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Chemokine expression and control of muscle cell migration during myogenesis

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Summary
Adult regenerative myogenesis is vital for restoring normal tissue structure after muscle injury. Muscle regeneration is dependent on progenitor satellite cells, which proliferate in response to injury, and their progeny differentiate and undergo cell–cell fusion to form regenerating myofibers. Myogenic progenitor cells must be precisely regulated and positioned for proper cell fusion to occur. Chemokines are secreted proteins that share both leukocyte chemoattractant and cytokine-like behavior and affect the physiology of a number of cell types. We investigated the steady-state mRNA levels of 84 chemokines, chemokine receptors and signaling molecules, to obtain a comprehensive view of chemokine expression by muscle cells during myogenesis in vitro. A large number of chemokines and chemokine receptors were expressed by primary mouse muscle cells, especially during times of extensive cell–cell fusion. Furthermore, muscle cells exhibited different migratory behavior 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. Given the large number of chemokines and chemokine receptors directly expressed by muscle cells, these proteins might have a greater role in myogenesis than previously appreciated.

Key words: Fusion, Myoblast, Myocyte, CXCR4, SDF-1α, Regeneration

Introduction
Skeletal muscle degeneration can occur as a result of 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 another or with existing myofibers to restore normal tissue architecture. In vitro studies demonstrate that migration is a key process during myogenesis. Migration is crucial 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). Identification of molecules that regulate cell migration might 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 have key roles during myogenesis (Bischoff, 1997; Corti et al., 2001; Lee et al., 1999; Robertson et al., 1993; Horsley et al., 2003; Lafreniere et al., 2006). 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 crucial for regulating cell migration during myogenesis (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 is required for proper migration of muscle cells.

Chemokines are secreted proteins, approximately 8–10 kDa in size, with 20–70% homology in amino acid sequences, that share both leukocyte chemoattractant and cytokine-like behavior (Baggiolini et al., 1995; Luster, 1998). Chemokines are important for the migration of muscle precursor cells during embryonic myogenesis (Vasyutina et al., 2005; Yusuf et al., 2006) 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 (Hirata et al., 2003; Porter et al., 2003; Sachidanandan et al., 2002; Warren et al., 2005; Warren et al., 2004; Civatte et al., 2005; Demoule et al., 2009). Finally, chemokines are known to regulate migration of several cell types postnatally, such as immune cells, sperm and metastasizing cancer cells (Kim, 2004; Kim, 2005; Stebler et al., 2004; Bleul et al., 1996; Isobe et al., 2002; Miyazaki et al., 2006; Vandercappellen et al., 2008; Muciaccia et al., 2005a; Muciaccia et al., 2005b). 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 behavior throughout myogenesis in vitro. One receptor–ligand pair, CXCR4–SDF-1α (CXCL12), regulated the 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
To determine which chemokine receptors and ligands are expressed by
muscle cells at different time points during myogenesis, pure
cultures of primary mouse muscle cells were used because they
follow a predictable time-course of myogenesis. Upon removal of
serum, myoblasts differentiate into myocytes that fuse to form
nascent myotubes, which are small and contain few nuclei.
Subsequently, myocytes fuse with nascent myotubes creating
mature myotubes, which are large and contain many nuclei (Fig.
1A). In our culture conditions, by 16 hours in differentiation
medium (DM), the majority of cells were terminally differentiated
myocytes as indicated by the high percentage of embryonic myosin-
heavy-chain-positive (eMyHC+) cells (Fig. 1B). After 24 hours in
DM, ~40% of myocytes were fused with each other to form nascent
myotubes. By 48 hours, ~70% of myocytes were fused, creating
mature myotubes (Fig. 1C). A real-time RT-PCR array was used to
investigate the mRNA steady-state levels of 84 chemokines,
chemokine receptors and signaling molecules, 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 in vitro. The steady-state
levels of these mRNAs varied drastically; a small subset of genes
had extremely high steady-state levels, ~10,000- to 1-million-fold
higher than other genes (supplementary material Table S1).
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 after
6 or 48 hours in DM; rather, most mRNA steady-state levels were
highest between 16 and 36 hours in DM (Table 1; Fig. 1D,E),
which were time points of extensive differentiation and fusion of
myocytes.

