Satellite cell activation in adult zebrafish (Danio rerio) single muscle fibre cultures

by

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A Thesis
Submitted to the Faculty of Graduate Studies of
The University of Manitoba in partial fulfilment of the
requirements of the degree of

Master of Science (MSc)

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Winnipeg, Manitoba

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Abstract

Satellite cells (SCs) are muscle stem cells that stay in a metabolically and mitotically quiescent state in adult skeletal muscle until activated. In mammals, SCs are activated and enter into the cell cycle for growth and regeneration. The mechanism initiating SC activation in vivo and in vitro, mediated by nitric oxide (NO) and hepatocyte growth factor (HGF) is described in the mouse model, but not in other species. Here, we assessed SC activation by counting bromodeoxyuridine (BrdU)-immuno-positive cells, and found that SC activation in zebrafish single muscle-fibre cultures is also NO and HGF dependent, peaking at 1 mM isosorbide dinitrate (ISDN, an NO donor drug) and 10 ng/mL HGF respectively, using dose-response experiments. Moreover, HGF signalling via the c-Met receptor is involved in the SC activation pathway and is considerably affected by temperature (i.e., 21 °C). Overall, understanding NO-HGF-c-Met signalling in SC activation gives new insights on fish muscle growth and conservation of regulatory pathways between species.

Acknowledgements

First and Foremost, I would like to express my deepest appreciation to my supervisor Dr. Judy Anderson, for her patience, continuous and generous support, encouragement, and inspiration throughout my research and study of the Master's degree. I am sincerely and deeply grateful to you for being such a great role model for me in my research career, more importantly, my personal life. Not only your ample knowledge, motivation and enthusiasm to science, but also your great personality were and will be affecting me throughout my whole life time.

I would also like to express my special thanks to my committee members: Dr. Gary Anderson and Dr. Jeff Leiter for their insightful comments and constructive suggestions, as well as hard questions. I am deeply appreciative to the Department of Biological Sciences for the financial support, as well as NSERC and GETS funding from Dr. J. Anderson and the Faculty of Graduate Studies.

I own my heartfelt gratitude to my wonderful lab colleagues: Ritika Uphadaya, Wanda Snow, Deanna Gigliotti, and Jeremiah Yarmie for their invaluable friendship, assistance and unconditional support.

Last but not least, I want to thank my families: Kaishuo Zhang, Rong Liu, Jiayu Zhang and Liyun Zhang for your unconditional love and support.

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Introduction

Skeletal muscle function in fish is of fundamental significance for both low speed swimming (use of red muscle) and high speed swimming (use of white muscle). Similar to mammals, red muscle is enriched in mitochondria and blood supply and uses aerobic metabolism to produce energy, while white muscle is committed to using glycolytic and more anaerobic metabolism and has fibres with bigger diameter but fatigues more quickly (Fauconneau et al., 1995). It is well known that while mammalian muscle has a ratio of red and white muscle fibres close to a 1:1 ratio on average, muscles in teleost fish are constituted predominantly by white muscle fibres (90-95%) (Fenichel, 1966; Johnston, 1999). Additionally, after birth, muscle fibres in mammals can only show an increase of fibre size by hypertrophy, while muscle in fish can grow by increases in both the number and the size of muscle fibres in a combination of hyperplasia and hypertrophy. The reasons underlying these phenomena (i.e., variations in muscle growth across taxa) are not well known, especially those involving satellite cells and the molecular mechanisms of myogenesis (muscle formation) between mammals and fish.

In mammals, satellite cells (SCs) are resident muscle stem cells which originate from embryonic development and maintain a quiescent state (G0 phase) after birth; they occupy a position between the sarcolemma (muscle fibre membrane) and the basal lamina (a layer of connective tissue) (Mauro, 1961; Schultz et al, 1978). Once receiving an activation signal, quiescent SCs enter the cell cycle and start dividing and are responsible for muscle growth or regeneration (after injury) since muscle fibre nuclei are post-mitotic and cannot divide. It is of particular importance to understand the mechanism(s) of SC activation that initiates subsequent steps of myogenesis (muscle growth or regeneration).

To date, chemical and mechanical mechanisms are known to activate SC in mammals, as studied using model systems developed from rat and mouse muscle (Tatsumi et al., 2002). Following SC activation and division, daughter cells, also named myoblasts, possess the capacity for further division before fusion with each other or with existing muscle fibres for muscle growth or repair.

Using zebrafish single muscle fibre cultures, established by methods modified from previous studies on mouse muscle, this project aimed to explore the potential conservation of the mechanisms of SC activation during myogenesis across species. The information on zebrafish SC activation in turn, provides an insight into the more well-known regulatory pathways understood to occur in the mouse model. Specifically in this thesis, following the review of literature on myogenesis in mammals and zebrafish and the method of single muscle fibre culture, mechanisms of SC activation by chemical (HGF-c-Met pathway) and mechanical stimuli (stretch-induced) were tested using fish single fibre cultures. Studies on the influence of temperature allowed further study of the interaction with the mechanisms of activation were examined. Results and discussion of the individual experiments, and consideration of future studies, conclude the thesis.

Chapter 1: Literature Review

1.1 Skeletal muscle in adult mammals

1.1.1 Overall relationship with the motor system

Skeletal muscle is a major component of the motor system, and is highly efficient at converting chemical energy directly into mechanical energy (Enoka, 1988). Muscles have both nerve and vascular supplies which are studied in the gross anatomy laboratory, and which are crucial for voluntary movement. Muscle contraction is regulated via nerve connections to each muscle fibre (see below, section 1.1.2) and feedback to the body goes through sensory nerves and reflexes at different levels in the nervous system (Rayment et al., 1993). Although these processes are clearly important in muscle function and in development, growth and regeneration, this thesis will consider nerve-independent processes related to growth and regeneration in skeletal muscle.

1.1.2 Muscle tissue

Skeletal muscle, the subject of this thesis, is one type of muscle in the body; the other two types are smooth muscle (around tubes such as gut and blood vessels) and cardiac muscle in the heart (Buckingham et al., 2003). Skeletal muscle is made up of muscle fibres and connective tissues (tendon, fascia, adipose tissue and ligaments) (Buckingham et al., 2003). The whole muscle is surrounded by epimysium which is a thickened layer of moderately dense connective tissue that connects the muscle to its tendons and then to the tendon attachments on bone, skin or cartilage. It protects the muscle, holding fibre bundles together, and in doing so, separates muscles from one another for relatively independent contraction (Gordon et al., 2000). Within a muscle,

there are fascicles or bundles of muscle fibres, which are wrapped by perimysium, a thinner layer of connective tissue that separates fibre bundles and allows blood vessels and nerves to reach into the muscle belly. Finally, single muscle fibres are covered by a very thin layer of connective tissue, referred to as endomysium (Squire et al., 2005). This allows each fibre to contract without interfering with other fibres and protects the fibre and its resident satellite cells. As well as the internal anatomy of muscle fibres in a muscle, which changes depending on which type of fibre is under examination (see section 1.3), the architecture of muscles also varies between different muscles in the body (Lefaucheur, 2010). For example, the flexor digitorum brevis muscle which has been used in many mouse-fibre culture studies because of its short fast-twitch fibres (see section 3), is multipennate; it has four different bellies of muscle, each with its own distal tendon, all connected to a single proximal tendon; this architecture regulates coordinated contraction of the digits in the foot. By comparison, the biceps muscle is bipennate and has two muscle bellies.

1.1.3 Muscle fibres

Skeletal muscle cells are long, cylindrical and multi-nucleated cells called muscle fibres due to their thread-like appearance under a microscope (Noguchi et al., 2000). Generally, they are only barely visible to the naked eye. Muscle fibres are formed in development or regeneration, by the fusion of many immature mono-nuclear cells which are undifferentiated cells called myoblasts (Fluck and Hoppeler, 2003). The plasma membrane and cytoplasm in muscle fibres are called the sarcolemma and sarcoplasm, respectively (Bottinelli and Reggiani, 2000). Similar to other cells, there are organelles in the sarcoplasm, including nuclei, mitochondria, rough endoplasmic

reticulum, the golgi apparatus, and the sarcoplasmic reticulum; unique to skeletal muscle, the sarcoplasm also contains T-tubules (see below). As well, there are myofibrils in the sarcoplasm (Bottinelli, 2001). Myofibrils are bundles of myofilaments. Two proteins, myosin and actin, also the primary myofilaments involved in contraction, and are called thick and thin filaments, respectively (Pette and Staron, 2001). They are organized in a very distinctive banding pattern that shows up as transverse "stripes" along the length of a fibre, in which the repeating pattern is called a sarcomere.

Sarcomeres are the basic unit of muscle contraction. They are organized between two Z-lines that cross (and attach to) actin filaments at the so-called H-line in the I-band; between two I-bands there is an intervening A-band of myosin filaments (Morgan and Proske, 2003). Myosin and actin filaments in the sarcomere cooperate or interact to produce contraction of a muscle fibre by sliding past one another using cross-bridges to pull the myosin filament along actin in the cross-bridge cycle involving calcium ions and myosin ATPase and using ATP to generate force (Pollard and Borisy, 2003). This sliding filament process shortens the sarcomere between the Z-lines in a concentric contraction, and lengthens the sarcomere in an eccentric contraction.

The contractile process is regulated by the structure inside a muscle fibre, and by calcium-release mechanisms. Each myofibril is surrounded by vesicles of sarcoplasmic reticulum. Those vesicles hold a reserve store of calcium ions that is used to produce muscle contraction by interaction with troponin, tropomyosins and ATPases (Chhabra and Higgs, 2007). The sarcoplasmic reticulum branches around fibrils and has distal "end sacs" called terminal cisternae. There are also T-tubules that project from the sarcolemma into the inside of a muscle fibre (Defranchi et al., 2005). Around the T-tubules as they

penetrate between fibrils and at particular sarcomere regions (A/I junction in mammalian muscle), two terminal cisternae surround the T-tubule and the three vesicles that appear in a section by electron microscopy are called a triad. The T tubule is a critically important component of the signalling pathway that transmits a depolarization of the sarcolemma from the action potential in a nerve via the nerve-muscle junction (NMJ) into the deeper regions of the muscle fibre. That depolarization stimulates the sarcoplasmic reticulum to release calcium and the increase in calcium ion concentration within the sarcoplasm leads to muscle fibre contraction (Bottinelli and Reggiani, 2000).

1.1.4 Myogenesis

The formation or development of skeletal muscle fibres is called myogenesis. Myogenesis occurs in embryonic development and also in regeneration of muscle fibres after injury in post-natal or adult muscle (Conboy and Rando, 2002). The stages of myogenesis are: formation of myoblasts, proliferation, migration, fusion into fibres, differentiation, development of satellite cells (SCs), innervation and growth of fibres. These will be considered, one at a time, in the following paragraphs. There are different molecular or cellular markers for detecting satellite cells or muscle status and these markers are continuously being revised as we understand more about each part of the many processes in muscle formation (Pownall et al., 2002). As well, this section begins with an overview of developing skeletal muscle fibres, up until the establishment of satellite cells on fibres. At that point, the details of satellite cell structure, function, activation and quiescence will be discussed in detail, followed by presentation of myogenesis in regeneration.

Formation of myoblasts

All the skeletal muscles except head muscles are derived from mesodermal precursor cells which originally come from the dorsal part of somites; this region is called the dermomyotome. Somites begin as epithelial spheres of paraxial mesoderm (Karalaki et al., 2009). Each somite develops specifically in time and space, based on the signals that are received from surrounding tissues which induce the expression of myogenic regulatory factors (MRFs) by precursor cells (Dockter, 2000). The dorsal and ventral parts of a somite will develop into the dermomyotome and sclerotome, respectively, after expression of the transcription factor, Pax3, in the more dorsal cells; this expression induces a transition from epithelia-mesenchyme toward dermomyotome (Dockter, 2000). The sclerotome will form ribs, whereas the epithelia-mesenchyme maintains an epithelial organization. A sheet of pseudo-stratified columnar cells is added to the epithelial-type cells, and together they form a region referred to as dermomyotome. With the expansion of the dermomyotome dorsomedially and ventrolaterally (due to cell proliferation), the edges of the dermomyotome adjacent to sclerotome produce myogenic cells that constitute a third layer within the somite (layered in the dorsoventral axis), named the myotome. The myotome is divided into the epaxial myotome that is near the neural tube and the hypaxial myotome that gives origin to myogenic cells that will become muscles of limb, trunk, diaphragm and neck (Dockter, 2000). However, in 2006, Devoto et al. found particular cells that they called ‘external cells’ that express Pax3 and Pax7 at the surface of the somite. These fairly superficial cells can also form a dermomyotome-like tissue and produce myoblasts and muscle fibres.

Myogenic cells expressing Myf5 and MyoD are referred to as myoblasts (Shi and Garry, 2006). The vast majority of myoblasts in mature muscle are myogenic precursor cells derived from activated satellite cells (SCs). Myoblasts can also be derived from other sources outside skeletal muscle, such as hematopoietic stem cells, but those cells are outside the scope of this thesis. After an injury to muscle, there are signals that induce SCs to become mobile and depart from their niche on the fibre (see section 1.5 below). Such signals originate from the damaged area of muscle and also from other non-damaged places along the fibres. SCs receiving these signals become activated and then are able to migrate to an injury site as they enter the cell cycle to become myoblasts (Schultz et al., 1985).

In development, there are three different populations of myoblasts and they are named by the order of their appearance in muscle tissue; these include embryonic, fetal and adult or satellite myoblasts (Evans et al., 1999). Embryonic myoblasts are responsible for the formation of primary muscle fibres whereas fetal myoblasts are distributed throughout a muscle to form secondary fibres that develop around and between primary fibres. Interestingly, the nuclei that are contained inside muscle fibres are not able to undergo mitosis once they develop into fibres following fusion of myoblasts. That means that SCs are the only myogenic cells in skeletal muscle postnatally, that are able to respond by mitosis to contribute to muscle growth and regeneration. SCs have multiple origins and fates which are complicated and understood only by a very detailed study of embryogenesis. SCs can be derived from the somite as well as from other types of cells (Cossu et al., 2000). In addition, SCs are determined as

multipotent stem cells and can differentiate into non-muscle tissues such as bone, adipose and connective tissues (Seale and Rudnicki, 2000).

Proliferation

Upon activation, SCs become cycling myoblasts and will upregulate the expression of two of the four genes called the primary myogenic regulatory factors (MRFs), i.e., Myf5 and MyoD. After muscle damage, mononuclear cells, including mainly myogenic SCs are activated through a process regulated by factors released from the injured muscle (McClung et al., 2007). Hepatocyte growth factor is this stimulatory factor, and is referred to as HGF. The release of HGF from injured muscle was first demonstrated by Tatsumi et al. in 1998 (Tatsumi et al., 1998). This mechanism was further investigated by Shi and Garry (2006). After release, HGF first binds to the c-Met receptor; that binding activates SCs through p38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (P13K) signaling pathways. Apart from that, macrophages are also reported to activate myogenic cells (Lescaudron et al., 1999).

Differentiation

After the proliferation phase in myogenic precursor cells or myoblasts, expression of myogenin and MRF4 (secondary MRF members) is upregulated in cells; this is an indication that terminal differentiation of myoblasts has begun. This is followed by completion of that cell cycle and permanent withdrawal from the cell cycle (Cornelison and Wold, 1997). As the differentiation program proceeds further, the cell begins to express muscle-specific proteins, such as myosin heavy chain (MHC) and muscle creatine kinase (MCK). Expression of these genes indicates that myoblasts have

terminally differentiated into myocytes (Karalaki et al., 2009). Eventually, mononuclear myocytes fuse to form a multinucleated syncytium, which finally matures into a contractile muscle fibre. The myocytes fuse to form new muscle fibres and also fuse into existing fibres to repair areas of damage in those fibres. In the course of muscle regeneration, molecules expressed by muscle cells and fibres are transported by nearby segments of surrounding fibres to a location between cells; these molecules include M-cadherin and M-calpain. They ensure adhesion between cells during fusion and also between satellite cells and muscle fibres. (Krauss et al., 2005) (see section 1.1.5, below).

1.1.5 Satellite cell development

The two paired-box transcription factors, Pax3 and Pax7 are expressed at the same time in the majority of myotomal cells of the somite in embryonic stages, including in SCs (Shi and Garry, 2006). Specifically, Pax3 is required for the migration of muscle precursor cells from the somite into the trunk or limbs during embryogenesis. Pax7 is regarded as necessary for satellite cell specification. Muscle satellite cells appear as a distinct population of muscle precursor cells at the end of embryonic development (Wagers and Conboy, 2005). In this process called SC lineage recruitment, Pax3 and Pax7 genes play an important role for the specification of progenitor cells that will contribute to the satellite cell pool (Wagers and Conboy, 2005). However, it is not clear whether Pax3 and Pax7 contribute only to the specification of these cells directly or also contribute to the survival of satellite-cell progenitors (Zammit et al., 2004).

1.1.6 Markers

To date, a large amount of research about SCs is based on distinct and specific SC markers; however, there are still many complications to the use of these markers, and many more processes are less understood and need to be identified and characterized at the different stages of myogenesis (Carvajal and Rigby, 2010). Moreover, researchers have developed methodologies, including single fibre isolation or whole muscle culture *in vitro* and also use *in vivo* experiments to investigate SCs by focusing on various levels of complexity and various parts of the process (Cornelison and Wold, 1997). Due to the availability of SC-specific markers such as expression of the c-Met receptor (since Cornelison and Wold's report), investigators have refined the understanding of SCs. Initially SCs were understood to be monopotential cells that occupy an exquisite niche between the basal lamina (endomysium) around fibres and the external fibre membrane called the sarcolemma. We now recognize the existence of other muscle stem cells, called the "side population" (SP) cells, which are multipotent and can contribute to become muscle precursor cells in certain microenvironments (Asakura et al., 2002; Tamaki et al., 2003). Therefore, understanding the processes of muscle development underlies the ability to use SC markers effectively; this plays a pivotal role in research on SC-related events in skeletal muscle. For example, once SCs are activated, they will enter the cell cycle and proceed through DNA synthesis. At the same time, they express different transcription factors, and we can study that expression to identify discrete phases of the process of myogenesis during the time from quiescent status of SCs to their proliferation and ultimate differentiation. As well, most of the cell-surface markers on SCs identified to date are proteins related to adhesion properties because the processes that activated

SCs need to ensure that the cells can depart from their unique niche on fibres and form myoblasts that are mobile. The same adhesion factors are then needed during the process of myocytes fusing together or fusing with existing fibres (Cornelison et al., 2000). First, the marker proteins that are typically used in studies of myogenesis will be described, and then satellite cells will be described in more detail, including their activation and proliferation.

Generally speaking, the most extensively used markers can be divided into the transcription factors (e.g., Pax7) and myogenic regulatory factors (MRFs, including MyoD, Myf5, myogenin and MRF4) that are described in previous sections, and cell-surface markers (c-Met, Syndecan-3, Syndecan-4, M-cadherin, CD34, VCAM-1, integrin, and myostatin) that are described below, in relation to transcription factors and MRFs.

Of specific interest is the c-Met receptor, a protein that is a tyrosine kinase receptor. The expression of this gene is present in quiescent SCs and also in activated SCs and myoblasts. This finding was first reported by Cornelison and Wold (1997) as a breakthrough in our understanding of skeletal muscle SCs. Note that myoblasts and SCs together comprise the population of myogenic precursor cells (MPCs) that are responsible for muscle growth and regeneration (Wozniak et al., 2003; Figeac et al., 2007). Although the distribution of SC populations is distinct in different types of muscle (with fast- and/or slow-twitch fibres), c-Met will be expressed in SCs on both fibre types (Sonnenberg et al., 1993). C-Met is also expressed in other types of cells including pericytes adjacent to blood vessels, mesangioblasts adjacent to the developing

aorta, hepatocytes and metastatic cells in the lung (Anderson 2000; Cornelison and Wold, 1997).

Syndecans are transmembrane heparin-sulphate proteoglycans on the surface of SCs, and syndecan3 and syndecan4 show expression that overlaps with c-Met expression by quiescent SCs and MPCs (Rapraeger, 2000; Cornelison et al., 2004).

M-cadherin, known as a calcium-dependent adhesion molecule, is expressed on quiescent SCs and activated myogenic precursors but not on differentiated myotubes (Moore and Walsh, 1993). M-cadherin is also expressed in conjunction with a hematopoietic stem-cell marker called CD34, which suggests the existence of heterogeneity within the SC compartment (Irintchev et al., 1994). Pax7 is expressed on quiescent SCs and proliferating MPCs and will be downregulated as differentiation proceeds (Seale et al., 2000). However, since Pax7 is not detected on non-myogenic cells, it is comprehensively recognized and employed as the specific marker for SCs (Halevy et al., 2004; Olguin and Olwin, 2004) in muscle unless fibres with SCs are separated from the other tissues in muscle; in that case, c-Met receptor protein is also valuable as a specific marker of SCs. However, studies that track cells using their expression of marker proteins that are only expressed in a subpopulation of quiescent or activated SCs or that are expressed in non-myogenic cells, require studies of combined expression with other factors, such as MyoD and Myf5 (that are expressed during proliferation) or myogenin (that is expressed in differentiation) to clarify the particular aspects of function in those cells. For instance, SCs are able to maintain their population through multiple rounds of damage and regeneration, and throughout growth up until old age. This "steady state" is known as self-renewal, and is thought to be regulated by the expression of myostatin,

which is a secreted signalling factor belonging to the TGF-β family (McCroskery et al., 2003; Amthor et al., 2004; Leiter and Anderson, 2010).

Despite the fact that there are distinct mechanisms of SC activation under examination in the literature, the above markers are in common use to study all but the very early, initial processes of SC activation. Studies of those very early pathways include use of marker proteins in the Wnt and Notch signalling pathways and the HGF-c-Met pathway, which is the focus of this proposal and is described in detail below in conjunction with the Notch signalling pathway (Luo et al., 2005).

1.1.7 Satellite cells

Satellite cells (SCs) are mononuclear cells that were first identified as muscle stem cells in1961 by Mauro. They were named by their special location, lying between the sarcolemma and external lamina of muscle fibres, as observed under the electron microscope. SCs are small spindle-shaped cells, with a prominent nucleus and a low volume of organelles. In adult muscle tissue, they are in a quiescent state, metabolically and mitotically (in the G0 phase of the cell cycle) (Schultz et al, 1978). SCs have an important function in the process of muscle regeneration. Before describing regeneration, the four phases of myogenesis will be described, including embryonic, fetal, neonatal and postnatal myogenesis; to some extent, these stages happen again in an overlapped fashion, throughout the whole lifetime of muscle growth and repair in an animal (Murphy and Kardon, 2011).

In the earliest stage of development, muscle fibres originally develop from multipotent progenitor cells in the somite. With the proliferation of those progenitors, the

developing population will become determined by particular gene expression, to become a muscle cell called a myoblast. After differentiation, the cell is a myocyte, and many myocytes fuse together into a multinucleated myofibre. This early fibre is also called a myotube as it displays myonuclei in the centre of the fibre along its length. As the fibre matures in developing muscle tissue, those myonuclei migrate from the central part of the fibre to a peripheral location. This process forms the postmitotic, multi-nucleated myofibres (Murphy and Kardon, 2011). These processes continue throughout the lifetime of an animal, whenever growth and injury of muscle occur. In 2005, Collins et al. demonstrated that the ability of SCs for self-renewal makes it possible for muscle regeneration to occur repeatedly throughout the lifetime of an organism. Work by Louboutin and colleagues (1995) demonstrated the ability for SCs to sustain very effective regeneration through multiple rounds of damage. There are also minor contributions by other non-satellite stem cells, such as cells in the muscle interstitium between fibres (Asakura et al, 2002; Polesskaya et al, 2003) and cells that are circulating and derived from bone marrow (LaBarge and Blau, 2002). Asakura et al. (2001) reported that SCs are multipotent stem cells, with the ability to differentiate into osteocytes and adipocytes in specific microenvironments, instead of "merely" being unipotent myocytes in muscle tissue. In addition, in 2002, the same researchers indicated that cells within the satellite niche are not equal to muscle-derived stem cells because they give rise to distinctive cell types. In fact, the multipotent muscle stem cells are able to produce extensive numbers of hematopoietic progenitors (when transplanted to the appropriate niche) whereas SCs cannot. However, muscle stem cells do have significant myogenic potential and support muscle growth and regeneration with the significant assistance of

myoblasts derived from multipotent SCs. The satellite-cell population in growing muscle can be separated into two groups: a fast-dividing population that undergoes limited replication before differentiating, and a slow-dividing population that might return to G0 between cycles and give rise to the fast-dividing population (Schultz, 1996). Indeed, cells from other sources, such as dorsal aorta progenitors, side populations and hematopoietic stem cells, can also contribute to the satellite cell niche (Asakura et al., 2002; LaBarge and Blau, 2002). Although it seems there is more than one source of MPCs in the body, the non-SC sources are considered a very small component of MPCs and do not contribute very much to muscle regeneration or growth (Collins et al., 2005). This topic is complicated and somewhat confusing in the literature. However, that confusion itself, illustrates the current uncertainty about how the different subpopulations of SCs are provided in development or maintained throughout lifetime. This is an ongoing and "hot" area of research today that is rapidly evolving.

Although SCs are present in all skeletal muscles, the size of their population (often expressed as a number of SCs per fibre and a percentage of fibres that display a SC in a tissue section or as the number on isolated fibres in culture) is different in each muscle and also differs in the frequency of SCs distributed on a population of fibres. More SCs are found adjacent to slow muscle fibres compared with fast muscle fibres within the same muscle. Moreover their number is higher at the neuromuscular junctions (NMJ) on fibres than away from those regions (Grand and Rudnicki, 2007).

Activation of satellite cells

To date, researchers have demonstrated that there are two major ways to initiate SC activation: through mechanical stimuli and chemical stimuli (Tatsumi et al., 2002). In addition, the chemical stimulation pathway, mediated mainly by nitric oxide (NO) and hepatocyte growth factor (HGF) can activate the SCs independently without involvement of mechanical stimuli, whereas mechanical stimuli, like stretch or injury, need the participation of those chemical molecules to transform the signal modality for stimulation first and then to trigger the activation of SCs (Tatsumi and Allen, 2008). Both activation mechanisms show the pivotal roles of NO and HGF. Interestingly, the release of HGF is indispensible in stretch-induced activation which can be independent of NO, but the NO-signalling pathway requires HGF as it is next in the cascade of activation (Tatsumi et al, 2002). HGF can be produced and released by single activated myogenic cells and by myotubes in culture (Sheehan et al., 2000) and by fibres *in vitro,* in culture and *in vivo*. Upon injury, HGF is released from the extracellular membrane (ECM) by an NO-dependent mechanism (Shi and Garry, 2006) that is also calcium-dependent (Hara et al., 2012). In fact, according to Tatsumi and Allen (2008) and modelled initially by illustration in Anderson (2000), the processes of SC activation can be interpreted as a cascade of events that includes an exercise or injury stimulus, NO production, matrix metalloproteinase (MMP) activation, HGF release and HGF binding to c-Met. In mammalian skeletal muscle, the NO is produced by nitric oxide synthase-μ (NOS-Iμ) by enzymatic conversion of L-arginine (LA) to L-citrulline. Today, it is understood that proteoglycans, collagen, metalloproteinases and other elements of the ECM play key

roles in myoblast activities during muscle development and SC events during regeneration (e.g., Osses and Brandan, 2002; Midwood et al., 2004; Yamada et al., 2008).

