

IDENTIFICATION OF FACTORS THAT PROMOTE SATELLITE CELL
MOTILITY

A Thesis

Presented to

The Faculty of the Graduate School

At the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

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MAY 2014

The undersigned, appointed by the dean of the Graduate School,

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IDENTIFICATION OF FACTORS THAT PROMOTE SATELLITE CELL MOTILITY

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A candidate for the degree of Master of Science,

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ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. D.D.W. Cornelison, for accepting me into her lab, and helping to guide me through this journey. I am thankful for her patience and understanding as I took the first steps in my career as a professional scientist.

I would also like to thank my co-mentor Dr. Elizabeth Bryda for her unwavering support and encouragement. I would not have been able to be here today without her belief in my fledgling abilities. Her encouragement and insistence that I should consider graduate school, gave me the confidence to do so.

Thanks to Drs. Lorson and Kannappan for serving in an advisory capacity on my committee. Your input at meetings was invaluable and the questions you asked helped to sharpen my attention on areas where my understanding was incomplete, or at times, completely wrong.

To the people who gave me my start in science, I would like to express my sincere gratitude. Drs. Zhang and Critser provided me with an opportunity to grow my technical skills within their labs as an undergraduate and in my first job. Their willingness to give me meaningful responsibilities within their labs allowed me to grow both professionally and personally, and I am eternally grateful for the faith you showed in me. You are both deeply missed by many people; may you rest in peace.

Thank you to my many lab mates and collaborators for your daily contributions to my work, your patience in dealing with my missteps and the support and companionship

you have provided me. In no particular order: Danny Stark, Dane Lund, Dave Roeseler, Matthew Salie, Maxwell Leslie, Ilker Ersoy, Chongbei Zhao, and Mozow Zuidema.

Finally, but most importantly, I would like to recognize my family for their unconditional support, especially my mother. For a long time it was just the two of us. Thank you for always putting my wellbeing at the forefront. To my wonderful fiancé, Lydia Cook, I thank my lucky stars every day that I met you, and somehow tricked you into agreeing to marry me. Without you, none of this is possible. With you, I look forward to a continued life of discovery, both professional and personal.

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ABSTRACT

An important but poorly understood aspect of stem cell-mediated skeletal muscle regeneration is stem cell recruitment and motility within the damaged tissue; a failure to spread after therapeutic engraftment is also a key stumbling block in cell-based therapies for muscle disease. Acute muscle damage results in the local release of diffusible factors, many of which have potential to promote either chemokinesis or chemotaxis, but to date no comprehensive survey of damage-induced motogens has been published. We generated crushed muscle extract (CME) to obtain a physiologically-relevant sample of secreted factors from damaged muscle; we propose that components of CME will promote satellite cell motility within the injured tissue in a concentration-dependent manner. Using *in vitro* tests of motility and chemotaxis, we have shown that total CME as well as known components such as HGF and SDF-1 are both motogenic and chemotactic for primary mouse satellite cells. To identify other active components, we used size-exclusion and affinity chromatography to fractionate CME and test the fractions for motogenic potential. Utilizing this approach we have identified and validated a novel factor, Wnt5b, as a satellite cell motogen *in vitro*. Wnt-5b is both chemokinetic and chemotactic for primary mouse satellite cells and this motility is dose dependent. Additional fractionation of CME using heparin affinity chromatography has identified Annexin A2 and Galectin-1 as putative motogens present in high activity fractions of CME. Ongoing work is aimed at validating Annexin A2 and Galectin-1 as additional novel satellite cell motogens. Identification of these signals and an understanding of their activity individually and collectively will add significantly to our understanding of skeletal muscle regeneration.

CHAPTER I - SATELLITE CELLS: STEM CELLS OF ADULT

SKELETAL MUSCLE

Adult Skeletal Muscle

Mammalian skeletal muscles account for an average of 38% of the total body mass in human adult males¹ and are anchored by tendons to bones in order to provide support for the body. The contraction of antagonistic groups of skeletal muscles generates the force for movement. Skeletal muscle, like most tissue types, is a highly ordered structure. At the cellular level, the primary actor in skeletal muscle is the myofiber. The myofiber is a rod shaped multinucleated cell, which in turn, is composed of subunits of myofibrils. The fundamental unit of a myofibril, the sarcomere, is composed of long actin and myosin filaments which bind in a calcium dependent manner to cause contraction. All the myofibrils in a myofiber contract in unison. Bundles of myofibers are anchored together by connective tissue to form discrete groups within the muscle tissue known as fascicles. Fascicles are also anchored together by connective tissue that is continuous with the tendon to form a complete muscle. It is this hierarchy of concentric structures that enables skeletal muscle to execute its function of support and locomotion for the body.

Developmental Origins of Skeletal Muscle

Early in embryogenesis the three primary germ layers of endoderm, ectoderm and mesoderm are formed. The paraxial mesoderm segments into somites which flank

the neural tube and notochord. In somitogenesis the hypaxial region of the dermomyotome generates migratory limb muscle precursor cells². These limb muscle precursor cells delaminate and migrate to colonize the developing limb bud and form the dorsal and ventral muscle masses³. Delamination and migration of the muscle precursor cells is dependent upon the transcription and expression of the c-met gene⁴, which is mediated by the transcription factor Pax3⁵. C-met encodes for a receptor tyrosine kinase which is bound by its associated ligand, hepatocyte growth factor (HGF). Extracellular concentration gradients of HGF determine the route of migration for the limb muscle precursor cells⁶. Establishment of the myogenic cell fate in limb muscle precursor cells is determined by the expression of basic helix-loop-helix (bHLH) transcription factors Myf5 and MyoD³. In addition to Myf5 and MyoD there are two other myogenic regulatory factors (MRF's), myogenin and MRF4, which function in muscle differentiation⁷. The activation of specific myogenic-associated bHLH transcription factors in the somite is induced by the combinatorial signaling of Wnts, originating from the neural tube, and sonic hedgehog (Shh), originating from the notochord/floor plate⁸. Although the migrating limb muscle precursor cells do not express Myf5 and MyoD until they reach the limb⁹, activation of these myogenic transcription factors occurs in a similar process. Wnt7a from the dorsal surface ectoderm³ and Shh released from the zone of polarizing activity triggers induction of Myf5 transcription and myogenesis¹⁰. Induction of the myogenic program in migrating limb muscle precursor cells triggers their differentiation into myoblasts. The limb myoblasts fuse to each other to form the first muscle fibers known as primary myofibers and a subsequent wave of proliferation gives rise to another population of myoblasts which fuse around the primary myofibers to form

secondary myofibers¹¹. Another muscle resident cell population, satellite cells, have progenitors that arise from the dermomyotome, and although no satellite cell progenitor specific molecular markers have been identified, it remains unclear whether these cells represent a distinct lineage from the dermomyotome derived limb muscle precursor cells¹².

Somatic Stem Cells in Tissue Maintenance and Regeneration

In normal, uninjured conditions, skeletal muscle is a very stable tissue, with limited turnover of existing myofibers. However, in response to injury, skeletal muscle is remarkably regenerative, able to completely repair damaged sections of muscle 15 days after injury¹³. In fact, muscle regeneration is so efficient, skeletal muscle is able to reconstitute its original configuration even after complete disruption¹⁴. The regeneration and maintenance of a damaged adult tissue is mediated by tissue-resident somatic stem cells. For a cell to be classified as a somatic stem cell, it must exhibit important physiological properties including: (1) residence in a specific microenvironment, known as the stem cell niche, (2) the ability to self-renew via successive rounds of proliferation, and (3) the ability to differentiate into some or all of the cell types resident to its host tissue or organ. Different types of cell division determine whether a given cell is fated for self-renewal or differentiation. In symmetric division, both daughter cells retain their stem-like characteristics, whereas in asymmetric division, only one daughter cell retains the stem-like characteristics. The other becomes a transit amplifying cell, with limited potential for self-renewal before commitment to differentiation. Somatic stem cells are well established, and found in a variety of tissue types including the brain, bone marrow, heart, gut and liver.

The Satellite Cell as a Somatic Stem Cell

Adult skeletal muscle contains a small, heterogeneous population of resident somatic stem cells known as satellite cells¹⁵, and they are absolutely required for proper muscle maintenance and regeneration after injury¹⁶⁻²⁰. Under uninjured conditions, satellite cells reside in a specialized niche on the periphery of the host myofiber between the basal lamina and sarcolemma²¹ in a mitotically quiescent state. Satellite cells can be readily identified by their expression of the transcription factors Pax7²² and Myf5²³, in addition to cell surface markers such as syndecan-4²⁴, CD34²³ and CXCR4²⁵. Assembly of the basal lamina around the satellite cell is mediated by Notch/Delta signaling²⁶ and is instrumental in the formation of the specialized niche that protects and isolates satellite cells from most extracellular signals²⁷. In response to injury or stress, satellite cells become “activated”, re-entering the cell cycle and emerging from their niche. Activated satellite cells are known as myoblasts. The source of the activating signal or signals has yet to be fully characterized²⁸, although at least 3 independent activating signals have been described [sphingosine-1-phosphate²⁹, HGF^{30,31}, and fibroblast growth factors (FGF)^{32,33}] highlighting the complex regulation of multiple signals in the transition between quiescence and activation. Activated satellite cells (myoblasts) will undergo successive rounds of division, where some of the daughter cells will re-colonize the satellite cell niche, while others will migrate to the site of injury and differentiate into myocytes³⁴. The differentiated myoblast, the myocyte, can either fuse to existing myofibers, or to other myocytes to produce new myofibers, to achieve regeneration of the damaged tissue³⁵.

The Satellite Cell as a Vector in Cell Based Therapies

In some cases where chronic damage to the myofibers occurs, such as Duchenne's muscular dystrophy (DMD), endogenous satellite cells are unable to maintain proper myofiber function, resulting in progressive muscle degeneration and death. Duchenne's muscular dystrophy, affecting 1 in every 3,500 males³⁶, is caused by an X-linked mutation in the dystrophin gene, which encodes the dystrophin protein^{37,38}. Dystrophin is a critical component of the dystrophin-associated glycoprotein (DAG) complex, which functions to anchor sarcomeres to the cellular membrane³⁹. While a comprehensive understanding of the underlying mechanism of how lack of functional dystrophin leads to muscle degeneration is incomplete, it is clear that lack of functional dystrophin is the critical component in the initiation and progression of DMD⁴⁰. Donor satellite cell derived myoblasts, expressing a normal version of the dystrophin gene, would seem to be ideal candidates for cell based therapies to treat DMD, as fusion of the transplanted myoblasts to the existing myofiber would deliver a functional copy of the dystrophin gene allowing for functional protein expression and restoration of normal myofiber function. This cell based therapy, known as myoblast transfer therapy (MTT), although conceptually straight forward, has met with little clinical success³⁶. One of the primary factors contributing to the ineffectiveness of MTT is failure of the donor cells to migrate away from the site of intramuscular injection⁴¹. The farthest myoblasts have been documented to migrate from an injection site is 200 μm ⁴², necessitating protocols which require 100 intramuscular injections per square cm and up to 4,000 injections for a given patient^{43,44}. Despite being more successful than previous therapeutics, this injection regimen, would clearly be more feasible if the donor myoblasts were able to migrate

farther, as fewer injections would be required. Comparatively little research has been done on to identify endogenous signals that promote myoblast migration. Identification of these signals and an understanding of their temporal and spatial regulation would represent a significant improvement over current MTT protocols.

