level of activation in satellite cells, despite the previous reports that c-met mRNA levels are correlated with c-met protein levels in glioblastoma cells (83). A more definitive measure of c-met activity would be to measure c-met receptor phosphorylation in these experiments, and relate changes in phosphorylation to changes in activation or satellite cell activity. If phosphorylation was observed to increase and that increase was correlated with increases in c-met mRNA in skeletal muscle, one could state more definitively that c-met-HGF signaling

was directly involved in the observed increase in satellite cell activation.

Finally, alternate pathways to activation are very likely to exist, given that the activation response is critical to growth as well as regeneration. However, here, the results of the experiments on alternate signaling pathways that involved studies of G-proteins, tyrosine kinases and Ca^{2+} channels were not reproducible. It is possible that the experimental design used in this thesis was not rigorous or specific enough to identify these pathways clearly. For example, although we confirmed that BrdU was able to diffuse into the core of an FDB muscle belly in culture conditions, ^{3}H-thymidine may diffuse less easily and therefore the labelling of new DNA synthesis would have missed a significant (and unknown) proportion of the satellite cells that could have been activated by the stimuli under examination. Additionally, although the concentrations or the inhibitors or stimulators used to study signaling pathways were tailored to fiber and muscle cultures from the literature reports in tissue culture experiments, the levels may not have been effective in changing the activation of satellite cells by the assay that was employed. In the future, earlier markers of activation such as c-met phosphorylation, activation of c-fos, c-jun or PKC, measurements of adenylate cyclase pathway byproducts, and other immediate/early genetic and biochemical events may be more effective in assessing the potential cascade from quiescence to activation through pathways other that the NO-HGF cascasde. It is also conceivable in normal muscle that it is not possible to identify alternate pathways when NO and HGF are readily available in the muscles. It would therefore be important to try inducing the use of alternate pathways in the presence of L-NAME and/or anti-HGF in normal muscle, or by testing for those pathways in *mdx* or NOS-1(-/-) muscles instead, especially since the latter two strains show

regeneration of muscle tissue after an injury (and in muscular dystrophy) despite the downregulation or absence of NOS-I. This may clarify the understanding of the role and impact of G-protein, tyrosine kinase and ion channel signaling pathways on satellite cell activation.

2.2 Strengths

The major strength of the work presented within this thesis is the new information regarding the control of satellite cell activation and quiescence. We have been able to identify a bimodal response of satellite cells to passive stretch, and that this activation occurs through a NO and/or HGF-dependent AND independent pathways. We have also shown that NO is involved in the regulation of both quiescence and activation, and that NO is released in real time in a pulsatile manner from myotubes. This new information will be important for the future development of therapies and for ongoing basic science investigations into satellite cell activation and muscle regeneration.

The major technical strength of the body of research presented in this thesis was our ability to maintain satellite cells in a quiescent state after the isolation of single fibers. This single feature, initially only a methodological novelty in comparison to other literature on single fibers, allowed us to examine the regulation of very early stages in activation. It also revealed subtle and previously unidentified changes in activation (i.e. the significant increase in activation after ½ hr of stretch) and gene expression (the change in the distribution of c-met+ satellite cells on normal single fibers) due to stimulation. The impact of these

observations is significant, given that it is paramount in experimental biology to have a control condition that is stable and changed only by the interventions that are designed into the experiments. Other indices of activation other than BrdU incorporation, such as immunostaining for the expression of a muscle regulatory gene such as myf5 or proliferating cell nuclear antigen, would also reveal activation from a prior stimulus. While they may provide a read-out of activation that is temporally closer to a "real-time" measure, their use prevents confirmation of stable quiescence over at least one cell cycle, as is possible for cultures exposed to BrdU, and interpretation of such observations may be subject to various definitions of the quiescent state (55;92). Experiments using BrdU incorporation into DNA of satellite cells on fibers in culture over 24 hours were therefore sensitive to changes in cycling behaviour due to earlier activation, and were established as specific to the interventions under examination.

Another major strength of this study was the model that we developed to examine the signaling cascades involved in satellite cell activation. The single fiber model mimics *in vivo* conditions to a greater extent than the isolated satellite cell model, without most of the non-muscle influences seen *in vivo*. The use of passive mechanical stretch that is within the physiological range (approximately 10%) to activate satellite cells in a manner representative of exercise, allowed greater confidence for the conclusion that NO and HGF are involved in satellite cell activation *in vivo* under conditions that a normal skeletal muscle would typically experience.

3. Stretch-activation of satellite cells on single fibers

Early in the time course experiments, we proposed that after a certain duration of stretch satellite cells on single fibers would activate, and that the level of activation would remain elevated for any period after that initial activating stimulus. Surprisingly, this idea was not supported by the observations. Results demonstrated that any duration of stretch other than ½ hr or 2 hr (out to 3 hrs) was followed by a return to activation levels seen in unstretched controls (82). There are 3 possible explanations for this increase and subsequent decrease in satellite cell activation.

The first is that satellite cells may be migrating away from the fiber between ½ hr and 1 hr (and 2 and 3 hrs) of stretch. Since we counted only those satellite cells that were attached to single fibers in the analysis, it is possible that counting missed assessing a population of cells that was no longer attached to fibers. However, there were very few single cells located on the plates rather than on fibers (in comparison to those reported by Pilipowicz and Anderson (112) in the initial report from this laboratory), and that number did not appear to change over any time point (Wozniak and Anderson, unpublished data). For this reason, we do not believe that satellite cell migration off of fibers can explain the change in activation seen after 1-1½ and 3 hrs of stretch.

One alternate explanation is that activation is not an all-or-nothing process. It is possible that satellite cells were activated at 1 hr of stretch, and that the expression of signaling molecules required for the transition of satellite cells from G1 into S phase was not optimal or sufficient to maintain the process leading to activation and cycling, such that satellite cells were not able to incorporate BrdU, as they did not enter into S-phase. In other words, cells were not "allowed" past the "check-point" between G1 and S phase. These

check-points are controlled by the binding of cyclins and cdks (25). If the cyclin and cdk for a specific checkpoint do not bind, or if binding is abnormal, cells are not able to move from one phase to another, and are in effect halted in one phase (25). Since BrdU is only incorporated into cells that have entered into S phase and initiated DNA synthesis, it is not possible to resolve the time points between withdrawal from G0 (quiescence) and entry into S phase. Thus the experiments can only conclude that the cells did not begin DNA synthesis. This idea could be tested in an experiment that assessed both BrdU incorporation, as well as much earlier markers of cell cycle progression, such as cdk and cyclin levels. If the levels of BrdU incorporation did not correlate with changes observed in cyclin and cdk levels, we could conclude that while cells were exiting from quiescence and entering into G1, they were not able to enter in S phase of the cell cycle. While this is a technically interesting question, the fact remains that only those cells that enter into S phase are those that contribute to new muscle fiber formation, and are arguably the only cells that need to be considered in the context of the experiments presented within this thesis.

The final explanation is that there is more than one satellite cell population present on fibers in normal muscle, and that each population has distinctive activation characteristics, including a typical threshold duration of stretching required to produce activation. This possibility has quite extensive support within the literature, where it has been suggested by many investigators that there is a population of cells on single muscle fibers that are more committed to the myogenic lineage, and another one (or more) that are more stem-like in nature (see Chapter 1 Section 3.2, 3.3). The time course data from *mdx* and NOS-1(-/-) fiber culture experiments also suggests that there are multiple populations of satellite cells, and

that one population of these satellite cells is absent on *mdx* fibers, possibly having been completely exhausted by the ongoing need to regenerate muscle following dystrophic injury. NOS-1(-/-) fibers show satellite cell *de*activation after ½ hr of stretch, and that persisted until 1 ½ hr of stretching, prior to a second reduction at 3 hrs of stretching. These 2 'dips' in activation are reminiscent of the 2 peaks of activation in normal fiber cultures, albeit slightly delayed by comparison to the normal muscle fiber time course experiments. The *mdx* fiber cultures however only show stretch-*de*activation after ½ hr of stretch.

Due to the support within the literature, the time course experiments, and other results presented in this thesis (differential activation requirements for the first and second activation peaks, and changes in c-met expression with stretch), this third explanation appears to be the most likely reason for the 2 peaks of activation seen in the stretch-activation time course.

The presence of only one population of satellite cells on *mdx* single fibers could indicate that one population was lost early in the dystrophic process, possibly due to the heavy demand for new muscle cells during the initial degeneration-regeneration cycles. It would be interesting to determine the time course of disappearance, since this would aid in determining when therapeutic treatments to decrease satellite cell activation, and perhaps rescue that satellite cell population, would be most effective in restoring the normal threshold for activation. In order to conduct such an experiment, it would first be necessary to determine whether the 2 populations of satellite cells are even present on dystrophic muscle fibers before the onset of the dystrophic process in *mdx* mice (before 5-6 weeks of age). It is quite possible that this population never exists in post-natal *mdx* dystrophic muscle, since the dystrophin mutation may affect cell migration or satellite cell specification in some

manner that relates to the formation of new fibers during development . If two populations are indeed present on post-natal muscle fibers prior to the onset of degeneration in muscular dystrophy, it may be possible to identify the second population using time course stretch experiments, by specifying the experimental design such that observations will reveal 2 decreases in satellite cell activation during stretching. At the same time, it would be useful to use c-met to mark the two populations, or changes in the populations, with and without stretch, since the expression of the c-met receptor appears to identify multiple distinct populations in normal muscle (see Section 7. below). It would also be very useful to include staining for pax7 protein, since that gene specifies satellite cells, and should identify the satellite cells that may not express c-met at the time of isolation.

4. The role of NO in activation and quiescence

The dependence of satellite cell activation on NO release was elucidated using experiments on normal, *mdx* and NOS-1(-/-) fiber cultures. Normal single fibers were treated with the NOS inhibitor L-NAME and either stretched or not for ½ hr and 2 hrs. The time course of the activation response in *mdx* and NOS-1(-/-) single fiber cultures was used as a means to examine how satellite cells respond to stretch when NOS-1 is either low or absent. While treatment of the cultures of *mdx* single fibers with a NOS inhibitor would have abrogated NOS activity, and would have served as a general confirmation of NO-dependence, we chose to use cultures of NOS-1(-/-) fibers for this comparison. The rationale for this selection was that NOS-1 downregulation in *mdx* muscle is not fully understood and

may involve variable upregulation of NOS-2 and/or NOS-3 in all or some sub-set of muscles and fibers. For this reason, satellite cells on NOS-1(-/-) fibers served as a "genetic negative-control" condition for comparison to satellite cells on normal single fibers that were examined during the non-specific NOS inhibition with L-NAME.

Together the results of the experiments on inhibition of activation using normal single fibers treated with L-NAME, along with the results of the time course studies on *mdx* and NOS-1(-/-) fiber cultures show that both satellite cell quiescence and activation are NO dependent. Stretching normal single fiber cultures for ½ hr or 2 hrs in the presence of L-NAME resulted in decreased activation compared to untreated, stretched controls. These data are interpreted to mean that normal stretch-activation is NO-dependent. The high levels of activation observed in unstretched *mdx* and NOS-1(-/-) cultures, and in unstretched L-NAME treated normal fiber cultures, supports the notion that NO is required for satellite cell quiescence. The observation that the level of activation was decreased with stretching in both *mdx* and NOS-1(-/-) fibers, suggests the possibility that NO production is increased due to upregulation of NOS during stretch (291), and that NOS manipulation in conjunction with mechanical stimulation could regain a degree of control over satellite cell hyperactivation. However, we have not experimentally confirmed that the decrease in activation was NO-dependent. It is known that activated satellite cells in *mdx* muscle express NOS-1μ despite the downregulation of NOS-1 in the *mdx* muscle fibers, and activated NOS-1(-/-) satellite cells have an as yet unidentified NOS activity (15). However, since NOS-1(-/-) fibers do not have any NOS-1, the NOS activity that is observed by enzyme histochemistry would have to be taken over by compensatory activity by one of the other NOS isoforms, likely NOS-3.

Interestingly, experiments using NOS-1(-/-) single fibers have revealed that upregulation of other NOS isoforms likely occurs in NOS-1(-/-) satellite cells, not in *mdx* satellite cells, although the original rationale for using NOS-1(-/-) fibers in these experiments was to control for this possibility in *mdx* satellite cells.

NO release from normal satellite cells in culture during stretch was visualized using the fluorescent compound DAF. This was the first direct evidence that NO is released due to stretching, and provided insight into the exquisite control over NOS activity over the relatively short time frame of 60 seconds following stretch. Experiments with *mdx* satellite cells in culture indicate that NO release was generally stable, albeit much reduced, compared to normal muscle in culture, and slightly increased during the period of time after stretching, as visualized by DAF (see Chapter 5 Figure 3B). This was a different response than that observed for cultures of normal satellite cells and myotubes after stretching, where there is an initial increase in NO and a subsequent decrease over time after stretch (see Chapter 5 Figure 3B). We postulate in Chapter 5 that the dysregulation of the enzyme over time after stretch may be partially responsible for satellite cell hyperactivity in *mdx* muscle. However, an alternate explanation would be that this response by *mdx* satellite cells of a sustained increase of NO release after stretching could actually counteract, at least in part, the hyperactivation of the satellite cells and help to restore or regain the normal regulation of activation by increasing NO concentrations. Perhaps this is a backup mechanism established by skeletal muscle, that partly compensates for the hyperactivity of satellite cells in the dystrophic process. If this is the case, this process would help to account for decreases in satellite cell activation with stretch in *mdx* single fiber cultures, due to sustained levels of NO

which could aid in returning satellite cells to the more quiescent state observed after ½ hr of stretch. Experiments on activation and inhibition of satellite cells on *mdx* single fibers (like those described above for normal fiber cultures) indicate that the control of satellite cell activation can be restored to more "normal" levels by increasing NO levels in the context of mechanical stimulation, since stretching in the presence of L-Arg is able to decrease activation significantly.

On a purely speculative note, it would be interesting to determine whether activated satellite cells in DMD patients express NOS-1μ. Since the expression of NOS-1μ in activated satellite cells of *mdx* muscle is a putative target of treatment to restore the regulation of satellite cell activation and quiescence and does help promote the effectiveness of muscle regeneration from dystrophic damage, it could be hypothesized that NOS-1μ is absent from DMD satellite cells. If this were the case, it may partly account for the lesser severity of dystrophy in *mdx* animals compared to human DMD, especially during initial stages of the disease.

It is important to note that the increased activation that was observed in muscle from *mdx* and NOS-1(-/-) animals is not definitive evidence that the loss of NOS-1μ is responsible for hyperactivation of satellite cells. However, taken together with the results from experiments where NOS inhibition with L-NAME caused an increase in satellite cell activation on unstretched normal fibers, the observations are highly suggestive of this possibility. To test this hypothesis specifically, it would be necessary to disrupt NOS-1μ from the sarcolemma of normal muscle fibers and see whether that induces hyperactivation under the conditions which normally support satellite cell quiescence. While the studies of

NOS and HGF inhibition in Chapter 4 have partly addressed this hypothesis, inhibitors are not specific and may not completely block the activity of the enzyme in question. Therefore, future studies on this subject should employ RNAi, transfection with a dominant-negative sequence, site-directed mutagenesis experiments or other comparable molecular biology techniques to test the hypothesis.

Taken together, these data led us to propose a hypothetical NO dose-response curve, which presents a model of satellite cell activation as a function of NO concentration (Chapter 4). The model suggests that a particular concentration of NO, which still has to be determined, maintains satellite cells as quiescent. At concentrations either higher or lower than this set point, activation increases. This model attempts to account for all of the experimental findings presented in this thesis. Validation of the model would require experiments to be repeated such that real-time levels of NO could be measured (such as using NO-entrapment methods and electrospin resonance spectroscopy techniques) and related directly to the levels of activation observed in the same muscles or fibers.

The results of this research suggest that NOS-1μ in muscle fibers has a dual role in satellite cell activation, namely to maintain quiescence under normal conditions, and activate satellite cells after stimulation. We originally hypothesized that if satellite cells were observed to be hyperactivated in *mdx* mouse muscle before the onset of dystrophic damage (as they are during the dystrophic process), there would be evidence that NO release from fibers was an absolute requirement for maintaining satellite cell quiescence. This evidence would effectively suggest that hyperactivation was due to NOS-1μ deficiency rather than the dystrophic processes extant in the muscle tissues. However, as presented in Chapter 7, this

hyperactivation was not observed in young *mdx* animals during growth and development prior to 3 weeks-of-age, which is the onset period of dystrophy in the limb muscles. The subsequent reconsideration of the nature of the growth and regeneration processes has led us to formulate a new hypothesis that pathological hyperactivation of satellite cells will only be observed in mature muscles, since those muscles require satellite cells to be responsive to stimuli and therefore able to enter into or exit quiescence, both of which require NO (Chapter 4). By comparison, during the period of active growth in young animals, satellite cells display repetitive normal cycling and the cells do not transition from G0 to G1 or G1 to G0, and therefore are not subject to changes in the level of NOS-1μ expression or activity. Experiments to test this notion include a more detailed study of satellite cell activation in dystrophic muscle over varying ages. In order to definitively say that the loss of NOS-1μ does not negatively affect muscle growth during development, the level of satellite cell activation in both normal and *mdx* muscle would need to be measured over time, especially during the ages of 2-3 weeks, as the dystrophic process is initiated during the 3rd week. If NOS-1μ is only absolutely required after satellite cells have become quiescent precursors, we would expect to see similar levels of activation in normal and *mdx* muscle before the onset of the dystrophic process. After the disease onset, satellite cell activation in *mdx* muscle should increase, due to increased demand for new muscle fibers, as well the inability of satellite cells to enter into a quiescent state due to the lack of NOS-1μ. In normal muscle we would expect to see a decrease in satellite cell activation as growth is completed and satellite cells withdraw from the cell cycle and take up their resident position on single fibers.