Quiescence of satellite cells

One significant characteristic of skeletal muscle is the ability to accomplish regeneration and repair after repeated injuries (Hirata et al., 2003). This capacity implies that after every regenerative process the SC population or pool is renewed. To maintain the SC pool, particularly the more stem-like group of SCs, it undergoes division asymmetrically during mitosis. This division results in one daughter cell that is committed to differentiation as muscle, whereas the other daughter cell will either continue to proliferate or become quiescent (Zammit et al., 2006; Shinin et al., 2006). In other words, the daughter cells that are not committed to the myogenic lineage will return to quiescence as SCs that maintain the stem-like population of SC precursors. At the same time, the population of daughter cells that is committed to become muscle, will go through proliferation (one or more times) and differentiate. This results in production of new fibres or fusion with existing fibres (Kuang et al., 2007).

1.1.8 Innervation

The innervation of skeletal muscle happens during the early part of the embryonic phase in development (Ontell and Feng, 1981). The initial nerves are attached on the outside of pre-muscle mass during the process of primary muscle development. After individual muscles are formed, the nerves can travel deep into the individual muscles (Ontell and Kozeka, 1984). Initially, each muscle fibre is innervated by axons from multiple motor neurons. However, a few weeks later, the number of neurons is

reduced to one single neuron that administers several fibres of the same fibre-type in a motor unit (Washabaugh et al., 1998; Washabaugh et al., 2007). In adult mammalian muscles, muscle fibre heterogeneity is influenced dramatically by innervation (Schiaffino and Reggiani, 1996). In the condition of denervation, the expression of mature phenotypes of both fast and slow fibres is reduced and fibres become much more homogeneous in contraction characteristics and size (Wilson and Harris, 1993).

1.1.9 Fibre types

Skeletal muscles are heterogeneous, meaning they are composed of different types of muscle fibres. This characteristic of distinct fibre phenotypes is significant in insuring that our body can perform distinct movements very smoothly, from a stand-still to running and jumping (Schiaffino and Reggiani, 2011). Fibre-type heterogeneity emerges early in the commitment of mesenchymal cells to their myogenic fate, before a cell becomes a muscle precursor (known as a myoblast). In the myoblast state, cell fate related to contractile function is determined by the members of the myosin heavy chain gene family that are expressed at the time when the myoblast differentiates into a myocyte or when it fuses into a muscle fibre (Karalaki et al., 2009). The original naming of fibre phenotypes, slow and fast-twitch fibres, is based on the expression of a particular myosin heavy chain (MyHC) gene. There are four isoforms of MyHCs in mammalian muscle, dependent on the particular properties of the globular-head region in interactions with ATP (during hydrolysis) and actin-binding sites that decide the contractile properties of muscle fibres (Weiss et al., 1999b). The isoforms include MyHC-I which found in fibres with a slow speed of contraction and MyHC-IIa, MyHC-IIX/d, and MyHC-IIb found in fast-contracting fibres.

1.2 Zebrafish muscle

Zebrafish (*Danio rerio*) is an ectothermic teleost fish that lives in tropical freshwater, and is used extensively as a vertebrate model in studies of embryonic development and human-disease research. There are many reasons for this extensive use. They are small in size (about 3-4 centimetres in length) when full grown. Zebrafish embryos develop quite quickly and will become adults three months after hatching. As a model, the zebrafish makes high-throughput assays of extrinsic or intrinsic factors possible because they are transparent during embryonic and larval stages and develop externally from the parent fish (ex utero) (Kishi, 2011). Further, zebrafish can reproduce sexually only three months after fertilization, and adults can produce large numbers of eggs with 50-350 offspring over 5-7 days (Shuji, 2011).

As well, in the past 20 years, we have learned that the zebrafish genome is highly conserved within the human genome. Along with the recent detailed characterization of the zebrafish genome, researchers have developed multiple mutant and transgenic phenotypes of zebrafish to model a broad range of diseases. Further, with specific gene-knockdown techniques enabled through the use of morpholino antisense oligonucleotide sequences, it is possible to investigate the potential mechanisms underlying particular conditions of physiology and pathology in zebrafish (Lieschke and Currie, 2007).

1.2.1 Development

According to Kimmel et al. (1995), the period from 48-72 hours post-fertilization (hpf) is referred to as the embryonic stage (after hatching). This stage will last until the appearance of a protruding mouth. From 72 hpf to one month is the larval period. When

larvae develop to the stage when they attain the external pattern of scales and lose all the fin folds typical of larvae, they reach what is known as the juvenile stage. About three months after hatching, zebrafish become adults with the ability to breed and produce viable gametes. Similar to other vertebrates during the period of embryonic development when the myotome (derived from somite) develops, the pre-somitic mesoderm produces adaxial cells (adaxial means away from the body axis, as these cells are laterally placed, along the outer aspect of the body). This feature is distinct from mammalian development of muscle, as adaxial cells are regarded as the original pool of cells that form slow muscle fibres (Norris et al., 2000).

As shown in previous studies (e.g., Fauconneau and Paboeuf, 2001), the body temperature of wild zebrafish ranges from 16.5-38.6 °C due to the variations of season and locations. Vergauwen and colleagues (2012) examined adult zebrafish physiology (i.e., oxygen uptake and ammonia excretion) under different temperatures (18, 26 and 34 °C) for 28 days. They found that with the restriction of fast swimming speed, lower temperatures resulted in a decreased metabolic rate, whereas a higher temperature resulted in an increased the metabolic rate. The highest body temperature of zebrafish exceeds the tolerance limitation of some other fish species, such as trout. However, the optimal conditions for zebrafish growth in a research laboratory typically are recommended at a water temperature of 28.5 °C (Osterauer and Kohler, 2008). In addition, zebrafish embryos can only grow normally, that is, with a proper distribution of fast and slow muscle precursor cells, when reared between the temperatures of 25 and 33 °C. Development of embryos in water below or above this temperature range will induce abnormal development including smaller body size (Sterneckert et al., 2005). In a study

of adult zebrafish phenotypic plasticity when embryonic and larval stages of zebrafish were subjected to different temperatures (22, 28 and 32 °C) for up to 13 days and kept feeding to five to 10 months, Georga and associates (2010) determined that thermal treatments, in particular, lower temperature (22 °C) exerted a considerable effect on adult fish structure, including fins, gill and jaw. Moreover, Johnston (2006) reviewed the influence of environmental factors (i.e., temperature and dissolved oxygen concentrations) on muscle fibre number and muscle growth in teleost fish, related to the alteration of gene expression pattern, metabolic rate and so forth. It was also pointed out that muscle plasticity depends on the developmental stage of a fish. Specifically, for early development, the influence of temperature variation is profound and critical, as a different temperature during an embryonic stage causes dramatic changes in the time and quality of muscle formation after hatching.

1.2.2 Muscle structure

The axial muscles of zebrafish are mainly composed of fast or so-called white muscle fibres. They are covered with a layer of intermediate-pink muscle fibres, and the thin layer of slow or red muscle fibres is further outward, and located just underneath the skin (Buckingham and Vincent, 2009). Slow and fast muscle fibres also have different orientations in the body and together form a “W”-shaped myotome when viewed in a cross section of the fish body. The slow muscle fibres are arranged superficially along the lateral line and run parallel to the body axis whereas the deeper fast muscle fibres form at angles up to 40 ° from the longitudinal body axis. The distinctive colours of muscles are due to the higher amount of myoglobin, greater mitochondrial density and population of muscle capillaries in red than white muscles. Depending on the frequency of fish

tailbeats, a population of fish can be divided into slow and fast swimmers, and in addition, some fish swim with a curved body shape and blend markedly whereas others possess a more rigid or stiffer body. These facts are all likely due to the population and distribution of red and white muscle fibres; this has led to a significant amount of research on slow and fast muscle function, and at the same time has left questions to be answered by further study. As mentioned earlier, white muscles are responsible for sprint swimming when fish are escaping from predators whereas slow (red/aerobic) muscles are in charge of steady swimming. However, whether the power of swimming is derived from a wave of contraction from anterior/rostral to posterior/caudal or vice visa is controversial. Coughlin and David (2002) reviewed and compared the power generation of slow muscle when fish were undergoing steady swimming, specifically in an anterior to posterior orientation, among different fish species, including scup (Perciformes), bass (Perciformes) and rainbow trout (Salmoniformes) which produce the force from posterior to anterior, whereas mackerels (Perciformes) and carp (Cypriniformes) generate muscle force from anterior myotome.

1.2.3 Fibre types

Consistent with skeletal muscles in mammals, in zebrafish there are slow-twitch and fast-twitch muscles. The nomenclature of muscle-type heterogeneity varies according to their metabolic properties in particular species, but generally they are named as slow-oxidative/aerobic, fast oxidative-glycolytic and fast-glycolytic muscle fibres (Stickney et al, 2000), similar in function to type I, IIa and IIb fibres in mammalian muscle. These fibre types function with aerobic metabolism and more anaerobic (glycolytic) metabolism, respectively, during contraction (Barresi et al., 2001). In 1982, van

Raamsdonk and colleagues first demonstrated three primary components of muscle fibre types in adult zebrafish, adult red, intermediate and white muscle. Interestingly, these adult muscle phenotypes are not directly derived from embryonic red and white muscles; in essence, adult muscle fibre types are the result of two minor populations, red muscle pioneers and scattered intermediate muscle. Moreover, the distinct distribution of muscle fibre types between the fish mid-body and the tail myotome is likely associated with discrete patterns of fish swimming (i.e., fast swimming or steady swimming). Ochi and Westerfield (2007) determined that, in fact, there are four types of zebrafish muscle fibres rather than the classic three that are identified in mouse muscle: the list includes slow muscle cells, muscle pioneer cells, medial fast fibre cells and fast muscle cells. However, unlike the intermixture of fast- and slow- twitch fibres in most individual muscles in mammals (for example, in extensor digitorum longus), slow and fast fibres in zebrafish are located in separate regions of the fish. As well, there are intermediate or type IIX fibres in mammalian muscle (Schiaffino and Reggiani, 1994; Smerdu et al., 1994). As described above, slow muscle fibres are located just underneath the skin, along the lateral aspect of the body, whereas the fast muscle fibres lie deeper in the fish body, taking up over 90% of total muscle mass. (Bird and Mabee, 2003; Buckingham and Vincent, 2009).

1.2.4 Zebrafish myogenesis

During myogenesis, all the myogenic progenitors in zebrafish development come from the paraxial mesoderm of the somite; the paraxial mesoderm then is segmented into distinct compartments along the anterior (rostral) to posterior (caudal) axis (Buckingham and Relaix, 2007). As previously stated, in mammalian muscle development, fibres largely only increase in size (by a hypertrophy process) after birth (i.e., a muscle

generally does not increase in fibre number). By comparison, zebrafish muscle formation can be divided into three phases: a) embryonic myogenesis, b) a period of stratified hyperplasia and c) mosaic hyperplasia, in the post-hatch period in which there is a combination of increases in number (hyperplasia) and size (hypertrophy) of muscle fibres (Johnston and Hall, 2004). The embryonic phase starts from the formation of adaxial slow muscle which is induced by signalling from the gene called Hedgehog (Hh). After this primary myotome formation, the second stratified growth phase of development begins with the addition of new fibres in areas of both fast and slow muscle. This stage also creates so-called germinal zones on the lateral surface of myotome. The third phase happens inside the myotome itself, as tertiary fibres formed by being inserted in a mosaic-like pattern between existing fibres in the previously developed regions (Johnston and Hall, 2004). Unlike mammals, in teleost fish it is still controversial when (i.e., at what stage of myogenesis) muscle fibres become capable of hyperplasia and/or if the ability for hypertrophy ceases at some time point. The majority of research on myogenesis has been performed on white muscle, due to its dominant role in terms of muscle mass compared to slow muscle fibres. Weatherley and colleagues (1988) compared growth of axial white muscle in ten different teleost fish (including five families) and first proposed that the hyperplasia stopped when fish fork length (from the very tip of the snout to the end of the middle caudal fins) reached 44% of the final body length in mature fish. In agreement with that, Veggetti et al. (1993) found that mosaic hyperplasia was reduced dramatically or even disappeared in fish where the ultimate body size is small, such as the guppy Poecilia reticulate. Indeed, fish body size and the underlying mechanism of myogenesis were affected fundamentally by environmental and

behavioural factors (e.g., extent of body curve when fish escape from predator). Rowlerson and colleagues (1998) compared muscle regeneration under mechanical stretch stimulation (monitoring body curve) for two teleost fish with dramatic differences in body size. One fish was sea beam, which possess more potential for hyperplasia in the post larval stage whereas the zebrafish had limited hyperplasia during growth. In an effort to explore the causes and possible mechanisms underlying their distinct body sizes, they found newly formed muscle fibres are derived from different sources. The experimental approach in this study used immunostaining for desmin and myosin isoforms in tissue sections. In other words, new fibres in the sea bream originated from post-larval white muscle compared to their origin from the monolayer of red muscle in zebrafish development, although the new fibres generated differently in the two species both ended up as white muscle fibres. Interestingly, in a comparison of muscle growth pattern between zebrafish and its close relative, the giant danio (Danio aequipinnatus), a fish which can reach 10-13 cm in body length when full grown, Biga and Goetz (2006) demonstrated the absence of mosaic hyperplasia in zebrafish. However, Patterson and associates (2008) illustrated the presence of mosaic hyperplasia at the 6 mm larval stage of zebrafish, and that process overlapped in time with stratified hyperplasia. This finding was slightly different from a previous report of Rowlerson and Veggetti (2001) that the last phase of mosaic hyperplasia starts after the larval stage.

1.2.5 Satellite cells

Since SCs have a specific niche outside the fibre sarcolemma and inside the basal lamina, they are vulnerable to factors affecting muscle fibres and those processes that affect the integrity of the basal lamina surrounding fibres. Intrinsic factors such as aging

and muscle fibre type can influence the ability or capacity for SC proliferation whereas extrinsic factors like feeding, food quality and temperature can affect the differentiation rate of SCs (Fauconneau and Paboeuf, 2001). Notably there is a significant difference between zebrafish and other vertebrates, in that myogenesis in zebrafish can still occur after hatching and can last for most of their lifetime (Fauconneau and Paboeuf, 2001). Other vertebrates, especially mammals, display myogenesis that essentially is completed post-hatch or post-natally, and which only restarts if muscle is injured and myogenesis is stimulated for regeneration. This post-hatch myogenesis of zebrafish is supported by the observation that the number of muscle fibres can increase in adult zebrafish; Koumans et al. (1993) referred to this as hyperplasia (meaning an increase in number of fibres). As well, it is noted that SCs isolated from mammalian fast-glycolytic and slow-aerobic muscle fibres are different populations, whereas in zebrafish, SCs isolated from the different muscles are essentially a single population and only show differences in the rate of proliferation that is higher in SCs derived from red muscle fibres (Feldman and Stockdale, 1991; Kryvi and Eide, 1977).

SCs in zebrafish have the same anatomical location as those in mammalian and avian fibres, and are similarly responsible for growth of existing fibres (hypertrophy, i.e. the growth of cell size) and for formation of new fibres (myogenesis) as in mammalian and bird muscles. SCs in mammals (Leiter and Anderson, 2010) and birds eventually lose the ability to respond to demands for fibre hypertrophy and regeneration. However, as described above, unlike other vertebrates, myogenesis in zebrafish continues after embryos hatch, and the fish go through most of their lifetime supporting the continuing formation of new fibres (fibre hyperplasia) (Fauconneau and Paboeuf, 2001). For these

reasons, and due to their ongoing myogenic capacity, SCs in zebrafish are much more similar to myoblasts than to SCs typical of adult mammalian muscles (Fauconneau and Paboeuf, 2001). In addition, SCs in mammals are sometimes described as distributed near muscle fibre nuclei (referred to as myonuclei) (particularly near NMJs in mammalian muscle) when seen in transverse sections of muscle. In zebrafish muscle, however, SCs are usually described as located at various places somewhat randomly distributed along the length of fibres and are more likely found at the "corners" of the polygon-shaped muscle fibres, as seen in transverse sections (Fauconneau and Paboeuf, 2001). However, this apparent distinction may be partly due to fixation conditions prior to sectioning, section thickness, and to observations made in particular muscles. Other factors such as magnification of observations, and use of SC-specific marker proteins or genes can also affect an observer's ability to discriminate SCs (largely by their nuclei) from the myonuclei in the fibre beneath the sarcolemma. In a comparative observational study of cell mobility properties between primary satellite cells (i.e., resident satellite cells adjacent to muscle fibre) of mice and myoblasts in cultures of different cell lines (i.e., C2C12 and MM14), Siegel and coworkers (2009) found that primary satellite cells are much more active and movable than other myoblasts, suggesting a considerable and critical signalling conducting role of satellite cells along/between muscle fibres, therefore promoting muscle growth or regeneration after injury. However, it is important to note that Siegel et al. (2009) isolated fibres by a fairly traumatic process that stimulates generalized activation of all SCs on the fibres, and thus the mobility of the SCs observed in the report was at a high level of activation that may not have been similarly stimulated in the cultured C2C12 or MM14 cells.

1.3 Culture methods used to study satellite activation on fibres

1.3.1 Historical studies of satellite cell activation *in vivo* in mice

To date, a diverse array of genes expressed in different stages of SC activation has been elucidated by *in vivo* experiments in mouse models. Tatsumi et al. (1998) explored the role of HGF in the process of SC activation *in vivo*; that report demonstrated the presence of HGF in skeletal muscle and that HGF can be released from injured muscle and activates SCs. In addition, Cooper et al. (1999) induced regeneration in skeletal muscle of mouse model by using cardiotoxin and found the expression of Myf5 and MyoD in activated SCs. Furthermore, in 2000, Anderson explored the injury-induced release of nitric oxide (NO) on SCs *in vivo* and detected a quick response to NO induced SC activation. By comparison with the results of activation studies *in vivo* from *mdx* dystrophic mice and NOS-I knockout mice, her results demonstrated a major role of NO on SC activation.

1.3.2 Historical studies of satellite cell activation *in vitro* in single-fibre cultures

The goal to isolate pure cultures of myogenic precursor cells was a very hot topic in muscle research prior to the identification of c-Met and pax7 as marker genes for SCs. That isolation process has been studied frequently and comprehensively, along with a broad variety of reports on isolation protocols, including collecting myogenic progenitors from adult zebrafish muscle (Alexander et al, 2011). In accordance with Alexander and colleagues, studies from that laboratory accumulated all the progenitors which potentially were able to develop to myoblast cells from dorsal myotome. In other words, the primary satellite cells, and also other cells from the stem cell pool, such as fat

tissue which can become myogenic when the microenvironment is altered, were isolated, and have potential to be beneficial for the growth of fish muscle. This led to a methodological restriction of isolating purified myogenic satellite cells. Studies of mouse muscle had led to the ability to culture pure myoblasts from isolated muscle fibres (e.g., Rosenblat et al., 1995).

It was a significant advance in muscle research to be able to use single-fibre cultures to model and explore the activation of SCs that occurs during the earliest stages of muscle regeneration *in vivo*. Herein, the current study focused specifically on the activation of the quiescent satellite cells that stay in the satellite-cell niche and which originally developed from embryonic stage. This single-fibre model is used extensively, as it can eliminate the disturbance from and impact of non-muscle cells (e.g., in a mixed culture isolated from homogenized muscle tissue) and still keep intact, the interactions between SCs and the muscle fibres where they are attached (Wozniak and Anderson, 2005). In addition, this singular environment for studying SCs using *in vitro* experiments is capable of keeping SCs in a quiescent state (non-proliferative), at least in fibres isolated from mice. This provides a necessary baseline for measuring SC activation under conditions of various stimuli. Originally, single-fibre cultures were first adopted by Bekoff and Betz (1977) and then developed further by Bischoff in 1986. Until now, this model has been modified to satisfy assumptions of distinct hypotheses. However, its use is inconsistent among different laboratories in regard to the standard (or proportion) of quiescent SCs on the fibres cultured in non-stimulated (control) conditions, due to the diverse protocols used to isolate and maintain fibres; those differences affect the comparability and reliability of data from different labs (Wozniak and Anderson, 2005).

1.3.3 Single-fibre culture method

According to the paper published by Wozniak and Anderson in 2005, fibres are prepared in proliferation medium (PM) and then maintained in basal growth medium (BGM). The small proportion of serum in PM is necessary to prevent the muscle cells from sticking to the glassware, but immediately after treatment in collagenase (0.2% in DMEM for 2.5 h at 37 ˚C and 5% CO2) and isolation of fibres by careful dissection, muscle fibres are transferred to BGM that contains a only a serum-replacement formulation. BGM (and the absence of serum containing hormones and other growth factors, including HGF) is necessary in this protocol to restrict or limit unintended SC activation. It is also important in the dissection process to handle muscle and fibres as little as possible, in order to avoid (or at least minimize) SC activation through trauma to the fibres. After isolating fibres, they are plated on dishes that are coated with a 100% collagen solution that gels at room temperature to ensure that muscle fibres attach well on the dish, since SCs on floating or suspended fibres display different behaviour and higher levels of activation than those that stay firmly attached to a culture dish. Lastly, the addition of bromodeoxyuridine (BrdU) to culture media is used to label cells that become activated and specifically enter the S-phase of mitosis during the period of exposure to BrdU. BrdU incorporation is identified using immunostaining techniques after acid–alcohol fixation of muscle fibres. A count of the number of BrdU+ cells per fibre is typically used to assay SC activation in fibre cultures (Anderson and Pilipowicz, 2002). This approach using a labelled nucleotide was originally reported in the classic papers by Bischoff (1986) who used thymidine and autoradiography techniques to assay the proliferative response of SCs in fibre cultures. With both isotope and non-isotope

incorporation into new DNA, the observer identifies a standard level of labelling or staining as a ‘positive’ marker of activation.

1.3.4 Stretching cultures to induce activation

Combining the method of Anderson et al. (1993) with cultures of myoblasts derived from rat SCs, Tatsumi et al. (2001) employed a FlexCell System to produce a stretch stimulus on isolated cells. In more recent studies (Wozniak et al., 2003), muscle fibres were isolated and plated in dishes coated with Vitrogen (100% collagen) with either a flexible or rigid (control) substrate. The dishes were then manipulated by imposing cycles of vacuum pressure from below that produced a 10% stretch (along the radius of the culture dish) at a frequency of 4 cycles per minute. Tatsumi et al. (2001) found that a stretching stimulus applied to single cells isolated from muscle of old rats (where SCs are refractory to activating stimuli for approximately 48 hr) can shorten the time before SCs enter S-phase in activation. Wozniak et al. (2003) first reported application of cyclical stretch to induce SC activation on fibres. In addition, Tatsumi and colleagues demonstrated that HGF was released into the medium by stretching cultures of SCs. These studies together, acted as the intellectual bridge to connect mechanical stretching with cell behaviour during SC activation. Further, in 2003, Wozniak et al. examined the involvement of the c-Met receptor protein, the ligand of HGF, by using in situ hybridization to track the expression of c-Met mRNA. That study confirmed that mechanical stretching can activate SCs on fibres and at the same time induce a rapid increase in the number of SCs on fibres that expressed the c-Met transcript. Further, the same paper reported that there are two peaks of SC activation, one after 30 minutes of stretching and another after two hours of the cyclical stretching stimulus. This result

suggested that there is heterogeneity of SC populations in mouse fibres in culture, according to differences in response time.

1.4 Rationale

A lot is known about mammalian satellite cell activation by the cascade of events involving the NO-HGF-c-Met signalling pathways. However, much less is known about this process in fish muscle. In addition, the huge dependence of human populations on fish for meat, suggests that we should learn more about these important processes involved in growing muscle via satellite cell activation. In addition, the evolution of the regulatory steps involved in satellite cell activation across species or taxa is not understood. The research proposed for this project was designed to supply information for future investigations of genes important in regulating evolutionary changes and to better understand the process of muscle growth in adult zebrafish. The following hypotheses and experiments were designed to begin to address some of the current deficiencies. After successfully isolating and culturing single fibres of zebrafish muscle in Dr. Anderson`s lab (Anderson et al., 2010), the following experiments were feasible using the zebrafish. This project builds on previous work by another student in Dr. Anderson's lab (Jacqueline Richelle) who successfully demonstrated the presence of Pax7 and c-Met receptor proteins in SCs of zebrafish muscle using immunostaining techniques to study satellite cells on single fibres. That student also showed that there are BrdU+ cells on zebrafish fibres after 24 hours of incubation with BrdU in culture, which was important preliminary information for measuring the outcome of the different experiments proposed below.

1.5 Hypotheses and Objectives

The overall goal of the proposed research was to understand the mechanisms involved in SC activation in cultured zebrafish muscle fibres. Experimentations on cultured zebrafish fibres were employed for comparison with the basic information that is now established regarding SC activation in mouse single-fibre cultures. Specific hypotheses are listed below (see Figure 1-1).

Hypothesis 1: Satellite cell activation in zebrafish muscle is dependent on NO and HGF.

Hypothesis 2: Responses to HGF are temperature-dependent

Hypothesis 3: Stretch-activation of zebrafish satellite cells on fibres acts via NO release.

1.6 Significance

The results of experiments included in testing these three hypotheses provide new information about the NO- and HGF-c-Met dependence of SC activation in zebrafish muscle fibre cultures, and the responsiveness of SC activation to mechanical stimulation and temperature. These experiments are the first studies of isolated zebrafish fibres to examine features of SC activation related to NO and HGF-c-Met signalling pathways using a physiological approach in vitro. This model separates treatment effects from those related to other systemic functions in the fish (e.g., innervation, vascular supply) and will be useful in initial comparisons with mammalian (mouse) fibre cultures and cultures of fibres from other animal species. The experiments conducted in this thesis project were restricted to studies of early activation assayed by BrdU incorporation into new DNA or c-Met staining, since there is a limited suite of commercially available

antibodies that are required to study other proteins specifically and sensitively in fish muscle SCs that are translated after changes in gene expression. Additional studies could be designed from the results of these experiments, to determine the critical genes involved in regulating SC activation, including whether there is a requirement for MyoD and/or Myf5 expression for example, and the signalling cascade downstream of c-Met tyrosine kinase activation.