Motility of Satellite Cells

An important, yet poorly understood aspect of satellite cell physiology is the migration of activated satellite cells within the damaged tissue. In order for any cell to migrate efficiently it must engage in a sequence of steps known as the cell motility cycle. The cell motility cycle is characterized by adoption an asymmetric morphology with leading and trailing edges while adhering to the underlying substrate using a suite of integrins^{45,46}. Intracellular signaling events localized in one region of the cell orients the leading edge, and subsequent contraction and release in other localized regions orchestrates cellular motility^{45,46}. Other types of somatic stem cells including neural stem cells⁴⁷ and hematopoietic stem cells⁴⁸ are motile within the context of their tissues and can actively home to areas of need by sensing signals within the environment. These pro-motility signals can come from a variety of different sources, and it is the origin of the signal that defines the type of motility the cell engages in. Haptotaxis occurs in response to differential adhesion kinetics in the underlying substrate or a guidance cue sequestered within the extracellular matrix⁴⁹. Durotaxis is a change in cell motility in response to the rigidity of the substrate⁵⁰. Electrotaxis, as the name implies, occurs in response to the presence of differential electric fields⁵¹. Finally, both chemokinesis and chemotaxis can occur in response to soluble signals, and it is important to define the differences between these two modes of soluble signal induced motility. Cell

chemokinesis occurs when a cell encounters a given soluble signal, either symmetrically or asymmetrically applied, and alters its normal pattern of cell motility. This alteration can be either speeding up or slowing down, but the signal does not dictate the direction of migration⁵². Cell chemotaxis occurs when a cell encounters an asymmetrically applied soluble signal and changes its motility to migrate directionally, according to the concentration gradient of the signal⁵². Accordingly, compounds that promote chemotaxis are of particular interest in the context of somatic stem cell motility. This is logical as somatic stem cells constitute a very small percentage of the total nuclei within a tissue (~4% with respect to satellite cells⁵³), and a motile, chemotactically responsive phenotype would presumably decrease the latency of regeneration by calling stem cells from distal areas towards the site of injury. Although satellite cell motility has long been noted *in vitro*⁵⁴, and more recently in our lab on *ex-vivo* myofibers⁵⁵; the question of whether motility occurred *in vivo* has only been answered within the last three years^{56,57}. The observation of satellite cell motility in injured limbs towards the site of injury, and a lack of motility in uninjured contra-lateral control limbs strongly suggests that satellite cell migration may be an important aspect of efficient skeletal muscle regeneration.

Known Satellite Cell Motogens

The repertoire of soluble factors that promote satellite cell motility is incompletely understood; however several factors have been identified and validated. Of particular interest is the multi-faceted role of HGF in mediating not only the switch from quiescence to activation, but also acting *in vivo* to regulate satellite cell migration towards an area of injury⁵⁸. HGF, a soluble growth factor, has been shown to be sufficient to induce activation of satellite cells through its endogenous receptor c-met^{59,60}. HGF is

deposited in discrete pockets in the extracellular matrix, and in response to injury, the muscle releases nitric oxide, which liberates HGF from the pockets allowing it to diffuse through the tissue^{30,31}. HGF binds to the single-pass transmembrane receptor tyrosine kinase c-met, which is expressed on all satellite cells, both quiescent and activated⁶¹. Binding of HGF to c-met induces homodimerization and trans-phosphorylation at tyrosine 1234/1235 in the catalytic domain of the receptor⁶². Trans-phosphorylation is critical for the initiation of an intra-cellular signaling cascade that produces multiple effects including proliferation and migration⁶³⁻⁶⁵. A seminal paper for the study of satellite cell motogens demonstrated that HGF, which had previously been established as a chemoattractant for myogenic cells in embryogenesis^{4,66}, could induce satellite cell chemotaxis in Boyden-type chambers in a concentration dependent manner⁵⁴. Later research, using conditional inactivation of c-met, demonstrated that mutant myoblasts exhibited slower migration in addition to decreased dispersal from the point of origin compared to controls⁶², underscoring the importance of the HGF/c-met signaling axis in myogenic cell migration.

The small chemokine Stromal Cell-derived Factor 1 (SDF-1 α) is strongly chemotactic for a variety of cell types including lymphocytes⁶⁷, endothelial progenitor cells⁶⁸, hematopoietic cells⁶⁹, primordial germ cells⁷⁰ and mesenchymal stem cells⁷¹. Indeed, the term chemokine was coined for cytokines that can induce chemotaxis in cells that express the appropriate receptor. Unique among members of the chemokine family, which are normally able to bind more than one receptor, the receptor for SDF-1 α is CXCR4 alone, implying an important biological role⁷². CXCR4 is used as a cell-surface marker for satellite cells²⁵ and has been reported to be expressed on 80% of freshly

isolated Pax7⁺ satellite cells⁷³. SDF-1 α is secreted by muscle derived fibroblasts that reside within the adult skeletal muscle and C2C12 cells (a common cell line for studying satellite cell biology) responded in a significantly chemotactic manner to conditioned media²⁵. Importantly, the same study showed reduced chemotaxis to conditioned media, statistically indistinguishable from controls, when cells were treated with an inhibitor of CXCR4, demonstrating that it was the presence of SDF-1 α in the conditioned media that was responsible for the chemotactic effect. Other studies have shown that SDF-1 α induces migration of myoblasts and myocytes in a concentration dependent manner^{74,75}; and that SDF-1 α mediated signaling is required for muscle development⁷⁶, establishing the SDF-1 α /CXCR4 axis as a critical player in the development of the myogenic lineage and myogenic cell migration.

Other secreted factors have been shown to elicit a significant chemotactic effect on satellite cells, their differentiated progeny, or immortalized cell lines including TGF- β ⁵⁴, VEGF⁷⁷, and a currently unknown soluble ligand to the MOR23 odorant receptor⁷⁸. However, the list of known soluble factors that are capable of significantly increasing satellite cell motility is noticeably short, and likely remains fertile ground for continued research. This may be due in part to previous studies taking a candidate based approach, whereby known factors that had the potential to be satellite cell motogens were evaluated. This approach is experimentally tedious, necessitating the testing of numerous factors that may not even be expressed at the right place (within the muscle) nor time (during regeneration). By focusing on identifying factors already present within the regenerating muscle tissue, and then testing their ability to promote satellite cell motility, potential false positives could be avoided.

Crushed Muscle Extract as a Physiological Source of Motogens

Acute damage to a muscle results in the production of a wound fluid which contains potent soluble factors that have the ability to regulate myogenesis. This was initially noted in 1986 when Bischoff generated saline extracts from crushed and uncrushed rat muscles and observed that the extracts from the crushed muscles were mitogenic for both embryonic rat myoblasts and adult rat satellite cells⁷⁹. Thus, it was demonstrated that the activating mitogen for satellite cells was present only in the saline extracts of the crushed muscles. These saline extracts were termed crushed muscle extracts (CME), and provides an excellent model system for investigating soluble factors released upon muscle injury, as these same factors are likely to be released during normal muscle injury⁸⁰. Although CME is a complex and largely undefined mixture of secreted factors, its utility in stimulating a mitogenic response in satellite cells lead to its use in many key studies important for sheading insight into activation and proliferation⁸¹⁻⁸⁴. Various attempts have been made to identify the source of the activity, but have met with limited success. One study, which utilized a candidate approach, concluded that CME contained factors that regulate mouse myogenesis which are distinct from basic fibroblast growth factor (bFGF), Insulin-like growth factor 1 (IGF-I), IGF-II, platelet-derived growth factor (PDGF), epidermal growth factor (EGF), adrenocorticotrophic hormone (ACTH), macrophage colony-stimulating factor (M-CSF) and transferrin (Tf)⁸⁰. Subsequent studies by the same group utilized biochemical fractionation of CME over a heparin-affinity column in an attempt to isolate the activity⁸⁵. This fractionation resulted in 4 distinct fractions (3 heparin-binding) which contained mitogenic activity, one of which eluted off the column at 0.9M NaCl and could not be identified using known

mitogenic factors of satellite cells. It was later shown that HGF is present within CME and is the primary activator and mitogen for satellite cells⁶⁰. Both HGF and CME have also been tested for chemotactic activity and shown to induce chemotaxis in rat satellite cells in a dose dependent manner⁵⁴. In an attempt to isolate the mitogenic activity of CME, the same study used heparin affinity chromatography to separate CME into two distinct fractions (heparin and non-heparin binding) and tested each fraction for chemotactic activity⁵⁴. In contrast to the mitogenic activity of CME residing in the heparin binding fraction, the non-heparin binding fraction was found to contain the chemotactic activity of CME, indicating that distinct factors may regulate motility and proliferation, although factors like HGF have been demonstrated to induce both effects⁵⁴. We propose that technologies have matured to a point that will allow for the identification and characterization of previously unknown protein components in CME that are mediators of satellite cell chemokinesis and/or chemotaxis. As no comprehensive survey of pro-motility signals in damaged tissue has been published, and it has been demonstrated that satellite cell migration occurs *in vivo* in response to an injury⁵⁶ or exogenous HGF⁵⁸, an understanding of the motility signals that regulate satellite cell migration is critical to understanding muscle regeneration *in vivo*. . This thesis constitutes a necessary first step in defining protein components of CME that mediate satellite cell motility, and provides an experimental framework for future studies to identify any additional factors that are present within CME.

CHAPTER II - WNT-5B PROMOTES INCREASED SATELLITE CELL MOTILITY

Abstract

Stem cell recruitment and motility within damaged adult skeletal muscle is an important but poorly understood aspect of stem cell mediated skeletal muscle regeneration.

Satellite cells are the resident stem cells of adult skeletal muscle, and are required for efficient regeneration. The local release of diffusible factors occurs as a result of acute muscle damage. These factors have the potential to promote either chemokinesis or chemotaxis, but to date no comprehensive survey of damage-induced motogens has been published. We generated a physiologically-relevant sample of secreted factors from damaged muscle by collecting crushed muscle extract (CME). We propose that components of CME will promote satellite cell motility in a concentration-dependent manner. Using in vitro tests of motility and chemotaxis, we have shown that total CME, in addition to known components such as, HGF and SDF-1 are both motogenic and chemotactic for primary mouse satellite cells. To identify other active components, we used size-exclusion and affinity chromatography to fractionate CME and test the fractions for motogenic potential. Utilizing this approach we have identified a novel candidate, Wnt-5b, as a satellite cell motogen in vitro. Moreover, Wnt-5b induced both chemokinesis and chemotaxis at rates higher than previously established satellite cell motogens (HGF and SDF-1). Collectively, these data provide evidence for a model

which involves multiple instructive cues released in different spatiotemporal patterns which modulate satellite cell motility to ensure efficient muscle regeneration.

Introduction

Satellite cells are the resident stem cells of skeletal muscle¹⁵, and are absolutely required as a source of reserve myogenic cells for muscle repair and regeneration after injury¹⁶⁻²⁰. In healthy, uninjured muscle, satellite cells are located between the basal lamina and sarcolemma of the host myofiber²¹ in a mitotically quiescent state⁸⁶. Upon injury, signals are released from both the damaged muscle tissue^{54,58,80,85,87-90} and various local cell types, including fibroblasts¹⁸, and macrophages^{90,91}, to dynamically regulate satellite cell activity²⁷. These signaling events lead to satellite cell activation (after which they may be referred to as myogenic progenitor cells or adult myoblasts), proliferation, migration and differentiation into myocytes³⁴. Myocytes, in turn, either fuse to the existing damaged myofiber or fuse to each other to form mature myofibers, thereby completing the regeneration of the injured muscle³⁵. The regenerative capacity of satellite cells is highlighted by observations that a single satellite cell is capable of generating new myofibers⁹² and that as few as seven satellite cells associated with a single donor myofiber, can generate more than 100 new myofibers, each containing thousands of myonuclei¹⁵. While significant advances have been made in our understanding of satellite cell proliferation and differentiation, significantly less is known regarding satellite cell motility. Because satellite cells constitute only a small fraction (~4%) of the total nuclei in adult skeletal muscle⁵³, homing of satellite cells from a distal location to sites of injury would presumably decrease the time required to complete regeneration. However, although satellite cells are motile when cultured *in vitro*, significant hurdles to

imaging satellite cell motility and migration towards a site of injury *in vivo* have made progress in this area difficult^{56,58}. Largely because of the intractability of the system, protein factors regulating satellite cell motility *in vivo* are not well characterized in either number or activity, and to date no comprehensive survey of pro-motility factors released from injured muscle has been published.