Overall, results presented in this thesis begin to paint a picture of the regulation of

satellite cell activation that is far from simple. The specific requirements for NOS activity and appropriate localization in fibers, and NO concentration and pulsatile release in regulating normal satellite cell activation and quiescence all appear to be critical, starting from the time when satellite cells are first established in their position as quiescent precursors. Compared to NOS-1 in fibers, the changes in the regulation of NOS-1 activity and NO production in satellite cells of dystrophic muscle may actually aid in controlling satellite cell activation to some degree. The partial attenuation of hyperactivation might partly offset the otherwise continuous cycling of satellite cells that rapidly leads to senescence. There also appear to be changes in the expression of other NOS isoforms as a response to the presence or absence of NOS-1; complete loss of NOS-1 appears to increase the ability to control hyperactivation of satellite cells, and could explain why the absence of NOS-1 is less detrimental to skeletal muscle than a downregulation of NOS-1 from fibers in dystrophic muscle. This may suggest that the alternate isoforms (NOS-2 and NOS-3) are subject to upregulation only when NOS-1 is completely absent or inactive. This is a testable hypothesis. Finally, the nature of NO in regulating both the quiescence and activation of satellite cells, which we propose is related to concentration gradients and the mechanisms and character of NO delivery, points to the existence of multiple checks and balances in normal muscle which combine to regulate, and finely tune, an extremely important process.

5. HGF and satellite cell activation

The role of HGF in satellite cell activation was examined using experiments in which

single fibers were exposed to exogenous HGF. In the absence of stretching, treatment with HGF resulted in high levels of activation (Chapter 4), as previously described (112). Surprisingly, parallel experiments in which fibers were stretched for either ½ hr or 2 hrs in the presence of HGF resulted in levels of activation that were significantly below values observed for untreated fiber cultures that were stretched. We proposed that this may be due to desensitization of the c-met receptor during exposure to stretching in the presence of additional HGF, each of which would be expected to upregulate activation and c-met receptor expression. This may result from the stretch-induced release of HGF from the extracellular matrix, shown by work from Tatsumi et al. (141). The dose response curve showing activation as a function of HGF concentration, and differences in that function with or without stretch, supports this hypothesis. The concentrations of HGF ranged from 0-30 ng/ml and showed increased activation with increasing concentrations in unstretched fiber cultures. Stretching in the presence of HGF, however, decreased activation with increasing HGF concentrations, suggesting c-met desensitization when stretching in the presence of high enough concentrations of HGF (Chapter 4). Studies done in stretched isolated satellite cells cultures also show desensitization, although at higher concentrations (Tatsumi, personal communication). These results indicate that HGF is involved in normal stretch-activation of satellite cells on single fibers, and that high concentrations of HGF can overwhelm the c-met receptor and desensitize it.

The results presented in this thesis show that both NO and HGF play a role in stretch-activation of normal satellite cells on fibers. One may reasonably ask whether the two signals are independent (parallel), or if they interact in the cascade of events leading to activation

(i.e., NO preceding HGF), as is the case for stretched satellite cells in culture (141). Experiments were designed to examine this question by affecting both signals together, through inhibition of NOS (with L-NAME) at the same time as exogenous HGF was added to the medium. From the experiments showing that activation increased in unstretched cultures treated with either L-NAME or HGF alone, we expected that a combined treatment with L-NAME and HGF together would also stimulate increased activation in unstretched cultures. This did not occur. As well, stretching for ½ hr did not affect activation in the presence of the combined treatment with L-NAME and HGF (compared to unstretched, untreated control cultures). However, after 2 hrs of stretch during the combined treatment, activation was increased over untreated stretched cultures, suggesting that the exogenous HGF was able to overcome NOS inhibition in the presence of a prolonged stretch stimulus. These results are interpreted to support the notion of two populations of satellite cells, one of which responds to shorter periods of mechanical stretching, while the other responds only after longer periods of stretching. This is consistent with the original and highly reproducible observation of 2 peaks of activation in the stretch-activation time course (Chapter 2). Together these findings suggest that activation of satellite cells by ½ hr of stretching has an absolute requirement for NO signaling, and that when fibers are stretched for longer periods (e.g., 2 hrs under the current experimental conditions), the requirement for NO is lost if there is significant amounts of HGF available to bind c-met and activate satellite cells. These "inhibition experiments" were therefore valuable in providing clues to the normal signaling cascade in satellite cell activation.

Parallel inhibition experiments using *mdx* fiber cultures confirmed that HGF-c-met

signaling is also involved in activation on dystrophic muscle fibers, as treatment with anti-HGF was able to decrease activation in unstretched *mdx* fiber cultures. However the kinetics of activation are not normal for *mdx* satellite cells. In the absence of stretch, treatment with anti-HGF produced a significant decrease in satellite cell activation (Chapter 7), and this blocking effect is interpreted to indicate that the already highly activated satellite cells were activated through an HGF-c-met pathway. While the character of the dose-response to HGF by satellite cells on dystrophic fibers is not known, the observed changes in *mdx* satellite cell activation with increased NO (with L-Arg treatment) or with HGF manipulation in *mdx* single fiber cultures each suggest that the same two signaling pathways are at work, albeit with different regulation properties due to the loss of NOS-1μ from dystrophic muscle.

When considering the potential real-world application of these findings, one major theme stands out. There are potential treatments that would decrease satellite cell activation in *mdx* animals, and potentially DMD patients, and they could be designed for 'titration', or tailored use based on the level of physical activity in an individual. Since NOS manipulation affects activation over the shorter periods of mechanical stretching activity, it seems possible that drugs designed to increase NOS, or NO concentration at the satellite cell could be used during daily activity. However, NOS manipulation did not appear to impact satellite cell activation on *mdx* fibers in the absence of stretch, and might be predicted to have little benefit for inactive muscle or subjects. By comparison, during these times of inactivity, one could speculate that it might be beneficial to inhibit the HGF-c-met signaling pathway (as seen here with anti-HGF). These ideas remain to be tested in *mdx* mice *in vivo*.

6. Other activation pathways

While the manipulation of NOS and HGF may be able to regulate or modify the level or time course of satellite cell activation in dystrophic muscle, it is very likely that there are additional pathways for satellite cell activation, since NO levels are low and skeletal muscle in dystrophic mice, and dystrophic muscles do demonstrate remarkably effective regeneration in young and adult animals (reviewed in (33)). Although numerous experiments were conducted, the results showed no significant effects of studies that attempted to interfere with the putative process of activation through alternative pathways in normal muscle. However, there were some interesting trends in the data. G-proteins appear to be working by influencing NOS, based on the observed changes in activation with Wortmannin treatment (Chapter 7 Figure 5A); by comparison, the pattern of findings with and without stretch during Wortmannin exposure appear to mimic the pattern observed with L-NAME treatment (Chapter 4 Figure 1). Therefore it is possible that, in the absence of NOS-1μ, activation of G-proteins may have some influence on satellite cell activation. This influence may be 'delivered' through a mechanism dependent on HGF, since G-proteins can activate adenylate cyclase pathways without influencing NOS activity in other tissues such as human skin (320) and fetal lung cells ((320) and reviewed in (321)). In experiments on tyrosine kinase inhibitors, data suggest that kinase activity is important for fiber survival (Chapter 7). Therefore, inhibition of tyrosine kinase activity may be directing nuclei toward apoptosis, which may account for the observation of a diffuse BrdU staining in all of the nuclei in a single fiber, indicative of small amounts of DNA synthesis during entry into apoptosis

(Chapter 7, Figure 5 inset; (333)). In experiments with the Ca^{2+} ionophore, in this case A23187, the treatment resulted in an increase in activation in conditions where satellite cells are normally quiescent. This is not a surprising result, since NOS-1μ activity is closely regulated by Ca^{2+} concentrations (see Chapter 1, Section 1.1.2.). Finally, the finding that Nifedipine treatment during stretching tended to increase satellite cell activation may due to hypersensitization of c-met, due to decreases in the release of HGF due to stretching.

It is important to keep in mind that these experiments were conducted using normal muscles, and that there are no data to suggest whether such alternative pathways exist or are used in normal muscle. In fact, it is possible that substantial 'alternate pathways' will be revealed only in experiments on fibers where there is little, to no, possibility of signaling through NO. For this reason, future experiments should include a repeat study where normal muscles are treated with L-NAME and/or anti-HGF to re-examine the possibility of alternative pathways for activation. Additional experiments on the signaling pathways should also be conducted using *mdx* and NOS-1(-/-) muscles, as in those systems, results may indicate more clearly whether such alternate pathways are actually functioning in conditions where a decrease (or complete absence) of NOS-1μ signaling occurs naturally within the muscle.

7. C-met: a marker of all satellite cells?

The activity of the c-met receptor appears to play a pivotal role in satellite cell

activation. This tyrosine kinase receptor for HGF is intimately linked with signaling pathways that promote migration, cell survival and proliferation in other tissues (334). In fact, in tumours, over-expression of c-met, which is a proto-oncogene, has been linked to high probability of metastasis, increased tumour aggressiveness and a decreased clinical prognosis (335). C-met also plays an important role in development of muscle and other tissues, as demonstrated by the embryonic lethality of a c-met knockout in mice. Based on an earlier report that c-met was expressed by skeletal muscle satellite cells under both quiescent and activated conditions, and that the literature had not established the number of satellite cells present on a typical FDB fiber, experiments were designed to count the number of c-met+ cells on a normal single fiber (82). Notably, knowledge of the population size of satellite cells on a fiber was important, since that number forms the denominator in any assay that might be used to examine experimental changes in satellite cell activation. Observations from these in situ hybridization experiments showed that the number of c-met+ cells per fiber ranged from 0 to 6, with an average of 1.38 c-met+ satellite cells/per fiber (82). These results were surprising, since it was not considered plausible that satellite cells would be absent from a single fiber isolated from normal muscle. In the context of the evidence from the initial time course studies which showed two peaks of activation (using BrdU incorporation as the index of earlier activation), the findings of a wide numerical range of satellite cells on a fiber under quiescent conditions, including 0 c-met+ cells per fiber, suggested that there may be more than one population of satellite cells present on a single, normal fiber. For this reason, it was hypothesized that not all cells in a satellite cell position were expressing c-met under quiescent conditions. To test this hypothesis, experiments were designed in which

whole muscles or single fibers were stretched for ½ hr, and immediately thereafter, the total level of c-met mRNA expression (in muscle tissues) and the distribution of c-met+ satellite cells on single fibers was measured. Results were compared between stretched and unstretched cultures of fibers and muscles, using two systems to test the hypothesis. Whole muscle culture experiments showed an overall increase in the level of c-met mRNA was produced by stretching (111). Experiments on single fibers in culture demonstrated that stretching for ½ hr is sufficient to induce a significant shift in the distribution of c-met+ satellite cells toward higher numbers of cells per fiber (Chapter 6, Figure 1). These two sets of experiments are strong evidence that there are some cells in a satellite position that do not express c-met under quiescent conditions and which respond to a mechanical stretching stimulus by displaying c-met expression very rapidly. These observations supported the hypothesis that there are at least 2 populations of satellite cells present on normal muscle fibers (reviewed in (33)). These results are consistent with the time course experiments on stretch-activation and the working hypothesis (82) on time-dependent responses in satellite cell activation. Together, the data suggest that "early-activating" satellite cells (activated after ½ hr of stretch) are those that express c-met during quiescence, and may be prepared in advance for activation signaling through the c-met pathway. The other "later-activating" satellite cells (those that are activated after 2 hrs of stretch), may be those cells that upregulate c-met (apparently *de novo*) after ½ hr of stretch, and by that upregulation are prepared for further mechanical stimulation. These findings were intriguing, and led to the studies of c-met as a potential early gene (see below).

It was very interesting to compare the results of changes in c-met expression during

activation, between normal muscle preparations and the preparations of whole muscle and single fibers from *mdx* mice. Data from dystrophic muscle preparations were quite different from wild type. Studies on whole muscle cultures demonstrated the same increase in the level of c-met mRNA in the tissues after activation. However, the distribution of c-met-expressing satellite cells (number of c-met+ satellite cells per fiber) did not change as a result of stretching *mdx* muscle fibers in culture. Based on the interpretation of an increase in the number of c-met+ cells as indicative of 2 populations on normal single fibers, the results of experiments on *mdx* fibers and muscles are consistent with the notion that one of the two populations is not present on *mdx* fibers by the time of their isolation in our experiments (5-8 weeks-of-age). This hypothesis of a missing population of satellite cells in *mdx* single muscle fibers is supported by time course experiments using *mdx* single fibers (See Section 3 above). Stretching *mdx* single fibers results in one decrease in activation after ½ hr of stretch. The time course of normal stretch-activation on the other hand shows 2 increases in activation, one after ½ hr, the other after 2 hrs of stretch. These two populations on normal single fibers show differential c-met expression; we postulate that those that activate after ½ hr of stretch express c-met under quiescent conditions, while those that activate after 2 hrs of stretch upregulate c-met after ½ hr of stretch perhaps in order to respond to later stimuli. This differential expression of c-met is not present in *mdx* single fibers after ½ hr of stretch, suggesting that the satellite cells present on *mdx* single fibers all express c-met under control conditions. Taken together, these observation are strongly supportive of the hypothesis that one population of satellite cells that is typically found in preparations of normal muscle fibers is missing from *mdx* muscle fibers prepared for culture.

It remains to be determined which of the two satellite cell populations is missing from *mdx* fibers, whether two populations ever exist in *mdx* muscle tissue, and whether there is a significant impact of the missing population in shaping the outcome of disease progression or regenerative capacity. Is the missing population the "early activating" population - the one that is absolutely dependent on NO for activation, or is it the second population - the one that does not require NO to activate if stimulated (by stretching) for long enough periods of time? Since the first population of satellite cells on normal single fibers is the early responding cells that are "ready" to activate (as suggested by c-met expression in these cells), it seems plausible that these are the cells that are lost from *mdx* fibers due to constant stimulation for regeneration. Also, since the satellite cells on *mdx* fibers are obviously activated according to physical characteristics in tissue sections and the time course studies conducted as part of this thesis, and this appears to relate to the loss of NOS-1μ from the underlying muscle fibers (according to the inhibition studies on *mdx* muscle fibers), it would make sense that the population of satellite cells which remains located on muscle fibers is that population that does not require NO to activate.

If it were the case that the early-activating population of satellite cells was missing from single fibers isolated from *mdx* muscle, it would mean that the satellite cell population which is revealed to demonstrate stretch-induced *de*activation in *mdx* fiber cultures is the second population of cells present on normal fibers. This population would then be deduced as the population in normal muscle fiber cultures which showed later stretch-activation after 2 hrs of stretching. This would suggest the reasoning that constant stimulation from the dystrophic environment to repair muscle fiber damage, together with the downregulation of

NOS-1μ expression and activity, would require that all satellite cells on *mdx* fibers should express c-met. This prediction agrees with lack of a shift toward increased numbers of c-met+ satellite cells after stretching (in the distribution studies on *mdx* muscle fibers) that was demonstrated here. This reasoning suggests that, at the time of isolation, the maximum number of satellite cells had already reached the state of c-met expression in activation, and is congruent with the idea that these already "activation-committed" cells are then only able to upregulate the amount of c-met mRNA expressed *per cell* in response to stretch, rather than the muscle fibers having another population of satellite cells available to recruit to activation by *de novo* expression of the c-met receptor.

The demonstration that c-met distribution in the population of satellite cells on normal fibers increases rapidly after only ½ hr of stretch, and our subsequent identification of c-met receptor as a delayed early gene in skeletal muscle provide novel insights into skeletal muscle satellite cell activation. These findings have advanced our knowledge of activation to a much earlier timepoint than was possible through the more fundamental experiments that used DNA synthesis to document full activation of satellite cells from quiescence. It will now be possible to use c-met expression as an early marker of satellite cell activation, without the complications of a marker that is only incorporated when cells enter into S phase (BrdU or ^{3}H thymidine) some time after a stimulus, and without the technical demands of staining for 2 proteins (as is the case with HGF and c-met double staining) to document co-localization (implying early activation, as reported by Tatsumi and colleagues (81) and Anderson (35)). The impact of NOS, one of the most tightly regulated enzymes in the body, to influence c-met expression, is shown by experiments using L-NAME

modulation of c-met expression under different conditions (111). These observations suggest that c-met expression is also tightly regulated. This is not surprising given that gene mutations to this receptor cause many types of cancer (336;337). This tight regulation of c-met expression by NOS in skeletal muscle may account for the very low incidence of tumors that originate in skeletal muscle. When those tumors do arise, over 60% of the cases are diagnosed in children under the age of 10, and are usually due to a translocation in pax7 (338), the gene which specifies skeletal muscle satellite cells in development. Even in *mdx* muscle, NOS regulates c-met expression (as shown by the inhibition studies on muscle cultures), which may account for the very low incidence of skeletal muscle tumors (rhabdomyosarcomas) in children with muscular dystrophy despite the very high rate of cell proliferation induced by repetitive cycles of degeneration and regeneration in the disease.

The original objectives of the thesis involved studies of gene expression, particularly for key myogenic genes (myf5 and MyoD), and a gene that is thought to be involved in quiescence (myostatin). However, for a number of reasons, the design requirements of the experiments did not allow us to make observations on the potential changes in expression of these genes. Samples for the experiments on gene expression were planned to have the same source (for comparative purposes) as those used for studies of c-met expression (i.e., muscles harvested after ½ hr of stretch). However, none of the genes (myf5, MyoD, myostatin) other than c-met are immediate, or delayed, early genes, so changes in the expression of mRNA or protein would be expected within that time frame, as for example, the expression of MyoD and myf5 is reported (and confirmed in this laboratory) to begin around 4-6 hrs after stimulation (77). The time course of changes in myostatin expression

after an intervention in vivo are not as well defined in the literature; however definitive changes would be expected over the 24 hr period following stimulation. The experimental design was revised in an attempt to accommodate the schedule of gene expression (i.e., by stretching the muscles for ½ hr and maintaining them in culture for 24 hrs). However, there was insufficient RNA isolated from the samples to conduct an assay for c-met (requiring RNAse protection assay), MyoD or myf5, and myostatin from the same muscle (or even 3 pooled muscles). As a result, the time course of any changes in mRNA expression for these genes of interest following a brief stretch (½ hr), which has the potential to reveal the activation cascade of the "early activating" population of satellite cells remains to be determined. In addition, the ensuing pattern of gene expression that occurs over the 24 hour period following 2 hrs of stimulation is also not included in the thesis, although the information on muscle regulatory gene expression is reported within the literature (as cited in Chapter 1 Section 3.4). It would certainly be interesting to see whether the time course of gene expression is the same for satellite cells in both normal and dystrophic muscle, including the genes mentioned here and other muscle-specific and non-myogenic genes (including pax-3 and -7, NOS-1-3, syndecan-3 and syndecan-4). The information would be valuable in making further approaches that have the potential to distinguish properties, shared and distinct, between the two apparent sub-populations of satellite cells.