Chapter 2: Methods

2.1 Morphological study on zebrafish

2.1.1 Tissue Sectioning

Zebrafish were reared at 27 °C under a natural photoperiod in the Animal Holding Facility of the University of Manitoba, and handled under protocol number F-12-034. Fish were fed once a day with a mixture of flaked food, frozen shrimp, blood worm (frozen) and commercial trout pellets. To prepare a zebrafish for sectioning and immunohistochemistry (IHC), one fish was anaesthetised by dissolving 80 mg (overdose) Tricaine methanesulfonate (MS-222) (obtained from Animal Holding Facility and stored at 4 °C) into 125 mL warmed (27 °C) tap water, and then fish length and weight were measured (see table 1 for fish body properties). All significant solutions used in this project are listed in detail in the appendix.

First, the head and fins of the fish were removed using fine scissors, while the fish skin and stomach contents were kept. The remaining part of the fish body was then lightly sprayed with 70% ethanol, and extra liquid was blotted off. The fish body was fixed by immersion in about 12 mL of 4% paraformaldehyde (PFA, four grams in 100 mL 0.01M phosphate buffered saline (PBS), see appendix) for 24 hours at 4 °C. Then decalcification was performed using a 50 mL solution of 0.5M ethylenediaminetetraacetic acid (EDTA, pH 8.0, at a solution to tissue ratio of 50:1) at room temperature for one week (Moore et al., 2002). In an effort to diminish the freezing damage later on, the fish body was incubated in a conical tube containing approximately 12 mL cryoprotectant (30 grams of sucrose in 100 mL of 0.1M phosphate buffer, PB) at

4 °C, until it sunk to the bottom (usually this required overnight incubation). The next day, the fish was removed from the tube and transferred onto filter paper to blot off extra liquid, and then cut into three parts longitudinally using a sharp blade.

Prior to embedding the tissue blocks for cryosectioning, isopentane (Fisher, Hampton, New Hampshire) was cooled to minus 50 °C using dry ice (this process took about 30 minutes). When the isopentane reached the optimal temperature, fish sections were embedded with distinct orientations into Shandon Cryomatrix (Fisher, Hampton, New Hampshire) in three separate cryomolds (Tissue-Tek, Sakura Finetek, Torrance, CA), then quickly flash frozen in isopentane (about five minutes). Next, tissue was sectioned using a cryostat at a thickness of 7 µm, ensuring that the cryostat temperature was below minus 21 °C during sectioning to avoid freeze artefacts. Tissue sections were collected on slides that had been previously silanated (to facilitate adhesion of the section to glass). Slides were left to air dry for 30 minutes after all sections were collected, then stored in a closed container in a freezer until staining in haematoxylin and eosin (H&E),as described below.

2.1.2 Haematoxylin and Eosin Staining

Prior to H&E staining, slides containing eight transversally or longitudinally oriented tissue sections per slide, were removed from the freezer and allowed to return to room temperature for 30 minutes. Next, slides were immersed in 100% ethanol for ten minutes followed by soaking in 95% ethanol for two minutes. Then slides were washed for five minutes in distilled water both prior to immersion in Harris' Haematoxylin (Fisher, Hampton, New Hampshire) (which was filtered prior to use) for four and half

minutes to label fibre nuclei. After slides were washed again in distilled water, the extra water was blotted off, and slides were dipped five times into a 1% acid-alcohol solution (concentrated HCl diluted by 70% ethanol), followed by five minutes of washing in distilled water. Slides were then immersed in saturated lithium carbonate for two minutes, submerged into eosin for two minutes, and washed again for five minutes with distilled water. Immediately after eosin staining, slides were dipped ten times in distilled water and then in increasing concentrations of ethanol, each step with distinct duration, starting with 70% ethanol for ten dips followed by 95% ethanol for 15 dips, and ending with 100% ethanol for four minutes. Slides were then incubated for four minutes in Slide Brite (Jones Scientific Products, Kitchener, CA), blotted and partially dried (for about three minutes) and then mounted with permount mounting medium (Vector Laboratories, Inc., Burlingame, CA). Tissue histology was observed using a light microscope (Olympus BHT-2) (Olympus America Inc., Parkway, PA) at magnifications that varied from 40-400X. Images were collected using a digital camera (Sony 3 chip colour CCD camera (Sony, US)).

2.2 Single fibre culture methods on zebrafish

2.2.1 Fish dissection

Fish muscle fibres were dissected according to Anderson et al., (2010). Zebrafish were anesthetised in a solution made by dissolving 160 mg MS-222 in 250 mL of tap water (pre-heated to 27 °C). Fish were blotted on a paper towel to absorb any extra liquid before measuring their body weight and total length (from the tip of the snout to the end of the caudal fin). Then each zebrafish was stabilized by pinning through the head

and tail into a layer of dental wax. Using very fine spring scissors, the dorsal fin, pelvic fin, anal fin and the pair of pectoral fins were removed. Following fin removal, an incomplete ventral to dorsal slit was made, just caudal to the head (behind the gill opening) and proceeding through three quarters of the body width using autoclaved fine scissors. To remove stomach contents, an additional cut was made along the ventral surface of the fish, from the initial cut toward the opening at the anal fin. Internal organs were carefully removed, avoiding contamination of muscle by body fluids or any contact of the muscle with scales and skin. Fish skin was then carefully removed by placing the flat aspect of the forceps alongside the body, to avoid damaging the muscle. Next, the pins were pulled out of the fish, the head was removed completely and the caudal fin was cut off and discarded. The skinless, gutted fish body was transferred carefully into a 15 mL plastic tube containing 10 mL of medium containing 0.2% collagenase (see appendix), using autoclaved forceps (Fine Science Tools Inc., North Vancouver, CA). The tube containing the fish was then incubated at 37 °C for two hours.

2.2.2 Fish muscle fibre isolation

To isolate fibres, the following procedures were used. The collagenase-containing medium was poured off and the fish body was gently poured from the tube into an autoclaved 60 mm glass Petri dish (VWR International, Radnor, US) containing about 4 mL fresh proliferation medium (PM). PM was prepared ahead of time and kept at 4 °C (see appendix). Fibres were isolated using the wide end of an autoclaved wide-bore pipette which was made in advance by breaking off and flame-polishing the thin pointed end of a Pasteur pipette (Fisher, Hampton, New Hampshire). The fish carcass was triturated gently in and out of the wide-bore pipette to separate fibres from ribs.

Specifically, visible debris (e.g. remaining scales, blood, and connective tissues) were removed using autoclaved fine forceps prior to trituration. In an effort to avoid over-triturating the superficial muscle fibres which detach earlier from fish ribs compared to deeper muscle fibres, a minimum of two triturations per fish (average of 15-20 times for each time) was performed during the process of fibre collection. Immediately following the first trituration, approximately 1/3 of the total amount of muscle fibres was separated from ribs and the remaining fish body was transferred back into collagenase-containing medium. Incubation at 37 °C was continued for an additional hour, to allow digestion of the deeper layer of muscle fibres and their separation by the second trituration. Care was taken to avoid making bubbles in the medium at all times (see Figure 2-1). After fibre separation, more debris, including bones, scales, fat, etc., was removed from the PM using autoclaved fine forceps while viewing under a dissection microscope.

Fibres were isolated from two fish for each experiment using the same dissection and fibre isolation procedures, one at a time. Although there were plenty of fibres released by the isolation procedure for one fish, and this was sufficient to provide fibres for 30-50 dishes or coverslips in an experiment, two fish were used together in order to avoid individual differences such as male vs. female and pregnant vs. not pregnant fish. Therefore, fibres from two individual fish were combined and poured carefully into a clean petri dish.

To clean the fibre preparation further, a second method of debris removal was accomplished by gravity sedimentation. Specifically, a 15 mL conical-bottom centrifuge tube (Falcon, Lincoln Park, New Jersey) was filled with 8 mL PM. Then fibres were transferred carefully from the Petri dish by using a clean wide-bore pipette to place them

gently on the surface of PM medium in the tube. Fibres were then allowed to fall down through the fluid column of PM and settle to the conical bottom for approximately four minutes, by which time a fibre pellet was observed on the bottom of column. In the fluid, other fibres and tissue debris, such as broken or damaged fibres and connective tissue elements such as blood vessels, were typically still visible in the fluid column above the pellet. Following the four-minute gravity sedimentation period, the column of PM above the pellet was slowly removed (and discarded) by using a wide-bore pipette (without creating turbulence in the column or re-suspending the pelleted fibres), until roughly 1mL of medium containing the pellet of fibres was left in the tube. Gently, fibres were re-suspended in the remaining medium. Next, the fibre-containing fluid was transferred into a clean 60 mm Petri dish with a wide-bore pipette. At this point, fibres were ready to plate on coverslips (Fisher, Hampton, New Hampshire). Prior to use, the coverslips were immersed in acetic acid for 15 minutes and both sides of each coverslip were rinsed well with tap water for 20 minutes, placed into a 150 mm glass Petri dish and autoclaved (see Figure 1 top panel).

2.2.3 Fish muscle fibre plating

For plating, the following steps were conducted inside a bio-safety cabinet (BSC). Plating refers to the process of spreading the muscle fibres evenly on a coverslip which had been pre-coated with collagen (Advanced BioMatrix, Inc., San Diego, CA) so fibres would adhere to the surface of the coverslip. Pre-cleaned and autoclaved coverslips were placed, one to a dish, in the bottom of 35 mm plastic Petri dishes (VWR International, Radnor, US). Before plating, collagen was removed from 4 °C and kept on ice during the process of fibre plating. Collagen was swirled gently before use and, due to

its viscosity, was withdrawn into a pipette fitted with a plastic tip that had been cut shorter (to widen the bore). Next, one corner of the bottom of each sterile Petri dish was labelled according to treatment and experiment code. A tray was filled with crushed ice and placed on top of another tray laid out with those labelled dishes. The surface of the crushed ice was flattened so that all the dishes were level (in the same plane). By doing so, fibres plated in each dish were able to spread out evenly on the coated coverslip and were prevented from clumping or clustering to one corner before the liquid collagen gelled and firmly embedded the fibres. Collagen in 50 µL aliquots was pipetted onto the centre of each coverslip. A custom-made glass pipette shaped like a hockey stick was prepared by heating the tip of a Pasteur pipette over a flame and bending the tip at a 90° angle with forceps, so the bent tip was as long as the width of a coverslip. The hockey stick was allowed to cool for a few seconds before the end of the tip was flame polished, and then autoclaved. These hockey sticks were used to spread out liquid collagen evenly and gently on the entire surface of coverslip, while working on ice (to prevent collagen from gelling). After coating the coverslips with collagen, 30 µl PM containing fibres (gently re-suspended in the minimal amount of PM) was pulled into a pipette tip that had been prepared by cutting off the pointed end and flaming briefly to prevent damage to fibres. Fibres were dispersed evenly onto the surface of collagen. Each Petri dish was then covered with the lid, and the tray with all dishes was moved carefully into a 27 °C incubator for 50 minutes so that the collagen could form a gel that would hold fibres adherent (see Figure 1 bottom panel).

2.2.4 Fish muscle fibre culturing

Once fibres were attached to the collagen gel on the coverslips, the tray was removed from the incubator and 500 µL basal growth medium (BGM, see appendix) supplemented with or without treatment(s) (as described below for individual experiments) was placed on the coverslip. Then, the tray of dishes was returned to the incubator at 27 °C for an additional 24 hours. Twelve hours after plating, the pH (using the coloured indicator in medium) and the amount of culture medium on each coverslip were checked to ensure fibres were covered and maintained with the appropriate condition pH of 7.4.

Depending on the number of treatments in an experiment and the number of dishes/coverslips required for the study, the medium containing fibres (collected after gravity sedimentation) was diluted with PM into the ideal volume for distribution. For example, for a dose-response experiment with six different drug concentrations, 4-5 dishes per group were pre-labelled (to provide for sufficient replication) with an additional five dishes without any treatment were prepared to use for negative controls (e.g., in an immunostaining assay, see below) or as back-up extra control dishes. This representative experiment would provide at least 35 dishes, each containing one coverslip of plated fibres (see below). Typically, each dish was plated with about 40 fibres. If there were fewer than 20-25 intact fibres on a coverslip, that coverslip would not be used in counting toward data collection. In other words, this means that in a group size of n=4 dishes for example, the mean and standard error of the mean (sem) represent the data collected from observing at least 80-100 fibres in that treatment group.

2.2.5 Fish muscle fibre fixation

All dishes were maintained for 24 hours in culture (timed from initial plating) in media containing BrdU to label DNA synthesis. After culturing for 24 hours, culture medium was discarded (after careful removal from the coverslips) and 2 mL of sterile 0.01M PBS was added to rinse each coverslip/dish. Fibres were fixed for 20 minutes with 2 mL of 5% acid alcohol (glacial acetic acid diluted with 100% ethanol) and then air dried for about 45 minutes inside the BSC. Fibres on coverslips were stored in the same dishes until immunohistochemistry (IHC) by adding 2 mL of 0.01M Tris-buffered saline (TBS, see appendix) containing 1% horse serum to each dish; dishes were kept at 4 °C in a clean, covered tray containing a wet Kim-Wipe (Fisher, Hampton, New Hampshire) to maintain humidity and prevent drying.

2.3 Stretching-induced activation on single muscle fibre cultures

Following muscle fibre isolation as reported above (see section 2.2.2), single fibres were plated on six-well FlexCell culture plates (see Figure 2-2) (FlexCell International Corporation, Hillsborough, North Carolina), and subjected to a cyclical stretching protocol (see below) by using a computer-controlled FlexCell International, Inc. vacuum system (called a "stretch system" in the text below) (see Figure 3). The FlexCell plates possess a silastic (flexible) bottom substrate. Similar to normal Petri dishes, fibres would only adhere stably in the dishes if the wells were pre-coated with a layer of collagen and then allowed to incubate at 27 °C for 50 minutes to gel. Indeed, this extra collagen cover was of critical importance to prevent fibres from detaching from the dish during stretching. The dishes containing plated fibres were placed on a vacuum mat and connected to the stretch system. Following the set-up of the FlexCell stretch system

apparatus, 500 µL of culture media, namely BGM with or without additional treatment(s), was added to each well prior to stretch.

The stretching parameters originated from work on the mouse model, reported by Wozniak et al., (2003). Mouse fibres in those previously reported experiments were subjected to two hours of cyclic strain using a 10% stretch (length change along the radius of a culture well) imposed at a frequency of four cycles per minute. Here, the same strain frequency was adopted; the length change was modified to a 20% stretch and the period of cyclic stretch was set at three hours (see Figure 4). These modifications to increase the proportionate elongation and extend the duration of stretching were made in consideration of the physiological environment of muscle in fish, in which there is a demand for continuous repetitive muscle contraction for swimming. This is particularly important in considering fast-start swimming when fish need to escape from a predator by generating robust and rapid, power swimming (Altringham and Johnston, 1990). After three hours of stretching, the vacuum mat was disconnected from the computer controls and fibres were incubated at 27 °C for a further 21 hours prior to fixation (see section 2.2.5). For each time point in an experiment, a set of FlexCell wells that did not receive stretch were included, and served as unstretched controls.

2.4 Immunohistochemistry for BrdU and c-Met

The incorporation of BrdU into SCs during S-phase was assayed using anti-BrdU antibodies according to immunohistochemistry (IHC) World protocols, reported by Mizunoya et al., 2011.

Specifically, fibres on each dish or coverslip were washed twice, 10 minutes per wash, with 2 mL 0.01M PBS (diluted from 0.1M PBS with distilled water). For dishes to be stained for BrdU, each dish was treated with 2mL of 2N HCl and dishes were incubated at 37 °C for one hour. Fibres were then washed twice again, 10 minutes per wash, with 0.01M TBS-Tween20 (TBS-T) (diluted from 0.1M TBS with 1% Tween20). Next fibres were incubated overnight at 4°C in a blocking solution of goat serum (Invitrogen Corporation, Carlsbad, US) containing one of goat anti-mouse biotin-unconjugated antibody (Jackson Immuno Research Laboratories, Inc., West Grove, US) for BrdU staining, or goat anti-rabbit biotin-unconjugated antibody (Jackson Immuno Research Laboratories, Inc., West Grove, US) for c-Met staining (both diluted at the ratio of 20:1). To prevent drying, coverslips were inverted on slides. The next day, slides were soaked in 0.01M TBS-T to rinse and remove coverslips and coverslips were placed on Parafilm (VWR International, Radnor, US) with tissue paper underneath to maintain humidity (three squares for five coverslips, on average). From here forward, most of the steps took place on this foil layer, including incubation with 0.001% avidin (Sigma-Aldrich Corporation, St. Louis, US) and 0.001% biotin (Sigma-Aldrich Corporation, St. Louis, US) for 15 minutes each. Following those two background blocking steps, fibres were incubated overnight in primary antibody (anti-BrdU antibody (Roche Applied Science, Penzberg, Germany) and anti-c-Met antibody (Santa Cruz Biotechnology, Inc., Dallas, US) diluted 1:50 with primary antibody dilution buffer, see appendix) (for this step, coverslips were inverted again onto clean glass slides to prevent drying). For those steps performed on foil film, the washing volume was 500-600 µL; and the incubation volume was 200 µL per coverslip. On the third day of the IHC protocol, i.e. after

incubating with primary antibody overnight, fibres were washed twice with 0.01M TBS-T, 10 minutes per wash, incubated with 3% H_2O_2 for 10 minutes, and then washed twice in 0.01M TBS-T again (10 minutes per wash). Then fibres were incubated in the secondary antibody, biotin-conjugated anti-mouse IgG (Jackson Immuno Research Laboratories, Inc., West Grove, US) for BrdU staining and biotin-conjugated anti-rabbit IgG (Jackson Immuno Research Laboratories, Inc., West Grove, US) for c-Met staining (both diluted 1:200 in secondary antibody dilution buffer, see appendix) at room temperature (RT) for one hour, then washed twice with 0.01M TBS-T for 10 minutes per wash. After washing, fibres were incubated with a 1:500 solution of streptavidin conjugated with horseradish peroxidase (HRP, Vector Laboratories Inc., Burlington, CA) in 0.01M PBS at RT for 20 minutes, and then washed twice with 0.01M TBS-T, 10 minutes per wash. Finally, fibres were incubated in a diaminobenzidine (DAB, Sigma Aldrich, St. Louis, MO) working solution (including 0.01M PBS, DAB, colour intensifier and 3% H_2O_2) which reacts with HRP and produces a dark brown stain over nuclei (BrdU staining) or cytoplasm (c-Met staining) (seen under a microscope) after developing for 5-7 minutes at RT. Then fibres were rinsed with tap water 7-8 times to stop the reaction, mounted with immu-mount (Thermo Electron Corporation, Waltham, Massachusetts) onto slides and dried before counting BrdU-positive (+) or c-Met-positive (+) staining.

2.5 Analysis and Statistics

The SC response to various treatments was evaluated by viewing under an Olympus BH2 light microscope (Olympus Canada Inc., Markham, Ontario) to make observations of BrdU+ staining indicating nucleotide uptake into SC nuclei. In scanning the coverslips or dishes, each field of view displayed a variety of fibres, including wide

and narrow, long and short, intact (with sarcomeres clearly visible) and contracted (including completely and partially shortened so that sarcomeres were not easily visible); some fibres (particularly in early dissections) were intertwined with connective tissue or clumped into groups (see Figure 5-1).

To evaluate the level of SC activation, defined by incorporation of BrdU in S-phase of the cell cycle, the number of SC per fibre that were stained positive for BrdU was counted. At least 20 fibres per dish were observed by scanning across the coverslip or dish, and counts were only recorded for the intact fibres that were separated from one another, without knowing the treatment group. Using 400X magnification or higher to assay BrdU+ staining, only the nuclei that were stained dark brown were counted as positively stained.

During analysis, cells with dark brown nuclei were counted as positive staining for BrdU incorporation (see Figure 5-2); cells with pale nuclei, in other words, cells that may have been about to enter or exit S-phase (with very little time for BrdU incorporation), were not counted. As well, background staining was low and the influence of possible background was removed by considering only cells with dark brown nuclei (i.e., interpreted as being in S-phase for sufficient time to result in a high level of BrdU incorporation). All the dishes were coded to avoid bias during counting. The data (number of BrdU-positive (+) SC per fibre for at least 20 fibres per dish) were compiled in an Excel spreadsheet for each experiment. The mean ± standard error of the mean (sem) for each treatment was calculated, using each dish or coverslip as n=1 (recalling that many fibres are plated on each coverslip, see Figure 1-2). The number of BrdU+ nuclei (activated SCs) was plotted on the y-axis against the different treatment groups on

the x-axis. The significance of the changes in SC activation with treatment was determined using appropriate statistical methods in JMP-SAS software or Excel. Analysis of variance (ANOVA) in a one-way design was used to study the effects of treatment concentration on activation. A two-way ANOVA design was used to study the effects of stretch and effects of treatment, time or temperature plus the interaction effects. Significant differences were indicated by $p< 0.05$. Post hoc Tukey's tests were used to determine differences in various pairs of means from the ANOVA. In other cases, a two-sample T-test with equal variance was used to compare the number of BrdU+ SCs between two specific groups. The possibility of treatment-induced changes in the frequency distribution of the number of BrdU+ or c-Met+ SCs per fibre was examined using Chi-squared statistics.

2.6 Specific experiments

2.6.1 An *in vivo* time-course study of SC activation

The *in vivo* time-course study was conducted to investigate whether a 24 hour culture period was adequate for detecting a cycle of BrdU incorporation into DNA of the S-phase of zebrafish muscle SCs. The 24-hour time point was utilized in previous mouse muscle studies (Bischoff, 1986; Yablonka-Reuveni and Rivera, 1994; Anderson and Pilipowicz, 2002; Wozniak et al., 2003). This time-course experiment was performed by injecting BrdU intraperitoneally (ip). Specifically, a zebrafish (maintained at 27 ºC) was brought from the animal holding facility and anaesthetized using MS-222 in a solution at 40 mg/125 mL. When the fish was anesthetised, it was removed quickly from the MS-222 solution and extra water was blotted off. The fish was weighted and its length was

measured (see table 1 for fish body properties). Fish-handling time out of the water was minimized to a few seconds. At an angle of approximately 45° between the caudal fins and anal vent, the fish was injected ip with 0.04 mL of a BrdU solution (1 mg/mL) (Joachim, 2010), with the needle pointing toward the fish head. Immediately following injection, the fish was returned to plain water and monitored for recovery, which always occurred within 30 seconds. After two and half hours, the fish was euthanized in a solution of MS-222 at a concentration of 80 mg/125 mL and single fibres were isolated and plated on coverslips in 35 mm petri dishes coated with collagen using the same methods described above (see section 2.2.2 and 2.2.3). Following fibre plating, dishes were incubated at 27 ºC for 50 minutes; during this time, fibres settled down and were partially or fully embedded in collagen while it turned into a gel state. Immediately following this incubation, four dishes were removed from the incubator and fixed with 5% acid alcohol, representing the first time point in the experiment. In the meantime, 500 µL BGM was added to each of the remaining dishes and they were incubated at 27 ºC for various times. Subsequent time points were created by removing and fixing three dishes every two hours in the same way as the first (zero time) group, up to a maximum of 24 hours incubation. Following fixation, 2 mL of 1% horse serum in 0.01 M TBS was added to each dish and dishes were stored at 4 ºC, awaiting processing for IHC. When all the dishes were fixed, anti-BrdU antibody IHC was performed as described above (see section 2.4). To assess SC activation, the number of BrdU+ SC per fibre in each dish from all time points was counted as described above (see section 2.5).

2.6.2 The population of SCs assessed over time by c-Met immunostaining

As mentioned previously (Cornelison and Wold, 1997), c-Met receptor is known to be expressed by both quiescent and activated SCs regardless of their activation state or phase in the cell cycle. Here, by employing immunostaining for c-Met protein and counting c-Met-positive (+) cells on zebrafish muscle fibres, the size of the SC pool on fish muscle fibres was assessed over a period of 24 hours in culture. This study was conducted to evaluate the population of SCs which is available to activation. In particular, the number of c-Met+ cells was compared with the number of BrdU+ cells at the time of plating (zero time) and after 24 hours of incubation. Experiments were conducted by dissecting two fish (see table 1 for fish body properties), followed by isolating and plating fibres as described above (see section 2.2.2 and 2.2.3). Once fibres were embedded in collagen, i.e., after incubation at 27 °C for 50 minutes, 500 µL BGM per dish was added to a set of five dishes to be used for c-Met staining. All five dishes were moved to the 27 °C incubator for 24 hours. Meanwhile, a second set of five dishes was fixed right after embedding fibres in collagen to represent the zero time point for c-Met staining. Following fibre culture over 24 hours, the first set of dishes were fixed and anti-c-Met immunostaining was performed in the appropriate dishes using the same approach described before (see section 2.2.5 and 2.4). Recalling that c-Met is expressed on the membrane of SCs, only cells displaying DAB precipitate (seen as a dark brown colour) around a pale or unstained nucleus were counted positive. Again, dishes were coded before counting to reduce bias.

2.6.3 Study of SC activation induced by NO and HGF

In vitro dose-response studies for NO and HGF

To explore whether SC activation on zebrafish muscle fibres is dependent on NO, fibres were cultured in media containing isosorbide dinitrate (ISDN, Sigma Aldrich, St. Louis, MO), a NO-donor drug known to activate SCs on mouse fibres in culture, at various concentrations (0-2.5 mM) in a dose-response experiment. Similarly, different HGF concentrations (0-25 ng/mL) (Sigma Aldrich, St. Louis, MO) were included in media to address whether SC activation on zebrafish muscle fibres shows a dose-dependent response to HGF. Specifically, single fibres from two fish (see table 1 for fish body properties) were isolated and plated following the same methods described above (see section 2.2.2 and 2.2.3). Immediately following 50 minutes incubation at 27 °C to embed fibres in collagen, different treatments were added to culture media (BGM containing 10 mg/mL of BrdU) and delivered by pipette to muscle fibres for culture. The initial doses of ISDN treatment were 0 mM, 0.5 mM, 1 mM, 1.5 mM, 2 mM, 2.5 mM and for HGF were 0 ng/ml, 5 ng/ml, 10 ng/ml, 15 ng/ml and 25 ng/ml (based on preliminary studies by J. Richelle and Dr. Anderson on zebrafish fibres, and previous reports on mouse cells and fibres by Tatsumi and colleagues and Wozniak et al., respectively, as referenced earlier). Within each treatment group, four duplicate cultures were ensured (n=4). All dishes were cultured at 27 °C for 24 hours; although CO_2 was not required, a tray filled with distilled water was critical to maintaining the humidity of the incubator that would prevent drying of the fish fibres during culture. After fibre incubation, all dishes were fixed and immunostained for BrdU as described above (see section 2.4). To determine the extent of SC activation under distinct ISDN and HGF concentrations, the

number of BrdU+ SCs on intact fibres was counted per culture (at least 20 fibres per culture) using light microscopy; again slides were coded without knowing treatment groups.

