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Christine Griffin	Date

Regulation of skeletal muscle regeneration by chemoreceptors and odorant receptors

By

Christine A. Griffin

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Biochemistry, Cell and Developmental Biology

Grace K. Pavlath _____

Advisor

S. Russ Price _____

Committee Member

Barry D. Shur _____

Committee Member

Charles Saxe _____

Committee Member

Winfield S. Saxe _____

Committee Member

Accepted:

_____

Lisa A. Tedesco, Ph. D.

Dean of the James T. Laney School of Graduate Studies

_____

Date

Regulation of skeletal muscle regeneration by chemoreceptors and odorant receptors

By

Christine A. Griffin
B.S., University of Washington, 2004

Advisor: Grace K. Pavlath, Ph.D.

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Abstract

Regulation of skeletal muscle regeneration by chemoreceptors and odorant receptors

By Christine A. Griffin

Skeletal muscle is a complex and highly organized tissue that is necessary for locomotion, metabolism and breathing. Myogenesis, the formation of skeletal muscle, occurs during muscle development, remodeling and regeneration in the adult. Many processes are required for proper myofiber formation, including migration and adhesion. In this dissertation, we demonstrated that many chemokines and chemokine receptors are expressed by muscle cells *in vitro*. The large number of migratory factors expressed by muscle cells suggests a complex temporal and spatial control of muscle cell migration during myogenesis. These data also determine a specific role for CXCR4 and SDF1α during myogenesis. The migration of both proliferating and terminally differentiated muscle cells is regulated by CXCR4 and SDF1α. Also, in this dissertation, we determined that at least 13 olfactory receptors (ORs) are expressed during myogenesis. Furthermore, one specific OR, MOR23 regulates both migration and adhesion, affecting proper myogenesis. MOR23 also affects a phenomenon known as myofiber branching, where a myofiber is contiguous with several smaller myofibers. Myofiber branching is increased with multiple injuries, aging and muscular dystrophies and decreases the contractile force of the myofiber.

Over-expression of MOR23 decreases the incidence of myofiber branching in regenerating muscle, suggesting potential options for treating various muscle diseases. Therefore, manipulation of either chemokines or ORs may allow for an increased efficiency of cell transplantation therapies for various muscle disorders.

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List of Abbreviations

ACIII: adenylyl cyclase III

ATP: adenosine triphosphate

BrdU: 5-bromo-2-deoxyuridine

cAMP: cyclic adenosine monophosphate

CM: conditioned media

CREB: cAMP-response element binding protein

DM: differentiation media

ECM: extracellular matrix

EMSA: electromobility shift assay

eMyHC: embryonic myosin heavy chain

FGF: fibroblast growth factor

GEF: guanine exchange factor

GM: growth media

Golf: G-alpha olfactory protein

GTPase: guanine triphosphate phosphatase

HGF: hepatocyte growth factor

HMGB1: high mobility group box 1

hOR: human olfactory receptor

IGF: insulin-like growth factor

IL-4: interleukin-4

MEF: myocyte enhancer binding factor

MMP: matrix-metalloproteinase

MOR23: mouse olfactory receptor 23

MR: mannose receptor

MRF: muscle regulatory factor

NFAT: nuclear factor of activated T cells

NO: nitric oxide

OR: olfactory receptor

Olfr: olfactory receptor

PDGF: platelet derived growth factor

PGI_2: prostaglandin I_2 or prostacyclin

PI: propidium iodide

PKA: cAMP-dependent protein kinase

RV: retrovirus

SDF1α: stromal derived factor 1 alpha

TA: tibialis anterior

uPA: urokinase plasminogen activator

XSA: cross-sectional area

Chapter 1: Introduction

Adult regenerative myogenesis is dependent on muscle progenitor cells called satellite cells. Satellite cells are normally mitotically quiescent, but proliferate in response to injury, and their progeny myoblasts differentiate and fuse either with each other or with existing myofibers to restore normal tissue architecture. Migration is critical to achieve cell-cell adhesion, which is necessary for differentiation (Kang et al., 2004) as well as formation and growth of myotubes *in vitro* (Bae et al., 2008; Jansen and Pavlath, 2006; Mylona et al., 2006; O'Connor et al., 2007). Identifying molecules which regulate muscle cell migration may reveal potential molecular targets for improving muscle regeneration and the efficiency of cell transplantation therapies (El Fahime et al., 2002; El Fahime et al., 2000; Galvez et al., 2006; Hill et al., 2006; Palumbo et al., 2004).

The research in this dissertation was largely based on identifying regulators of migration of muscle cells during myogenesis. As outlined in Chapter 1, many cytokines are found within muscle, both embryonic and adult. The central goal of the research outlined in this dissertation was to **identify migratory factors which are necessary for later stages of myogenesis.** We formulated two specific aims to address this goal, which are addressed in Chapters 4-5.

Previous work in the field suggested that migration is an important process for muscle cells. However, muscle cells exist both as proliferating myoblasts and terminally differentiated myocytes, both of which are present during myogenesis and capable of migration. Thus, our first aim was **to test the hypothesis that migration of myoblasts and myocytes are regulated by distinct chemokines**

and receptors (Chapter 4). To address this hypothesis, we first examined migration of muscle cells during the process of *in vitro* myogenesis. We also examined expression of chemokines, chemokine receptors and signaling factors. Our results support a model in which proliferating and differentiated muscle cells have distinct migratory behaviors.

Odorant receptors (ORs) are known to regulate migration of cells. We hypothesized that ORs modulate processes during myogenesis. Preliminary analysis in the Pavlath lab indicated that at least one OR, MOR23, was expressed during *in vitro* myogenesis. Therefore, our second aim was **to determine the expression of multiple ORs and the role of a specific OR, MOR23 during myogenesis** (Chapter 5). To address this aim, we examined expression of several ORs (19 total) consistently found in published skeletal muscle microarrays during myogenesis; mRNA for 13 of these ORs was expressed, many with distinct patterns. Furthermore, MOR23-dependent migration and adhesion of muscle cells was necessary for proper skeletal muscle myogenesis, both *in vivo* and *in vitro*, through affects on myofiber cross-sectional area, number and branching. Although branched myofibers have been noted in the literature for 100 years (Schmalbruch, 1976; Volkmann, 1893), no molecule is known to regulate their formation. Our data indicate an important novel finding that MOR23 expression levels affect myofiber branching.

The research presented in this dissertation identifies distinct regulators of muscle cell migration during myogenesis. These studies conclude that many chemokine receptor/ligand pairs are differentially expressed during myogenesis,

and necessary for proper muscle cell fusion. In addition, our work provides conclusive evidence that MOR23 functions outside of the olfactory epithelium during adult regenerative myogenesis *in vivo*. Cell migration within muscle is a major issue in transplantation of cells for treatment of muscular dystrophy (Skuk and Tremblay, 2003; Smythe et al., 2001). Study of the receptor-ligand pairs that regulate migration and/or adhesion of muscle cells may allow for more efficient therapeutic strategies. In addition, further studies on ORs may reveal additional unexpected functions in various tissues and tissue repair.

Chapter 2: Background and Significance

Skeletal muscle structure and function

Skeletal muscle is a complex and highly organized tissue that comprises approximately 40-45% of human body mass, which is necessary for locomotion, metabolism and breathing (Lieber, 1992). The primary cellular component of skeletal muscle is the long, cylindrical single muscle cell, or myofiber, which contain hundreds of myonuclei. Myofibers are bundled together within the muscle tissue and contract in concert to generate force. Myofibrils within the myofiber contain myosin, actin, and titin which are arranged in repetitive, force-generating units, called sarcomeres. The plasma membrane surrounding each myofiber is referred to as a sarcolemma and is encased by an outer basal lamina. In addition to myofibers, muscle tissues also contain a network of blood vessels that deliver essential systemic factors to the tissue. Motor neurons are also located within the tissue, interacting with individual myofibers at neuromuscular junctions.

Myogenesis, the formation of skeletal muscle, occurs during muscle development, remodeling and regeneration in the adult. Myofibers are generated during development via the fusion of mononucleated muscle precursor cells with one another. Although muscle development takes place at only discrete stages, muscle remodeling occurs every day. Furthermore, muscle is lost due to common occurances such as aging, acute injuries and disease. As myonuclei are post-mitotic, postnatal muscle growth and regeneration requires additional myonuclei from outside the myofiber (Allen et al., 1999). Muscle precursor cells, or satellite cells, located outside the sarcolemma and beneath the basal lamina (Mauro, 1961), serve as the primary source for new myonuclei in the adult (Moss

and Leblond, 1971; Zammit et al., 2006). Upon certain stimuli, these cells are stimulated to undergo myogenesis and fuse with one another or existing myofibers to promote muscle growth or repair.

Myogenesis in the embryo and adult

The majority of skeletal muscle tissue in the adult originates during embryogenesis from the somites, which are paired structures containing both epidermal and mesodermal cells located along the neural tube (Buckingham, 2006). Highly proliferative myogenic precursor cells are found within the dermomyotome of each somite (Kalcheim and Ben-Yair, 2005). These cells will undergo proliferation and differentiation, which are highly regulated by both temporal and spatial cues. Muscles of the back and body wall develop first (Buckingham et al., 2003), while other myogenic cells remain undifferentiated and continue to proliferate. At later stages, these undifferentiated cells migrate out of the somites and into the limb buds in response to multiple cues, such as hepatocyte growth factor (HGF) and N-cadherin, where they will differentiate into the limb musculature (Brand-Saberi et al., 1996a; Brand-Saberi et al., 1996b; Heymann et al., 1996; Houzelstein et al., 1999; Sze et al., 1995). The first limb muscles form at approximately E11-E14 in the mouse and are referred to as primary myofibers. A second wave of myogenesis takes place around E14-E16, leading to the formation of secondary myofibers. Basal lamina then forms around the secondary myofibers (Cossu and Biressi, 2005), and becomes populated with satellite cells, which are also somatic in origin (Relaix et al., 2005).

Satellite cells are necessary for remodeling and repair of muscle in the adult. Myonuclei turnover in adult rats occurs at a rate of approximately 1-2% per week (Schmalbruch and Lewis, 2000), suggesting that even uninjured muscle is not completely static. Radiolabeling experiments have determined that satellite cells are normally quiescent (Schultz et al., 1978), but become mitotically active following injury and serve as the primary source for new myonuclei (Moss and Leblond, 1971; Snow, 1978). Interestingly, only a few satellite cells are necessary as donor satellite cells associated with a single donor myofiber are sufficient to replenish the entire satellite cell pool and provide the host muscle with robust regenerative capacity (Collins et al., 2005; Collins and Partridge, 2005; Sacco et al., 2008). Indeed, progeny from donor muscle cells post-transplantation can be re-isolated from the host muscle tissue, and transplanted into a new host muscle where they contribute to myogenic cells located underneath the basal lamina (Sacco et al., 2008). As satellite cells are defined by their position underneath the basal lamina, these data suggest that a muscle satellite cell can self-renew. As satellite cells can be induced to express markers of adipogenic, osteogenic, and smooth muscle cells *in vitro*, they may also be considered multipotent stem cells (Asakura et al., 2001; Shefer et al., 2004). Satellite cells are capable of asymmetric cell division, another property of stem cells (Kuang et al., 2007). For example, the Notch signaling repressor, Numb, is preferentially divided to a proportion of activated satellite cells during cell division (Conboy and Rando, 2002). Also, some satellite cells preferentially divide parental DNA into a single daughter cell (Conboy et al., 2007; Cossu and

Tajbakhsh, 2007; Shinin et al., 2006; Shinin et al., 2009). Based on these experiments, satellite cells can be considered muscle stem cells.

Recent studies of satellite cells have focused on the heterogeneity of the satellite cell population. As mentioned above, satellite cells can undergo asymmetric cell division, which creates two different cells from a single progenitor. Many labs have utilized FACS to separate different populations of satellite cells based on the expression of cell surface markers. A population of satellite cells that are $CD45^{-}Sca1^{-}Mac1^{-}CXCR4^{+}\beta1intregrin^{+}$ are capable of differentiation into myotubes autonomously, without exogenous signals (Sherwood et al., 2004). Upon transplantation, these satellite cells are also significantly more efficient at engrafting into dystrophic host muscle tissue and the muscle satellilte cell niche compared to satellite cells lacking these markers. These data suggest that the $CXCR4^{+}\beta1intregrin^{+}$ cell population can proliferate extensively, demonstrating the heterogeneity of the satellite cell population. Use of other cell surface markers, such as Syndecan-4, a heparan sulfate proteoglycan, and ABCG2, an ATP-binding cassette transporter, yields similar findings, where specific populations retain the ability to spontaneously differentiate and engraft into muscle (Tanaka et al., 2009). Interestingly, a single $\alpha7integrin^{+}CD34^{+}$ satellite cell appears able to engraft a large proportion of the host muscle tissue (Sacco et al., 2008). Overall, these data suggest the presence of multiple populations of muscle satellite cells, all with different properties.

Different stages of myogenesis are associated with the expression of muscle regulatory factors (MRFs) from the basic helix-loop-helix class, including MyoD,

Myf5, myogenin, and MRF4, as well as another family of transcription factors, which includes the myocyte enhancer binding factor-2 (MEF-2) (Charge and Rudnicki, 2004; Le Grand and Rudnicki, 2007; Rudnicki et al., 2008). These transcription factors activate the transcription of muscle-specific genes in response to stimuli, such as hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) and nitric oxide (NO) (Anderson et al., 1991; Tatsumi et al., 2002; Wozniak and Anderson, 2007). Proliferating muscle cells which express MyoD and Myf5 (Fuchtbauer and Westphal, 1992; Jin et al., 2000; Megeney et al., 1996; Nicolas et al., 1996; Rudnicki et al., 1993) as well as proliferation markers, such as Ki67 (Gerdes et al., 1991), will be referred to as myoblasts in this dissertation. Myogenin expression marks the first, committed step of myogenic differentiation in which myoblasts exit the cell cycle; mononucleated muscle cells which have exited the cell cycle will be referred to as myocytes. Throughout this dissertation, the term “muscle cells” will refer to mononucleated muscle cells, including quiescent satellite cells, also called muscle precursor cells, myoblasts, and myocytes. Interestingly, myogenin^{+} myocytes can still be stimulated to re-enter the cell cycle (Andres and Walsh, 1996). Nuclear accumulation of the cell cycle inhibitor p21 marks terminal differentiation, which is followed by the expression of contractile proteins such as embryonic myosin heavy chain (eMyHC) and fusion of myocytes with each other to form multinucleated cells, called myotubes *in vitro* and myofibers *in vivo* (Andres and Walsh, 1996). *In vivo*, expression of eMyHC by the myofiber is followed by the expression of more mature MyHC isoforms (Pette and Staron, 1988; Pette and

Staron, 2000; Schiaffino and Reggiani, 1994). Therefore, myogenesis in the adult is complex and occurs in several stages.

Despite the linear progression of proliferation, differentiation, and fusion during adult regenerative myogenesis, signs of myogenic differentiation can occur before satellite cell proliferation. Some muscle cells begin expressing proteins associated with differentiation, including desmin, MyoD and myogenin, within 4 to 8 hrs after injury (Rantanen et al., 1995). In contrast, muscle cell proliferation, as determined by incorporation of bromodeoxyuridine (BrdU), is not apparent until 24 hrs after injury (Rantanen et al., 1995). Furthermore, proteins that inhibit proliferation and enhance terminal differentiation are necessary for myogenesis (Hawke et al., 2003). Several other studies indicate proliferating myoblasts and terminally differentiated myocytes may co-exist during regenerative myogenesis (Andres and Walsh, 1996; Garry et al., 2000; Goetsch et al., 2003; Grounds et al., 1992; Ishido et al., 2004a; Ishido et al., 2004b; Megeney et al., 1996; Nicolas et al., 1996; Rantanen et al., 1995; Turk et al., 2005). The co-existence of these cell types has yet to be determined decisively, which complicates results of studies *in vivo*.

Muscle cells in the adult depend upon the actions of other cell types to promote myogenesis. Immediately following injury to skeletal muscle, neutrophils invade the site of damage, aid in the phagocytosis of cellular debris, and secrete proinflammatory cytokines (Fielding et al., 1993). Macrophages also enter the site of injury within a few days and remain in the area for up to a week (Tidball, 1995). Together with neutrophils, macrophages clear cellular debris produced by

the injury (Teixeira et al., 2003). Macrophages also secrete a variety of cytokines and growth factors that regulate myoblast proliferation, migration, and survival during regeneration (Cantini and Carraro, 1995; Chazaud et al., 2003; Robertson et al., 1993; Sandri et al., 2001) and many of these factors are discussed later in this dissertation. Pericytes, which are vascular endothelium associated cells, are another group of cells capable of participating in muscle regeneration. Pericytes are capable of differentiating into multiple cell types, including skeletal muscle and are a potential group of muscle progenitor cells (Andersen et al., 2008; Brachvogel et al., 2005; Crisan et al., 2008; Dellavalle et al., 2007). Lastly, another group of cells, referred to as side-population cells expressing high levels of MMP-2, are capable of promoting muscle regeneration through stimulating proliferation and migration of myoblasts (Motohashi et al., 2008). These studies suggest that non-muscle cells are necessary for muscle regeneration and can affect muscle proliferation, migration, and survival.

The fusion of myocytes to one another and into nascent myofibers to form mature myofibers is a critical step in the formation of skeletal muscle. Myocyte fusion occurs throughout adulthood, as skeletal muscle growth and regeneration require the accumulation of additional nuclei within myofibers. Two molecularly distinct stages of cell fusion occur in mammalian muscle cells (Horsley et al., 2001). Myocyte-myocyte fusion occurs to generate the initial multinucleated cell in the primary stage, commonly called nascent myotubes, which are small with few nuclei. During the secondary stage of fusion, additional myocytes fuse with the nascent myotube leading to mature myotubes with increased myonuclear

number and cell size. The phenomenon of dual stages of myogenesis has also been observed in *Drosophila melanogaster* (Menon and Chia, 2001; Rau et al., 2001). While many molecules regulating myocyte fusion have been identified (Charlton et al., 2000; Fortier et al., 2008; Horsley and Pavlath, 2004; Jansen and Pavlath, 2008; Rau et al., 2001; Richardson et al., 2007; Sohn et al., 2009; Swailes et al., 2004), the precise mechanism by which these molecules act in concert to control fusion remains to be elucidated. The molecules discussed in this dissertation are in general limited to those that regulate myofiber formation through migration and adhesion processes.

Branched myofibers during myogenesis

An interesting process occurs during adult regenerative myogenesis, called myofiber branching, bifurcation or splitting, which was first observed late in the 19th century (Volkmann, 1893). Myofiber branching is characterized by a single myofiber having a plasma membrane contiguous with several smaller myofibers. Later descriptions of muscle regeneration hypothesized why such branches may occur, suggesting that satellite cells would fuse to one another alongside a myofiber but would be unable to fuse with the damaged myofiber (Schmalbruch, 1976). Furthermore, electron microscopy studies suggest that myofiber to myofiber fusion is possible *in vitro*, and *in vivo* (Rash and Fambrough, 1973; Robertson et al., 1990). Unfortunately, whether myocyte fusion is specific to distinct regions of myofibers is not well understood. Studies of prenatal myofiber growth suggests that myocytes preferentially fuse to the ends of myofibers (Aziz

and Goldspink, 1974; Kitiyakara and Angevine, 1963; Zhang and McLennan, 1995); however, these studies do not address localized fusion during adult regenerative myogenesis. If fusion can only occur in specific regions along the myofiber, than defects in migration could yield branched myofibers.

Investigations into muscle regeneration after extreme exercise, which causes disruption of the muscle tissue architecture, using serial sections and electron microscopy, suggested that branching normally occurs during the regeneration of muscle (Hall-Craggs and Lawrence, 1969; Hall-Craggs and Lawrence, 1970). Other studies, using denervated dystrophic muscles found that myofiber branches have a contiguous cytoplasm, but the plasma membrane and basal lamina separate around these branches (Ontell and Feng, 1981). An increase in branched myofibers is correlated with transplantation (Bourke and Ontell, 1984; Ontell, 1986; Ontell et al., 1982), compensatory skeletal muscle hypertrophy (Tamaki et al., 1996), age (Bockhold et al., 1998) and severe or repetitive injuries to muscles (Anderson, 2000; Hurme et al., 1991; Tamaki and Akatsuka, 1994). In frogs, these branched myofibers are not associated with dysfunction of the muscle, however studies in mammals suggest that muscles with branched myofibers are weaker (Brown et al., 1982; Chan et al., 2007; Lovering et al., 2009; Tamaki et al., 1997). As significant amounts of branched myofibers are found within dystrophic muscle (Martin and Ontell, 1988; Schmalbruch, 1984; Tamaki et al., 1993), these myofibers may contribute to the loss of strength associated with muscular dystrophy (Stedman et al., 1991). Furthermore, one study indicates that these branches may not fully fuse with one another to form

continuous myofibers even after extended time periods (Vaittinen et al., 2002). Despite the large number of studies that have observed branched myofibers, no molecules are known to regulate this phenomenon, although branches are hypothesized to occur due to changes in adhesion during skeletal muscle regeneration (Hurme and Kalimo, 1992). Investigations into molecules which regulate formation or resolution of myofiber branching may provide insight into how and why branching occurs.

Migration and adhesion during myogenesis

Migration during embryogenesis

During embryogenesis, myogenic cells located at the lateral edge of the dermomyotome migrate to the limb buds and eventually give rise to the limb musculature (Brand-Saberi et al., 1996b; Houzelstein et al., 1999); cell migration is thus a key phase of embryonic myogenesis. The muscle cells that migrate to the limb buds are not yet differentiated (Wang et al., 1995). In fact, premature differentiation of muscle cells induced by ectopic sonic hedgehog actually disrupts migration of muscle cells in chicks (Chazaud et al., 2000). A substantial body of research has suggested that HGF binding to the c-met receptor is the primary molecular mechanism driving muscle progenitor cell migration to the limb buds (Gorza and Vitadello, 2000). HGF is expressed by tissues along the routes through which these muscle cells migrate (Doherty et al., 2005). In mice lacking HGF or c-met, muscle precursor cells are properly specified, but fail to exit the

somites (Entwistle et al., 1988). Additionally, ectopic HGF promotes the migration of muscle cells away from the somite (Krause et al., 1995). Other factors necessary for migration of muscle cells during embryogenesis include FGF and platelet derived growth factor (PDGF) (Webb and Lee, 1997; Webb et al., 1997). Overall, many factors induce muscle cell migration from somites into limb bubs, allowing myogenesis.

Many studies of factors which regulate migration during myogenesis *in vivo* were investigated using *Drosophila* as a model, where embryogenesis can be easily imaged using time-lapse microscopy. Unfortunately many of these studies do not differentiate between migration and adhesion of muscle cells during myogenesis, and instead focus on fusion, which is downstream of both processes. During myoblast fusion in *Drosophila*, a single myoblast is established as a founder cell for each muscle and the founder myoblast recruits fusion competent myoblasts to form multinucleated myofibers (Baylies et al., 1995; Paululat et al., 1995; Rushton et al., 1995). Founder cells express the protein dumbfounded (duf) which is part of the Ig superfamily, and loss of duf causes decreased adhesion of fusion competent cells with founder cells, decreasing fusion (Ruiz-Gomez et al., 2000). The fusion competent cells in *Drosophila* express sticks and stones (sns, also called kin of irre) another member of the Ig superfamily, which binds to duf on founder cells (Sohn et al., 2009). The homolog for sns in zebrafish and mice, called nephrin, regulates development of muscle in both of these systems, which suggests a possible functional conservation for these proteins of the Ig superfamily. Other proteins

capable of regulating migration and necessary for fusion of *Drosophila* muscle cells include the Robo/Slit family (Piper and Little, 2003), FGF (Beiman et al., 1996; Michelson et al., 1998), and laminin alpha chains (Martin et al., 1999). Furthermore, nothing is known about muscle regeneration in *Drosophila* and most studies on regenerative myogenesis are performed in the mouse model.

Migration during adult regenerative myogenesis

The extent to which cell migration occurs during mammalian skeletal muscle regeneration is a current controversy within the skeletal muscle field. However, several groups have shown that migration is necessary for differentiation of muscle cells *in vitro*. Cell-cell contact creates what is called a promyogenic complex of proteins that increases differentiation (Krauss et al., 2005). The extracellular matrix proteins (ECM) and ECM associated proteins, such as decorin and N-cadherin, are necessary for differentiation during myogenesis (Brand-Saberi et al., 1996a; Olguin et al., 2003). Matrix-metalloproteinases (MMPs) are also necessary for migration of muscle cells during myogenesis, affecting differentiation (Lluri and Jaworski, 2005; Lluri et al., 2008). One of the proteins that regulates migration and differentiation is CD44 (Mylona et al., 2006). Lastly, loss of NFAT5, a transcription factor, changes expression of other proteins yielding a decrease in both migration and differentiation (O'Connor et al., 2007). All of these studies suggest that migration is necessary for differentiation *in vitro*, but leave open the question of *in vivo* migration.

There are many studies that suggest migration may occur during *in vivo* adult regenerative myogenesis. Muscle cells throughout a muscle will activate and proliferate in response to a focal injury (Schultz et al., 1985). These results suggest that damage signals are present along the entire length of the muscle despite local injury. Activation of muscle cells distal from injury would be necessary if they are expected to contribute to regeneration of the muscle. Secondly, more muscle cells concentrate at an injury site than can be contributed to proliferation (Grounds et al., 1992). The current hypothesis for clustering of muscle cells to an injury is that muscle cells distal from the injury migrate towards the regenerating portion to contribute to repair. These data suggest that muscle cell migration may occur during adult regenerative myogenesis.

Other groups suggest that migration of muscle cells during adult regenerative myogenesis may be rare. For example, muscle cells from one myofiber may not repair adjoining myofibers frequently; suggesting that although muscle cells are capable of migrating through the basal lamina (Borisov et al., 2005; Hughes and Blau, 1990), such events happen infrequently. Unfortunately, other cell types reside in the interstium, such as pericytes, that are capable of transdifferentiating into muscle cells and fusing into myofibers (Galvez et al., 2006), clouding the study of muscle cell movement out of the basal lamina. Another example of rare muscle cell migration uses the mdx dystrophic mouse model, which contains a nonsense mutation in the open reading frame of dystrophin, and is often used to study clonal expansion of muscle cells. In the mdx mouse, sporadic clusters of myofibers express dystrophin, called revertent fibers, which are due to exon

skipping resulting in correction of the nonsense mutation and expression of functional dystrophin (Lu et al., 2003b; Lu et al., 2000; Thanh et al., 1995). The area of revertent fibers expands both longitudinally and laterally throughout the muscle, possibly due to clonal expansion of revertent muscle satellite cells during muscle regeneration (Yokota et al., 2006). Longitudinal expansion could be due to migration of muscle cells along the basal lamina. Lateral expansion of revertent myofibers would be dependent on migration of revertent satellite cells from one myofiber to another, crossing both basal laminas. As the expansion of reverent myofibers does not occur quickly or frequently, these studies might suggest that muscle cell migration occurs infrequently. However, the mdx mouse may not be the most ideal model to use for studies on muscle cell migration as the entire muscle is undergoing degeneration and regeneration, rather than specific localized injuries. Unfortunately, all of these studies only correlate presence of the cells to migration, none study migration directly.

Migration of transplanted muscle cells

Although studying migration of endogenous muscle cells *in vivo* is technically difficult, the migration of transplanted donor muscle cells, either as part of an engrafted muscle tissue or transplanted without associated myofibers, is better documented. Many studies have focused on muscle grafts as a source of donor muscle cells. When donor muscle tissue grafts are transplanted into a host animal, donor muscle cells disappear from the core of the graft, and appear at the periphery of the donor muscle tissue (Schultz et al., 1988). These changes

occur after 18-24 hrs of transplantation and are not concurrent with cell death, suggesting that donor muscle cells are capable of migrating within the engrafted tissue. Studies also suggest that donor cells transplanted with muscle tissue grafts can migrate out of the donor tissue into injured host muscle tissue (Phillips et al., 1990). Similar studies also suggested that muscle cells can emigrate from the graft into the host (Jockusch and Voigt, 2003). Therefore, the donor muscle cells are not lost, but move out of the donor graft tissue. Furthermore, adjacent muscles can share muscle cells; if a donor muscle is engrafted, a nearby host muscle can receive donor muscle cells upon injury (Watt et al., 1987). These data suggest that muscle cells can migrate out of one muscle and into another muscle. Although, some researchers believe that the distribution of donor muscle cells throughout the host muscle tissue can be contributed solely to proliferation; other researchers suggest that migration may also play a role in the dispersal of muscle cells throughout a tissue. Interestingly, large grafts of donor muscle tissue containing muscle cells are not necessary to support engraftment of donor muscle cells into the host muscle tissue. For example, transplanting a single wild-type fiber with only 7-10 donor muscle cells into an mdx host muscle tissue yields large numbers of dystrophin^{+} myofibers throughout the host tissue (Collins et al., 2005). Studies suggest muscle cells persist long term and participate in fusion events necessary to repopulate muscle after injury. Donor muscle cells may populate the satellite cell niche, as they can contribute to regeneration upon subsequent re-injury of host muscle. These studies suggest that muscle cells are capable of migrating over a distance of several millimeters.

Further studies suggest that muscle cells can migrate throughout the tissue after injection. Unfortunately, the longer muscle cells are cultured *in vitro*, the more likely the cells are to be rejected upon transplantation into the host (Smythe and Grounds, 2000). Muscle cells transplanted into the tibialis anterior (TA) migrate away from the injection site and penetrate the basal lamina of muscle fibers (Lipton and Schultz, 1979). When wild-type cells are transplanted into mdx muscle, they can migrate into adjacent muscles (Morgan et al., 1993; Watt et al., 1993). Although muscle cells may migrate away from the injection site, fusion of transplanted cells is highest near the injection site and decreases away from the site (Rando et al., 1995). Another study suggests that several parameters are required to allow migration of muscle cells (Schultz et al., 1986), as a physical bridge between the adjacent muscles where the connective tissues were disrupted was necessary to allow migration. Therefore, cell migration and damage to connective tissue are both necessary for engrafted cells to emigrate from donor muscle tissue into host muscle. To determine the efficacy of transplanting muscle cells into muscle tissue of larger animals, donor cells were injected into muscle of non-human primates. These studies determined that donor muscle cells did not migrate farther than 1mm from the injection site in the host muscle (Skuk et al., 2002). The distance muscle cells can migrate in these studies may be the minimum and can possibly be altered, or increased with changing several factors either injected with or in the muscle cells.

Few studies have focused on factors capable of changing the migration of transplanted muscle cells within muscle tissue. One report utilized the

longitudinal distance of the muscle to determine migration; donor cells were injected into the distal portion of the host muscle and the proximal end of the host muscle was injured (Neuhaus et al., 2003). Fibroblast growth factor (FGF) was capable of regulating the migration of the transplanted muscle cells, as muscle cells isolated from $FGF^{-/-}$ mice showed decreased migration towards the injury. This report also indicates a second important aspect of migration, donor muscle cells migrated towards the muscle injury more often than away from the injury; indicating injured muscle may secrete factors that increase migration towards the area. One factor known to be released upon muscle injury is hepatocyte growth factor (HGF) (Allen et al., 2003). HGF can also regulate migration of muscle cells *in vivo*, as donor muscle cells transplanted with a scaffold that contains both HGF and FGF can increase migration of muscle cells out of the scaffold into host muscle (Hill et al., 2006). Lastly, IL4 enhances migration of muscle cells out of a transplanted microtube and into the surrounding host muscle tissue (Lafreniere et al., 2006). These studies suggest that muscle cell migration can be affected by both soluble secreted factors and cell-surface receptors.

Other studies on cell migration and muscle utilize a class of vessel-associated stem cells called mesoangioblasts (Dellavalle et al., 2007; Sampaolesi et al., 2006; Sampaolesi et al., 2003), which can differentiate into muscle after transplantation. These cells can be injected intra-arterially, and will correctly home to muscle tissue (Sampaolesi et al., 2003). To determine factors which regulate migration of mesoangioblasts to muscle, beads containing chemoattractants were injected into the muscle. High mobility group box 1

(HMGB1), an abundant chromatin protein that acts as a cytokine when released by cellular damage, increased homing of mesoangioblasts into muscle tissue (Palumbo et al., 2004). Furthermore, pretreatment of mesoangioblasts with the chemokines SDF1α or TNFα increases their migratory abilities *in vitro* and transplantation of mesoangioblasts *in vivo* (Galvez et al., 2006). These studies indicate that muscle cells with enhanced migratory abilities engraft into muscle tissue with a higher efficiency. Therefore, factors which enhance muscle cell migration are of therapeutic interest.

Migratory factors for muscle cells

As muscle cells can migrate to injured areas within muscle, factors that can attract muscle cells must exist within muscle tissue. Indeed, there are many possible sources of chemoattractants within muscle such as the ECM, which releases a variety of possible chemoattractants after injury and during muscle regeneration (Dourdin et al., 1999; Dourdin et al., 1997). Another possible source of muscle cell chemoattractants are the immune cells, some of which normally reside within the muscle and some that infiltrate the site of injury. For example, conditioned media from macrophage cultures can cause muscle cell migration (Cantini and Carraro, 1995). Interestingly, crushed muscle extract from injured muscle can attract macrophages using *in vitro* assays (Chazaud et al., 2003; Robertson et al., 1993) implicating cross-talk occurs between muscle and the immune system. Also, platelet extract contains at least one chemoattractant capable of increasing muscle cell migration; this factor is most likely TGF-β as

incubating the extract with a TGF-β neutralizing antibody decreased migration (Bischoff, 1986). Another source of chemoattractants are the myofibers of the muscle cells themselves, as a muscle secretome has been elucidated that suggests muscle can secrete many factors (Bortoluzzi et al., 2006); however, studies were not done to determine if these factors regulate migration of muscle cells. More interestingly, time-lapse microscopy on fusing muscle cell cultures indicates that muscle cells move into areas that form myotubes (Chazaud et al., 1998). These data suggest that some muscle cells must attract other muscle cells in order to form myotubes, causing migration. Media removed from fusing muscle cell cultures increases muscle cell migration, suggesting that muscle cells themselves secrete the factors that regulate their migration (Bondesen et al., 2007; Kramerova et al., 2004). Therefore, a combination of factors produced by the ECM, immune cells, injured myofibers, and muscle cells are thought to serve as chemoattractants that recruit myoblasts to the site of injury.