8. Conclusions and future directions

The work presented in this thesis has shown that NO and HGF are involved in the

activation of satellite cells, and that NO precedes HGF in the stretch-activation cascade. The potential for the involvement of 2 unique or partly overlapping populations of satellite cells in the time course of an activation cascade was also demonstrated. One population is absolutely dependent on NO for activation, expresses c-met under quiescent conditions and appears to be an "early-activating" population. The second is able to bypass the requirement for NO release from fibers, and instead responds to HGF that is released from the extracellular matrix of skeletal muscle following a somewhat longer period of stretch, or to exogenous HGF in combination with 2 hrs of stretching, upregulates c-met mRNA expression after ½ hr of stretch and appears to be a "later-activating" population. These results suggest that the loss of a satellite cell population in *mdx* muscle is likley affecting the population that is dependent on NO for activation (i.e., the "early-activating" population). It also appears that the complete absence of NOS-1μ provides satellite cells more flexibility or capacity to respond effectively (i.e., with a capacity reflecting two populations and with a timing more similar to normal) to demands for activation and muscle regeneration than the downregulation of NOS-1μ that is extant in dystrophic muscle. It is possible that this flexibility or capacity of satellite cells in muscle from NOS-1 knockout mice is due to a compensatory upregulation of the other NOS isoforms. Applications of the system of whole muscle cultures, which is one step closer to modeling *in vivo* conditions in a muscle than the single fiber model, as well as a method by which to visualize NO release which we have demonstrated in normal and dystrophic muscle cells in culture, will allow us to explore further the impact of potential new therapeutic compounds on satellite cell activation and NOS regulation in dystrophic muscle.

The research presented here can now be extended to examine how satellite cell activation is altered with aging. Results indicated that the population of satellite cells on *mdx* fibers, which is already a highly activated and cycling population, do not show an increase in the number of c-met+ cells after stretching. We presume this is due to prior loss of the population that is dependent on NO for activation. We proposed the idea that all of the satellite cells on *mdx* fibers already express c-met and are "ready" to activate. By comparison, we do not know the status of satellite cells in aging muscle. It is possible that in aged muscle, instead of finding that all satellite cells express c-met (as seen in *mdx* fiber cultures) or that only one population expresses c-met in quiescence (as seen in normal fiber cultures), that the whole satellite cell population may have shifted so that most satellite cells do not express c-met. This would theoretically mean that only a small number of satellite cells would be "ready" to activate after an initial stimulation event. This condition would fit with published accounts that satellite cells in aged muscle are resistant to activation by stimuli that normally activate satellite cells in muscle from younger animals (See Chapter 1 Section 7.1). There is some evidence to support this from experiments with hind limb suspension models of disuse atrophy, in which there was no expression of c-met or other myogenic markers after 2 weeks of hind limb suspension. Since the level of pax7 expression did not change during hind limb suspension, these data were interpreted to show that the total number of satellite cells did not change although the general level of activation was substantially decreased by suspension (212). It is still not known whether aged muscle would show a shift of the remaining satellite cell populations (i.e., remaining after active living and ongoing small levels of regeneration) into the second "later-activating" category (which is

possibly more stem-like); this would be in contrast to younger muscle that has satellite cells in the more committed category (i.e., expressing c-met and "ready" for activation, with a sub-population that is "on call" and will express c-met after an activating stimulus. The approach to this area of research would first involve establishing that pax7 expression does not change with stretch, to ensure that it is indeed a good marker of satellite cells regardless of activation state. Then experiments could focus on the distribution of c-met+ cells on fibers from young, mature and old normal muscle, before stretch and after different durations of stretch. If the data on these proposed experiments were to support the hypothesis regarding the potential differential character of the 2 satellite cell populations, we would expect to see a significant difference in the distribution of c-met+ cells on single fibers between muscles from young and old animals. With stretching, one might anticipate a relatively rapid shift toward higher numbers of c-met+ satellite cells in the distribution of c-met+ cells on young fibers after brief stimulation, and a slower shift in the distribution of c-met+ cells on fibers from muscle of mature and older animals, after longer periods of stretch.

It will also be important to identify whether or not NOS-1μ is present in activated satellite cells of DMD patients. This knowledge would allow for the use of *mdx* satellite cells and DAF technology as tools for drug development, and additionally may reveal potential reasons for the relatively effective recovery from initial dystrophic degeneration by *mdx* mice compared to DMD.

A definitive identification of the two populations of satellite cells on normal fibers, particularly through gene expression profiling, is of great consequence. With this information, we would be able to establish the nature of satellite cell populations in young

dystrophic muscles. It is important to learn whether *mdx* muscle fibers, for example, originally begin with 2 populations of satellite cells and exhaust or lose one population through the dystrophic process, since that would have implications for strategically designing new therapies. Furthermore, if the precursor cell populations extant on muscle fibers are eventually observed to shift toward a more 'reserve' or less committed level as a result of the aging process, that information would allow for specific targeting of drug or rehabilitation therapies in a continuum or spectrum of approaches to treatment for DMD and age-related atrophy.

Finally, identifying how this research could be applied to a clinical setting is of great importance. Before this can happen however, there are many questions that need to be answered or issues that need clarification, including: the type/magnitude of the response by satellite cells to other magnitudes, frequencies and types of stretching, how stretching protocols used *in vitro* would translate *in vivo*, and the thresholds of activity for satellite cell activation in humans. Also, targeting drugs to skeletal muscle specifically will be necessary in order to avoid unwanted side effects of a NO-based therapy. Data from our laboratory have shown that skeletal muscle satellite cells have a different cell surface charge compared to other cell types (Bock and Anderson, unpublished data), suggesting that this could be a mechanism by which skeletal muscle could be targeted.

In summary, the experiments in this thesis have revealed novel information on stretch-activation, and the differential regulation of that activation process by NO, HGF, and the expression of c-met receptor in satellite cells of normal and dystrophic *mdx* muscle. We are excited to consider that future investigations will, in conjunction with other avenues of

therapy, advance the treatment of conditions in skeletal muscle that are so limiting to human function or are lethal.

Chapter 9.
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Appendix 1.
Signaling satellite-cell activation in skeletal muscle: markers, models, stretch, and potential alternate pathways

INVITED REVIEW

ABSTRACT: Activation of skeletal muscle satellite cells, defined as entry to the cell cycle from a quiescent state, is essential for normal growth and for regeneration of tissue damaged by injury or disease. This review focuses on early events of activation by signaling through nitric oxide and hepatocyte growth factor, and by mechanical stimuli. The impact of various model systems used to study activation and the regulation of satellite-cell quiescence are placed in the context of activation events in other tissues, concluding with a speculative model of alternate pathways signaling satellite-cell activation.

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SIGNALING SATELLITE-CELL ACTIVATION IN SKELETAL MUSCLE: MARKERS, MODELS, STRETCH, AND POTENTIAL ALTERNATE PATHWAYS

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Satellite cells, first identified using electron microscopy by Mauro in 1961,[95] are muscle precursor cells that lie between the external lamina and sarcolemma of skeletal muscle fibers (Fig. 1). In young muscle, satellite cells display organelles including ribosomes, rough endoplasmic reticulum, and Golgi complexes, indicative of cells that are metabolically active.[129] In normal adult muscle, these organelles are much reduced in volume, and cells are mitotically and metabolically quiescent.[129] With appropriate environmental signals, satellite cells enter into the cell cycle to provide the precursors needed for new muscle formation in growth and repair.[132] Only during these periods of growth and repair from injury or in disease conditions, such as in Duchenne muscular dystrophy (DMD), do the satellite cells resemble those of young muscle.[127]

Abbreviations: AP-1, activating protein-1; ATP, adenosine triphosphate; BrdU, bromodeoxyuridine; CME, crushed-muscle extract; DMD, Duchenne muscular dystrophy; DNA, deoxyribonucleic acid; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; FDB, flexor digitorum brevis muscle; GC, guanylate cyclase; cGMP, cyclic guanosine monophosphate; GTP, guanosine triphosphate; HGF, hepatocyte growth factor; JNK, c-Jun-N-terminal kinase; L-NAME, N-nitro L-arginine methyl ester; MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; NO, nitric oxide; NOS, NO synthase; PIP2, phosphatidylinositol bisphosphate; PKB, protein kinase B; PKC, protein kinase C; SHP-2, SH2 domain containing protein phosphatase

Keywords: c-met; HGF; hepatocyte growth factor; nitric oxide; regeneration; single fiber

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The capacity of satellite cells to increase muscle mass and repair muscle after damage makes them essential for the normal adaptive functions of skeletal muscle. In addition, the ability of skeletal muscle, and possibly activators from other sources, to regulate when, how many, and which satellite cells enter the cell cycle provides evidence of an extensive control system. At this time, the regulatory systems that control entry to and departure from the cell cycle during growth and repair are only partially understood from studies of activation and quiescence, although there is an expanding suite of satellite-cell markers and experimental research methods for their investigation.

For the purposes of this review, attention is focused on satellite-cell activation from fiber-derived signals until the time hepatocyte growth factor (HGF) binds to the c-met receptor, defining activation as the entry of satellite cells into the cell cycle and mobilization from a quiescent state (G0). Cell-cycle entry or withdrawal from G0 begins the process of preparing for the synthesis of deoxyribonucleic acid (DNA). In addition, the term *satellite cell* will refer here to those myogenic precursor cells that reside in the satellite-cell position on fibers. We distinguish satellite cells from the myogenic populations that may reside in the interstitial space in a muscle, and those circulating or bone marrow–resident stem cells that may have the capability of entering the myogenic lineage, as reviewed elsewhere.[2,33,40,41,44,55,62,65,66,88,101,118,125,138,139,148,158] There

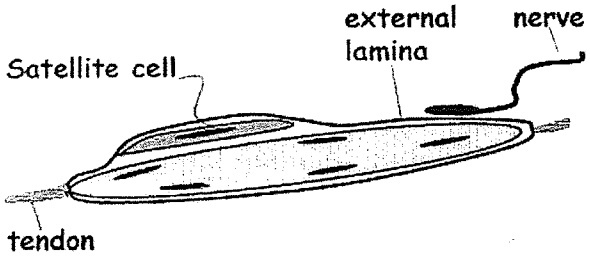

FIGURE 1. A schematic showing a satellite cell in close juxtaposition with the sarcolemma of a skeletal muscle fiber. Note the close association of satellite cells, overlying a highly regular and minute cleft above the sarcolemma and the subjacent layer of NOS-Iμ, localized by the dystrophin-associated protein complex in normal skeletal muscle.

may be additional nonmyogenic or otherwise undifferentiated stem cells, also located in the satellite position on fibers,[20] or in the interstitial space. The activation process includes hypertrophy of the satellite cell,[9, 97,127] as well as an increase in motility, resulting in migration away from the parent fiber.[6,131] The explicit definition of activation necessitates experiments that are designed to examine the very early stages of activation from quiescence.[15] There are, however, various working definitions for activation within the field, including overlap with the activity of satellite cells and myogenic precursors. For this reason, it is essential that the markers of satellite cells are considered within the context of the definition of activation applied in a particular report.

SATELLITE-CELL MARKERS

Satellite cells can be identified by position or by immunostaining for various gene transcripts and proteins. Satellite cells are positioned between the fiber membrane, outlined by dystrophin in the fiber cytoskeleton and laminin in the extracellular matrix (ECM) of normal muscle,[102–104] or within the laminin surrounding dystrophin-deficient fibers.[62,135] Electron microscopy, for many years the gold standard tool for identifying satellite cells, is an important, albeit technically lengthy method for experiments on satellite-cell dynamics during development and regeneration.[7,12,95,96,127,129,132,134] Even ultrastructural studies of satellite cells can be confounded by partial sections of infiltrating macrophages or the absence of a nucleus from a cell process observed between the sarcolemma and external lamina of a given fiber, and tangential sections that blur the discrete ECM layer external to a putative satellite cell. By fluorescence microscopy, satellite cells are typically located by position and, in relation to their activation state, can be identified by additional immunostaining for one of the proteins that identifies a satellite-cell product, a protein that specifies the myogenic lineage of precursors, or an important receptor. Satellite cells, both quiescent and activated, are typically positioned just external to the layer of m-cadherin protein that is found in the very narrow cleft between satellite cell and muscle fiber,[9,34,74] although there may be cells in the satellite-cell position that do not overlie m-cadherin.[34] Localization of the family of proteins expressed by the muscle regulatory factor genes myf5, MyoD, myogenin, and MRF4 in nuclei of activated satellite cells, and transcripts of muscle regulatory factors such as myf5 in perinuclear cytoplasm are also used effectively to identify satellite cells in regenerating muscle, and myogenic precursor populations between fibers.[11,20,34,61,82,99,123,157,161] Satellite cells also deposit syndecan-3 and syndecan-4 proteins in the ECM,[31,36] and pax7 expression specifies the satellite-cell population in development and persists in mature muscle.[133,134]

The expression of c-met receptor protein and mRNA expression is an additional marker that can be used to identify satellite cells, because c-met is expressed in both quiescent and activated satellite cells.[38] Unfortunately, immunostaining for c-met protein, the receptor for HGF, is not reproducible across laboratories, possibly due to differences in the immunostaining protocol. C-met is observed in the attenuated cytoplasm of satellite cells in normal adult muscle; in the hypertrophic cytoplasm of satellite cells in dystrophic muscle; in mononuclear, probable myogenic precursors between fibers; and in the smallest new myotubes in regenerating muscle, prior to the formation of satellite cells on those new fibers.[8,9,18,38,109,145,156] Notably, there is no reported test of the hypothesis that there should be a precise one-to-one correlation between satellite cells in vivo, identified by electron microscopy and by c-met immunodetection methods.

It is widely recognized that c-met protein is not exclusive to skeletal muscle satellite cells. Rather, c-met expression is reported in a wide variety of epithelial and mesodermal tissues, including liver, retina, smooth muscle, and lung, and additionally in tumor and metastases of the prostate, thyroid, brain, and breast tissues,[1,30,42,88,90,94,113,117,119,124,142] where it is used to identify cells of stem-like or multipotent capability. However, in studies of muscle in normal, regenerating, and disease states, c-met expression is useful as it marks both quiescent and activated satellite cells. It is also expressed by myogenic precursor cells outside the satellite compartment in muscle.

The variety of model systems (single-fiber cultures, dispersed cell cultures, muscle sections, muscle homogenates, whole-mount muscles or embryos, and intact, regenerating, or pathological muscle in various wild-type strains or transgenic mice on a variety of genetic backgrounds), isolation procedures (enzymatic digestion, flow cytometry, migration assays, dissection), and experimental designs (e.g., including notexin or cardiotoxin injection, irradiation, injury, aging, and exercise) reported in characterizing satellite-cell c-met expression and localization have probably contributed to the divergent observations reported in the literature.

Each of the above proteins or the respective transcripts can be usefully employed with variable specificity to identify satellite cells, noting the important and sometimes subtle distinctions drawn from studies in various laboratories, and the specific hypotheses under examination. Indeed, any population of cells, satellite cells being one of many, would typically be characterized by a range of each property, such as protein expression or phenotype, distributed across the types and states of a normal tissue such as muscle. It should again be emphasized that satellite cells are defined differently among laboratories, to include or exclude stem-like cells in a satellite position. In view of the differences in precursor position, and changes effected in satellite cells by processes used to isolate myogenic populations, the expression and the functional profile of expression of each putative marker protein will also vary and require characterization for each model, design, and hypothesis. To a relatively large extent, use of the single muscle-fiber model allows a much cleaner visualization of cells in the satellite position that express c-met or muscle regulatory factor genes, although the literature lacks consensus on whether CD34 or muscle regulatory factor genes are expressed by quiescent satellite cells, and on expression of CD34 by activated satellite or stem cells, and myogenic cells in the interstitium.[20,142,144]

Further discrimination of the profile of gene expression in satellite cells in the fiber model will advance the understanding of those genes that regulate satellite-cell quiescence in vivo; current information suggests that experimental results need to be interpreted to accommodate the possibility of multiple populations of myogenic precursors and also of heterogeneity among satellite cells.

HETEROGENEITY OF MUSCLE PRECURSORS

Although the ability to identify satellite cells using histological methods will remain controversial until their characteristics and activities are further elucidated, it is recognized that the existence of multiple populations of myogenic precursor cells would allow muscle tissue to respond differentially to a particular stimulus, type of injury, or physiological demand, and thereby enable a highly controlled response. The range of characteristics displayed by muscle precursor cells is most often available experimentally from differences among cells isolated from muscle. For example, different lineages of muscle precursors can be isolated from the variety of slow- and fast-twitch muscles in typical proportions, and each can differentiate to express distinct profiles of protein isoforms typical of slow and fast muscle.[114] It is also demonstrated that muscle precursor-cell populations within craniofacial muscles are distinct from those of limb muscles, which may influence their ability to regenerate after an injury.[115]

Not only are myogenic and satellite-cell populations observed to differ in characterized lineages and potential among various muscles of the body, there is additional evidence to suggest that more than one type of precursor cell with myogenic capacity is involved in regeneration within a single type of skeletal muscle.[20,66,78,107,118,120,161] By identifying the differential efflux of Hoechst dye 33342 from cells using fluorescence-activated cell-sorting analyses,[75] different populations of precursor cells in muscle have been separated into a conventional or main population of myogenic cells such as is characterized in normal muscle regeneration, and another that comprises the side-population cells.[118] Alternative terminology has referred respectively to these two cell populations, as one that is relatively more committed to the myogenic lineage and another that is more primitive[78] and multipotent or stem-like. However, the function or identity of these two populations is in dispute.[20,142–144] Interestingly, similar populations can be sorted from hematopoietic and other tissues, and side-population cells can develop into different types of tissue, depending on culture conditions or the tissue environment in vivo after transplantation.