Early studies into the potential for satellite cell motility *in vivo* showed that following focal crush injury, satellite cells distal to the injury were activated and the number of satellite cells in distal areas decreased, suggesting satellite cell migration from those areas towards the injury⁹³. To identify molecules released by injured muscle that had activating and/or motogenic effects, saline extracts of muscles gently crushed after excision from the mouse (termed crushed muscle extract or CME) were generated⁷⁹. When tested on immortalized or primary myoblasts, CME had mitogenic, motogenic, and chemotactic activity^{85, 54} and separation by heparin affinity chromatography suggested that the mitogenic and motogenic activities of CME were present in different fractions (where the motogenic fraction was non heparin-binding). Subsequent candidate screening approaches identified both hepatocyte growth factor (HGF) and transforming growth factor-beta (TGF- β)⁵⁴ as components of CME that are chemotactic for satellite cells *in vitro*; later work showed that HGF is the activation signal for satellite cells *in vivo*⁶⁰. HGF is present in CME⁵⁹ as well as in localized deposits on the muscle fiber in uninjured muscle, and the HGF receptor c-met is expressed on all satellite cells during quiescence, activation, and proliferation⁶¹. It is proposed that HGF is released (not secreted) upon injury³¹ in order to stimulate satellite cell activation, proliferation, differentiation, and migration^{54,94}. In this study, we used 2D timelapse microscopy and an automated *in vitro*

chemotactic assay to screen for satellite cell motogens and/or chemotactic factors in CME. Whole CME is, as expected, both motogenic and chemotactic, and biochemical separation of CME by size and native charge also fractionated the activity. By LC-MS/MS analysis, the fraction with the highest motogenic activity contained a non-canonical Wnt (Wnt-5b), and recombinant Wnt-5b increases both total and directional satellite cell motility in a concentration dependent manner.

Methods

Muscle satellite cell isolation and culture

Satellite cells were isolated from female mice (B6D2F1; Jackson Labs) between the ages of 70 and 175 days according to our previously published methods²⁴. Briefly, hindlimb muscles were excised in PBS, minced, and digested in 400 U/mL collagenase type I (Worthington Biochemical) diluted in Ham's F-12 medium (Invitrogen). Tissue slurries were filtered and pelleted, then resuspended and plated in Ham's F-12 supplemented with 15% horse serum (Equitech), 0.5 nM rhFGF2 and 1% penicillin/streptomycin (Sigma) on gelatin-coated plates. Cells were maintained at 37° C and 5% CO₂ in a humidified incubator for 4 days before use.

Crushed Muscle Extract

The quadriceps, gastrocnemius and tibialis anterior were dissected out, weighed, and submerged in an equal volume of ice-cold PBS, then gently squeezed with blunt forceps. Samples were rocked for 2 hours at 4° C then the muscle was removed. The supernatant was centrifuged for 5 minutes at 1,000 RPM to sediment any remaining muscle tissue, sterile filtered using a 0.22 µm filter, then stored in aliquots at -80° C.

Protein concentration of each batch of CME was determined by Bradford assay (Bio-Rad).

2-D time-lapse microscopy and postimaging analysis

Satellite cells were collected 4 days after isolation and resuspended in Ham's F-12 without serum. 5,000 cells/well were added to 48 well plates (Corning) coated in 10 $\mu\text{g/mL}$ laminin (Sigma) per well. Experimental wells were supplemented with individual candidate motility factors [32 $\mu\text{g/mL}$ total CME, 1 $\mu\text{g/mL}$ Wnt-5b (R&D Systems), 80 ng/mL HGF (R&D Systems), 200ng/mL SDF-1 (R&D Systems), 5 $\mu\text{g/mL}$ Fractionated CME]. Each condition was run in triplicate. Three 10x fields of each well were imaged every 5 minutes for 12 hours with Metamorph (Molecular Devices) on a Leica DMI 4000B microscope equipped with a stagetop incubator (Live Cell Instrument) and a Retiga 2000R camera (QImaging)

Individual .tiff images generated by Metamorph were arranged in sequential order and collapsed into stacks. Cell movement from frame to frame was measured using digital pixel trace measurements. Cells selected for tracking remained viable for the entire 12 hour duration and remained within the field of view. If a cell selected for tracking proliferated during the 12 hour track, one daughter cell was selected at random to continue the trace. Velocities for each cell were calculated and mean velocities were determined for each condition.

Real-time cell migration assay

Satellite cell chemotaxis was analyzed in real-time using the xCELLigence DP system (Roche) following the manufacturer's instructions for growth factor-mediated cell migration with the following modifications. The top and bottom of the membrane was coated with 10 µg/mL laminin (Sigma). Top and bottom chambers were filled with Ham's F-12 (Invitrogen). Top chambers were seeded with 30,000 satellite cells in growth medium. Bottom chambers were loaded with candidate chemotactic factors as above [32 µg/mL CME, 200 ng/mL Wnt-5b (R&D Systems), 1 µg/mL Wnt-5b (R&D Systems), 5 µg/mL Wnt-5b (R&D Systems), 80 ng/mL HGF (R&D Systems)]. Individual assays were run in quadruplicate. Electrical impedance was measured every 5 minutes for 72 hours using the RTCA DP Analyzer and Software (Roche Diagnostics). Readouts for each condition were averaged among replicates then each experimental timepoint was normalized using the corresponding control (minimal media only) average as baseline.

Chromatography and Mass Spectrometry

2 mg of CME (pooled from three independent preparations) was separated by size by fast protein liquid chromatography (FPLC) on an AKTAprime Plus (GE) over a Superdex 75 gel filtration column (Pharmacia) and eluted in 500 µl fractions. Size separation of 20 sequential fractions containing over 95% of the protein was confirmed by PAGE. Four fractions were chosen to cover the full size range and separated by charge over a HiTrap-Q anion exchange column (GE Life Sciences) with a linear gradient of NaCl (25 - 750mM). Protein-containing fractions were desalted using Amicon Ultra-2 concentrating columns (Millipore) and tested for *in vitro* activity.

Mass spectroscopy analysis was done at the University of Missouri Proteomics Core. The most active fraction was precipitated with 4 volumes of 10% trichloroacetic acid in acetone and the pellet washed three times with 80% acetone (in water). Final pellet was resuspended in 10 μ L of 6M urea, 100mM HEPES, pH 7.8, reduced and alkylated, and digested with trypsin. Following acidification and lyophilization, the sample was resuspended in 10 μ L 5% acetonitrile, 1% (v/v) formic acid in milliQ water. Sample was centrifuged at max speed for 10 minutes and 9 μ L removed to an autosampler vial. The vials were placed in a thermostatted (4C) autosampler. An aliquot (5 μ L) was loaded onto a C8 trap column (C8 captrap – Michrom Bioresources). Bound peptides were eluted from this trap column onto a 10.5 cm, 150 μ m i.d. pulled-needle analytical column packed with Magic C18 reversed phase resin (Michrom Bioresources). Peptides were separated and eluted from the analytical column with a continuous gradient of acetonitrile from 5 to 40% (in 0.1% formic acid) over 70 minutes, data across a total of 80 minutes of elution were collected. The Proxeon Easy nLC system is attached to an LTQ Orbitrap mass spectrometer. Following a high-resolution FTMS scan of the eluting peptides, each cycle, the 9 most abundant peptides were subjected to peptide fragmentation (CID in iontrap). The data were searched against the NCBI nr database limited to Mus species using Sorcerer2 IDA. Results (identifications) were examined using the Scaffold program. Full details of data processing and search parameters are available on request.

Western Blots

Four independent samples of CME (10 µg each) and 33.33 ng recombinant mouse Wnt-5b (R&D Systems) were loaded onto a 4-12% Bis-Tris Gel (Invitrogen), transferred to a polyvinylidene difluoride (PVDF) membrane and blocked in StartingBlock Blocking Buffer (Thermo Scientific). Wnt-5b primary antibody (GeneTex) was diluted 1:500 and incubated 1 hour at room temperature, followed by horseradish peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotech) diluted 1:50,000 for 1 hour at room temperature. Chemiluminescent substrate (Pierce SuperSignal West) was detected with a LAS3000 imager (Fujifilm).

Immunofluorescence

Frozen sections of mouse tibialis anterior muscles were fixed with 4% PFA, blocked for 1 hour at room temperature with 10% normal goat serum/ 1% Nonidet-P40, and incubated with anti-Wnt-5b (GeneTex) at 1:250 overnight at 4° C. Sections were washed with PBS, incubated with goat anti-rabbit Alexa 555 (Invitrogen) at 1:500 for 1 hour at room temperature, then washed in PBS and reblocked for 1 hour at room temperature with 10% rabbit serum to mask the available binding sites of the anti-rabbit secondary antibody. They were then incubated with anti-laminin (Sigma Aldrich) at 1:400 for 40 minutes at room temperature, washed and incubated with goat anti-rabbit Alexa 488 (Invitrogen) at 1:500 for 20 minutes at room temperature. Sections were mounted using Vectashield (Vector Labs) with DAPI and imaged on a BX61 microscope (Olympus) using SlideBook (Intelligent Imaging Innovations) and µManager

(www.micro-manager.org). Fluorescence was normalized using no-primary controls as background for imaging.

RT-PCR

Total RNA was extracted from primary mouse satellite cells using RNeasy Mini Kit (Qiagen) and reverse transcribed using SuperScriptIII (Invitrogen). 250ng cDNA was used as template for amplification. Primer sequences used are listed in Table 2-1. All frizzled primer sequences are previously published by other groups (Fzd1-3 and Fzd5⁹⁵, Fzd6⁹⁶, Fzd7,8,10⁹⁷, Fzd9⁹⁸) except Fzd4. GAPDH was amplified as a control.

Statistical Analyses

Data are presented as mean + SE unless otherwise noted. Comparisons between control and CME were made using a 1-tailed Welsh's t-test. Comparisons between control and treated samples were made using a 1-way ANOVA with Dunnett's post hoc test.

Results

CME is motogenic and chemotactic for primary mouse satellite cells

We used 2-D time-lapse microscopy to measure general motility (chemokinesis)⁹⁹ and a real-time migration assay to measure chemotaxis. A model depicting the difference between these two types of movement is shown in Figure 2-1 (A and B). For timelapse, satellite cells on laminin-coated plates were cultured in either minimal medium (MM, Ham's F-12) or medium supplemented with CME then imaged every 5 minutes for 12 hours. Satellite cells are viable (based on propidium iodide

staining, not shown) and proliferative even in the absence of serum or exogenous factors, but are not highly motile (Figure 2-1C, D), while cultures supplemented with CME had significantly increased motility (86.7 $\mu\text{m/hr}$ vs. 67.35 $\mu\text{m/hr}$ in MM) (Figure 2-1C, D). Similarly, real-time migration assays using CME as a chemoattractant showed increased satellite cell chemotaxis compared to MM, with the fastest rates of migration occurring early, when the chemotactic gradient is steepest (Figure 2-1E). Both of these results are consistent with previous descriptions of CME-induced motility and chemotaxis⁵⁴.

Wnt-5b is present in both CME and uninjured muscle sections

To separate and simplify the protein components of CME, we first separated by size using gel filtration chromatography, which was validated by SDS-PAGE (data not shown) and all of the fractions with detectable concentrations of protein were tested for motogenic potential as above. Some size-separated fractions significantly increased satellite cell movement, and some did not promote motility above baseline (data not shown). The fraction with the highest motogenic activity was separated a second time by charge, and the motility assay was repeated. When this fraction was analyzed by LC-MS/MS, one of the peptides identified was Wnt-5b, which we chose to follow up.

To confirm that Wnt-5b is a component of CME, four independently-collected samples of CME were compared by Western blot; recombinant Wnt-5b was used as a positive control. Wnt-5b was detected in all four samples of CME at similar concentrations, which were calculated based on comparing sample band intensity to that of recombinant Wnt-5b (Figure 2-2A). When we looked for expression and localization of Wnt-5b protein in uninjured tibialis anterior (TA) muscle cross-sections, we saw that

Wnt5b (red) expression was localized to the interstitial space, between the laminin (green) of adjacent myofibers (Figure 2-2B). Wnt5b is considered a non-canonical Wnt¹⁰⁰, which would be expected to signal through Wnt receptors (Frizzleds)¹⁰¹. Activated satellite cells *in vitro* detectably expressed mRNA for all ten Frizzled receptors (Figure 2-5). We also noted that cells adhered to laminin in particular upregulated Fzd1, 2, 3, and 5, while Fzd 7, 8 and 9 were highly transcribed regardless of substrate (Figure 2-5A).