Due to the difficulty of examining the roles of putative muscle cell chemoattractants *in vivo*, much of our knowledge on post-natal myoblast migration has been gained by performing migration assays *in vitro*. Several different types of muscle cells are used in migration assays, including primary muscle cells from human, quail, mouse and rat muscle, and immortalized muscle cell lines such as MM14, C2C12 and L6. Furthermore, many assays are utilized to study migration *in vitro*, including traditional Boyden chambers, Boyden chambers modified with Matrigel or endothelial cells, Dunn chambers, wound healing assays, cell dispersion assays and time lapse microscopy (Allen et al.,

2003; Bischoff, 1997; Corti et al., 2001; El Fahime et al., 2002; Lafreniere et al., 2004; Mylona et al., 2006; Neuhaus et al., 2003; Robertson et al., 1993; Suzuki et al., 2000; Villena and Brandan, 2004). Unfortunately, comparing results from studies using different muscle cells and migratory assays is very difficult. Furthermore, traditional Boyden chambers are end-point assays, which do not differentiate between an increase in cell velocity, known as chemokinesis, or directed migration. Boyden chambers modified with either Matrigel or endothelial cells measure invasiveness and migration. Finally, wound healing assays measure directed migration, but are most suitable for studies on the effect of cell-matrix and cell-cell interactions (Rodriguez et al., 2005). Despite these issues, many secreted factors are considered confirmed chemoattractants for proliferating myoblasts (Table 1.1): FGF (Allen et al., 2003; Corti et al., 2001; El Fahime et al., 2002; Lafreniere et al., 2009; Lafreniere et al., 2004; Mylona et al., 2006; Neuhaus et al., 2003; Robertson et al., 1993; Suzuki et al., 2000; Villena and Brandan, 2004), PDGF (Allen et al., 2003; Bischoff, 1997; Corti et al., 2001; El Fahime et al., 2002; Robertson et al., 1993), EGF (Bischoff, 1997; Corti et al., 2001), HGF (Allen et al., 2003; Alvarez et al., 2008; Bischoff, 1997; Corti et al., 2001; El Fahime et al., 2002; Germani et al., 2003; Kawamura et al., 2004; Mylona et al., 2006; Neuhaus et al., 2006b; Robertson et al., 1993; Suzuki et al., 2000), IGF (Allen et al., 2003; Becciolini et al., 2006; Lafreniere et al., 2004; Neuhaus et al., 2003; Suzuki et al., 2000), and VEGF (Germani et al., 2003). Several other factors regulate migration through either Matrigel or endothelial cell layers: TGF-β (Allen et al., 2003; Bischoff, 1997; Kawamura et al., 2004; Villena

and Brandan, 2004), TNFα (Allen et al., 2003; Corti et al., 2001), IFNγ (Corti et al., 2001), RANTES (Corti et al., 2001); suggesting that they may regulate invasiveness, as well as migration. The presence of so many secreted chemoattractants suggests a complex temporal and spatial regulation of muscle cell migration.

Although many secreted migratory factors are expressed by muscle and muscle tissue, other types of factors are also capable of regulating muscle cell migration, such as intracellular factors which are not normally secreted. For example, HMGB1, a chromatin remodeling factor, which is released from necrotic tissues, causes rat L6 cells to migrate in Boyden chamber assays (Riuzzi et al., 2006). Also, saline solution alone is enough to attract muscle cells (Corti et al., 2001), although this result may be indirect as ion channels are necessary for migration (van Lunteren et al., 2002). Therefore, factors released by necrotic tissue may also regulate muscle cell migration.

Interestingly, secreted factors also decrease migration of muscle cells. Loss of the PGI_2 receptor IP increases migration in Boyden chambers to conditioned media collected from muscle cells during time points of extensive fusion, whereas addition of iloprost, a stable PGI_2 analog, decreases migration to conditioned media (Bondesen et al., 2007). These data suggest that PGI_2 can decrease migration during myogenesis. Sphingosine 1-P was shown to decrease migration to IGF-1 in Boyden chambers modified with Matrigel (Becciolini et al., 2006). With the large number of molecules that increase migration, why muscle would also contain factors to decrease migration is perplexing. One model

suggests that muscle cells must slow down in order to adhere and fuse, much like immune cells slowing to adhere and invade through blood vessels.

Unfortunately, it remains to be determined whether the chemoattractants identified by *in vitro* experiments also promote muscle cell migration *in vivo*. Due to the aforementioned technical issues with migration *in vivo*, new techniques will have to be developed in order to study migration *in vivo*. Furthermore, the differentiation status of muscle cells has a significant impact on cell migration (Dourdin et al., 1999; Elamrani et al., 1995), and chemoattractants identified for proliferating myoblasts may provoke a divergent effect on the migration of differentiating muscle cells. Overall, a large number of factors regulate migration of muscle cells, suggesting complex spatial and temporal control during myogenesis.

Table 1.1

Factors involved in muscle cell migration *in vitro*

	Factor	Cells	Assays	Reference
Media and Extracts	Conditioned Media from muscle cells in culture	primary mouse muscle cells, primary mouse IP-/- muscle cells	Boyden chamber, Time-lapse microscopy	(Bondesen et al., 2007)
	CME post injury	Macrophages	Boyden chamber	(Robertson, 1993 #146)
	Crushed muscle extract (Pimorady-Esfahani et al.)	primary mouse cells, primary rat muscle cells, C2C12	Boyden chamber	(Bischoff, 1986), (Robertson, 1993 #146)
	Macrophage media from macrophage cells in culture	primary mouse muscle cells, C2C12	Boyden chamber	(Robertson, 1993 #146)
	Platelet extract (also with TGF beta antibody)	Primary rat muscle cells	Boyden chamber	(Bischoff, 1986)
Secreted Factors	Cyr61	primary mouse muscle cells, primary NFAT5 -/- cells	Time-lapse microscopy	(O'Connor et al., 2007)
	EGF	primary mouse muscle cells, C2C12, primary rat muscle cells	Boyden chamber, Boyden chamber modified with endothelial cells, wound healing	(Bischoff, 1997), (Corti et al., 2001)
	FGF	primary mouse muscle cells, primary FGF -/-, primary CD44-/-, C2C12, primary human muscle cells, primary rat muscle cells	Boyden chamber, Boyden chamber modified with Matrigel or endothelial cells, wound healing, in vivo microtube or freeze injury	(Allen et al., 2003), (Bischoff, 1997), (Corti et al., 2001), (El Fahime et al., 2002), (Lafreniere et al., 2006; Lafreniere et al., 2004), (Mylona et al., 2006), (Neuhaus et al., 2003), (Robertson, 1993 #146), (Suzuki et al., 2000), (Villena and Brandan, 2004)

	Factor	Cells	Assays	Reference
Secreted factors con't	IFN gamma	primary mouse muscle cells, C2C12	Boyden chamber modified with endothelial cells, wound healing	(Corti et al., 2001)
	IGF1	primary mouse muscle cells, C2C12, primary human muscle cells	Boyden chamber, Boyden chamber modified with Matrigel	(Allen et al., 2003), (Becciolini et al., 2006), (Lafreniere et al., 2006), (Neuhaus et al., 2003), (Suzuki et al., 2000)
	IL4	primary mouse muscle cells, primary IL4R-/-, primary human muscle cells	Boyden chamber, Time-lapse microscopy, Dunn chamber	(Lafreniere et al., 2006), (Jansen and Pavlath, 2006)
	Iloprost	primary mouse muscle cells, primary IP -/- muscle cells	Boyden chamber, Time-lapse microscopy	(Bondesen et al., 2007)
	LIF	primary mouse muscle cells, C2C12	Boyden chamber	(Robertson, 1993 #146)
	PDGF	primary mouse muscle cells, C2C12, primary rat muscle cells	Boyden chamber, Boyden chamber modified with Matrigel or endothelial cells, wound healing, in vivo microtube	(Allen et al., 2003), (Bischoff, 1997), (Corti et al., 2001), (El Fahime et al., 2002), (Robertson, 1993 #146)
	RANTES	primary mouse muscle cells, C2C12	Boyden chamber modified with endothelial cells, wound healing	(Corti et al., 2001)
	SDF	C2C12, primary rat muscle cells	Boyden chamber	(Bae et al., 2008), (Odemis et al., 2007)
	Sphingosine 1-P	C2C12	Boyden chamber modified with Matrigel	(Becciolini et al., 2006)
	TGF beta	primary mouse muscle cells, C2C12, primary rat muscle cells	Boyden chamber, Boyden chamber modified with Matrigel	(Allen et al., 2003), (Bischoff, 1997), (Kawamura et al., 2004), (Villena and Brandan, 2004)

	Factor	Cells	Assays	Reference
Secreted factors	VEGF	primary mouse muscle cells, C2C12	Boyden chamber	(Germani et al., 2003)
Extracellular matrix and associated proteins	alpha 5 integrin	primary quail embryo muscle cells	Wound Healing, Time-lapse	(Huttenlocher et al., 1998)
	Collagen type IV	primary rat muscle cells	Time-lapse microscopy	(Funanage et al., 1992)
	Decorin	primary mouse muscle cells, primary decorin -/- muscle cells, C2C12	cell aggregate dispersion	(Olguin et al., 2003)
	Dermatan Sulfate	C2C12	Boyden chamber	(Villena and Brandan, 2004)
	Entactin	primary rat muscle cells	Time-lapse microscopy	(Funanage et al., 1992)
	Fibronectin	primary rat muscle cells,rat L8E63, C2C12 and MM14 muscle cells, primary mouse embryo muscle cells	Time-lapse microscopy	(Crawley et al., 1997)(Funanage et al., 1992)(Goodman et al., 1989)(Ocalan et al., 1988)(Turner et al., 1983)(Yao et al., 1996)
	Heparin sulfate proteoglycan	primary rat muscle cells	Time-lapse microscopy	(Funanage et al., 1992)
	Laminin	rat L8E63 muscle cells, C2C12, MM14	Time-lapse microscopy	(Crawley et al., 1997)(Goodman et al., 1989)(Ocalan et al., 1988)(Yao et al., 1996)
	MMP9	C2C12, rat L6, primary human muscle cells	Boyden chamber, Boyden chamber modified with Matrigel	(Lewis et al., 2000), (Riuzzi et al., 2006)
	N-cadherin	primary quail embryo muscle cells	Wound Healing, Time-lapse microscopy	(Huttenlocher et al., 1998)

	Factor	Cells	Assays	Reference
ECM con't	uPA	primary human muscle cells	Boyden chamber, Boyden chamber modified with Matrigel, Time-lapse microscopy	(Chazaud et al., 2000), (Mills et al., 2007)
Other	HMGB1	rat L6	Boyden chamber	(Riuzzi et al., 2006)
	Saline	primary mouse muscle cells, C2C12	Boyden chamber modifed with endothelial cells, wound healing	(Corti et al., 2001)

Migration signaling during myogenesis

Cell migration is a complex process that is essential for a wide range of processes, including embryonic myogenesis and adult tissue repair. The assembly and disassembly of either actin or myosin filaments regulate migration of a variety of cell types. In the case of muscle cells, actin filaments are assumed to regulate muscle cell migration (Costa et al., 2008; Dedieu et al., 2004; Kuwahara et al., 2005). In response to directional migration cues, cells may adopt polarized morphologies, which are characterized by a leading edge of membrane protrusion due to actin polymerization (Boettiger et al., 1995). Actin may polymerize in several ways; for instance, the Arp2/3 protein complex promotes extension of new actin filaments branching from existing filaments, whereas formin complexes promote the linear extension of existing actin filaments (Schwander et al., 2003). Regardless of whether polymerization occurs in branches or extensions, the nucleating complexes are regulated by the activity of a variety of kinases, phosphatases, and GTPases. Localized activation of the Arp2/3 complex promotes directed cell migration and is regulated by signaling cascades that include the Wiskott-Aldrich syndrome proteins, WASP-family verprolin-homologous (WAVE) proteins, and Rho GTPases Rac, RhoA, and Cdc42 (Bompard and Caron, 2004; Miki and Takenawa, 2003; Tachibana and Hemler, 1999; Takenawa and Suetsugu, 2007). Although skeletal muscle formation is dependent upon myoblast migration, current studies disagree about which intracellular signaling cascades are necessary to direct myoblast migration.

One study suggests that depolarization of muscle cells may enhance their migratory ability, as potassium channels are necessary for proper migration of rat muscle cells in wound healing assays (van Lunteren et al., 2002). Unfortunately, further assays have not been done on the regulation of muscle cell migration by ion channels. Another set of studies have focused on GTPases and their regulating guanine exchange factors (GEF). For example, proliferating myoblasts migrate to HGF, FGF and IGF using the downstream signaling molecules GEF Ras and Ral GTPases. Interestingly, these signals are not regulated through PI3K (Suzuki et al., 2000), which is a more canonical signaling molecule for chemotaxis (Firtel and Chung, 2000). These data suggest that myoblast migration may be regulated by a unique or specific pathway, rather than through canonical signaling mechanisms. In contrast, other labs suggest that proliferating myoblasts migrate to HGF through PI3K-regulated signaling molecules including N-WASP and WAVE2 (Kawamura et al., 2004), proteins necessary for actin polymerization. Although both studies utilized myoblasts, the results are directly opposite to each other. Due to the differences in techniques, the signaling mechanisms which are used by myoblasts or myocytes are unclear. The second study is more compatible with studies done in *Drosophila*, where SCAR/WAVE and ARP2/3 are needed for fusion events to occur (Richardson et al., 2007). Other actin-regulating proteins are necessary for myogenesis, such as the actin binding protein, Xin, which is expressed during myogenesis *in vivo*, and loss of Xin negatively affects myogenesis (Hawke et al., 2007). Interestingly, cytoskeleton proteins like non-muscle myosins can also regulate both alignment

and fusion events (Swailes et al., 2006), suggesting that actin may not act alone in myogenesis. Nevertheless, these studies suggest actin-dependent migration is necessary for myogenesis.

Actin may also regulate migration of cells through changes in focal adhesions, which connect the cytoskeletal machinery to the extracellular matrix (ECM). Several studies suggest the importance of focal adhesions during muscle cell migration. For instance, the skeletal muscle LIM protein 1 (SLIM1), a molecule found in focal adhesion machinery, has distinct effects in myocytes versus quiescent muscle cells. Increasing expression of SLIM1 induces hyperelongation of myocytes, whereas quiescent muscle cells exhibit multiple protrusions of the cytoplasm, both through the α5β1 integrin receptor (McGrath et al., 2003). Both hyperelongation and the increase in protrusions effect adhesion of the cells to the extracellular matrix, therefore affecting migration. Other focal adhesion machinery proteins are also important for muscle cell migration. The loss of the ubiquitous μ-calpain or m-calpain, calcium activated neutral cysteine proteases, decreased migration of C2C12 cells (Dedieu et al., 2003). An increase in the inhibitor of calpain, calpastatin, yielded similar results (Dedieu et al., 2004). The decrease in calpain caused a subsequent change in the sub-cellular localization of MARCKS, a myristoylated alanine rich C kinase substrate protein that is part of the focal adhesion complex machinery, increasing the ratio of cytosolic-MARCKS versus membrane bound-MARCKS (Dedieu et al., 2003; Dedieu et al., 2004). The cells used in these experiments were pre-incubated in DMEM containing 0.1% FBS, these cells were considered to be “quiescent” by the

authors; however C2C12 cells in low serum conditions will differentiate (Yaffe and Saxel, 1977); therefore, the actual differentiation state of these cells is unknown. By changing the focal adhesions, calpains and MARCKS are involved in cell attachment and cytoskeletal machinery, both of which are dynamic during cell migration. Other focal adhesion proteins are also implicated in migration of muscle cells, such as integrin cytoplasmic domain-associated protein-1, ICAP-1, which is involved in the migration of C2C12 cells on β1-integrin. Knockdown of ICAP-1 by RNAi or inhibition of ROCK-1 kinase yields a decrease in migration of C2C12 cells to HGF (Swailes et al., 2004), again implicating proteins involved in focal adhesions in regulation of muscle cell migration. Furthermore, ROCK signaling is controlled by RhoE, which regulates both RhoA and ROCK during fusion events (Fortier et al., 2008). Other factors regulated by the RhoA pathway include phospholipase D (PLD) and phosphatidic acid, both of which are necessary for remodeling of the actin cytoskeleton for fusion of muscle cells (Komati et al., 2005). Focal adhesions are formed after muscle cells adhere to an extracellular matrix, which promote spreading and migration, through downstream signals such as the skeletal muscle LIM protein 1 (SLIM1) (Robinson et al., 2003), desmin and actin proteins (Costa et al., 2008). As many of these proteins are found in the basal lamina, and muscle cells can migrate along these factors, the basal lamina of myofibers may then be a sort of highway for muscle cells, allowing them to migrate along the length of the myofiber. Although these experiments suggest many regulatory factors and signaling cascades affect migration during myogenesis. Unfortunately, due to their

inconsistencies in these studies, more work is necessary to determine the specific signaling cascades necessary for migration during myogenesis.

Regulation of migration by the extracellular matrix

Although secreted factors can act as chemoattractants for muscle cells, they can be bound to the extracellular matrix (ECM), allowing regulation of their activity. For instance, Cyr61, a secreted cysteine-rich heparin-binding protein, binds to the ECM, and can increase migration of muscle cells (O'Connor et al., 2007). Proteins that remodel the ECM can release factors or activate them (Bischoff, 1997; Chen and Quinn, 1992), creating gradients from molecules already present. For example, *in vivo*, HGF is expressed in an inactive form by muscle cells and then bound by the ECM until cleaved and the active form is released (Allen et al., 2003). Therefore, secreted factors can be regulated by the ECM.

Rearrangement of the ECM by matrix metalloproteinases (MMP) is a key step during the formation of new myofibers (Miller et al., 2003). Matrix metalloproteinases (MMP) 2 and 9 and urokinase-type plasminogen activator (uPA) all increase migration in Boyden chambers (Chazaud et al., 2000; Lewis et al., 2000; Mills et al., 2007; Riuzzi et al., 2006). They also increase migration in Boyden chambers modified with Matrigel suggesting that they may allow muscle cells to more efficiently migrate across a barrier. Several MMPs are expressed by C2C12 cells, including MMP2, 3, 7, 9 and 10 (Lluri and Jaworski, 2005; Lluri et al., 2008; Nishimura et al., 2008). MMPs inhibitors decrease migration of muscle

cells, while increasing their directed migration, (Nishimura et al., 2008) and inhibit fusion of C2C12 muscle cells without affecting biochemical differentiation (Ohtake et al., 2006). The activity of membrane type I-MMP (MT1-MMP) is important for fusion, as shRNA knockdown of MT1-MMP expression inhibits fusion and MT1-MMP null mice undergo impaired myofiber formation *in vivo* (Ohtake et al., 2006). MMP1 regulates migration and differentiation of muscle cells, and can increase engraftment of donor muscle cells into dystrophic host muscle tissue (Wang et al., 2009). Furthermore, growth factors are capable of stimulating expression of MMP9 by muscle cells, increasing their invasion and migration abilities *in vitro* (Allen et al., 2003). These data suggest migration of muscle cells is regulated by MMPs and are necessary for myogenesis.

The urokinase system is composed of the cell surface receptor urokinase plasminogen activator receptor (uPAR), the serine proteinase urokinase plasminogen activator (uPA) and the inhibitory molecules plasminogen activator inhibitor (PAI)-1 and PAI-2. Cell surface bound uPA cleaves plasminogen to plasmin, which degrades components of the ECM surrounding the cell, thereby aiding in cell migration (McLennan, 1991). uPA, uPAR and PAI-1 are localized to the leading edge of migrating cells where they have been postulated to play a role in cytoskeletal reorganizations necessary for migration by mediating mechanical force transfer across the plasma membrane (Bonavaud et al., 1997; Wang et al., 1995). Recent investigations indicated mannose receptor was required for migration of muscle cells and played an important role in the clearance of collagen from the ECM (Jansen and Pavlath, 2006). Together, these

studies indicate the ECM is an important source and regulator of chemoattractants for muscle cells.

The ECM contains a number of different types of proteins which are capable of regulating migration and adhesion of muscle cells to the matrix. Dermatan sulfate, an ECM protein, increases migration to growth factors in Boyden chambers (Villena and Brandan, 2004). Perhaps dermatan sulfate creates a matrix across which cells can migrate more efficiently. Decorin, another ECM protein, actually decreases migration in a cell dispersion assay (Olguin et al., 2003). In contrast, cells on laminin migrate much faster than cells on fibronectin, which suggested muscle cells may adhere more strongly to fibronectin (Ocalan et al., 1988). Later studies proved that muscle cells adhere more efficiently to fibronectin, as the cytoskeleton of muscle cells is stabilized by fibronectin, but not by laminin (Goodman et al., 1989). Fibronectin can also guide muscle cells to create long unbranching myotubes, through the migration of muscle cells along fibronectin crystals (Turner et al., 1983). Cells will adhere to a diluted Matrigel substrate, which contains laminin, type IV collagen, heparan sulfate proteoglycan, and entactin. Blocking either laminin or entactin impairs satellite cell adherence to the matrix (Funanage et al., 1992). Muscle cells interact with the extracellular matrix through cell surface receptors, for example, the alpha 7 beta 1 integrin receptor mediates interactions with laminin (Crawley et al., 1997). Loss of this receptor blocks adhesion and motility on specific isoforms of laminin (Yao et al., 1996). Other receptors include the alpha 5 integrin receptor that binds fibronectin, and loss of this receptor affects cell migration, formation of myotubes

and alignment of nuclei within a myotube (Boettiger et al., 1995). Recent evidence supports this conclusion, as time lapse microscopy of intact myofibers revealed that muscle satellite cells migrate efficiently along the length of the myofiber *in vitro* (Siegel et al., 2009). These studies provided strong evidence for the possibly of migration of mononucleated muscle cells. All of these experiments demonstrate that cell migration and adhesion are critical for the development of cell-cell contacts necessary for myoblast fusion, a theme that is repeated with several other molecules to be discussed later.

Role of chemokine ligands and receptors during myogenesis

Chemokines are 8-to-10-kd secreted proteins with 20 to 70 percent homology in amino acid sequences that share both leukocyte chemoattractant and cytokine-like behaviors (Baggiolini et al., 1995; Luster, 1998). When initially identified, these proteins had no known biologic activity but instead, were associated with inflammatory disease, such as human platelet factor 4 (Deuel et al., 1977). Since then, a set of nomenclature has been created on the basis of the relative position of their cysteine residues: C, CC, CXC and CX_2C (Baggiolini et al., 1994; Mackay, 1997).

Chemokines can regulate cells in a number of different ways. They can regulate cell number by influencing survival and proliferation (Miyazaki et al., 2006; Schober and Zernecke, 2007). Furthermore, chemokines can act in either a paracrine or autocrine manner. In the immune system, chemokines act in a mostly paracrine manner, allowing the recruitment of immune cells to damaged

tissue cells (Bleul et al., 1996; Loetscher et al., 1996; Weber et al., 1995). However, autocrine regulation by chemokines is also possible, as it occurs in many cancer models (McManus et al., 2000; Menten et al., 2002; Wang et al., 2008). Chemokines also regulate migration of cells, as discussed below.

Chemokines and migration

Chemokines regulate the migration of a number of different inflammatory cells in tissues including eosinophils, basophils, monocytes, both activated and resting T cells, dendritic cells, neutrophils and natural killer cells (Bleul et al., 1996; Loetscher et al., 1996; Weber et al., 1995), and therefore, play an important role in inflammation. Chemokines also regulate migration of non-immune cells, such as sperm and metastasizing cancer cells (Isobe et al., 2002; Miyazaki et al., 2006; Muciaccia et al., 2005a; Muciaccia et al., 2005b; Stebler et al., 2004; Vandercappellen et al., 2008; Wang et al., 2008).

Chemokines bind to specific G-protein coupled receptors (GPCR) on target cells and induce cell migration (Murphy, 1994). Most chemokine receptors bind to more than one chemokine and vice versa, yielding redundant systems. The downstream signaling of chemokine receptors is linked to phospholipases through G proteins yielding an increase in inositol phosphate, the release of intracellular Ca^{2+} and activation of protein kinase C (Luster, 1998; Springer, 1994). However, chemokines are also known to activate small GTP-binding proteins of the Ras and Rho families (Laudanna et al., 1996), which are

important for cell migration processes such as membrane ruffling, formation of pseudopods and focal adhesion complex assembly.

Similar to other secreted factors, chemokines are capable of binding to extracellular matrix proteins, such as heparin and heparan sulfate, as they are highly basic. Trapping chemokines in the extracellular matrix is hypothesized to create a local concentration of chemokines which helps in gradient formation (Luster et al., 1995; Rot, 1992; Rot, 1993). As such, chemokines and their receptors are important regulators of migration during homeostasis, disease and injury.

Chemokines and muscle

Chemokines and their receptors are upregulated in several types of myopathies. Inflammatory myopathies are clinically defined as muscle weakness with inflammatory infiltrates in muscle tissue, due to upregulation of several receptor/ligand pairs: CCL2, CCL3, CCL4, CCL5 (RANTES) and their receptors CCR3, CCR1, CCR5 (Civatte et al., 2005) CCL20/CCR6 (Page et al., 2004), CCL19, CCL21 and their receptor CCR7 (Tateyama et al., 2009). The expression of a number of chemokine receptor/ligand pairs, including CCL5/CCR1, was increased with infection of muscle tissue by live *Pseudomonas aeruginosa* bacteria (Demoule et al., 2009). Studies on genes differentially expressed by mdx mice, a model of dystrophin-deficient muscular dystrophy, suggest that several chemokine receptors are upregulated by dystrophic muscles: CCR1, CCR7, CCR8, CCR9 (Turk et al., 2005), CCL2, CCL5, CCL6, CCL7, CCL8,

CCL9 and their receptors CCR2, CCR1 and CCR5 (Porter et al., 2003). Dystrophic muscle fibers express both CCL2 and CCL6, suggesting that the myofibers themselves can regulate chemotaxis of immune cells (Porter et al., 2003). The upregulation of chemokines and their receptors in several types of myopathies suggests these molecules may be required for the inflammatory processes necessary for healing diseased muscle.

Expression of chemokines is also increased in normal muscle, through either damage or even muscle use. A study which utilized a cardiotoxin muscle injury model also indicated upregulation of several chemokine receptor/ligand pairs at 48 hours after injury: CCL3, CCL9, CCL7, CCL12, CCR1, CCR2, CCR5, and CXCR4 (Hirata et al., 2003). Exercise can increase the expression of CXCR2 by skeletal muscle (Frydelund-Larsen et al., 2007). Finally, even in the absence of other cell types, cultured skeletal muscle cells that have undergone mechanical stress upregulated a number of cytokines that cause neutrophil chemotaxis, including IL8 (Peterson and Pizza, 2009), CXCL1 and CXCL5 (Nedachi et al., 2009). The upregulation of chemokines and their receptors after muscle use or injury suggests the participation of chemokines in muscle cell repair.

Chemokines can affect cell types other than muscle cells within muscle tissue. Chemokines play an important role in adult regenerative myogenesis as muscle damage induces macrophage infiltration into the tissue through release of chemokines (McLennan, 1996; Pimorady-Esfahani et al., 1997; Robertson et al., 1993). Macrophages then remove necrotic tissue, which is necessary for subsequent muscle regeneration (McLennan, 1996; Pimorady-Esfahani et al.,

1997). Furthermore, loss of CCR5 during muscle regeneration after freeze injury yields an increase in adipose cells (Warren et al., 2004). In contrast, loss of CCR2 causes increased adipogenesis and fibrosis (Warren et al., 2005; Warren et al., 2004). Another injury model, using ischemia to damage the muscle, suggested a role for CCR2 in immune cell recruitment, fat deposition and muscle regeneration (Contreras-Shannon et al., 2007). Therefore, chemokines can regulate muscle regeneration indirectly through effects on immune cells and other cells within muscle tissue.

Chemokines also regulate adult regenerative myogenesis more directly. For example, immune cells may play a larger role in muscle regeneration as several studies indicate that co-culture of these cells with muscle enhances proliferation of the muscle cells (Cantini and Carraro, 1995; Chazaud et al., 2003; Merly et al., 1999). CCR2 was expressed by differentiating muscle cells in regenerating muscle and loss of CCR2 may result in impaired fusion of muscle cells (Warren et al., 2005). Interleukin 4 (IL-4) is important for recruitment of muscle cells into nascent myotubes to form mature myotubes (Horsley et al., 2003). Later studies have indicated that IL-4 is necessary for migration of muscle cells both *in vitro* and *in vivo* (Lafreniere et al., 2006). Therefore, chemokines may directly regulate muscle cells within muscle tissue. Together, these studies indicate that chemokine receptor/ligand pairs play important roles in muscle biology.

CXCR4/SDF1α axis in muscle

The CXCR4 and SDF1α (also known as CXCL12) axis is an important regulator of migration for many cell types. Hematopoietic progenitor cells increase migration in response to exposure to gradients of SDF1α (Kim and Broxmeyer, 1998). Bone marrow expresses SDF1α, and hematopoietic stem cells express CXCR4. Under normal conditions, the expression of SDF1α by bone marrow maintains the hematopoietic stem cell niche (Katayama et al., 2006), causing the stem cells to stay within the marrow. Suppression of SDF1α signaling in the bone marrow enhances mobilization of progenitor cells into the circulation (Katayama et al., 2006). Early investigations focused on the link between CXCR4 and migration of hematopoietic stem cells and circulating muscle stem cells to bone marrow and muscle tissue, respectively (Pituch-Noworolska et al., 2003; Ratajczak et al., 2003), suggesting that expression of CXCR4 allows cells to home to specific tissues. Germ cell migration during embryogenesis is also regulated by SDF1α (Doitsidou et al., 2002; Molyneaux et al., 2003; Stebler et al., 2004). SDF1α also modulates negative regulators of axon guidance in effector motor neurons and is necessary for proper axonal path-finding (Chalasani et al., 2003; Chalasani et al., 2007). Therefore CXCR4 and SDF1α are capable of regulating cell migration.

CXCR4 and SDF1α also regulate muscle cells on a number of levels, although current literature contains conflicting evidence for the role of CXCR4 and SDF1α during myogenesis. During embryogenesis, muscle progenitor cells express CXCR4, and SDF1α is expressed by cells along the pathway from

somites into limb buds and head muscles (Vasyutina et al., 2005). Loss of CXCR4/SDF1α signaling during embryogenesis yields a decreased number of muscle cells, and smaller myofibers in both mice and zebrafish (Chong et al., 2007; Odemis et al., 2005). Some investigators also noted a decrease in immunostaining of muscle sections for desmin and myogenin, markers for muscle cells (Odemis et al., 2005). Unfortunately, quantification of muscle proteins was not performed, although these researchers hypothesized that differentiation of muscle cells could be impaired by loss of CXCR4/SDF1α signaling. Later studies, utilizing the immortalized C2C12 muscle cell line, indicated SDF1α enhances migration and proliferation of these cells *in vitro* (Odemis et al., 2007). Studies on C2C12s also suggest decreased expression of differentiation-specific muscle proteins, such as MyoD, myogenin and myosin heavy chain, at both the protein and mRNA levels (Odemis et al., 2007). Interestingly, this study suggests an almost complete abrogation of muscle cell differentiation, although only 15% of C2C12's express CXCR4 *in vitro* (Odemis et al., 2007). No hypotheses were suggested for how the loss of SDF1α decreases differentiation in cells that do not express the CXCR4 receptor. Another study, using similar cell lines, suggests that loss of CD164, a cell surface sialomucin that targets CXCR4 to endosomes and lysosomes via its intracellular region, is necessary for proper migration and myotube formation, and not proliferation of muscle cells (Bae et al., 2008). Interestingly, $CXCR4^{+}$ muscle stem cells are more efficient engrafters into host muscle during transplantation (Cerletti et al., 2008), and mesoangioblasts pre-treated with SDF1 are more effective at

engrafting into host muscle (Galvez et al., 2006). These data suggest the CXCR4/SDF1 axis may enhance adult myogenesis *in vivo*, possibly through increasing migration of the muscle cells. In order to determine the processes during adult regenerative myogenesis regulated by CXCR4 and SDF1α, many of these assays should be repeated using primary muscle cells or adult muscle tissue as was performed and discussed later in this dissertation.

Cell-cell adhesion during myogenesis

Fusion of muscle cells requires several important processes including cell-cell recognition and adhesion. Prior to undergoing fusion, differentiated myocytes recognize and adhere to one another and to nascent myotubes. Although several adhesion molecules have been suggested to function in this process (Charlton et al., 2000; Dickson et al., 1990; Knudsen et al., 1990a; Knudsen et al., 1990b; Ruiz-Gomez et al., 2000), the precise relationship between these molecules remains elusive. Early studies indicated both calcium-dependent and calcium-independent fusion molecules are necessary for cell-cell adhesion, although specific molecules were not identified at this time (Gibralter and Turner, 1985; Knudsen and Horwitz, 1977). Later studies have determined that calcium-independent adhesion is associated with the neural cell adhesion molecule (NCAM), which is expressed by muscle cells and mediates muscle cell interactions necessary for myotube formation (Knudsen et al., 1990a). Muscle cells that over-express a specific glycosylphosphatidylinositol-linked NCAM isoform display increased fusion compared to controls (Dickson et al., 1990).

Interestingly, although NCAM can enhance fusion, loss of NCAM did not reduce myoblast fusion (Charlton et al., 2000), and therefore is not essential for proper myogenesis.

In contrast, another set of molecules are associated with the calcium-dependent adhesion, such as the glycoprotein N-cadherin, which regulates myoblast adhesion, and loss of N-cadherin inhibits myotube formation (Knudsen et al., 1990b). Loss of M-cadherin, a close family member of N-cadherin has no affect on myotube fusion, suggesting that N-cadherin may have a specific role in muscle regeneration (Hollnagel et al., 2002). Cadherins form a complex at the plasma membrane with several other promyogenic Ig superfamily members, such as CDO (CAM-related/down-regulated by oncogenes) and BOC (brother of CDO) (Kang et al., 2004). This complex also contains neogenin, a receptor for the secreted factor netrin-3, which regulates myoblast differentiation and myotube formation (Kang et al., 2004). Another group of adhesion molecules associated with muscle are integrins, which although they normally associated with adhesion to the extracellular matrix, can regulate cell-cell adhesion, both integrin very late antigen-4 (VLA-4) and its counter receptor vascular cell adhesion molecule-1 (VCAM-1) are expressed by muscle cells. VLA-4 expression is specific to forming myotubes, whereas VCAM-1 is present on both myocytes and myotubes (Rosen et al., 1992). Loss of either protein inhibits fusion of muscle cells into myotubes. The recognition and adhesion of myocytes prior to fusion likely involves several adhesion molecules, and the interplay between such molecules should be further investigated in the future.

Characteristics and functions of olfactory receptors

The mammalian olfactory receptor (OR) family was first discovered in the early 1990s and is comprised of G-protein coupled receptors which are structurally related to one another (Buck and Axel, 1991). Later studies indicated that over 1,209 mouse OR genes exist, of which over 900 have uninterrupted open reading frames and are expected to encode functional ORs (Godfrey et al., 2004). These intact ORs are very diverse, exhibiting between 30 and 99% sequence identity and are found on 17 chromosomes in the mouse genome (Olender et al., 2008). Similar studies of the human ORs indicate that there are over 300 functional ORs in the human genome (Malnic et al., 2004). Several different nomenclatures have been used for ORs depending on the original group which identified a particular OR; for example, mouse odorant receptor 23 (MOR23) is also known as Olfr16 and MOR267-13. In this dissertation, all ORs will be referred to by the nomenclature found most often in the literature for that particular OR.

Two classes of ORs have been identified, Class I and Class II. Class I ORs were originally identified in fish (Ngai et al., 1993) and make up approximately 10% of the intact OR genes in mammalian genomes (Buettner et al., 1998; Bulger et al., 1999; Feingold et al., 1999; Fuchs et al., 2001; Glusman et al., 2000a; Glusman et al., 2000b; Niimura and Nei, 2007). Although some Class II ORs are expressed in fish, the expansion of Class II ORs occurred in amphibian species (Freitag et al., 1995) and are commonly referred to as mammalian ORs.

The majority of ORs investigated in this dissertation were Class II ORs however two ORs, Olfr78 and Olfr66, expressed by skeletal muscle were Class I ORs.

ORs are highly expressed in the olfactory epithelium where they function to respond to small volatile ligands we perceive as smell. ORs can be activated by multiple ligands and each ligand can interact with multiple ORs, suggesting that it is the unique combination of olfactory receptors activated by a ligand which specifies the odor (Malnic et al., 1999). Ligand binding has been mapped to transmembrane domains 3-6 which form a 3-dimensional binding pocket within the plasma membrane (Khafizov et al., 2007).