Study of the HGF-c-Met pathway

This experiment was performed to explore whether signalling through the pathway of HGF binding to its corresponding receptor, c-Met on the SC surface, is required for SC activation. To test this HGF-c-Met pathway hypothesis, anti-c-Met neutralizing antibody (Santa Cruz Biotechnology, Inc., Dallas, US) was employed to block the activity of the c-Met receptor on SCs, as previously reported by Tatsumi et al., (1998) and SC activation in this group was compared to an untreated control group. In half of each of these two groups (control and anti-c-Met), HGF at 10 ng/mL was added to the media (based on the results of the HGF dose-response study) in order to establish HGF-induced SC activation and blocked signalling of activation, respectively.

Experimentation was conducted using the same fibre isolation and plating methods described above (see section 2.2.2 and 2.2.3). When fibres collected from two fishes (see table 1 for fish body properties), were stabilized in collagen-coated dishes, dishes were divided into four groups randomly, with at least four dishes per group; dishes were labelled as group A, B, C and D. Dishes in group A were regarded as control group, with only 30 µL/mL BrdU (10 mg/mL) in the 500 µL of BGM. Dishes in group B, C and D also had culture media including BrdU, and in addition, 10 ng/mL HGF, 1 µg/mL anti-c-Met antibody, and both HGF and anti-c-Met antibody, respectively were added into dishes corresponding to groups B, C and D. All dishes were moved to the 27 °C

incubator and cultured for 24 hours. After incubation, fibre fixation and anti-BrdU immunostaining were conducted following the descriptions above (see section 2.4). The level of SC activation under discrete treatments was recorded from counting the number of BrdU+ SCs per fibre.

Study on the influence of temperature on HGF-induced activation

To better understand HGF-induced SC activation, the influence of a reduced temperature (i.e., 21 °C) was examined. Fibres from two fish (see table 1 for fish body properties) were isolated and plated as described before (see section 2.2.2 and 2.2.3). Following 50 minutes incubation to ensure fibres were anchored in the collagen layer, dishes were divided randomly into two groups, with a minimum of ten dishes per group, titled group A and B. Culture media (30 µL/mL BrdU (10 mg/mL) in 500 µL BGM were added into group-A dishes, representing the control group. HGF (10 ng/mL) was added to BGM containing BrdU to constitute group B. Next, five dishes from each of group A and group B were selected randomly, and renamed as groups C and D. Following this arrangement of groups, groups A and B were moved to a 21 °C incubator, whereas group C and D were moved to a 27 °C incubator. All dishes were cultured for 24 hours. After incubation, fibres were fixed as described above (see section 2.2.5) and stained for BrdU. The number of BrdU+ SCs was recorded, without knowledge of treatment groups, to examine the level of SC activation under distinct temperatures and the possibility of temperature effects on HGF-induced SC activation.

2.6.4 Study of stretching-induced activation and NOS inhibition

As reported in the mouse model (Wozniak et al., 2003), SC activation resulting from stretch stimulation was demonstrated to be NO dependent using the nonspecific NOS inhibitor, N^{ω}-nitro-L-Arginine methyl ester (L-NAME, Sigma Aldrich, St. Louis, MO). To explore whether cells on fish muscle fibres would have a similar response to the NOS inhibitor during stretching, fibres from two fish (see table 1 for fish body properties) were isolated and embedded in 100% collagen spread on FlexCell 6-well BioFlex culture plates with flexible silicone elastomer membrane on the bottom of each well. Following fibre preparation, plates were labelled according to one of five assigned treatment groups. Specifically, one plate with six wells of fibres was designated as the control group that would receive no stretching; all the other wells (different plates) were subjected to a stretch stimulus. Moreover, stretch-treated wells were further divided randomly into four groups, with four wells per group. All five groups were incubated in BGM media (500 µL) containing 30 µL/mL BrdU per well. Culture medium in a stretch-only group did not contain any additional treatment. The other three groups were treated with either 0.2 µg/mL of L-NAME, 1 mM of ISDN or both 0.2 µg/mL of L-NAME and 1 mM of ISDN. Following the addition of culture medium, stretch was applied as described earlier (see section 2.3) to the four non-control groups. After three hours of stretch, fibre plates were maintained in culture for an additional 21 hours (i.e., 24 hours in total) at the same temperature (27 °C) before being fixed and immunostaining. SC activation was assessed by counting the number of BrdU+ SCs per fibre, as described above (see section 2.5).

2.6.5 Changes in c-Met protein as a result of stretch

In an attempt to further explore the influence of stretch on the number of SC resident on fibres, c-Met immunostaining was performed on dishes of muscle fibres allocated randomly to the following five groups: 1) zero hour of incubation (right after plating) without stretch, n=5; 2) three hours of incubation with stretch, n=4; 3) three hours of incubation without stretch, n=4; 4) 24 hours of incubation with the first three hours under stretch conditions, n=4; and 5) 24 hours of incubation without stretch, n=4. The parameter of stretch was consistent throughout, as described earlier (i.e., 20% cyclical elongation for three hours), regardless of the incubation time. For this experiment, muscle fibres were harvested from two fish (see table 1 for body properties). The fibre isolation and plating approaches were in accordance with the method described previously (see section 2.2.2 and 2.2.3). Following 0, 3 or 24 hours of incubation with appropriate treatments for each of the groups described above, fibres in individual dishes were fixed and immunostained using anti-c-Met antibody, as reported before (see section 2.2.5 and 2.4). Using a light microscope at 200-400X magnification, the number of c-Met+ SCs per fibre was counted without knowledge of treatment group (blinded); data were compiled in an Excel spreadsheet. After statistical analysis, the code was broken.

Chapter 3: Results

Zebrafish body properties (i.e., body length and weight) from all zebrafish used in experimentation were recorded (see table 1). In an attempt to harvest intact single fibres without damaging the fibres or stimulating the satellite cells during trituration, the total length (from the tip of snout to the end of the longer lobe of the caudal fin) at about 35 ± 3 mm was regarded as the optimal fish length for isolating fibres, regardless of fish body weight which varies considerably as a result of diet, sex and maturity.

3.1 Morphological study of zebrafish

Figure 6 shows a representative micrograph of H&E staining of a transverse (a) and a longitudinal (b) fish muscle section. The right side of panel (a) represents the lateral side of the fish body with skin attached (seen as folded dark red colour). Fibre diameter increased markedly from superficial muscle fibres to deeper muscle fibres. This observation is consistent with the distribution of red (superficial) and white (deep) muscle fibres in which white muscle fibres possess larger diameters compared to red muscle fibres. From panel (b) showing a longitudinal section of the fish tail, the ventral side of the fish body is seen at the bottom of the picture. From the ventral to the dorsal side of the fish body, muscle fibres were parallel and bundles were separated from each other by myosepta. Fibres in this portion of the fish body had similar dimensions of length and width.

3.2 An *in vivo* time-course study of SC activation

Fish were euthanized 2.5 hours after injection with BrdU. After isolating and plating fibres and placing the dishes in the incubator, culture dishes were removed from

the incubator in a time-course from 0-24 hr later and fixed. For each time point, 80-100 intact muscle fibres were observed under the microscope and analysed in four or more culture dishes. The number of BrdU+ SCs per fibre was plotted against the incubation time in hours (see Figure 7). As shown in the line graph, the number of BrdU+ SCs per fibre varied over time. The level of SC activation (observed as cells that had incorporated BrdU in S-phase) increased gradually from 1.8 ± 0.2 (mean ± sem) to 2.4 ± 0.2, reaching the first peak at five hours of incubation. Then the number of BrdU+ SCs decreased to a minimum of 1.1 ± 0.2 at 12 hours of incubation. Subsequently, a second peak in the level of SC activation occurred at 20 hours of incubation. To further explore pair-wise differences between groups, a two-sample T-test with equal variance was performed. Results showed that the level of BrdU+ SC per fibre was significantly different between 0 and 5 hours of incubation ($p<0.03$), and between 0 and 20 hours of incubation ($p<0.03$). No significant difference was detected between these two maximum values, although the mean of the second peak (2.1 ± 0.2) appeared to be lower than that from the first peak (2.4 ± 0.2).

3.3 The population of SCs assessed over time by c-Met immunostaining

Fibres were observed under 400X magnification, and the number of c-Met positive (+) SCs was counted per fibre. For analysis, only intact (non-retracted, complete) fibres were observed. Cells in the satellite position on fibres that contained a pale nucleus surrounded by dark brown staining peripherally were regarded as c-Met+ were counted to analyse the population of SCs per fibre, regardless of activation state, at 0 hour and 24 hours after plating (no treatment). The average number of muscle fibres counted per dish was 22, with 4-5 replicate dishes at each of 0 hours and 24 hours of

incubation. As indicated in Figure 8, the number of c-Met+ SCs at zero hours of incubation was 6.4 ± 1.4 (mean ± sem). The number showed approximately a 1.5-fold increase after 24 hours of incubation, reaching a peak of 9.0 ± 1.9 c-Met+ SCs per fibre. A two-sample paired T-test was conducted and showed that the increase in activation between zero hour and 24 hours of incubation was significant ($p<0.04$).

3.4 Study of SC activation induced by NO and HGF

3.4.1 In vitro dose-response studies for NO and HGF

Graphs representing dose-response curves were created by plotting the number of BrdU+ SCs per fibre (mean ± sem), indicating the activation level of SC, against a range of treatments with ISDN (0-2.5 mM) or HGF (0-25 ng/mL) concentrations (see Figure 9). There were four dishes for each treatment, consisting of 20-25 intact fibres per dish. For the control group (exposed to neither ISDN nor HGF), there were six dishes, including counts from 135 intact fibres. Results displayed changes in the number of BrdU+ SCs per fibre depending on the concentration of either ISDN or HGF; in other words, the level of SC activation (indicated by the mean number of BrdU+ SCs per fibre) showed a significant response to ISDN and HGF ($p<0.0001$ and $p<0.0001$, respectively; ANOVAs). The level of activation peaked at 1 mM ISDN and 10 ng/mL HGF. The maximum values for BrdU+ SCs per fibre under these two ISDN and HGF concentrations were 3.7 ± 0.5 and 3.8 ± 0.4, respectively, compared to their corresponding controls (3.0 ± 0.7 BrdU+ SCs per fibre).

3.4.2 Study of the HGF-c-Met pathway

This experiment was derived from consideration that if c-Met receptor has a critical role in signal transduction to SCs, which subsequently activate a downstream signalling pathway and initiate mobility of SCs, blocking c-Met activity should result in a decrease in the number of activated SCs. In agreement with the hypothesis, data gathered by counting the number of BrdU+ SCs per fibre showed that culturing fibres in the presence of anti-c-Met antibody had an inhibitory effect on SC activation (see Figure 10). As seen in figure 10, the number of BrdU+ SCs per fibre in the control group was 3.0 ± 0.6 in the absence of any treatment. With the addition of 10 ng/mL HGF, the number of BrdU+ SCs showed a significant increase ($p<0.0001$, two-sample T-test with equal variance), reaching a maximum level of activation at 3.8 ± 0.8 BrdU+ cells per fibre. However, fibres treated by HGF plus anti-c-Met antibody together showed no stimulation of activation by HGF; in other words, the number of BrdU+ SCs in this group was not significantly different from the control group. In an effort to avoid the possible influence from anti-c-Met antibody alone, the group of fibres cultured with only anti-c-Met antibody was analysed and also revealed no difference compared to the control group in the number of BrdU+ SCs per fibre.

3.4.3 Study of temperature influence on HGF-induced activation

To explore the influence of a lower temperature on SC activation, the optimal concentration of HGF (i.e., 10 ng/mL) was employed to establish the level of SC activation at 27 °C as an “activation baseline”. Results here were consistent compared to the previous HGF dose-response study at 27 °C (see Figure 11); fibres exposed to

10 ng/mL HGF at 27 °C exhibited a significant increase in the level of SC activation (3.8 ± 0.8) (by analysing the number of BrdU+ SCs per fibre) compared to the corresponding control group (3.0 ± 0.6) at 27 °C ($p<0.0001$, two-sample T-test with equal variance). Interestingly, when fibres were cultured at the lower temperature of 21 °C, fibres treated with the same concentration of HGF (10 ng/mL) also displayed a significant increase in SC activation compared to the untreated control group without HGF at 21 °C ($p<0.0001$, two sample T-test with equal variance). However, the increase in activation was smaller than at 27 °C as the number of BrdU+ SCs per fibre increased from 2.6 ± 0.5 in the control group only to 3.1 ± 0.6 when fibres were treated by HGF at 21 °C. For ease of comparison, the percentage increase in the mean SC activation by HGF was 119% at 21 °C and 126% at 27 °C. In an attempt to further explore the effect of treatment (HGF) and/or the effect of temperature to SC activation, a two-way ANOVA was applied and found HGF alone increased SC activation significantly regardless of temperature ($p<0.0001$); by comparison, a lower temperature decreased SC activation by HGF significantly regardless of treatment ($p<0.0001$). Moreover, the interaction of temperature and HGF together had a significant effect on SC activation ($p<0.001$).

3.5 Study of stretch-induced SC activation and NOS inhibitor

This experiment was established to test the effect of stretch on SC activation on zebrafish fibres and also whether NOS activity was essential for activation of SC on fibres in culture at 27 °C. There were five groups in this experiment, including a control group (no stretch or treatment), stretch control, stretch plus ISDN, stretch plus L-NAME, stretch plus ISDN and L-NAME.

As observed in Figure 12, the number of BrdU+ SCs per fibre increased dramatically in the presence of stretch, reaching a value of 3.5 ± 0.4 in the stretch-only group. However, this augmentation of BrdU+ SCs per fibre by stretch was not as high as that (3.9 ± 0.4) in the group of fibres treated with 1 mM ISDN (optimal concentration for SC activation based on dose-response study) together with stretch. In contrast, the number of BrdU+ SCs in the stretched group, with the addition of L-NAME showed a lower level of SC activation than the stretch only group, reaching only 3.3 ± 0.4 BrdU+ SCs per fibre. Interestingly, when both L-NAME and ISDN were added to fibre cultures together, the degree of SC activation was 3.6 ± 0.4 BrdU+ SCs per fibre, close to the level of stimulation in fibre dishes treated by ISDN only.

One-way ANOVA showed that there was a significant difference among five treatment groups including stretched and unstretched fibre cultures ($p<0.0001$). To determine further, whether and where the significant pair-wise differences were among these groups, Tukey's post hoc analysis was conducted with the confidence level of $p<0.05$ (calculated to require a q value ≥ 4.3). There were significant differences between the following groups: all stretch-treated groups vs. the unstretched control group ($q\geq 5.7$); the stretched control group vs. the stretch plus ISDN group ($q=5.35$); the stretch plus ISDN and L-NAME group vs. the stretch plus L-NAME group ($q=6.18$).

3.6 Changes in c-Met expression as a result of stretch

Figure 13 shows the number of c-Met+ SCs per fibre (y-axis, mean ± sem) plotted for each of the groups (zero hour, and three or 24 hours of incubation with and without stretch). One-way ANOVA was applied and found a significant difference

among the five groups ($p<0.0001$). The follow-up Tukey's post hoc analysis was utilized to determine differences between two groups at the level of significance defined as $p<0.05$ ($q \geq 4.51$). In line with previous results regarding c-Met expression over 24 hours of incubation in the absence of stretch, the number of c-Met+ SCs per fibre increased significantly after 24 hours of incubation in both stretched and unstretched groups ($q=12.97$ and 12.65). The number of c-Met+ SC's per fibre started from 5.5 ± 0.5 (mean ± sem) at zero hour of incubation, and reached a maximum of 8.7 ± 0.7 and 8.6 ± 0.6 in stretched and unstretched groups, respectively, after 24 hours of incubation. Interestingly, the number of c-Met+ SCs on fibres that underwent three hours of incubation with the presence of stretch (7.1 ± 0.5) was significantly higher compared to the group immediately post-plating at zero hour of incubation ($q=6.63$). However, there was no significant difference between the group incubated for three hours without stretch (6.3 ± 0.5) compared to its counterpart three-hour stretch group ($q=3.31$), or compared with the group that was fixed immediately after plating at zero hour incubation ($q=3.31$). Moreover, the number of c-Met+ SC's per fibre showed a significant increase between three and 24 hours of incubation for both the stretched and unstretched groups, i.e., over a further 21 hours incubation ($q= 6.55$ and 9.33, respectively).

Chi-square statistics were performed to analyze the distributions of c-Met+ SCs per fibre over 24 hours with or without stimulation by stretching. With the degrees of freedom established at 16 (some groups were pooled to satisfy the need to have numbers greater than or equal to 5 in each cell), Chi-square values that were greater than 26.3 were regarded as significant ($p<0.05$). Generally, the distribution of c-Met+ SCs per fibre among the five groups together (i.e., 0 hour, 3 hours stretch or unstretched, 3 hours with

or without stretch plus additional 21 hours of incubation) was significant among the groups, with the total Chi-square value of 69.6. Specific pairwise comparisons showed that the population of fibres was shifted toward fibres with higher numbers of c-Met+ SC per fibre after 24 hours incubation compared to zero hours, in the group with stretch (Chi-square=47.1, $p<0.01$) and without stretch (Chi-square=46.2, $p<0.01$). This shift was due to increased proportions of fibres with higher number of positive c-Met cells. The distribution of the fibre population was not significantly different from zero hours incubation after three hours incubation, although this was nearly at the level of significance (Chi-square=24.4; critical value = 26.3 for $p<0.05$). However, the distributions did not differ after 24 hours of incubation, between those fibres that received stretching or not for the first three hours of incubation (calculated Chi-square = 3.13).

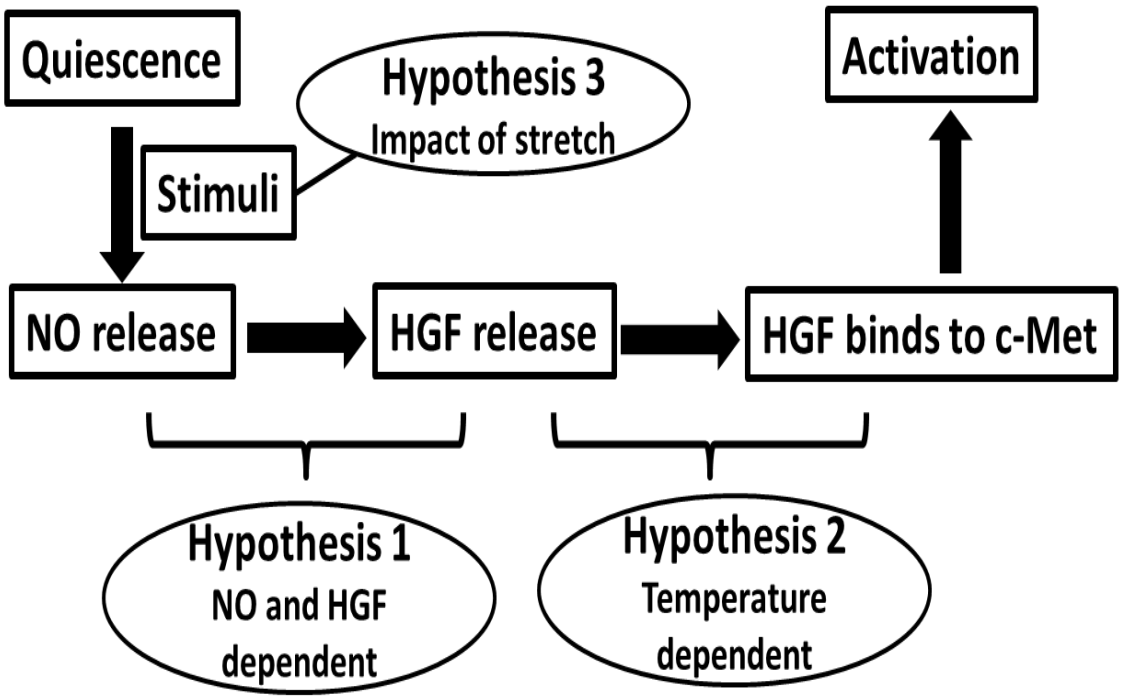


Figure 1-1. Flowchart of the satellite cell activation pathway and the hypotheses in the current project. In the mouse muscle-fibre culture model, satellite cells can be activated and enter the cell cycle. This activation process is a cascade of events, including NO release, HGF release, and HGF binding to the c-Met receptor; this triggers satellite cell activation. Three hypotheses were examined in this thesis by using fish fibre cultures: 1) Satellite cell activation is dependent on NO and HGF; 2) HGF-induced satellite cell activation is temperature dependent; 3) Stretch can activate satellite cells via NO release.

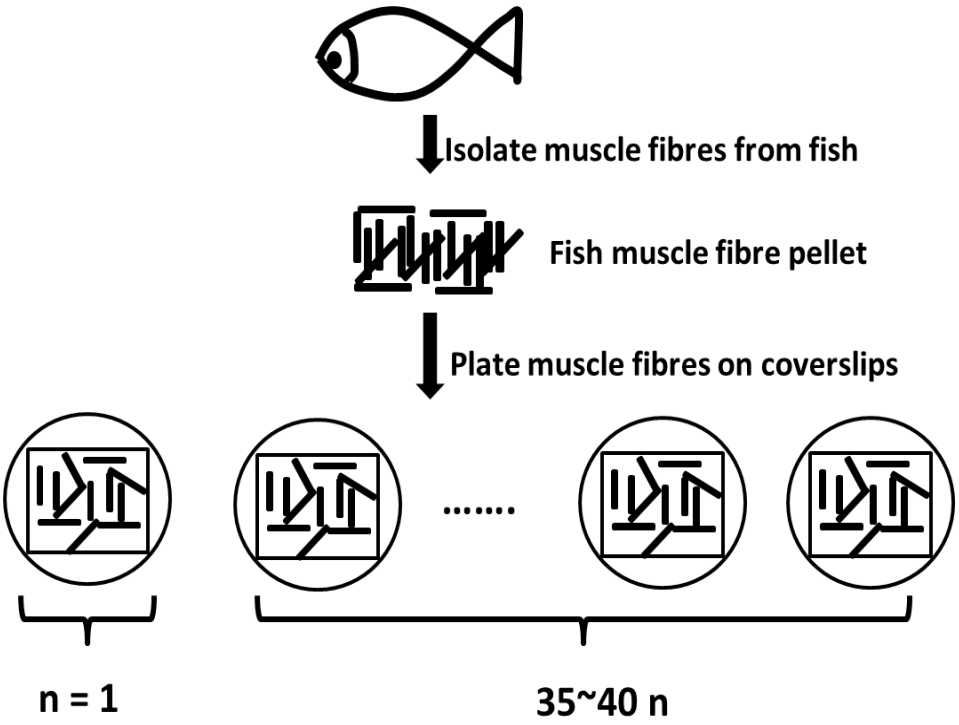


Figure 1-2. Illustration of the experimental design that is available from isolation of fish single muscle fibres for culture. Each individual fish can generate, on average, thousands of single muscle fibres; this forms a pellet of muscle fibres in a conical tube used for gravity sedimentation during isolation. These muscle fibres from a single zebrafish can be plated on 35~40 coverslips pre-coated in collagen, each coverslip receiving at least 20-25 fibres; coverslips are then placed in 35mm Petri dishes and covered with medium. Each coverslip/Petri dish was considered as n=1 for analysis; the mean number of satellite cells positive for BrdU or c-Met was calculated from counts of 20-25 fibres per coverslip/dish.

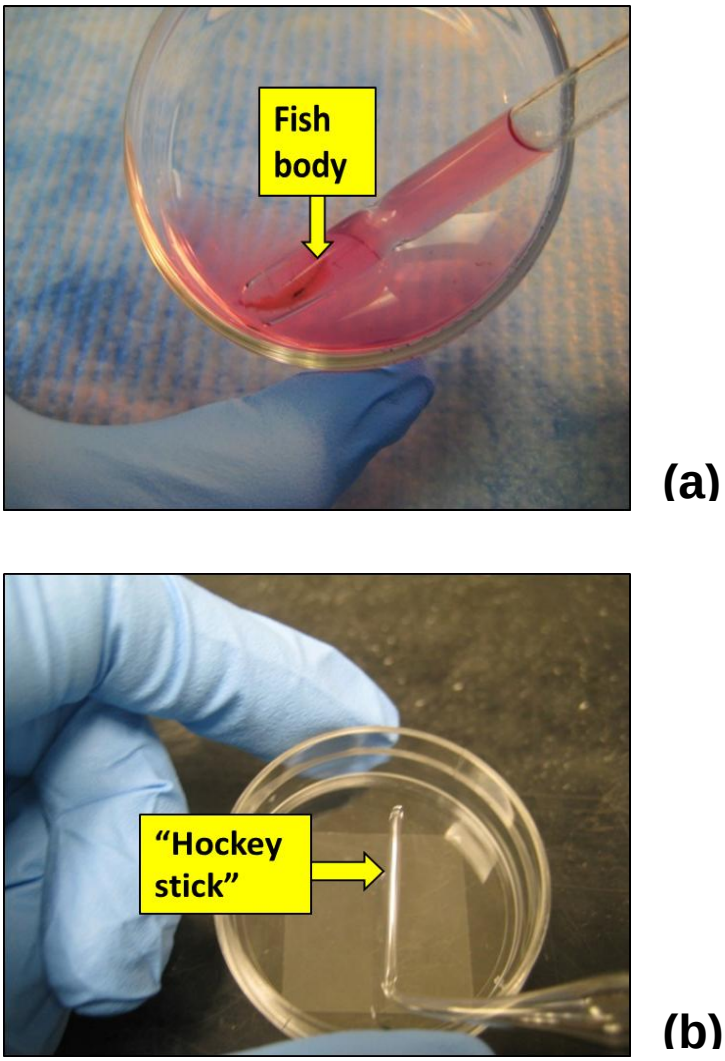


Figure 1-3. Fish muscle fibre isolation through trituration and fibre plating on a coverslip. Photographs of fish fibre isolation via trituration (top image, a) and fibre plating onto collagen coated coverslips (bottom image, b). The body size of the fish must fit inside the wide bore pipette used to triturate and separate the muscle fibres to avoid damaging or injuring the fibres. The "hockey stick" was used to evenly coat the coverslip with collagen.

Figure 2. BioFlex plate with a silastic (elastic) bottom. Six-well FlexCell BioFlex plate with silicone elastomer bottoms, which was inserted into a mat and connected to a strain unit. There was a hole underneath each well which allowed air to go in and out. When the air became compressed, the elastic membrane stretched and went down toward the mat bottom; meanwhile, fibres adhered to the flexible membrane, thereby achieving a radial stretching stimulus within each well. The wells were stretched to 20% elongation of the radial axis.