Many chemokine receptors and ligands known to be expressed
by skeletal muscle cells or tissue were shown in this assay to be
expressed directly by muscle cells (Bischoff, 1997; Chazaud et
al., 2003; Chong et al., 2007; Civatte et al., 2005; 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 myogenesis in vitro and in vivo
(Horsley et al., 2003; Lafreniere et al., 2006), was identified
by this chemokine array (Table 1). 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.

Table 1. Chemokines and chemokine receptors expressed
during in vitro myogenesisa

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aReal-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.
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. 2A). Myocytes displayed distinct differences in migration compared with 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. 2A). The proportion of slow-moving cells also increased during myogenesis (Fig. 2B), causing a concomitant decrease in mean velocity from 56 μm/hour at 0 hours to 22 μm/hour at 48 hours in DM. The diminished velocity of myocytes at 48 hours was not due to a loss in cell motility or viability because the addition of fresh serum-free DM increased cell migration (data not shown). The enhancement of cell migration by fresh DM might be due to elimination of inhibitory factors secreted by cells into the medium during myogenesis. Thus, muscle cells are migratory throughout myogenesis; as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; as most investigations have focused on myoblast 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. Both HGF and PDGF greatly enhancing the migration of myoblasts; however, neither factor stimulated myocyte migration (Fig. 3), suggesting that 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 with control medium (Fig. 3). Migration to CM suggests that migratory factors, 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 might not regulate myocyte migration during myogenesis in vitro.

Myocytes exist during muscle regeneration

We next 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, p21 and eMyHC and then fuse with each other and with myofibers to restore normal tissue architecture. Mononucleated cells were isolated from injured mouse muscles and analyzed by flow cytometry (Fig. 4A). Muscle cells were defined as α7-integrin-positive 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 (Schultz et al., 1978) and in days immediately following injury, the majority of mononucleated cells in muscle tissue are immune cells (Allbrook, 1981; McLennan, 1996; Tidball, 2005); day 3 was the earliest time point analyzed. At later time points, myogenic cells are fusing into newly regenerating myofibers (Allbrook, 1981), 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. 4B). To determine whether differentiated α7-integrin+ CD31− CD45− muscle cells exist during regeneration, cells were also immunostained for p21, which marks terminally differentiated cells (Andres and Walsh, 1996). The peak percentage of terminally differentiated p21+ myogenic cells was observed at day 5 after injury (Fig. 4C,D).

We used several 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 because 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 after injury by FACS, and immunostained for myogenin and eMyHC in vitro (Fig. 4E). Approximately 60% of myogenic cells were myogenin+ and 18% were eMyHC+ (Fig. 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 to be involved in the regulation of differentiating myogenic cells in vivo.

**CXCR4 and SDF-1α 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 SDF-1α, in more detail. The receptor CXCR4 and ligand SDF-1α were of specific interest because several studies have shown expression of these proteins by muscle cells or tissue, but conflicting reports exist regarding their role during myogenesis (Bae et al., 2008; Chong et al., 2007; Melchionna et al., 2010; Odemis et al., 2007; Odemis et al., 2005; Vasyutina et al., 2005; Yusuf al., 2003; Bischoff, 1986). To confirm expression of CXCR4 at the protein level, flow cytometry was used to determine the percentage of CXCR4+ cells in pure cultures of primary mouse myoblasts and myocytes; ~30% of myoblasts were CXCR4+ compared with ~60% of myocytes (Fig. 5A,B). Furthermore, myocytes contained ~twofold more CXCR4 per cell (Fig. 5C,D), yet myocytes were only 18% larger than myoblasts (Fig. 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. 1E). To determine whether CXCR4 and SDF1α are expressed during adult regenerative myogenesis, the percentage of CXCR4+ myogenic cells that express CXCR4 in regenerating muscle at day 3 is lower than the 80% CXCR4+ cells observed in freshly isolated quiescent Pax7 satellite cells on myofibers from uninjured muscle (Cerletti et al., 2008). This discrepancy might be due in part to the marker used for positive selection of myogenic cells in our studies, but is also probably due to modulation of CXCR4-expressing cells during regeneration. Together, these data demonstrate that CXCR4 and SDF1α proteins are expressed by primary mouse muscle cells during myogenesis in vitro. As CXCR4 was expressed by mononucleated muscle cells during adult regenerative...
myogenesis, and SDF-1α was isolated from muscle tissue, this receptor–ligand pair might regulate myogenesis.