After ligand binding, olfactory receptors activate a G protein, the olfactory trimeric G protein (G_{olf}) (Belluscio et al., 1998; Jones and Reed, 1989). A specific olfactory isoform of adenylate cyclase, ACIII is then activated (Wong et al., 2000), boosting the local concentration of cyclic AMP. In olfactory neurons, cyclic nucleotide gated calcium channels are opened (Lynch and Lindemann, 1994), and the levels of intracellular calcium are drastically increased. Inactivation of ligand-bound ORs occurs through both the regulator of G protein signaling 3 (RGS3) (Norlin and Berghard, 2001) and β-arrestin (Dawson et al., 1993).

Types of ligands for ORs

Screening for OR ligands requires finding a specific system with which to assay for ligands and has been problematic. The first OR and ligand pair, rat ORI17 and octanal, was confirmed by using an adenovirus to express a single OR in rat nasal epithelium (Zhao et al., 1998), allowing the entire epithelial

muscosa to be assayed. However, this approach requires an adenoviral system, as well as many live animals. Heterologous expression systems, where the OR is expressed in cells that are easily cultured, are effective if the appropriate downstream signaling proteins are either endogenously available or co-expressed with the odorant receptor. Also, due to incorrect folding and lack of binding partners, many ORs are caught in the endoplasmic reticulum (Lu et al., 2003a; McClintock and Sammeta, 2003), and require recombinant proteins, such as the N-terminal region of rhodopsin, to effectively recruit ORs to the plasma membrane. Experiments using heterologous expression of ORs and G-proteins identified a number of mouse OR ligands, most of which are also small volatile molecules like octanal (Krautwurst et al., 1998). Later studies have also used *Saccharomyces cerevisiae*, *Xenopus laevis* oocytes, COS-7 cells and *Caenorhabditis elegans* neurons to investigate ligand/OR interactions (Minic et al., 2005; Mombaerts, 2004; Shirokova et al., 2005). Interestingly, several antagonists of OR-signaling have also been identified along with agonists, such as the antagonist undecanal for the human OR17-4 (Spehr et al., 2003). Confirmed OR/ligand interactions will allow further studies of ORs in both olfaction and in other tissues.

ORs and axon guidance

Within the olfactory epithelium, one OR is expressed per olfactory neuron, and neurons expressing the same receptor will converge into a glomerulus within the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994). Both genetic and

functional evidence suggest that ORs play a role in guidance of axons to a glomerulus. Replacement of one OR with a second OR caused convergence of axons into different glomeruli (Bozza et al., 2002; Feinstein et al., 2004; Feinstein and Mombaerts, 2004; Mombaerts, 1996; Mombaerts, 2001; Wang et al., 1998). In addition, ORs are also expressed on the axonal processes of olfactory neurons (Strotmann et al., 2004), suggesting their involvement in axon guidance. Furthermore, the canonical OR signaling proteins G_{α}olf, membrane ACIII and cAMP are all involved in axon guidance (Chesler et al., 2007; Imai et al., 2006; Zou et al., 2007). Therefore, ORs and their downstream signaling cascades may be important for the targeting of axons into specific glomeruli.

Several proteins are associated with adhesion and migration processes necessary for axon guidance, many of which are also associated with myogenesis. Loss of BIG-2, a member of the immunoglobulin superfamily (Yoshihara et al., 1995), yields loss of axon targeting into appropriate glomeruli (Kaneko-Goto et al., 2008) and is also associated with the neuromuscular junction (Compton et al., 2008). Another member of the immunoglobulin superfamily, Kirrel2, was decreased when axons are incorrectly targeted (Serizawa et al., 2006), along with ephrin, and kirrel3. Homologs of Kirrel2 are characterized as adhesive cell-recognition molecules in both the nervous system (Schneider et al., 1995; Shen and Bargmann, 2003) and myogenesis (Ruiz-Gomez et al., 2000). The immunoglobulin/fibronectin domain Robo receptor (Kidd et al., 1998) was also correlated to changes in the targeting of axons into glomeruli (Cho et al., 2007). This receptor and its ligand, Slit, are necessary for

adhesion and migration processes in myogenesis (Kang et al., 1998). Another protein family that regulates olfactory axon migration into glomeruli are the classical cadherins and protocadherins (Akins et al., 2007; Akins and Greer, 2006; Hasegawa et al., 2008). The cadherin family proteins are also known to regulate adhesion in other cell types, such as muscle (Akins et al., 2007; Akins and Greer, 2006; Brand-Saberi et al., 1996a; Hasegawa et al., 2008; Hollnagel et al., 2002; Huttenlocher et al., 1998; Knudsen et al., 1990b; Kramerova et al., 2006).

Other types of receptors and ligands are also known to regulate axon targeting. The eph and ephrin receptor tyrosine kinases (Hirai et al., 1987) are part of the olfactory axon migration into glomeruli (Hirata et al., 2001; Serizawa et al., 2006) and are necessary for migration and adhesion in muscle (Swartz et al., 2001). Also, the neuropilin/semaphorin axis regulates both axon migration and myogenesis (de Castro et al., 1999; Schwarting et al., 2000; Schwarting et al., 2004; Wu et al., 2007). Finally, the chemokine receptor/ligand pair SDF1α/CXCR4 is necessary for both axonal targeting in olfaction (Miyasaka et al., 2007), and migration during muscle formation as discussed earlier (Odemis et al., 2007). Overall, these data suggest proteins which regulate migration and adhesion are conserved in multiple tissues, including olfactory epithelium and muscle.

Ectopic expression of ORs

For many years the expression of ORs was assumed to be exclusive to the olfactory epithelium. One of the first indications that ORs may not be restricted to the olfactory epithelium, was transient expression of a putative OR in the notochord of developing chick embryos (Nef and Nef, 1997). Another group reported expression of ORs, G_{α}olf and adenylyl cyclase in the heart (Ferrand et al., 1999). The evidence for global OR expression is more recent (Feldmesser et al., 2006), and suggests that ORs can be found in the majority of mammalian tissues. Microarrays indicating the widespread expression of ORs suggest that these proteins are not restricted to olfaction but also may regulate functions in other tissues.

ORs appear to have a homeostatic function in the kidney, as loss of OR signaling correlates to significantly decreased glomerular filtration rate and decreased levels of renin in the plasma (Pluznick et al., 2009). At least six ORs are expressed by the kidney: Olfr78, Olfr90, Olfr1373, Olfr1392, Olfr1393, and Olfr NP_TR6JSE50FPA). The necessary signaling proteins, ACIII and G_{α}olf are also found within the distal tubules of nephrons. Unfortunately, no functions for specific ORs were identified within the kidney.

A human OR, OR51E2, was also known as the prostate-specific G-protein coupled receptor (PSGR) due to its reported upregulation in prostate cancer (Neuhaus et al., 2009). A synthetic ligand for the receptor has been identified as the odorant β-ionone. Activation of OR51E2 in prostate cancer cells by this ligand yielded an increase in intracellular Ca^{2+}, activation of members of the MAPK

family and inhibition of cell proliferation. Ligands for OR51E2 might be potential candidates for prostate cancer treatment. However, no function was identified for the OR51E2 within normal prostate cells.

ORs and sperm

Most work on ORs outside of the olfactory epithelial has focused on the expression of ORs in testis and sperm. The first evidence for expression of ORs in mammalian sperm came from studies of dog sperm and testis (Parmentier et al., 1992; Vanderhaeghen et al., 1993). Later studies of 5' UTRs in OR mRNAs suggested that distinct promoters are used for expression of ORs in testis and the olfactory epithelium (Asai et al., 1996; Spehr et al., 2004a). However, only expression of the ORs was determined and functions were unknown at this time.

Later studies have determined a role for ORs in sperm chemotaxis, as activation of the human olfactory receptor 17-4 (hOR17-4) by bourgeonal causes chemotaxis of sperm up a gradient of the odorant (Spehr et al., 2003), indicating the first function for ORs in sperm. Investigations into mouse ORs indicated that the mouse olfactory receptor 23 (MOR23) is expressed in testis and sperm (Fukuda et al., 2004). The synthetic ligand for MOR23, lyral, caused elevation of intracellular Ca^{2+} levels in both HEK cells expressing MOR23 and sperm cells (Touhara et al., 1999). Activation of MOR23 by lyral induced sperm chemotaxis, likely through these affects on Ca^{2+} (Fukuda et al., 2004). Using sperm from transgenic mice over-expressing MOR23, lyral-induced sperm chemotaxis was deemed to be specific to MOR23. Although none of these studies have indicated

an *in vivo* ligand or an *in vivo* function for ORs in sperm, investigators hypothesize that cumulous cells surrounding an egg may secrete a ligand (Spehr and Hatt, 2004; Spehr et al., 2004a), which regulates chemotaxis of sperm to fertilizable eggs.

The OR signaling mechanisms were also found to be expressed by sperm. For example, adenylyl cyclases, a component for OR signaling, are expressed by sperm and necessary for their chemotaxis (Livera et al., 2005; Spehr et al., 2003; Spehr et al., 2004b). Previous investigations suggest that sperm chemotaxis is activated though Ca^{2+} and cAMP-dependent effects on microtubule sliding (Si and Okuno, 1995), both of which were increased with OR activation. Proteins that inhibit downstream signaling from ORs, such as β-arrestin2, are also expressed by testis and sperm (Walensky et al., 1995). Further studies of hOR17-4 revealed down-regulation of the receptor after activation by β-arrestin2; after binding with hOR17-4, β-arrestin2 translocates into the sperm nucleus, whereupon it may affect transcription post-fertilization (Neuhaus et al., 2006), indicating a role for OR signaling cascades in regulating transcription of downstream genes. These studies suggest that ORs may be capable of canonical OR signaling in testis and sperm and affecting both chemotaxis and transcription.

As ORs are known to regulate migration, both in axons of olfactory neurons and sperm, we further investigated the role of ORs in adult regenerative myogenesis.

Chapter 3: Materials and Methods

Animals and muscle injuries

Adult mice between 8–24 weeks of age were used and handled in accordance with the institutional guidelines of Emory University. To induce regeneration, gastrocnemius muscles of male C57BL/6 mice were injected with 40μL of 1.2% $BaCl_2$ (O'Connor et al., 2007) and collected as described for RNA, protein, flow cytometry, cross-sectional area, or single myofiber analysis (see specific sections below).

Primary muscle cell culture, differentiation and fusion assays

Primary myoblasts were derived from the hindlimb muscles of Balb/C mice, except when percoll gradient was used, then C57 mice. For cell culture, all muscles were isolated, for flow cytometry or immunochemistry, gastrocnemius muscles were isolated post-injury. Cells isolated from muscle by mechanical and enzymatic dissociation. Muscles were excised, separated from fat and excess connective tissue, minced into a coarse slurry, and then digested for 1 hour with 0.1% pronase (Calbiochem), or collangase class II (1%, Sigma) and dispase grade II (2.4U/mL, Sigma) in DMEM at 37°C with mild agitation. Pronase digestion was used for all experiments except for analysis of CXCR4, where collagenase and dispase were used to decrease digestion of epitopes. The digest was then mechanically dissociated by repeated trituration followed by filtration through a 100 μm vacuum filter (Millipore).

For flow cytometry or immediate immunostaining, the filtered digest was centrifuged through an isotonic Percoll gradient (60% overlaid with 20%) as

described (Yablonka-Reuveni et al., 1987). For immunostaining, mononucleated cells were collected from the Percoll interface, resuspended in Ham's F10 supplemented with 20% fetal bovine serum, 5 ng/mL bFGF, 100 U/mL penicillin G, and 100 µg/mL streptomycin, and seeded onto collagen-coated Permanox chamber slides (Fisher Scientific). After 24 hours, cells were washed with PBS and fixed for 10 min in 3.7% formaldehyde. For immunostaining protocols, see below.

For primary cell culture, cells were suspended in growth medium (GM: Ham's F10, 20% FBS, 5 ng/ml bFGF, 100 U/ml penicillin G, 100 µg/ml streptomycin) and grown on collagen-coated dishes in a humidified 5% CO_2 incubator at 37° C. Primary cultures were enriched for myogenic cells to >99% purity using the preplating technique as described previously and by MyoD immunostaining (Jansen and Pavlath, 2006).

Primary myoblasts were seeded on entactin-collagen IV-laminin (E-C-L; Upstate Biotechnology) coated 6-well dishes at a density of 2 x 10^5 cells/well in GM. Cells were allowed to adhere to the dish for approximately 1 hr before switching to DM (DM: DME, 1% Insulin-Transferrin-Selenium-A supplement [Invitrogen], 100 U/mL penicillin G and 100µg/mL streptomycin). Following 24, 48, or 72 hrs in DM, cells were fixed in 3.7% formaldehyde for 10 min and subsequently immunostained with an antibody against embryonic myosin heavy chain (eMyHC, F1.652, Developmental Studies Hybridoma Bank) as described previously (Horsley et al., 2001). The differentiation index was determined by dividing the total number of nuclei in eMyHC-positive cells by the total number of

nuclei counted. The average number of nuclei per myotube was determined by dividing the total number of nuclei in myotubes (≥2 nuclei) by the total number of myotubes counted. The fusion index was determined by dividing the total number of nuclei in myotubes by the total number of nuclei counted. AMD3100 (Sigma) was dissolved in PBS and used at 10 μM in DM. At least 100 myotubes and 500 nuclei per condition were analyzed for each assay.

Cell proliferation analysis

To assess cell proliferation, differentiating myotubes were incubated in 25 μM 5-Bromo-2'-deoxyuridine (BrdU, Sigma) for 24 hrs. Cells were subsequently fixed in 2% paraformaldehyde and immunostained using a BrdU antibody (Accurate Chemical and Scientific Corp) at a dilution of 1:500. At least 500 myoblasts from three independent isolates were analyzed for each condition.

Cell mixing experiments

Cell mixing experiments were performed as described previously with minor modifications. Primary myoblasts were grown at low density ($0.5x10^5$ cells per well of a 6 well plate) or high density ($2x10^5$ cells per well of a 6 well plate) in DM for 24 hrs to generate differentiated mononucleated cells or nascent myotubes, respectively. Nascent myotubes were incubated with CellTracker Orange CMTMR (5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethylrhodamine) (Molecular Probes) diluted in DM (2.5 μM) and mononucleated cells were incubated with CellTracker Green CMFDA (5-chloromethyl-7-hydroxycoumarin)

(Molecular Probes) diluted in DM (0.5 μM) for 10 mins at 37°C. Cells were washed twice with PBS, trypsinized, mixed at equal cell number, and plated to give a final cell number of $2x10^5$ cells per well of a 6 well E-C-L-coated plate. Following 24 hrs in DM, the cells were fixed for 10 mins in 3.7% formaldehyde. The presence of dual label was analyzed in 50-100 myotubes with ≥3 nuclei. Mixing experiments were performed in triplicate from three independent cell isolates.

Plasmid production and retroviral infection

Oligonucleotides (Ambion; MOR23 2: sense 5' GATCCCCGGAACAAACAACTC AAAGATTCAAGAGATCTTTGACTTCTTTGTTCCTTTTTA, antisense 5' AGCTT AAAAAGGAACAAAGAAGTCAAAGATCTCTTGAATCTTTGACTTCTTTGTTCCG GG; MOR23 3: sense 5' GATCCCCGGCCAGAAGAAAGCCTTGTCAAGAGACA AAGGCTTTCTTCTGGCCTTTTTA, antisense 5'AGCTTAAAAAGGCCAGAAGA AAGCCTTTGTCTCTTGAACAAAGGCTTTCTTCTGGCCGGG) encoding siRNA (underlined) to MOR23 were cloned into the retroviral plasmid pSUPER.retro.puro according to OligoEngine protocol. The control plasmid contained a scrambled siRNA sequence. Retroviral production and infection were performed as described (Abbott et al., 1998) and cells were used 48 hrs later in experiments. The efficiency of retroviral-mediated gene transfer was >95%, based on cell survival in the presence of puromycin following two rounds of retroviral infection.

To generate a MOR23 retroviral expression vector, a cDNA fragment

encoding a FLAG-Rho-MOR23 was subcloned from the pME18S construct (Katada et al., 2003) into the retroviral vector pTJ84 using the AvrII and SalI/XhoI sites (Abbott et al., 2000). pTJ84 was used as the control vector.

Semi-quantitative RT-PCR

RNA was isolated using Trizol Reagent (Life Technologies) from muscle cells. RT-PCR was performed for each sample using primers specific for MOR23 (Fukuda et al., 2004) (accession number: NM_008763.1) (sense, 5′-TCCATAGA ACAGAATGCAGAG-3′; antisense, 5′-GCTTGAGCTAAAGTTCTCCTG-3′). All RT reactions were performed using 2.5 µg of total RNA. 18S rRNA was used as control for each sample using QuantumRNA 18S primers (Ambion). MOR23 cDNA was amplified using Expand High Fidelity PCR system (Roche) by incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and termination at 72°C for 5 min. Then, 5 µl of the initial PCR reaction was removed, and PCR reagents were replenished for a second round of amplification. A portion of total RNA was also tested by PCR for DNA contamination, using the same protocol. The amplicons were resolved by electrophoresis on a 1.5% agarose gel and visualized by ethidium bromide staining.

Real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies). All RNA was DNase treated (Invitrogen) and a portion of DNase-treated RNA was reverse

transcribed. Real-time PCR was performed, and results were analyzed by using the iCycler iQ Real-Time Detection System and software (Bio-Rad). cDNA (1µl from each sample) was amplified by using gene-specific primers (primer sequences acquired from SABiosciences are proprietary, see Table 5.1 for catalog numbers and accession numbers) in a 25µl reaction containing the appropriate primer pair and iQ SYBRgreen Supermix (Bio-Rad). Samples were incubated at 95°C for 4 min, followed by 40 cycles (30 s each) of denaturation, annealing, and extension at 95°C, 55°C, and 72°C, respectively. SYBRgreen fluorescence was measured at the end of the extension step of each cycle. Amplification of 18S ribosomal RNA using QuantumRNA primers (Ambion) was used as an input control for all reactions. All reactions were run in duplicate or triplicate, and PCR product size was verified by melt curve analysis.

Real-time PCR reactions were also performed on 2µL of each DNase-treated RNA sample to confirm the absence of contaminating genomic DNA. Samples were considered to be uncontaminated if the C_T value of the DNase-treated RNA was either above 35 cycles or more than 5 cycles from the C_T value of the cDNA. All samples were normalized to 18S rRNA due to the extreme variability in most genes during myogenesis and muscle regeneration, although similar results were obtained with use of HPRT for normalization. MOR23 mRNA was quantified in reference to MOR23-pME18S plasmid standard.

For SABiosciences chemokine array, total RNA was isolated using TRIzol reagent (Life Technologies). All RNA was DNase treated (Invitrogen) and a portion of DNase-treated RNA was reverse transcribed. Real-time PCR was

performed, and results were analyzed by using the iCycler iQ Real-Time Detection System and software (Bio-Rad). cDNA (1 µl from each sample) was amplified by using gene-specific primers in a 96-well SABiosciences chemokine array (Chemokines & Receptors PCR Array, Mouse, PAMM-022) and iQ SYBRgreen Supermix (Bio-Rad) in a 25 µl reaction. Samples were incubated at 95°C for 4 minutes, followed by 40 cycles (30 seconds each) of denaturation, annealing, and extension at 95°C, 55°C, and 72°C, respectively. SYBRgreen fluorescence was measured at the end of the extension step of each cycle. All reactions were run in triplicate, and PCR product size was verified by melt curve analysis. All samples were normalized using Hypoxanthine guanine phosphoribosyl transferase 1 (HPRT).

Flow cytometry

To determine level of MOR23 mRNA in muscle cells, FACS was performed as described (Kafadar et al., 2009) using cells isolated from day 5 regenerating gastrocnemius muscles. Mononucleated cells were isolated from the gastrocnemius muscles of 6 week old male C57BL/6 mice at day 5 (n = 7 for each time point) following $BaCl_2$ injection, dissociated, and immunostained with antibodies to CD31 and CD45 (FITC), and alpha-7-integrin (PE). Two populations were isolated: $CD31^{-}CD45^{-}$ alpha-7-integrin^{+} and $CD31^{+}CD45^{+}$ alpha-7-integrin^{-}. Propidium iodide staining was used to gate out dead cells. Cells were sorted in a BD FACSVantage SE and placed into Trizol Reagent (Invitrogen) for RNA isolation.

To analyze proliferation during regeneration, the gastrocnemius muscles of C57B/6 mice were induced to regenerate by $BaCl_2$ injection and electroporated with control or MOR23 siRNA two days later (see below for electroporation details). Mice were given twice daily injections of BrdU (100μg/gm weight) starting the day after electroporation to allow the siRNA time to knockdown MOR23. Either 3 or 5 days post-electroporation the muscles were harvested, and mononucleated cells isolated as described, with red blood cells removed using a Percoll gradient (Kafadar et al., 2009). Cells were immunostained using FITC-conjugated α-CD45 (1:100; BD Biosciences), FITC-α-CD31 (1:100; eBiosciences), and PE-conjugated α-alpha7-integrin (1:200; gift of Fabio Rossi, University of British Columbia). The BrdU Flow kit (BD Biosciences) was used to immunostain for BrdU (APC). Propidium iodide was included to gate out dead cells. The percentage of $\alpha7\text{-integrin}^{+}CD31^{-}CD45^{-}$ cells that was $BrdU^{+}$ was calculated. For each sample, 10,000 cells were analyzed on a FACSCalibur (Becton-Dickinson), and propidium iodide was used to gate out dead cells.

To analyze CXCR4 expression *in vitro* by flow cytometry, primary myoblasts were immunostained with anti-CXCR4-APC antibody (1:100; BD Pharmigen) and analyzed on a FACSCalibur (Becton-Dickinson). For analysis of CXCR4 expression during regeneration, mononucleated cells were dissociated from gastrocnemius muscles of mice at the indicated times after $BaCl_2$ injection (N=4 for each time point), and immunostained with antibodies to CD31-FITC (1:100; eBiosciences), CD45-FITC (1:100; BD Biosciences), alpha-7-integrin-PE (1:200) and CXCR4-APC (1:100; BD Pharmigen). $CD31^{-}CD45^{-}$ cells were analyzed for

alpha-7-integrin-PE (1:200; gift of Fabio Rossi, University of British Columbia) and CXCR4 expression.

For analysis of p21 expression during regeneration, mononucleated cells were dissociated from gastrocnemius muscles of mice at the indicated times after $BaCl_2$ injection (N=10 for each time point), fixed with cold 70% ethanol overnight at -20°C and immunostained with antibodies to CD31-APC (1:100; eBiosciences), CD45-APC (1:100; BD Biosciences), alpha-7-integrin-PE and p21 (1:100; Lifespan Biosciences). To detect p21, cells were incubated with biotin-conjugated donkey anti-goat (1:100; Jackson ImmunoResearch Lab., Inc.) for 20 min, then FITC-conjugated strepavidin (1:100; Jackson ImmunoResearch Lab., Inc.) for 20 min. $CD31^{-}CD45^{-}$ cells were analyzed for alpha-7-integrin and p21 expression (N=10 for each time point). For each sample, 10,000 cells were analyzed, and propidium iodide was used to gate out dead cells. Isotype controls were used to determine gating. All data analysis was performed using FlowJo v. 6.2.1 (TreeStar, Inc.).

Immunostaining

MOR23 immunostaining was performed using a TSA Rhodamine Tyramide Signal Amplification kit (PerkinElmer). Gastrocnemius muscles isolated 5 days post-injury were fixed for 48 hrs in 4% paraformaldehyde (PFA) and cryoprotected overnight in 20% sucrose at 4°C before sectioning. Alternatively, $\alpha7\text{-integrin}^{+}CD31^{-}CD45^{-}$ muscle cells isolated from gastrocnemius muscles by

FACS were plated then fixed in 4% PFA for 10 minutes. Sections or cells were treated with 3% H_2O_2, citric acid retrieval buffer (90°C), biotin/strepavidin blocking kits (Vector Lab, Inc.), mouse IgG (M.O.M kit, Vector Labs Inc.) and then blocking buffer containing 4% BSA in PBS for 1 hour. Afterwards, muscle sections or cells were incubated overnight at 4°C with MOR23 antiserum or purified rabbit IgG (Genetex Inc.) diluted 1:100 in blocking buffer. Following successive washes in PBS with 0.1% BSA, cells or sections were incubated with biotin-conjugated donkey anti-rabbit $F(ab)_2$ fragments (Jackson ImmunoResearch Lab., Inc.) diluted 1:200 in PBS with 4% BSA for 1 hour. Following repeated washes in 0.1% BSA, the cells or sections were incubated in HRP-conjugated streptavidin diluted 1:200 in TNB (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl and 0.5% blocking reagent) for 30 minutes followed by TSA Rhodamine, diluted 1:200 in amplification diluent for 5 minutes. Nuclei were counterstained with 25 μM 4,6-diamidino-2-phenylindole (Ottonello et al.) in 0.1% BSA. All staining was performed at room temperature unless stated otherwise. Fluorescence images were acquired using an Axiovert 200M microscope (ZEISS) with a 0.3 NA 10X ZEISS Plan-Neofluar objective and Qimaging camera with OpenLab (version 3.1.4) software. Images were assembled using Adobe Photoshop (version 7.0) software and were not modified with the exception of equal adjustments in size, brightness, and contrast.

Myogenin and eMyHC immunostaining were performed using a VectaStain kit (Vector labs). α7-integrin$^+$CD31$^-$CD45$^-$ muscle cells isolated from gastrocnemius muscles by FACS were plated then fixed in 4% PFA for 10 minutes. Cells were

treated with 3% H_2O_2, biotin/strepavidin blocking kits (Vector Lab, Inc.), mouse IgG (M.O.M kit, Vector Labs Inc.) and then blocking buffer containing 4% BSA in PBS for 1 hour. Afterwards, cells were incubated overnight at 4°C with anti-myogenin (diluted 1:10 in blocking buffer, F5D, developed by W. Wright, cells were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242), or anti-eMyHC supernatant (F1.652; Developmental Studies Hybridoma Bank) or appropriate IgG (diluted 1:100 in blocking buffer, Genetex Inc.). Following successive washes in PBS with 0.1% BSA, cells were incubated with donkey anti-mouse IgG (Jackson ImmunoResearch Lab., Inc.) diluted 1:200 in PBS with 4% BSA for 1 hour. Following repeated washes in 0.1% BSA in PBS, the cells were incubated in HRP-conjugated streptavidin (VectorLabs) for 30 minutes followed by visualization with diaminobenzidene (DAB). All immunostaining was performed at room temperature unless stated otherwise.

Immunoblotting

Muscles were harvested in RIPA-2 buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitors (Mini Complete; Roche). Lysates were spun at 21,000x g for 15 min at 4ºC. Protein concentration was determined using the Bradford assay (Bio-Rad) and total protein was separated by SDS-PAGE. Following transfer to a PVDF membrane (Millipore), specific proteins were detected using appropriate primary

antibodies (α-MOR23 at 1:500, GeneTex; α-G_{olf}, α-mACIII and α-tubulin at 1:5,000, Abcam, Inc.; myogenin at 1:10, F5D, developed by W. Wright, Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) and detected with appropriate secondary HRP-conjugated antibodies diluted at 1:5,000 (Jackson ImmunoResearch Lab., Inc.) (Friday and Pavlath, 2001). Membranes were stained with Ponceau (Bio-Rad) to confirm equal loading.

Cell surface biotinylation was performed as described (Salazar and Gonzalez, 2002). Sulfo-NHS-SS-Biotin was used at 500μM (Pierce) and protein was isolated in RIPA-2. Immunoprecipitation was performed with NeutrAvidin Agarose Resins (Pierce) with 300μg of protein at 4°C overnight.

SDF1α ELISA assay

SDF1α was detected using the ELISA Kit for Mouse SDF1α kit (RayBiotech, Inc.). Conditioned media was isolated as above. Crushed muscle extract (Pimorady-Esfahani et al.) was created as described (Chen and Quinn, 1992) using gastrocnemius muscles from C57BL/6 mice (N=10). Briefly, the muscles were dissected, pressed 7–10 times with forceps, pooled, and incubated in TBS (Tris-buffered saline; 20 mM Tris pH 7.6, 137mMNaCl; 1 mL of TBS was used for the muscles of each mouse) for 90 minutes at 4 °C on a rotator. The extract was centrifuged at 176,000 ×g for 30 minutes followed by filtration through a 0.2 μm filter and stored at -80°C. Protein concentration was determined using the

Bradford assay (Bio-Rad) and equal amounts of protein were used.

Transfection with RNAi

Primary myoblasts were seeded onto 6-well plates ($1.5x10^5$ cells/well) and transfected with 30 nM each of 3 control scrambled siRNAs or 3 siRNAs targeting the OR of interest (Stealth siRNA; Invitrogen) using Lipofectamine 2000 (Invitrogen). Myoblasts were used in experiments 24 hrs after transfection.

Cell migration and adhesion assays

Migration of muscle cells was quantified using time-lapse microscopy as described (Jansen and Pavlath, 2006). Images were recorded (QImaging Camera and OpenLab 3.1.4 software) every 5 min for 3 hours. Cell velocities were calculated in μm/hr using ImageJ software by tracking the paths of mononucleated cells. Approximately 20 mononucleated cells were tracked for each experiment.

Migration analysis using Boyden chambers

Boyden chambers were performed as described (Mylona et al., 2006). Primary myoblasts were seeded on 150-mm plates at low density ($9 X 10^5$ cells/plate) and switched to DM for 24 hrs to generate myocytes in the absence of myotube formation. Cells ($7.5 X 10^4$ cells in 200μL DM) were loaded in the upper wells of the Boyden chamber and incubated at 37°C for 5 hrs. Migrated cells were fixed, stained and counted. HGF and PDGF were used at 100 ng/mL in DMEM with 1%

BSA, SDF1α at 10-200 ng/mL in DM (Sigma). To prepare CM, myoblasts were incubated in DM for 24 hrs; the media, which had been "conditioned" with secreted factors, was then collected, filtered (0.45 μm), flash frozen, and stored at -80°C until use. Crushed muscle extract was created as described (Chen and Quinn, 1992) using gastrocnemius muscles from C57BL/6 mice (N=10) and stored at -80°C.

Dunn chamber analysis of directional migration

Dunn chambers were performed as described (Jansen and Pavlath, 2006). Permanox plastic cell culture slides (Nunc) were cut into 6-cm^2 squares, and an ~ 1-cm^2 region of each slide was coated with E-C-L for 1 h at 37°C. Primary myoblasts were then seeded at a density of 5 x 10^3 cells per slide in GM. Cells were allowed to adhere for 1 h, and GM was replaced with DM. The low density at which the cells were plated ensured that cells underwent myogenic differentiation with limited cell fusion. After 24 h in DM, the Dunn chamber was assembled as described previously (Zicha et al., 1991). To set up gradient experiments, both concentric wells of the chemotaxis chamber were filled with control DM (supplemented with 25 mM Hepes), and the slide containing differentiating cells was inverted onto the chamber to cover both wells. The slide was sealed onto the chamber with a hot 1:1:1 mixture of paraffin wax, beeswax, and petroleum jelly, leaving a small slit of the outer well open. DM was removed from the outer well and replaced with DM or 10^{-7}M lyral in DM, and the slit was sealed. SQ22536 (Sigma) was used at 2.5mM. After allowing the gradient to

establish for 30 min at 37°C, a small region over the annular bridge was visualized and cell migration was analyzed by time-lapse microscopy as above. Statistical analyses of directional data were performed to assess the chemotactic response of the cells as described previously (Zicha et al., 1997). Each cell path was converted to a trajectory originating from (0,0) on an x-y axis. A horizon distance for each condition was established by determining the distance passed by 50% of the cells in a straight line from their starting point. The horizon method is designed to assess the directionality of cell movement without influence from differences in cell motility. Cells that fail to reach the horizon distance were excluded from directional analysis. A trajectory angle for each cell was calculated as the direction of each cell from its starting point to the point at which the cell crossed the horizon distance. The directional data were summarized as circular histograms in which the area of each sector represents the proportion of trajectory angles located within each 18° interval. The Rayleigh test for unimodal clustering was applied with $P < 0.05$ as the criterion for rejecting the null hypothesis of uniform distribution. Where unimodal clustering was observed, a mean direction and 95% confidence interval were calculated. Statistical analysis was performed using Oriana 2.0 (RockWare). Dunn chamber assays were performed using three independent cell isolates and at least 15 cells per assay.

Cell-cell adhesion analysis

Cell-cell adhesion in suspension was analyzed by incubating myocytes at $2x10^5$ cells in 2mL of DM after lifting using Cell Dissociation Buffer Enzyme-Free

PBS-Based (Invitrogen). SQ22536 (Sigma) was used at 2.5mM. Duplicate 50µL aliquots of cells were taken at regular intervals for 60 min. This cell concentration was used to permit counting of individual cells within clusters using phase-contrast microscopy. Trypan blue staining was used at 60 min to determine cell viability.

In vivo electroporation

Gastrocnemius muscles of C57BL/6 mice were injected with $BaCl_2$ to induce muscle regeneration. Two days later the muscles were injected with the indicated plasmid (25µg DNA; 1µg/µL in PBS) and electroporated as described (O'Connor et al., 2007). Intramuscular injection of 25 µg DNA (1 µg/µl in PBS) was accompanied by several square wave pulses (200 V/cm) generated by a Grass S8800 stimulator. Pulse duration of 50 milliseconds and pulse interval of 270 milliseconds were used. The electrodes were a two-needle array fixed 5 mm apart (BTX) and inserted into the muscles. Post-electroporation, muscles were collected and homogenized in RIPA-2 for protein isolation or frozen and sectioned as described (Abbott et al., 1998). In preliminary experiments, electroporation of a lacZ plasmid was utilized to determine efficiency of gene delivery to $MyoD^+$ cells in the gastrocnemius muscle; 70% of adherent cells were $lacZ^+$, 80% were $MyoD^+$. Breifly, cells were stained with X-gal (5-bromo-4-chloro-3-indolyl-beta-Dgalactopyranoside, 1 mg/mL in 5 mM K3Fe(CN)6, 5 mM K4Fe(CN) 3H2O, 2 mM MgCl2 in PBS) and the percentage of β-galactosidase+ cells determined. Serial 14µm cross-sections were stained with hematoxylin and

eosin and analyzed by quantifying myofiber number and cross-sectional area (XSA) for three 307,200μm^2 fields in the core of the regenerating muscle (O'Connor et al., 2007). All analysis were performed blinded with N=4-8 mice per condition and 90-100 myofibers per mouse.

Single Myofiber Isolation

Single myofibers were isolated from gastrocnemius muscles as described (Mitchell et al., 2005). Briefly, the gastrocnemius was dissected and digested in DMEM containing 25 mM HEPES and 0.1% collagenase (type I, Worthington) for 90 min with gentle agitation. Single myofibers were extracted individually into 24-well plates pre-coated with 10% growth factor reduced Matrigel (BD Biosciences). Following plating, myofibers were centrifuged at 1100 ×g to facilitate adhesion to the Matrigel and fixed immediately upon plating with 3.75% formaldehyde, and DAPI-stained.

Statistics

To determine significance between two groups, comparisons were made using Student's *t*-tests or the Mann Whitney test (*in vivo*). Analyses of multiple groups were performed using a one-way or two-way analysis of variance with Bonferroni's posttest as appropriate. Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad). For all statistical tests, a confidence interval of $p < 0.05$ was accepted for statistical significance.