Wnt-5b promotes satellite cell motility

To ask whether Wnt-5b promotes satellite cell motility, we first tested a range of Wnt-5b concentrations for chemotactic activity. Total cell motility increased in a dose dependent manner (Figure 2-3B) with a strong and intermediate effect at 1 $\mu\text{g/mL}$, which is similar to the concentrations of Wnt-5b we detected in our CME samples (Figure 2-2A). We next compared satellite cells in MM (control) against wells treated with 32 $\mu\text{g/mL}$ CME, 80 ng/mL HGF, 200 ng/mL SDF-1, and 1 $\mu\text{g/mL}$ Wnt-5b (Figure 2-3A). HGF is known to stimulate satellite cell motility and chemotaxis⁵⁴ and SDF-1 has been shown to affect C2C12 motility²⁵; both are present in CME⁹⁹ so they were chosen as positive controls. All three additions increased satellite cell chemokinesis above MM (Figure 2-3A). Treatment with Wnt-5b also increased motility, more than either HGF or SDF-1 at the concentrations tested (Figure 2-3A) and comparable to CME. When we compared the range of cell velocities scored in each condition (Figure 2-4) we noted that the increase in average velocity in CME and Wnt-5b treated cells resulted primarily from shifting the lowest limit upwards without significantly affecting the upper limit. These data suggest that Wnt-5b contributes to the motogenic effect of CME on satellite cells

Wnt-5b promotes satellite cell chemotaxis

To ask whether Wnt-5b promotes satellite cell chemotaxis, we challenged primary mouse satellite cells with a positive gradient of 1 $\mu\text{g/ml}$ Wnt-5b. We used MM in both chambers (Figure 2-3B,C,D) or 1 $\mu\text{g/ml}$ Wnt-5b in both chambers (Figure 2-3D) as negative controls. As positive controls, we also tested CME and HGF (Figure 2-3C,D). We found that both CME and Wnt-5b promoted chemotaxis, as did HGF albeit to a lesser extent. The comparatively low effects of HGF compared to CME are surprising, since HGF is generally considered to be a potent chemoattractor for satellite cells⁵⁴; it also suggests that the chemokinetic and chemotactic activities of CME are due to different component proteins. Wnt-5b proved more chemotactic than HGF, although unlike its effect on total motility it did not equal CME. These data support the conclusion that Wnt-5b also contributes to the chemotactic effect of CME on satellite cells.

Discussion

The role of cell motility and migration in satellite cell-mediated muscle regeneration remains poorly understood, although several recent studies have identified motility as a component of efficient muscle regeneration^{57,62,74,75,78,90,102-104}. The extent to which general motility (chemokinesis) vs. directed motility (chemotaxis) each contribute, and whether total and directed motility have a cooperative effect, is also unclear. In other systems, when tissue-specific stem cells are activated and mobilized to respond to damage or other stimuli, homing from either local sources or the circulation is an essential component of a successful response. In muscle, exogenous HGF acts *in vivo* to

recruit cells towards areas of increasing HGF concentration, with higher migration rates corresponding to higher doses of HGF⁵⁸.

Studies aimed at identification of secreted signaling proteins modulating muscle satellite cell activation, proliferation, migration, and differentiation *in vivo* generally fall into two classes, each having advantages and disadvantages. Candidate approaches manipulate the presence or absence of a specific ligand or receptor, while more unbiased approaches report on the presence of, or changes in, multiple proteins (which are frequently sifted through for potential factors to be tested in a candidate approach). Crushed muscle extract or CME has been the starting material that led to the identification of several key modulators of satellite cell activity including HGF⁵⁴, TGF- β ⁵⁴, and bFGF⁸⁰. In this study, we set out to identify physiologically-relevant signaling molecules that promote total and/or directional activity in primary muscle satellite cells. We identified Wnt-5b, a noncanonical Wnt ligand, as a potent promoter of both, and conclude that Wnt-5b activity contributes to the known properties of CME *in vitro*. We propose that Wnt-5b *in vivo* acts to mobilize and attract satellite cells from areas distal to the damaged tissue, which would increase the efficiency of muscle regeneration.

Mammalian Wnts are a family of 19 secreted glycoproteins which act through Frizzled (Fzd) receptors¹⁰¹. Some Wnt/Fzd interactions lead to activation of the canonical pathway, promoting stabilization of β -catenin which can then transit to the nucleus to act as a transcriptional co-activator¹⁰⁵. Other Wnts and their cellular receptors participate in β -catenin-independent non-canonical pathways, with downstream effects such as cytoskeletal reorganization for migration¹⁰⁵ and symmetrical versus asymmetrical stem cell division¹⁰⁶. During vertebrate development, Wnt5b promotes gastrulation

movements¹⁰⁷, somite patterning¹⁰⁸, MyoD expression¹⁰⁹, and neural tube closure¹¹⁰ (the *curly tail* mouse mutant and *pipetail* zebrafish mutant are both deficient in Wnt5b.) Wnt-5b (as well as other family members) is transcribed in uninjured skeletal muscle¹¹¹; after injury Wnt-5b expression increases and peaks at 4 days post-injury¹¹¹, suggesting its potential involvement in satellite cell-mediated muscle regeneration. However, these RT-PCR data do not identify the cellular source of the Wnt proteins, which would provide a framework to explore possible mechanisms.

Various members of the Wnt family have different spatiotemporal expression during regeneration. For example, Wnt-7a (which has recently been shown to act at multiple steps in satellite cell-mediated muscle regeneration^{106,112}) expression is undetectable in uninjured muscle but is robustly expressed by nascent and regenerating myofibers; consistent with its effects promoting symmetric expansion and maintenance of the satellite cell compartment¹⁰⁶, hypertrophy of myofibers¹¹² and enhanced regeneration by Frzd7-positive activated satellite cells during active regeneration. Wnt-5b, on the other hand, is present even in the absence of muscle injury¹¹¹ and its expression is limited to a population of interstitial cells (Figure 3B). Its presence in the muscle tissue prior to injury makes it a good candidate for an immediately-released (rather than induced and secreted) diffusible signal to indicate sites of local muscle trauma. SDF-1/CXCL12, a small chemokine associated with chemotaxis in many types of adult stem cells⁴⁸, is secreted by muscle fibroblasts following injury and exerts a positive chemotactic influence on immortalized C2C12 myoblasts²⁵; our data show that SDF-1 is also a component of CME and is chemokinetic and chemotactic to primary satellite cells. These data reinforce a model in which multiple protein factors that modulate satellite cell motility and

migration, released by different cell types at different points in the regeneration process, will act in concert to activate, attract, and position satellite cells to ensure efficient and effective muscle repair.

Figures

Figure 2-1. Satellite cells display increased chemotaxis and chemokinesis when treated with CME in vitro.

(A,B) Diagrams illustrating the difference between chemokinesis and chemotaxis. (A) Chemokinesis is a change in the pattern of normal cell motility in response to a compound but independent of its concentration gradient. (B) Chemotaxis is a change in motility along a vector in response to the concentration gradient of a compound. (C) Static images from representative 2-D time-lapse microscopy treatments depicting individual satellite cell chemokinesis as different colored lines. Scale bars: 200 μm . (D) Quantification of satellite cell motility when treated with MM (n=111) or CME (n=135). Error bars, +SE. Statistical significance was determined by a 1-tailed Welch's t-test; * $p < 0.05$. (E) Real-time cell migration assay showing a differential response in satellite cells ability to migrate across a barrier in the absence (MM) or presence (32 mg/mL CME) of a concentration gradient. Data was normalized at 40 minutes to allow for settling. Normal satellite cell motility (MM) was set as the baseline to measure increased/decreased chemotaxis (n=3).

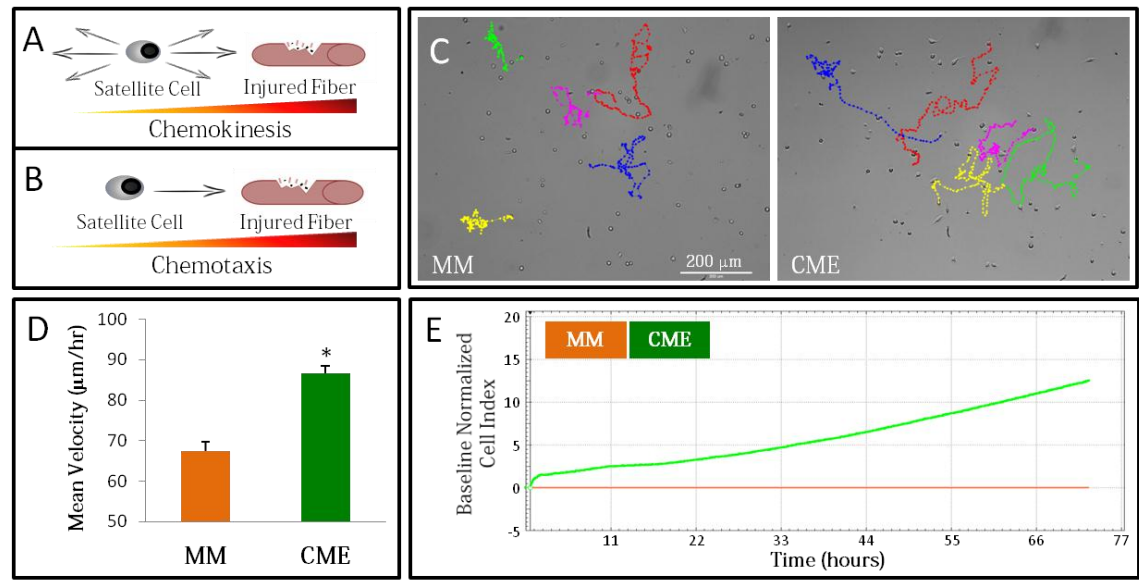


Figure 2-2. Wnt-5b is detectable in whole CME and in vivo.

(A) Four independent preparations of CME and rWnt-5b were immunoblotted with Wnt-5b antibody. Relative intensity and intensity profile of each primary band was determined compared to rWnt-5b to ensure sample and loading consistency. Concentrations of Wnt-5b present within each sample of CME were calculated based off the relative intensity and the concentration of rWnt-5b used. (B) Immunofluorescence analysis of murine tibialis anterior muscle cross-sections reveals interstitial localization of Wnt-5b. Wnt-5b (red), Laminin (green), Nuclei (blue). White boxes correspond to the adjacent sections which have been enlarged for detail.