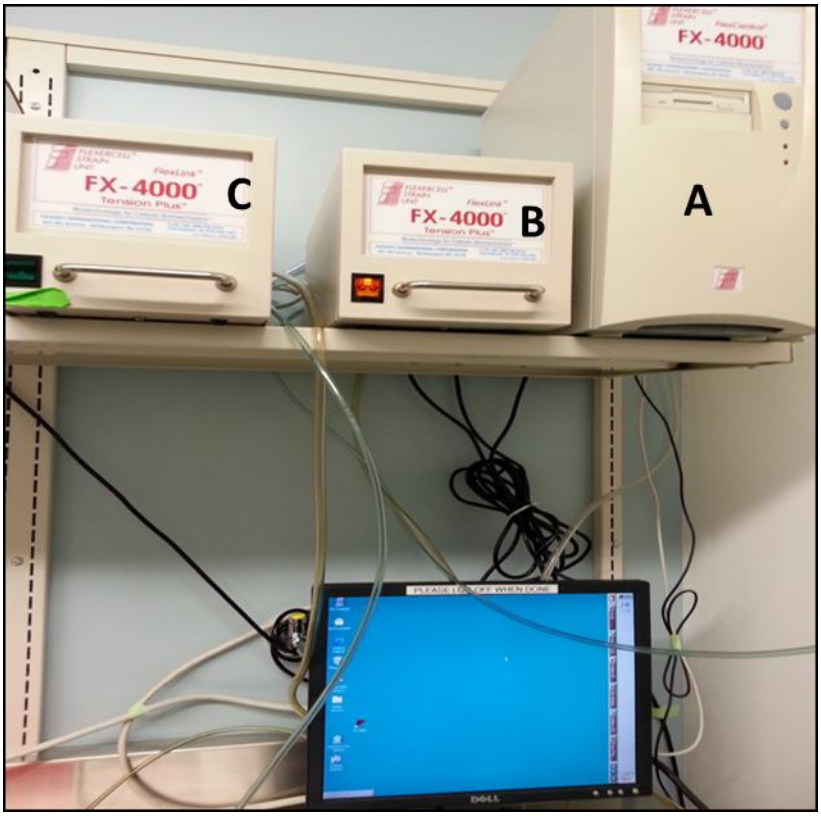
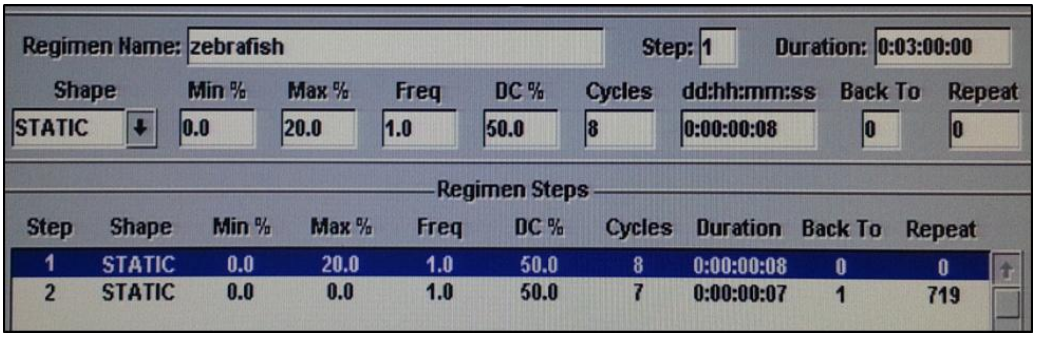


Figure 3. Computer-controlled FlexCell tension system. A computer-regulated FlexCell tension system (FX-4000), with the components of a computer which connects to the central controller (labeled as A) and two tension units (labeled as B and C) which transduce the vacuum pressure to the mats where the FlexCell plates are mounted. Mats can be placed into an incubator at appropriate temperatures as required and consistent or variable strains can be applied to individual mats using input to the computer program.


Regimen Name: zebrafish
Step: 1
Duration: 0:03:00:00
Shape
Min %
Max %
Freq
DC %
Cycles
dd:hh:mm:ss
Back To
Repeat
STATIC
0.0
20.0
1.0
50.0
8
0:00:00:08
0
0
Regimen Steps
Step Shape Min % Max % Freq DC % Cycles Duration Back To Repeat
1 STATIC 0.0 20.0 1.0 50.0 8 0:00:00:08 0 0
2 STATIC 0.0 0.0 1.0 50.0 7 0:00:00:07 1 719

(a)


Flexcell International FX-4000 (V4.0)
Controllers
FlexCentral
FlexLink #1
FlexLink #2
FlexLink #3
Plot Units
% Elong
kPa
Strain
mStrain
uStrain
Smoothing
None
Moving Avg
X:
Y:
User: Helia
Regimen: zebrafish
Baseplate: BFlx Plate, no Loading
Run Time: 0:00:01:12 0:02:58:48
8 sec
7 sec
Pressure (% Elong)
Time (Sec)
30.0
27.0
24.0
21.0
18.0
15.0
12.0
9.0
6.0
3.0
0.0
0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0

(b)

Figure 4. Stretch parameters with 20% elongation for three hours. Screenshot of the program output, indicating cyclic stretch parameters (20% elongation at 0.067 Hz), which consists of two static (or stable) strains (see panel a) (of 20% elongation for eight seconds each on either side of a seven second period (center of the tracing, see panel b) at 0% elongation). The program output was limited to the frequency range of 0.1 Hz to 3 Hz.

	Total length(mm)		Weight(g)	
Section 2.1.1	**34**		**0.3876**	
Section 2.6.1	**33**		**0.2883**	
Section 2.6.2	**Fish 1** **29**	**Fish 2** **34**	**Fish 1** **0.2608**	**Fish 2** **0.2961**
Section 2.6.3 (a)	**35**	**35**	**0.4046**	**0.4243**
Section 2.6.3 (b)	**34**	**33**	**0.2476**	**0.2874**
Section 2.6.3 (c)	**38**	**36**	**0.4487**	**0.3854**
Section 2.6.4	**34**	**35**	**0.2630**	**0.3427**
Section 2.6.5	**36**	**32**	**0.6677**	**0.3759**

Table 1. Fish body properties. Total length indicates the length from the tip of snout to the end of the longer lobe of the caudal fin. Sections in the table refer to each individual experiment described in the text.

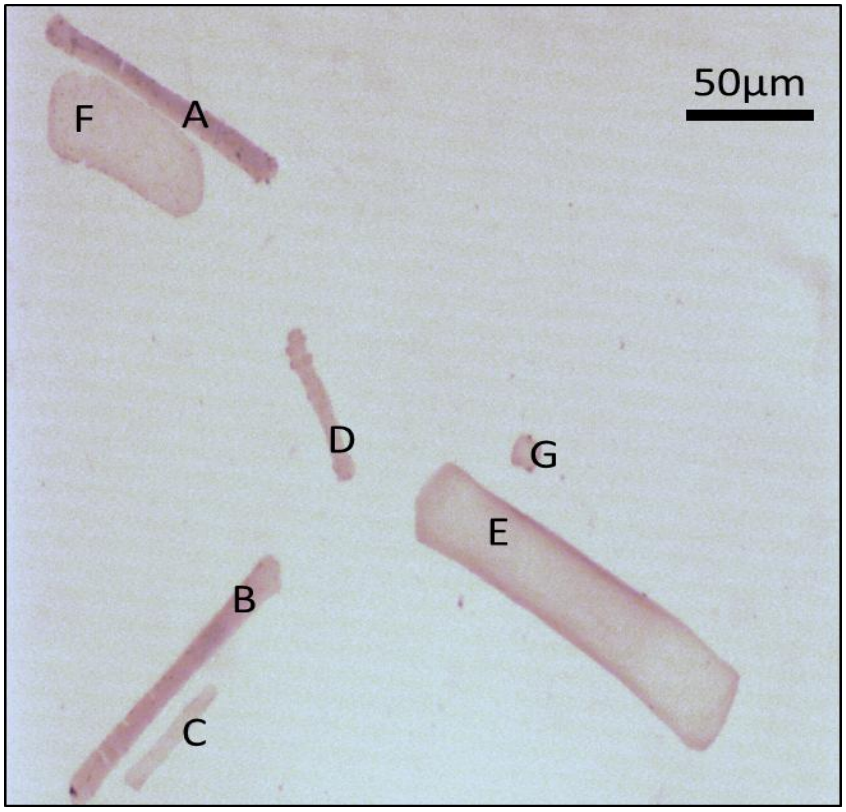
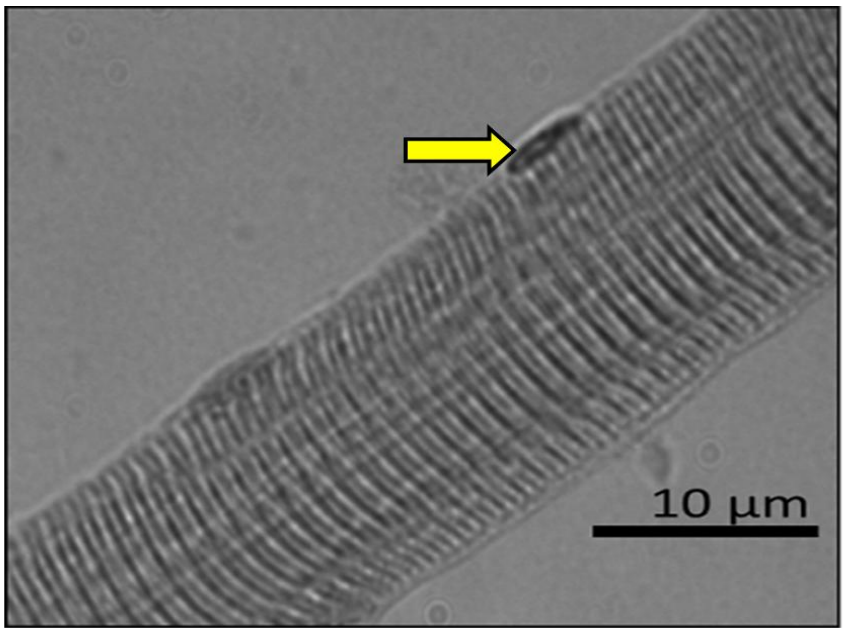
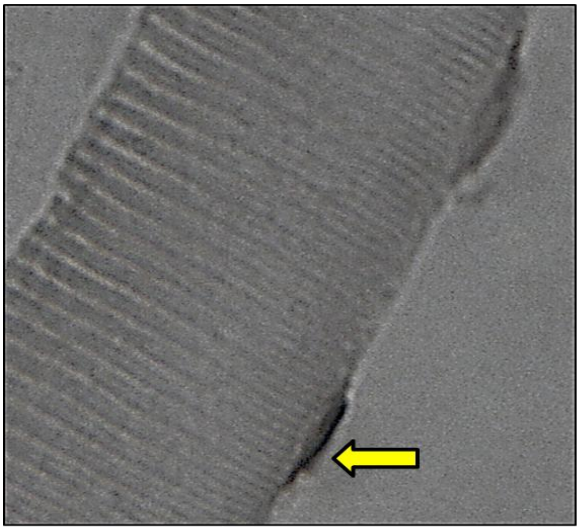


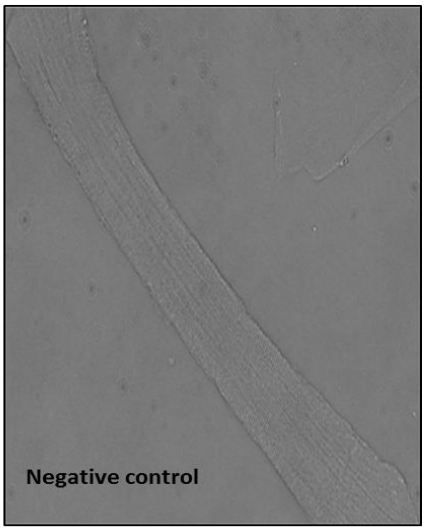
Figure 5-1. Representative muscle fibres after plating, as viewed under the microscope. Representative micrograph of fibres with distinct lengths and widths, captured with the 40X total magnification on a light microscope. Within one dish there was a wide variety of fibre dimensions; some are long and thin (labeled as A and B), some are short and thin (labeled as C), some are partially contracted (labeled as D), some have a large width but different lengths (labeled as E and F), and some of them are broken pieces (labeled as G). In this regard, fibres A, B, C, E and F are intact fibres.



(a)

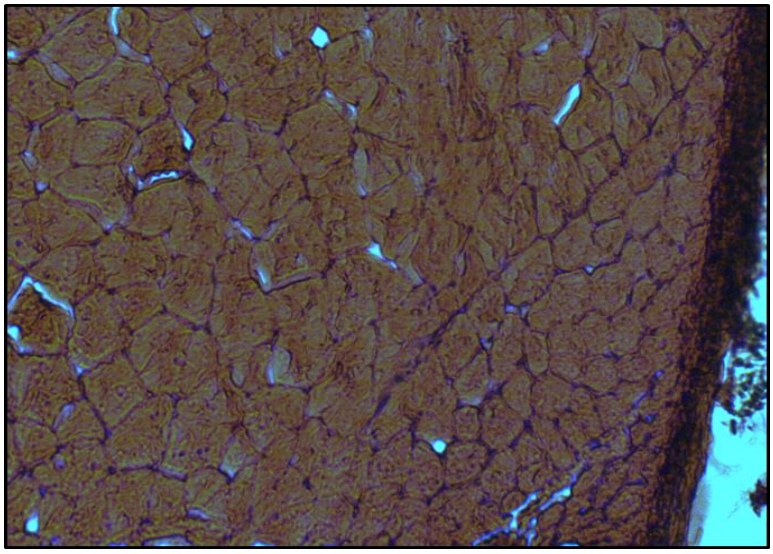


(b)

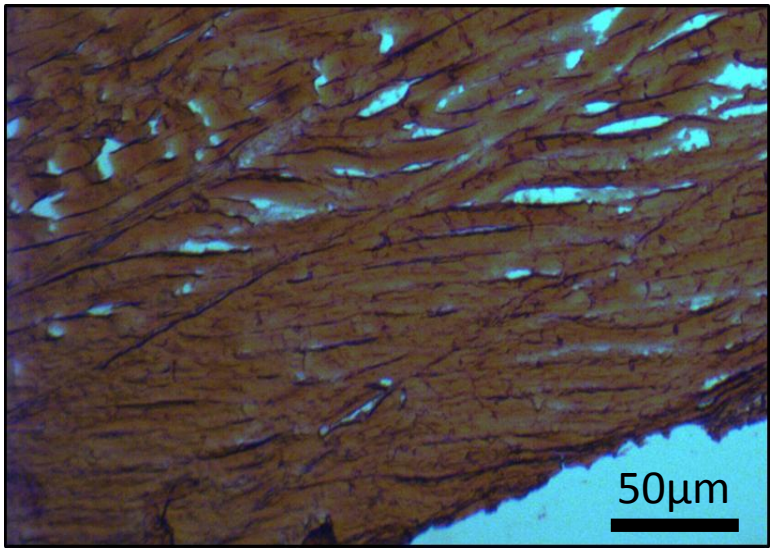


(c)

Figure 5-2. Representative images of fibres immunostained for BrdU and c-Met. Photos above were representative micrographs of BrdU+ SC (panel a); a c-Met+ SC (panel b); and a negative control fibre (panel c). Panel a and panel b were captured at 400X total magnification. A dark brown staining (from the HRP-DAB method of immunostaining) is located over the nucleus for BrdU+ staining and over the cytoplasm of a SC for c-Met+ staining. Note the unstained SC (c-Met-negative) just above the c-Met+ SC indicated in panel b. Panel c was visualized using 100X light microscope.



(a)



(b)

Figure 6. Representative cross and longitudinal sections of H&E staining of muscle histology in the zebrafish. Representative micrographs of H&E staining for transverse (top panel, a) and longitudinal (bottom panel, b) sections. Photos were captured at 40X total magnification. Nuclei are stained as blue and cytoplasm is stained as a red colour. Note in panel a that the slow fibres just under the layer of skin (on the right of the section) are smaller in diameter than the white muscle fibres deeper in the fish body that are seen at the left of the section.

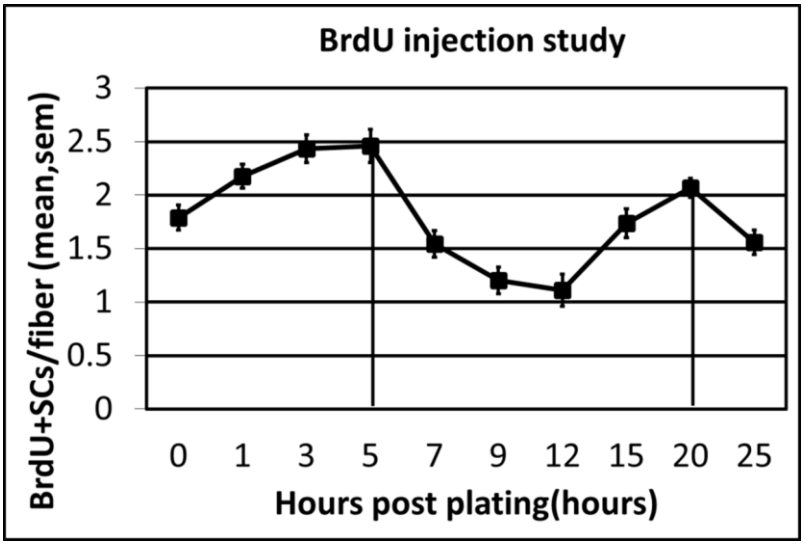


Figure 7. *In vivo* time-course study of satellite cell cycling on zebrafish muscle fibres. Line graph of BrdU+ SCs per fibre versus hours of incubation for the *in vivo* time-course study of BrdU incorporation following whole fish injection. Group size (n) is indicated for each time point, and the number of fibres assessed in each group varied as follows: 0 hr = 62 (n=3), 1 hr = 68 (n=3), 3 hr = 65 (n=3), 5 hr = 99 (n=4), 7 hr = 55 (n=3), 9 hr = 54 (n=3), 12 hr = 45 (n=2), 15 hr = 57 (n=3), 20 hr = 131 (n=5), 24 hr = 66 (n=3). Two peaks were observed at five hours (2.4 ± 0.2) and 20 hours (2.1 ± 0.2) of incubation. These peaks were interpreted as representing the first and second population of cell activation; they both were significantly different from 0 hour of incubation ($p<0.03$), suggesting a cell-cycle period of 15 hours.

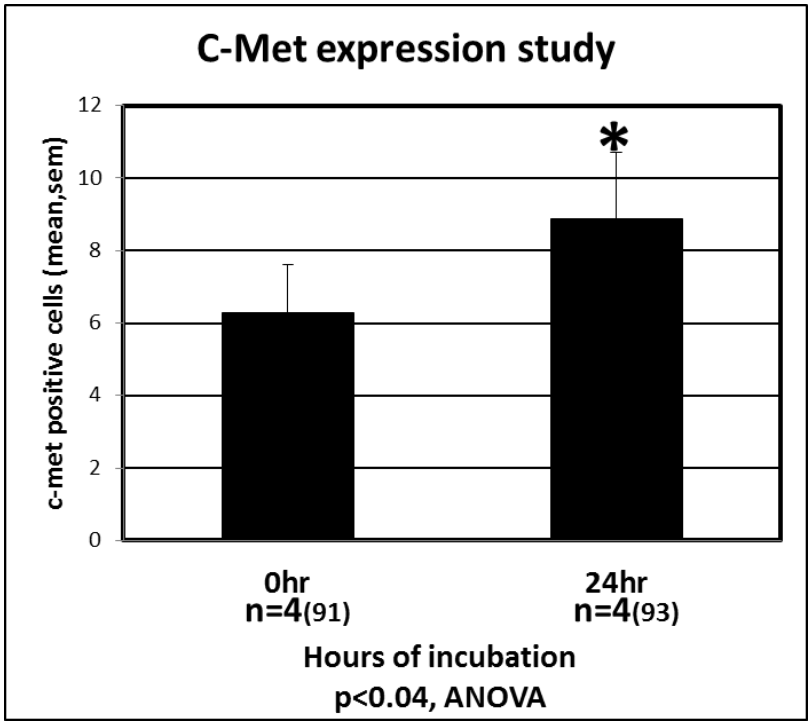
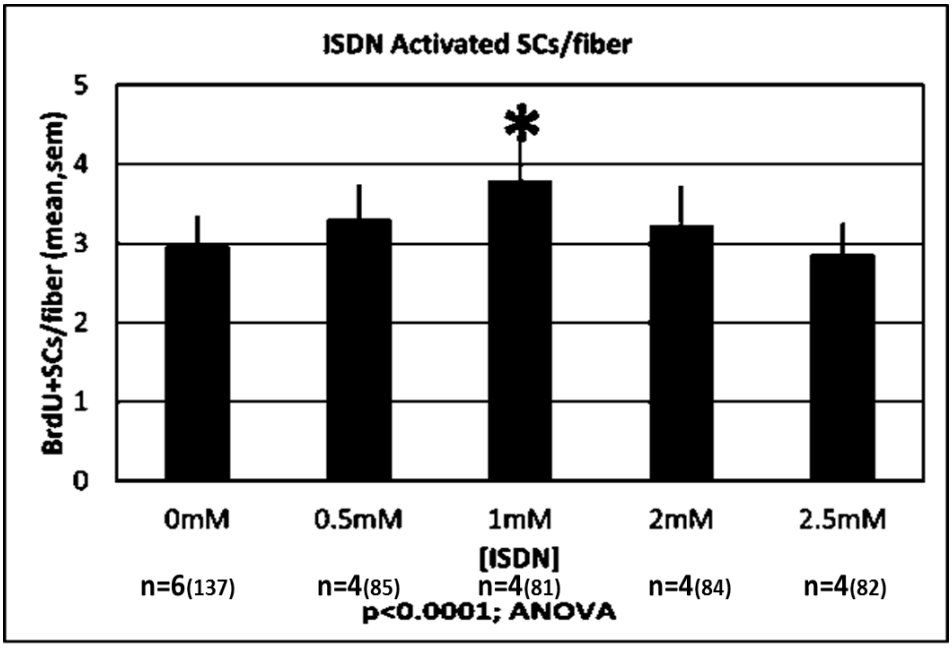


Figure 8. Immunostaining of c-Met protein expression over 24 hours. Histogram of c-Met staining at zero hour and 24 hours of incubation. There was a significant 1.5-fold increase (shown by the *) in the number of c-Met+ SC per fibre (from 6.4 ± 1.4 to 9.0 ± 1.9, $p<0.04$), assessed by immunostaining for c-Met protein (the HGF receptor). For each time point, there were four dishes (n=4) and 20-25 intact fibres per dish were included in the counts. The increase in the number of c-Met+ SCs over 24 hours in culture suggested that some cells were proliferating during incubation in the absence of any treatment.


ISDN Activated SCs/fiber
BrdU+SCs/fiber (mean,sem)
0
1
2
3
4
5
*
0mM
0.5mM
1mM
2mM
2.5mM
[ISDN]
n=6(137)
n=4(85)
n=4(81)
n=4(84)
n=4(82)
p<0.0001; ANOVA

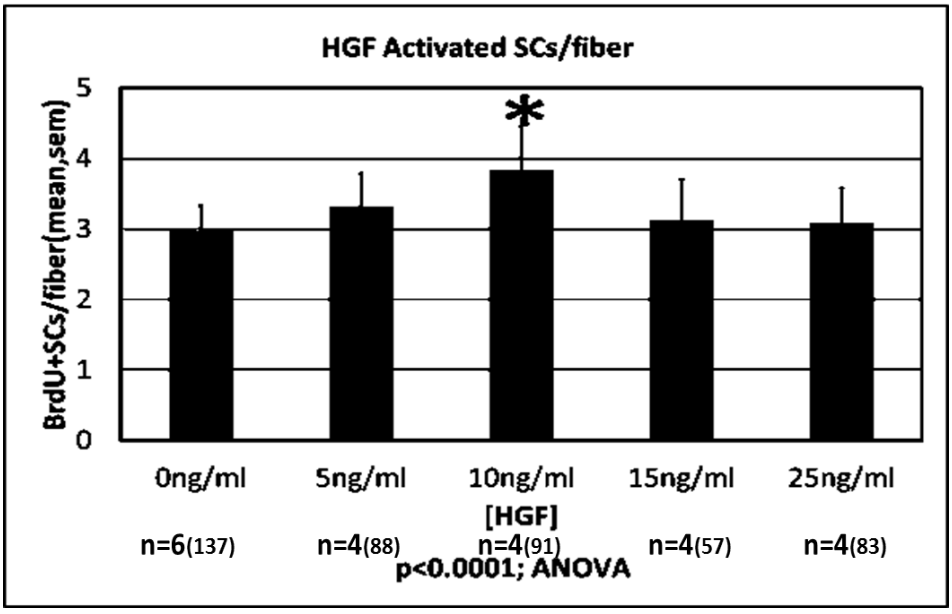

HGF Activated SCs/fiber
BrdU+SCs/fiber(mean,sem)
0
1
2
3
4
5
*
0ng/ml
5ng/ml
10ng/ml
15ng/ml
25ng/ml
[HGF]
n=6(137)
n=4(88)
n=4(91)
n=4(57)
n=4(83)
p<0.0001; ANOVA

Figure 9. ISDN and HGF dose-response graphs. Bar graphs showing the dose-response studies of the level of activation resulting from treatment with various concentrations of ISDN (upper panel, 0-2.5 mM) and HGF (lower panel, 0-25 ng/mL) plotted against the number of BrdU+ SCs per fibre (y-axis). There was a dose-dependent response of SC activation (the number of BrdU+ SCs per fibre) to ISDN and HGF, with significant differences ($p<0.0001$; ANOVAs) at 1 mM ISDN and 10 ng/mL HGF (shown by the *). There were at least 4 dishes per treatment group, and at least 20 intact fibres were counted per dish.

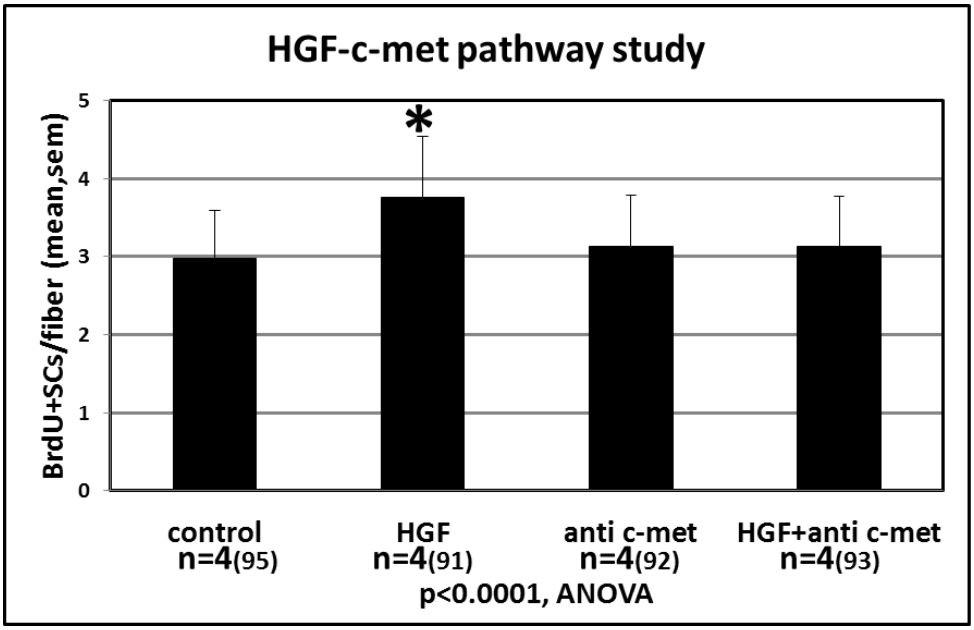


Figure 10. Study of HGF-c-Met pathway by neutralizing anti-c-Met antibody. Bar graph illustrating the experiment related to the study of the HGF-c-Met signalling pathway with comparison of groups treated with 10 ng/mL HGF and/or anti-c-Met antibody (1 µg/mL) compared to the untreated control group. The number of BrdU+ SCs per fibre was counted in a minimum number of 20-25 intact fibres per dish, with at least four replicate dishes per group. One-way ANOVA showed a significant difference among groups ($p<0.0001$, shown by the *). Tukey's post hoc tests found no significant difference between groups treated with HGF plus anti-c-Met compared to the control group, indicating the effect of anti-c-Met antibody significantly prevented HGF-induced activation.