Heterogeneity among myogenic precursors is also examined by other methods. Three potential subpopulations of myogenic cells have been identified, based on their ability to withstand different levels of irradiation,[66] and two populations have been isolated from regenerating or normal muscle, based on morphology, proliferative capacity, and ability to differentiate in vitro.[107] In the latter study, myogenic cells were all able to differentiate and contribute to forming myotubes, and desmin expression increased as myogenic cells within regenerating muscle became activated and differentiated. Those

distinctive populations may also differ with respect to their myogenic commitment, if the results are considered in the context of reports[33,41,73,77,78,88,118] in which the adhesion behavior of myogenic cell populations (selected by a serial preplating technique) was combined with study of myogenic potential and the expression of genes such as CD34, CD45, and Sca-1. Although reports on myogenic cell heterogeneity have not typically examined satellite cells specifically, the differences among these populations and marker proteins identified by different reports probably reflect the contributions of satellite cells (likely to be in the large majority), interstitial and circulating myogenic precursors, and also multipotential stem or stem-like cells resident in muscle tissue.

Satellite-Cell Heterogeneity. There are a few studies that directly implicate cells in the satellite position on fibers as being heterogeneous in nature. Satellite cells in extraocular muscle, identified by position and expression, are significantly different from those in limb muscles, and display slow, continuous proliferation and ongoing fusion to fibers, suggested as remodeling, even in normal animals.[102] Studies of stretch-induced activation of satellite cells on single fibers from our laboratory found a bimodal, nonuniform behavior of activation among satellite cells on fibers. Satellite cells were activated to enter the cell cycle and incorporated bromodeoxyuridine (BrdU) at two distinct times after the start of cyclical stretching.[156] This observation is consistent with there being two populations of satellite cells that each show distinct activation characteristics. The idea of differential thresholds for satellite-cell activation by stretching would allow skeletal muscle mechanical activity, injury, or disease processes to recruit distinct populations of cells into the cell cycle, based on the satellite-cell responsiveness to activating stimuli, and then to migrate away from fibers.[6] Two types of satellite cells have also been reported using immunostaining, the majority of which were defined by CD34 or myf5 expression in combination with m-cadherin, and a minority (about 5%) that were negative for both CD34 and myf5,[20] although there is general understanding that myf5 is only expressed by activated satellite cells.[34,53,57,140]

The literature therefore describes a wide range of muscle-derived muscle precursor cells, satellite cells, more stem-like cells, and nonsatellite myogenic cells, which have varying profiles of gene expression and behavior.[40,62,75,76,78,88,118,122,142] Differences in experimental design, staining protocols, and models at least partly preclude comparison of data from various reports and suggest that an international collaboration would fruitfully cross-reference the character of such cell populations by each of the commonly applied methods. However, although it is not known how many functionally distinct satellite-cell populations are contained within skeletal muscle and have functionally significant capacity to contribute to its growth and regeneration, there are reports that provide evidence for heterogeneity among satellite cells and among myogenic precursors, based on generally well-characterized markers of function, activation state, position, and gene expression. Integrating the functional distinctions between satellite and stem cells is challenging: to date, the latter are mostly isolated from muscle prior to their characterization, and observations of cell behavior can vary according to markers of satellite-cell identification, their unknown origins, modeling, and potential differences related to muscle disease and regeneration.

Models Used To Study Satellite Cells. Models used to examine satellite-cell activation from quiescence include cell cultures,[146,147] single fibers,[6,22,23,156] and in vivo studies in various experimental protocols.[9,24,34,60,145] Each model is capable of studying satellite cells to a variable degree of complexity. However, the maintenance of satellite-cell quiescence during isolation procedures is essential to enabling a study that addresses the timing or nature of satellite-cell withdrawal from G0 and entry to cycling during activation.[15] This was accomplished in the cell-culture model by isolating satellite cells (and probably other myogenic precursors) from older animals, because those cells have a longer latent period of quiescence that is maintained after an activating stimulus, before they make DNA, compared to satellite cells from younger animals.[146,147] The single-fiber culture model involves isolating myofibers, usually from the flexor digitorum brevis muscle (FDB) of mice and rats,[23,163] although fibers can be prepared from other muscles. This process of isolating fibers should maintain satellite cells in the normal, quiescent state, and in their characteristic position between the basal lamina and sarcolemma of skeletal muscle fibers. However, protocols for methods of isolation, digestion, plating, and culture vary among laboratories, and variations may cause inadvertent activation of satellite cells that may not be revealed by the markers of function, expression, or position that are selected for study. Finally, in vivo studies can provide the most comprehensive picture of satellite cells during activation in situ in muscle.[8,9,145] However the effects of treatments directed to satellite cells require careful interpretation, due to the com-

plex contributions from nonmuscle tissues, perfusion-dependent changes, constraints of tissue sampling, animal activity, and systemic physiology that each may introduce significant variability to indices under study.

Skeletal Muscle Regeneration. The complexity of the skeletal muscle regeneration process in repairing muscle tissue damage and forming new fibers has been explored carefully for many years.[96] Regeneration begins simultaneously with key events such as satellite-cell activation and the inflammatory response to damaged fibers.[21] As the removal of damaged tissue begins, muscle fiber regeneration ensues under extensive controls by the state of both the external lamina and sarcolemma,[24] growth factors,[68] and mechanical events affecting fibers in the ECM-wrapped complex with satellite cells.[21] Muscle regeneration can be usefully considered in four stages, apart from events in nonmyogenic tissues in muscle that also contribute to tissue repair: (1) satellite-cell activation, (2) myoblast or precursor proliferation, (3) differentiation, and (4) return to quiescence, as reviewed briefly in the following paragraphs.

Stage 1: Satellite-Cell Activation. Satellite-cell activation is the process by which satellite cells exit G0 and enter into the cell cycle. This activation can be measured using a wide variety of methods. Observations of changes in morphology and contents have been extensively used to develop structural criteria for the activation state of satellite cells. A quiescent satellite cell is a spindle-shaped cell with little cytoplasm and few organelles,[132] whereas an activated satellite cell has hypertrophied organelles and an expanded cytoplasm.[9] Another method that is used widely to mark activation is the incorporation of tritiated-thymidine or BrdU into new DNA.[132] Although this is a very useful method, it requires that cells enter into S phase, and therefore does not mark activation as it begins, but rather uses DNA synthesis as an effective marker of earlier activation. Application of this technique requires sufficient controls to maintain a baseline quiescence among a nonstimulated population and necessitates catching the appropriate interval when DNA synthesis will start or progress in one cycle, but not enter a second cell cycle. Proliferating cell nuclear antigen, which appears in cells during DNA synthesis,[79] can also be used as a marker for prior activation, as can the onset of muscle regulatory factor gene and protein expression.[57] Further investigations are needed to distinguish among specific markers of activation in satellite cells, and those, such as c-met, which are accepted as marking satellite cells independent of their activation state. At present, the earliest known marker of activation in progress in satellite cells is the co-localization of c-met with its ligand, HGF.[9,145] Finally the expression of immediate early genes c-fos and c-jun can also be used to mark prior activation, as the proteins are present in satellite cells 3 to 6 h after injury.[83] Interestingly, c-fos expression is prominent in normal diaphragm muscle satellite cells in vivo, which appear to be more activated without injury than satellite cells in limb muscle, likely by respiratory movements and fiber shear.[13] Interestingly, in liver tissue, c-fos mRNA expression is increased as soon as 15 min after activation of liver cells.[126]

Stage 2: Satellite-Cell Proliferation. The second stage in skeletal muscle regeneration is the proliferation of satellite cells and myogenic precursor cells.[59,130] Daughter cells (myoblasts) then commit to enter the myogenic lineage by the expression of myf5 and MyoD approximately 6 h after activation in vivo.[56] Committed myoblasts continue to proliferate and express muscle regulatory genes, including myogenin, until the balance of protein expression pushes the cells toward differentiation.[99,123] p27Kip,[1] a negative cell-cycle regulator, is suggested as a regulator of satellite-cell proliferation,[141] such that the inhibition of progression in cycling effectively decreases satellite-cell proliferation.

It is a major limitation in studies of muscle regeneration that the expression of myogenic marker genes and proteins cannot be used alone to determine whether all satellite cells that are initially stimulated to activation will ultimately progress to proliferation. Such information would be interesting evidence of the homogeneity or heterogeneity among satellite cells in vivo. For this reason, in studying satellite-cell and muscle-precursor behavior, it can be very effective to combine nucleotide incorporation into new DNA with techniques to localize protein or gene expression as tandem markers of proliferation in myogenic cells,[10,99] often revealed in a nonuniform distribution among cells in a tissue.

Stage 3: Precursor Differentiation. Differentiation is the process whereby proliferating myoblasts derived from activated satellite cells and other myogenic precursors will withdraw from the cell cycle and either fuse to existing fibers in repair of damaged segments or to each other to form new fibers.[46] Fusion events occur after cells exit mitosis and enter into G1.[26] The onset of differentiation in cell or fiber cultures can be marked by the expression of the myogenic regulatory genes myogenin and MRF4,[123,153,157,161] although in vivo even myogenin can be expressed in the late proliferation stages of

myoblasts during regeneration.[99] Early differentiation can also be measured by an increase in creatine kinase activity in muscle tissue,[46] particularly the isoform creatine kinase BB,[14,25] and later by the shift in expression of contractile protein isoforms from immature or developmental isoforms to adult isoforms.[16,116] The requirements for and initiators of myoblast differentiation and fusion as new fibers grow and subsequently become innervated, have been the focus of extensive research.[4,37,46,123,140,159,161,163] Because satellite cells are self-renewing and satellite-derived myoblasts do not uniformly fuse into fibers, at least some myoblasts likely maintain, resume, or take up the satellite-cell position, for example, as evidenced by retention of markers of earlier DNA synthesis,[12,58,165] and may ultimately return to quiescence.[39,45]

Stage 4: Return To Quiescence. The ability of satellite cells to return to quiescence has been less thoroughly investigated than activation, proliferation, and regeneration. Under normal conditions the proportion of satellite cells that actually reside in G0 rather than in a long lag phase in G1 is not known, as are many important aspects of satellite-cell quiescence. For example, it is uncertain whether the same satellite cells that originally respond to an activating stimulus will return to quiescence, or whether the satellite-cell compartment is only repopulated by daughter cells. It is also not established whether the requirements for maintaining quiescence in the absence of an activating stimulus are the same as those for a possible return to quiescence after activation and cell division. The details of these events likely have impact on the extent and decline of life-long regenerative capacity and relate to regulatory signals during development that may or may not be affected by the transgenic mutation strategies used for study. It is likely, however, that the expression of particular genes and proteins must be upregulated during late differentiation in order to return satellite cells to quiescence.

Myostatin is proposed as a key regulator of quiescence because its overexpression blocks regeneration.[98] Satellite-cell release from the effects of myostatin expression in the myostatin knockout mouse enables significant increases in muscle growth and regeneration.[105,150] The precise identity and characterization of these quiescence factors, including myostatin and other members of the transforming growth factor-β superfamily, and their interaction with genes that are upregulated during early activation, have yet to be determined. It would be interesting to examine coincident, hypothetically inverse changes in the level and timing of myostatin expression and BrdU incorporation in satellite cells on fibers in culture during a stretch-activation experiment. In the discussion of regulators of satellite-cell activation that follows, this review is restricted to those studies that demonstrate satellite-cell quiescence in control conditions.

Satellite-Cell Activators. *Growth Factors.* According to studies of proliferation, measured using BrdU or tritiated-thymidine incorporation into DNA, HGF activates satellite cells from quiescence in cell cultures,[5,136,146,147] in fiber cultures[6] and in vivo.[145] Bischoff[22] showed that satellite cells on single fibers become activated by an extract of crushed muscle (CME). The HGF contained in CME[145] is released upon injury from skeletal muscle, and is responsible for inducing activation of quiescent satellite cells in vivo.

The nature of the activation induced by HGF or CME can also be examined using frequency distributions of $BrdU^+$ satellite cells on fibers (Fig. 2; Pilipowicz and Anderson, unpublished observations). After exposure to HGF or CME treatment, the distribution of activated satellite cells on fibers was shifted higher compared to fiber cultures maintained in basal conditions without HGF or CME. However, fibers did not show a uniform response to these stimuli. In the population of FDB fibers, 45% to 55% had no $BrdU^+$ cells attached; and 30% to 35% of fibers had only one $BrdU^+$ satellite cell. Comparing these data with the distribution of fibers

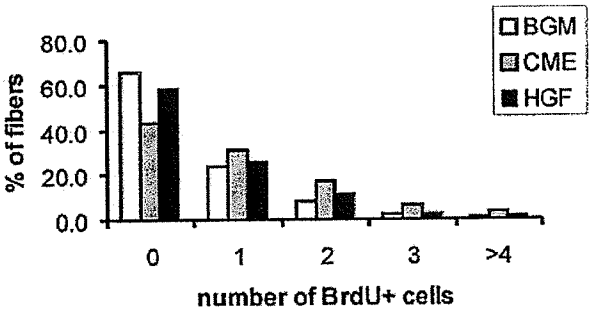

FIGURE 2. Activation of satellite cells on single fibers by CME and HGF (Pilipowicz and Anderson, unpublished observations). Frequency distributions showing the proportion (%) of total fibers that demonstrated activated satellite cells (0, 1, 2, 3, >4) on isolated fibers. Cultures were maintained in basal growth medium (BGM) as reported[6] (white bars, n = 5785 fibers), or treated with CME (grey bars, n = 3093 fibers) or HGF (black bars, n = 2892 fibers), and cultured for 48 h. Activation was determined according to immunostaining for the incorporation of BrdU into DNA. The distribution of fibers was shifted to significantly higher numbers of activated satellite cells by CME and HGF compared to basal conditions ($P < 0.001$). All three distributions were lower than the distribution of c-met$^+$ quiescent satellite cells shown in Wozniak et al.[156]

with data on c-met$^+$ satellite cells[156] from another series of experiments, the distribution of fibers with c-met$^+$ cells (thought at this time to represent the entire population of satellite cells in a nonstimulated culture) appears higher than the distribution of fibers with BrdU$^+$ satellite cells that were activated by HGF or CME. The difference in distribution was most obvious in comparing the 25% of fibers with 2 c-met$^+$ cells in basal, nonactivating conditions, to the 10% to 15% of fibers with 2 BrdU$^+$ cells observed after activation with HGF or CME. These general comparisons suggested that activating conditions do not uniformly engage the whole population of c-met$^+$ satellite cells that presumably is available for activation. A similar conclusion of satellite-cell heterogeneity was drawn from experiments that showed two peaks of activation were induced by stretching fibers for 30 min and 2 h.[156]

Activation by HGF occurs through the HGF receptor, c-met, expressed by satellite cells.[38] The binding of HGF to c-met initiates multiple signaling cascades including the mitogen-activated protein kinase (MAPK) and phosphatidyl inositol 3 kinase pathways[51] that are involved in driving the transcription of genes required for growth and cell division.[149]

Other growth factors, including fibroblast growth factor, platelet-derived growth factor, and insulin-like growth factor are not demonstrated to induce satellite-cell activation de novo from a quiescent state.[80] Rather these growth factors appear to be involved in promoting the proliferation and differentiation of satellite cells that were already activated at the time of exposure to those proteins. In the absence of an effective marker for quiescence, it is important to demonstrate in control conditions the maintenance of satellite cells in a quiescent, stable, nonproliferative state during the same interval in which growth factor or other stimulation induces proliferation. Fibroblast growth factor stimulates an increase in the proliferation of already activated satellite cells, and represses their ability to differentiate; insulin-like growth factor, by comparison, is known to promote both processes.[68]

Nitric Oxide. Nitric oxide (NO) was first implicated in satellite-cell activation in vivo in this laboratory.[9] A huge body of prior literature is built on the discovery of NO as endothelium-derived relaxation factor.[50,70–72] In muscle, NO is produced by nitric oxide synthase-Iμ (NOS-Iμ), a skeletal muscle-specific isoform of neuronal NOS, as one product of the conversion of L-arginine to citrulline.[108,121] Nitric oxide and an important biometabolite of NO, reactive nitrogen species, act on guanylate cyclase.[69,152] Many types of NOS inhibitors have widely variable specificity amongst the three NOS isoforms (neuronal, or type I; inducible, or type II; and endothelial, or type III), a property often incorrectly tested outside the optimal range of conditions, cells, or tissues, or in vivo.[3]

In muscle in vivo, the systemic exposure to a nonspecific NOS inhibitor, N-nitro, L-arginine methyl ester (L-NAME), just prior to a crush injury inhibited the rapid component of satellite-cell activation by NO[9]. L-NAME treatment also prevented the hypertrophy of satellite cells and colocalization of HGF and c-met proteins in satellite cells, one of the earliest signs that activation was inhibited by NOS inhibition. Electron microscopic studies revealed that satellite-cell cytoplasm, karyoplasms, and heterochromatin areas remained more condensed (i.e., electron dense) 10 min after an activating injury stimulus under NOS inhibition than under the control (saline injection) conditions (Fig. 3; Kong and Anderson, unpublished data). In this study, whole satellite cells exhibiting a nucleus were identified by two independent observers as satellite cells from electron micrographs without knowledge of source, and integrated areal density was measured (NIH Image software). Tibialis anterior muscles were sampled from mice injected with saline (controls) or the NOS inhibitor L-NAME. An intraperitoneal injection was delivered 15 min prior to crush injury of the right tibialis anterior muscle, and muscles were sampled in pairs (left and right) from each