**The CXCR4–SDF-1α axis is important for proper muscle cell fusion**

To examine the role of the CXCR4–SDF-1α axis in myogenesis, we used primary mouse muscle cells in vitro, because direct effects on muscle cells can be analyzed in the absence of other cell types. To determine whether the CXCR4–SDF-1α axis regulates migration during myogenesis, myoblasts and myocytes were allowed to migrate to several concentrations of SDF-1α in Boyden chambers (Fig. 6A). Interestingly, while both cell types were attracted to SDF-1α, 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, SDF-1α affects migration of both myoblasts and myocytes, although myocytes exhibit a greater sensitivity to SDF-1α.

To determine whether CXCR4-dependent processes are necessary for myogenisis, a pharmacological inhibitor of CXCR4, AMD3100 (De Clercq, 2005), 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. 6B). However, neither the number of cells per field nor the number of nuclei in differentiated cells was affected (data not shown). Rather, addition of AMD decreased the fusion index, or the total number of nuclei in myotubes, by ~30% compared with the control (Fig. 6C). We also examined myogenesis in vitro in cells containing siRNA to knock down CXCR4. CXCR4 protein levels were decreased by ~45% by Cxcr4 siRNA (Fig. 6D). After 24 or 48 hours in DM, cells were immunostained for eMyHC; at both time points, Cxcr4 siRNA cultures contained smaller myotubes compared with the control (Fig. 6E). This defect in myotube formation was not due to a decrease in the total number of nuclei (Fig. 6F), nor to an affect on differentiation, as measured by the percentage of nuclei found in eMyHC+ cells (Fig. 6G). Rather, Cxcr4 siRNA myocytes exhibited a clear defect in cell fusion (Fig. 6H), because the fusion index was decreased 36% and 24%, at 24 and 48 hours, respectively, in Cxcr4 siRNA cultures (Fig. 6H). Together, these data support the hypothesis that the CXCR4–SDF-1α axis is necessary for proper myogenesis.
in vitro. The predominant role for CXCR4–SDF-1α during myogenesis might be to regulate the migration of muscle cells, which affects downstream fusion events.

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 is complex.

Several chemokines and chemokine receptors we identified were not previously known to be expressed by skeletal muscle cells or 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), however, these molecules have known roles in other tissue (Civatte et al., 2005; De Rossi et al., 2000; Demoule et al., 2003; Sachidanandan et al., 2002; Warren et al., 2005; Warren 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., 2009). A key question is why so many chemokines and chemokine receptors are expressed directly by muscle cells during myogenesis in vitro. As muscle cells are heterogeneous (Asakura et al., 2002; Motohashi et al., 2008; Relaix et al., 2005; Tanaka et al., 2009), subpopulations of muscle cells might express a single receptor or ligand. Alternatively, several of these molecules might 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 several receptors are expressed by a single cell, specific chemokine receptors might be used in a spatial-temporal manner. Alternatively, a redundant system might 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 in vitro might 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, might regulate myoblast proliferation or survival. Also, because muscle cells must interact directly with one another for terminal differentiation to occur (Krauss et al., 2005), chemokines might also regulate migration of myoblasts. Our data suggest that multiple chemokine receptor–ligand pairs 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 in vivo might not only have a direct effect on myogenesis, but may also 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 crucial for muscle regeneration (Arnold et al., 2007). Therefore, chemokines might regulate myogenesis through several distinct processes.