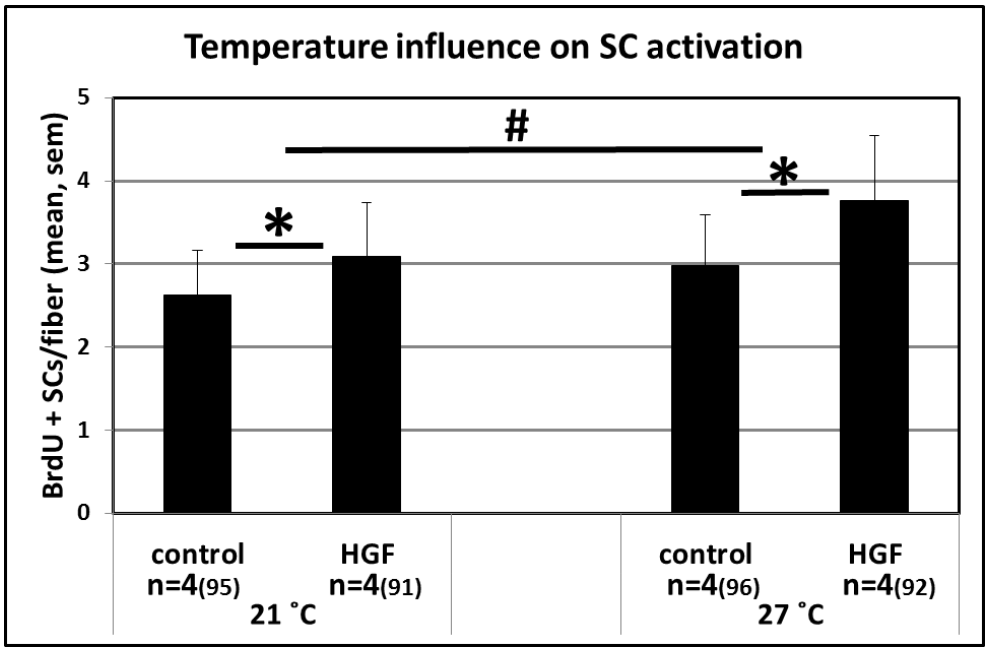


Figure 11. Temperature study on HGF-induced satellite cell activation. Bar graph of the study of temperature influence on HGF-induced SC activation. One group of dishes each temperature was treated with 10 ng/mL HGF and compared to the control group. The mean number of BrdU+ SCs per fibre was calculated. A repeated measures two-way ANOVA was performed and there was a significant effect of HGF to increase activation regardless of temperature (* indicates $p<0.0001$). This finding suggested HGF can still promote SC activation significantly at lower temperature, although the degree of activation induced by HGF was attenuated compare to that at 27 °C. Moreover, a lower temperature (21 ˚C) itself, regardless of the treatment, decreased the level of SC activation ($p<0.0001$). Also, the interaction of both temperature and HGF together showed a significant difference on SC activation (# indicates $p<0.001$).

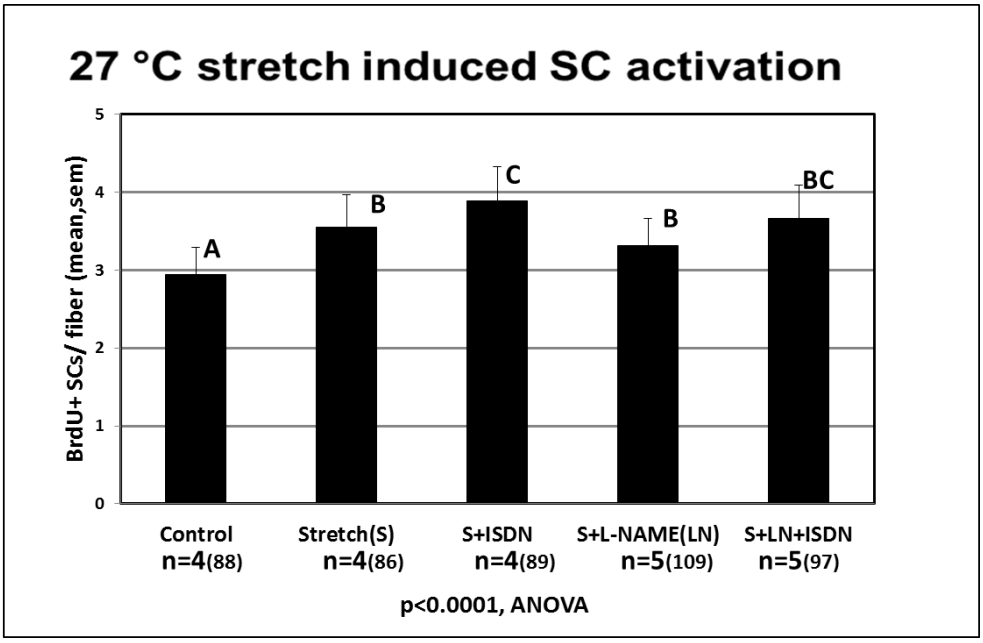


Figure 12. Stretch-induced SC activation study and NOS inhibition. Bar graph of the study on stretch-activation. The mean number of BrdU+ SCs per fibre (y-axis) is plotted for groups as follows: control, stretch only, stretch (S) plus ISDN, stretch plus NOS inhibition with L-NAME (LN) and stretch plus L-NAME plus ISDN. A one-way ANOVA found a significant difference among all treatments ($p<0.0001$). Further post hoc analysis by Tukey's test, found significant differences between the stretch-treated groups vs. the control group without stretch; the stretch-only group vs. the stretch plus ISDN group; and the stretch plus ISDN group vs. stretch plus L-NAME group. Groups with different letters (A-C) are significantly different by Tukey's test ($p<0.05$).

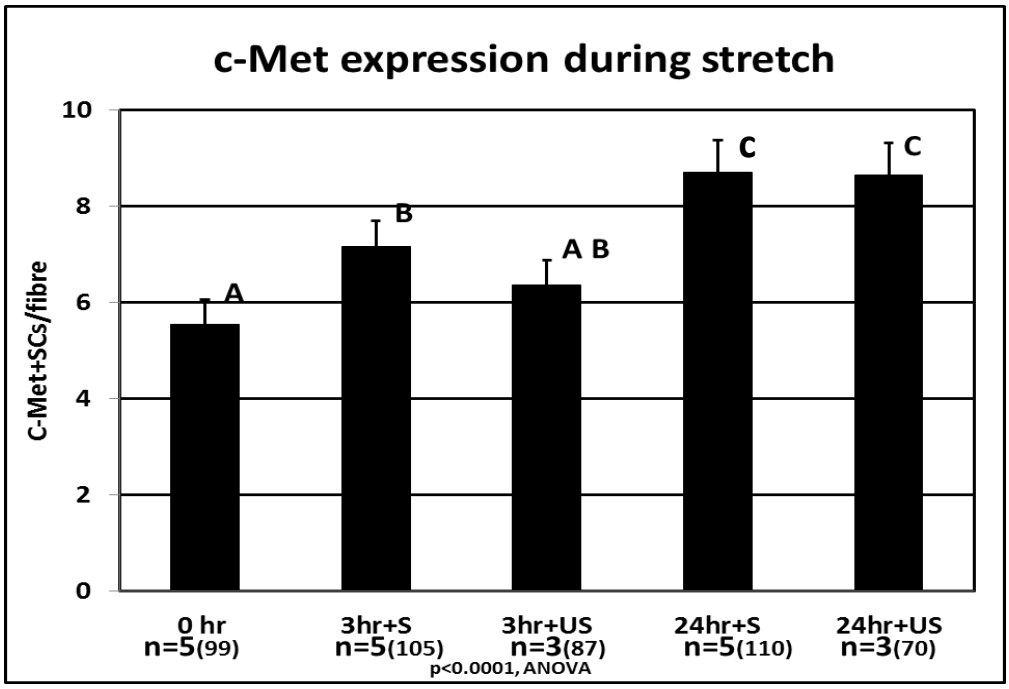
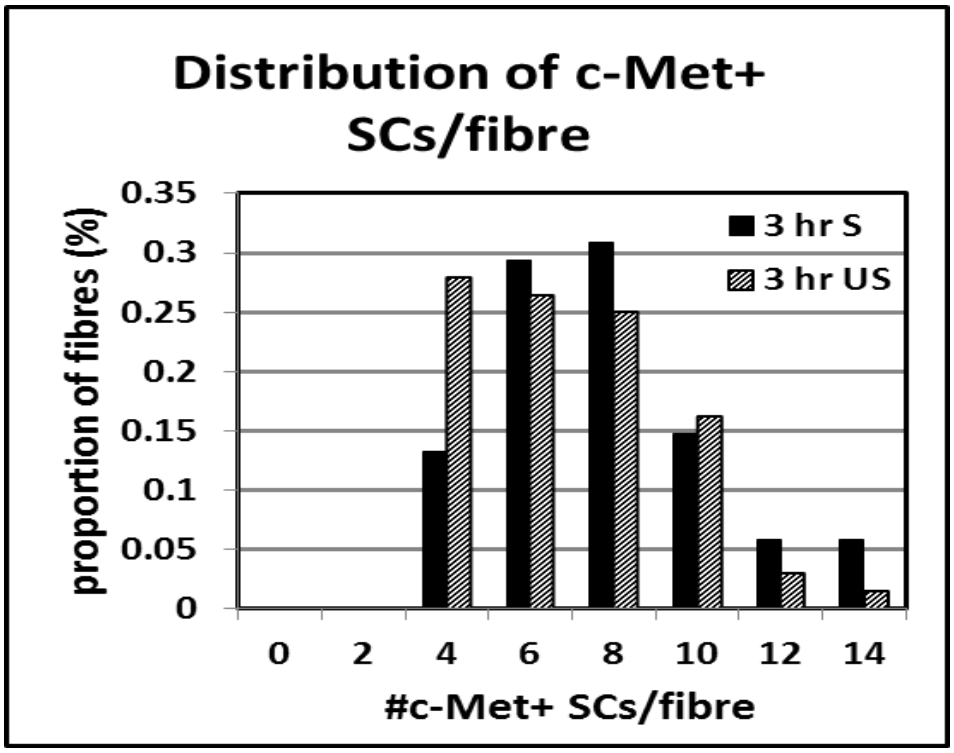
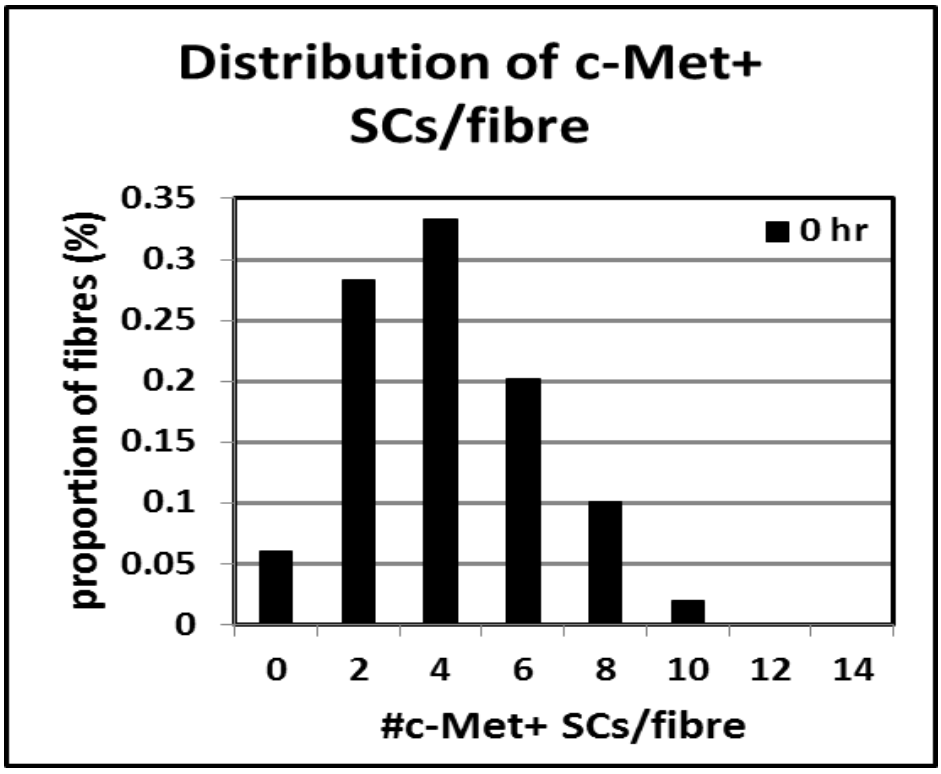


Figure 13. Immunostaing of c-Met protein expression during stretch over 24 hours. Bar graph of the number of c-Met+ SCs on fibre, detected using immunostaining, in groups with or without 3-hours of stretch (S) over 0-24 hours of incubation, as indicated. The mean value of c-Met+ SCs per fibre (y-axis) was plotted for five discrete groups (x-axis), including zero hour post-plating group without stretch (shown in graph as "0 hr"); three hours of incubation with stretch (shown in graph as "3hr+S"); three hours of incubation without stretch (shown in graph as "3hr+US"); 24 hours of incubation involving three hours of stretch (shown in graph as "24hr+S"); and 24 hours of incubation without stretch (shown in graph as "24hr+US"). One-way ANOVA found a significant difference among the above groups ($p<0.0001$). Tukey's test was performed and detected significant differences between following pairs of groups: 0 hr vs. 3hr+S; 0 hr vs. 24hr+S and 24hr+US; 3hr+S vs. 24hr+S and 24hr+US; 3hr+US vs. 24hr+S and 24hr+US. Groups marked with different letters (A-C) are significantly different ($p<0.05$).


Distribution of c-Met+ SCs/fibre
proportion of fibres (%)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
3 hr S
3 hr US
0
2
4
6
8
10
12
14
#c-Met+ SCs/fibre

(a)


Distribution of c-Met+ SCs/fibre
proportion of fibres (%)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 hr
0
2
4
6
8
10
12
14
#c-Met+ SCs/fibre

(b)

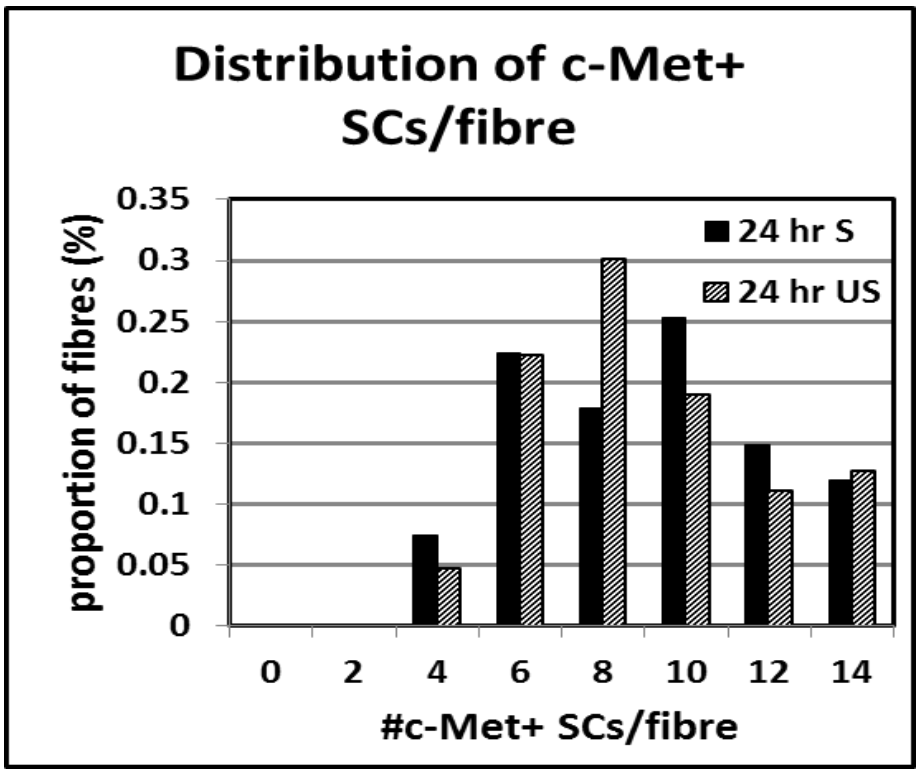


Figure 14. Distributions of c-Met positive SCs per fibre during stretch over 24 hours. Graphs above (a, b and c) present the proportion of fibres (y axis) plotted against the number of c-Met+ SCs per fibre (x axis) to compare the different groups represented in the bar graphs of mean ± sem in Figure 13. Panel a shows the distribution of c-Met+ SCs per fibre at 0 hour of incubation. Panel b compares the distribution of c-Met+ SCs per fibre at 3 hours of incubation between the groups with stretch (black bar) or without stretch (grey bar). Panel c exhibits the distribution of c-Met+ SCs per fibre comparing the two group after 24 hours of incubation, either with 3 hours of stretch then incubated an additional 21 hours (black bar) or without stretch then incubated for an additional 21 hours (grey bar).

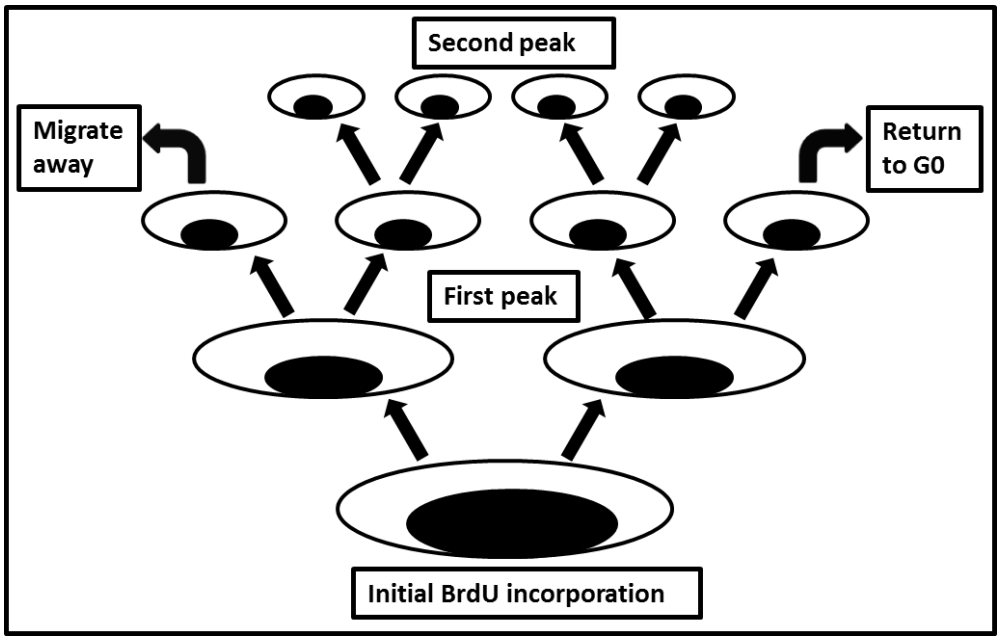


Figure 15. Schematic of BrdU incorporation over 24 hours for *in vivo* time course study. Schematic illustration of the cell division process in zebrafish fibre cultures over 24 hours post-plating, after BrdU incorporation into S-phase in vivo. The peaks of BrdU+ SCs detected in the *in vivo* labelling study, were interpreted as resulting from the first and second generation of daughter cells produced by cell division. Due to the absence of BrdU in the culture media (the only source was from the BrdU injection 2.5 hours before euthanasia), no additional BrdU incorporation was possible. Therefore, a portion of activated satellite cells may have migrated away from the muscle fibres to result in a decline in the number of BrdU+ SCs per fibre that was found after the peak of SC activation measured at 5 hours of incubation. Note that process of isolation, gravity sedimentation and plating of fibres from 1-2 fish took approximately 5 hours from the time of euthanasia, so the total time from BrdU exposure to the start of culture (0 hours) was 7.5 hours (2.5 hours from injection to euthanasia plus 5 hours).

Chapter 4: Discussion

The overall goal of this thesis project was to examine features of SC activation related to NO and HGF-c-Met signalling pathways using the zebrafish muscle-fibre culture system. The SC activation process is important in mammals for initiating muscle growth after birth and regeneration in adults because without the addition of new nuclei, muscle fibres can only undergo hypertrophy (only the size of the muscle fibres increases). This is seen in adult mammalian muscle. By comparison, fish muscle undergoes hypertrophy together with hyperplasia (both the size of fibres and the number of muscle fibres increase) for the entire lifetime of the fish. For this reason, the significance of SC activation in zebrafish could be considered even more important than in mammalian muscle, although prior to this project, the regulation of SC activation in fish was uncertain. Results obtained here were used to uncover the regulatory mechanisms involved in SC activation, to allow the comparison of these processes across species, namely fish and mammals. In particular, several novel findings were gathered and are discussed below. Moreover, this evidence provides useful information for future research to better understand SC-mediated muscle growth and repair in zebrafish and other fish.

4.1 NO- and HGF-induced SC activation in zebrafish single muscle-fibre cultures

Before testing the proposed hypotheses, two separate experiments were performed to establish a baseline for pursuing the overall goal. First, in an effort to analyse the level of SC activation in zebrafish muscle fibres over 24 hours post-plating, BrdU was injected into the fish prior to fibre isolation. This allowed cell activation and proliferation to be tracked using BrdU immunostaining at different time points in culture. Results showed that cells were dividing spontaneously (in the absence of stimuli) over

the first five hours of incubation, although the number of BrdU+ SCs at the first peak was not double that at the zero hour time point. This suggested that only 67% cells that had incorporated BrdU *in vivo*, had divided by 5 hours after plating. By comparison, in a study of SC proliferation in one-month-old rats, Schultz (1996) reported about 80% of SCs were dividing *in vivo*. The continuous increase in BrdU+ SCs in the first five hours was likely a result of an initially mixed population of SCs on fibres (SCs that had incorporated BrdU+ at different times during S-phase), which (when cultured with plain media without BrdU) already had begun DNA replication. The period of available BrdU in the circulation is only 15-30 minutes before it is excreted from the body (Mizunoya et al., 2011). This replication process continued before giving rise to a second, larger generation of BrdU+ daughter SCs (see Figure 15). Following the first peak, a marked diminishment of BrdU+ SCs was observed, reaching a trough on the graph at a value of 1.1 ± 0.2. This level was even lower than the group sampled at the zero hour time point.

This observed drop between the two peaks could be due to two factors. First, when cells had undergone activation and proliferation, a portion may have migrated away from the muscle fibre, therefore decreasing the number of BrdU+ SCs per fibre after the first peak. Shefer and associates (2004) provided a possible interpretation for this migration phenomenon. In that report using isolated SCs from mouse muscle, it was reasoned that SC have mescenchymal plasticity, meaning they can develop into or yield non-myogenic cells, such as adipocytes and osteoblasts; however none of these cell types was observed in zebrafish fibre cultures. Second, since there was no additional source of BrdU in the media, cells that were activated and prolilferating following incubation but were not derived from the same cells as the initially dividing cells with BrdU

incorporated (i.e., a different lineage), did not stain. Interestingly, a second peak in the number of BrdU+ SCs was observed at 20 hours of incubation, although the activation level (2.1 ± 0.2) appeared to be less than the first peak. The apparent difference in the value of these maxima may be because, as previously mentioned, some of BrdU+ SCs may have migrated away from the fibres, and thus were out of consideration; however the two maximum values were not significantly different, so any migration would have been very minor. The second peak presumably resulted from a subsequent generation of daughter cells generated from the initial group of BrdU+ SC labelled in vivo. For this reason, the graph was interpreted as suggesting that the duration of the cell cycle in zebrafish muscle SC is 15 hours. Another possible explanation for this phenomenon of the second peak in the *in vivo* time-course study is that there may be a second population of SC in existence, which were slower to activate than those in the initial peak. This second set of satellite cells may have also incorporated BrdU into their DNA before fibre plating but then developed condensed DNA and divided later, resulting in the second peak, or at least contributing to it. This would be possible as only the darkly stained nuclei were counted as BrdU+, so the cells that had incorporated BrdU in S-phase and proceeded to G2 and M phases more slowly, were not counted until their DNA was condensed. Overall, since the cell cycle of zebrafish muscle SC was concluded to be approximately 15 hours, a period of 24 hours of incubation for single muscle-fibre cultures from zebrafish (derived from the previous studies in the mouse single-fibre model) was used as an adequate period for the experiments detecting proliferating cells that had been in S-phase at the time of exposure to BrdU.