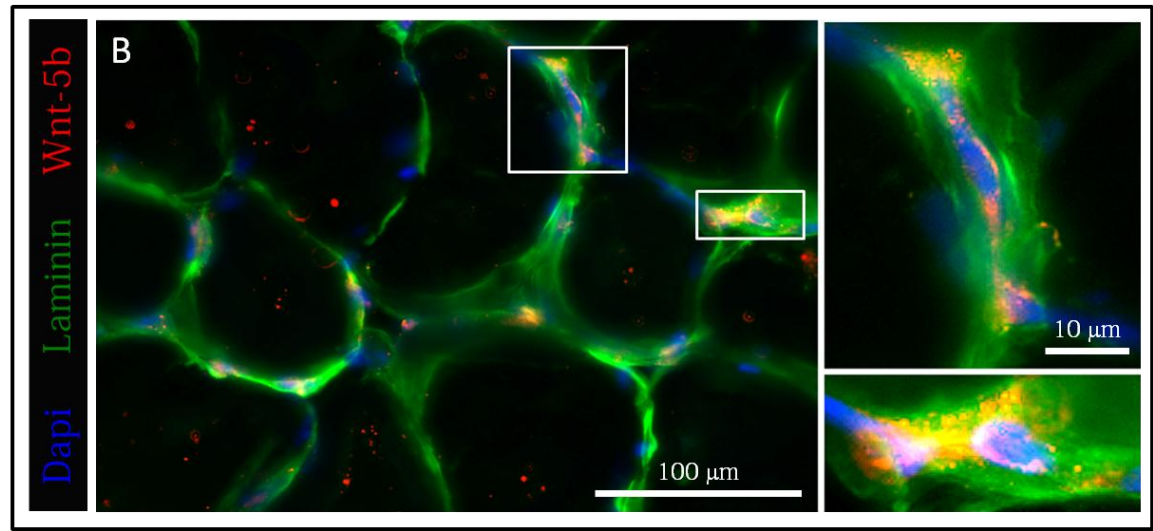
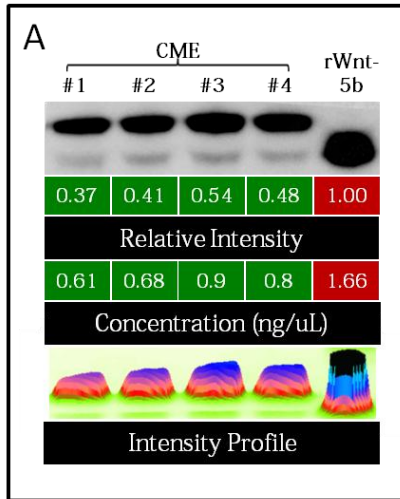
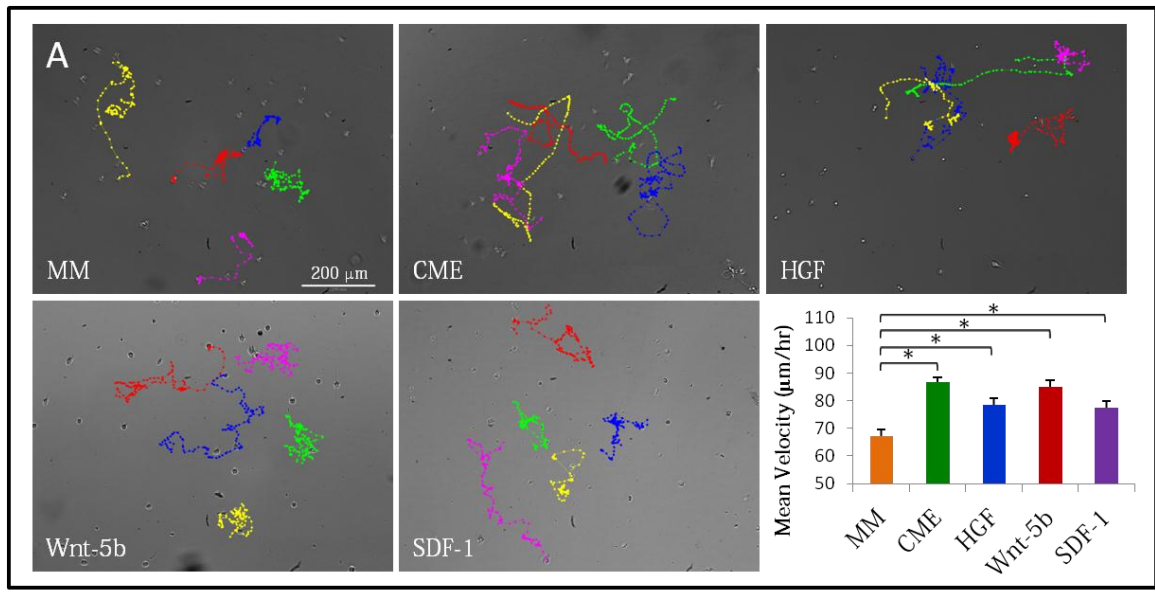


Figure 2-3. Satellite cells display dose dependent increased chemotaxis and chemokinesis when treated with Wnt-5b in vitro.

(A) Static images from representative 2-D time-lapse microscopy treatments depicting individual satellite cell chemokinesis as different colored lines. Scale bars: 200 μ m. Quantification of satellite cell motility when treated with MM (n=111), CME (n=135), HGF (n=131), Wnt-5b (n=107) or SDF-1 (n=94). Error bars, +SE. Statistical significance was determined by a 1-way ANOVA with Dunnett's post hoc test; *p < 0.05. (B) Real-time cell migration assay showing a dose dependent response in satellite cells ability to migrate across a barrier in the absence (MM) or presence (0.2 mg/mL Wnt-5b, 1 mg/mL Wnt-5b, 5 mg/mL Wnt-5b respectively) of a concentration gradient over a 72 hour period. Data was normalized at 40 minutes to allow for settling and normal satellite cell motility (MM) was set as the baseline to measure increased/decreased chemotaxis. (C) Assay demonstrating a differential response in satellite cells ability to migrate across a barrier in the absence (MM) or presence (CME, HGF, or Wnt-5b respectively) of a concentration gradient. Data was normalized as before (n=3). (D) Assay showing a lack of Wnt-5b gradient results in satellite cell migration similar to no gradient controls (MM) over a 72 hour period. Data was normalized at 40 minutes to allow for settling and normal satellite cell motility (MM) was set as the baseline to measure increased/decreased chemotaxis.



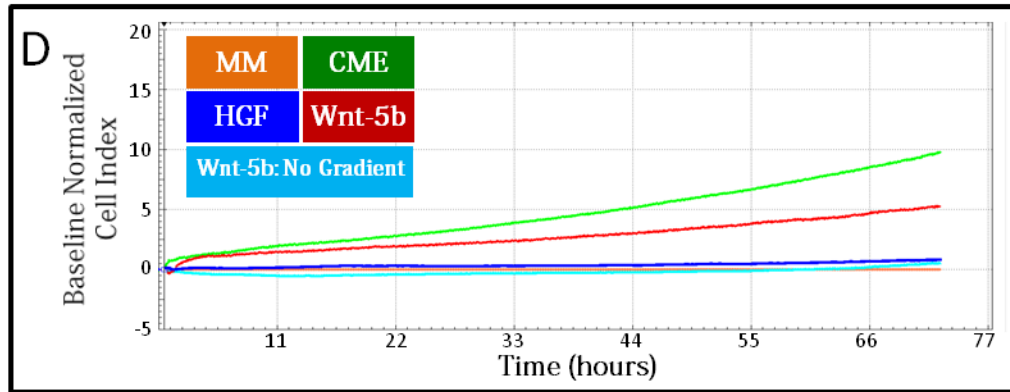
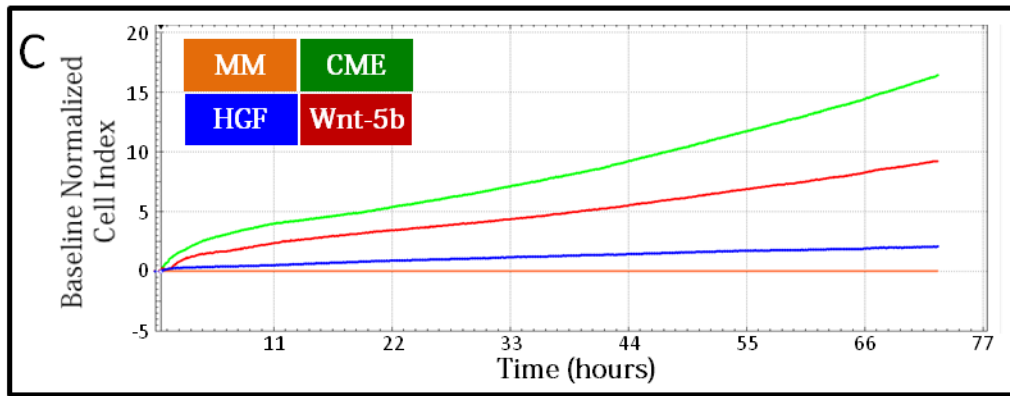
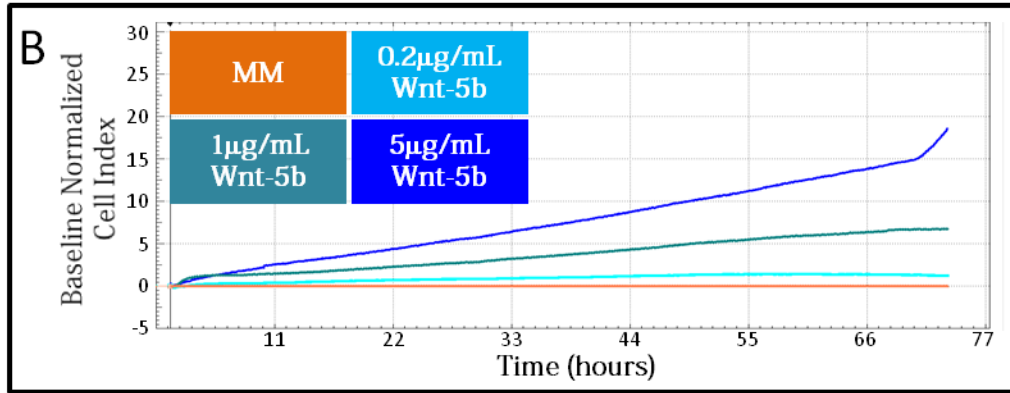


Figure 2-4. Treatment specific changes in satellite cell population chemokinesis.

(A-E) All tracked satellite cells for a given treatment binned into groups of similar mean velocities to illustrate treatment induced motility changes [MM (n=111), CME (n=135), HGF (n=131), Wnt-5b (n=107), SDF-1 (n=94)]. (F) Quantification of A-E depicting treatment-specific motility differences among satellite cell populations. Black bars depict treatment median velocities. Top and bottom of each box represents the third and first quartile, respectively. Whiskers represent minimum and maximum velocities observed. Statistical significance was determined by a 1-way ANOVA with Dunnett's post hoc test; *p < 0.05.

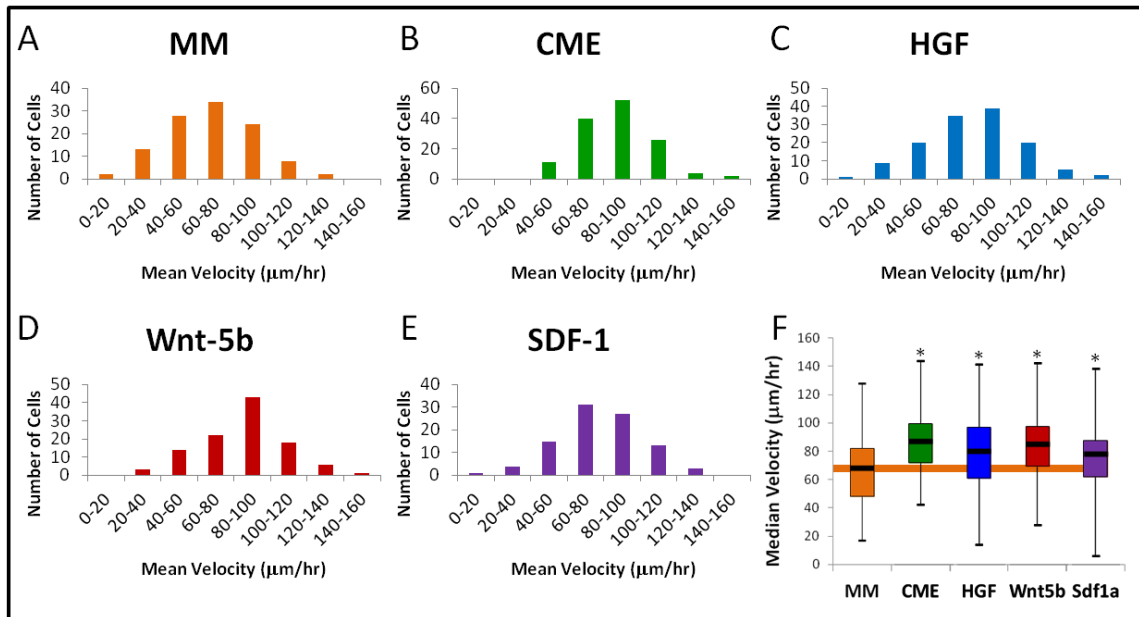


Figure 2-5. Satellite cells express all known frizzled receptors.