Chapter 4: Myoblasts versus myocytes paper

Data presented in this chapter are published as:

Contributions:

K. K. Long assisted with isolation and FACS analysis of mono-nucleated muscle cells (Figures X.Y and X.Y).

Introduction

Skeletal muscle degeneration can occur due to disease or injury; however, this tissue has an extensive ability to regenerate. Adult regenerative myogenesis is dependent on progenitor cells called satellite cells. Satellite cells are normally quiescent, but proliferate in response to injury, and their progeny myoblasts differentiate into fusion-competent myocytes, which fuse with one another or with existing myofibers to restore normal tissue architecture. In vitro studies demonstrate that migration is a key process during myogenesis. Migration is critical to achieve cell-cell adhesion, which is necessary for differentiation (Kang et al., 2004), as well as formation and growth of myotubes in vitro (Bae et al., 2008; Jansen and Pavlath, 2006; Mylona et al., 2006; O'Connor et al., 2007). Identifying molecules which regulate cell migration may reveal potential molecular targets for improving muscle regeneration and the efficiency of cell transplantation therapies (Galvez et al., 2006; Hill et al., 2006; Palumbo et al., 2004).

A number of extracellular molecules are known to regulate muscle cell migration in vitro. Secreted factors such as hepatocyte growth factor, fibroblast growth factor, platelet-derived growth factor, and IL-4 play key roles during myogenesis (Bischoff, 1997; Corti et al., 2001; Horsley et al., 2003; Lafreniere et al., 2006; Lee et al., 1999; Robertson et al., 1993; Villena and Brandan, 2004). In addition, extracellular matrix (ECM) proteins and ECM associated molecules, such as laminin, fibronectin, CD44, decorin and N-cadherin, as well as matrix-metalloproteinases, are critical for regulating cell migration during myogenesis

(Brand-Saberi et al., 1996a; Echtermeyer et al., 1996; Lluri and Jaworski, 2005; Lluri et al., 2008; Mylona et al., 2006; Ocalan et al., 1988; Olguin et al., 2003; Yao et al., 1996). Overall, a complex interplay among many types of proteins are required for proper migration of muscle cells.

Chemokines are secreted proteins, approximately 8-to-10-kd in size, with 20 to 70% homology in amino acid sequences, that share both leukocyte chemoattractant and cytokine-like behaviors (Baggiolini et al., 1995; Luster, 1998). Chemokines are important for the migration of muscle precursor cells during embryonic myogenesis (Vasyutina et al., 2005; Watchorn et al., 2001) and for macrophage infiltration into damaged muscle tissue (McLennan, 1996; Robertson et al., 1993). Furthermore, chemokines and their receptors are expressed by diseased or regenerating muscle tissue (Civatte et al., 2005; De Rossi et al., 2000; Demoule et al., 2009; Hirata et al., 2003; Peterson and Pizza, 2009; Porter et al., 2003; Sachidanandan et al., 2002; Warren et al., 2005; Warren et al., 2004). Lastly, chemokines are known to regulate migration of several cell types post-natally, such as immune cells, sperm and metastasizing cancer cells (Bleul et al., 1996; Isobe et al., 2002; Kim, 2004; Kim, 2005; Miyazaki et al., 2006; Muciaccia et al., 2005a; Muciaccia et al., 2005b; Stebler et al., 2004; Vandercappellen et al., 2008). However, no studies have comprehensively examined the expression of these molecules specifically by muscle cells at different phases of myogenesis.

Our studies indicate that a large number of chemokines and chemokine receptors are expressed by primary mouse muscle cells in vitro, especially during

times of extensive cell-cell fusion. Furthermore, muscle cells exhibited different migratory behaviors throughout myogenesis in vitro. One receptor/ligand pair, CXCR4/SDF-1α (CXCL12), regulated migration of both proliferating and terminally differentiated muscle cells, and was necessary for proper fusion of muscle cells.

Results

Many chemokines and their receptors are expressed during myogenesis

In order to determine which chemokine receptors and ligands are expressed at different time points during myogenesis, primary mouse muscle cells were utilized as they follow a predictable time course of myogenesis in vitro (Fig. 4.1A). By 16 hours in differentiation media (DM) the majority of cells are terminally differentiated myocytes as indicated by the high percentage of embryonic myosin heavy chain^{+} (eMyHC) cells (Fig. 4.1B). After 24 hours in DM, ~40% of myocytes are fused with each other to form nascent myotubes, which are small and contain few nuclei. By 48 hours, ~ 70% of myocytes are fused, creating mature myotubes, which are large and contain many nuclei (Fig. 4.1C). A Real Time RT-PCR array was used to investigate the mRNA steady-state levels of 84 chemokines, chemokine receptors and signaling molecules, in order to obtain a comprehensive view of chemokine expression during myogenesis. Approximately 80 of these mRNAs were detected during myogenesis, indicating that many chemokine receptors and ligands are expressed directly by muscle cells. The steady-state levels of these mRNAs varied drastically, with a small subset of genes, having extremely high steady-state levels, ~10,000 to 1 million fold higher than other genes (Table 4.1). Furthermore, no genes were constitutively expressed at a stable level throughout myogenesis; instead the mRNA levels of all genes increased after differentiation. Very few mRNAs were present at 6 or 48 hours in DM; rather, most mRNA steady-state levels were

highest between 16 and 36 hours in DM (Table 4.2, Fig. 4.1D, E), time points of extensive differentiation and fusion of myocytes.

Many chemokine receptors and ligands known to be expressed in skeletal muscle tissue were show in this assay to be expressed directly by muscle cells (Bischoff, 1997; Chazaud et al., 2003; Chong et al., 2007; Civatte et al., 2005; Corti et al., 2001; De Rossi et al., 2000; Hirata et al., 2003; Odemis et al., 2007; Peterson and Pizza, 2009; Porter et al., 2003; Ratajczak et al., 2003; Sachidanandan et al., 2002; Summan et al., 2003; Warren et al., 2005; Warren et al., 2004). For example, IL4, an important pro-myogenic factor expressed during in vitro myogenesis (Horsley et al., 2003; Lafreniere et al., 2006), was identified by this chemokine array (Table 4.2). However, a few chemokine receptors and ligands not previously known to be expressed by skeletal muscle cells or tissue were also identified, including angiotensin receptor like-1 (AGTRL1, Aplnr, apelin receptor), bone morphogenic protein-10 (BMP10), CXCL13, and its receptor CXCR5 (Burkitt's lymphoma receptor 1, BLR1). The large number of chemokine receptor/ligand pairs expressed directly by muscle cells suggests a complex spatial and temporal control of migration during myogenesis.

The migratory behavior of muscle cells changes during myogenesis

To conduct an in depth analysis of the migratory behavior of muscle cells during myogenesis, time-lapse microscopy was performed for 3 hours at different time points (Fig. 4.2A). Myocytes displayed distinct differences in migration compared to myoblasts. At 0 hours, myoblasts migrated far from their point of

origin, whereas over the course of myogenesis, myocytes stayed progressively closer to their point of origin (Fig. 4.2A). The proportion of slow moving cells also increased during myogenesis (Fig. 4.2B), causing a concomitant decrease in mean velocity from 56 μm/hour at 0 hours to 22 μm/hr at 48 hours in DM. The diminished velocity of myocytes at 48 hours was not due to a loss in cell motility or viability as the addition of fresh media increased cell velocity (data not shown). Thus, muscle cells are migratory throughout myogenesis; as most investigations have focused on myoblast migration, the majority of receptor-ligand pairs that regulate myocyte migration are unknown.

Myoblasts and myocytes migrate to distinct factors

To determine whether myocytes migrate in response to canonical myoblast chemoattractants, cell migration was analyzed in Boyden chambers using hepatocyte growth factor (HGF) and platelet derived growth factor (PDGF), potent myoblast chemoattractants (Bischoff, 1997; Corti et al., 2001). We enriched for myocytes by culturing cells in DM for 24 hours at low density to prevent myotube formation yielding 96% of nuclei in eMyHC^{+} cells, and only 7% of nuclei in myotubes. Despite both HGF and PDGF greatly enhancing the migration of myoblasts, neither factor stimulated myocyte migration (Fig. 4.3), suggesting intrinsic differences exist between the two cell types, such as differential expression of chemoattractant receptors. However, myocytes exhibited a 65-fold increase in migration to conditioned media (CM), which contains the factors secreted by muscle cells during differentiation and fusion,

compared to control media (Fig. 4.3). Migration to CM suggests that myocyte chemoattractants, such as chemokines, are secreted during myogenesis and control migration during the process of cell fusion to form myotubes. Together, these data suggest that factors which regulate myoblast migration may not regulate myocyte migration during myogenesis in vitro.

Myocytes exist during muscle regeneration

We quantified the percentage of myocytes during adult regenerative myogenesis in vivo. Regenerative myogenesis is an asynchronous process that requires both spatial and temporal coordination. Upon injury satellite cells proliferate and then terminally differentiate to become fusion-competent myocytes, which express differentiation-specific proteins such as myogenin (myog) and eMyHC and then fuse into myofibers. Mononucleated cells were isolated from injured mouse muscles and analyzed by flow cytometry (Fig. 4.4A). Muscle cells were defined as α7-integrin^{+} cells which were also negative for endothelial and hematopoietic lineage markers (CD31 and CD45) (Blanco-Bose et al., 2001; Kafadar et al., 2009). As muscle cells are quiescent before injury, and in days immediately following injury, the majority of mononucleated cells in muscle tissue are immune cells; therefore, day 3 was the earliest time point analyzed. At later time points myogenic cells are fusing into newly regenerating myofibers, therefore day 7 was the latest time point assayed. The relative percentage of mononucleated muscle cells did not change during these time points of regeneration (Fig. 4.4B). To determine whether differentiated α7-

integrin^{+}CD31^{-}CD45^{-} muscle cells exist during regeneration, cells were immunostained for p21, which marks terminally differentiated cells (Andres and Walsh, 1996). Terminally differentiated p21^{+} myogenic cells increased by day 5 after injury, but only 25% of myogenic cells were p21^{+} (Fig. 4.4C, D).

We used multiple markers to determine the progression of muscle cells through the continuum of differentiation. As muscle cells progress through differentiation, first myogenin is expressed, then p21, and finally MyHC (Andres and Walsh, 1996). Therefore, cells at later stages of differentiation are myogenin^{+}p21^{+}eMyHC^{+} and these cells are not likely to accumulate as they should be fusing to form newly regenerated myofibers. To determine the percentage of muscle cells at early and late stages of differentiation, myogenic cells were isolated from gastrocnemius muscles at day 5 post-injury by FACS and immunostained for myogenin and eMyHC in vitro (Fig. 4.4E). Approximately 60% of myogenic cells were myogenin^{+} and 18% were eMyHC^{+} (Fig. 4.4F). Therefore, regenerating muscle tissue at day 5 is a mixture of myogenic cells at various stages of differentiation. As the expression of chemokine receptor/ligand pairs increased after differentiation of muscle cells in vitro, these factors are likely candidates for regulating differentiating myogenic cells in vivo.

CXCR4 and SDF1α are expressed during myogenesis in vitro and in vivo

We examined the role of the most highly expressed chemokine receptor, CXCR4, and its ligand, CXCL12 or SDF1α, more in depth. The receptor CXCR4 and ligand SDF1α were of specific interest, as several studies have shown

expression of these proteins by muscle tissue, but conflicting reports exist regarding their role during myogenesis (Bae et al., 2008; Chong et al., 2007; Molyneaux et al., 2003; Odemis et al., 2005; Ratajczak et al., 2003; Vasyutina et al., 2005). To confirm expression of CXCR4 at the protein level, flow cytometry was used to determine the percentage of $CXCR4^+$ cells in primary mouse myoblast and myocyte cultures; ~30% of myoblasts were $CXCR4^+$ compared to ~60% of myocytes (Fig. 4.5A, B). Furthermore, myocytes contained ~2-fold more CXCR4 per cell (Fig. 4.5C, D), yet myocytes were only 18% larger than myoblasts (Fig. 4.5E), suggesting that myocytes have a higher density of CXCR4 at the plasma membrane. The increased level of CXCR4 protein in myocytes correlated to the increased mRNA levels of CXCR4 at 24 hours in DM (Fig. 4.1E). To determine whether CXCR4 and SDF1α are expressed during adult regenerative myogenesis, the percentage of $CXCR4^+$α7-$integrin^+CD31^-CD45^-$ myogenic cells was determined at both days 3 and 5 after injury (Fig. 4.5F, G). From day 3 to day 5, the percentage of myogenic $CXCR4^+$ cells increased from ~45% to 77% (Fig. 4.5H). In addition, the amount of CXCR4 per muscle cell was increased ~2.5 fold at day 5 compared to day 3 (Fig. 4.5I), with no change in cell size (data not shown).

To validate expression of SDF1α at the protein level, ELISA assays were performed using control DM and 24 hours CM; significant levels of SDF1α were detected in CM (Fig. 4.5J). The levels of SDF1α in crushed muscle extract, which contains released soluble protein by control and regenerating muscles, were also determined (Bischoff, 1986; Chen and Quinn, 1992). Muscles at day 3 after injury

contained significantly higher levels of SDF1α, compared to uninjured muscles, or muscles at day 5 after injury (Fig. 4.5K). Therefore, SDF1α may be released under certain conditions after injury. These data demonstrate that CXCR4 and SDF1α proteins are expressed by primary mouse muscle cells during myogenesis. As CXCR4 was expressed by mononucleated muscle cells during adult regenerative myogenesis and SDF1α was isolated from muscle tissue, this receptor/ligand pair may regulate myogenesis.

CXCR4/SDF1α axis is important for proper muscle cell fusion

To examine the role of the CXCR4/SDF1α axis in myogenesis, we used primary mouse muscle cells in vitro, as direct effects on muscle cells can be analyzed in the absence of other cell types. To determine whether the CXCR4/SDF1α axis may regulate migration during myogenesis, myoblasts and myocytes were allowed to migrate to multiple concentrations of SDF1α in Boyden chambers (Fig. 4.6A). Interestingly, while both cell types were attracted to SDF1α, myoblasts required a 20-fold higher concentration than myocytes to achieve a similar level of migration. This difference is likely due not only to the greater percentage of $CXCR4^+$ cells in the myocyte population, but also to the increased CXCR4 per myocyte. Thus, SDF1α is a chemoattractant for both myoblasts and myocytes, although myocytes exhibit a greater sensitivity to SDF1α.

To determine whether CXCR4-dependent processes are necessary for myogenesis, a specific pharmacologic inhibitor of CXCR4, AMD3100 (AMD), was

added to cells at the start of differentiation. Nascent myotubes in cultures treated with AMD appeared smaller than vehicle-treated cells at 24 hours in DM (Fig. 4.6B). However, the number of cells per field was not affected, suggesting that neither proliferation nor apoptosis of muscle cells was affected by inhibition of CXCR4 (Fig. 4.6C). Furthermore, the number of nuclei within differentiated cells was not affected (Fig. 4.6D), as determined by immunostaining for eMyHC, suggesting that differentiation is not affected by CXCR4 inhibition. Addition of AMD decreased the fusion index, or the total number of nuclei in myotubes, by ~30% compared to control (Fig. 4.6E). These data support the hypothesis that the CXCR4/SDF1α axis is necessary for proper myogenesis in vitro, but has no effect on proliferation, survival or differentiation of primary mouse muscle cells. Therefore, the predominant role for CXCR4/SDF1α during myogenesis may be in regulating migration of muscle cells, which affects fusion.

Discussion

Adult regenerative myogenesis is vital for restoring normal myofiber structure after muscle injury. Myogenic progenitor cells must be precisely regulated and positioned in order for proper cell fusion to occur. Using a cell culture model of myogenesis, we demonstrated that a large number of chemokines and chemokine receptors were upregulated during myogenesis when terminally differentiated myocytes were fusing. Differences in migratory behavior were noted between myoblasts and myocytes. These results suggest that regulation of cell migration during myogenesis and tissue repair is likely complex.

Several chemokines and chemokine receptors we identified were not previously known to be expressed in skeletal muscle (Civatte et al., 2005; De Rossi et al., 2000; Demoule et al., 2009; Hirata et al., 2003; Peterson and Pizza, 2009; Porter et al., 2003; Sachidanandan et al., 2002; Warren et al., 2005; Warren et al., 2004), however these molecules have known roles in other muscle types. For example, the angiotensin receptor like-1 (AGTRL1, Aplnr, apelin receptor) has protective effects in ischemic heart disease (O'Donnell et al., 2007) and bone morphogenetic protein-10 (BMP10) regulates hypertropic growth in heart muscle (Chen et al., 2006). Neither of these proteins has identified functions in skeletal muscle but may regulate skeletal muscle growth or repair given their role in smooth and cardiac muscle. Another novel gene we identified expressed during myogenesis, Burkitt's lymphoma receptor 1 (BLR1, CXCR5), regulates migration of B cells into ischemia-damaged intestinal tissue through expression of CXCL13 by the damaged areas (Chen et al., 2009), but lacks an

identified role during injury-repair in skeletal muscle. These results suggest new avenues of research into chemokine-mediated regulation of adult regenerative myogenesis.

A key question is why so many chemokines and chemokine receptors are expressed directly by muscle cells during myogenesis. As muscle cells are heterogenous (Andersen et al., 2008; Asakura et al., 2002; Motohashi et al., 2008; Relaix et al., 2005; Tanaka et al., 2009), sub-populations of muscle cells may express a single receptor or ligand. Alternatively, several of these molecules may be expressed by each muscle cell as occurs in the immune system (Civatte et al., 2005; Porter et al., 2003; Warren et al., 2004). If multiple receptors are expressed by a single cell, specific chemokine receptors may be utilized in a spatial-temporal manner. Alternatively, a redundant system may exist, allowing the substitution of one receptor/ligand pair for another. Such a system would allow disruption of a single receptor/ligand pair without serious detriment to myogenesis. Interestingly, our results demonstrate that myocytes did not migrate in response to canonical myoblast migration factors. Instead, myocytes migrated to factors secreted by fusing muscle cells. Thus, regulation of cell migration during different phases of myogenesis is differentially controlled.

The multitude of chemokines and chemokine receptors expressed during myogenesis may regulate similar or distinct processes. Chemokines regulate cell number at several levels, including survival and proliferation (Miyazaki et al., 2006; Schober and Zernecke, 2007); thus, chemokines expressed early during myogenesis, may regulate myoblast proliferation or survival. Also, as muscle

cells must interact directly with one another for terminal differentiation to occur (Krauss et al., 2005), chemokines may also regulate migration of myoblasts. Our data suggest that multiple chemokine receptor/ligand pairs may regulate later stages of myogenesis, such as migration and fusion, as these molecules are not expressed at high levels until the majority of cells are terminally differentiated myocytes. Curiously, the expression levels of these molecules were highest during periods of myogenesis in which the myocytes were progressively moving slower as measured by time-lapse microscopy. Chemokines not only regulate cell velocity, but also directional migration of cells (Kim, 2004). Perhaps chemokines at these later stages of myogenesis are key for positioning myocytes in the correct spatial patterns necessary for cell fusion to occur with other myocytes and with nascent myotubes, rather than acting to enhance cell velocity. Chemokines expressed by muscle cells may not only have a direct effect on myogenesis, but also may act in a paracrine manner. Chemokines regulate the recruitment of immune cells to damaged tissues (Bleul et al., 1996; Loetscher et al., 1996; Weber et al., 1995), including injured muscle (Robertson et al., 1993); immune cells such as macrophages are critical for muscle regeneration (Robertson et al., 1993). Therefore, chemokines may regulate adult regenerative myogenesis through multiple cell types and processes.

The investigation of a single receptor/ligand pair, CXCR4 and SDF1α indicated that some chemokines can regulate migration during myogenesis. The current literature contains conflicting evidence for the role of CXCR4 and SDF1α during myogenesis. Studies utilizing the immortalized C2C12 muscle cell line,

indicated SDF1α enhances migration and proliferation of these cells (Odemis et al., 2007). In contrast, our work indicates that although SDF1α does increase migration of primary muscle cells, it has no apparent effect on proliferation during *in vitro* myogenesis. Further investigations on C2C12s also suggested loss of CXCR4 leads to decreased expression of differentiation-specific muscle proteins, such as myogenin and myosin heavy chain (Odemis et al., 2007). Interestingly, an almost complete abrogation of muscle cell differentiation was observed with loss of CXCR4, despite only 15% of C2C12 cells expressing CXCR4 (Odemis et al., 2007). While our investigations using primary muscle cells also suggest only a low percentage of myoblasts express CXCR4 (~30%), no differentiation defect was observed during *in vitro* myogenesis. Interestingly, CD164, a cell surface sialomucin that targets CXCR4 to endosomes and lysosomes via its intracellular region, was necessary for proper migration and myotube formation, and not proliferation of muscle cells (Bae et al., 2008). Our results indicate CXCR4 is expressed by both myoblasts and myocytes, and its ligand SDF1α can increase migration of both cell types, albeit at different concentrations. However, despite inhibition of CXCR4, primary muscle cells proliferate and differentiate at a similar rate to untreated cells. Loss of CXCR4-dependent signaling decreases fusion, suggesting CXCR4 is necessary for migration of muscle cells to one another, which is required for normal fusion.

CXCR4 is also of specific interest as a subset of muscle satellite cells which are $CXCR4^{+}$ can be engrafted into injured muscle tissue with a high efficiency (Cerletti et al., 2008; Sherwood et al., 2004). As CXCR4 regulates migration of

muscle cells, the increased engraftment may be due to an increased migratory ability of these cells. Furthermore, SDF1α enhances migration, yielding a positive effect on engraftment of cells into damaged muscle (Galvez et al., 2006). These data suggest that CXCR4/SDF1α-dependent migration enhances the engraftment of cells into damaged muscle, which may enhance cell transplantation therapies necessary for many myopathies.

The large number of chemokine receptors and ligands expressed by muscle cells during myogenesis suggests these proteins may play a larger role in myogenesis than previously appreciated. Manipulation of these molecules may allow for an increased efficiency of cell transplantation therapies for various muscle disorders.

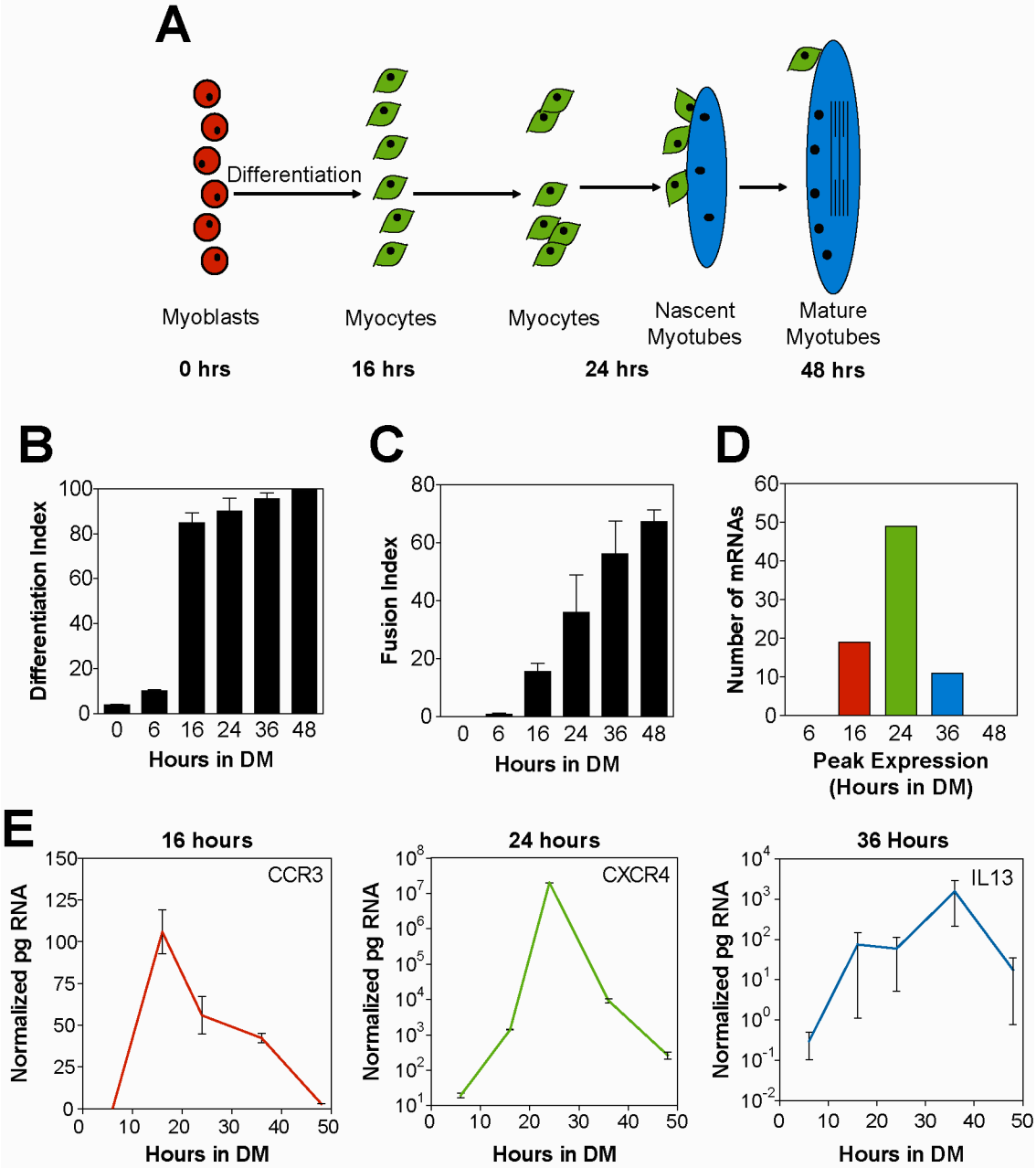

A
Differentiation
Myoblasts
Myocytes
Myocytes
Nascent Myotubes
Mature Myotubes
0 hrs
16 hrs
24 hrs
48 hrs
B
Differentiation Index
Hours in DM
C
Fusion Index
Hours in DM
D
Number of mRNAs
Peak Expression (Hours in DM)
E
16 hours
CCR3
24 hours
CXCR4
36 Hours
IL13
Normalized pg RNA
Hours in DM

Figure 4.1

Chemokines and their receptors are expressed during *in vitro* myogenesis. A) During myotube formation the majority of myoblasts (red) terminally differentiate into myocytes (green) which migrate, adhere and fuse with one another to form small nascent myotubes with few nuclei (blue). Subsequently, nascent myotubes fuse with myocytes to form large mature myotubes with many nuclei (blue). B) Primary mouse muscle cells were immunostained for eMyHC at different times in DM and the percentage of nuclei within $eMyHC^+$ cells (differentiation index) was determined. By 16 hours in DM, most nuclei were in $eMyHC^+$ cells. C) The fusion index, or percentage of nuclei in myotubes, increased with time, and by 48 hours the majority of nuclei were within myotubes. D) A Real Time RT-PCR array was used to analyze the time-course of expression in vitro for 84 genes pertaining to chemokines. Positive results were obtained for 80 genes. Three patterns of expression were observed with mRNA steady state levels peaking at 16, 24 or 36 hours in DM, times of extensive differentiation and fusion. The number of genes with peak expression levels at each time point is shown. E) Time course of expression for 3 representative genes peaking at 16 (CCR3), 24 (CXCR4), or 36 hours (IL13) in DM. Data are mean ± s.e.m., N=3.

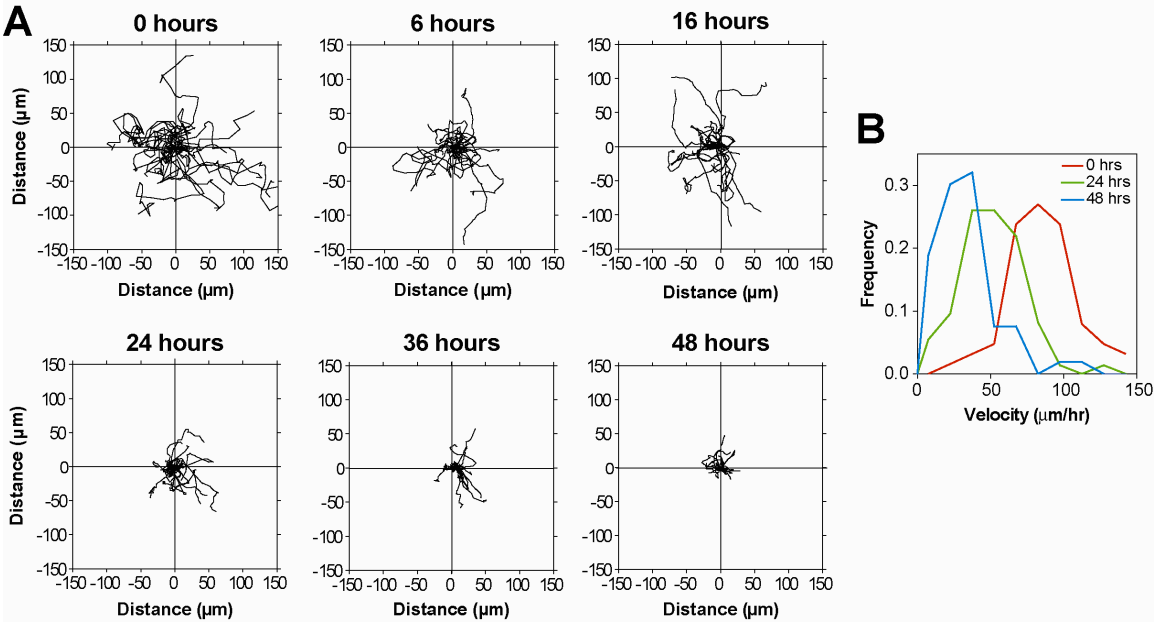


Figure 4.2

Changes in migratory behavior with muscle cell differentiation. A) Migratory paths of mononucleated primary mouse muscle cells at 0, 6, 16, 24, 36 and 48 hours in DM. Tracks were taken from 3 hours of time-lapse microscopy with pictures every 5 minutes. Representative graphs are shown from one of 3 independent isolates with 20 cells each. B) Frequency distribution of cell velocity at different times in myogenesis. A total of 60 cells were analyzed. Data are N=3.

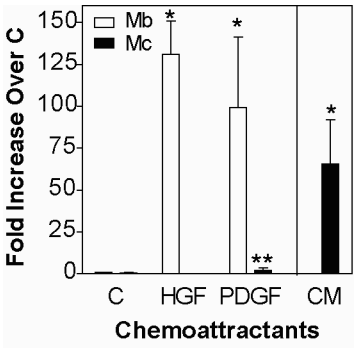


Figure 4.3

Myocytes do not migrate to canonical myoblast migratory factors. Primary mouse myoblasts (Mb) and myocytes (Mc, 24 hours in DM) were allowed to migrate in Boyden chambers to control media (C) or media containing 100 ng/mL HGF or PDGF for 5 hrs. Myocyte migration to conditioned media (CM) from cultures in DM for 24 hours was also tested. Data are mean ± s.e.m., N=3-5 (* $p<0.05$ from control; ** $p<0.05$ from myoblasts).

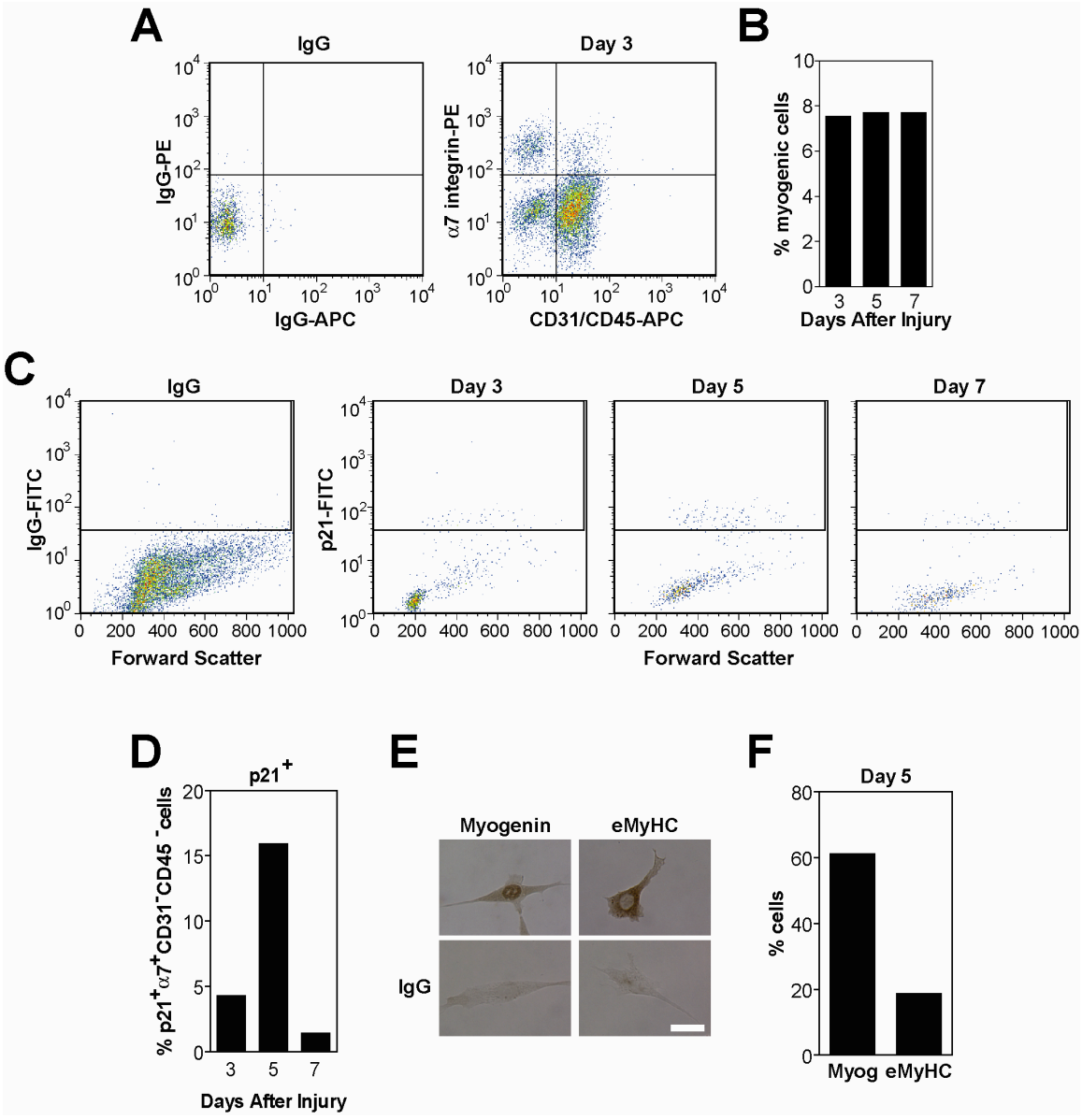

A
IgG
Day 3
IgG-PE
IgG-APC
α7 integrin-PE
CD31/CD45-APC
B
% myogenic cells
Days After Injury
C
IgG
Day 3
Day 5
Day 7
IgG-FITC
p21-FITC
Forward Scatter
D
p21+
% p21+α7+CD31-CD45- cells
Days After Injury
E
Myogenin
eMyHC
IgG
F
Day 5
% cells
Myog
eMyHC

Figure 4.4

Myocytes exist during muscle regeneration. A) Mononucleated cells were isolated from gastrocnemius muscles at days 3, 5, and 7 post-injury and immunostained with antibodies to CD31 (APC), CD45 (APC), and α7-integrin (PE): $CD31^{+}CD45^{+}$, to identify endothelial and immune cells and α7-$integrin^{+}CD31^{-}CD45^{-}$ for myogenic cells. Myogenic cells constituted ~8% of the total mononucleated cells at day 3. Isotype controls were used to determine proper gating (left panel). B) The percentage of myogenic cells remained stable during muscle regeneration. C) Mononucleated cells were isolated from gastrocnemius muscles at indicated days post-injury and immunostained with antibodies to CD31 (APC), CD45 (APC), α7-integrin (PE) and p21 (FITC) to identify terminally differentiated muscle cells. Myogenic α7-$integrin^{+}CD31^{-}CD45^{-}$ cells were analyzed for p21. Isotype control was used to determine proper gating (left panel). D) The percentage of $p21^{+}$ myogenic cells was highest at day 5 post-injury. E) Mononucleated α7-$integrin^{+}CD31^{-}CD45^{-}$ myogenic cells isolated from gastrocnemius muscles 5 days post-injury were plated in vitro and immunostained for differentiation markers, myogenin (top left panel) and eMyHC (top right panel) or appropriate IgG controls (bottom panels) (Bar=10 μm). F) The percentage of $myogenin^{+}$ and $eMyHC^{+}$ cells in (E) was analyzed; ~60% of cells were $myogenin^{+}$ a marker for earlier stages of differentiation, and ~20% were $eMyHC^{+}$, a marker for later stages of differentiation. All data are mean with ten mice.