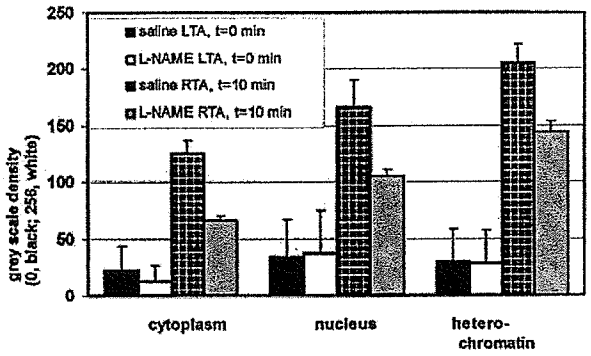

FIGURE 3. Histogram showing the density of three regions of electron micrographs of satellite cells in crush-injured (right tibialis anterior; RTA) or intact (left tibialis anterior; LTA) muscles of normal mice, sampled 0 min or 10 min after injection with saline (controls) or the NOS inhibitor, L-NAME, as reported.[9] Density (mean ± standard deviation) is represented by grey scale measures from 0 (black) to 256 (white), for integrated areas of the cytoplasm, nucleus, or heterochromatin in 5–8 satellite cells per group. The change in density from 0 to 10 min after injury decreased (shifted to a higher grey scale reading) significantly more in control muscles than during NOS inhibition, consistent with a rapid, NO-dependent satellite-cell hypertrophy.

animal, either immediately after injury (n = 2 mice) or 10 min later (n = 2 mice). Satellite-cell hypertrophy, colocalization of HGF with c-met and satellite-cell activation were observed in the injured muscle of saline-treated mice at 0 and 10 min, but were delayed 10 min or more by the L-NAME treatment.[9] Without NOS inhibition, satellite cells showed rapid, NO-dependent cell and organellar hypertrophy, colocalization of HGF and c-met proteins, and reduced adhesion to fibers.[9]

In vivo studies have also shown that short-term treatment with L-arginine, the NOS substrate, resulted in an increase in activation, as marked by increased DNA synthesis in *mdx* animals. Animals were treated for 3.5 days with L-arginine in drinking water (3.75 mg/ml), and injected with tritiated-thymidine 24 h before muscles were collected (Fig. 4, Bock and Anderson, unpublished data). Further studies of satellite cells resident on single fibers have confirmed the involvement of NO. Although activation of individual cells is considered an all-or-none process when defined by BrdU staining (positive or negative), the satellite-cell population showed a dose-related response to the activating stimulus of L-arginine.[6] Nitric oxide is also known to regulate the release of HGF from satellite-cell cultures after mechanical stretching.[146]

These observations suggest that there may be one preferred pathway to activation (Fig. 5, pathway A), with alternate mechanisms for activation under conditions where NO levels are suboptimal or nonexistent (one possibility being pathway B; Fig. 5). This notion is supported by data from NOS-I$^{-/-}$ mice, which show delayed satellite-cell activation after muscle injury, but were still able to regenerate.[9] The timing of the delay seen in muscles of NOS-I$^{-/-}$ mice is the same as that seen in *mdx* muscles after injury, where fibers have significantly reduced levels of NOS-Iμ.[9] Although neither muscles from NOS-I$^{-/-}$ or *mdx* mice appear deficient in development as a result of this delay, and *mdx* mouse muscle can regenerate effectively, it is interesting that satellite cells are hypertrophic in muscles from *mdx* mice and demonstrate NOS-Iμ expression.[13] By comparison, the underlying dystrophic fibers in *mdx* mice do not show NOS-Iμ expression due to the disruption of the dystrophin–cytoskeleton complex. Additionally, there is NOS activity (although not related to NOS-I) in activated satellite cells of NOS-I$^{-/-}$ mice.[13] Muscles from *mdx* mice demonstrate effective regeneration compared to normal muscle[100,116,165] when examined by histopathological markers of myoblast proliferation, myotube growth, and differentiation. The regeneration is also partially prevented by NOS inhibition.[9,13] This observation suggests that NOS-Iμ activity in satellite cells of *mdx* mice, in the absence of NOS-Iμ activity in underlying fibers, may shift satellite cells toward the preferential pathway for

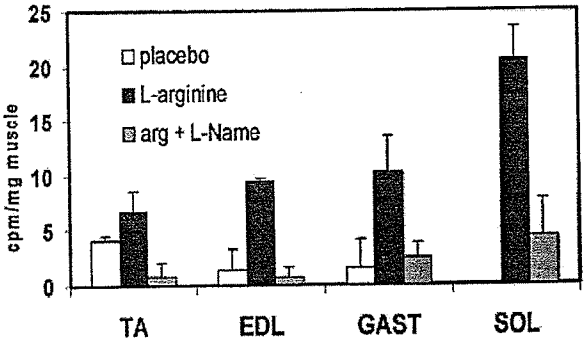

FIGURE 4. A histogram showing data for an NO-dependent increase in tritiated-thymidine incorporation into DNA in four muscles sampled from *mdx* mice (n = 6 per group). Animals were treated for 3.5 days with placebo (drinking water), L-arginine, or L-arginine plus L-NAME according to reported methods.[9] DNA synthesis (cpm/mg muscle ± standard deviation) increased significantly in tibialis anterior (TA), extensor digitorum longus (EDL), gastrocnemius (GAST), and, especially, soleus (SOL) after L-arginine. The increase was prevented by the combined treatment with L-arginine + L-NAME. No change in DNA synthesis was noted in normal or *mdx* muscles after 1 day of these treatments. Muscles from normal mice treated for 3.5 days showed similar but nonsignificant trends in DNA synthesis.

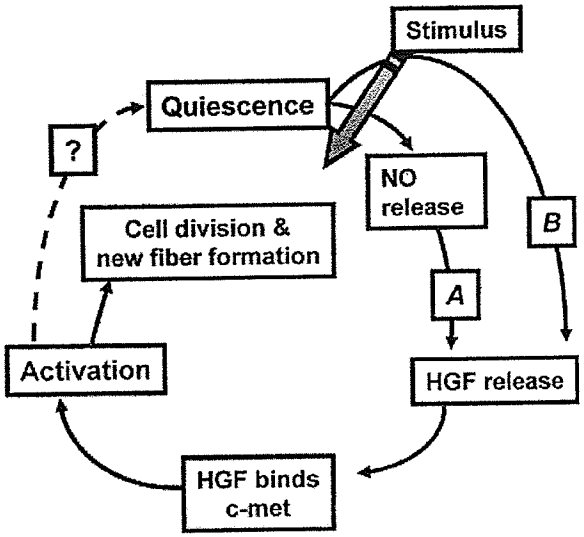

FIGURE 5. The signaling cascade for satellite-cell activation that includes pathway A, in which a stimulus-dependent release of NO in turn initiates the release of HGF from the extracellular matrix. A possible alternative pathway, B, bypasses the release of NO and potentially involves HGF release through an NO-independent mechanism, as suggested by the regeneration capacity of muscle that lacks NOS-Iμ expression in fibers in NOS-I$^{-/-}$ mice.

activation (A), albeit by a somewhat delayed sequence compared to normal.[9] It is possible that only the complete loss of NOS-Iμ expression from fibers and satellite cells, as in NOS-I$^{-/-}$ mice, may enable an alternate pathway to activation (B). This could involve alternate sources of NO from NOS-II and NOS-III, which are also expressed in skeletal muscle, and other ligands, but this idea is not tested. Even though muscle regeneration in *mdx* and NOS-I$^{-/-}$ mice is relatively normal in young animals as assessed by current markers, it is important to note that satellite-cell activation is not. Satellite cells in both mutant strains show a deficiency in the early phase of activation. The impact of this altered activation on the aging process in muscles, in which capacity for satellite-cell activation declines, and on the longer term outcome of progressive disease, in which activation is continuously employed for repair, is unknown and under exploration by a number of laboratories.

Mechanical Stimulation. A mechanical stretch stimulus applied to satellite cells in culture induces activation after 2 h, as visualized by the subsequent incorporation of BrdU into new DNA.[147] This mechanical stimulus is now known to induce HGF release into the medium in an NO-dependent manner, because release is inhibited by L-NAME.[146] The release allows HGF to interact with, and bind to, the c-met receptor, which then initiates signals that lead to DNA synthesis.

Experiments on the early activation of satellite cells on single fibers by mechanical stretching indicated that satellite cells are generally maintained at a low level of spontaneous activation.[156] The features and threshold for a stimulatory mechanical stretch (e.g., single vs. multiple; percentage of fiber lengthening; duration of stretch) are not yet characterized for satellite cells on fibers. Satellite-cell activation was significantly increased when fiber cultures were subjected to stretch for as little as 30 min, and there was a second rise in activation above the control level after 2 h of stretch. The presence of two peaks of activation was not reported previously, and suggests that activation may not be an all-or-none process. Alternatively, satellite cells may be heterogeneous in their precursor phenotype (committed vs. stem-like), in their original state at the time of isolation, in their responsiveness to stretch, or in the kinetics of their activation, as mentioned earlier.

A new model to study activation was recently developed in this laboratory to scale up studies of gene expression during activation using whole mouse muscles in culture (FDB and extensor digitorum longus). Muscles were isolated, cleaned, and then pinned at resting length on flexible or rigid culture plates and subjected to a stretch stimulus for 30 minutes and 2 h in the presence of BrdU. After a total of 24 h from the start of stretching, muscles were collected for DNA isolation. Isolated DNA was run in a SouthWestern preparation, in which DNA was run in an agarose gel, blotted onto nitrocellulose, and immunostained for BrdU incorporation. Results showed an increase in satellite-cell activation (indicated by an increase in BrdU incorporation) after 30 min and 2 h of stretching (Fig. 6; Wozniak and Anderson, unpublished observations). The results indicated that satellite cells in whole muscles in culture, at least the small muscles from normal mice, responded similarly to those on single fibers, and were generally quiescent in the absence of the stretch stimulus. The magnitude of the increase in BrdU incorporation in the whole-muscle culture model was approximately the same as that seen in the single-fiber model. This muscle culture model will be useful for studying gene expression in satellite-cell activation and quiescence, because satellite cells are maintained in a quiescent state in conditions even closer to the in vivo state than in fiber cultures. It may be possible with this model to examine the reversible withdrawal of satellite cells from

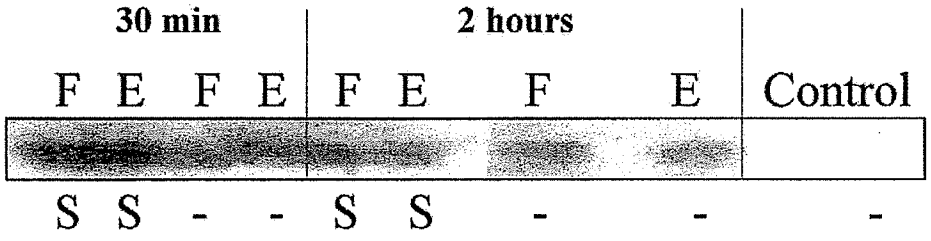

FIGURE 6. Whole muscle cultures of FDB (F) and extensor digitorum longus (E) muscles were pinned in rigid or flexible culture dishes and stretched (S) or not (—) for 30 min and 2 h in the presence of BrdU. Muscles were cultured for 24 h after adding BrdU, unpinned, and DNA isolated. DNA samples were run on an agarose gel, blotted, and immunostained for BrdU (negative control, Control). An increase in BrdU incorporation was observed in FDB and extensor digitorum longus muscles after 2 h of stretch (2.5× and 2.6×, respectively).

cycling after an activating stimulus, as demonstrated in modelling the return to quiescence by myogenic cells in dispersed cultures.[106]

Physiological Stimuli by Exercise. Muscles exposed to physical activity and exercise are reported to experience traumatic microtears. The trauma can be either direct or indirect,[21] and results in activation of normally quiescent satellite cells upon sufficient disruption of the sarcolemma.[21,24] This was demonstrated by a variety of indices. After resistance exercise induced by motor nerve stimulation, satellite-cell activity increased: by 5 h after a single bout of exercise, intracellular signaling via phosphorylation of extracellular signal-regulated kinase-2 (ERK2) and p70 S6 kinase-1 (S6K1) had increased; by 10–24 h after exercise, there was higher mRNA expression of insulin-like growth factor-I and the variant isoform, mechanogrowth factor; cyclin D1; and myogenic regulatory genes (myogenin).[64]

In vivo, satellite cells are known to proliferate in response to exercise, stretching, and weight loading, and their proliferation is reduced by loss of weight-bearing in the suspension model of microgravity.[47,110,128] It is not known, however, whether exercise is able to induce widespread activation of satellite cells or whether it is a more localized signal that might induce a response near the neuromuscular or muscle–tendon junctions or in a region within the muscle architecture defined by the highest strain on muscle fibers or segments.[32,84] With increasing age, the impact of exercise in human subjects induces significantly lower responses from stress- and damage-responsive or inflammatory genes, suggesting exercise regulation of the muscle-hypertrophy response adapts to aging.[81] In aged rats, reloading after hind-limb suspension also induces less satellite-cell activation than in young adults (Dupont-Versteegden, personal communication), similar to the profound atrophy and resistance to exercise-induced activation during space flight. Further information on these interactions of exercise and loading with the biology of satellite-cell activation will be essential in developing strategies for maintaining muscle mass (fiber diameter) and contractile strength, and preventing serious atrophy with disuse or age.

Mechanisms by which the mechanical stimuli of exercise are able to induce satellite-cell activation are not well understood. It has been proposed that decreased pH, reduced calcium cycling, glycogen depletion, and ATP depletion may be involved in the transduction of the exercise signal into a response.[154] It is also known that one bout of exercise is sufficient to stimulate a more than 20-fold increase in c-fos mRNA within 4 min.[32] Because c-fos expression can be used as a marker of activation that occurred a short time earlier, this information suggests that satellite cells have a rapid response to exercise in vivo, consistent with experiments on single cells, muscle fibers, and muscles that are stretched in culture.

Other Potential Activation Pathways. The signaling cascade involved in activating skeletal muscle satellite cells is only partly available from direct data. However, there are extensive reports on cell activation, including activation of stem-like precursors cells, in nonmuscle tissues, which offer, through speculation, some potentially informative parallels in relation to muscle. A concept map of pathways that may be involved in signaling satellite-cell activation was compiled from both direct information and indirectly from observations of other systems and tissues, based on the presence in skeletal muscle of the mRNA and proteins involved in those other cascades. To date, the main pathway for satellite-cell activation from quiescence, in bold italics in Figure 7, involves the release of NO after a stimulus, which leads to HGF release and subsequent binding to the c-met tyrosine kinase receptor.[6,146]

The release of NO that initiates activation after a stimulus must be due to an increase in the activity of NOS-I or an increase in the number of NOS-I enzyme molecules that are dimerized (the active state of the enzyme that is stabilized by L-arginine and pterin) and therefore are producing NO. Such activation of NOS-I was shown to be induced by dephosphorylation by SH2 domain containing tyrosine phosphatase (SHP-2) in Chinese hamster ovary cells.[35] SHP-2 is a tyrosine phosphatase that positively regulates signals from several growth factors, and is activated by the $\beta\gamma$ subunit of G-proteins. This may be a mechanism by which NOS-Iμ activity is increased in skeletal muscle fibers after an activating stimulus.

MAPK activity increases in isolated plantaris muscles held under tension for 5 min, such that the rise is quantitatively related to the amount of applied tension.[92] Initially the rise in MAPK activity is from ERK and c-Jun-N-terminal kinase (JNK) pathways, with a later response from p38 MAPK. All three MAPK pathways are also activated in the diaphragm by mechanical stresses applied axially and transversely across the muscle.[86] MAPK activation occurred as soon as 5 min after a continuous mechanical stress and reached maximum levels at 15 min of loading.[86] Whole soleus muscles also showed increased MAPK activity as soon as 5 min (p38 and ERK) and 10 min (JNK) after contraction induced

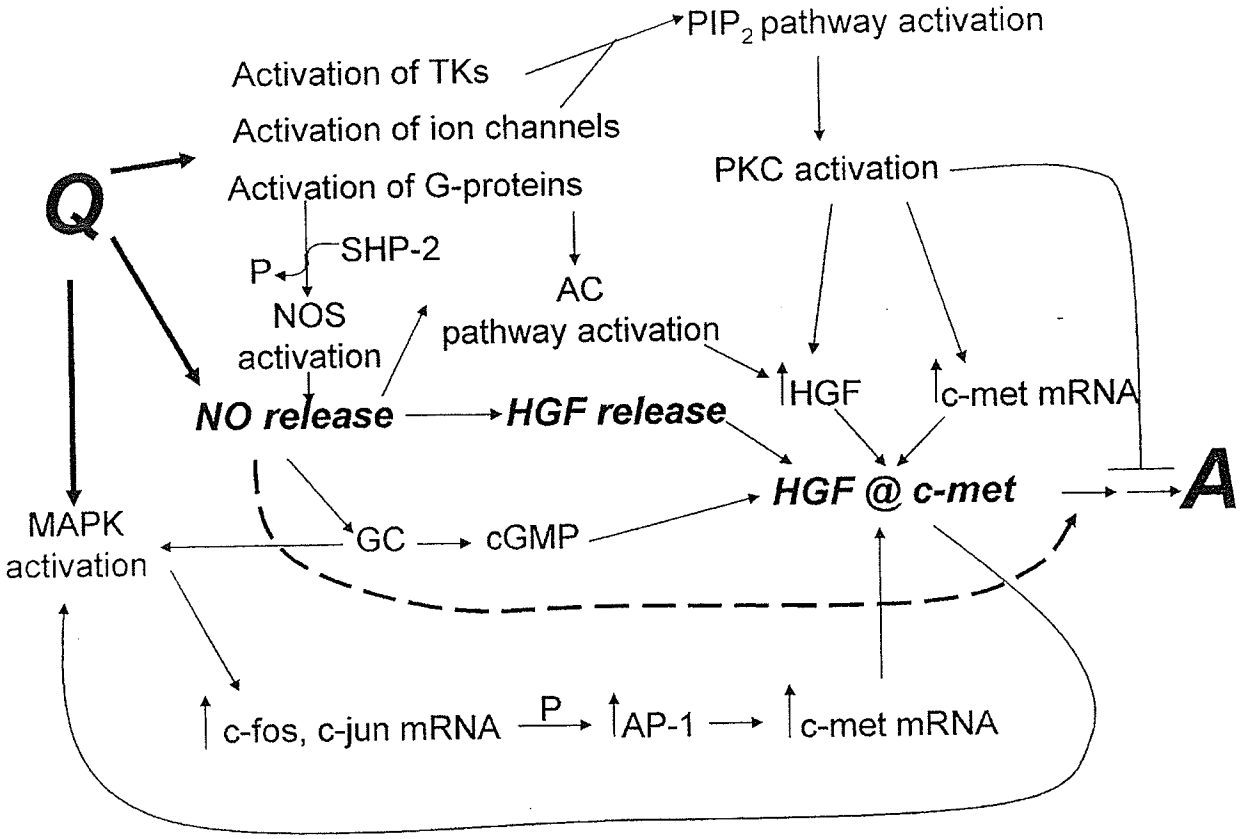

FIGURE 7. A schematic cascade of signaling pathways that represent known (denoted by text in bold italics) and potential alternate (normal text) mechanisms for the activation of skeletal muscle satellite cells from quiescence. The dashed arrow represents another potential pathway that may bypass HGF ligand-binding to c-met receptor for activation. Abbreviations: A, activated state; AC, adenylate cyclase; AP-1, activating protein-1; cGMP, cyclic guanosine monophosphate; GC, guanylate cyclase; HGF, hepatocyte growth factor; HGF @ c-met, HGF ligand-binding with c-met receptor; MAPK, mitogen-activated protein kinase; mRNA, messenger ribonucleic acid; NOS, nitric oxide synthase; P, phosphate; PIP_2, phosphatidylinositol bisphosphate; PKC, protein kinase C; Q, quiescent state; SHP-2, SH2 domain containing tyrosine phosphatase; TKs, tyrosine kinases.