(A) RT-PCR analysis of Fzd1-10 using cDNA prepared from satellite cells plated on laminin (L), collagen (C) or gelatin (G). Uterus cDNA was used as a positive control (+).
 (B) Whole gels from A

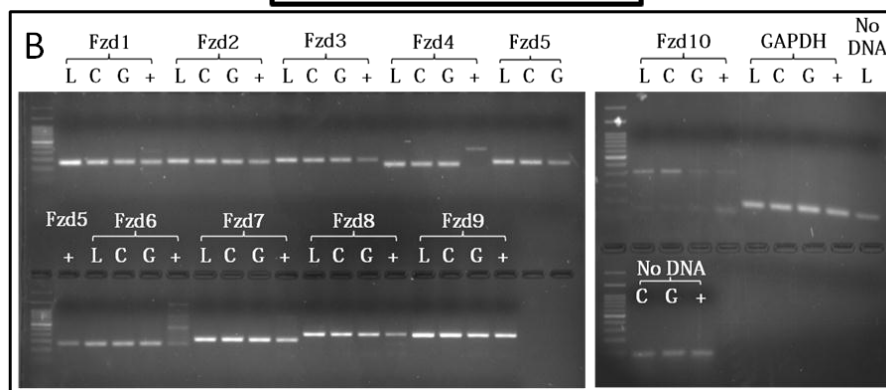
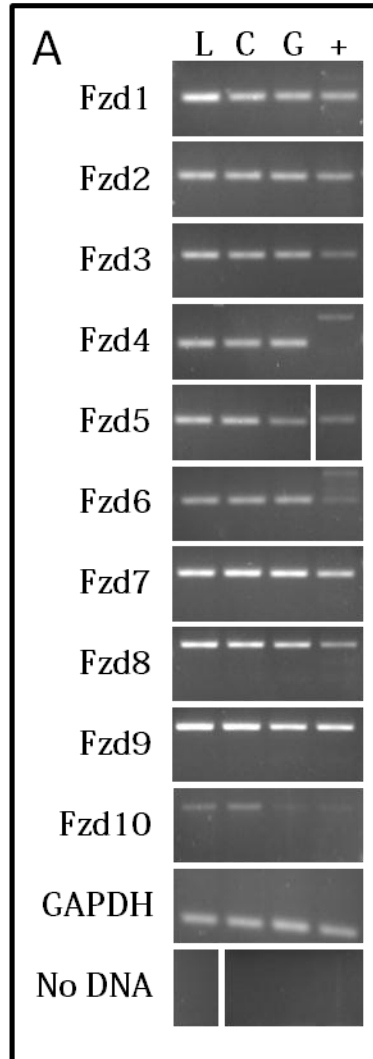


Table 2-1. Primer sequences for Frizzled 1-10 and GAPDH.

Forward and reverse primer sequences used for Fzd and GAPDH amplification along with expected amplicon length.

	Forward	Reverse	Expected Amplicon
Fzd1	5' - CACCTGGATAGGCATCTGGT-3'	5' - CAGAAAGCCAGCGATGTAGG-3'	163 nt
Fzd2	5' - CCGACGGCTCTATGTTCTTC-3'	5' - TAGCAGCCGGACAGAAAGAT-3'	172 nt
Fzd3	5' - AGCGTGCCTATAGCGAGTGT-3'	5' - TCTCTGGGACACCAAAAACC-3'	193 nt
Fzd4	5' - AACCTCGGCTACAACGTGAC-3'	5' - CCGAACAAAGGAAGAACTGC-3'	139 nt
Fzd5	5' - TCTTGTCTGCGTGTACCTG-3'	5' - GGCCATGCCAAAGAAATAGA-3'	167 nt
Fzd6	5' - TGAAGGAGAGAAGCAATGGATC-3'	5' - TGAACAGGCAGAGATGTGGAG-3'	160 nt
Fzd7	5' - GCTTCTAGGTGAGCGTGAC-3'	5' - AACCCGACAGGAAGATGATG-3'	216 nt
Fzd8	5' - TTACATGCCCAACCAGTTCA-3'	5' - CGGTTGTAGTCCATGCACAG-3'	306 nt
Fzd9	5' - CGGAGTCTTTTCCATCCTTTACAC-3'	5' - GCCATTTTTCGGTAGCACAGG-3'	294 nt
Fzd10	5' - AGATTCCCATGTGCAAGGAC-3'	5' - AGTTGGGTCTTCTTGTG-3'	321 nt
GAPDH	5' - CAAGGTCATCCATGACAACCTTG-3'	5' - GGGCCATCCACAGTCTTCTG-3'	90 nt

CHAPTER III - IDENTIFICATION OF ANNEXIN A-2 AND GALECTIN-1

Introduction

Skeletal muscle, like many other adult tissues, has a resident population of stem cells known as satellite cells¹⁵ which are absolutely required for normal muscle repair and regeneration after injury¹⁶⁻²⁰. Between periods of regeneration satellite cells are quiescent⁸⁶ and reside in a specialized location between the basal lamina and sarcolemma of the host myofiber²¹. Quiescent satellite cells express the paired-box transcription factor Pax7²² and the myogenic regulatory factor (MRF) Myf5²³. Upon injury, satellite cell activity is dynamically regulated by a plethora of local signals released from infiltrating immune cells^{90,91}, muscle resident fibroblasts¹⁸ and the damaged myofiber itself^{54,58,80,85,87-90}. Myotrauma triggers satellite cell activation (most likely through interdependence on nitric oxide and HGF^{30,31}), and expression of an additional MRF, MyoD. MyoD⁺ satellite cells are known as myoblasts which undergo proliferation, migration and differentiation into myocytes; which, through fusion events either with other myocytes or damaged myofibers produce new/regenerated myofibers³⁵. Skeletal muscle displays a remarkable capacity for regeneration as it is able to reconstitute its original architecture even after complete disruption¹⁴, with a single satellite cell capable of generating new myofibers⁹². While the field has made significant advances in understanding the mechanisms regulating proliferation and differentiation, the role of satellite cell motility remains largely uninvestigated. As satellite cells are comparatively

rare in adult skeletal muscle, accounting for ~4% of the total nuclei⁵³, mobilization of satellite cells in uninjured portions and targeted migration to sites of injury, would presumably speed up the regenerative process. It has recently been shown that satellite cells are motile *in vivo*^{56,57}, and that migrating cells within adult skeletal muscle alter their motility in response to varying concentrations of HGF⁵⁸. However, data remains sparse on which protein factors regulate satellite cell motility *in vivo*, necessitating a comprehensive survey of motility factors released from muscle in the context of an injury.

A role for satellite cell motility and recruitment in regeneration was suggested early based upon observations of satellite cell movement from distal uninjured areas towards a site of focal crush⁹³, as well as noting that satellite cells can migrate from the viable to necrotic half of a longitudinally split myofiber autograft¹¹³. To investigate factors present within injured muscle that may influence myogenesis, researchers began to collect saline extracts of muscles subjected to mechanical crush after removal from rats or mice⁷⁹. The resultant extracts, termed crushed muscle extract (CME) were tested on immortalized or primary myoblasts and shown to contain mitogenic and chemotactic factors^{54,79,80,85,89}. Attempts to isolate the source of the myogenic effects by heparin affinity chromatography, revealed that that mitogenic proteins were mostly present within the heparin binding fractions⁸⁵, while the chemotactic proteins were non-heparin binding⁵⁴, implicating different proteins are involved in each of these processes. In a previous study, we demonstrated that HGF and SDF-1 (known components of CME) promote satellite cell motility and used biochemical fractionation of CME by size and native charge to isolate the motogenic content. Subsequent analysis of the most motile

fraction by LC-MS/MS identified a novel motility factor, Wnt-5b, which elicited both chemokinesis and chemotaxis at higher rates than previously known motogens. In this study, we used the same techniques, but fractionated by heparin affinity, to identify two factors present within high motility fractions previously implicated in motility phenotypes. These putative satellite cell motogens, Annexin A-2 and Galectin-1, are currently being tested for their ability to induce *in vitro* chemokinesis and chemotaxis.

Methods

Muscle satellite cell isolation and culture

Satellite cells were isolated from female mice (B6D2F1; Jackson Labs) between the ages of 75 and 130 days according to our previously published methods²⁴. Briefly, hindlimb muscles were excised in PBS, minced, and digested in 400 U/mL collagenase type I (Worthington Biochemical) diluted in Ham's F-12 medium (Invitrogen). Tissue slurries were filtered and pelleted, then resuspended and plated in Ham's F-12 supplemented with 15% horse serum (Equitech), 0.5 nM rhFGF2 and 1% penicillin/streptomycin (Sigma) on gelatin-coated plates. Cells were maintained at 37° C and 5% CO₂ in a humidified incubator for 4 days before use.

Crushed Muscle Extract

The quadriceps, gastrocnemius and tibialis anterior were dissected out, weighed, and submerged in an equal volume of ice-cold PBS, then gently squeezed with blunt forceps. Samples were rocked for 2 hours at 4° C then the muscle was removed. The supernatant was centrifuged for 5 minutes at 1,000 RPM to sediment any remaining muscle tissue, sterile filtered using a 0.22 µm filter, then stored in aliquots at -80° C.

Protein concentration of each batch of CME was determined by Bradford assay (Bio-Rad).

2-D time-lapse microscopy and postimaging analysis

Satellite cells were collected 4 days after isolation and resuspended in Ham's F-12 without serum. 5,000 cells/well were added to 48 well plates (Corning) coated in 10 µg/mL laminin (Sigma) per well. Experimental wells were supplemented with fractions of and whole CME [32 µg/mL whole CME, 5 µg/mL Fractionated CME (A-G)]. Each condition was run in triplicate. Three 10x fields of each well were imaged every 5 minutes for 12 hours with Metamorph (Molecular Devices) on a Leica DMI 4000B microscope equipped with a stagetop incubator (Live Cell Instrument) and a Retiga 2000R camera (QImaging)

Individual .tiff images generated by Metamorph were arranged in sequential order and collapsed into stacks. Cell movement from frame to frame was measured using digital pixel trace measurements. Cells selected for tracking remained viable for the entire 12 hour duration and remained within the field of view. If a cell selected for tracking proliferated during the 12 hour track, one daughter cell was selected at random to continue the trace. Velocities for each cell were calculated and mean velocities were determined for each condition.

Real-time cell migration assay

Satellite cell chemotaxis was analyzed in real-time using the xCELLigence DP system (Roche) following the manufacturer's instructions for growth factor-mediated cell migration with the following modifications. The top and bottom of the membrane was

coated with 10 µg/mL laminin (Sigma). Top and bottom chambers were filled with Ham's F-12 (Invitrogen). Top chambers were seeded with 30,000 satellite cells in growth medium. Bottom chambers were loaded with fractions of and whole CME [32 µg/mL CME, 5 µg/mL Fractionated CME (A-G)] Individual assays were run in quadruplicate. Electrical impedance was measured every 5 minutes for 72 hours using the RTCA DP Analyzer and Software (Roche Diagnostics). Readouts for each condition were averaged among replicates then each experimental timepoint was normalized using the corresponding control (minimal media only) average as baseline.

Chromatography and Mass Spectrometry

2 mg of CME (pooled from three independent preparations) was separated by heparin affinity by fast protein liquid chromatography (FPLC) on an AKTApriime Plus (GE) over a HiTrap Heparin HP column (GE Life Sciences) with a linear gradient of NaCl (0-1M) and eluted in 2mL fractions. Size separation of 8 sequential fractions was confirmed by SDS-PAGE. All resultant fractions were desalted using Amicon Ultra-2 concentrating columns (Millipore) and tested for *in vitro* activity.