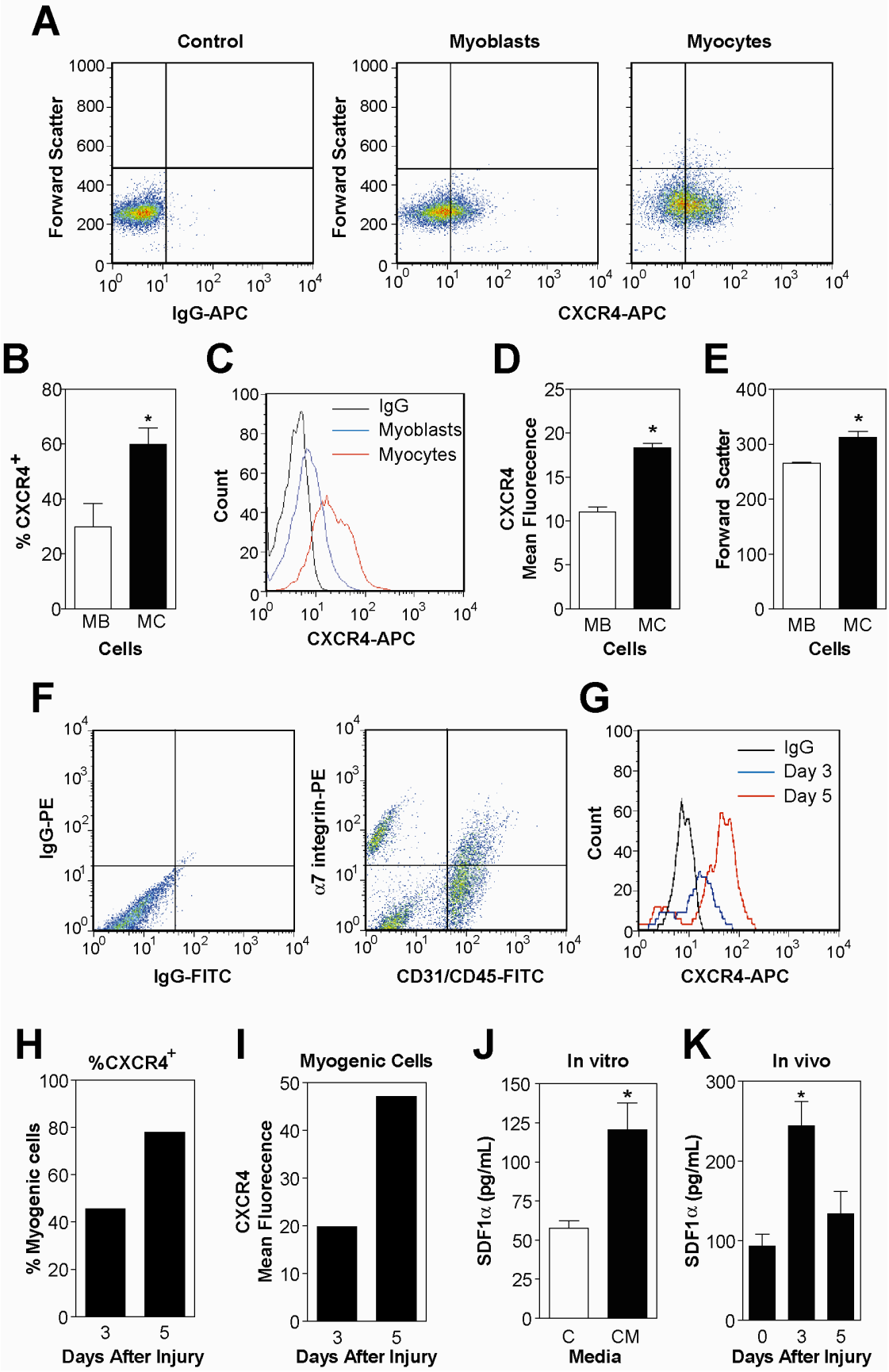

A
Control
Myoblasts
Myocytes
Forward Scatter
IgG-APC
CXCR4-APC
B
% CXCR4+
MB
MC
Cells
C
Count
IgG
Myoblasts
Myocytes
CXCR4-APC
D
CXCR4 Mean Fluorecence
E
Forward Scatter
F
IgG-PE
IgG-FITC
α7 integrin-PE
CD31/CD45-FITC
G
Day 3
Day 5
H
%CXCR4+
% Myogenic cells
Days After Injury
I
Myogenic Cells
J
In vitro
SDF1α (pg/mL)
C
CM
Media
K
In vivo

Figure 4.5

CXCR4 and SDF1-α are expressed during myogenesis in vitro and in vivo. A) Primary mouse myoblasts and myocytes were immunostained with antibodies against CXCR4 (APC) in vitro. B) The percentage of $CXCR4^+$ cells was quantified; a significantly higher percentage of myocytes were $CXCR4^+$. C) Representative histogram is shown; the level of CXCR4 per cell was also increased between myoblasts and myocytes. D) The mean fluorescence intensity of CXCR4 per cell was quantified; myocytes contained almost twice as much CXCR4 per cell. E) Myocytes were 18% larger than myoblasts. F) Mononucleated cells were isolated from gastrocnemius muscles at days 3 and 5 post-injury and immunostained with antibodies to CD31 (FITC), CD45 (FITC), α7-integrin (PE) and CXCR4 (APC). Cells were analyzed with the following criteria: $CD31^+CD45^+$, to identify endothelial and immune cells and $\alpha7\text{-integrin}^+CD31^-CD45^-$ for myogenic cells. Day 5 is shown. G) Myogenic $\alpha7\text{-integrin}^+CD31^-CD45^-$ cells were analyzed for CXCR4 and a representative histogram is shown. H) The percentage of $CXCR4^+$ myogenic cells was quantified; a higher percentage of myogenic cells were $CXCR4^+$ at day 5. I) The mean fluorescence intensity of CXCR4 per cell was also increased between day 3 and 5; myocytes contained almost twice as much CXCR4 per cell. J) The level of SDF1α secreted by primary mouse muscle cells in vitro during myogenesis (24 hours CM) was determined by ELISA. K) The level of SDF1α in crushed muscle extract was also determined by ELISA. The level of SDF1α was increased at day 3. In all flow cytometry experiments: propidium iodide $(PI)^-$ was used to remove dead cells

from analysis, representative flow plots are shown and isotype controls were used to determine proper gating. Data are mean ± s.e.m, N=3 (* $p<0.05$ from Mb, control or 0 days as appropriate).

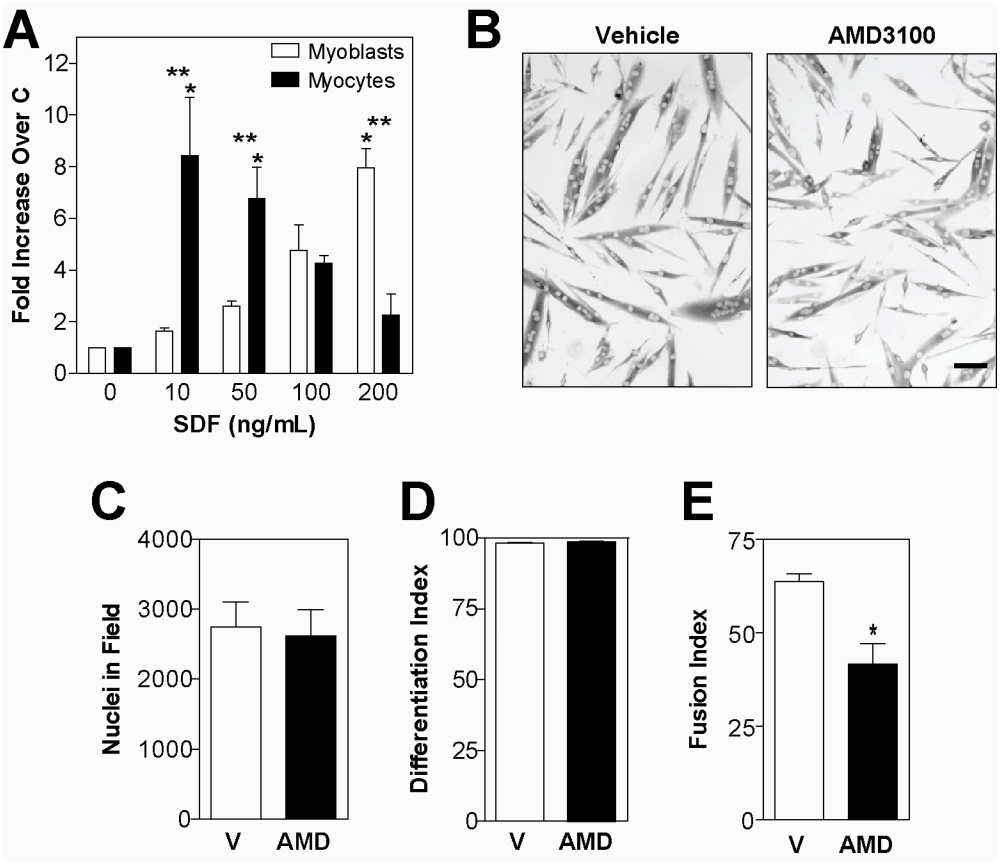

A
Myoblasts
Myocytes
Fold Increase Over C
SDF (ng/mL)
0
10
50
100
200
B
Vehicle
AMD3100
C
Nuclei in Field
V
AMD
D
Differentiation Index
E
Fusion Index

Figure 4.6

CXCR4 and SDF1-α regulate migration of myoblasts and myocytes, and are necessary for myogenesis. A) Boyden chamber experiments were performed with primary mouse myoblasts (Mb) and myocytes (Mc) with varying concentrations of SDF1-α. Myoblasts exhibited peak migration to 200 ng/mL, whereas myocytes migrated to 10-50 ng/mL. B) AMD3100 (AMD) or vehicle (V) was added to cultures with differentiation media (DM). Cultures were fixed and immunostained for embryonic myosin heavy chain (eMyHC) at 24 hours in DM (Bar=50μm). C) The total number of nuclei in 10 fields was calculated. No difference between control and AMD cultures was observed, indicating that proliferation and apoptosis during differentiation were not affected by AMD. D) Differentiation index was calculated as the number of nuclei in $eMyHC^{+}$ cells divided by the total number of nuclei. No difference was observed, suggesting that terminal differentiation was not affected by AMD. E) Fusion index was calculated as the number of nuclei in myotubes divided by the total number of nuclei. Addition of AMD decreased fusion at 24 hrs in DM. Data are mean ± s.e.m., N=3 (* $p<0.05$ from control or Mb, ** $p<0.05$ from Mb at same concentration).

Table 4.1

mRNA levels of 84 genes pertaining to chemokines during myogenesis.

	Gene	6	16	24	36	48
16 hours	Cmtm6	-	8.9E+06 ± 5.5E+04	-	12.7 ± 0.6	-
	Ccr1	-	271.7 ± 59.5	36.4 ± 7.9	208.1 ± 64.0	15.9 ± 5.9
	Ccr3	-	105.9 ± 22.8	55.9 ± 19.4	42.3 ± 4.7	-
	Bmp10	-	90.7 ± 11.6	13.0 ± 9.7	27.8 ± 5.6	-
	Cmtm3	11.6 ± 2.3	84.8 ± 28.3	19.3 ± 4.6	31.6 ± 11.9	-
	Csf2	-	49.9 ± 10.5	11.1 ± 3.1	45.8 ± 10.7	-
	Cx3cr1	-	47.7 ± 8.7	5.3 ± 2.2	40.7 ± 13.1	-
	Cxcl11	-	31.3 ± 9.3	5.7 ± 0.3	29.5 ± 7.8	-
	Cxcl10	-	25.3 ± 4.0	23.2 ± 3.8	19.0 ± 2.8	-
	Csf1	21.5 ± 6.3	24.5 ± 6.0	-	21.7 ± 6.7	-
	Ccl17	-	23.1 ± 7.9	-	-	-
	Ccl2	-	22.6 ± 4.3	13.6 ± 2.4	13.5 ± 1.4	-
	Ccbp2	-	22.3 ± 5.3	12.9 ± 4.2	8.6 ± 1.1	-
	Ccl12	-	21.1 ± 2.9	23.7 ± 2.6	8.2 ± 0.9	-
	Cx3cl1	-	8.9 ± 1.9	-	-	-
	Ccr9	-	8.2 ± 1.6	-	-	-
24 hours	Cxcr4	19.6 ± 5.3	1.4E+03 ± 5.9E+01	2.0E+07 ± 2.9E+04	9.3E+03 ± 2.4E+03	265.2 ± 97.5
	Bdnf	-	6.3E+03 ± 2.2E+03	5.9E+05 ± 4.5E+04	6.2E+03 ± 2.3E+03	243.5 ± 89.8
	Cxcl15	6.5 ± 2.1	3.0E+03 ± 8.2E+01	3.3E+05 ± 5.1E+04	7.8E+03 ± 2.9E+03	296.1 ± 111.6
	Ccr6	-	1.9E+03 ± 5.4E+02	2.5E+05 ± 9.5E+04	4.5E+03 ± 2.5E+02	79.5 ± 29.7
	Trem1	-	35.1 ± 1.1	5.5E+04 ± 5.6E+03	84.8 ± 27.7	-
	Il8ra	6.1 ± 2.2	-	2.6E+04 ± 6.1E+02	76.9 ± 27.7	-
	Tnfsf14	-	-	9.4E+03 ± 2.3E+03	-	-
	Il8rb	-	458.3 ± 173.5	8.1E+03 ± 2.0E+03	402.7 ± 97.6	-
	Il1a	-	42.3 ± 12.5	7.7E+03 ± 2.9E+03	112.9 ± 38.1	-
	Ccl4	-	96.4 ± 36.0	2.2E+03 ± 5.4E+02	484.1 ± 117.1	-
	Ccl9	-	-	2.1E+03 ± 5.2E+02	79.7 ± 112.7	-
	Cmklr1	-	56.6 ± 21.4	1.8E+03 ± 6.9E+02	534.6 ± 129.3	-
	Xcl1	-	23.4 ± 4.0	1.3E+03 ± 4.7E+02	18.1 ± 3.1	-
	Ccl19	-	-	879.6 ± 333.1	105.5 ± 25.2	-
	Tnfrsf1a	5.4 ± 1.5	9.9 ± 1.0	789.8 ± 208.4	21.2 ± 1.8	-
	Il4	-	75.8 ± 27.8	600.3 ± 218.4	221.5 ± 49.1	-
	Cxcl1	-	50.3 ± 18.0	383.2 ± 92.9	77.1 ± 17.8	-
	Cmtm	-	21.4 ± 1.5	364.3 ± 112.9	38.6 ± 9.0	-

	2a																		
	Ecgf1	-			27.0	±	6.3		306.0	±	115.0		38.4	±	13.5		-		
	Cxcl5	-			69.9	±	16.9		301.1	±	73.0		241.7	±	58.1		-		
	Ccrl2	-			56.3	±	4.1		285.0	±	107.8		37.5	±	11.7		-		
	Tnf	-			12.3	±	3.0		227.2	±	85.9		41.9	±	9.5		-		
	Cxcl2	-			15.3	±	2.9		191.8	±	70.7		75.4	±	25.4		-		
	Cxcl7	-			21.0	±	6.1		166.5	±	60.5		97.9	±	34.7		-		
	Cmtm5	-			6.5	±	1.0		147.5	±	41.7		8.3	±	1.8		-		
	Ccl5	-			26.3	±	0.5		135.1	±	15.5		21.0	±	2.0		-		
	Ccl11	-			31.4	±	2.8		123.3	±	29.6		37.7	±	8.8		-		
	Cxcr3	-			18.0	±	1.1		109.7	±	37.6		35.5	±	8.0		-		
	Ccr8	-			30.4	±	2.8		101.5	±	34.3		26.6	±	0.5		-		
	Bmp15	-			28.8	±	3.6		96.4	±	35.5		31.3	±	6.7		-		
	Ccl8	-			17.4	±	0.2		93.5	±	21.9		31.6	±	4.2		-		
	Agtrl1	-			16.0	±	1.6		92.3	±	6.7		27.8	±	3.4		-		
	Ccl1	-			22.3	±	6.7		71.4	±	17.1		31.3	±	11.1		-		
	Ccr5	-			30.3	±	2.0		58.4	±	20.1		29.4	±	5.2		-		
	Rgs3	-			12.2	±	3.4		56.7	±	2.8		23.7	±	0.3		-		
	Ltb4r2	-			19.9	±	4.8		42.2	±	5.0		12.8	±	1.7		-		
	Cxcl9	-			12.1	±	1.2		40.8	±	12.7		22.6	±	3.6		-		
	Ccr1l1	-			33.6	±	3.5		37.5	±	10.0		22.3	±	1.3		-		
	Ccr4	-			11.6	±	2.4		36.3	±	2.1		9.6	±	3.3		-		
	Ccl6	-			8.0	±	1.1		35.7	±	13.3		19.7	±	6.1		-		
	Ccl7	-			14.7	±	4.7		33.3	±	12.3		26.7	±	5.2		-		
	Cxcl13	-			8.4	±	2.9		28.8	±	6.9		11.9	±	2.7		-		
	Ccl20	-			5.8	±	3.1		25.7	±	8.0		12.4	±	0.2		-		
	Cxcl4	-			7.6	±	2.8		19.7	±	7.1		6.3	±	1.1		-		
	Il18	-			-				19.2	±	4.1		5.9	±	0.3		-		
	Gpr2	-			-				18.7	±	4.4		8.0	±	1.7		-		
	Ccr7	-			7.2	±	0.4		17.5	±	6.2		12.4	±	0.2		-		
	Gdf5	-			-				17.1	±	3.4		-				-		
	Il16	-			8.7	±	1.2		13.4	±	3.9		-				-		
	Gpr81	-			-				10.6	±	3.5		6.6	±	0.5		-		
	Slit2	-			-				8.9	±	1.4		6.1	±	0.4		-		
	Ccrl1	-			-				8.1	±	2.9		6.0	±	2.3		-		
36 hours	Inhbb	-			3.2E+03	±	3.8E+02		33.0	±	8.7		7.9E+03	±	2.5E+03		102.0	±	37.1
	Blr1	-			1.4E+03	±	4.6E+02		1.2E+03	±	4.6E+02		2.1E+03	±	8.0E+02		66.1	±	25.0
	Il13	-			101.1	±	37.3		77.1	±	24.6		1.9E+03	±	5.4E+02		23.6	±	8.2
	Bmp6	-			1.1E+03	±	3.5E+02		111.6	±	36.7		1.5E+03	±	5.4E+02		88.4	±	33.4
	Cmtm4	-			76.9	±	6.6		72.6	±	23.9		540.3	±	47.5		16.3	±	1.3
	Nfkb1	-			-				-				121.6	±	38.7		6.3	±	2.1
	Ccr2	-			55.4	±	7.7		20.6	±	1.9		74.9	±	25.6		6.9	±	2.4
	Cxcl12	7.1	±	2.0	60.1	±	9.9		15.9	±	1.6		66.1	±	24.8		-		

	Tlr4	-	-	-	42.7 ± 12.5	-
	Hif1a	-	-	-	25.9 ± 0.2	-
	Cmkor 1	-	22.9 ± 6.7	-	25.8 ± 8.9	-
	Myd88	-	-	8.8 ± 0.3	25.6 ± 8.4	-
	Mmp2	-	-	-	-	-
	Cxcr6	-	-	-	-	-
	Lif	-	-	-	-	-
	Inha	-	-	-	-	-

Table 4.1: mRNA levels of 84 genes pertaining to chemokines during myogenesis. Real Time RT-PCR was used to analyze the mRNA levels of 84 genes pertaining to chemokines in primary mouse muscle cells at 6, 16, 24, 36 and 48 hours in DM. Data are mean ± s.d, N=3.

Table 4.2

Chemokines and chemokine receptors expressed during in vitro myogenesis.

16 hours	24 hours			36 hours	Not expressed
Bmp10	Agtrl1	Ccr8	Gdf5	Blr1	Cxcr6
Ccbp2	Bdnf	Ccrl1	Gpr2	Bmp6	Inha
Ccl12	Bmp15	Ccrl2	Gpr81	Ccr2	Lif
Ccl17	Ccl1	Cmklr1	Il16	Cmkor1	Mmp2
Ccl2	Ccl11	Cmtm2a	Il18	Cmtm4	
Ccr1	Ccl19	Cmtm5	Il1a	Cxcl12	
Ccr3	Ccl20	Cxcl1	Il4	Hif1a	
Ccr9	Ccl4	Cxcl13	Il8ra	Il13	
Cmtm3	Ccl5	Cxcl15	Il8rb	Inhbb	
Cmtm6	Ccl6	Cxcl2	Ltb4r2	Myd88	
Csf1	Ccl7	Cxcl4	Rgs3	Nfkb1	
Csf2	Ccl8	Cxcl5	Slit2	Tlr4	
Cx3cl1	Ccl9	Cxcl7	Tnf		
Cx3cr1	Ccr1l1	Cxcl9	Tnfrsf1a		
Cxcl10	Ccr4	Cxcr3	Tnfsf14		
Cxcl11	Ccr5	Cxcr4	Trem1		
	Ccr6	Ecgf1	Xcl1		
	Ccr7				

Table 4.2: Chemokines and chemokine receptors expressed during in vitro myogenesis. Real Time RT-PCR was used to analyze the mRNA levels of 84 genes pertaining to chemokines in primary mouse muscle cells at 6, 16, 24, 36 and 48 hours in DM. Genes are shown at the peak expression time point (hours in DM) with N=3.

Chapter 5: MOR23 promotes muscle regeneration and regulates cell adhesion and migration

Introduction

Odorant receptors (ORs) are G-protein-coupled receptors (GPCR) expressed within the olfactory epithelium of the nose where they function as chemosensors to detect small molecules we perceive as smell. ORs are the largest receptor family in mammals comprising 3-5% of all genes (Young and Trask, 2002), with approximately 913 potential OR genes in the mouse genome, and 390 in the human genome (Olender et al., 2008; Zhang and Firestein, 2002). Microarray analyses suggest ORs are expressed in many tissues (Feldmesser et al., 2006), but functions of these receptors are known for only three: chemosensing in olfactory epithelium, proliferation in prostate cancer and chemotaxis in sperm of humans and mice (Fukuda et al., 2004; Neuhaus et al., 2009; Spehr et al., 2003).

One of the ORs that regulates sperm chemotaxis is mouse OR23 (MOR23, Olfr16) (Fukuda et al., 2004). We unexpectedly observed an increase in MOR23 expression in a microarray analysis performed during myogenesis of primary cultured mouse muscle cells (unpublished data). This increase occurred when muscle cells were undergoing extensive cell migration, adhesion and fusion to form multinucleated cells. In adult skeletal muscle, myogenesis occurs to regenerate muscle after injury. This process is dependent on satellite cells, which are normally quiescent, but in response to injury proliferate, and their progeny myoblasts differentiate and fuse with each other or with existing myofibers to restore normal tissue architecture. Migration and cell-cell adhesion are necessary for muscle cell differentiation (Kang et al., 2004) and fusion *in vitro* (Jansen and Pavlath, 2006). Therefore, identifying the molecules that control muscle cell

adhesion and migration may reveal potential molecular targets for improving muscle regeneration. As MOR23 regulates migration of sperm, we hypothesized that ORs may affect similar processes during myogenesis.

Here, we demonstrate that MOR23 is an important regulator of myogenesis *in vitro* and *in vivo* through affects on cell migration and adhesion, influencing downstream fusion events. In addition, our data suggest a MOR23 ligand is secreted by muscle cells *in vitro* and is also present in muscle tissue. Furthermore, a number of ORs were also expressed by regenerating muscle, which may suggest a larger role for ORs in tissue repair.

Results

Multiple ORs, with distinct patterns, are expressed during myogenesis

Multinucleated myofibers form through fusion of muscle cells, which requires cell migration and adhesion factors (Jansen and Pavlath, 2008). Myogenesis *in vitro* occurs in distinct phases (Figure 5.1A): proliferating myoblasts terminally differentiate to become fusion-competent myocytes in differentiation media (DM). By 24 hrs in DM, myocytes fuse with one another to form small, nascent myotubes with few nuclei. Further rounds of fusion create mature myotubes with many nuclei, by 48 hrs in DM. By microarray analysis, MOR23 mRNA was upregulated at 24 hrs of myogenesis (unpublished data). To validate the expression of MOR23 and also to determine whether other ORs are expressed during myogenesis *in vitro* and *in vivo*, we performed Real Time RT-PCR. Given the large numbers of mouse ORs, we choose 18 ORs previously identified in

microarray screens of skeletal muscle (Beggs et al., 2004; Melcon et al., 2006; Tseng et al., 2002; Zhao et al., 2002). Olfactory bulb RNA was used as a positive control for OR mRNA (Table 5.1). Thirteen ORs were expressed during myogenesis (Table 5.2 and 5.3, Figure 5.1). The majority of ORs expressed *in vitro* were most highly expressed at 0 hrs, when the muscle cells are proliferating myoblasts (Table 5.2, Figure 5.1B, C). A smaller number of ORs were increased with terminal differentiation, and most highly expressed at 24 hrs in DM, when myocytes are fusing. Interestingly, no ORs were upregulated or exclusively expressed at 48 hrs in DM, when most cells have already fused.

Expression of these ORs was also highly regulated during skeletal muscle regeneration (Table 5.3, Figure 5.1D, E). Only one OR was most highly expressed in uninjured muscle tissue, whereas the majority of ORs were upregulated at day 5 of regeneration, when muscle cells are proliferating, differentiating and fusing into nascent myofibers. The ORs expressed at day 5 were those detected at both 0 and 24 hrs *in vitro*. Two ORs were most highly expressed at day 10 after regeneration, when most muscle cell fusion had already occurred. The mRNA for most ORs was expressed both *in vitro* and *in vivo*, except for two. Olfr70 was expressed at very low levels *in vitro* and was not apparent *in vivo*. This discrepancy may be due to problems with sensitivity of detection or time points chosen *in vivo* or may be related to cell culture conditions. Olfr49, expressed only *in vivo*, might be expressed by cells other than muscle, or in a sub-population of muscle cells that was not retained *in vitro*. Together, our results demonstrate multiple ORs display distinct expression

patterns during myogenesis, suggesting that individual ORs may have non-redundant functions. ORs increased after differentiation and during fusion may have similar functions to ORs in sperm and olfactory neurons, regulating migration or adhesion. This subset of ORs was of specific interest as they might have conserved functions in muscle.

MOR23 is expressed during myogenesis and regulates myocyte migration

To investigate the role of a specific OR, MOR23 was selected, as its mRNA was upregulated during times of cell fusion and its expression was verified by use of a second primer pair (Table 5.2 and 5.3, Figure 5.1B, D, 2A, Figure 5.4); similarly immunoblot analysis revealed increased amounts of MOR23 protein at 24 hrs in DM (Figure 5.2B). The MOR23 antibody likely cross-reacts with the closely related olfactory receptor, Olfr1403, which shares 85% homology to MOR23 and whose mRNA is most highly expressed in myoblasts (Figure 5.3A, B). Based on the mRNA expression pattern for Olfr1403 most of the antibody reactive band at 24 hrs in DM likely reflects MOR23 and not Olfr1403.

In both olfactory neurons and sperm activation of ORs leads to similar downstream signaling events: activation of $G\alpha_{olf}$, a G-protein specific to odorant receptors, initiates signal transduction through membrane adenylyl cyclase III (mACIII) (Spehr et al., 2004b). We observed both isoforms of $G\alpha_{olf}$, and mACIII in muscle cells at 24 hrs in DM by immunoblotting (Figure 5.2B) suggesting that canonical OR signaling may occur within muscle cells.

Given that MOR23 is upregulated when myocytes are migrating and fusing, we hypothesized that MOR23 may have a conserved migratory function in myocytes. Thus, we examined the migration of myocytes in which MOR23 expression was knocked down by siRNA. First, to determine efficiency of knockdown, RNA was isolated from cells retrovirally infected with control or one of two distinct MOR23 siRNAs at 24 hr in DM and RT-PCR was performed; no MOR23 mRNA was observed with either MOR23 siRNA (Figure 5.4). Since both MOR23 siRNAs resulted in similar knockdown, subsequent experiments were carried out using MOR23 siRNA 3, unless otherwise indicated. To determine whether expression of other ORs was affected by MOR23 siRNA, RNA was isolated from cells infected with control or MOR23 siRNA and Real Time RT-PCR was performed; no affect on mRNAs for other ORs was observed with MOR23 siRNA (Table 5.4). By immunoblotting, cells infected with MOR23 siRNA exhibited at least a 58% decrease in MOR23 protein although some of the residual antibody reactive band could have been Olfr1403, and hence a greater decrease in MOR23 protein actually occurred (Figure 5.2C). To determine if MOR23 signaling was abrogated due to MOR23 siRNA, control and MOR23 siRNA cells were exposed to the synthetic MOR23 ligand lyral (Fukuda et al., 2004), and the levels of cAMP were detected by ELISA (Figure 5.2D). Levels of cAMP were increased 60% in control siRNA cells and MOR23 siRNA abrogated this effect, indicating MOR23 protein levels are significantly decreased in MOR23 siRNA cells as they do not increase cAMP in response to lyral.

Subsequently, Boyden chamber experiments were performed using control and MOR23 siRNA cells with conditioned media collected from muscle cells at 24 hrs in DM as the chemoattractant (Figure 5.2E). A 55% decrease in migration to CM was observed with each MOR23 siRNA. In order to determine if MOR23 siRNA cells had a general motility defect that could account for these results, time-lapse microscopy was performed at 24 hrs in DM in regular culture dishes (Figure 5.2F). MOR23 siRNA did not significantly affect cell velocity when compared to control siRNA cells. To determine if removal of any OR was sufficient to alter migration, we focused on the following two ORs: Olfr1508 and Olfr1403; Olfr1508 was the only OR to share a similar expression pattern to MOR23, both *in vivo* and *in vitro*, (Figure 5.5A, B), and Olfr1403 has the highest homology to MOR23. Knockdown of either Olfr1403 (Figure 5.3D) or Olfr1508 (Figure 5.5C) did not affect migration to CM (Figure 5.3E, 5.5D). Hence, decreased migration to CM was specific to loss of MOR23 and not simply due to loss of any OR.

Migration was also examined in cells expressing recombinant MOR23. First, to determine whether recombinant MOR23 was properly trafficked to the plasma membrane, cell surface biotinylation was used. The subsequent biotin pull-down was assayed for MOR23 by immunoblotting; a 3-fold increase in cell surface MOR23 was observed (Figure 5.2G). We performed Boyden chamber experiments using diluted CM to determine if these MOR23 over-expressing (OE) cells were more sensitive to CM. As the CM was diluted, less migration occurred in both control and MOR23 OE cells; however, the MOR23 OE cells

exhibited higher levels of migration at all dilutions (Figure 5.2H). Thus, increasing MOR23 levels alone is sufficient to change the migratory behavior of myocytes. Together, these data indicate that MOR23 regulates myocyte migration and suggest that an endogenous MOR23 ligand is secreted by fusing muscle cultures.

To discern whether a MOR23 ligand is present in muscle tissue *in vivo*, Boyden chamber experiments were performed using crushed muscle extract. CME resulted in a 9-fold increase of myocyte migration, which was abrogated in MOR23 siRNA cells (Figure 5.2I). In order to test the migration of MOR23 OE cells, a different optimal concentration of CME was used based on preliminary dose response curves. With MOR23 OE a 3-fold increase in myocyte migration occurred (Figure 5.2J). These data suggest that a ligand(s) or its precursor is present within muscle tissue prior to induction of muscle regeneration.

MOR23 regulates directed migration

The decreased migration of MOR23 siRNA cells to CM likely results from a defect in directed migration as no general motility defect was observed. To address this question, we used the Dunn chemotaxis chamber to determine if MOR23 siRNA cells respond to a gradient of lyral. Time-lapse microscopy was performed for 3 hrs, the paths of individual cells tracked and the final location of each cell in relation to its origin determined (Figure 5.6A). Myocytes migrated toward lyral, yielding migration paths that ended on the lyral side of the chamber. To statistically analyze the pattern of migratory paths in response to lyral,

directional data were summarized in circular histograms. Rayleigh's statistical test for clustering revealed that control cells (Figure 5.6B) but not MOR23 siRNA cells (Figure 5.6C) migrated toward lyral. A rescue experiment in which myocytes expressed both MOR23 siRNA and recombinant MOR23 (Figure 5.7) demonstrated directed cell migration toward lyral (Figure 5.6D), indicating that migration to lyral is specific to MOR23. To determine whether migration towards lyral requires mACIII, an inhibitor of membrane adenylyl cyclases, SQ22536, was used (Figure 5.8). Addition of the mAC inhibitor abrogated migration towards lyral (Figure 5.6E), indicating that canonical OR signaling is used in lyral-directed migration. As a control, to determine if lyral-dependent migration was specific to MOR23, myoblasts which express the highly homologous Olfr1403 and the lyral-responsive Olfr15 (Saito et al., 2009) but not MOR23, were tested in Dunn chambers. These cells did not undergo directed migration towards 10^{-7}M lyral (Figure 5.6F). In addition, knockdown of Olfr1403 or Olfr1508 in myocytes did not affect migration to lyral (Figure 5.3F, 5.5E), revealing that migration of myocytes to lyral is specific to MOR23 and occurs in a directed manner.

Loss of MOR23 alters cell-cell adhesion

Recent evidence suggests that ORs may regulate axon guidance of olfactory neurons via downstream changes in the expression of cell-cell adhesion molecules (Serizawa et al., 2006). To determine whether loss of MOR23 alters cell adhesion, a solution based cell adhesion assay was utilized in which control or MOR23 siRNA myocytes were suspended in media and aliquots taken over 60

min to count the number of adhered and un-adhered cells using phase-contrast microscopy. Myocytes infected with MOR23 siRNA did not efficiently adhere to one another (Figure 5.9A). Although no difference in adhesion was observed at early time points, at later time points, a significant increase occurred in un-adhered MOR23 siRNA cells (Figure 5.9B). At later times MOR23 siRNA cells remained in small clusters compared to control siRNA cells (Figure 5.9C). Cell viability was ~99% at 60 min in all conditions (data not shown). Canonical OR signaling is also necessary for axonal migration and adhesion (Imai et al., 2006), and to determine whether it regulates cell-cell adhesion of myocytes, cells were treated with mAC inhibitor SQ22536, or vehicle (Figure 5.9D). At early time points, no difference was observed, however at later time points a significant increase occurred in un-adhered cells with SQ22535 (Figure 5.9E). Furthermore, SQ22536-treated cells formed smaller clusters than vehicle (Figure 5.9F). Knockdown of Olfr1403 or Olfr1508 did not affect adhesion (Figure 5.3G, 5.5F). Hence, adhesion affects were specific to loss of MOR23 and not simply due to loss of any OR. Together, these data suggest that MOR23 signaling affects adhesion of myocytes to one another likely through a downstream adhesion molecule.