by electrical stimulation in vitro.[29] Activation of MAPK leads to an increase in activating protein-1 (AP-1) binding to DNA,[86] presumably due to increases in c-jun and c-fos levels, which are both upregulated in rat muscle after contraction in situ.[17,137] AP-1 rose most dramatically when muscle fibers were stretched transversely rather than axially, indicating that fiber orientation plays a role in mechanical transduction.[86] The type of stretch may also determine which of the MAPK pathways are activated, as static stretching increases JNK activity in soleus and extensor digitorum longus and p38 activity in soleus to a greater extent than either achieved after contraction and relaxation.[29] Because stretch also induces satellite-cell activation on fibers,[156] these reports suggest the possibility that MAPK signaling plays a role in that activation, similar to the role of MAPK in c-fos/c-jun expression in glioblastoma cells[1] and vascular smooth muscle.[89] MAPK activation may involve mechanical transduction through the β-dystroglycan component of the dystrophin-associated cytoskeletal protein complex, as the adapter protein growth factor receptor bound protein (Grb2) is known to link this complex to the Ras/MAPK pathway. HGF release may also directly activate MAPK in satellite cells, as reported in glioblastoma cells,[1] where increased c-fos/c-jun mRNA leads to an increase in AP-1, and subsequently to increased expression of c-met mRNA and protein. In vascular smooth muscle, mechanical stimulation increases MAPK and protein kinase C (PKC) activity, c-jun and c-fos expression, and AP-1 activity.[89] Preliminary data indicate that c-fos mRNA expression in satellite cells is rapidly upregulated by stretch in single-fiber cultures. All three MAPK pathways (ERK, p38, and JNK) are likely involved in the satellite-cell activation cascade, as reported for maximal stretch-induction of c-fos mRNA in myometrial smooth muscle,[111] diaphragm,[86] and soleus muscle.[29]

G-proteins may also play an extensive role in the activation of satellite cells. G-proteins are involved in initiating DNA synthesis in fibroblasts cultured from human skin and fetal lung[91,93] via activation of the adenylate cyclase pathway, and in cardiomyocytes by unknown mechanisms.[48] In skin and lung, adenylate cyclase activation resulted in increased expression of HGF mRNA and HGF secretion. If similar steps were involved in muscle, the ensuing increase in HGF release would enhance ligand binding to c-met and lead to satellite-cell activation. Adenylate cyclase is also modulated by NO signals through G-protein–coupled receptors.[85] Activation of NOS-I was achieved in CHO cells within 30 s of treatment with cholecystokinin, a hormone known to operate through G-proteins.[35] Such rapid increases in NOS-Iμ activity would be required for, and consistent with, the hypothesis that a bolus release of NO activates satellite cells in normal muscle.[9] The possible involvement of G-proteins in the activation of PKC via the phosphatidylinositol bisphosphate (PIP2) pathway in muscle is suggested by data from activation studies on cultured human alveolar cells,[164] human skin fibroblasts,[54] and epithelial cells,[119] although these pathways may also be activated by tyrosine kinases that are extensively involved in initiating DNA synthesis in many systems.[119]

Although the reports above did not distinguish between the two types of receptors involved, PKC activation induced notable increases in HGF[54,164] and expression of c-met mRNA.[27] The mechanism by which HGF increases c-met gene expression, possibly through stimulation of c-fos and c-jun,[92,110] may account for signal amplification that recruits more than one satellite cell after a single stimulus such as brief stretching, and could explain some of the apparent heterogeneity in the satellite-cell population response to stretch.[156] PKC activation is also able to induce MAPK activation in vascular smooth-muscle cells[89] and possibly in a lung epithelial cell-line.[27] MAPK activation would also augment c-met mRNA expression[1,27] in a positive regulatory loop, and potentiate the satellite-cell response to a second activating stimulus.

It is very likely that there are balancing mechanisms to prevent or halt such amplification when activation signals stop or decline in intensity. Such downregulation or attenuation may occur via a PKC-mediated pathway, because PKC can block the autophosphorylation of c-met on its tyrosine residues in gastric carcinoma cell-lines.[52] The blockade in c-met phosphorylation would thereby prevent recruitment of other signaling molecules that normally advance the cascade toward DNA synthesis and cell proliferation. This balancing effect via PKC would be important in muscle for controlling the extent of the response to a single activating stimulus and preventing early satellite-cell senescence.

Stretch-activated ion channels may also be involved in activating satellite cells, because ion channel activity can induce PKC activation via the PIP2 pathway in vascular smooth-muscle cells.[91] The regulation of NOS activity by calcium–calmodulin, for example in endothelial cells,[43,49,87] suggests the possibility of a similar pathway in skeletal muscle fibers or in satellite cells themselves, because NOS-I is expressed in satellite cells upon activation in vitro[14-16] and in vivo, even in the absence of the stable localization of NOS-I normally afforded by dystrophin.[13] The calcium influx that occurs with fiber damage also leads to hypercontraction and generates shear forces that are potent signals in many tissues. Shear stress acting on endothelial cells increases activity by protein kinase B (PKB/Akt) and NOS.[43] In skeletal muscle, hypercontraction results in NO-dependent satellite-cell activation in vivo,[9] and in liver after partial hepatectomy, shear increases hepatocyte c-fos expression and cell proliferation.[126,151] Calcium–calmodulin also reverses caveolin3-dependent inhibition of NOS activity. L-type calcium channel opening is stimulated by S-nitrosation via reactive nitrogen species, whereas NO itself inhibits calcium channel activity.[67] Therefore, the movement and concentration gradients of calcium ions may play a substantial role in activation, acting via shear or directly on NOS-Iμ, to release NO and activate satellite cells in skeletal muscle.

Nitric oxide also can signal via guanylate cyclase (GC). GC is a major NO receptor and its dimerization is required to catalyze GTP to cyclic guanosine monophosphate (cGMP). As a receptor, GC has tissue-specific isoforms which vary in structure at four domains: an extracellular ligand-binding domain, transmembrane domain, an intracellular protein kinase-homology domain, and a catalytic domain with cyclase homology.[152] Although there are huge cell–cell variations in the GC-cGMP interval of NO signaling, other large variations in cell-type specific phosphodiesterases can increase NO over a short interval (90 s) and simulate a rapid rise in cGMP. Cyclic GMP acts internally in cells that are stimulated by shear, interleukin-1β, sodium nitroprusside (an NO donor molecule), and L-arginine (the NO substrate) to release NO via phosphodiesterase and PKB/Akt signaling pathways. Importantly, cGMP can also be exported to the extracellular space and induce paracrine activation in neighbouring cells.[63] In vascular

smooth muscle, excess cGMP can block vasoconstriction (in response to potassium, norepinephrine, angiotensin II, and serotonin), and cause dilation via cGMP-dependent potassium and calcium channels. Although not yet examined in skeletal muscle, these GC pathways also have the potential to mediate NOS activity and NO-dependent satellite-cell activation, and affect other possible pathways in NO-independent activation in muscle fibers and satellite cells. Collectively, GC pathways may have a major impact on activation of satellite cells and the recruitment of neighbouring quiescent satellite or stem-cell precursors.

The potential signaling pathways of satellite-cell activation discussed above, although not exhaustive, are compiled in Figure 7. Some pathways in the schematic are known from other tissue systems and may ultimately be excluded from a role in satellite-cell activation in skeletal muscle. However, the capacity for muscle regeneration in mutant animals (although delayed in the absence of NOS-I expression and signaling from NOS-Iμ in fibers[9]), is strong evidence to support the hypothesis that there is more than one pathway for satellite-cell activation. Together, the observations that a single stimulus recruits more than one satellite cell into activation, and recruits an apparently heterogeneous satellite-cell population with distinct courses in time,[156] and the tight control of activation and quiescence shown by satellite cells are suggestive that multiple pathways engage various positive and negative regulatory loops in that control.

FUTURE DIRECTIONS

Although the precise mechanisms of satellite-cell activation from quiescence are not known, a number of points will guide further investigations. The definition of activation is important. Without accommodating the potential for extremely rapid changes in morphological, biochemical, or functional assays in satellite cells in vivo, on isolated fibers or in cell cultures, many steps may be missed by sampling error. The original discovery of NO-mediated satellite-cell activation in vivo was predicated on tests that could reveal a rapid change immediately upon injury, rather than requiring a delay to await eventual changes in expression of a later marker gene or protein, DNA synthesis, and cell proliferation. Activation can be usefully defined as withdrawal from G0, satellite-cell hypertrophy, HGF–c-met colocalization, entry to the cell cycle, and satellite-cell mobilization. This description serves to elaborate expectations of experiments to study a rapid sequence in real time.

In studying satellite-cell activation, it is essential to demonstrate a baseline quiescent state in unstimulated preparations. Without this baseline as a control, a net stimulatory effect and the threshold and timing of an activation response are all left in question. With special attention, quiescence can be maintained in cultures of cells, fibers, and muscles, and by in vivo conditions.

According to criteria that define satellite-cell activation from quiescence, only one growth factor, HGF, and one molecule, NO, have been demonstrated to activate satellite cells. As reviewed elsewhere,[19,28,61,112,155,160–162] other growth factors including insulinlike, fibroblast, and platelet-derived growth factors, strongly promote proliferation and growth that ensue from satellite-cell activation. Further understanding of activation pathways will help in developing methods to regulate specific aspects of the steps from activation to proliferation and growth in muscle tissue.

New data suggest that there is more than one type or population of satellite cells, even on a single fiber. Alternatively, the apparent population heterogeneity among satellite cells may derive from the broad spectrum of physiological and pathological stimuli that singly and together appear to mediate activation. The roles of NO and HGF in satellite-cell activation are likely complex, in view of the many constituents of the ECM, heparan sulfate proteoglycan, syndecan-3, aquaporin, the dystrophin-associated protein complex, and the many sources of NO derived from the three NOS isoforms expressed by various cells and tissues. Nonetheless, new results in the field of satellite-cell activation in skeletal muscle are revealing strong similarities to activation in other systems. The unique system that regulates satellite-cell activation is achieved by morphological and molecular specializations at the topographically intimate juxtaposition of satellite cells and fibers. The parallels between signaling pathways in muscle and nonmuscle tissue offer the potential for future discoveries of drug families that can be targeted to act on specific aspects of the same pathways in particular in skeletal muscle and in disease conditions that affect muscle with such serious consequences to life, health, activity, and independence.

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Appendix 2.
Satellite cell activation on fibers: modeling events in vivo - an invited review

REVIEW / SYNTHÈSE

Satellite cell activation on fibers: modeling events in vivo — an invited review

Judy E. Anderson and Ashley C. Wozniak

Abstract: Knowledge of the events underlying satellite cell activation and the counterpart maintenance of quiescence is essential for planning therapies that will promote the growth and regeneration of skeletal muscle in healthy, disease and aging. By modeling those events of satellite cell activation in studies of single muscle fibers or muscles in culture, the roles of mechanical stretching and nitric oxide are becoming understood. Recent studies demonstrated that stretch-induced activation is very rapid and exhibits some features of satellite cell heterogeneity. As well, gene expression studies showed that expression of the c-met receptor gene rises rapidly after stretching muscles in culture compared to those without stretch. This change in gene expression during activation, and the maintenance of quiescence in both normal and dystrophic muscles are dependent on NO, as they are blocked by inhibition of nitric oxide synthase (NOS). Mechanical, contractile activity is the defining feature of muscle function. Therefore, ongoing studies of stretch effects in satellite cell activation and quiescence in quiescent fiber and muscle cultures provides appropriate models by which to explore the regulatory steps in muscle in vivo under many conditions related to disease, repair, rehabilitation, growth and the prevention or treatment of atrophy.

Key words: regeneration, stretch, myofiber culture, muscular dystrophy, quiescence.

Résumé : Une connaissance des événements qui sous-tendent l'activation des cellules satellites ou, au contraire, le maintien à l'état de quiescence est essentielle pour planifier les traitements qui favoriseront la croissance et la régénération du muscle squelettique normal, pathologique et vieillissant. La modélisation de ces événements dans des études de muscles ou de fibres musculaires isolées en culture améliore la compréhension des rôles de l'étirement mécanique et du monoxyde d'azote. De récentes études ont démontré que l'activation induite par l'étirement est très rapide et présente certaines caractéristiques de l'hétérogénicité des cellules satellites. De même, des études de l'expression génique ont montré que l'expression du récepteur c-met augmente rapidement après l'étirement des muscles en culture comparativement à celle des muscles non étirés. Cette modification de l'expression génique durant l'activation de même que le maintien de la quiescence dans les muscles normaux et dystrophiques sont dépendants du NO, étant donné qu'ils sont bloqués par l'inhibition de la monoxyde d'azote synthase (NOS). L'activité contractile mécanique est la caractéristique déterminante de la fonction du muscle. Ainsi, les études en cours des effets de l'étirement sur l'activation des cellules satellites et la quiescence dans les cultures de fibres et de muscles quiescents fournissent des modèles appropriés pour examiner les étapes régulatrices dans le muscle in vivo dans de nombreuses conditions liées à la maladie, à la réparation, à la réadaptation, à la croissance ainsi qu'à la prévention ou au traitement d'une atrophie.

Mots clés : régénération, étirement, culture de fibre musculaire, dystrophie musculaire, quiescence.

[Traduit par la Rédaction]

Introduction

Satellite cells are quiescent precursors that occupy a "satellite" position immediately outside muscle fibers (Mauro 1961). Satellite cell activation is defined, here, as entry into G1 from quiescence and mobilization (Schultz and McCormick 1994; Anderson 2000). Activation is pivotal to initiating muscle growth and repair (Mauro 1961). Without activation, strength declines, muscles atrophy, and disability ensues. The disabling weakness in age-related muscle atrophy (Dedkov et al. 2003; Welle 2002; Kamel 2003; Thornell et al. 2003) is attributed to profound satellite cell quiescence (Desplanches 1997; Darr and Schultz 1989). In contrast, satellite cells are more hyperactivated and hypertrophic in dystrophic muscle (Anderson 1998, 2000). Initially, satellite cells can repair the muscle fibers damaged by dystrophy, but ultimately, they become senescent (Decary et al. 1996, 1997). Therefore, dysregulated satellite cell activation, either

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low or high, has an enormous impact, limiting muscle function in aging and neuromuscular disease. By learning how satellite cell activation is controlled, we will be able to design drugs that specifically target activation to promote growth and repair.

This laboratory demonstrated that nitric oxide (NO) directly and very rapidly mediates the activation of satellite cells in vivo. Expression or activity of NO synthase I (NOS-I), decreased by pharmacologic treatments, directly from or secondary to genetic alteration, markedly altered satellite cell activation by muscle injury. Such interventions also delayed the interaction between hepatocyte growth factor (HGF) and the c-met receptor expressed by satellite cells (Anderson 2000). However, in vivo studies were unable to determine, in detail, the timing and interaction of NO and HGF signals, were unable to evaluate the need for HGF in activation, and were unable to establish the mechanism by which dystrophin deficiency or muscle aging affected activation. Although subsequent studies using cultures of satellite cells isolated from muscle tissue (Tatsumi et al. 2001, 2002) supported our initial work on satellite cell activation from quiescence in vivo, further advances in the field require more appropriate modeling. The topography of satellite cells on muscle fibers (Anderson 2000), which are the normal source of NO and HGF, is not maintained in cultures of muscle cells, because fibers are removed during cell isolation. This means that satellite cells can't be isolated or cultured in a stable quiescent state; upon attachment to a culture substrate they become activated, albeit more slowly in cultures derived from aged muscle (Tatsumi et al. 1998), and then synthesize NO and HGF (Tatsumi et al. 1998, 2001, 2002). For these reasons, cultured satellite cells are not effective in comprehensively modeling the nature of activation in vivo by NO and HGF.

We recently used cultures of single muscle fibers to study satellite cell activation (Anderson and Pilipowicz 2002; Wozniak et al. 2003). In the fiber model, satellite cells can be maintained in quiescence unless stimulated by treatment, making this model is the best available for studies of activation in vivo (Allen et al. 1997).

This review concerns stretch-induced signaling by NO and HGF, the only factors that have been established to rapidly activate satellite cells *from established quiescence*. Mechanical stretch is a hallmark feature of muscle function. This review will provide an overview of NO and HGF/c-met signaling in normal stretch-activation, and will discuss the impact of dystrophin deficiency and muscle age on activation and NO biology in muscle. Ongoing studies of stretch-induced satellite cell activation on fibers in culture are elucidating the interplay between NO and HGF in activation in normal, dystrophic, and aged muscles. Recent research suggests that NO and HGF/c-met signals are both essential for stretch-induced activation on fibers, and that NO signaling precedes HGF in transducing that activation.