Mass spectroscopy analysis was done with the help of Matt Salie in Dr. Jay Thelen's lab . To prepare the protein extracts for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, 50uL basic buffer (8M Urea, 50mM Tris Base) was added to a 50uL aliquot of each protein extract. The protein extracts were then subjected to in-solution trypsin digestion. Briefly, protein extracts were reduced with DTT (10mM DTT, 37°C, 30 min), alkylated with iodoacetamide (50mM iodoacetamide, room temperature, dark, 30 min), and digested with sequencing grade trypsin (Promega,

Madison, WI) added at a concentration of 1/50 the total extract concentration (37°C, 16 h). Tryptic peptides were lyophilized and stored at -80°C until LC-MS/MS analysis. Protein samples were analyzed by LC-MS/MS as described previously¹¹⁴ with the following exceptions: 35uL aliquots of each protein extract fraction were added to a polypropylene 96-well plate, three separate 10uL (~1.5ug protein) injections were analyzed on an LTQ Orbitrap XL ETD instrument (Thermo Fisher, San Jose, CA), and peptides were eluted from the in-line C₈ Captrap (Michrom Bioresources, Auburn, CA) using a 50 min 2-95% acetonitrile gradient. Tryptic peptides were fragmented using collision induced dissociation (CID). Nanospray ionization source parameter settings were ion spray voltage (kV), 1.51; capillary temperature (°C), 200; capillary voltage (V), 22; and tube lens (V), 125. Precursor masses were scanned with the analyzer set to FTMS; mass range, normal; resolution, 15000; scan type, positive mode; data type, profile; and a scan range of 200-2000 *m/z*. The 4 most abundant ions from the precursor scans were selected for subsequent fragmentation using the ion trap-analyzer, normal mass range, normal scan rate, and centroid data type. Charge-state screening and monoisotopic precursor-selection modes were enabled. Unassigned charge states and masses with a charge state of +1 were not analyzed. The CID data-dependent scan settings were as described previously¹¹⁵, except with an isolation width of 1.0 *m/z*. Dynamic exclusion was enabled with a repeat count of 3, repeat duration of 15 s, exclusion list size of 50, and exclusion duration of 10 s. Acquired spectra were searched against the mouse proteome database (Mus musculus, 59,234 entries, downloaded 10/28/2011) concatenated to a reverse decoy database using the SEQUEST algorithm (Proteome Discoverer 1.3, Thermo Fisher, San Jose, CA). Results were filtered to <1%

FDR using the filters mass deviation, 10 ppm; Xcorr/Charge State; 2 peptide minimum; and protein grouping enabled. Only proteins that passed these filters were considered present in the sample. Additional search parameters were maximum missed cleavages, 2; precursor mass tolerance, 1000 ppm; fragment mass tolerance, 1 Da; dynamic modification of Met oxidation (+15.995 Da); static modification of Cys carbamidomethylation (+57.021 Da). Identified peptides were validated using Percolator (Proteome Discoverer 1.3, Thermo Fisher, San Jose, CA) set to the default settings. Full details of data processing and search parameters are available on request.

Statistical Analyses

Data are presented as mean + SE unless otherwise noted. Comparisons between control and treated samples were made using a 1-way ANOVA with Dunnett's post hoc test.

Results

Fractionation by heparin-affinity yields 3 fractions with chemokinetic effects

To isolate the motogenic components of CME, we separated whole CME by heparin affinity using heparin affinity chromatography (Figure 3-1C), and validated effective separation of protein content by SDS-PAGE (Figure 3-1B). A diagram representing the heparin fractionation is shown in Figure 3-1A. After concentration and desalting, all fractions were tested for chemokinetic effects using 2-D time-lapse microscopy. Satellite cells were seeded on laminin-coated plates and cultured in either minimal medium (MM, Ham's F-12), MM supplemented with whole CME or MM supplemented with fractions of CME (A-G) and imaged every 5 minutes for 12 hours

(Figure 3-2A). As expected satellite cells treated with whole CME displayed elevated chemokinesis compared to controls (MM), while cultures supplemented with three distinct fractions (A,B and F) had significantly increased chemokinesis (Figure 3-2B). Interestingly, two of the fractions (B and F) that elevated chemokinesis were heparin-binding, although it is important to note that proteins present in fraction B were eluted at very low concentrations of NaCl, and there was a large degree of overlap in protein content between the non-heparin binding fraction (A) and fraction B. Additionally, fraction F which eluted off the column at the highest concentration of NaCl used did not increase chemokinesis nearly as much as fractions A and B. Surprisingly, we also noted an increase in the upper limits of motility for satellite cells treated with fractions A and B (Figure 3-2C). Analysis of CME fractions using a real-time migration assay proved more difficult than in previous attempts, as consistent results between experiments could not be obtained, although fraction B did consistently stimulate satellite cell chemotaxis at higher levels than whole CME (Figure 3-3). These data suggest effective separation of the motogenic content in CME based on heparin-affinity.

Secreted proteins Annexin A-2 and Galectin-1 are present in high motility fractions

The fractions that promoted chemokinesis as well as fractions that did not significantly increase chemokinesis above controls were analyzed by LC-MS/MS. The resultant lists were cross-referenced against each other to eliminate any proteins that were present in both the significant and non-significant fractions. Two proteins in particular stood out, annexin a-2 (resident to fraction F) and galectin-1 (resident to fraction B). Annexin A-2 is known to induce pro-motility effects including the recruitment of immune cells to sites of infection¹¹⁶, and is dysregulated in many forms of metastatic

cancer¹¹⁷. Similarly, galectin-1 promotes neutrophil migration¹¹⁸, is highly expressed in motile malignant astrocytoma cell lines¹¹⁹, and is known to be secreted by myoblasts as they differentiate into myotubes¹²⁰⁻¹²³. Ongoing work in our laboratory is aimed at determining if either of these two proteins contributes to the motogenic effect of the CME fractions they were found in.

Discussion

Although *in vivo* motility of satellite cells has recently been confirmed^{56,57}, the factors regulating this behavior and the extent to which general vs. directed motility contribute to the migration observed remain poorly understood. CME, being composed entirely of soluble factors released from the muscle in response to damage, makes it an appealing system for investigating modulators of satellite cell activity^{54,79,80,85,89}. Our previous attempt at identifying novel satellite cell motogens present within CME resulted in the successful validation of a non-canonical wnt, Wnt-5b, as both chemokinetic and chemotactic to mouse primary satellite cells *in vitro* and suggests a novel role for wnt signaling during adult skeletal muscle regeneration. This study constitutes our second attempt at identifying soluble pro-motility factors present within CME.

Annexins are a family of calcium and membrane binding proteins which are divided into 5 distinct groups; the A group, which is expressed in vertebrates, contains 12 members¹¹⁷. Annexin A-2 contains an actin-binding domain, demonstrating its ability to interact with the cytoskeleton, and annexin a-2 has recently been observed to localize in the pseudopodia of invasive glioma cells¹²⁴ suggesting it may play a role in modulating cell migration. Although annexin a-2 is best characterized as an intracellular protein¹²⁵,

soluble annexin a-2 is found in the sera of healthy people at low levels¹²⁶ and a number of reports document annexin secretion by non-classical mechanisms¹²⁷⁻¹³⁰. Importantly, annexin a-2 has been implicated in promoting motility in a variety of cell types, although admittedly, these studies used siRNA to knockdown expression of the intracellular protein^{116,131-133}. Nevertheless, the detection of soluble annexin a-2 in high motility fractions of CME warrants further investigation into a possible role for promoting satellite cell motility.

Galectins are a family of 15 soluble lectins which bind to β -galactoside sugars¹³⁴. Several members of the galectin family, including galectin-1 are known chemoattractants for a variety of immune cells both *in vitro* and *in vivo*^{118,119,135-138} which suggests they may also serve as pro-motility factors to other cell types. Interestingly, within the context of muscle, galectin-1 has been observed to be exported in a regulated process which accompanies myoblast differentiation¹²¹. Additional studies observed galectin-1 aggregation at the leading edges of myoblasts migrating on laminin¹²³, providing a clue to their potential involvement in motility. As the exact role galectin-1 plays in myogenesis is currently unknown, further investigation into a possible role mediating satellite cell motility appears warranted.

Figure 3-1. Effective fractionation of motogenic components of CME by heparin-affinity chromatography

(A) Diagram illustrating fractionation of CME by heparin-affinity chromatography over an increasing gradient of NaCl. (B) Coomassie stain of protein content of resultant fractions separated on SDS-PAGE. (C) FPLC read-out demonstrating fractionation. Blue line represents protein eluted from the column. Yellow line represents concentration of NaCl gradient. Hash marks on x-axis denote individual fraction cut off points. Note the majority of the protein eluted was in the non-heparin binding fraction, while a minority of protein eluted was strongly heparin binding.

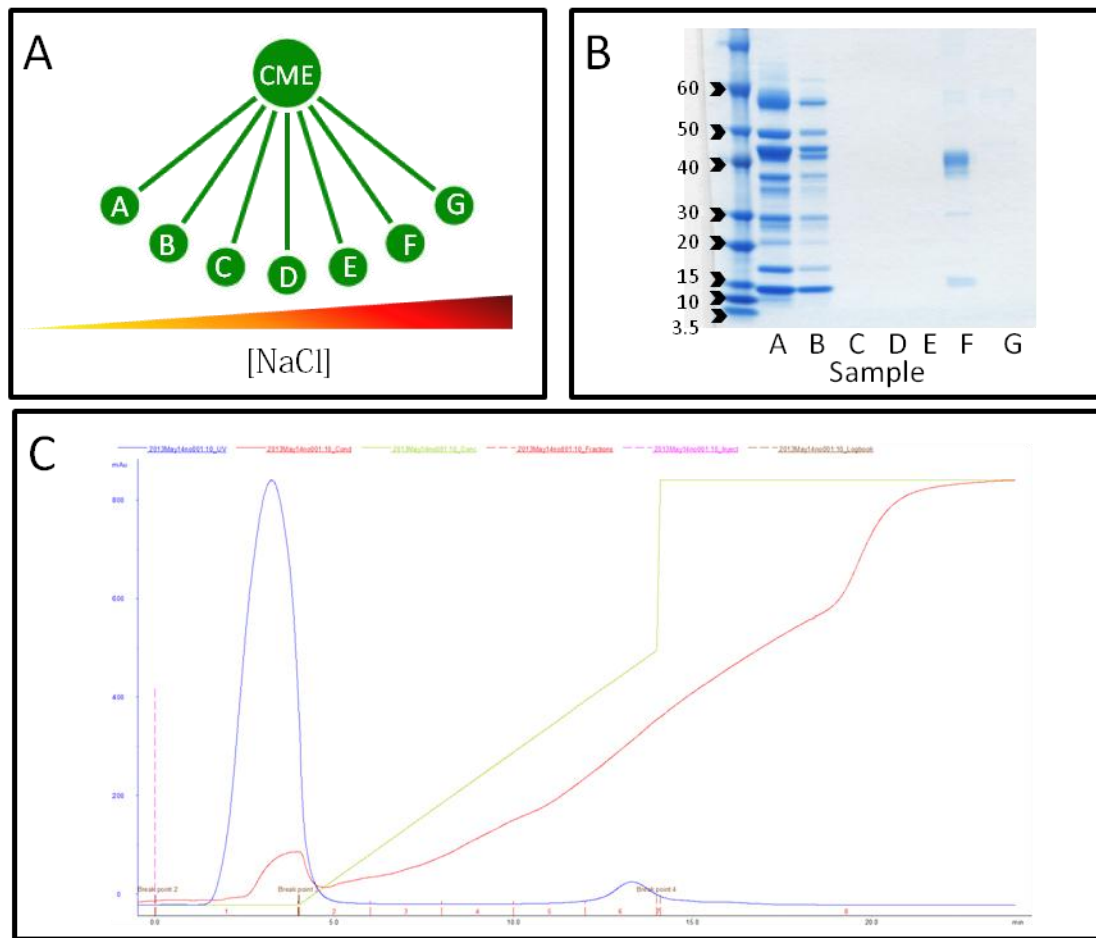
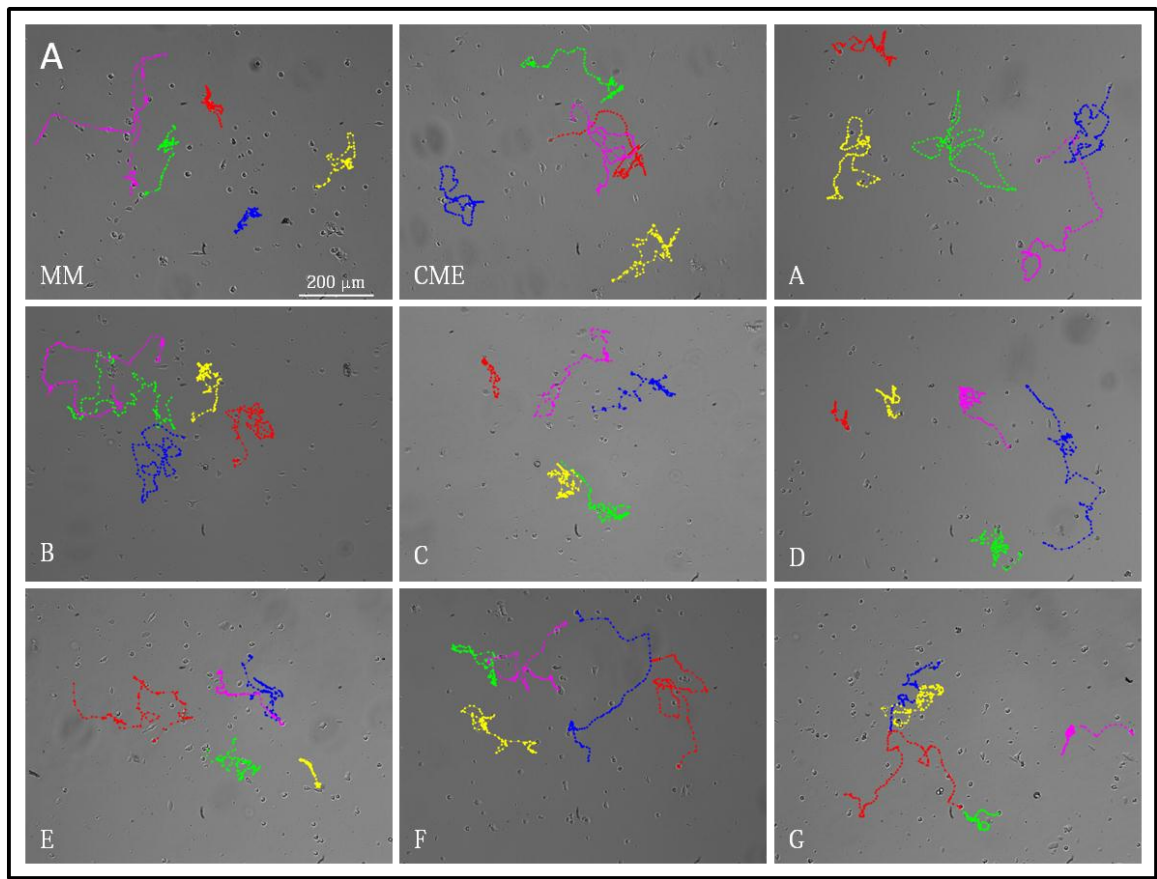