Myotube formation is dependent on MOR23

To test whether MOR23-dependent migration and/or adhesion is involved in muscle cell differentiation or fusion, we examined myogenesis *in vitro* in cells containing one of two MOR23 siRNAs. After 24 or 48 hr in DM, cells were

immunostained using an antibody against embryonic myosin heavy chain (eMyHC). After 24 hrs in DM, both MOR23 siRNA cultures contained fewer and smaller nascent myotubes compared to control (Figure 5.10A). This defect in myotube formation was not due to an affect on differentiation, as measured by the percentage of nuclei found in $eMyHC^{+}$ cells (Figure 5.10B), nor to a decrease in the total number of nuclei (data not shown). Rather, MOR23 siRNA myocytes exhibited a clear defect in cell fusion (Figure 5.10C-E). The fusion index, as well as the number of myotubes, was transiently decreased 33% and 26% respectively in MOR23 siRNA cultures (Figure 5.10C, D). A 15% decrease in myonuclear number was also noted at 24 hrs in DM in MOR23 siRNA cultures, which remained virtually unchanged at 48 hrs (Figure 5.10E) and 72 hrs (data not shown). The transient fusion defects in MOR23 siRNA cultures may be due to compensation from other factors which regulate fusion.

To determine whether the impaired ability of MOR23 siRNA cultures to fuse is specific to the loss of MOR23; assays using MOR23 OE were also performed. MOR23 OE resulted in an increased number of nascent myotubes at 24 hrs in DM (Figure 5.10F), with no change in differentiation (Figure 5.10G). Fusion assays revealed a 30% increase in the fusion index and a 40% increase in the number of myotubes at 24 hrs in DM, with smaller increases in both parameters still noted at 48 hrs and 72 hrs (Figure 5.10H, I and data not shown). Myonuclear number was also increased ~10% at both time-points in MOR23 OE cultures (Figure 5.10J). Together these data suggest that MOR23-dependent migration and/or adhesion contributes to the fusion of myocytes to form nascent myotubes.

Changes in myonuclear number with MOR23 siRNA or OE at 48 hrs suggest that MOR23-dependent migration and/or adhesion may also regulate formation of mature myotubes from fusion of myocytes with nascent myotubes. Since the formation of nascent myotubes was affected in previous assays, we could not independently determine a role for MOR23 in this later stage of fusion. To analyze if MOR23 affects the fusion of myocytes with nascent myotubes (Figure 5.1A), control and MOR23 siRNA nascent myotubes and myocytes were created separately, labeled with green or orange fluorescent dye, co-cultured for 24 hrs (Figure 5.10K), and the number of dual labeled myotubes was quantified. With MOR23 siRNA in myocytes the percentage of myotubes with dual label was decreased 20% compared to control (Figure 5.10L), whereas MOR23 siRNA in myotubes had no effect on dual labeling. To determine whether increased expression of MOR23 on myocytes could enhance fusion, control or MOR23 OE myocytes were co-cultured with control nascent myotubes (Figure 5.10M). MOR23 OE in myocytes increased dual labeling 35% (Figure 5.10N), indicating MOR23 expression on myocytes alone is sufficient to increase fusion of myocytes with nascent myotubes. Together, these data indicate that MOR23 is functionally required on myocytes. Therefore, MOR23-dependent processes can affect myonuclear addition at later stages of myogenesis.

MOR23 regulates myogenesis *in vivo*

To determine the detailed expression pattern of MOR23 during adult regenerative myogenesis, RNA was isolated from mouse gastrocnemius muscles

at different times after injury. Using Real Time RT-PCR, MOR23 mRNA was induced 4-fold during days 3-7 post-injury (Figure 5.11A), a time of extensive cell fusion. By 14 days post-injury, when regeneration is nearly complete, MOR23 mRNA levels had returned to uninjured control levels. MOR23 protein levels were increased at day 5 post-injury, a time when fusion is ongoing and nascent myofibers are present, as determined by immunoblot (Figure 5.11B, G). Olfr1403 mRNA was not increased during muscle regeneration at these times (Figure 5.3C). Sections from gastrocnemius muscles at day 5 post-injury were immunostained with an antibody against MOR23 (Figure 5.11C). Regenerating myofibers, identified by their centrally located nuclei and small size, contained MOR23. To determine whether mononucleated muscle cells express MOR23 during regeneration, cells were isolated from regenerating muscle and myogenic cells (α7-integrin^{+}CD31^{-}CD45^{-} (Kafadar et al., 2009)) were separated from immune and endothelial cells (α7-integrin^{-}CD31^{+}CD45^{+}) by FACS (Figure 5.12). Real Time RT-PCR revealed expression of MOR23 mRNA in α7-integrin^{+}CD31^{-} CD45^{-} cells, which also expressed myogenin mRNA, confirming their myogenicity (Figure 5.11D). MOR23 mRNA was not apparent in the α7-integrin^{-}CD31^{+}CD45^{+} fraction; this fraction did express Mac1 mRNA, suggesting many of these cells are immune cells. Furthermore, immunostaining for MOR23 in α7-integrin^{+}CD31^{-} CD45^{-} cells indicated ~86% of muscle cells express MOR23 (Figure 5.11E). The myogenic purity of these cells was >99% as determined by the expression of Myf5. Thus, MOR23 expression is induced in myogenic cells during muscle regeneration when myofibers are forming. Overall these data suggest that the

induction of MOR23 expression may be tied to migration and/or adhesion events that occur during cell fusion in muscle regeneration.

To analyze MOR23 function during muscle regeneration, gastrocnemius muscles were induced to regenerate with $BaCl_2$ two days prior to electroporation of either MOR23 siRNA or OE plasmids. Induction of regeneration allowed myoblasts to take up plasmid, as determined in preliminary experiments. Gastrocnemius muscles were isolated 5, 10 or 20 days post-electroporation (Figure 5.11F). Knockdown of MOR23 protein was determined by immunoblot at day 5, and an 85% reduction of MOR23 was observed (Figure 5.11G), suggesting that the majority of the immunoreactive band is MOR23 and not Olfr1403. Although little to no difference existed in proliferation or differentiation in muscles that received MOR23 siRNA compared to control (Figure 5.13), abnormalities in muscle regeneration were noted in MOR23 siRNA muscles at all time points (Figure 5.11H). The mean cross-sectional area (XSA) of regenerating myofibers in MOR23 siRNA muscles was decreased 26-38% (Figure 5.11I) with a corresponding 30% increase in the number of myofibers smaller than 1500μm^2 at day 20 (Figure 5.11J). In addition, the number of regenerating myofibers per field increased ~50% with MOR23 siRNA (Figure 5.11K). In contrast, MOR23 OE *in vivo* resulted in a 5-fold increase in MOR23 mRNA (Figure 5.14), and increased myofiber XSA ~50% (Figure 5.11L), with a corresponding 39% decrease in the number of myofibers smaller than 1500μm^2 at day 20 (Figure 5.11M). In addition, the number of regenerating myofibers per field decreased

21-36% with MOR23 OE (Figure 5.11N). These data suggest that optimal muscle regeneration is dependent on MOR23.

MOR23 affects myofiber branching *in vivo*

Small clusters of myofibers in MOR23 siRNA muscles were observed, which suggested that myofiber branching may have occurred with loss of MOR23. Myofiber branching is characterized by a single myofiber having a plasma membrane contiguous with several smaller myofibers (Figure 5.15A). Myofiber branching is an important phenomenon as it is increased with multiple injuries, aging, and muscular dystrophy (Ontell et al., 1982). The molecules which regulate this process are unknown, although cell-cell adhesion molecules are hypothesized to play a role fusing branched myofibers to form mature myofibers. We hypothesized that the increase in small myofibers observed in MOR23 siRNA muscles may be due to an increase in unresolved myofiber branches, yielding the appearance of many small myofibers rather than large myofibers apparent in control siRNA muscles. To determine whether MOR23 may regulate myofiber branching, cross-sections 170μm apart were isolated from gastrocnemius muscles 5 days after electroporation. Myofiber branching was quantified by determining the number of regenerating myofibers that were a single myofiber in one section and multiple fibers occupying the same area in a second section (Figure 5.15B). Muscles with MOR23 siRNA exhibited a 40% increase in the percentage of branching myofibers (Figure 5.15C), and more branches per myofiber (Figure 5.15D). MOR23 OE did not result in changes in myofiber

branching at day 5 (Figure 5.15E). In order to visualize the entire myofiber, single myofibers were isolated to determine the extent of branching. We were unable to isolate intact regenerating myofibers at day 5; however myofiber branches could be visualized at day 10 and 20 using phase-contrast microscopy and DAPI-staining was used to identify regenerating myofibers by the presence of centrally located nuclei (Figure 5.15F). Myofibers with multiple tracks of central nuclei, but unbranched cytoplasm, are mature regenerated myofibers (Figure 5.15F, arrowheads). Muscles electroporated with MOR23 siRNA exhibited a 60% increase in regenerating myofibers with branches (Figure 5.15G) and 90% more branches per myofiber (Figure 5.15H). In contrast, a 50% decrease in the number of branched myofibers (Figure 5.15I) occurred in MOR23 OE muscles at day 20 with 70% fewer branches per myofiber (Figure 5.15J). These data suggest that myofiber branching is regulated by MOR23, possibly through its effects on migration and adhesion of muscle cells.

Discussion

In many systems, tissue repair requires cell migration and adhesion. Our results suggest these processes are also necessary for proper regeneration of muscle tissue. We show multiple ORs are expressed by muscle cells during myogenesis *in vitro* and muscle regeneration *in vivo*. In depth studies on one of these ORs, MOR23, revealed a role in regulating migration and adhesion of muscle cells, which are critical for cell fusion. Importantly, these studies identify an unexpected functional role for MOR23 in skeletal muscle regeneration, demonstrating a novel function for an OR in tissue repair.

MOR23 was expressed by terminally differentiated myocytes *in vitro*, functioning to regulate their migration. Interestingly, MOR23 also regulates migration of mouse sperm *in vitro* (Fukuda et al., 2004) and ORs are hypothesized to play a role in axonal growth cone migration (Feinstein et al., 2004), suggesting that MOR23 has a conserved function in migration of multiple cell types. Although synthetic ligands are known for many odorant receptors, endogenous ligands have not been identified. Both crushed muscle extract and conditioned media contained a ligand that stimulated myocyte migration in a MOR23-dependent manner. CME was prepared from uninjured muscle tissue, which indicates that the MOR23 ligand is present prior to the onset of muscle injury. The ligand may be present in an inactive form in normal muscle tissue and become activated by the “crushing” used in preparing CME. *In vivo*, HGF is expressed in an inactive form, which is expressed by muscle cells and then bound by the extracellular matrix until matrix metalloproteases cleave and

release the active form (Allen et al., 2003). Alternatively, the ligand may be stored within myofibers and released upon myofiber degeneration. CM collected from differentiating muscle cultures *in vitro* also contained the MOR23 ligand, demonstrating that muscle cells themselves can be the source of the ligand. These results do not rule out that other cell types may also express the MOR23 ligand *in vivo*. Preliminary data indicate the MOR23 ligand is likely not sensitive to heat or Proteinase K digestion (data not shown). Identification of sources for the MOR23 ligand will allow future purification of an endogenous OR ligand from a readily available source, as opposed to follicular fluid, which is the only other hypothesized source of an endogenous OR ligand (Spehr and Hatt, 2004). If the ligand for MOR23 can be isolated, it may give insight into the nature of endogenous ligands for other odorant receptors, including the human odorant receptor found in sperm.

MOR23 also regulated cell-cell adhesion, indicating another conserved function for ORs between muscle and olfactory neurons. In olfactory neurons, OR signaling is required for expression of adhesion molecules, which correlates with altered axon guidance into specific glomeruli (Imai et al., 2006). However, changes in the adhesiveness of the olfactory neurons were not assayed. In contrast, we performed adhesion assays which demonstrated that loss of MOR23 or OR signaling decreased cell-cell adhesion, suggesting that MOR23 regulates adhesion indirectly, possibly through regulating expression of specific downstream adhesion molecules. Further studies are needed to determine which adhesion molecules may be affected by MOR23 signaling.

MOR23 expression was induced with muscle regeneration and manipulating the levels of MOR23 affected several aspects of muscle regeneration, including myofiber cross-sectional area, number and branching. Although branched myofibers have been noted in the literature for 100 years (Schmalbruch, 1976; Volkmann, 1893), no molecule is known to regulate their formation. We show that loss of MOR23 increased myofiber branching. Expression of MOR23 by mononucleated muscle cells and regenerating myofibers *in vivo* suggests both migration and adhesion could contribute to these effects. If myocyte migration is required for fusion with existing regenerating myofibers, then defects in migration would increase the formation of *de novo* myofibers. Alternatively, formation of small myofibers may normally occur, which adhere to one another, and then fuse to form larger myofibers, and when adhesion is disrupted, branched myofibers occur. Therefore, loss of MOR23 may disrupt myofiber formation on several levels. Since muscles containing unbranched myofibers are stronger than those with extensive branching (Chan et al., 2007) and MOR23 OE can decrease the occurrence of branching, MOR23 may be an important target for therapies directed at increasing strength of dystrophic muscle.

As MOR23 functions in the repair of skeletal muscle, and multiple ORs are expressed during myogenesis, other ORs may regulate similar or distinct processes. Recent evidence suggests that activation of an OR inhibits proliferation of prostate cancer cells (Neuhaus et al., 2009), therefore, ORs that were upregulated in myoblasts may regulate proliferation. Also, as muscle cells must interact directly with one another for terminal differentiation to occur (Krauss

et al., 2005), ORs in addition to MOR23 may play a role in the migration or adhesion of these cells. As Olfr49 was expressed only in muscle tissue, other cell types present in muscle, such as immune cells, endothelial cells, fibroblasts, or neurons may also express ORs. Finally, ORs present in uninjured muscle or at later time points in regeneration may be necessary for quiescent satellite cells, either to keep these cells in their niche or to affect their proliferation state. Our data suggest that multiple ORs may regulate myogenesis, and therefore, may be necessary for proper tissue repair. Additional studies will be necessary to determine the specific effects of each of these ORs both *in vitro* and *in vivo*.

Interestingly, in the olfactory neuron field, only one OR is expressed in each cell (Touhara et al., 1999). As multiple ORs were expressed by muscle cultures, sub-populations of muscle cells may each express a single OR. Potentially the "one cell, one OR" hypothesis may not hold true for muscle cells, and several ORs are expressed by each muscle cell. A redundant system may exist, so that even if one OR signal is somehow blocked, other ORs may substitute. Alternatively, cells expressing multiple ORs may utilize specific ORs in a spatial-temporal manner. Additional studies may elucidate whether multiple populations of muscle cells each express a single OR, or whether each muscle cell expresses several ORs.

In summary, MOR23 is a key regulator of myogenesis through its actions on cell migration and adhesion, affecting downstream fusion *in vitro* and muscle regeneration *in vivo*. Cell migration within muscle is a major issue in transplantation of cells for treatment of muscular dystrophy (Skuk and Tremblay,

2003; Smythe et al., 2001). Study of the receptor-ligand pairs that regulate migration and/or adhesion of muscle cells may allow for more efficient therapeutic strategies. In addition, further studies on ORs may reveal additional unexpected functions in various tissues.

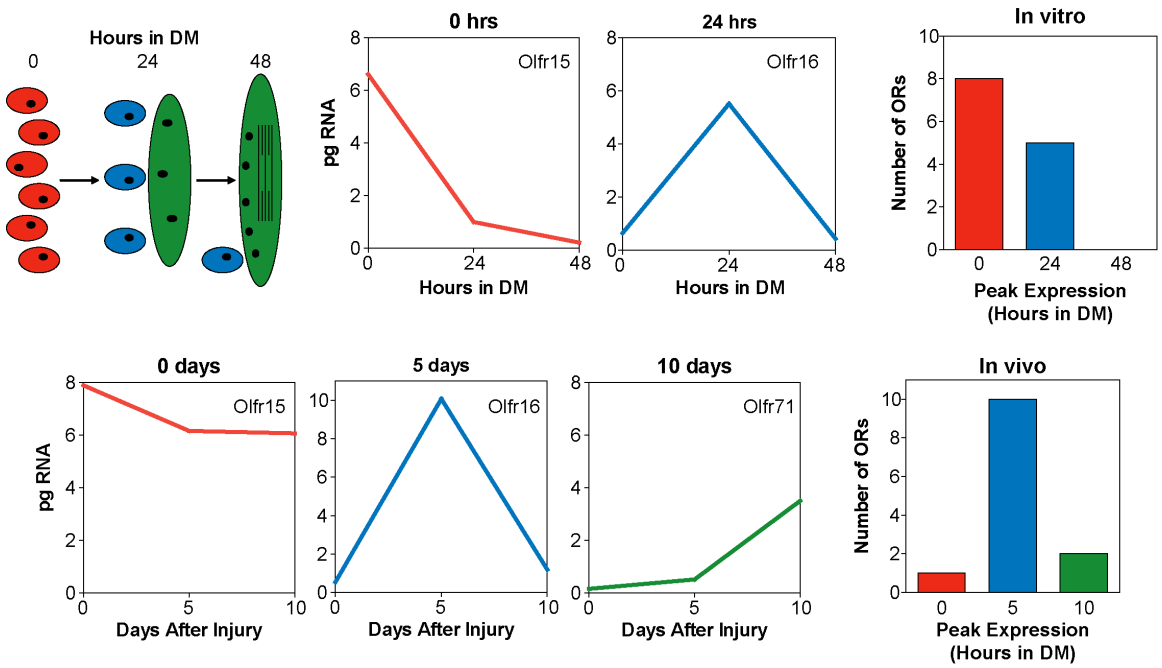

Hours in DM
0
24
48
0 hrs
Olfr15
pg RNA
Hours in DM
24 hrs
Olfr16
Hours in DM
In vitro
Number of ORs
Peak Expression
(Hours in DM)
0 days
Olfr15
pg RNA
Days After Injury
5 days
Olfr16
Days After Injury
10 days
Olfr71
Days After Injury
In vivo
Number of ORs
Peak Expression
(Hours in DM)

Figure 5.1

Multiple ORs are expressed during *in vitro* myogenesis and muscle regeneration. Real Time RT-PCR was used to analyze the time-course of expression both *in vitro* and *in vivo* for 19 ORs. A) During myotube formation the majority of myoblasts (red) differentiate into myocytes (blue). Myocytes fuse with one another to form small nascent myotubes with few nuclei. Subsequently, nascent myotubes fuse with myocytes to form large myotubes with many nuclei. B) Two patterns of expression were observed *in vitro*: peak expression either during proliferation or after terminal differentiation. Olfr15 and Olfr16 (MOR23) are shown. C) The number of ORs with highest expression levels at each time point *in vitro*. The majority of ORs are expressed in proliferating cells at 0 hrs in DM; however several ORs are also expressed at 24 hrs in DM, during the fusion process. D) Three patterns were observed *in vivo*: peak expression in uninjured muscle and increased expression day 5 or day 10 after injury. Olfr15, Olfr16 (MOR23) and Olfr71 are shown. E) The number of ORs with highest expression levels at each time point *in vivo*. The majority of ORs demonstrated peak expression at day 5 after injury, with few ORs showing peak expression either earlier or later. All data are mean ± SEM.

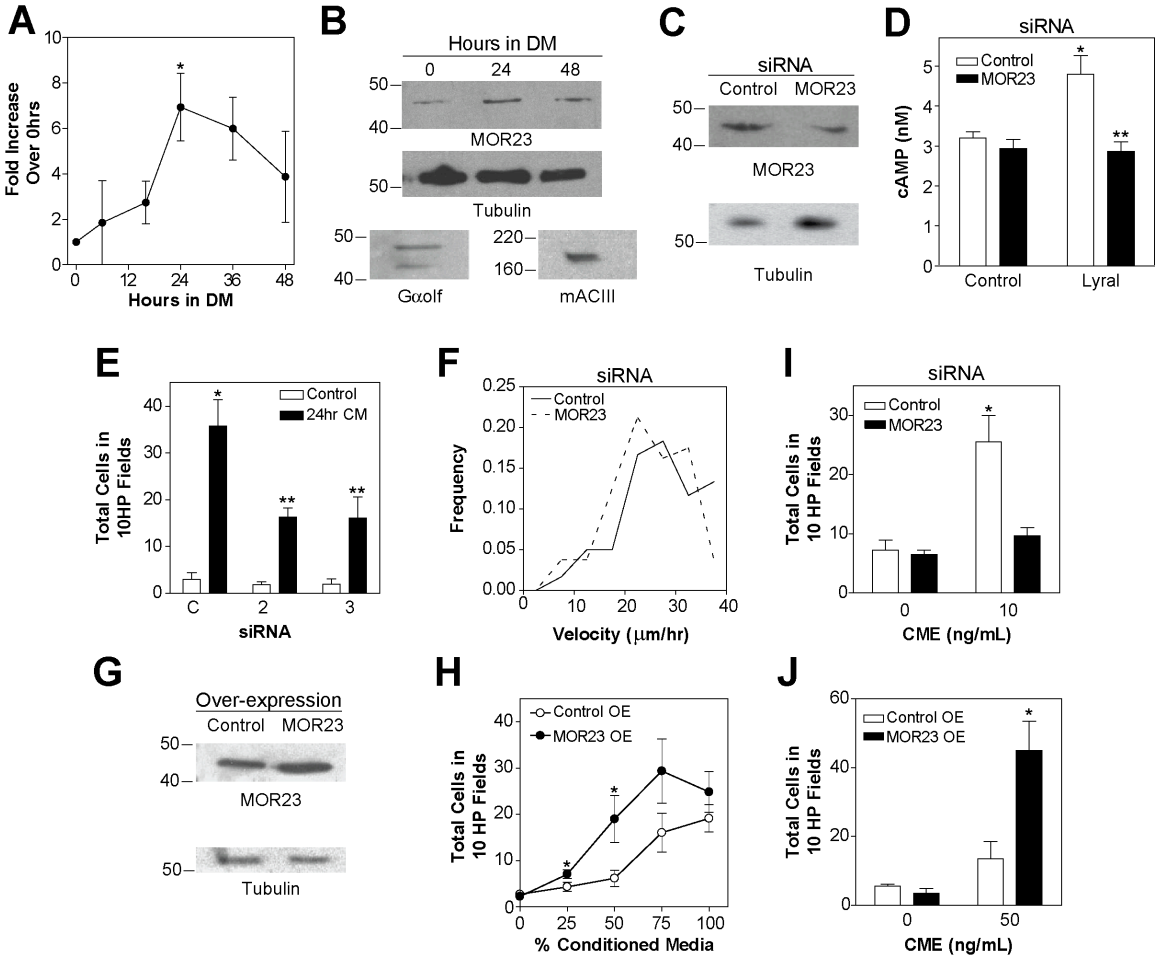

A
Fold Increase Over 0hrs
Hours in DM
B
Hours in DM
0
24
48
MOR23
Tubulin
Gαolf
mACIII
C
siRNA
Control
MOR23
MOR23
Tubulin
D
siRNA
Control
MOR23
cAMP (nM)
Control
Lyral
E
Control
24hr CM
Total Cells in 10HP Fields
C
2
3
siRNA
F
siRNA
Control
MOR23
Frequency
Velocity (μm/hr)
I
siRNA
Control
MOR23
Total Cells in 10 HP Fields
CME (ng/mL)
G
Over-expression
Control
MOR23
MOR23
Tubulin
H
Control OE
MOR23 OE
Total Cells in 10 HP Fields
% Conditioned Media
J
Control OE
MOR23 OE
Total Cells in 10 HP Fields
CME (ng/mL)

Figure 5.2

MOR23 regulates myocyte migration during myogenesis. A) Real Time RT-PCR indicates MOR23 mRNA was significantly increased at 24 hrs in DM. Data were normalized to 18S rRNA (*p<0.05 from 0). B) Immunoblots for MOR23 and OR signaling proteins; $G\alpha_{olf}$ and membrane adenylyl cyclase III (mACIII) at 24 hrs in DM. Tubulin was used as a loading control. C) Immunoblots indicate MOR23 protein was decreased at least 58% by MOR23 siRNA. D) Myocytes from control or MOR23 siRNA cultures were treated with lyral and levels of cAMP were determined (* p<0.05 from control; ** p<0.05 from lyral). E) Myocytes from control or MOR23 siRNA cultures were allowed to migrate to conditioned media. MOR23 siRNA decreased migration by 55% compared to control (* p<0.05; ** p<0.01 from control). F) Myocyte velocity was calculated from 3 hrs of time-lapse microscopy with 20 cells analyzed in each of 3 isolates. Minor decreases in velocity were observed in MOR23 siRNA cultures at 24 hrs in DM. G) MOR23 OE cells at 24 hr in DM demonstrated increased levels of cell surface MOR23 compared to control; immunoblots were performed after cell surface biotinylation and biotin pull-down. H) Myocytes from control or MOR23 OE cells were allowed to migrate to dilutions of CM. MOR23 OE myocytes exhibited increased migration at all concentrations of CM compared to control (* p<0.05). I) Control or MOR23 siRNA myocytes were allowed to migrate in Boyden chambers to crushed muscle extract. Control siRNA myocytes increased migration to CME 2.5-fold, but MOR23 siRNA myocytes did not. J) Control or MOR23 OE myocytes were allowed to migrate in Boyden chambers to CME. Migration of MOR23 OE

myocytes to CME was increased 3-fold compared to control myocytes (* $p<0.05$). All data are mean ± SEM.

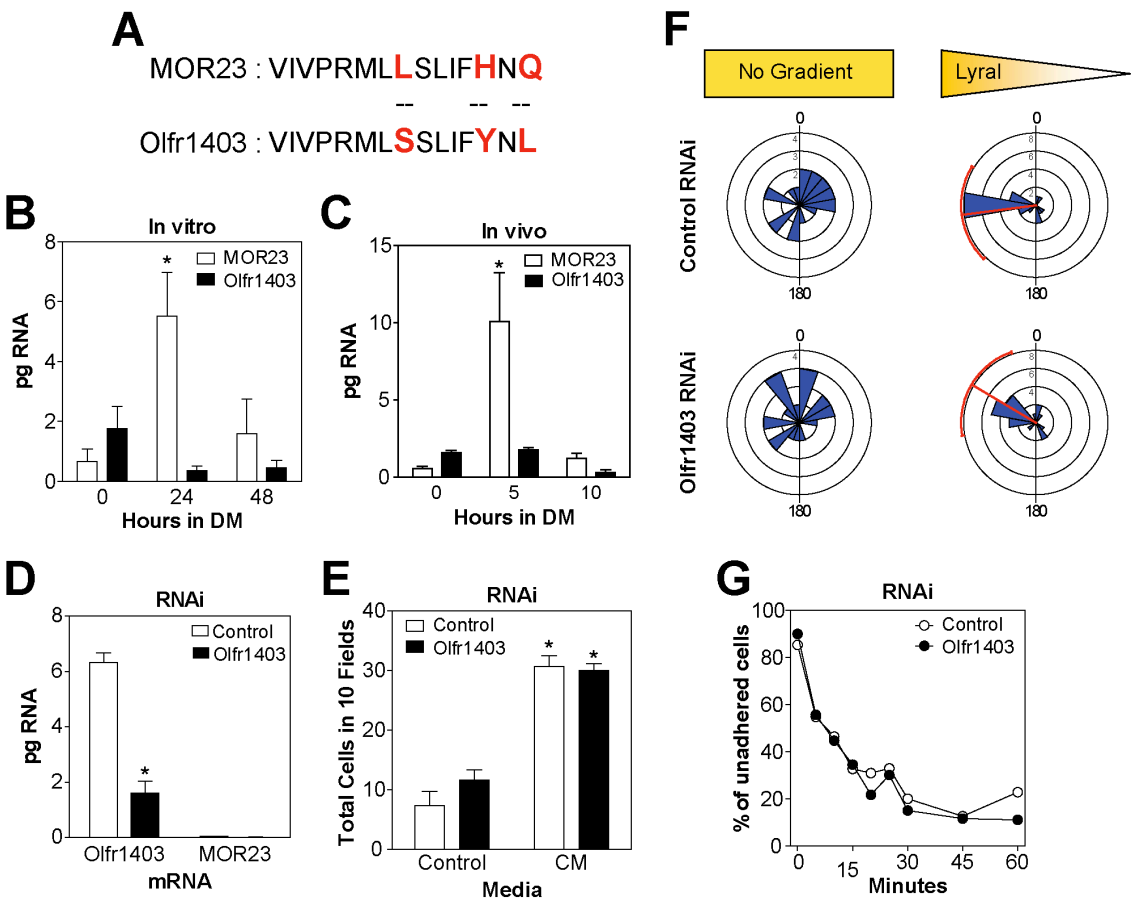

A
MOR23 : VIVPRMLLSLIFHNQ
-- -- --
Olfr1403 : VIVPRMLSSLIFYNL
B
In vitro
MOR23
Olfr1403
pg RNA
Hours in DM
C
In vivo
MOR23
Olfr1403
pg RNA
Hours in DM
D
RNAi
Control
Olfr1403
pg RNA
Olfr1403
MOR23
mRNA
E
RNAi
Control
Olfr1403
Total Cells in 10 Fields
Control
CM
Media
F
No Gradient
Lyral
Control RNAi
Olfr1403 RNAi
G
RNAi
Control
Olfr1403
% of unadhered cells
Minutes

Figure 5.3

Olfactory receptor 1403 is expressed by muscle cells *in vitro* and during muscle regeneration, and does not regulate similar processes as MOR23.

A) The MOR23 antibody likely cross-reacts with the closely related olfactory receptor, Olfr1403, as the 15 amino acid epitope only contains three differences between the two proteins (dashes and red letters). B) Real Time RT-PCR for MOR23 and Olfr1403 mRNA during myogenesis *in vitro* (*, $p<0.05$ from 0). MOR23 mRNA was most highly expressed at 24 hrs in DM, whereas Olfr1403 mRNA was highest at 0 hrs. These data suggest that the antibody reactive band at 0 hrs is Olfr1403, whereas at 24 hrs in DM the band should be mostly MOR23 (Fig. 2B). C) Real Time RT-PCR for MOR23 and Olfr1403 mRNA in gastrocnemius muscles at different times post-injury (*, $p<0.05$ from 0). Olfr1403 mRNA was present in both uninjured muscle and 3 days after injury; at 5 days post-injury, levels of Olfr1403 mRNA had decreased and MOR23 mRNA had increased. These data indicate that at day 5 post-injury most of the MOR23 antibody reactive band is likely MOR23, as opposed to Olfr1403 (Fig. 6B). D) RNAi against Olfr1403 resulted in decreased levels of Olfr1403 mRNA at 0 hrs in DM, with no effect on MOR23 mRNA. Data are normalized to 18S rRNA (*$p<0.05$ from C). E) Myocytes from control or Olfr1403 RNAi cultures were allowed to migrate to CM in Boyden chambers. No change in migration to CM was observed with Olfr1403 RNAi. F) Circular histogram plots summarizing Dunn chamber data with 15-20 cells analyzed in each experiment. Lyral gradient was highest at left side. Red line and arc indicate the mean direction and 99% confidence interval

for conditions in which significant clustering of cell migration occurred. No change in migration to lyral was observed with Olfr1403 RNAi. G) Myocytes with control or Olfr1403 RNAi were incubated in suspension for 60 min with aliquots taken regularly, to determine the percentage of un-adhered and adhered cells by phase-contrast microscopy. Olfr1403 RNAi did not affect adhesion of cells to one another. All data are mean ± SD of a single experiment performed in triplicate.

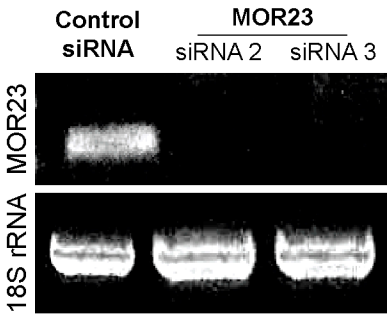


Figure 5.4

MOR23 mRNA is decreased with two distinct MOR23 siRNAs. Semi-quantitative RT-PCR for MOR23 mRNA and 18S rRNA was performed on muscle cells infected with control or one of two MOR23 siRNA retroviruses. Both MOR23 siRNAs yielded loss of MOR23 mRNA.

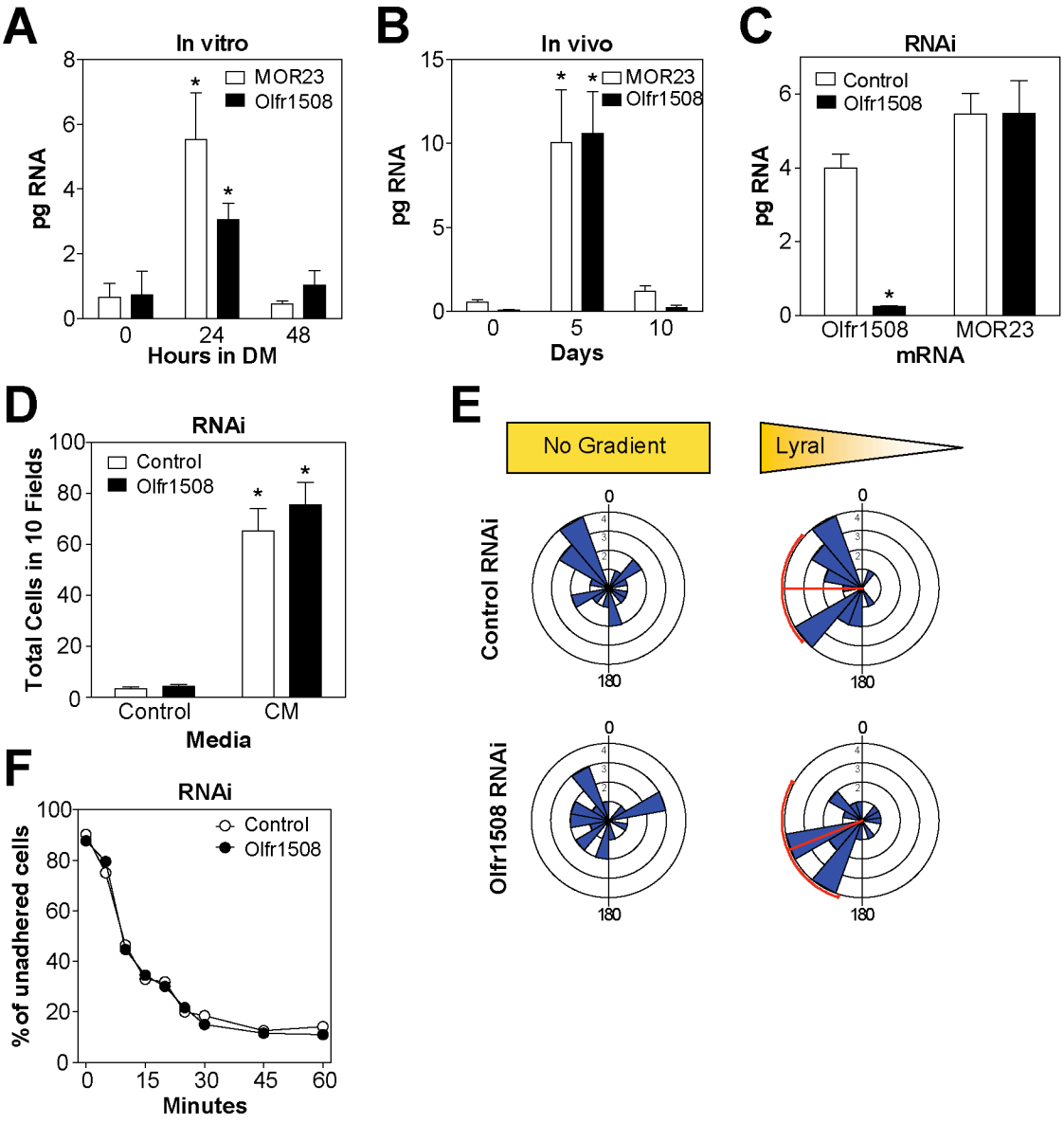

A
In vitro
MOR23
Olfr1508
pg RNA
Hours in DM
B
In vivo
MOR23
Olfr1508
pg RNA
Days
C
RNAi
Control
Olfr1508
pg RNA
Olfr1508
MOR23
mRNA
D
RNAi
Control
Olfr1508
Total Cells in 10 Fields
Control
CM
Media
E
No Gradient
Lyral
Control RNAi
Olfr1508 RNAi
F
RNAi
Control
Olfr1508
% of unadhered cells
Minutes

Figure 5.5

Olfactory receptor 1508 is expressed by muscle cells *in vitro* and during muscle regeneration, and does not regulate similar processes as MOR23. A) Real Time RT-PCR for MOR23 and Olfr1508 mRNA during myogenesis *in vitro*. Both were increased at 24 hrs in DM (*p<0.05 from 0). B) Real Time RT-PCR for MOR23 and Olfr1508 mRNA in gastrocnemius muscles at different times post-injury. Both were increased at day 5. All data were normalized to 18S rRNA (*p<0.05 from 0). C) RNAi against Olfr1508 resulted in decreased levels of Olfr1508 mRNA at 24 hrs in DM (*p<0.05 from C), with no effect on MOR23. D) Myocytes from control or Olfr1508 RNAi cultures were allowed to migrate to CM in Boyden chambers (*p<0.05 from C). No change in migration to CM was observed with Olfr1508 RNAi. E) Circular histogram plots summarizing Dunn chamber data with 15-20 cells analyzed in each experiment. Lyral gradient was highest at left side. Red line and arc indicate the mean direction and 99% confidence interval for conditions in which significant clustering of cell migration occurred. No change in migration to lyral was observed with Olfr1508 RNAi. F) Myocytes with control or Olfr1508 RNAi were incubated in suspension for 60 min with aliquots taken regularly, to determine the percentage of un-adhered and adhered cells by phase-contrast microscopy. Olfr1508 RNAi did not affect cell-cell adhesion. All data are mean ± SD of a single experiment performed in triplicate.