Second, to better understand how many quiescent SCs were available on the fibres to activation over the next 24 hours of incubation in the absence of treatment, anti-c-Met immunostaining was conducted on muscle fibres at zero hour and 24 hours of incubation. This idea was based on the finding from Cornelison and Wold (1997) underlying satellite cell gene co-expression during quiescence and then after the transition to an activated state during a time course study. C-Met receptor tyrosine kinase was the first marker identified for quiescent SCs, and also served as a reliable marker of SCs that were activated. The results of the current study showed an approximate 1.5X increase (i.e., a 150% increase) in the number of c-Met+ cells in the satellite position after 24 hours of culturing. This indicated that cells were dividing and underwent proliferation during 24 hours. Interestingly, the number of SC stained positive for c-Met protein started from 6.4 ± 1.4 per fibre prior to incubation (zero hour time point, immediately after plating), whereas the number of BrdU+ cells from the *in vivo* time-course study was 1.8 ± 0.2 at zero hours of incubation (again, immediately after plating). The difference between these two numbers (i.e., about 4.6 satellite cells) theoretically would have consisted of three distinct SC populations with each population in a different state, namely fully quiescent SCs in G0, activated SCs in G1 phase prior to their entry into S-phase and activated cycling SCs which had exited S-phase. The fourth population, SC in S-phase of the cell cycle, would have been labelled by BrdU incorporation *in vivo* in the fish, prior to plating and would be represented as the zero hour time point in the time-course study. This finding was important to determine the potential size of the quiescent SC population on fish muscle fibres for comparison to fibres in the mouse model organism, that were exposed to the same stimuli in culture. There are two likely

interpretations of the rise in the number of c-Met+ cells between zero hour and 24 hours of culturing. On one side, the accumulation of c-Met+ cells was perhaps a result of several generations of a few initially activated SCs (i.e., those with a high proliferation rate that stayed as c-Met+ cells). On the other side, this circumstance could be possible if more quiescent SCs became activated, but had produced fewer daughter cells (i.e., possibly through a long cell-cycle time) due to a large potential for self-renewal as a more stem-cell like population (Collins et al., 2005). Collins and colleagues transplanted a single myofibre with seven SCs into an irradiated host-muscle tissue where the SC niche was disrupted but the myofibers and non-proliferating SC were intact. Their results found that the transplanted fibre was able to yield 100 new myofibers containing thousands of nuclei (indicating huge potential for cycling and for self-renewal). Although results of the present study are consistent with the two possible explanations outlined above, it is not known whether these scenarios are present in fish.

The first hypothesis of this project was to examine NO and HGF involvement in the SC activation pathway; therefore three sets of experiments were conducted. The first experiment was designed to study the dose-response pattern of SC activation to NO (ISDN was adopted as a NO-donor) and HGF by adding increasing concentrations of ISDN and HGF exogenously. Analysis of the number of BrdU+ SCs per fibre was used to generate the dose-response patterns. Results showed a bell-curve shape for both ISDN and HGF effects, with the optimal concentration for SC activation revealed as 1 mM ISDN and 10 ng/mL HGF, respectively. This indicates now, in the context of the time-course study, that the majority of SCs was activated under those circumstances. Compared to the previous studies on activation of SCs on single mouse-muscle fibres in

culture (Anderson and Pilipowicz, 2002; Wozniak and Anderson, 2007), quiescent SCs showed a peak-activation response to lower ISDN and higher HGF levels on zebrafish fibres in culture. Interestingly, Yamada and colleagues (2010) demonstrated that high concentrations of HGF (10-500 ng/ml) applied on isolated SCs can prevent SC proliferation by increasing the expression of myostatin; this was in comparison to effects of peak activation on those mouse fibres which used an HGF concentration of only 2.5 ng/ml. Tatsumi and colleagues have also reported that satellite cells in culture (not on fibres) also show a decrease in activation at high levels of HGF (Yamada et al., 2010). So future studies of SC activation could usefully examine activation on fish muscle fibres by using a high concentration of HGF and detect whether the dose-response has a similar pattern, on the size of the population of BrdU+ SC per fibre. Alternatively, an experiment can be designed to test the influence of endogenous HGF on SC activation by adding purified crushed muscle extract, according to Tatsumi and associates (1998) or over-expressing HGF in the fibre myonuclei through use of transgenic mice or in vitro transfection methods. It would be interesting if the activating effect of endogenous HGF (according to the assay of the number of BrdU+ cells on fibres) was different from the effect of the exogenous level of HGF added into the culture media.

The second experiment was to address whether HGF signalling was acting through interaction with the c-Met receptor on the SC membrane; this ligand-receptor binding conducts the activation signal into SCs. HGF is understood as required or essential for SC activation in mouse muscle-fibre cultures. Results showed that blocking the c-Met receptor using anti-c-Met antibody prevented SC from being activated by 10 ng/mL HGF treatment; this result suggests that HGF binding to the c-Met receptor was

essential in SC activation. Further experiments in which neutralizing anti-HGF antibody was added as a different type of blocker, or anti-HGF antibody was used to pre-neutralize the activity of exogenous HGF prior to its addition to medium, would provide further support for the concrete need for HGF-c-Met signalling during SC activation. In addition to the HGF-c-Met pathway, there is a Notch-mediated SC activation pathway (Conboy and Rando, 2002) in mouse muscle. Once a muscle gets injured, Delta-1, which is the ligand that binds to the Notch receptor, would up-regulate, then activate the Notch receptor signaling cascade on quiescent SC, even those SCs located on a region of sarcolemma that is far away from the location of the injury.

Owing to the fact that fish belong to a cold blooded species, whereas mammals (e.g., mice) are warm-blooded, it was interesting to study the influence of temperature on SC activation, in order to determine whether or how much the activation-signalling pathway would be affected at a lower temperature (i.e., 21 °C). Interestingly, results revealed that a lower temperature decreased the number of activated BrdU+ SCs on fibres in both control and HGF-treated groups at 21 °C, compared to the same treatment groups at 27 °C, although both at 21 ° C and 27 °C, the number of BrdU+ SCs per fibre in the HGF treatment group was significantly higher compared to the corresponding control groups. In this regard, it is interesting to consider that, although fish are able to decrease their metabolic rate to adjust to lower temperatures under physiological situations, here, the single-fibre culture experiments were performed without innervation or blood supply that *in vivo* would provide higher-level or systemic influences on the response. It is surprising that SC activation experienced a considerable decrease at the lower temperature and that this was observed in both the control and treatment groups. One

possible explanation of this finding is that the lower temperature exerted an effect on an innate factor prior to or further along the HGF-c-Met pathway, in other words, the activity of one or more enzymes upstream or downstream of ligand binding. Regarding the generation of HGF and its release from the extracellular matrix following muscle fibre stimulation (e.g., by stretch or ISDN), the enzyme, matrix metalloproteinase 3 (MMP-3), which undergoes a process of phosphorylation must be activated (Tatsumi and Allen, 2008). Lower temperature likely reduces HGF production and/or release by diminishing MMP-3 activity; therefore, fewer SCs would receive this extracellular activation signal through HGF binding to the SC surface receptor, c-Met. Moreover, following the HGF-c-Met pathway, a series of enzyme-dependent intracellular signalling takes place which further transmits the activation signal into the SC nucleus and transduces the activation signal. Among them, the pathway that is most likely involved following HGF-induced activation is the RAS pathway which initiates GTPase activation followed by activation of mitogen-activated protein kinase (MAPK) (Marshall, 1995; O'Brien et al., 2004). Beside the temperature influence on distinct enzyme activities along the SC activation pathway, the affinity of HGF binding to the c-Met receptor is also likely affected by a reduced temperature. In a study of the affinity of HGF binding to c-Met in mammalian cells, Holmes and colleagues (2007) demonstrated that only kringle domains (K) of HGF are adequate and efficient for stimulating c-Met activity, although the N-terminal and the serine proteinase homology domain (SP) of HGF also have the capacity of binding to c-Met. Overall, the findings of the present study indicated that the HGF-c-Met signalling pathway is fundamental for SC activation and that an appropriate temperature is important to activation.

4.2 Stretching-induced SC activation on fish single muscle fibre culture

While separate chemical and mechanical stimuli can each activate SCs from quiescence, the relationship between them was not elucidated prior to this project. To explored further, whether stretch can still activate SCs in the absence of endogenous NO, the NOS inhibitor, L-NAME was added to culture media and culture wells were stretched using the FlexCell apparatus. Results showed that SC activation was increased due to the effects of stretch on the fibre cultures; however, with the addition of 1 mM ISDN together with L-NAME to a different group of dishes, the number of BrdU+ SCs per fibre was restored to the level in the ISDN-treated and stretched group; this compensated for NOS inhibition by exogenous ISDN (i.e., NO) and showed a level of activation that was significantly different from the stretch-only group ($p<0.001$). Results of experiments reviewed by Tatsumi and colleagues (2010) indicated that the activation mechanism is a cascade of events including influx of calcium ions, calcium-calmodulin complex formation, NO synthase activation, NO radical production, MMP-3 activation, HGF release and HGF binding to c-Met; results of this thesis project are consistent with a similar cascade working in activation of SCs on zebrafish fibres.

According to the previous study of stretch-induced SC activation, the number of BrdU+ SCs per fibre increased in the presence of 20% stretch for three hours in fish fibres incubated for 24 hours, compared to the control group without stretch or any treatment. This confirmed SCs at 24 hours of incubation had responded to stretch by proliferating, since the mean number of c-Met+ SCs per fibre in the current experiment increased significantly over 24 hours of incubation. It was of particular interest that following *three* hours of incubation with stretch, there was also a significantly higher

number of c-Met+ SCs per fibre compared to the counterpart group without stretch. This time point is too early for the response to have been due to proliferation. However, the response indicated that the stretch stimulus indeed influenced SC activation, and promoted the processes up to translation of c-Met protein in SCs as a result of stretch. In mouse, Wozniak et al. (2003) showed that stretch for 30 minutes was sufficient to increase c-Met mRNA expression so that there were more c-Met-expressing SCs per fibre after 30 minutes of stretch. In the current study, it is not clear whether transcription has changed as a result of stretching for three hours, since c-Met protein was used as the assay by immunostaining. All SCs counted as positive, had the same level of staining through the experiments. It is puzzling that there was no significant difference between stretched and unstretched cultures in the number of c-Met+ SCs per fibre after 24 hours in culture. One explanation is that c-Met+ SCs could have migrated away from fibres once they became activated, and then would not have been counted. This movement was reported as explaining similar findings by Anderson and Pilipowicz (2002). However, there were very few if any c-Met+ single cells (or BrdU+ cells in the other experiments) observed to be located on the surface of the dishes, away from fibres. Another possibility is that the c-Met+ SCs in the stretched group, had differentiated faster or further toward myocytes that would fuse into fibers, than those in the unstretched group; that could result in finding that incubation for another 21 hours after stretching had pushed the differentiated cells to begin decreasing the amount of c-Met protein in each cell (too low to be counted as positive). This type of down-regulation is known to happen in pax7-expressing myogenic cells in which pax7 expression decreases starting with the onset of myogenin expression (Seale et al., 2000). Such a down-regulation, if it happened for c-

Met mRNA, could have counteracted the expected increase in the proliferation rate after stretch, according to the assay by immunostaining for c-Met. Alternatively, if SCs on fibres in both the stretched and unstretched groups possess similar properties of differentiation by 24 hours of incubation, it is possible that the number of c-Met+ SCs per fibre would differ between the two groups if fibres were allowed to incubate beyond 24 hours. By 36 hours, for example, one might expect the stretched group to show a higher number of c-Met+ SCs per fibre than the group that did not receive the stretch stimulus. These possibilities can be tested by future experiments. For example, it would be interesting to study the population of c-Met+/myogenin+ SCs per fibre in stretched vs. unstretched cultures of fibres using double immunostaining methods.

Results of the current thesis project demonstrate strong consistency with studies of SC activation on mouse fibres, with differences related to the size of the c-Met+ population, the optimal level of ISDN and HGF to produce activation in the dose-response curves, and the significant effect of lower temperature to reduce the time-dependent activation in the absence of stimulation and also for HGF-induced activation in culture.

4.3 Limitations

First of all, the present study used assays of absolute values of individual markers (e.g., BrdU and c-Met staining). This meant it was impossible to determine the activation state and characterize the nature of the cell (c-Met expression) together. In an effort to assay the response of quiescent SCs to distinct stimuli that result in activation, it would be better to analyse the percentage of activated cells characterized as SCs by

performing double immunostaining of BrdU and c-Met on the same fibres. This use of double staining should be extended to study the state of differentiation in the activated SC population, for instance by studying MRF proteins in BrdU+ SCs. By doing so, the capability for activation by different factors (i.e., HGF, NO or stretch) can be compared among the different stages of differentiation. This would help us in understanding further, which stimulating factors are most influential at the different stages of differentiation in zebrafish muscle fibres. Secondly, in an attempt to explore the chronological relation between HGF and NO in the cascade of SC activation on fish muscle fibres, experiments could endogenously introduce or inhibit the NO source. For example, studies could use L-arginine to promote NO production by NOS in the muscle fibres, and alternatively silence NOS activity (by inhibitors that are more specific and/or more potent than L-NAME against NOS-1 activity) or NOS-1 gene expression (by genetic studies to down-regulate mRNA expression *in vitro*) to overcome endogenous NO generation. These experiments would be ideal for detecting HGF-induced activation that did not depend on NO production by the fibre. Thirdly, this model of SC activation using zebrafish muscle fibre cultures, established the condition that the nerve and blood supplies are not involved in SC activation. It would be interesting to introduce an electric pulse while fibres were involved in responding to chemical (NO, HGF) and mechanical (stretch) signals for activation, and then study the interaction of electrical with the two types of activating stimuli, for instance by examining potential changes in proteins such as typical biomarkers of the resident SC adherent to fibres (e.g., M-cadherin) that may be reduced more rapidly as SCs become mobile immediate after activation.

4.4 Future directions

In an attempt to make a concrete conclusion about the destination or fate of the daughter cells produced as a result of SC activation, it would be interesting to perform future experiments based on the report from Cornelison and Wold (1997) in which four myogenic regulatory factors (MRFs, also called MyoD family members) were used to identify the time-course of proliferation and differentiation by SCs once activated. Specifically, activated SCs first express either myf5 or MyoD, then co-express both of them; this stage of proliferation was then followed by the expression of myogenin and then MRF4 in the initiation of myoblast differentiation to a myocyte.

Future experiments can be designed to address the alternative explanations as discussed before, related to the possibility of different populations of SCs that can proliferate at a rapid vs. a slow rate, or the stage of the cell cycle at which those different populations can be affected by an activating stimulus (or even a stimulus that would drive SCs into quiescence). If there are distinct populations of SCs on zebrafish fibres as there are on mouse fibres, some of those populations may be able to detect and respond to a second activating stimulus when they are early in a cycle of proliferation from a prior stimulus (e.g., expressing myf5 or MyoD, but not if they’re expressing myogenin or MRF4). Others may not be able to respond to activating stimuli unless there are no MRFs expressed, or only if they’re expressing the later MRFs during the differentiation phase. Alternatively, it will be of particular interest to trace myogenin expression by using double staining of BrdU and myogenin in fibres that are isolated following *in vivo* BrdU injection and incubated for 24 hours.

The *in vivo* time-course study after BrdU injection of fish was a preliminary study to the experiments described in this thesis, and would help design future research. For example, in an effort to explore the activation of SCs undergoing different stimuli, it is critical to study a particular period of time, reducing the influence from previous cycles of cell division that have already activated SCs (through any type of treatment). Cells could be cultured for five hours, and then treatments could be added into culture media containing BrdU and BrdU+ SCs monitored afterwards. Additionally, if the operation procedure for injection was regarded as one of the elements that could activate quiescent SCs, this study would give a reference value, to act as a baseline, for interpreting the implications of the treatment effect.

SC cycle time in one-month-old rats was 32 hours, and S-phase accounted for 14 hours of that time (Schultz, 1996); that study showed that the cycle time was the same in fast- and slow-twitch fibres, and results were confirmed using injections of two DNA-synthesis markers, tritiated-thymidine and BrdU, with an interval time of 6 hours. Although there were no differences between the cell-cycle time of fast and slow fibres, Lagord et al. (1998) illustrated that fast and slow fibres possess SCs with distinct activation characteristics and capacities for proliferation and differentiation. Therefore, whether the two peaks of SC activation observed in the current study were derived from two different populations, or more specifically, were associated with distinct fibre types, is not known. In addition to this question, Schultz also determined that some daughter cell progeny generated from primary SC proliferation would stay mitotic and continue dividing whereas others, albeit at the same generation rate, would return to the G0-phase and return to a quiescent state. This type of information would be exciting to determine

for SCs on zebrafish muscle fibers as it would relate to the overall growth capacity in the fish, and the need for fiber hyperplasia during the life span of the organism.

It was shown in the mouse model that a mechanical stimulus such as stretch was capable of activating SCs (Wozniak et al., 2003); the results of experiments on fish muscle fibres here were consistent with that finding in mouse fibres. However, while SC activation was assayed by counting the number of BrdU+ cells in both this and the previous mouse studies, the proportionate SC activation was not as dramatic in fish fibres as that in mouse fibres. In other words, SCs on mouse fibres were activated by the same stretch stimulus, to a level 200% (two-fold) higher than that in the mouse control group, whereas in fish muscle fibres, the number of BrdU+ SCs increased to a lesser extent, approximately 50% more after stretch compared to the non-stretched control group. Both types of fibres (mouse and zebrafish) seemed to display similar numbers of SC, typically from 1-7 BrdU+ cells per fibre, although the variability in the number of BrdU+ cells was higher in fish than in mouse. This may have obscured some of the effects of stretch-activation. This possibility could be explored best in a single experiment on mouse and fish fibres side by side, by comparing the frequency distributions of the number of BrdU+ SCs per fibre induced by stretching. It would also be interesting to study the proportion of proliferating SCs using double-staining experiments to identify BrdU incorporation and c-Met staining. This experiment would compare the frequency distribution of three combinations of staining, i.e., BrdU+/c-Met+ and BrdU-/c-Met+ SCs that represent activated and quiescent SCs, whereas BrdU+/c-Met-negative cells would indicate proliferating non-myogenic cells. If cultured long enough, it may be possible to study the

fate of the daughter cells generated from SC proliferation, by examining the level of BrdU+ myonuclei inside cultured fibres.

References

Alexander, M. S., Kawahara, G., Kho, A. T., Howell, M. H., Pusack, T. J., Myers, J. A., Kunkel, L. M. (2011). Isolation and transcriptome analysis of adult zebrafish cells enriched for skeletal muscle progenitors. *Muscle & Nerve, 43*(5), 741-750. doi: 10.1002/mus.21972

Altringham, J. D., & Johnston, I. A. (1990). Modeling muscle power output in a swimming fish. *Journal of Experimental Biology, 148*, 395-402.

Amsterdam, A., Burgess, S., Golling, G., Chen, W. B., Sun, Z. X., Townsend, K., Hopkins, N. (1999). A large-scale insertional mutagenesis screen in zebrafish. *Genes & Development, 13*(20), 2713-2724. doi: 10.1101/gad.13.20.2713

Amthor, H., Nicholas, G., McKinnell, I., Kemp, C. F., Sharma, M., Kambadur, R., & Patel, K. (2004). Follistatin complexes myostatin and antagonises myostatin-mediated inhibition of myogenesis. *Developmental Biology, 270*(1), 19-30. doi: 10.1016/j.ydbio.2004.01.046

Anderson, J., & Pilipowicz, O. (2002). Activation of muscle satellite cells in single-fiber cultures. *Nitric Oxide-Biology and Chemistry, 7*(1), 36-41. doi: 10.1016/S1089-8603(02)00011-3

Anderson, J. E. (2000). A role for nitric oxide in muscle repair: Nitric oxide-mediated activation of muscle satellite cells. *Molecular Biology of the Cell, 11*(5), 1859-1874.

Anderson, J. E., Carvalho, R. S., Yen, E., & Scott, J. E. (1993). Measurement of strain in cultured bone and fetal muscle and lung cells. *In Vitro Cellular & Developmental Biology : Journal of the Tissue Culture Association, 29A*(3 Pt 1), 183-186.

Anderson, J. E., Wozniak, A. C., & Mizunoya, W. (2012). Single muscle-fiber isolation and culture for cellular, molecular, pharmacological, and evolutionary studies. *Methods in Molecular Biology (Clifton, N.J.), 798*, 85-102. doi: 10.1007/978-1-61779-343-1_6; 10.1007/978-1-61779-343-1_6

Asakura, A., Komaki, M., & Rudnicki, M. (2001). Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation. *Differentiation; Research in Biological Diversity, 68*(4-5), 245-253.

Asakura, A., Seale, P., Girgis-Gabardo, A., & Rudnicki, M. A. (2002). Myogenic specification of side population cells in skeletal muscle. *The Journal of Cell Biology, 159*(1), 123-134. doi: 10.1083/jcb.200202092

Barresi, M. J. F., D'Angelo, J. A., Hernandez, L. P., & Devoto, S. H. (2001). Distinct mechanisms regulate slow-muscle development. *Current Biology, 11*(18), 1432-1438. doi: 10.1016/S0960-9822(01)00428-6

Bekoff, A., & Betz, W. (1977). Properties of isolated adult rat muscle-fibers maintained in tissue-culture. *Journal of Physiology-London, 271*(2), 537-&.

Biga, P. R., & Goetz, F. W. (2006). Zebrafish and giant danio as models for muscle growth: Determinate vs. indeterminate growth as determined by morphometric analysis. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 291*(5), R1327-R1337. doi: 10.1152/ajpregu.00905.2005

Bird, N. C., & Mabee, P. M. (2003). Developmental morphology of the axial skeleton of the zebrafish, danio rerio (ostariophysi : Cyprinidae). *Developmental Dynamics, 228*(3), 337-357. doi: 10.1002/dvdy.10387

Bottinelli, R. (2001). Functional heterogeneity of mammalian single muscle fibres: Do myosin isoforms tell the whole story? *Pflugers Archiv-European Journal of Physiology, 443*(1), 6-17. doi: 10.1007/s004240100700

Bottinelli, R., & Reggiani, C. (2000). Human skeletal muscle fibres: Molecular and functional diversity. *Progress in Biophysics & Molecular Biology, 73*(2-4), 195-262. doi: 10.1016/S0079-6107(00)00006-7

Brodeur, J. C., Calvo, J., Clarke, A., & Johnston, I. A. (2003). Myogenic cell cycle duration in harpagifer species with sub-antarctic and antarctic distributions: Evidence for cold compensation. *Journal of Experimental Biology, 206*(6), 1011-1016. doi: 10.1242/jeb.00204

Buckingham, M., Bajard, L., Chang, T., Daubas, P., Hadchouel, J., Meilhac, S., . . . Relaix, F. (2003). The formation of skeletal muscle: From somite to limb. *Journal of Anatomy, 202*(1), 59-68. doi: 10.1046/j.1469-7580.2003.00139.x

Buckingham, M., & Relaix, F. (2007). The role of pax genes in the development of tissues and organs: Pax3 and Pax7 regulate muscle progenitor cell functions. *Annual Review of Cell and Developmental Biology, 23*, 645-673. doi: 10.1146/annurev.cellbio.23.090506.123438

Buckingham, M., & Vincent, S. D. (2009). Distinct and dynamic myogenic populations in the vertebrate embryo. *Current Opinion in Genetics & Development, 19*(5), 444-453. doi: 10.1016/j.gde.2009.08.001

Chhabra, E. S., & Higgs, H. N. (2007). The many faces of actin: Matching assembly factors with cellular structures. *Nature Cell Biology, 9*(10), 1110-1121. doi: 10.1038/ncb1007-1110

Collins, C. A., Olsen, I., Zammit, P. S., Heslop, L., Petrie, A., Partridge, T. A., & Morgan, J. E. (2005). Stem cell function, self-renewal, and behavioral heterogeneity of cells from the adult muscle satellite cell niche. *Cell, 122*(2), 289-301. doi: 10.1016/j.cell.2005.05.010

Conboy, I. M., & Rando, T. A. (2002). The regulation of notch signaling controls satellite cell activation and cell fate determination in postnatal myogenesis. *Developmental Cell, 3*(3), 397-409. doi: 10.1016/S1534-5807(02)00254-X

Cornelison, D. D., & Wold, B. J. (1997). Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. *Developmental Biology, 191*(2), 270-283. doi: 10.1006/dbio.1997.8721

Cornelison, D. D. W., Olwin, B. B., Rudnicki, M. A., & Wold, B. J. (2000). MyoD(-/-) satellite cells in single-fiber culture are differentiation defective and MRF4 deficient. *Developmental Biology, 224*(2), 122-137. doi: 10.1006/dbio.2000.9682

Cornelison, D. D. W., Wilcox-Adelman, S. A., Goetinck, P. F., Rauvala, H., Rapraeger, A. C., & Olwin, B. B. (2004). Essential and separable roles for syndecan-3 and syndecan-4 in skeletal muscle development and regeneration. *Genes & Development, 18*(18), 2231-2236. doi: 10.1101/gad.1214204

Cossins, A. R., & Crawford, D. L. (2005). Opinion - fish as models for environmental genomics. *Nature Reviews Genetics, 6*(4), 324-333. doi: 10.1038/nrg1580

Cossu, G., De Angelis, L., Borello, U., Berarducci, B., Buffa, V., Sonnino, C., . . . Molinaro, M. (2000). Determination, diversification and multipotency of mammalian myogenic cells. *International Journal of Developmental Biology, 44*(6), 699-706.

Coughlin, D. J. (2002). Aerobic muscle function during steady swimming in fish. *Fish and Fisheries (Oxford), 3*(2), 63-78. doi: 10.1046/j.1467-2979.2002.00069.x

Defranchi, E., Bonaccurso, E., Tedesco, M., Canato, M., Pavan, E., Raiteri, R., & Reggiani, C. (2005). Imaging and elasticity measurements of the sarcolemma of fully differentiated skeletal muscle fibres. *Microscopy Research and Technique, 67*(1), 27-35. doi: 10.1002/jemt.20177

Dockter, J. L. (2000). Sclerotome induction and differentiation. *Current Topics in Developmental Biology, Vol 48, 48*, 77-127.

Driever, W., SolnicaKrezel, L., Schier, A. F., Neuhauss, S. C. F., Malicki, J., Stemple, D. L., . . . Boggs, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development, 123*, 37-46.

Enoka, R. M. (1988). Muscle strength and its development - new perspectives. *Sports Medicine, 6*(3), 146-168. doi: 10.2165/00007256-198806030-00003

Evans, D. J. R., Britland, S., & Wigmore, P. M. (1999). Differential response of fetal and neonatal myoblasts to topographical guidance cues in vitro. *Development Genes and Evolution, 209*(7), 438-442. doi: 10.1007/s004270050275

Fauconneau, B., & Paboeuf, G. (2001). Muscle satellite cells in fish. *Fish Physiology, 18*, 73-101.

Fauconneau, B., Alamidurante, H., Laroche, M., Marcel, J., & Vallot, D. (1995). Growth and meat quality relations in carp. *Aquaculture, 129*(1-4), 265-297. doi: 10.1016/0044-8486(94)00309-C

Fenichel, G. (1966). A histochemical study of developing human skeletal muscle. *Neurology, 16*(8), 741-&.

Feldman, J. L., & Stockdale, F. E. (1991). Skeletal-muscle satellite cell diversity - satellite cells form fibers of different types in cell-culture. *Developmental Biology, 143*(2), 320-334. doi: 10.1016/0012-1606(91)90083-F

Figeac, N., Daczewska, M., Marcelle, C., & Jagla, K. (2007). Muscle stem cells and model systems for their investigation. *Developmental Dynamics, 236*(12), 3332-3342. doi: 10.1002/dvdy.21345

Fluck, M., & Hoppeler, H. (2003). Molecular basis of skeletal muscle plasticity-from gene to form and function. *Reviews of Physiology, Biochemistry and Pharmacology, Vol 146, 146*, 159-216. doi: 10.1007/s10254-002-0004-7

Georga, I., & Koumoundouros, G. (2010). Thermally induced plasticity of body shape in adult zebrafish danio rerio (hamilton, 1822). *Journal of Morphology, 271*(11), 1319-1327. doi: 10.1002/jmor.10874

Gordon, A. M., Homsher, E., & Regnier, M. (2000). Regulation of contraction in striated muscle. *Physiological Reviews, 80*(2), 853-924.