Figure 3-2. Satellite cell chemokinesis when treated with different fractions of CME

(A) Static images from representative 2-D time-lapse microscopy treatments depicting individual satellite cell chemokinesis as different colored lines. Scale bars: 200 μ m. (B) Quantification of satellite cell motility when treated with MM (n=115), CME (n=140), A (n=123), B (n=125), C (n=114), D (n=116), E (n=119), F (n=125) or G (n=74). Readout from Figure 3-1C is shown in background. Error bars, +SE. Statistical significance was determined by a 1-way ANOVA with Dunnett's post hoc test; *p < 0.05. (C) Quantification of fraction A-G treatment-specific motility differences among satellite cell populations. Black bars depict treatment median velocities. Top and bottom of each box represents the third and first quartile, respectively. Whiskers represent minimum and maximum velocities observed. Statistical significance was determined by a 1-way ANOVA with Dunnett's post hoc test; *p < 0.05.



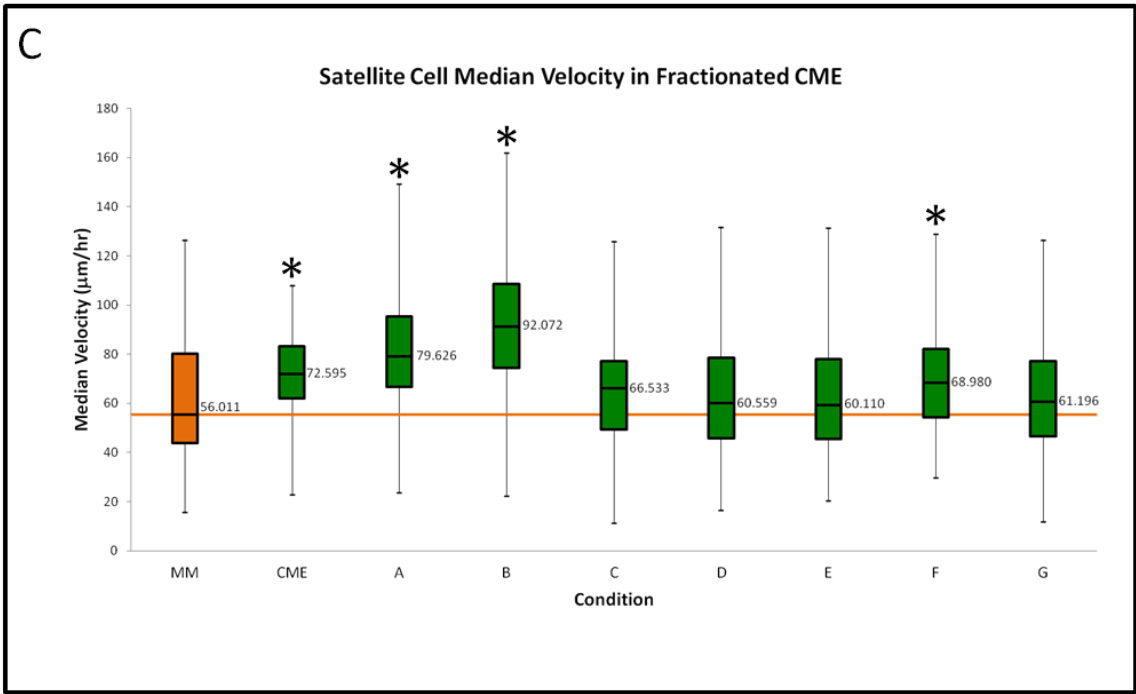
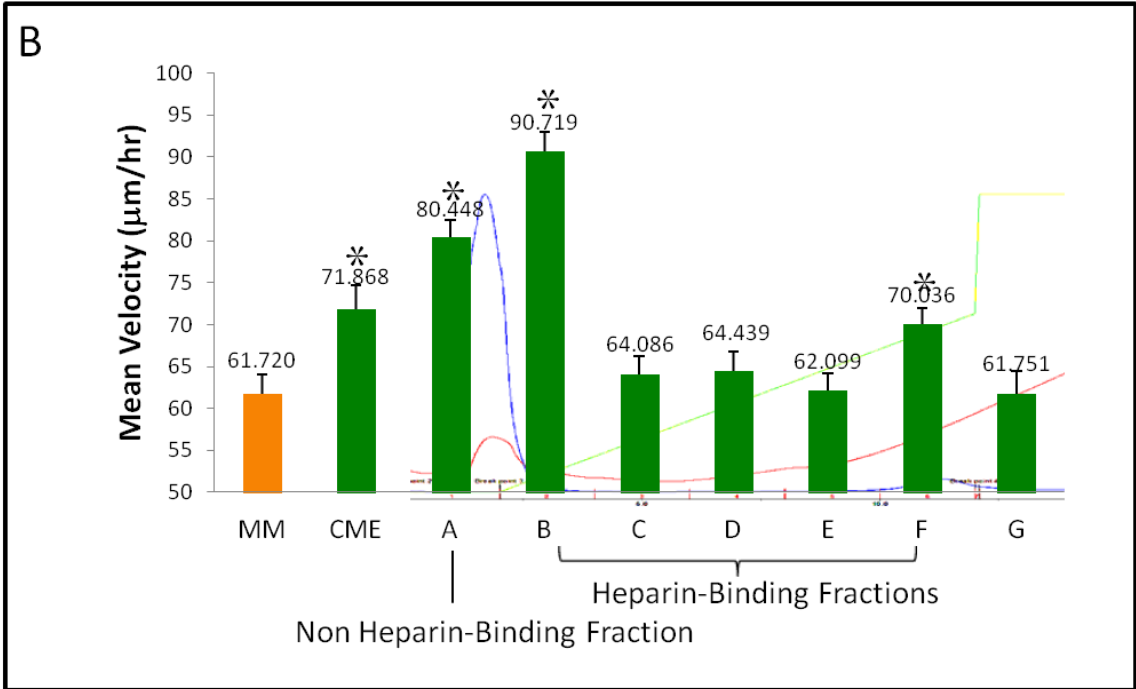
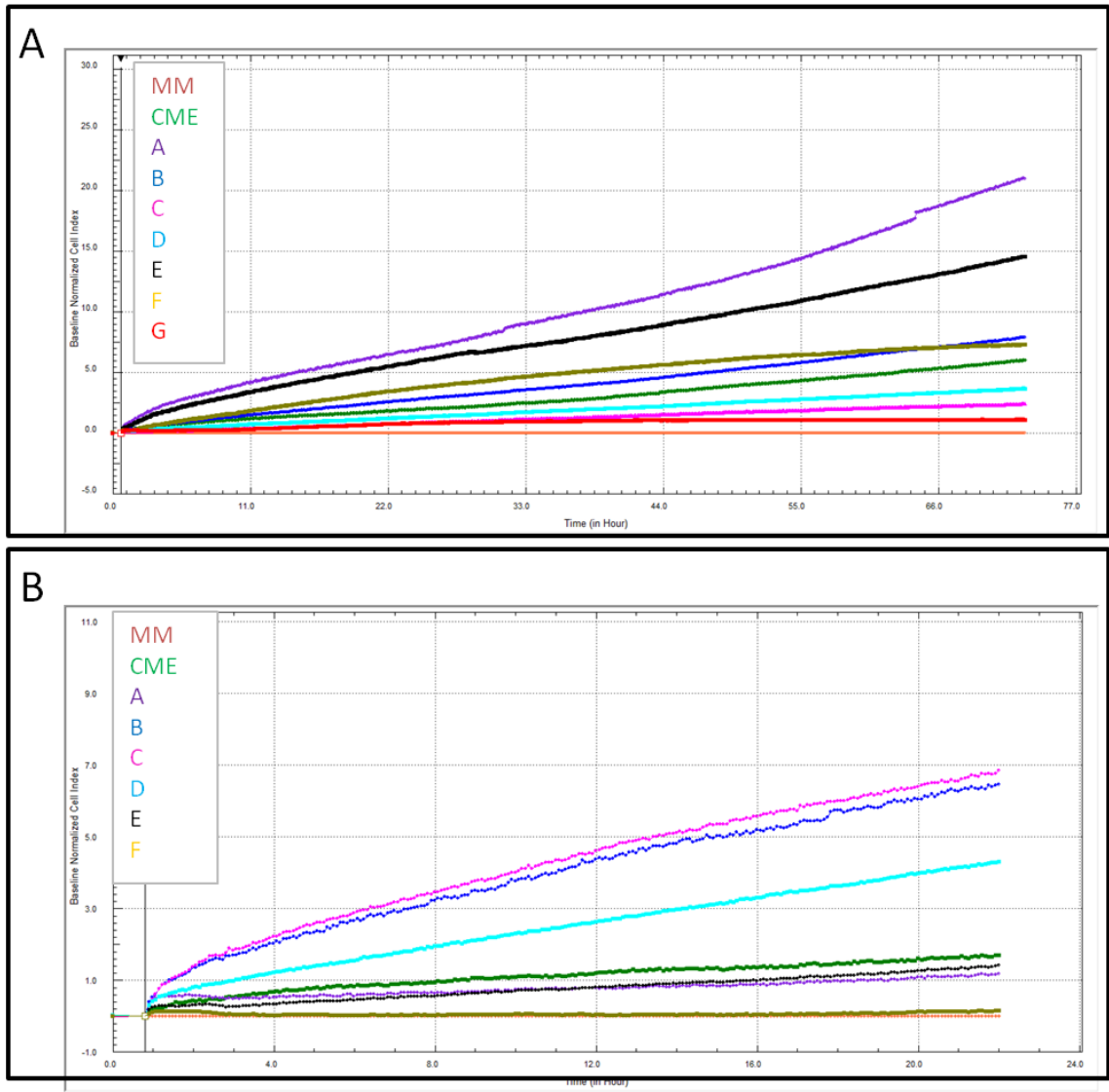


Figure 3-3. Satellite cell chemotaxis when treated with different fractions of CME

Assays demonstrating a differential response in satellite cells ability to migrate across a barrier in the absence (MM) or presence (CME, fractions A-G respectively) of a concentration gradient. Results were inconsistent due to high well to well variability, however fraction B consistently increased satellite cell chemotaxis. Data was normalized at 40 minutes to allow for settling and normal satellite cell motility (MM) was set as the baseline to measure increased/decreased chemotaxis.



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