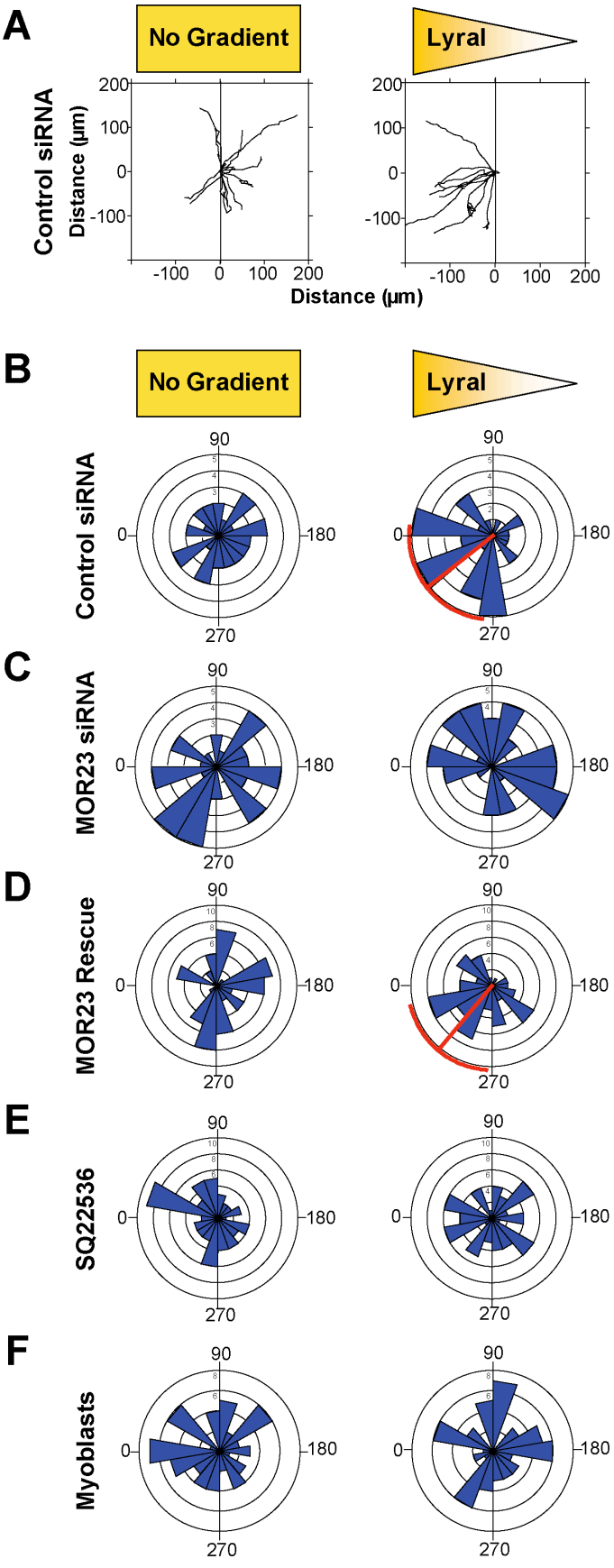

A
No Gradient
Lyral
Control siRNA
Distance (µm)
200
100
0
-100
-100
0
100
200
Distance (µm)
B
No Gradient
Lyral
Control siRNA
C
MOR23 siRNA
D
MOR23 Rescue
E
SQ22536
F
Myoblasts
90
0
180
270

Figure 5.6

MOR23 regulates directed migration of myocytes to lyral. A) Migratory paths of myocytes tracked for 3 hrs with pictures every 5 min in a representative Dunn chamber experiment with 15-20 cells in each graph. Lyral gradient was highest at left side. B-F) Circular histogram plots summarizing Dunn chamber data from 3-5 independent cell isolates with 15-20 cells analyzed in each experiment. Lyral gradient was highest at left side. Red line and arc indicate the mean direction and 99% confidence interval for conditions in which significant clustering of cell migration occurred. B, C) Control cells exhibited directed migration to lyral which was abolished in MOR23 siRNA cells. D) Migration to lyral is MOR23-specific as MOR23 siRNA cells rescued by MOR23 OE exhibited directed migration. E) Migration is dependent on membrane adenylyl cyclase function as inhibitor SQ22536 abrogated directed migration to lyral. F) Myoblasts, which express Olfr1403 but not MOR23, did not exhibit directed migration towards lyral.

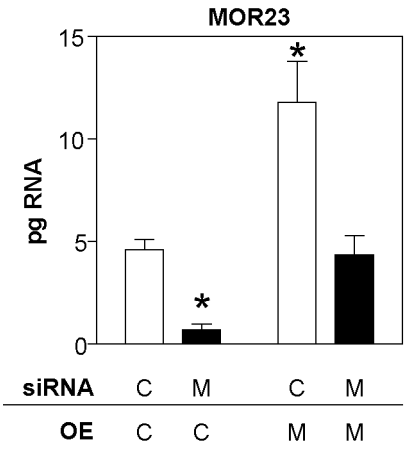


Figure 5.7

Rescue of MOR23 expression in MOR23 siRNA cells *in vitro*. Muscle cells from control or MOR23 siRNA cultures were infected with MOR23 or control retrovirus and levels of MOR23 mRNA at 24 hrs in DM were determined by Real Time RT-PCR. MOR23 siRNA significantly decreased MOR23 mRNA, whereas MOR23 OE significantly increased MOR23 mRNA. Infection of MOR23 siRNA cells by MOR23 retrovirus led to rescue of normal levels of MOR23 mRNA. All data were normalized to 18S rRNA and expressed as mean ± SEM of triplicate samples (*$p<0.05$ from control).

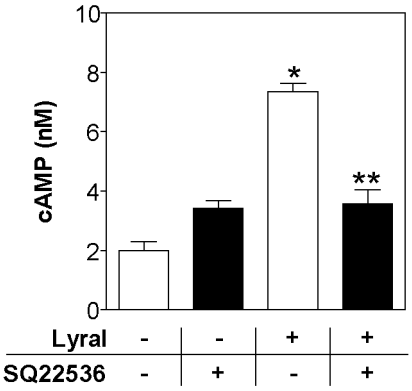


Figure 5.8

Membrane adenylyl cyclase inhibitor, SQ22536, decreases cAMP response to lyral. Myocytes were treated with 10^{-7}M lyral, in the presence or absence of 2.5mM SQ22536, and levels of cAMP were determined (* $p<0.05$ from control; ** $p<0.05$ from lyral). Presence of the mAC inhibitor abrogates the cAMP response to lyral. All data are mean ± SEM, N=3.

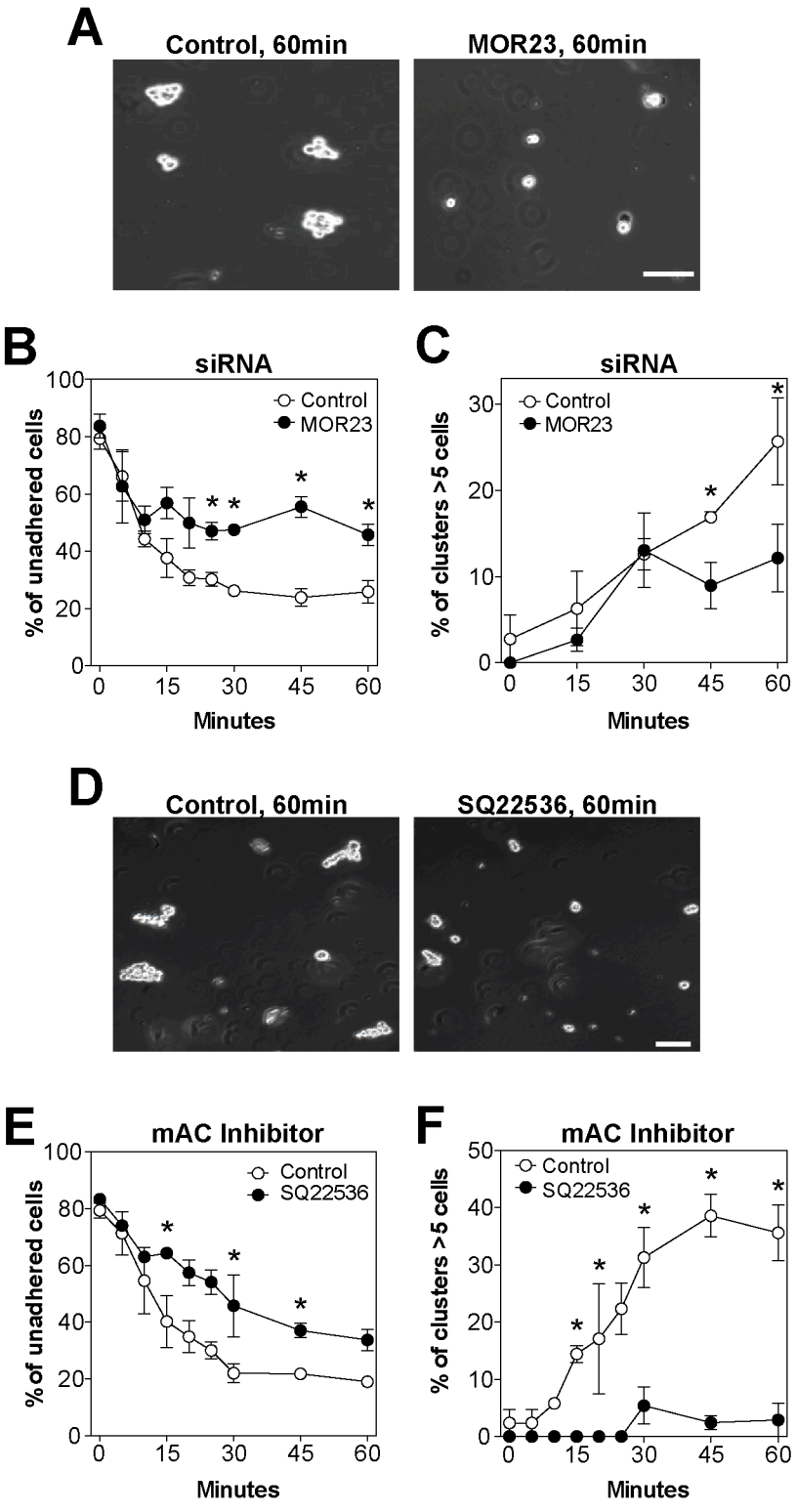

A
Control, 60min
MOR23, 60min
B
siRNA
Control
MOR23
% of unadhered cells
Minutes
C
siRNA
Control
MOR23
% of clusters >5 cells
Minutes
D
Control, 60min
SQ22536, 60min
E
mAC Inhibitor
Control
SQ22536
% of unadhered cells
Minutes
F
mAC Inhibitor
Control
SQ22536
% of clusters >5 cells
Minutes

Figure 5.9

Loss of MOR23 or OR signaling alters cell-cell adhesion. A) Myocytes infected with control or MOR23 siRNA were incubated in suspension for 60 min with aliquots taken regularly, to determine the percentage of un-adhered and adhered cells by phase-contrast microscopy (Bar=50μm). B) MOR23 siRNA cells displayed a significantly higher percentage of un-adhered cells at later time points than control (* $p<0.05$). C) MOR23 siRNA cells exhibited fewer clusters with > 5 cells (* $p<0.05$). D) Myocytes were suspended in media containing a mAC inhibitor, SQ22536, or vehicle (Bar=50μm). E) SQ22536-treated cells displayed a significantly higher percentage of un-adhered cells at later time points (* $p<0.05$). F) SQ22536-treated cells formed small clusters (* $p<0.05$). All data are mean ± SEM.

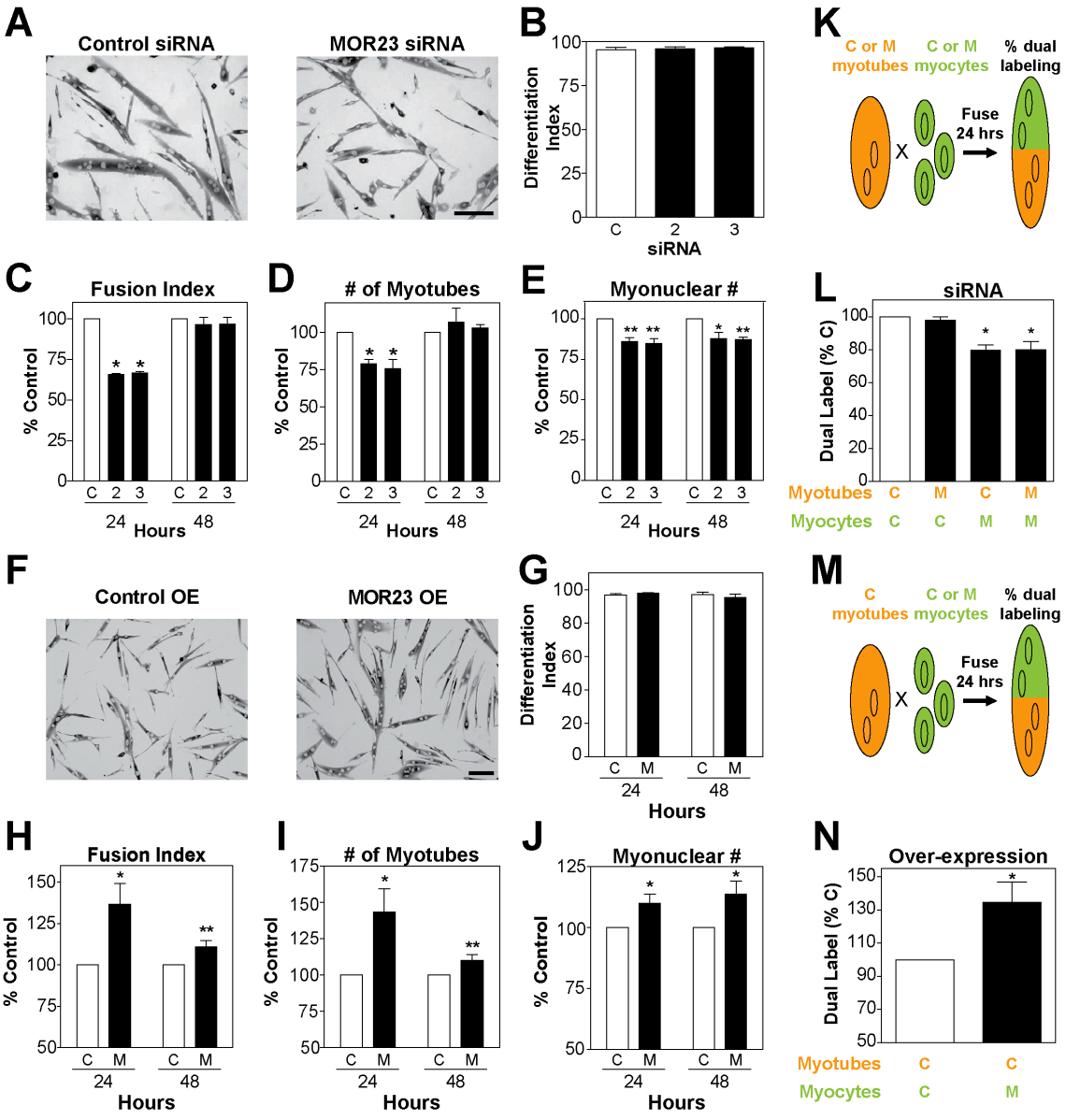

A
Control siRNA
MOR23 siRNA
B
Differentiation Index
siRNA
K
C or M myotubes
C or M myocytes
% dual labeling
Fuse 24 hrs
C
Fusion Index
% Control
Hours
D
# of Myotubes
E
Myonuclear #
L
siRNA
Dual Label (% C)
Myotubes
Myocytes
F
Control OE
MOR23 OE
G
M
H
I
J
N
Over-expression

Figure 5.10

Myotube formation is regulated by MOR23. A) Control or MOR23 siRNA 2 and 3 cells immunostained for eMyHC at 24 hrs in DM (Bar=50μm). B) No difference was observed in the percentage of nuclei in eMyHC$^+$ cells at 24 hrs in DM (differentiation index). C) The fusion index in MOR23 siRNA cultures was transiently decreased at 24 hrs in DM (* $p<0.001$). D) The number of myotubes in MOR23 siRNA cultures was transiently decreased at 24 hrs in DM (* $p<0.001$). E) Myonuclear number in MOR23 siRNA cultures was decreased at both 24 and 48 hrs in DM (* $p<0.05$; ** $p<0.01$). F) Control (C) or MOR23 OE (M) cells were immunostained for eMyHC at 24 hrs in DM (Bar=50μm). G) No difference was observed in the percentage of nuclei in eMyHC$^+$ cells at 24 or 48 hrs in DM. H) The fusion index in MOR23 OE cultures was increased at 24 and 48 hours in DM (* $p<0.05$; ** $p<0.01$). I) The number of myotubes in MOR23 OE cultures was increased at 24 and 48 hrs in DM (* $p<0.05$; ** $p<0.01$). J) Myonuclear number in MOR23 OE cultures was increased at 24 and 48 hrs in DM (* $p<0.01$). K) Nascent myotubes infected with control or MOR23 siRNA were labeled orange and mixed with control or MOR23 siRNA myocytes labeled green. After 24 hrs of co-culture, cultures were fixed and myotubes analyzed for dual labeling. L) With MOR23 siRNA in myocytes the percentage of myotubes with dual label was decreased 20% relative to control; however, MOR23 siRNA in myotubes had no affect (* $p<0.001$). M) Control nascent myotubes were mixed with control or MOR23 OE myocytes and analyzed as in K. N) With MOR23 OE in myocytes,

the percentage of myotubes with dual label was increased 35% relative to control (* $p<0.05$). All data are mean ± SEM.

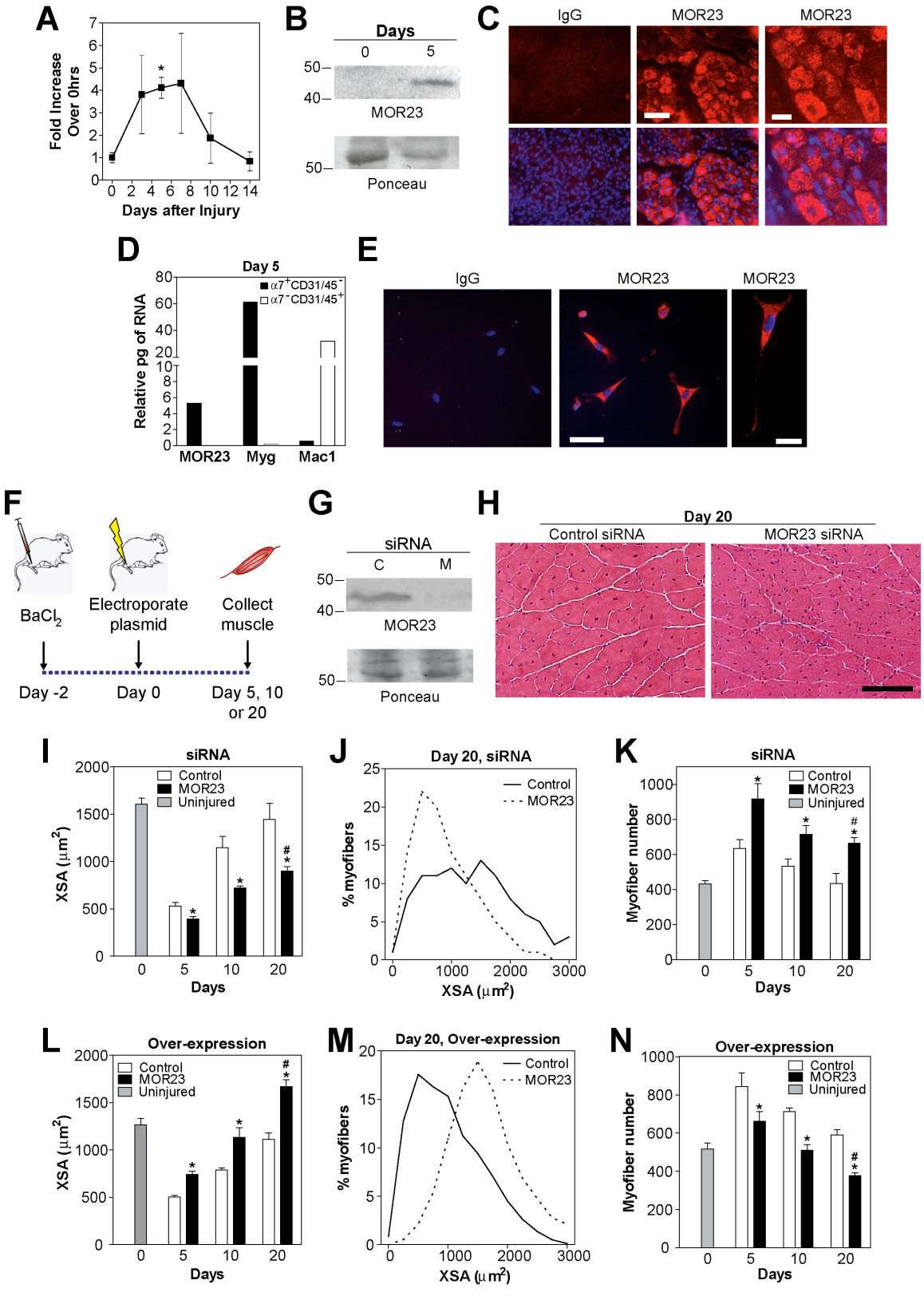

A
Fold Increase Over 0hrs
Days after Injury
B
Days
0
5
MOR23
Ponceau
C
IgG
MOR23
MOR23
D
Day 5
Relative pg of RNA
MOR23
Myg
Mac1
E
IgG
MOR23
MOR23
F
BaCl2
Electroporate plasmid
Collect muscle
Day -2
Day 0
Day 5, 10 or 20
G
siRNA
C
M
MOR23
Ponceau
H
Day 20
Control siRNA
MOR23 siRNA
I
siRNA
Control
MOR23
Uninjured
XSA (μm2)
Days
J
Day 20, siRNA
Control
MOR23
% myofibers
XSA (μm2)
K
siRNA
Control
MOR23
Uninjured
Myofiber number
Days
L
Over-expression
Control
MOR23
Uninjured
XSA (μm2)
Days
M
Day 20, Over-expression
Control
MOR23
% myofibers
XSA (μm2)
N
Over-expression
Control
MOR23
Uninjured
Myofiber number
Days

Figure 5.11

Changes in MOR23 expression affect muscle regeneration. A) Real Time RT-PCR for MOR23 mRNA in gastrocnemius muscles at different times post-injury. All days are normalized to 18S rRNA and expressed as fold increase over 0 days (* $p<0.01$). B) Immunoblot for MOR23 at 0 and 5 days with a portion of the Ponceau-stained blot shown as control. C) Muscles 5 days post-injury immunostained for MOR23 or control IgG (Bar=25μm). $MOR23^+$ regenerating myofibers are shown at higher magnification in last panels (Bar=10μm). Overlay in bottom panels indicates DAPI-stained nuclei. D) Mononucleated cells were isolated from gastrocnemius muscles 5 days post-injury and immunostained with antibodies to CD31 (FITC), CD45 (FITC) and α7-integrin (PE): $CD31^+CD45^+$, to identify endothelial and immune cells and $\alpha7\text{-integrin}^+CD31^-CD45^-$ for myogenic cells. Real Time RT-PCR for MOR23, myogenin (Myg), and Mac1 mRNA in $\alpha7\text{-integrin}^+CD31^-CD45^-$ cells or $\alpha7\text{-integrin}^-CD31^+CD45^+$ cells. All genes are normalized to 18S rRNA and expressed as mean pg of RNA from N=2; 7 mice each. E) Mononucleated $\alpha7\text{-integrin}^+CD31^-CD45^-$ muscle cells isolated from gastrocnemius muscles 5 days post-injury, were plated and immunostained for MOR23. A single $MOR23^+$ cells is shown at higher magnification in right panel (Bars=10μm). F) Gastrocnemius muscles were induced to regenerate using $BaCl_2$ 2 days prior to electroporation of control or MOR23 plasmids. Muscles were isolated 5, 10 or 20 days post-electroporation; uninjured muscles were collected from contra-lateral day 20 legs. G) Immunoblot demonstrating knockdown of MOR23 protein at day 5 with section of Ponceau-stained blot

shown as control. H) Control or MOR23 siRNA muscles stained with hematoxylin and eosin (Bar=100μm). I) The cross-sectional area (XSA) of regenerating myofibers was decreased with MOR23 siRNA (* $p<0.05$ from control, # $p<0.05$ from uninjured). J) The frequency of small regenerating myofibers increased with MOR23 siRNA. K) The number of regenerating myofibers increased with MOR23 siRNA (* $p<0.05$ from control, # $p<0.05$ from uninjured). L) The cross-sectional area (XSA) of regenerating myofibers was increased with MOR23 OE (* $p<0.05$ from control, # $p<0.05$ from uninjured). M) The frequency of small regenerating myofibers decreased with MOR23 OE. N) The number of regenerating myofibers decreased with MOR23 OE (* $p<0.05$ from control, # $p<0.05$ from uninjured). All data are mean ± SEM unless stated otherwise.

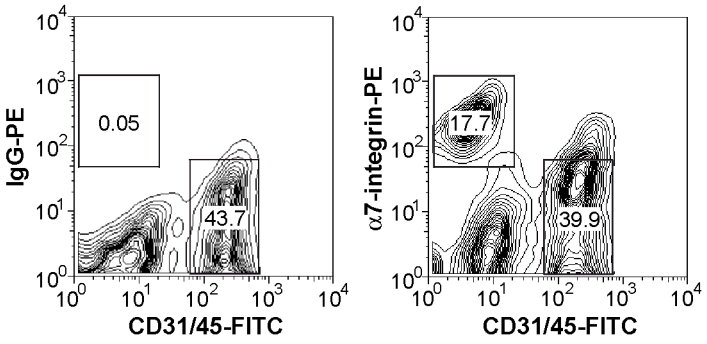
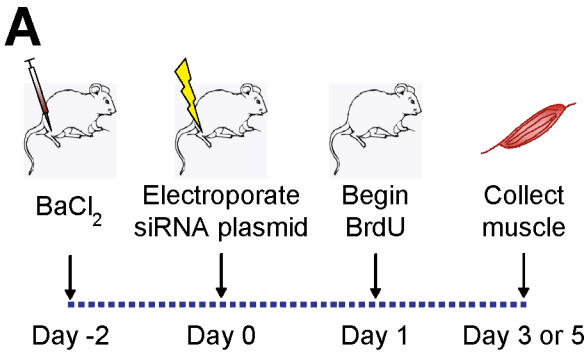
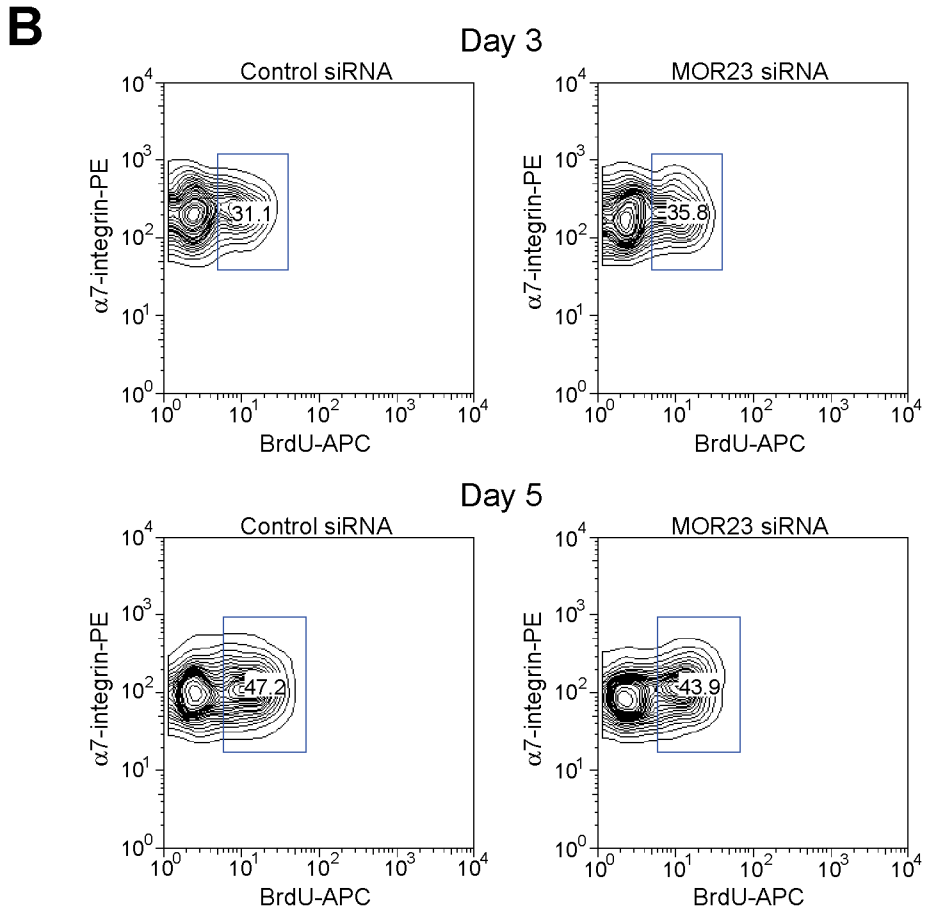


Figure 5.12

Isolation of muscle cells from gastrocnemius muscles by FACS.

Mononucleated cells were isolated from gastrocnemius muscles 5 days post-injury and immunostained with antibodies to CD31 (FITC), CD45 (FITC) and α7-integrin (PE). Cells were sorted with the following criteria: propidium iodide $(PI)^-$ to remove dead cells, $CD31^+CD45^+$, to identify endothelial and immune cells and $\alpha7\text{-integrin}^+CD31^-CD45^-$ to identify myogenic cells. Isotype controls were used to determine proper gating. N=2; 7 mice each.


A
BaCl2
Electroporate siRNA plasmid
Begin BrdU
Collect muscle
Day -2
Day 0
Day 1
Day 3 or 5


B
Day 3
Control siRNA
MOR23 siRNA
α7-integrin-PE
BrdU-APC
31.1
35.8
Day 5
Control siRNA
MOR23 siRNA
47.2
43.9

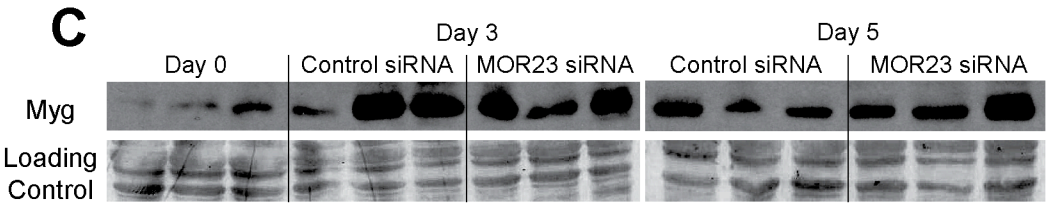

C
Day 3
Day 5
Day 0
Control siRNA
MOR23 siRNA
Control siRNA
MOR23 siRNA
Myg
Loading Control

Figure 5.13

Loss of MOR23 does not affect proliferation or differentiation of muscle cells during regeneration. A) Gastrocnemius muscles were induced to regenerate using $BaCl_2$ 2 days prior to electroporation of either control or MOR23 siRNA plasmids. BrdU injections were started one day after electroporation and continued until muscles were isolated 3 or 5 days post-electroporation. B) Mononucleated cells were isolated and then immunostained with antibodies to CD31 (FITC), CD45 (FITC), α7-integrin (PE) and BrdU (APC). Cells were sorted with the following criteria: propidium iodide $(PI)^-$ to remove dead cells, $CD31^+CD45^+$ to identify endothelial and immune cells and $\alpha7\text{-integrin}^+CD31^-CD45^-$ to identify myogenic cells. Representative flow plots are shown. Isotype controls were used to determine proper gating and 4 mice were used for each condition. Uninjured contralateral legs contained 1.29% $BrdU^+\alpha7\text{-integrin}^+CD31^-CD45^-$ cells; therefore the increase in $BrdU^+$ cells was due to injury. The percentage of $BrdU^+\alpha7\text{-integrin}^+CD31^-CD45^-$ cells differed little between control and MOR23 siRNA muscles, indicating MOR23 does not regulate proliferation of myoblasts during regeneration. C) For differentiation assays, muscles were collected 3 or 5 days post-electroporation. Immunoblot demonstrates no change in the amount of myogenin in MOR23 siRNA muscles at day 3 or 5 with section of total protein shown as control; therefore MOR23 does not regulate differentiation.

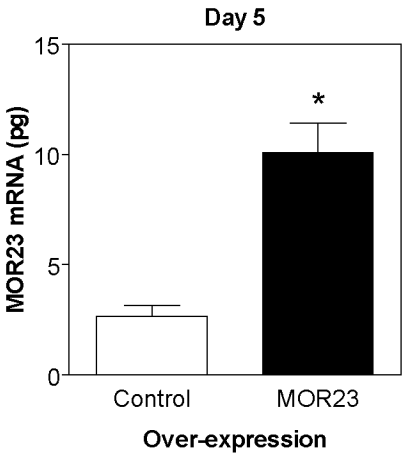


Figure 5.14

Over-expression of MOR23 *in vivo* by plasmid electroporation. The levels of MOR23 were determined by Real Time RT-PCR using RNA from control or MOR23 OE muscles at day 5 after electroporation. Electroporation of MOR23 plasmid significantly increased MOR23 mRNA (*$p<0.05$ from control). All data are normalized to 18S rRNA, N=4, mean ± SEM.