Halevy, O., Piestun, Y., Allouh, M. Z., Rosser, B. W. C., Rinkevich, Y., Reshef, R., . . . Yablonka-Reuveni, Z. (2004). Pattern of Pax7 expression during myogenesis in the posthatch chicken establishes a model for satellite cell differentiation and renewal. *Developmental Dynamics, 231*(3), 489-502. doi: 10.1002/dvdy.20151

Hara, M., Tabata, K., Suzuki, T., Do, M. Q., Mizunoya, W., Nakamura, M., . . . Tatsumi, R. (2012). Calcium influx through a possible coupling of cation channels impacts skeletal

muscle satellite cell activation in response to mechanical stretch. *American Journal of Physiology-Cell Physiology, 302*(12), C1741-C1750. doi: 10.1152/ajpcell.00068.2012

Higashijima, S., Okamoto, H., Ueno, N., Hotta, Y., & Eguchi, G. (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. *Developmental Biology, 192*(2), 289-299. doi: 10.1006/dbio.1997.8779

Hill, A. J., Teraoka, H., Heideman, W., & Peterson, R. E. (2005). Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicological Sciences, 86*(1), 6-19. doi: 10.1093/toxsci/kfi110

Hirata, Y., Atsumi, M., Ohizumi, Y., & Nakahata, N. (2003). Mastoparan binds to glycogen phosphorylase to regulate sarcoplasmic reticular Ca2+ release in skeletal muscle. *Biochemical Journal, 371*, 81-88. doi: 10.1042/BJ20021844

Irintchev, A., Zeschnigk, M., Starzinskipowitz, A., & Wernig, A. (1994). Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. *Developmental Dynamics, 199*(4), 326-337.

Johnston, I. A. (1999). Muscle development and growth: Potential implications for flesh quality in fish. *Aquaculture, 177*(1-4), 99-115. doi: 10.1016/S0044-8486(99)00072-1

Johnston, I. A. (2006). Environment and plasticity of myogenesis in teleost fish. *The Journal of Experimental Biology, 209*(Pt 12), 2249-2264. doi: 10.1242/jeb.02153

Johnston, I. A., Bower, N. I., & Macqueen, D. J. (2011). Growth and the regulation of myotomal muscle mass in teleost fish. *The Journal of Experimental Biology, 214*(Pt 10), 1617-1628. doi: 10.1242/jeb.038620; 10.1242/jeb.038620

Johnston, I. A., & Hall, T. E. (2004). Mechanisms of muscle development and responses to temperature change in fish larvae. *Development of Form and Function in Fishes and the Question of Larval Adaptation, 40*, 85-116.

Johnston, I. A., Lee, H., Macqueen, D. J., Paranthaman, K., Kawashima, C., Anwar, A., . . . Dalmay, T. (2009). Embryonic temperature affects muscle fibre recruitment in adult zebrafish: Genome-wide changes in gene and microRNA expression associated with the transition from hyperplastic to hypertrophic growth phenotypes. *Journal of Experimental Biology, 212*(12), 1781-1793. doi: 10.1242/jeb.029918

Karalaki, M., Fili, S., Philippou, A., & Koutsilieris, M. (2009). Muscle regeneration: Cellular and molecular events. *In Vivo, 23*(5), 779-796.

Kimmel, C. B. (1989). Genetics and early development of zebrafish. *Trends in Genetics, 5*(8), 283-288. doi: 10.1016/0168-9525(89)90103-0

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B., & Schilling, T. F. (1995). Stages of embryonic-development of the zebrafish. *Developmental Dynamics, 203*(3), 253-310.

Kishi, S. (2011). The search for evolutionary developmental origins of aging in zebrafish: A novel intersection of developmental and senescence biology in the zebrafish model system. *Birth Defects Research Part C-Embryo Today-Reviews, 93*(3), 229-248. doi: 10.1002/bdrc.20217

Kishi, S. (2011). The search for evolutionary developmental origins of aging in zebrafish: A novel intersection of developmental and senescence biology in the zebrafish model system. *Birth Defects Research Part C-Embryo Today-Reviews, 93*(3), 229-248. doi: 10.1002/bdrc.20217

Koumans, J. T. M., Akster, H. A., Booms, R. G. H., & Osse, J. W. M. (1993). Influence of fish size on proliferation and differentiation of cultured myosatellite cells of white axial muscle of carp (cyprinus-carpio L). *Differentiation, 53*(1), 1-6. doi: 10.1111/j.1432-0436.1993.tb00639.x

Krauss, R. S., Cole, F., Gaio, U., Takaesu, G., Zhang, W., & Kang, J. S. (2005). Close encounters: Regulation of vertebrate skeletal myogenesis by cell-cell contact. *Journal of Cell Science, 118*(11), 2355-2362. doi: 10.1242/jcs.02397

Kronnie, G. T., & Reggiani, C. (2002). Skeletal muscle fibre type specification during embryonic development. *Journal of Muscle Research and Cell Motility, 23*(1), 65-69.

Kryvi, H., & Eide, A. (1977). Morphometric and autoradiographic studies on growth of red and white axial muscle-fibers in shark etmopterus-spinax. *Anatomy and Embryology, 151*(1), 17-28. doi: 10.1007/BF00315294

Kuang, S., Kuroda, K., Le Grand, F., & Rudnicki, M. A. (2007). Asymmetric self-renewal and commitment of satellite stem cells in muscle. *Cell, 129*(5), 999-1010. doi: 10.1016/j.cell.2007.03.044

LaBarge, M. A., & Blau, H. M. (2002). Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell, 111*(4), 589-601. doi: 10.1016/S0092-8674(02)01078-4

Le Grand, F., & Rudnicki, M. A. (2007). Skeletal muscle satellite cells and adult myogenesis. *Current Opinion in Cell Biology, 19*(6), 628-633. doi: 10.1016/j.ceb.2007.09.012

Lefaucheur, L. (2010). A second look into fibre typing - relation to meat quality. *Meat Science, 84*(2), 257-270. doi: 10.1016/j.meatsci.2009.05.004

Leiter, J. R. S., & Anderson, J. E. (2010). Satellite cells are increasingly refractory to activation by nitric oxide and stretch in aged mouse-muscle cultures. *International Journal of Biochemistry & Cell Biology, 42*(1), 132-136. doi: 10.1016/j.biocel.2009.09.021

Lescaudron, L., Peltekian, E., Fontaine-Perus, J., Paulin, D., Zampieri, M., Garcia, L., & Parrish, E. (1999). Blood borne macrophages are essential for the triggering of muscle regeneration following muscle transplant. *Neuromuscular Disorders, 9*(2), 72-80. doi: 10.1016/S0960-8966(98)00111-4

Lieschke, G. J., & Currie, P. D. (2007). Animal models of human disease: Zebrafish swim into view. *Nature Reviews Genetics, 8*(5), 353-367. doi: 10.1038/nrg2091

Louboutin, J. P., Fichtergagnepain, V., Pastoret, C., Thaon, E., Noireaud, J., Sebille, A., & Fardeau, M. (1995). Morphological and functional-study of extensor digitorum longus muscle regeneration after iterative crush lesions in mdx mouse. *Neuromuscular Disorders, 5*(6), 489-500. doi: 10.1016/0960-8966(95)00006-9

Luo, D., Renault, V. M., & Rando, T. A. (2005). The regulation of notch signaling in muscle stem cell activation and postnatal myogenesis. *Seminars in Cell & Developmental Biology, 16*(4-5), 612-622. doi: 10.1016/j.semcdb.2005.07.002

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling - transient versus sustained extracellular signal-regulated kinase activation. *Cell, 80*(2), 179-185. doi: 10.1016/0092-8674(95)90401-8

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. *Journal of Biophysical and Biochemical Cytology, 9*(2), 493-&. doi: 10.1083/jcb.9.2.493

McClung, J. M., Davis, J. M., & Carson, J. A. (2007). Ovarian hormone status and skeletal muscle inflammation during recovery from disuse in rats. *Experimental Physiology, 92*(1), 219-232. doi: 10.1113/expphysiol.2006.035071

McCroskery, S., Thomas, M., Maxwell, L., Sharma, M., & Kambadur, R. (2003). Myostatin negatively regulates satellite cell activation and self-renewal. *Journal of Cell Biology, 162*(6), 1135-1147. doi: 10.1083/jcb.200207056

Midwood, K. S., Williams, L. V., & Schwarzbauer, J. E. (2004). Tissue repair and the dynamics of the extracellular matrix. *International Journal of Biochemistry & Cell Biology, 36*(6), 1031-1037. doi: 10.1016/j.biocel.2003.12.003

Mizunoya, W., Upadhaya, R., Burczynski, F. J., Wang, G., & Anderson, J. E. (2011). Nitric oxide donors improve prednisone effects on muscular dystrophy in the mdx mouse diaphragm. *American Journal of Physiology.Cell Physiology, 300*(5), C1065-77. doi: 10.1152/ajpcell.00482.2010; 10.1152/ajpcell.00482.2010

Moore, J. L., Aros, M., Steudel, K. G., & Cheng, K. C. (2002). Fixation and decalcification of adult zebrafish for histological, immunocytochemical, and genotypic analysis. *Biotechniques, 32*(2), 296-+.

Moore, R., & Walsh, F. S. (1993). The cell-adhesion molecule M-cadherin is specifically expressed in developing and regenerating, but not denervated skeletal-muscle. *Development, 117*(4), 1409-1420.

Morgan, D. L., & Proske, U. (2004). Popping sarcomere hypothesis explains stretch-induced muscle damage. *Clinical and Experimental Pharmacology and Physiology, 31*(8), 541-545. doi: 10.1111/j.1440-1681.2004.04029.x

Murphy, M., & Kardon, G. (2011). Origin of vertebrate limb muscle: The role of progenitor and myoblast populations. *Current Topics in Developmental Biology: Myogenesis, 96*, 1-32. doi: 10.1016/B978-0-12-385940-2.00001-2

Noguchi, S., Wakabayashi, E., Imamura, M., Yoshida, M., & Ozawa, E. (2000). Formation of sarcoglycan complex with differentiation in cultured myocytes. *European Journal of Biochemistry, 267*(3), 640-648. doi: 10.1046/j.1432-1327.2000.00998.x

Norris, W., Neyt, C., Ingham, P. W., & Currie, P. D. (2000). Slow muscle induction by hedgehog signalling in vitro. *Journal of Cell Science, 113*(15), 2695-2703.

O'Brien, L. E., Tang, K., Kats, E. S., Schutz-Geschwender, A., Lipschutz, J. H., & Mostov, K. E. (2004). ERK and MMPs sequentially regulate distinct stages of epithelial tubule development. *Developmental Cell, 7*(1), 21-32. doi: 10.1016/j.devcel.2004.06.001

Ochi, H., & Westerfield, M. (2007). Signaling networks that regulate muscle development: Lessons from zebrafish. *Development Growth & Differentiation, 49*(1), 1-11. doi: 10.1111/j.1440-169x.2007.00905.x

Olguin, H. C., & Olwin, B. B. (2004). Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: A potential mechanism for self-renewal. *Developmental Biology, 275*(2), 375-388. doi: 10.1016/j.ydbio.2004.08.015

Ontell, M., & Feng, K. C. (1981). The 3-dimensional cytoarchitecture and pattern of motor innervation of branched striated myotubes. *Anatomical Record, 200*(1), 11-31. doi: 10.1002/ar.1092000103

Ontell, M., & Kozeka, K. (1984). The organogenesis of murine striated-muscle - a cytoarchitectural study. *American Journal of Anatomy, 171*(2), 133-148. doi: 10.1002/aja.1001710202

Osses, N., & Brandan, E. (2002). ECM is required for skeletal muscle differentiation independently of muscle regulatory factor expression. *American Journal of Physiology-Cell Physiology, 282*(2), C383-C394.

Osterauer, R., & Koehler, H. (2008). Temperature-dependent effects of the pesticides thiacloprid and diazinon on the embryonic development of zebrafish (danio rerio). *Aquatic Toxicology, 86*(4), 485-494. doi: 10.1016/j.aquatox.2007.12.013

Patterson, S. E., Mook, L. B., & Devoto, S. H. (2008). Growth in the larval zebrafish pectoral fin and trunk musculature. *Developmental Dynamics, 237*(2), 307-315. doi: 10.1002/dvdy.21400

Pette, D., & Staron, R. S. (2001). Transitions of muscle fiber phenotypic profiles. *Histochemistry and Cell Biology, 115*(5), 359-372.

Polesskaya, A., Seale, P., & Rudnicki, M. A. (2003). Wnt signaling induces the myogenic specification of resident CD45(+) adult stem cells during muscle regeneration. *Cell, 113*(7), 841-852. doi: 10.1016/S0092-8674(03)00437-9

Pollard, T. D., & Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell, 112*(4), 453-465. doi: 10.1016/S0092-8674(03)00120-X

Pownall, M. E., Gustafsson, M. K., & Emerson, C. P. (2002). Myogenic regulatory factors and the specification of muscle progenitors in vertebrate embryos. *Annual Review of Cell and Developmental Biology, 18*, 747-783. doi: 10.1146/annurev.cellbio.18.012502.105758

Rapraeger, A. C. (2000). Syndecan-regulated receptor signaling. *Journal of Cell Biology, 149*(5), 995-997. doi: 10.1083/jcb.149.5.995

Rayment, I., Holden, H. M., Whittaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., & Milligan, R. A. (1993). Structure of the actin-myosin complex and its implications for muscle-contraction. *Science, 261*(5117), 58-65. doi: 10.1126/science.8316858

Rosenblatt, J. D., Lunt, A. I., Parry, D. J., & Partridge, T. A. (1995). Culturing satellite cells from living single muscle-fiber explants. *In Vitro Cellular & Developmental Biology-Animal, 31*(10), 773-779.

Rowlerson, A., Radaelli, G., Mascarello, F., & Veggetti, A. (1997). Regeneration of skeletal muscle in two teleost fish: Sparus aurata and brachydanio rerio. *Cell and Tissue Research, 289*(2), 311-322. doi: 10.1007/s004410050878

Rowlerson, A., & Veggetti, A. (2001). Cellular mechanisms of post-embryonic muscle growth in aquaculture species. *Fish Physiology, 18*, 103-140.

Schiaffino, S., & Reggiani, C. (1994). Myosin isoforms in mammalian skeletal-muscle. *Journal of Applied Physiology, 77*(2), 493-501.

Schiaffino, S., & Reggiani, C. (1996). Molecular diversity of myofibrillar proteins: Gene regulation and functional significance. *Physiological Reviews, 76*(2), 371-423.

Schiaffino, S., & Reggiani, C. (2011). Fiber types in mammalian skeletal muscles. *Physiological Reviews, 91*(4), 1447-1531. doi: 10.1152/physrev.00031.2010

Schultz, E. (1996). Satellite cell proliferative compartments in growing skeletal muscles. *Developmental Biology, 175*(1), 84-94. doi: 10.1006/dbio.1996.0097

Schultz, E., & Jaryszak, D. L. (1985). Effects of skeletal-muscle regeneration on the proliferation potential of satellite cells. *Mechanisms of Ageing and Development, 30*(1), 63-72. doi: 10.1016/0047-6374(85)90059-4

Seale, P., & Rudnicki, M. A. (2000). A new look at the origin, function, and "stem-cell" status of muscle satellite cells. *Developmental Biology, 218*(2), 115-124. doi: 10.1006/dbio.1999.9565

Seale, P., Sabourin, L. A., Girgis-Gabardo, A., Mansouri, A., Gruss, P., & Rudnicki, M. A. (2000). Pax7 is required for the specification of myogenic satellite cells. *Cell, 102*(6), 777-786. doi: 10.1016/S0092-8674(00)00066-0

Sheehan, S. M., Tatsumi, R., Temm-Grove, C. J., & Allen, R. E. (2000). HGF is an autocrine growth factor for skeletal muscle satellite cells in vitro. *Muscle & Nerve, 23*(2), 239-245. doi: 10.1002/(SICI)1097-4598(200002)23:2<239::AID-MUS15>3.0.CO;2-U

Shefer, G., Wleklinski-Lee, M., & Yablonka-Reuveni, Z. (2004). Skeletal muscle satellite cells can spontaneously enter an alternative mesenchymal pathway. *Journal of Cell Science, 117*(Pt 22), 5393-5404. doi: 10.1242/jcs.01419

Shi, X., & Garry, D. J. (2006). Muscle stem cells in development, regeneration, and disease. *Genes & Development, 20*(13), 1692-1708. doi: 10.1101/gad.1419406

Shinin, V., Gayraud-Morel, B., Gomes, D., & Tajbakhsh, S. (2006). Asymmetric division and cosegregation of template DNA strands in adult muscle satellite cells. *Nature Cell Biology, 8*(7), 677-U69. doi: 10.1038/ncb1425

Siegel, A. L., Atchison, K., Fisher, K. E., Davis, G. E., & Cornelison, D. D. W. (2009). 3D timelapse analysis of muscle satellite cell motility. *Stem Cells, 27*(10), 2527-2538. doi: 10.1002/stem.178

Smerdu, V., Karschmizrachi, I., Campione, M., Leinwand, L., & Schiaffino, S. (1994). Type-iix myosin heavy-chain transcripts are expressed in type iib fibers of human skeletal-muscle. *American Journal of Physiology-Cell Physiology, 267*(6), C1723-C1728.

Sonnenberg, E., Meyer, D., Weidner, K. M., & Birchmeier, C. (1993). Scatter factor hepatocyte growth-factor and its receptor, the C-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *Journal of Cell Biology, 123*(1), 223-235. doi: 10.1083/jcb.123.1.223

Squire, J. M., Al-Khayat, H. A., Knupp, C., & Luther, P. K. (2005). Molecular architecture in muscle contractile assemblies. *Fibrous Proteins: Muscle and Molecular Motors, 71*, 17-+. doi: 10.1016/S0065-3233(04)71002-5

Steinbacher, P., Marschallinger, J., Obermayer, A., Neuhofer, A., Saenger, A. M., & Stoiber, W. (2011). Temperature-dependent modification of muscle precursor cell behaviour is an underlying reason for lasting effects on muscle cellularity and body growth of teleost fish. *Journal of Experimental Biology, 214*(11), 1791-1801. doi: 10.1242/jeb.050096

Sterneckert, J. L., Hill, C. M., Palmer, R., & Gearhart, J. D. (2005). Bone morphogenetic proteins produced by cells within embryoid bodies inhibit ventral directed differentiation by sonic hedgehog. *Cloning and Stem Cells, 7*(1), 27-34. doi: 10.1089/clo.2005.7.27

Stickney, H. L., Barresi, M. J. F., & Devoto, S. H. (2000). Somite development in zebrafish. *Developmental Dynamics, 219*(3), 287-303. doi: 10.1002/1097-0177(2000)9999:9999<::AID-DVDY1065>3.3.CO;2-1

Tamaki, T., Akatsuka, A., Okada, Y., Matsuzaki, Y., Okano, H., & Kimura, M. (2003). Growth and differentiation potential of main- and side-population cells derived from murine skeletal muscle. *Experimental Cell Research, 291*(1), 83-90. doi: 10.1016/S0014-4827(03)00378-8

Tatsumi, R., Hattori, A., Ikeuchi, Y., Anderson, J. E., & Allen, R. E. (2002). Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. *Molecular Biology of the Cell, 13*(8), 2909-2918. doi: 10.1091/mbc.E02-01-0062

Tatsumi, R., Sheehan, S. M., Iwasaki, H., Hattori, A., & Allen, R. E. (2001). Mechanical stretch induces activation of skeletal muscle satellite cells in vitro. *Experimental Cell Research, 267*(1), 107-114. doi: 10.1006/excr.2001.5252

Tatsumi, R., & Allen, R. E. (2008). Mechano-biology of resident myogenic stem cells: Molecular mechanism of stretch-induced activation of satellite cells. *Animal Science Journal, 79*(3), 279-290. doi: 10.1111/j.1740-0929.2008.00528.x

Vanraamsdonk, W., Vantveer, L., Veeken, K., Heyting, C., & Pool, C. W. (1982). Differentiation of muscle-fiber types in the teleost brachydanio-rerio, the zebrafish - posthatching development. *Anatomy and Embryology, 164*(1), 51-62.

Veggetti, A., Mascarello, F., Scapolo, P. A., Rowlerson, A., & Carnevali, M. D. C. (1993). Muscle growth and myosin isoform transitions during development of a small teleost fish, poecilia-reticulata (peters) (atheriniformes, poeciliidae) - a histochemical, immunohistochemical, ultrastructural and morphometric study. *Anatomy and Embryology, 187*(4), 353-361.

Vergauwen, L., Knapen, D., Hagenaars, A., De Boeck, G., & Blust, R. (2013). Assessing the impact of thermal acclimation on physiological condition in the zebrafish model. *Journal of Comparative Physiology.B, Biochemical, Systemic, and Environmental Physiology, 183*(1), 109-121. doi: 10.1007/s00360-012-0691-6; 10.1007/s00360-012-0691-6

Washabaugh, C. H., Ontell, M. P., Shan, Z., Hoffman, E. P., & Ontell, M. (1998). Role of the nerve in determining fetal skeletal muscle phenotype. *Developmental Dynamics, 211*(2), 177-190. doi: 10.1002/(SICI)1097-0177(199802)211:2<177::AID-AJA6>3.0.CO;2-E

Washabaugh, C. H., Ontell, M. P., Shand, S. H., Bradbury, N., Kant, J. A., & Ontell, M. (2007). Neuronal control of myogenic regulatory factor accumulation in fetal muscle. *Developmental Dynamics, 236*(3), 732-745. doi: 10.1002/dvdy.21078

Wagers, A. J., & Conboy, I. M. (2005). Cellular and molecular signatures of muscle regeneration: Current concepts and controversies in adult myogenesis. *Cell, 122*(5), 659-667. doi: 10.1016/j.cell.2005.08.021

Weatherley, A., GILL, H., & LOBO, A. (1988). Recruitment and maximal diameter of axial muscle-fibers in teleosts and their relationship to somatic growth and ultimate size. *Journal of Fish Biology, 33*(6), 851-859. doi: 10.1111/j.1095-8649.1988.tb05532.x

Wilson, S. J., & Harris, A. J. (1993). Formation of myotubes in aneural rat muscles. *Developmental Biology, 156*(2), 509-518. doi: 10.1006/dbio.1993.1097

Wozniak, A. C., & Anderson, J. E. (2005). Single-fiber isolation and maintenance of satellite cell quiescence. *Biochemistry and Cell Biology-Biochimie Et Biologie Cellulaire, 83*(5), 674-676. doi: 10.1139/005-046

Wozniak, A. C., & Anderson, J. E. (2007). Nitric oxide-dependence of satellite stem cell activation and quiescence on normal skeletal muscle fibers. *Developmental Dynamics : An Official Publication of the American Association of Anatomists, 236*(1), 240-250. doi: 10.1002/dvdy.21012

Wozniak, A. C., Pilipowicz, O., Yablonka-Reuveni, Z., Greenway, S., Craven, S., Scott, E., & Anderson, J. E. (2003). C-met expression and mechanical activation of satellite cells on cultured muscle fibers. *Journal of Histochemistry & Cytochemistry, 51*(11), 1437-1445.

Yablonkareuveni, Z., & Rivera, A. J. (1994). Temporal expression of regulatory and structural muscle proteins during myogenesis of satellite cells on isolated adult-rat fibers. *Developmental Biology, 164*(2), 588-603. doi: 10.1006/dbio.1994.1226

Yamada, M., Tatsumi, R., Yamanouchi, K., Hosoyama, T., Shiratsuchi, S., Sato, A., . . . Allen, R. E. (2010). High concentrations of HGF inhibit skeletal muscle satellite cell proliferation in vitro by inducing expression of myostatin: A possible mechanism for reestablishing satellite cell quiescence in vivo. *American Journal of Physiology.Cell Physiology, 298*(3), C465-76. doi: 10.1152/ajpcell.00449.2009; 10.1152/ajpcell.00449.2009

Yamada, M., Sankoda, Y., Tatsumi, R., Mizunoya, W., Ikeuchi, Y., Sunagawa, K., & Allen, R. E. (2008). Matrix metalloproteinase-2 mediates stretch-induced activation of skeletal muscle satellite cells in a nitric oxide-dependent manner. *International Journal of Biochemistry & Cell Biology, 40*(10), 2183-2191. doi: 10.1016/j.bioce1.2008.02.017

Zammit, P. S., Golding, J. P., Nagata, Y., Hudon, V., Partridge, T. A., & Beauchamp, J. R. (2004). Muscle satellite cells adopt divergent fates: A mechanism for self-renewal? *Journal of Cell Biology, 166*(3), 347-357. doi: 10.1083/jcb.200312007

Zammit, P. S., Relaix, F., Nagata, Y., Ruiz, A. P., Collins, C. A., Partridge, T. A., & Beauchamp, J. R. (2006). Pax7 and myogenic progression in skeletal muscle satellite cells. *Journal of Cell Science, 119*(9), 1824-1832. doi: 10.1242/jcs.02908

Appendix

Collagenase medium: 0.2% collagenase, 1X Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS), 2% chick embryo extract (CEE), 1% antibiotic/antimycotic and 0.1% gentamycin.

Proliferation medium: L-15 medium, 10% FBS, 2% CEE, 1% antibiotic/antimycotic and 0.1% gentamycin.

Basal growth medium: L-15 medium, 20% controlled replacement serum-2 (50X) (Sigma Aldrich, St. Louis, MO), 1% FBS, 1% antibiotic/antimycotic and 0.1% gentamycin.

5% acid alcohol: 90% absolute ethanol, 5% glacial acetic acid, 5% dd H_2O.

0.1M Phosphate buffered saline: 80 g NaCl, 2 g KCl, 26.8 g $Na_2HPO_4.7H_2O$, 2.4 g KH_2PO_4 in 800 mL of ddH2O and adjust pH to 7.4. Add volume to 1L with ddH_2O.

0.1M Tris buffered saline: 61g tris base and 90g NaCl in 800 mL ddH_2O and pH is adjusted to 8.4 using concentrated HCl. Add volume to 1L with ddH_2O.

Goat serum blocking solution: 2% goat serum, 1% bovine serum albumin (BSA), 0.1% cold fish skin gelatin, 0.1% Triton X-100, 0.05% Tween 20, 0.05% sodium azide.

Primary antibody buffer solution: 1%BSA, 0.1% cold fish skin gelatin, 0.5% Triton X-100, 0.05% sodium azide.

Secondary antibody buffer solution: 0.01M PBS, 0.05% Tween 20, pH7.4.