Satellite cell quiescence and activation

Satellite cell activation is triggered by injury, exercise, stretch, or hypertrophy (Anderson 2000). Focal activating signals are usually relayed to satellite cells along the length of fibers, such as occurs after injury to muscle (Grounds and McGeachie 1987; McIntosh and Anderson 1995), although this is not always the case (personal communication, Anderson and Wozniak, 2004). The final outcome of activation is marked by satellite cell DNA synthesis, 24–30 h later (or up to 48 h later in aged muscle). However, the early steps between a mechanical activating stimulus and DNA synthesis are not well established. The literature is rich with microscopy studies of tissues that have revealed the sequence that follows activation by injury. Initially, satellite cell size and shape change, nuclei enlarge, chromatin disperses, and cell volume swells as organelles hypertrophy (for example, Snow 1977, 1990; Schultz et al. 1978, 1985). More recent work, using the tools of cell biology, has established that the HGF, made by fibers and stored or sequestered in the surrounding extracellular matrix (ECM), rapidly colocalizes with the c-met receptor on satellite cells upon injury to normal muscle in vivo (Tatsumi et al. 1998). As well, we showed that, in vivo, NOS inhibition or the absence of NOS-I (from gene mutation in muscle from NOS-I$^{-/-}$ mice, or secondary to dystrophin deficiency, as in mdx mouse muscle) prevented the suite of rapid events marking activation, and determined that NO directly mediates normal activation (Anderson 2000).

The single-fiber culture system retains the ECM on fibers, and is the only model that maintains satellite cells in quiescence under control conditions (Cornelison et al. 2001; Wozniak et al. 2003). Fibers can be isolated and maintained in a serum-replacement medium (Yablonka-Reuveni and Rivera 1994; Yablonka-Reuveni et al. 1999; Anderson and Pilipowicz 2002; Wozniak et al. 2003). Early fiber experiments have shown that proliferation results from activation, and that growth factors, such as insulin-like growth factor (IGF-I) and fibroblast growth factor (FGF), do not activate satellite cells on fibers (Bischoff 1986*a*, 1986*b*,1990*a*, 1990*b*). Rather, IGF-I and FGF increase the proliferation of satellite cells once they are activated, even when that activation results during isolation (i.e., prior to plating fibers for culture). Although IGF-I is often reported to activate satellite cells, those reports typically used cultures of muscle cells that were not quiescent (Papy-Garcia et al. 2002). Others report the *metabolic or molecular activity* of satellite cells, rather than activation from quiescence, and examine satellite cell proliferation or gene expression from a few days to a week after IGF exposure or injury (Hill et al. 2003; Hill and Goldspink 2003; Rabinovsky et al. 2003). Recent experiments on fibers in this laboratory have examined events 0–3 h after an activating stimulus, which has been determined to be a change in DNA synthesis that occurs within 24 h of the stimulation. Applying the definition of activation from quiescence, which we identified as a very rapid process (Anderson 2000; Wozniak et al. 2003), work by Bischoff (Bischoff 1997; Bischoff and Heintz 1994) found that only an extract of gently compressed or crushed muscle caused dose-dependent activation of quiescent satellite cells on fibers. The causative factor in that extract was later found to be HGF (Tatsumi et al. 1998).

At a molecular level, satellite cell activation can be traced in vivo by sequential gene expression after injury. Early immediate genes c-fos and c-jun show increased expression by 3 h (Weiss 1994; Kami et al. 1995), with muscle-specific regulatory genes, such as myf5, showing increased expression by 6 h (Grounds et al. 1992; Cornelison and Wold

1997). Consequently, expression of c-fos and myf5 are valuable markers of *prior* satellite cell activation and can therefore be used to determine the level of activation and repair subsequent to an injury or disease process in muscle (McIntosh et al. 1998; Anderson and Vargas 2003). The sequence of satellite cell gene expression, including muscle regulatory factors and contractile proteins, can be demonstrated using the single-fiber model (Yablonka-Reuveni and Rivera 1994; Cornelison and Wold 1997; Yablonka-Reuveni et al. 1999; Kastner et al. 2000; Cornelison et al. 2000). With activation, expression of FGF and HGF genes rises (Husmann et al. 1996), Notch protein is activated (Conboy and Rando 2002), the IGF-1 isoform changes (Hill et al. 2003), and the expression of MSX1 and myostatin repressor genes declines (Cornelison and Wold 1997; Cornelison et al. 2000; Hawke and Garry 2001). Experiments designed specifically to examine activation from quiescence show findings distinct from those investigating the promotion of proliferation over differentiation, which may involve the same growth factors (Jeanplong et al. 2003; Kamanga-Sollo et al. 2003).

Satellite cells can now be detected in muscle by immunostaining for a small number of proteins restricted to satellite cells. Earlier studies required the painstaking employment of morphology, positional markers of satellite cells, and incorporation of nucleotides during DNA synthesis. While still informative, these studies often had a retrospective design that labeled nuclei in new fibers, for instance, that were derived after proliferation, from cells that once were satellite precursors (Grounds and McGeachie 1987; Anderson et al. 1987).

c-met proto-oncogene is the tyrosine kinase receptor for HGF. In normal muscle, it is only found in quiescent and activated satellite cells (Allen et al. 1995; Cornelison and Wold 1997; Tatsumi et al. 1998). Expression of syndecan 3 and syndecan 4 by satellite cells facilitates tyrosine kinase receptor binding (Cornelison et al. 2001); m-cadherin is a calcium-dependent adhesion molecule made by satellite cells (Irintchev et al. 1994; Beauchamp et al. 2000). These protein markers are functionally relevant because they mediate adhesion, movement, and mitosis. Pax7 specifies satellite cells in development (Seale et al. 2000).

Satellite cell activation, HGF, and c-met

In vivo, HGF activates satellite cells and enhances muscle repair (Tatsumi et al. 1998; Miller et al. 2000). HGF is made by muscle and nonmuscle tissues, and is sequestered in the ECM around fibers. Upon NO-dependent release of HGF from the ECM in muscle, HGF binds to the oncogene c-met. In cells from many tissues, including muscle, HGF/c-met binding induces cells to proliferate and scatter or migrate in culture (Rubin et al. 1993; Bischoff 1997). The c-met receptor is highly expressed in many types of cancer, and appears to be a checkpoint in such activation, interacting with integrins and cytoskeleton proteins to initiate growth, motility, and differentiation. PD098059 and geldanamycins nonspecifically inhibit HGF/c-met signaling in tumor cell cultures (Birchmeier and Brohmann 2000; Birchmeier and Gherardi 1998; Webb et al. 2000; Xie et al. 2001; Maulik et al. 2002; Leshem et al. 2002). HGF/c-met signaling is important in the formation, metastasis, and targeted therapy of cancer (Wang et al. 2001; Kitamura et al. 2000; Ma et al. 2003), but other signaling pathways also contribute to attributes, such as cell mobility and plasticity (Janda et al. 2002). Details of the mechanisms of satellite cell activation on fibers via HGF/c-met or alternate pathways are under currently investigation in a number of laboratories.

The role of HGF in early activation was first described by Allen and colleagues (1995). Subsequent experiments by Tatsumi and colleagues (1998) further characterized that role in vivo in muscle and in vitro. HGF is released from muscle ECM, and rapidly colocalizes with c-met in satellite cells, which then migrate from fibers. In satellite cell cultures, HGF release from cell ECM is NO-dependent, and inhibition of NO synthase (NOS) prevents that release and stretch-activation (Sheehan et al. 2000; Tatsumi et al. 2002). In vivo, NOS inhibition prevents HGF/c-met colocalization and structural changes of satellite cell activation (Anderson 2000). Once activated, satellite cells synthesize both HGF and c-met (Tatsumi et al. 1998; Anderson 1998). Although it is not clear whether NO can mediate activation independent of HGF/c-met signaling, we have established that without NO in vivo, the activation-repair sequence is altered. For example, in muscles that lack neuronal NOS (NOS-Iμ) expression, the early phase of activation, during which c-met-expressing cells are loosened from their attachment to muscle fibers, is lost or delayed (Anderson 2000). The ensuing repair demonstrates alterations in myotube formation and regeneration, even after a period of NOS inhibition restricted to the time of muscle injury (Anderson 2000). It is not known whether NO or HGF/c-met signaling are altered and might contribute to age-related atrophy or sarcopenia, where satellite cells are highly resistant to activation (Muller et al. 2002; Welle 2002; Roubenoff 2003: Kamel 2003).

Satellite cell activation and NO

The potential for the topography of flattened, attenuated satellite cells on fibers, intimately positioned over a large surface area next to the sarcolemma and beneath the surrounding external lamina, has been highlighted by the theoretically large role of shear (layers shifting past one another) in muscle during contraction, as observed in other systems (Dimmeler et al. 1999; Wang and Lautt 1998; Schoen et al. 2001). That topographic relationship has suggested that NO may activate satellite cells (Anderson 2000).

Earlier work has shown that NO is made by NOS-Iμ in muscle (Bredt 1999) and that the protein is linked via PDZ domains to α1-syntrophin in the dystrophin-associated protein complex (Grozdanovic and Baumgarten 1999; Cohn and Campbell 2000; Adams et al. 2000; Albrecht and Froehner 2002; Jones et al. 2003). Without dystrophin, NOS-Iμ gene and protein expression are very low (Brenman et al. 1995); in normal conditions, regulation of NOS-I is highly complex (Wang et al. 1999). NO produced from the activity of inducible and endothelial NOS proteins plays a role in inflammation and tissue perfusion (Reid 1998; Kaminski and Andrade 2001). NO synthesis and release are regulated by tissue structure and mechanical forces, such as shear (Lancaster 1997; Busse and Fleming1998; Dimmeler et al. 1999). In muscle, satellite cells located between fibers and the ECM

are ideally positioned to respond to shear-induced NO release from NOS-Iμ, previously outlined in our working model of NO-mediated satellite cell activation in normal muscle in vivo (Anderson 2000). One of the features most notable in dystrophin-deficient muscle is the susceptibility to mechanical stresses induced by muscle contraction, first demonstrated by Petrof and colleagues (1993).

NO is synthesized by NOS (Alderton et al. 2001), which converts the substrate L-arginine to L-citrulline. NO, a free radical, is rapidly oxidized to form nitrites and nitrates. NO production is often measured as the total NO oxidation product, using a modified Griess reaction (Marzinzig et al. 1997), although there are other and more recently developed strategies for measuring and visualizing NO release (Kojima et al. 1998).

Although satellite cells and satellite-derived myoblasts are typically examined in cell cultures for properties of gene expression and proliferation related to lineage determination and regulators of differentiation, direct evidence of satellite cell activation from quiescence, upon muscle injury, was reported from this laboratory (Anderson 2000). Because activated satellite cells are rapidly mobilized due to HGF/c-met binding (Tatsumi et al. 1998), activation was assayed 0–30 min after injury, as the yield of satellite cells loosened in a single whole muscle. Activation was calculated as a ratio of satellite cell yield from an injured muscle/the yield from the intact contralateral muscle. Without injury, the ratio is theoretically equal to one. Experiments have shown that activation doubled immediately after injury and was mediated by NO. This was an assay based on basic concepts in cell biology, but subsequent studies have been consistent in demonstrating NO-dependent activation in vivo, as well as later steps in muscle regeneration, which is outside the scope of this review.

NO-mediated activation was demonstrated by blocking activation with L-NAME, a NOS inhibitor. HGF/c-met colocalization and satellite cell enlargement were also delayed by NOS inhibition. Consistent with inhibition studies, early activation was completely absent in muscle without NOS-Iμ expression (mdx, NOS-I$^{-/-}$ transgenic mice); it would be interesting to learn whether NOS-I$^{-/-}$ mice exhibit muscle wasting that increases with age or exercise. Experiments have demonstrated that the outcome of injury-induced repair in normal and dystrophic muscle was poor after NOS inhibition (Anderson 2000; Anderson and Vargas 2003). That work provided strong evidence that NO mediates satellite cell activation and repair in vivo, and it has since been supported by other in vivo studies that showed that increased NO in mdx mice reduced the severity of dystrophy and promoted muscle growth and repair (Wehling et al. 2001; Anderson and Vargas 2003).

Satellite cell activation and aging muscle

In old age, muscle demonstrates atrophy (a loss of cross-sectional fiber area called sarcopenia) (Kamel 2003; Welle 2002) and significant, progressive weakness (Lynch et al. 2001; Fluckey et al. 2002). Satellite cells from aged muscle show a long latent period between activation stimulus and proliferation (Tatsumi et al. 1998; Yablonka-Reuveni et al. 1999). This "mitotic nadir," when satellite cells are profoundly quiescent (Desplanches et al. 1987; Desplanches 1997), is a sensitive predictor of primary muscle atrophy in aging (Darr and Schultz 1989). Microgravity and hindlimb suspension are used to model aging muscle (Norman et al. 2000; Jozsi et al. 2001; Fluckey et al. 2002; Biolo et al. 2003). The functional indication of the status of aged muscle is the reduction in satellite cell quiescence that occurs with intermittent reloading and exercise, although activation does not attenuate atrophy or weakness (Evans 2002). The explanation for persistent atrophy and quiescence, which resist stimulation by exercise, are not apparent; however, it is well established that loss of telomere length and restricted or temporally altered activity of mitogen-activated protein kinase are not limiting factors (Thornell et al. 2003; Yablonka-Reuveni et al. 1999). Indeed, satellite cells in aged muscle activate normally in response to denervation (Dedkov et al. 2003). Future studies of NO and HGF/c-met signaling in aged normal muscle will reveal how profoundly quiescent satellite cells in age-related atrophy can be activated to treat or prevent this disabling condition.

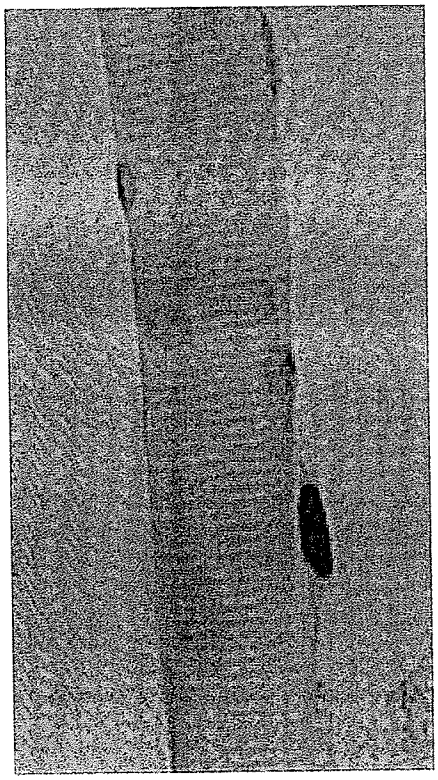
Satellite cells, muscular dystrophy and NO

Dystrophin protein is absent from muscle in human Duchenne and mdx mouse muscular dystrophy. As a result, muscle-fiber membranes are susceptible to injury from normal activity (Petrof et al. 1993; Brussée et al. 1997). Dystrophic muscles progressively weaken from repetitive fiber damage, inflammation, and fiber loss, even though muscle repair is initially effective in mdx mice, as has been previously demonstrated (Anderson et al. 1987, 1988, 1998; Zacharias and Anderson 1991). Weakness is lethal in Duchenne muscular dystrophy and shortens lifespan in mdx mice (Crawford et al. 2000). Relevant to this review, dystrophin deficiency causes downregulation of NOS-Iμ gene expression and protein in the dystrophin-associated protein complex (Cohn and Campbell 2000). In consequence, the time-course of satellite cell activation by NOS inhibition after injury in mdx muscle is identical to that produced in normal muscle (Anderson 2000). Dystrophic cardiomyopathy also involves disruptions of NOS-I and induction of NOS-II (Bia et al. 1999). The persistent absence of NO release from fibers in mdx muscle accompanies increased satellite cell activation. For this reason, it has been proposed that NO in normal muscle helps to maintain quiescence, in addition to stimulating activation (Anderson 2000). Further study of satellite cell activation in mdx muscle is required to understand how dysregulated activation and NOS-Iμ gene expression could be used to treat muscular dystrophy (Anderson and Vargas 2003).

Recent studies on satellite cell activation

Recent experiments have employed the single-fiber culture model to examine satellite cell activation in cell cultures, and to integrate our in vivo model with new data on activation via NO and HGF/c-met signals in stretch.

Fig. 1. A segment of a single muscle fiber isolated from the flexor digitorum brevis muscle. A satellite cell is prominent, adhered to the fiber and slightly elevated above the sarcolemma. The nucleus is positively stained for BrdU incorporation. The fiber was cultured in controlled serum-replacement medium in the presence of BrdU, subjected to 30 min of cyclical mechanical stretching, and maintained for a further 23.5 h before fixation and staining. As reported (Wozniak et al. 2003), stretch-induced activation from quiescence is rapid, and appears to be bimodal over a 2 hour time-course. In the absence of stretch, a very low level of activation demonstrated the baseline quiescence in these experiments.



NO and HGF activate satellite cells associated with isolated single fibers

We used the single-fiber model to study activation by NO and HGF (Anderson and Pilipowicz 2002). Fibers isolated from flexor digitorum brevis muscles (FDB) were plated on Vitrogen collagen. Bromodeoxyuridine (BrdU) was added to the media to label DNA synthesis. Activated satellite cells in these experiments were prominently elevated from the sarcolemma of fibers, and show BrdU-positive nuclei (Fig. 1). In these fiber cultures, satellite cell quiescence was maintained by culturing in 20% serum-replacement media (CSR-2, Sigma, St. Louis, Mo.) in DMEM (Anderson and Pilipowicz 2002; Wozniak et al. 2003), without the high serum concentrations that are typically used to stimulate proliferation (Conboy and Rando 2002). Activated satellite cells ($BrdU^+$ cells/fiber) were counted. Activation was demonstrated to be dependent on the levels of NO (related to level of the substrate L-arginine) and HGF in the medium, and was prevented by NOS inhibition using L-NAME, the non-specific inhibitor, consistent with earlier in vivo and cell culture data (Anderson 2000; Tatsumi et al. 2001; Sheehan et al. 2000). This was the first study to report on early satellite cell activation from a quiescent state measured on fibers under the control conditions.