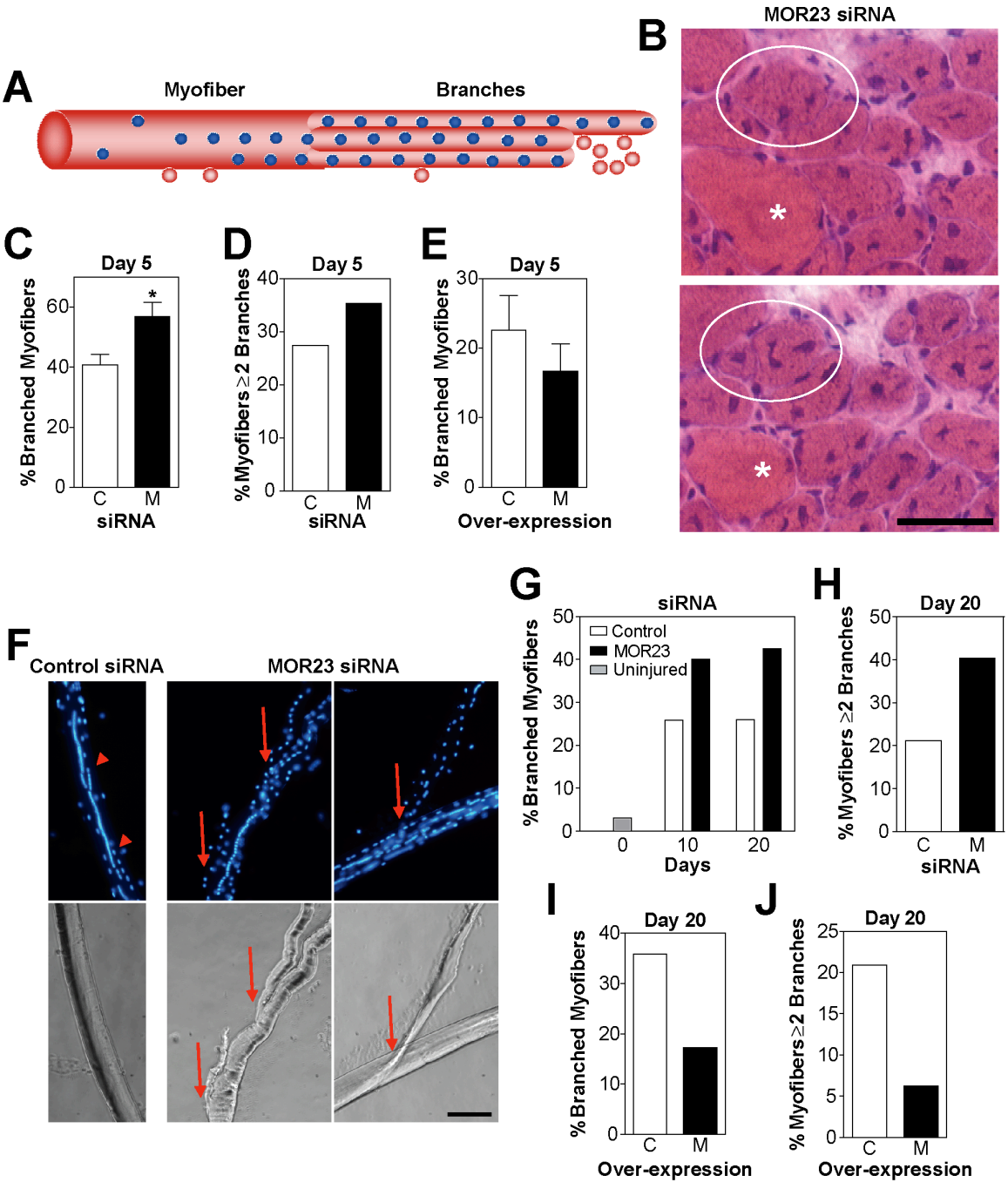

A
Myofiber
Branches
B
MOR23 siRNA
C
Day 5
% Branched Myofibers
siRNA
D
Day 5
% Myofibers ≥2 Branches
siRNA
E
Day 5
% Branched Myofibers
Over-expression
F
Control siRNA
MOR23 siRNA
G
siRNA
Control
MOR23
Uninjured
% Branched Myofibers
Days
H
Day 20
% Myofibers ≥2 Branches
siRNA
I
Day 20
% Branched Myofibers
Over-expression
J
Day 20
% Myofibers≥2 Branches
Over-expression

Figure 5.15

MOR23 regulates myofiber branching. A) Schematic of myofiber branching in which a single myofiber is contiguous with several smaller myofibers. Nuclei are blue and mono-nucleated cells are small red circles. B) Gastrocnemius muscles isolated 5 days after electroporation of plasmid were analyzed for myofibers that were a single myofiber in one section and multiple myofibers occupying the same area in a second section, 170μm away. Stars indicate the same myofiber in both sections, a branched myofiber is circled (Bar=50μm). C) Myofiber branching was increased with MOR23 siRNA. D) The number of branches per myofiber increased with MOR23 siRNA. Data are percentage of total myofibers. E) Myofiber branching was not affected at day 5 with MOR23 OE. F) Myofibers were isolated from gastrocnemius muscles 10 or 20 days after electroporation of plasmid. DAPI-stained centrally located nuclei and phase-contrast microscopy determined if regenerating myofibers exhibited branching. Arrows indicate a branched regenerating myofiber, arrow-head an unbranched regenerating myofiber (Bar=50μm). G) Muscles with MOR23 siRNA contained more branched regenerating myofibers. H) The number of branches per myofiber increased with MOR23 siRNA. I) Muscles with MOR23 OE contained fewer branched regenerating myofibers. Data are percentage of total myofibers. J) The number of branches per myofiber decreased with MOR23 OE. All data are mean ± SEM unless stated otherwise.

Table 5.1
Levels of OR mRNA in olfactory bulbs

Olfactory Receptor	Olfactory Bulb*	SABiosciences Primer	Accession Number
MOR23	25.7 ± 8.5	PPM60724A	NM_008763.1
Olfr2	24.2 ± 6.3	PPM34712A	NM_010983
Olfr15	27.3 ± 2.9	PPM60723A	NM_008762.2
Olfr49	34.7 ± 1.8	PPM60726A	NM_010991.1
Olfr66	25.1 ± 1.4	PPM60284A	NM_013618.3
Olfr70	34.8 ± 22.4	PPM40960A	NM_019485
Olfr71	28.1 ± 0.7	PPM40961A	NM_019486.1
Olfr78	37.4 ± 3.7	PPM05210A	NM_130866.3
Olfr138	24.0 ± 7.1	PPM59182A	NM_130868.1
Olfr140	8.8 ± 6.36	PPM40825A	NM_020515.1
Olfr155	20.0 ± 9.0	PPM30316A	NM_019473
Olfr156	13.1 ± 6.7	PPM60744A	NM_019474
Olfr157	12.7 ± 5.4	PPM40214A	NM_019475
Olfr159	18.4 ± 3.8	PPM30317A	NM_019476.1
Olfr480	10.2 ± 2.4	PPM60753A	NM_020291.1
Olfr1264	34.9 ± 19.1	PPM61038A	NM_021368.1
Olfr1507	46.5 ± 10.1	PPM30312A	NM_020512
Olfr1508	12.5 ± 8.5	PPM42070A	NM_020513.2
Olfr1509	29.4 ± 4.7	PPM30313A	NM_020514.1

*, values are mean pg of RNA ± SEM

Table 5.1

Levels of OR mRNA in olfactory bulbs. Real Time RT-PCR was used to analyze the levels of mRNA of 19 ORs in mouse olfactory bulbs, as a positive control for all OR primers. The SABiosciences catalog numbers are included for all primers. Data were normalized to 18S rRNA, N=3 mice.

Table 5.2
OR expression during *in vitro* myogenesis

In vitro				
Highest Expression	**Odorant Receptor**	**0 hours***	**24 hours***	**48 hours***
0 hours	Olfr15	6.6 ± 0.1	-	-
	Olfr66	2.1 ± 1.2	1.6 ± 1.2	-
	Olfr78	4.6 ± 0.5	-	-
	Olfr155	4.2 ± 2.5	-	-
	Olfr156	1.1 ± 0.4	-	-
	Olfr480	3.6 ± 1.0	-	-
	Olfr1264	33.2 ± 14.4	-	-
	Olfr1507	2.1 ± 0.8	-	-
24 hours	Olfr70	-	1.54 ± 0.1	-
	Olfr71	2.12 ± 0.2	2.4 ± 1.4	-
	Olfr157	-	7.0 ± 3.5	2.2 ± 0.6
	Olfr1508	-	3.1 ± 0.7	-
	MOR23 (Olfr16)	-	5.5 ± 2.1	-
Not expressed in vitro	Olfr2	-	-	-
	Olfr49**	-	-	-
	Olfr138	-	-	-
	Olfr140	-	-	-
	Olfr159	-	-	-
	Olfr1509	-	-	-

*, values are mean pg of RNA ± SEM; **, detected *in vivo*; -, less than 1 pg detected

Table 5.2

OR expression during *in vitro* myogenesis. Real Time RT-PCR was used to analyze the mRNA levels of 19 ORs in primary mouse muscle cells at 0, 24, and 48 hrs in DM. Data were normalized to 18S rRNA, N=3 independent cell isolates.

Table 5.3
OR expression during muscle regeneration

In vivo				
Highest Expression	**Odorant Receptor**	**0 days***	**5 days***	**10 days***
0 days	Olfr15	7.89 ± 6.9	6.16 ± 4.4	6.06 ± 1.9
5 days	MOR23 (Olfr16)	-	10.1 ± 5.4	1.2 ± 0.6
	Olfr49	-	1.6 ± 0.4	-
	Olfr66	-	6.4 ± 1.5	1.1 ± 0.4
	Olfr78	1.7 ± 1.6	22.8 ± 2.8	5.8 ± 3.5
	Olfr155	-	3.4 ± 1.1	-
	Olfr156	-	3.4 ± 2.1	-
	Olfr480	-	7.0 ± 3.4	-
	Olfr1264	3.76 ± 0.1	29.5 ± 20.1	6.6 ± 3.1
	Olfr1507	-	11.5 ± 5.3	1.6 ± 0.3
	Olfr1508	-	10.6 ± 4.4	-
10 days	Olfr71	-	-	3.5 ± 1.7
	Olfr157	1.13 ± 0.4	-	2.39 ± 1.4
Not expressed in vivo	Olfr2	-	-	-
	Olfr70**	-	-	-
	Olfr138	-	-	-
	Olfr140	-	-	-
	Olfr159	-	-	-
	Olfr1509	-	-	-

*, values are mean pg of RNA ± SEM; **, detected *in vitro*; -, less than 1 pg detected

Table 5.3

OR expression during muscle regeneration. Real Time RT-PCR was used to analyze mRNA levels of 19 ORs in gastrocnemius muscles 0, 5 or 10 days after injury. Data were normalized to 18S rRNA, N=3 mice.

Table 5.4

Expression of other ORs is not altered with MOR23 siRNA.

Highest Expression	Odorant Receptor	Control siRNA*	MOR23 siRNA*
0 hours	Olfr15	6.2 ± 4.9	6.7 ± 0.2
	Olfr66	4 ± 0.2	5.7 ± 0.8
	Olfr78	5.8 ± 2.9	7.2 ± 1.4
	Olfr155	3.2 ± 0.2	4.8 ± 2.6
	Olfr156	5 ± 1.1	5.5 ± 1.2
	Olfr480	5.3 ± 0.4	6.4 ± 2.5
	Olfr1264	38.7 ± 0.8	32.9 ± 8.9
	Olfr1507	4.7 ± 0.6	5.3 ± 1.3
24 hours	1.7 ± 0.3	1.8 ± 0.7	1.7 ± 0.3
	6.1 ± 1.3	6.6 ± 2.3	6.1 ± 1.3
	5.1 ± 0.3	6.6 ± 2.4	5.1 ± 0.3
	0.9 ± 0.1	1.1 ± 0.4	0.9 ± 0.1
	MOR23	**16.1 ± 2.9**	**3.3 ± 1.8**

*, values are mean pg of RNA ± SD

Table 5.4

Expression of other ORs is not altered with MOR23 siRNA. The mRNA levels of ORs expressed during *in vitro* myogenesis were examined for changes in response to MOR23 siRNA. No significant changes were observed in other ORs with MOR23 siRNA, although MOR23 mRNA was decreased. One experiment performed in triplicate.

Chapter 6: Discussion

Molecular regulation of adult regenerative myogenesis

Skeletal muscle growth and regeneration are necessary for human health. However the molecular mechanisms that regulate skeletal muscle growth are not well understood. The broad goal of this dissertation was to better understand the role of migration and adhesion during myogenesis. Prior work in the Pavlath lab had determined that molecules which regulate migration are necessary for myogenesis. The first aim of this dissertation was to determine whether chemokines and chemokine receptors are expressed by muscle cells as these factors can regulate cell migration in a number of cell types. Furthermore, many previous studies suggested chemokines are upregulated by damaged or diseased muscle tissue. The data presented in Chapter 4 demonstrate many chemokines and chemokine receptors are expressed by muscle cells *in vitro*. These data also determine a specific role for CXCR4 and SDF1α during myogenesis. In Chapter 4, we also quantified the number of terminally differentiated myocytes found in regenerating muscle tissue *in vivo*. Although earlier studies indentified the presence of both cell types, their relative numbers over the course of adult regenerative myogenesis have been never determined. The second aim of this dissertation was to determine whether olfactory receptors (ORs) are expressed and functional during myogenesis. The data in Chapter 5 suggest that at least 13 ORs are expressed by muscle during myogenesis, both *in vitro* and *in vivo*. Furthermore, one specific OR, MOR23 regulates both migration and adhesion, affecting proper myogenesis. MOR23 also affects a phenomenon known as myofiber branching, where a myofiber is contiguous with

several smaller myofibers. Myofiber branching is increased with multiple injuries, aging and muscular dystrophies and decreases the contractile force of the myofiber. Over-expression of MOR23 decreases the incidence of myofiber branching in regenerating muscle. The data presented in this dissertation characterize two classes of molecules which regulate cell migration during myogenesis.

Migration is dynamic during myogenesis *in vitro*

These studies have several implications for the regulation of muscle cell migration during myogenesis. Migration of muscle cells is extremely dynamic, as evidenced by changes in velocity which occur during myogenesis. Mononucleated muscle cells move more slowly at later stages of myogenesis, despite the expression of many chemoattractants. At early stages of myogenesis, muscle cells may increase velocity in order to scatter rather than migrate to specific locations. At later stages, muscle cells undergoing fusion may increase directed migration and decrease chemokinesis, in order to fuse in specific locations. Furthermore, studies presented in this dissertation suggest that migration of muscle cells at later stages of myogenesis is regulated by different molecules than those that regulate migration of cells at earlier stages. Many studies of migrating muscle cells have focused on the effect of migration on processes earlier during myogenesis, such as proliferation, survival or differentiation. However, our studies suggest that molecules which regulate

migration can affect fusion without affecting these earlier events. Migration is therefore a critical and dynamic process during myogenesis *in vitro*.

Migration of muscle cell *in vivo*

This dissertation presented two molecular regulators of migration during myogenesis. While migration is necessary for myogenesis *in vitro*, whether muscle cell migration normally occurs during adult regenerative myogenesis is unknown. *In vitro* studies of myogenesis have a 2-dimensional matrix, rather than a 3-dimensional matrix, and occur in an absence of other cell types present in muscle tissue; therefore, these studies can not be directly related to migration *in vivo*. Furthermore, although many chemoattractants are known to regulate migration of muscle cells *in vitro*, their standard function may be to regulate migration of other cell types within muscle tissue *in vivo*, such as immune or endothelial cells. Therefore, the ability of many chemoattractants expressed by muscle to regulate migration of muscle cells may not be relevant to myogenesis in the adult.

In order to determine whether muscle cell migration is necessary *in vivo*, new techniques are necessary. Current techniques focus on the migration of transplanted donor muscle cells rather than endogenous muscle cells and only yield snap shots of cell dispersion during myogenesis. Migration of endogenous lymphocytes within lymph nodes can be visualized using explants of lymph nodes, still connected to a living mouse, placed on microscope slides (Miller et al., 2003). Unfortunately, this technique requires a relatively thin and translucent

tissue which can be explanted and most muscles are both thick and opaque. Furthermore, myofibers tend to contract when stretched or released from their tendons, and therefore will be difficult to move onto a slide while still connected to the living mouse. Muscle cells also migrate at a much slower velocity than lymphocytes, and therefore time-lapse microscopy would have to occur over a period of several hours, rather than less than one hour. Another technique for visualization of endogenous cell migration is being developed, and utilizes micro-endoscopes placed under the skin which can track fluorescently-tagged cells using two-photon microscopy (Myaing et al., 2003; Thomas et al., 2004; Ye et al., 2002). Currently, no protocol has been developed with which to use such a technique within muscle tissue. Further studies should determine whether muscle cells migrate during adult regenerative myogenesis.

The necessity for multiple chemoattractants

The data in this dissertation demonstrate that muscle cells express many factors capable of regulating migration. These factors would allow mammalian muscle cells to attract one another similar to the founder cells in *Drosophila* which nucleate myotubes by attracting fusion competent myoblasts. A subset of muscle cells may express the ligand and recruit muscle cells which express the appropriate receptor; similar to the founder cells in *Drosophila* which nucleate myotubes by attracting fusion competent myoblasts. This process may be similar to how cells must find specific positions or locations to properly form specialized tissues. The area code hypothesis suggests that a set of receptors allows a cell

to determine a unique location within an embryo or tissue (Dreyer, 1998; Dreyer and Roman-Dreyer, 1999; Hood et al., 1977). While a single receptor/ligand pair gives one point of reference and describes a single dimension, a set of receptors would allow for localization based on several points of reference, describing a location within two or three dimensions. Area code molecules must be displayed on the surface of cells, and capable of providing specificity for both cell-cell interactions and sensing diffusible differentiation signals provided by hormones. Both the immune system and the olfactory receptors were previously suggested to act as area code molecules during embryonic development (Dreyer, 1998). The area code hypothesis is also used to explain the remodeling and repair of bones in adulthood. Chemokines govern the movement of osteoclasts, which are a class of leukocytes that reabsorb bone as part of bone repair, into specific areas of bone (Parfitt, 1998). Therefore, the expression of a large number of chemokines and ORs would be capable of regulating the locations of muscle cells during adult regenerative myogenesis. If the area code hypothesis functions during adult regenerative myogenesis, then further investigations will be required to determine whether both chemokines and ORs are components of the area code in muscle repair.

Expression of chemokines and chemokine receptors during myogenesis

The data presented in Chapter 4 demonstrate that many chemokines and chemokine receptors are expressed during myogenesis by muscle cells. The large number of factors and receptors expressed suggests complex temporal and

spatial control during myogenesis. Many of these factors are also expressed by other cells in regenerating muscle, such as cells of the immune system (Civatte et al., 2005; De Rossi et al., 2000; Porter et al., 2003). Therefore, chemokines and chemokine receptors may allow for crosstalk between muscle and immune cells.

Although protein expression of CXCR4 and SDF1α was verified *in vivo*, expression of some of the chemokine receptor/ligand pairs during *in vitro* myogenesis may be due to culture conditions. Studies will be necessary to determine the protein levels of chemokine receptor/ligand pairs by muscle cells during adult regenerative myogenesis *in vivo*. To determine the presence of soluble ligands, ELISAs for the different chemokines should be performed on crushed muscle extract. The presence of receptors on muscle cells may be elucidated with FACS, while their presence on myofibers can be determined by immunohistochemistry.

Chemokines and chemokine receptors also regulate proliferation, differentiation and survival (Chazaud et al., 2003; Nedachi et al., 2009; Odemis et al., 2007). These factors may regulate similar processes during myogenesis, without affecting migration of muscle cells. Our results do not address the affects chemokines and chemokine receptors may have on functions other than migration. As many chemokines and chemokine receptors were expressed during myogenesis, further studies will be necessary to determine their various roles.

Chemokines may regulate processes in either a paracrine or autocrine manner. A subset of muscle cells may express the chemokine and affect these processes in cells which express the receptor. Or a cell might express both the chemokine and the appropriate receptor, allowing autocrine regulation. If the receptor/ligand pair regulates cells in an autocrine manner, then immunocytochemistry should reveal expression of both the receptor and ligand in the same cells. However, if the receptor/ligand pair regulates cells in an paracrine manner than the receptor and ligand should be expressed in distinct populations of muscle cells. Identifying chemokines and chemokine receptors which regulate myogenesis may help identify which receptor/ligand pairs may have roles in tissue repair.

CXCR4/SDF1α regulation of migration during myogenesis

The data presented in Chapter 4 of this thesis demonstrate that the chemokine receptor/ligand pair CXCR4/SDF1α regulates migration of both myoblasts and myocytes, and inhibition of CXCR4 affected proper myogenesis. The role of CXCR4-dependent migration in myoblasts has yet to be elucidated as inhibition of CXCR4 did not affect proliferation, survival or differentiation of muscle cells. Perhaps CXCR4 regulates the position of myoblasts, as in the bone marrow, where the niche-supporting cells secrete SDF1α and the hematopoetic cells express CXCR4. Loss of SDF1α signaling within the niche allows release of these cells from the bone marrow and their subsequent homing to tissues expressing SDF1α (Pituch-Noworolska et al., 2003; Ratajczak et al., 2003).

Recent evidence has revealed that muscle cells can migrate along myofibers *in vitro* (Siegel et al., 2009). Therefore, immunohistochemistry studies should be performed to determine if myofibers express SDF1α, as previous studies have already determined that quiescent muscle cells express CXCR4 (Ratajczak et al., 2003). These studies may elucidate whether the CXCR4/SDF1α axis is available to regulate the position of muscle cells on myofibers.

CXCR4-dependent migration of myocytes is necessary for fusion of myocytes to create myotubes, as loss of CXCR4-dependent migration decreases fusion. However, these studies have not determined whether CXCR4 affects the velocity of cells or their directed migration. Interestingly, the results presented in Chapter 4 suggest that high concentrations of SDF1α do not increase migration of myocytes towards the SDF1α gradient. This may be due to fugetaxis, where cells migrate down a gradient, rather than up a gradient. CXCR4 and SDF1α are known to cause fugetaxis, as at high concentrations of SDF1α specific subpopulations of T-cells migrate away from the gradient (Brainard et al., 2004). Dunn chamber analyses will allow researchers to determine whether chemokinesis, directed migration or fugetaxis are regulated by CXCR4 in different muscle cells at specific concentrations of SDF1α. CXCR4 may have other, unidentified functions in muscle cells, which are also necessary for fusion, such as regulation of important cell-cell adhesion molecules.

As immune cells express CXCR4 during muscle regeneration, this protein is an ideal candidate for cross-talk between muscle and immune cells. In order to determine whether CXCR4 may regulate cross-talk between these cells types,

muscle and immune cells should be co-cultured. After loss of CXCR4 is induced in one cell type, studies should be performed on the other cell type. Researchers should pay specific attention to proliferation, survival, differentiation, migration and fusion, as these processes are known to be affected by CXCR4 or chemokines. These studies should allow researchers to determine which cell type requires CXCR4 in order for cross-talk to occur between muscle and immune cells during myogenesis.

Although the work presented in this dissertation established that CXCR4 is required for migration and fusion of myocytes during myogenesis *in vitro*, the role of CXCR4 during adult regenerative myogenesis *in vivo* has not been elucidated. As CXCR4 is a crucial regulator of embryonic myogenesis and regulates both hematopoetic stem cells and immune cells, CXCR4 knockout mice are non-viable after birth. Also, as the immune system plays an important role in adult regenerative myogenesis, through clearing of damaged myofibers, inducible non-tissue-specific CXCR4 knockout mice are also not ideal as the effects from loss of CXCR4 in muscle cells may be masked by the loss of CXCR4 in immune cells. Therefore, a muscle-specific inducible CXCR4 knockout mouse would be necessary to determine the effect of CXCR4 in muscle cells during regenerative myogenesis. If possible, different types of muscle-specific inducible CXCR4 knockouts should be created, in order to resolve the specific role of CXCR4 in myoblasts and myocytes. These studies will allow researchers to determine the role of CXCR4 in muscle cells during adult regenerative myogenesis.

The regulation of myogenesis by odorant receptors

Data presented in Chapter 5 of this dissertation reveal that 13 ORs out of 19 investigated were expressed by muscle during myogenesis, both *in vitro* and *in vivo*. The steady-state levels of these ORs varied, as some OR mRNAs peaked at the myoblast stage, others at the myocyte stage, and some had a relatively even level throughout myogenesis. Recent reports suggest ORs are capable of regulating proliferation of prostate cancer cells (Neuhaus et al., 2009). Therefore different ORs may regulate proliferation, differentiation, migration or adhesion of muscle cells. The differential expression levels suggest ORs may have many varied functions in muscle cells. Further studies are necessary to determine the role of multiple ORs during myogenesis.

The studies in this dissertation specifically focused on MOR23, as this OR was known to regulate sperm migration (Fukuda et al., 2004) and the guidance of olfactory neurons axons (Vassalli et al., 2002). There are separate transcriptional start sites for MOR23 depending on expression in either olfactory epithelium or testis (Asai et al., 1996). The testis transcript contains a single exon; however, the olfactory epithelium transcript also contains an untranscribed upstream exon. At this juncture, we do not know which site is utilized for expression of MOR23 in muscle cells. Preliminary data suggest that mRNA isolated from muscle cells does not contain the upstream exon which is part of the olfactory epithelium MOR23 transcript. Furthermore, there are two myocyte enhancer binding factor 2 (MEF-2) binding sites in the kilobase upstream of the testis transcription start

site. These data suggest that in muscle, the transcript start site is likely the same as in the testis. In order to determine whether MEF-2 regulates MOR23 expression, MEF-2 and the appropriate upstream region of MOR23 should be used in EMSA. If MEF-2 can bind to the promoter region of MOR23, then mutations in this region should be created to determine if the MEF-2 binding sites are necessary for MOR23 expression. These studies should allow researchers to determine how MOR23 expression is regulated in muscle.

No endogenous ligand for any OR has been isolated. However, as MOR23-dependent migration occurs to both conditioned media and crushed muscle extract, these data suggest that muscle cells may be a possible source for the MOR23 ligand. Preliminary studies suggest that the MOR23 ligand is neither heat-labile nor protease-sensitive. Furthermore, communications from investigators studying the human odorant recepteor 17-4 ligand suggest that the endogenous ligand may be a steroid hormone breakdown product. Such a molecule might not be heat-labile or protease-sensitive, and therefore studies into a MOR23 ligand should focus on such molecules. Unfortunately, fractionation studies to date have not been effective as diluted conditioned media is ineffective at increasing MOR23-dependent migration of muscle cells, suggesting that the ligand is present in low concentrations. Therefore, other more sensitive assays, such as detection of cAMP or Ca^{2+} levels, should be used after fractionation. Heterologous expression of MOR23 in HEK cells may be preferable in order to decrease non-specific effects of other ligands within conditioned

media. Finding an endogenous ligand for MOR23 may allow for easier characterization of other endogenous ligands for ORs.

Studies in this dissertation determined that MOR23 regulates migration mainly through affects on the directional migration of myocytes, rather than affects on the velocity of myocytes. The canonical OR signaling mechanisms, including membrane adenylyl cyclase III, are also necessary for MOR23-dependent migration. In sperm cells, MOR23 regulates migration through myosin proteins and although this dissertation did not address the specific filaments necessary for myocyte migration, many groups suggest that muscle cells migrate using actin filaments and not myosin (Dedieu et al., 2004; Hawke et al., 2007; Kawamura et al., 2004; Kuwahara et al., 2005; Louis et al., 2008). The difference in which filaments are regulated by MOR23 may explain the differential effects ORs have on velocity in sperm and muscle cells. The signaling mechanism between ORs and actin filaments has not been determined, and future studies will be necessary to determine the necessary mechanism. Potentially, the over-expression of MOR23 could be used with different inhibitors of cell signaling pathways, as inhibitors which bring fusion back down to control levels should be necessary for MOR23-dependent mechanisms. Screening of inhibitors in the absence of MOR23 OE, would likely find many pathways that are important for either migration or adhesion, and do not have a direct role in MOR23-dependent processes. Pathways which utilize either cAMP or Ca^{2+} as second messengers should be given priority, as ORs can utilize both of these molecules as messengers. Furthermore, a microarray comparing MOR23 siRNA, MOR23 OE

and control muscle cells should be conducted to determine downstream molecules affected by MOR23.

Recent evidence suggests that ORs can regulate adhesion of the axons of olfactory neurons. Our work indicates MOR23 is necessary for adhesion of muscle cells and that membrane adenylyl cyclase III function is also required. In olfactory neurons, expression of some adhesion molecules such as Kirrel2, Kirrel3, Ephrin-A3 and Ephrin-A5, is dependent on odorant receptor activity (Serizawa et al., 2006) and changes in their expression were correlated to altered axon guidance into specific glomeruli (Imai et al., 2006). Interestingly, homologues of kirrel proteins regulate myogenesis in both fruit flies and zebrafish (Srinivas et al., 2007). Preliminary experiments suggest that MOR23 may regulate Kirrel2, as both Real-Time RT-PCR and western blots suggest down-regulation of Kirrel2 with MOR23 siRNA. Specific studies into the role of Kirrel2 should be continued to determine whether Kirrel2 is in fact downstream of MOR23 in muscle cells.

There are several hypotheses for how ORs may regulate gene transcription. For instance, ligand binding increases intracellular cAMP concentration, which is known to activate cAMP-dependent protein kinase (PKA) and causes translocation of catalytic subunits of the kinase to the nucleus (Sands and Palmer, 2008). Nuclear PKA phosphorylates the transcription factor cAMP-response element binding protein (CREB), allowing for changes in the transcriptional program of the cell (Sands and Palmer, 2008). Therefore, changes in the cAMP intracellular concentration of the cell caused by OR activation, may

yield transcriptional changes through CREB. Another hypothesis is that ORs may regulate transcription through β-arrestin. ORs are inactivated by phosphorylation and subsequent binding of β-arrestin to the OR (Dawson et al., 1993). In sperm, this is followed by translocation of β-arrestin to the nucleus, where β-arrestin may affect the transcription of genes (Neuhaus et al., 2006). As β-arrestin is also expressed by muscle cells, a similar process may occur after MOR23 activation. To determine which of these mechanisms regulates MOR23-dependent transcription, further experiments will be necessary. If CREB regulates MOR23-dependent transcription, then replacement of wild-type CREB with a mutated amino acid at the Ser133 position should mimic effects of MOR23-siRNA, as Ser133 is essential for activation of CREB in response to cAMP (Mayr and Montminy, 2001). However, if β-arrestin regulates MOR23-dependent transcription, then loss of β-arrestin should mimic the effects of MOR23 siRNA. These studies may allow researchers to determine which transcription factors are necessary for regulation of other genes by ORs.

Although there is no evidence to suggest that ORs may regulate the sub-cellular localization of other membrane-bound proteins, MOR23 may exist within a complex of such proteins. Therefore, loss of MOR23 from the plasma membrane may result in down-regulation of the entire complex of proteins. As Kirrel2 levels are affected by MOR23 siRNA, Kirrel2 is one possible member of a MOR23 protein complex. To determine whether Kirrel2 and MOR23 exist in a complex together, changes in sub-cellular localization of Kirrel2 may be determined through comparison of MOR23 siRNA and control cells using

confocal microscopy and immunofluorescence. Co-immunoprecipitation experiments should also be performed to determine whether these two proteins exist as a complex. These studies may help determine whether MOR23 can regulate the sub-cellular localization of other membrane-bound proteins.

MOR23-dependent migration and adhesion of myocytes are necessary for proper fusion during myogenesis *in vitro*; however, whether these processes are necessary for *in vivo* myogenesis has not been determined. Also, MOR23 affects a phenomenon known as myofiber branching, where the cytoplasm of one myofiber is contiguous with the cytoplasm of several smaller myofibers. Current models of branching suggest that adhesion molecules regulate myofiber branching, suggesting that MOR23 has an indirect effect on branching. Further studies will be necessary to elucidate the processes that create or resolve branched myofibers.

Therapeutic interests

The data presented in this dissertation also may have important therapeutic implications. Skeletal muscle growth and maintenance are essential for metabolism, mobility and strength, and can be adversely affected by muscular diseases, such as dystrophies, injury and aging. The identification of molecules that regulate skeletal muscle growth may lead to novel therapies for muscle growth and maintenance.

Some forms of muscular dystrophy result from mutations of molecules within the dystrophin-glycoprotein complex that connects contractile molecules to the

ECM. Muscular dystrophies are normally characterized by extensive muscle damage and continuous rounds of muscle degeneration and regeneration. Many studies have focused on the transplantation of wild type muscle stem cells to ameliorate muscular degeneration. Unfortunately, there are many issues with transplantation; including donor muscle stem cells injected into host muscle do not migrate far from the injection site. Therefore, several groups have focused on ways to increase the migration of muscle stem cells throughout the host tissue. As most canonical migratory factors for muscle stem cells are also growth factors for other cells, they may cause formation of tumors; therefore, these molecules are not ideal for increasing migration of muscle cells within muscle tissue. Instead, focusing on increased odorant receptor expression may allow manipulation of migration without also increasing inappropriate proliferation of other cell types. The expression of chemokines and chemokine receptors are constitutively increased with muscular dystrophies and are correlated with increased immune cells within the muscle (Porter et al., 2003). Therefore, modulation of these molecules may decrease host muscle damage through decreasing activity of immune cells, and increase donor cell migration. Through isolating molecules which regulate migration and fusion of muscle cells, we may identify more appropriate target molecules for increasing the migration of transplanted muscle cells within host muscle tissue.

An interesting connection between MOR23 and muscular dystrophy is myofiber branching. A dystrophic mouse model, the mdx mouse which contains a mutant form of dystrophin, has significantly increased levels of branched

myofibers compared to wild type mice. Other labs report almost 100% of myofibers in the gastrocnemius muscle of mdx mice are branched by 3 months of age (Bockhold et al., 1998). Our preliminarily data suggests that ~30% of myofibers from the gastrocnemius muscle of mdx mice are branched by 2 months of age. In comparison, less than 1% of myofibers from the gastrocnemius muscle in wild type mice are branched at these ages. Importantly, branched myofibers are significantly weaker than unbranched myofibers. Furthermore, after long time periods, myofiber branching is not resolved (Vaittinen et al., 2002). In Chapter 5, the overexpression of MOR23 is shown to decrease the incidence of myofiber branching after injury. Furthermore, preliminary data suggest that MOR23 is constitutively expressed by mdx mice, and is not upregulated upon induced muscle injury. These data indicate that MOR23 is dysregulated with muscular dystrophy and that perhaps increasing the expression of MOR23 may decrease the incidence of myofiber branching that occurs due to muscular dystrophy.

Summary

In summary, the data presented in this dissertation identify a larger role for chemokine receptor/ligand pairs in adult regenerative myogenesis and a novel role for ORs in tissue repair. Many chemokines and chemokine receptors are expressed during time points of extensive fusion during myogenesis by muscle cells. We have elucidated the role of CXCR4 and SDF1α in myogenesis of primary muscle cells. CXCR4 regulates migration of both myoblasts and

myocytes, and is necessary for proper myogenesis. In addition, we have identified a functional role for MOR23 during adult regenerative myogenesis. MOR23 regulates migration and adhesion, affecting downstream fusion events and possibly myofiber branching. Finally, this work has identified the first molecule capable of regulating myofiber branching, and may have implications for the therapeutic potential of MOR23 in muscle dystrophies.

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