Mechanical stretching rapidly activates satellite cells on fibers

Our work was also the first to use the single-fiber model to study stretch-activation (Wozniak et al. 2003). Fibers were plated in dishes with either a flexible or rigid (control) substrate (FlexCell Inc.) and put in vacuum mats, which apply and release a vacuum, producing a cyclical 10% stretch (Anderson et al. 1993) at 4 cycles per min (Tatsumi et al. 2001). BrdU was added to the serum-replaced media (0 time) and fibers were stretched (or not) for 0–3 h. Cultures were maintained in BrdU for 24 h, so any cell entering S-phase during that time could be identified from BrdU-positive immunostaining. The number of $BrdU^+$ cells/fiber indicated the level of satellite cell activation. Fibers were resilient to stretch and retained satellite cells. Activation of satellite cells in unstretched (control) fiber cultures was very low, and it stayed low, indicating stable satellite cell quiescence in these cultures. We also found there were two peaks of 2-fold activation, one after 30 min of stretch, and a second after 2 h of stretch. It was speculated that this time-course profile of activation may be derived from the heterogeneity of early and later responder satellite cells. Distinctive populations of satellite cells may ultimately be identified by their activation through different signaling pathways. Alternatively, activation may be a sequential or gradual cascade of events, rather than a binary or off–on process.

In the same study (Wozniak et al. 2003), in situ hybridization for c-met mRNA in satellite cells was used to examine the whole population of satellite cells resident on a population of fibers. One deficiency of the fiber model is that nonactivated satellite cells are not readily identified, so these experiments were designed to quantify the population of satellite cells resident on a normal fiber. However, a positive aspect of the fiber model is that it allows many hundreds of intact fibers to be isolated from a single FDB muscle, providing a powerful sample of a population, useful in statistical analyses. We found a mean of approximately 1.4 $c\text{-}met^+$ satellite cells resident on a single fiber. According to identification with c-met mRNA expression, the satellite cell population ranged from 0–6 cells per fiber. Assessment of the total satellite cell population, using fiber-by-fiber counting, will allow future studies of the population response to stretch. Early data (personal communication, Wozniak and Anderson, 2004) suggest that the number of $c\text{-}met^+$ satellite cells may increase rapidly after a brief period of stretch, which could account for the second peak of stretch-activation observed, and define the second subset of satellite cells proposed in fiber experiments (Wozniak et al. 2003) and in vivo studies. Alternatively, there could be a threshold of stretch required to reinforce the activating signal in a single population. Both possibilities would have a direct impact on the interpretation of activation kinetics and signaling via HGF/c-met interaction. It is not known how the level of expression by other relevant genes, such as NOS-Iμ, c-fos, pax7, myf5, and m-cadherin, might respond to stretch-

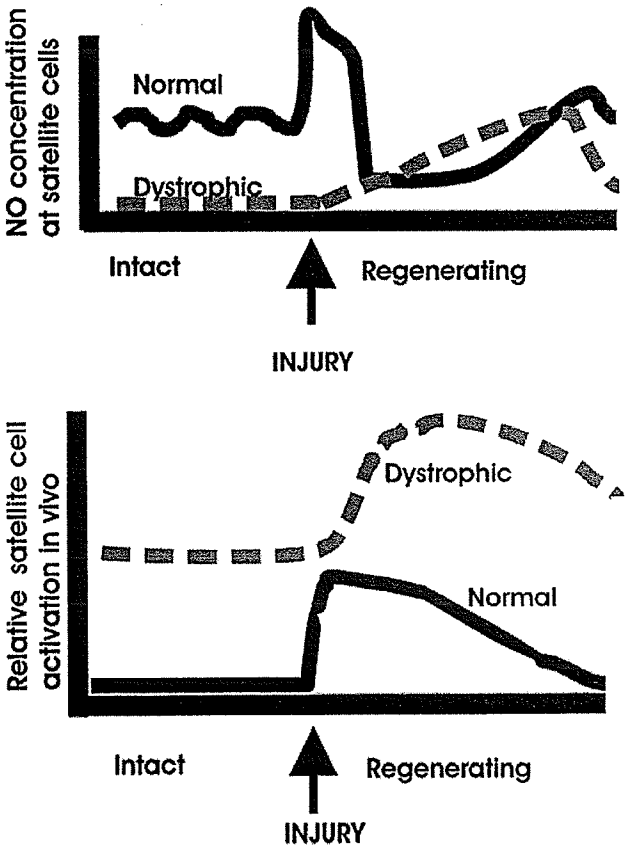
activation, or whether they are differentially expressed by normal, dystrophic, and aged fibers.

Mechanical stretch-activation is NO-dependent

Although the requisite level of signaling traffic between a fiber and resident satellite cells during quiescence is unknown, the presence of intact fibers is likely to be a critical modifier of satellite cell activation by signals from NO, HGF, and stretch. Early data support that notion, and show that normal fibers play a role in maintaining quiescence and initiating satellite cell activation (personal communication, Wozniak and Anderson, 2004). Stretch resulted in increased activation by satellite cells in cultures of normal fibers, and L-NAME prevented stretch-activation, in accordance with earlier reports of NO-dependent HGF release and activation in cell cultures (Tatsumi et al. 2001, 2002). However, NOS inhibition actually increased activation *in the absence of stretch*. Although counterintuitive, this suggests that NO plays a role in maintaining quiescence, as predicted earlier (Anderson 2000). On fibers, HGF increased activation without stretch. Teleologically, it makes sense that a muscle fiber would provide a regulatory mechanism to maintain quiescence that is distinct from that required to activate satellite cells, even if it uses the similar "NO currency." Other molecules are likely involved in regulating and restoring quiescence, and are not reviewed here. Interestingly, the observation that NO contributed to maintaining quiescence of satellite cells on fibers was made in the same set of experiments and at the same time as we observed that stretch also increased c-met expression. These experiments used a model of whole muscle cultures that we recently developed to enable more comprehensive assays of gene expression during satellite cell activation (see below). Understanding the differences between satellite cell activation on fibers and in cell cultures, and the roles of adhesion and reversible satellite cell arrest (Milasincic et al. 1996) is essential to understanding the nature of activation events in vivo.

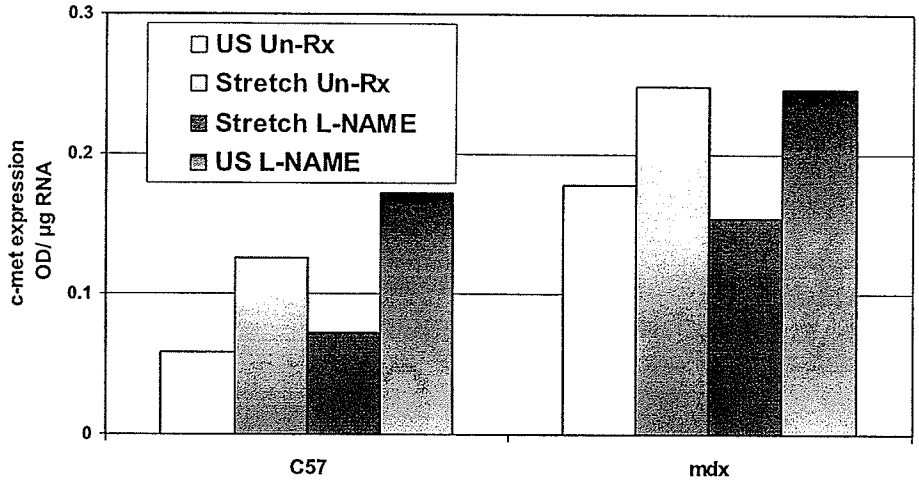
The theoretical time course of NO concentration acting on satellite cells, and the corresponding level of activation in normal and dystrophic muscle, is presented in Fig. 2, which was generated using the original model of NO-mediated activation and recent data. Mdx fibers lack NOS-Iμ because dystrophin is absent and a dystrophin-associated protein complex is deficient. It has been proposed that continuous absence of NO may permit a "hair-trigger" or overactivation of satellite cells on mdx fibers. This was suggested after hypertrophic satellite cells were observed in mdx muscles, a feature common to activation in the absence of injury (Anderson 1998). With this new schematic comparing levels of activation and NO concentration in normal and mdx muscle, we suggest that satellite cells in mdx muscle are hyperactivated in the absence of injury and respond to injury somewhat later than in normal muscle (Anderson 2000). This latter notion is attributed to the finding that the NOS-Iμ gene is expressed and active in producing NO in activated satellite cells, as well as in normal muscle fibers (Anderson and Vargas 2003). The steps in regeneration that follow injury and activation include proliferation and amplification of satellite cells, where NOS-Iμ and c-met are both expressed. Subsequently, muscle precursors fuse into multinucleated myotubes; this interval is accompanied by a rapid down-

Fig. 2. A hypothetical time-course of NO levels at the satellite cell, as released from NOS-Iμ (top panel), and the extent of satellite cell activation (lower panel) extant in normal (solid black lines) and dystrophin-deficient, dystrophic muscle (dashed grey lines) muscle, based on the proposed model of activation (Anderson 2000). Quiescent satellite cells in intact muscle are subject to pulses of NO, which are thought to help maintain quiescence. At the time of injury, a large NO-transient appears (a large bolus of NO followed by absence of NO), which initiates satellite cell activation. Levels of activation continue to rise as satellite cells are exposed to cytokines, growth factors, and other molecules. As satellite cells proliferate, NO levels increase (produced by myoblasts (Tatsumi et al. 2001)) and then become pulsatile as new myotubes and regenerated fiber segments are formed. In dystrophic muscle, the level of NO in the region of satellite cells is low because of the absence of dystrophin and NOS-Iμ down-regulation. In this condition, satellite cells are hyperactivated, compared with those in uninjured normal muscle. At the time of injury, and in the absence of an NO-transient, activation increases further in response to fiber damage. Activated satellite cells proliferate and the level of NO rises in that environment, because NOS-Iμ is expressed by satellite cells and myoblasts in the absence of dystrophin. Once new myotubes and regenerated segments of dystrophic fibers are formed, the NO level drops and activation returns to the typical hyperactivation state (e.g., hypertrophy (Anderson 1998)) observed in dystrophic muscles.



regulation of c-met gene expression, while NOS-Iμ is directed by dystrophin to become localized with the dystrophin-associated protein complex inside the sarcolem-

Fig. 3. A histogram showing results of a recent experiment that examined c-met gene expression in normal and dystrophic mdx EDLs. EDLs were maintained in culture overnight in controlled serum-replacement medium, and exposed to one of 4 treatments: control condition, unstretched, untreated (US-UnRx,), mechanical stretch alone for 30 min (Stretch-UnRx), stretch + the NOS inhibitor L-NAME (Stretch L-NAME), and L-NAME alone (US L-NAME). RNA was isolated from muscles immediately after the 30-min treatment period, and relative expression was quantified by RNAse protection assay (Anderson and Vargas 2003). The level of c-met gene expression was higher in mdx than normal muscle, and was increased by stretch and by L-NAME in both types of muscle. Results show that the stretch-induced increase in c-met expression was NO-dependent; c-met levels were reduced by stretching in the presence of L-NAME.



ma. We propose that this process of repair in normal and dystrophic muscles may gradually restore the characteristic levels of NO and either quiescence in normal muscle or typical levels of hyperactivation in dystrophic muscle.

The mechanism by which the satellite cell population is renewed by some selective or stochasitic sequestration, presumably derived from cells that were previously satellite cells or, alternatively, are stem cells, is not known. The stem cell literature is reviewed elsewhere, with much discussion of the putative functional markers of this small population in muscle (Beauchamp et al. 1999; Zammit and Beauchamp 2001; Seale et al. 2001; Hawke and Garry 2001; Blau et al. 2001; Deasy and Huard 2002; O'Brien et al. 2002; Goldring et al. 2002; Morgan and Partridge 2003; Asakura 2003; Chen and Goldhamer 2003). An increasing amount of evidence suggests that stem cell therapies will eventually find clinical applications (Tamaki et al. 2003; Brazelton et al. 2003; Cossu and Bianco 2003; Ikezawa et al. 2003; Cooper et al. 2003). However, it is not known what signals are used to mediate activation and quiescence by the putative stem cells that may lie resident in skeletal muscle (either within and (or) external to the external lamina around fibers). In part, this is likely related to the rarity of the stem-like cells that are not yet reproducibly defined by marker genes and that are just beginning to be identified in situ in muscle, and during regeneration.

Whole muscle cultures scale-up stretch-activation studies

Although the detailed pathways regulating satellite cell activation on fibers are still under investigation, we have developed a model whereby whole intact muscles, most easily isolated when they display two anatomically discrete tendons, were cultured under the same conditions as the fiber model to maintain satellite cell quiescence over at least 48 h. Evidence from experiments on normal and dystrophic (mdx) muscle cultures was consistent with the idea that NO mediates quiescence. Figure 3 shows that, without stretching the muscle cultures, c-met expression by satellite cells (Wozniak et al. 2003) was higher in mdx muscle than in normal muscle. Since mdx muscle lacks NOS-Iμ, these data agree with the idea that quiescence is maintained in normal muscle by a pulsatile NO release.

Recent studies on stretch-activation have used such cultures of whole FDB or extensor digitorum longus (EDL) fast-twitch muscles, pinned in FlexCell plates, to scale up studies of gene expression and stretch. After stretch, normal muscles showed a 2.5-fold increase in BrdU uptake, assessed using Southwestern blots of DNA extracts, immunoprobed for BrdU. The level of stretch-activation was similar to that observed for stretched normal fibers (Wozniak et al. 2003). Histology was normal after 36 h in culture muscles, and BrdU-positive satellite cell nuclei were found deep in the muscles. Figure 3 shows that stretch induced a rapid increase in expression of c-met gene transcripts in muscle cultures from normal and mdx mice. That increase was NO-dependent (it was prevented by L-NAME), indicating that NO plays a role in satellite cell quiescence. As well, a role for fibers and NO in maintaining normal quiescence was evidenced by data showing that in the absence of stretch, a decrease in NO provided by exposure to L-NAME induced an increase in c-met gene expression. The potential for NO to provide a fulcrum or balance setpoint in regulating the transition from quiescence to activation, and possibly back to quiescence, is intriguing, and currently under investigation.

Summary

Satellite cell activation is essential to muscle for growth

and regeneration after damage caused by trauma, ischemia, infection, or acquired or genetic neuromuscular disease. Without activation in aged muscle, strength declines, muscles atrophy, and disability ensues. In dystrophy, where satellite cell activation is dysregulated by a loss of NOS-Iμ, such as occurs in Duchenne's muscular dystrophy, progressive loss of regenerative capacity, which parallels decreased replicative potential of satellite cells (Decary et al. 1996, 1997; Heslop et al. 2000; Renault et al. 2002; Cooper et al. 2003), muscle weakness progresses to lethality. Satellite cell activation is, therefore, an essential step in muscle repair and adaptative functions.

By studying satellite cell activation on fibers and in muscles, we determined that single fibers and whole muscles in culture can be used effectively to study early activation events. We found that a mechanical stretch stimulus activates satellite cells on fibers (and in single cell cultures) in 2 distinct peaks over 2 h, suggesting a heterogeneous satellite cell population. As well, expression of the c-met gene appeared to be rapidly increased by stretching, and that increase was NO-dependent. Therefore, NO appears to be important in enabling stretch-induced satellite cell activation and also in maintaining satellite cell quiescence on skeletal muscle fibers. The latter observation will require studies of single fibers and muscles to determine in vivo differences between control and cell culture activation and quiescence, and also to determine the distinctive features regulating the switch from quiescence to activation (and potentially back to quiescence) of satellite cells in normal and dystrophic muscle.

Ultimately, studies of the fundamentals of quiescence and activation in skeletal muscle satellite cells, and the features that distinguish the mechanisms regulating those events in normal and dystrophic muscle, will direct research toward finding new ways to treat neuromuscular disease and to increase repair and growth of skeletal muscle. A thorough understanding of the NO signals that transduce mechanical stretching into satellite cell activation will allow us to design drugs that specifically promote muscle growth and repair in neuromuscular disease and aged muscle, with other potential applications in agriculture, rehabilitation, and exercise or sports medicine.

Contraction and stretch are defining functions in normal muscle. The study of stretch effects on satellite cell activation on single fibers and in muscle cultures will enhance our understanding of the role of NO signaling in activation. The best possible models, to date, for studying activation in vivo, are those in which satellite cells can be maintained in a quiescent state on fibers until stimulated to activate. On fibers and in muscles in culture, satellite cells maintain the ability to participate in the transmembrane signaling cascades contributed by proteins in the ECM, membrane, and muscle cytoskeleton (Geiger et al. 2001).

Interestingly, the idea that NO release from fibers by shear, stretch, or other stimuli, has a significant impact on normal satellite cell activation (Anderson 2000) clearly does not rule out NOS-independent pathways for activation and regeneration. This is well demonstrated by the capacity for regeneration in muscles of mdx and NOS-I$^{-/-}$ mice. However, those alternative and potentially parallel pathways have not yet been identified. It is also not known whether conditions such as age-related atrophy reflect a change in the expression or activity of NOS-Iμ or c-met genes. In aged animals, there is an increased resistence of satellite cells to activating stimuli in muscle. The same resistence has been noted in persistent atrophy after reloading exercise paradigms, following experimental limb suspension in aged animals. Both are broadly similar to the muscle atrophy observed after a period of microgravity. However, the mechanisms underlying the common feature, and a progressive functional deficit in muscle strength that accompanies that resistence to activation, and remain open for discovery.

Conclusion

Future research will extend our understanding of mechanical activity and stretch as a stimulus that activates satellite cells, and explore the role signals play in NO release and c-met gene expression and activity during activation and quiescence. Cultures of fibers and muscles with quiescent satellite cells can be used very effectively to model activation in vivo. In the future, they will be used to address the earliest events that signal activation, which will provide new information on activation in dystrophin-deficient neuromuscular disease and in age-related atrophy.

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