The investigation of a single receptor–ligand pair, CXCR4 and SDF-1α, indicated that some chemokines identified in this study do regulate migration during myogenesis in vitro. We show that CXCR4 is expressed by both primary mouse myoblasts and myocytes, and its ligand SDF-1α can increase migration of both cell types, albeit at different concentrations. However, despite inhibition of CXCR4 by two different methods, primary muscle cells differentiate similarly to untreated cells, but are unable to undergo fusion as efficiently. Together, these results suggest that CXCR4 is necessary for migration of muscle cells to one another, which is required for normal fusion. Our studies expand on previous CXCR4 studies in the field. The majority of in vitro CXCR4 studies use the immortalized C2C12 mouse muscle cell line (Melchionna et al., 2010; Odemis et al., 2007; Ratajczak et al., 2003). Similarly to our results, the CXCR4–SDF-1α axis enhances migration of C2C12 myoblasts (Odemis et al., 2007; Ratajczak et al., 2003). However, in contrast to our studies, investigations on C2C12 cells suggest that loss of CXCR4 leads to an inhibition of differentiation as measured by decreased expression of differentiation-specific muscle proteins, such as myogenin and/or myosin heavy chain (Melchionna et al., 2010; Odemis et al., 2007). In one study, an almost complete abrogation of muscle cell differentiation was observed with loss of CXCR4, despite the fact that only 15% of C2C12 cells express CXCR4 (Odemis et al., 2007). Differences between primary muscle cells and established cell lines could contribute to some of the differences between our studies and those with C2C12 cells. Interestingly, loss of CD164, a sialomucin that interacts with CXCR4, on the cell surface where it probably functions as a component of a CXCR4 receptor complex (Bae et al., 2008; Forde et al., 2007), also affected migration and myotube formation, but not differentiation of C2C12 cells, similarly to our experiments (Bae et al., 2008). The CXCR4–SDF-1α axis is known to have a role in embryonic muscle development. Most studies that analyze CXCR4 function during embryonic myogenesis in mice, zebrafish and chick suggest that perturbation of CXCR4 signaling alters limb-muscle development mainly as a result of deficiencies in migration of myogenic precursor cells from the somites to the limb buds (Chong et al., 2007; Vasyutina et al., 2005; Yusa et al., 2006). Since terminal differentiation and fusion occur downstream of migration, defects in these later processes could not be analyzed during embryonic development independently of migration defects. However, one study of embryonic muscle development in Cxcr4-null mice did not observe defects in migration of muscle precursor cells to the limb buds but defects in muscle mass were noted; no mechanism was determined for this loss of muscle mass (Odemis et al., 2005). No studies of the CXCR4–SDF-1α axis have been performed in adult regenerative myogenesis.

CXCR4 is of specific interest to cell-therapy approaches for various muscular disorders. A subset of muscle satellite cells that are CXCR4+ can be engrafted into injured muscle tissue with a high efficiency (Cerletti et al., 2008). As CXCR4 regulates migration of muscle cells both in vitro and in vivo, the increased engraftment might be due to an increased migratory ability of these cells. Furthermore, treatment with SDF-1α enhances migration of myogenic precursors, yielding a positive effect on engraftment of cells into damaged muscle (Galvez et al., 2006). These data suggest that CXCR4–SDF-1α-dependent migration enhances the engraftment of cells into damaged muscle. The large number of chemokine receptors and ligands expressed by muscle cells during myogenesis in vitro suggests further avenues of research to be explored during adult regenerative myogenesis. Further studies of chemokines in vivo might lead to manipulation of these molecules and allow for an increased efficiency of cell-transplantation therapies for various muscle disorders.

Materials and Methods

Animals and muscle injuries

Adult mice between 8 and 12 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 BaCl2 (O’Connor et al., 2007) and collected as described (Abbott et al., 1998).

Primary muscle cell culture, differentiation and fusion assays

Primary myoblasts were derived from the hindlimb muscles of Balb/c mice (Bondesen et al., 2004; Mitchell and Pavlath, 2001) and cultures were >99% myogenic as assessed by MyoD immunostaining (Jansen and Pavlath, 2006). For all experiments 3–5 independent isolates were analyzed. To induce differentiation, primary myoblasts were seeded at a density of 2–5 × 10⁴ cells/well on dishes coated with enaetin, collagen IV and laminin (E-C-L; Upstate Biotechnology) and switched to differentiation media [DM: DME, 1% insulin-transferrin-selenium-A supplement (Invitrogen), 100 U/ml penicillin G and 100 µg/ml streptomycin]. At indicated time points, cells were immunostained with an eMyHC antibody (F1.652; Developmental Studies Hybridoma Bank) and analyzed as described (Horsley et al., 2001). AMD3100 (Sigma) was dissolved in PBS and used at 10 µM in DM. At least 500 nuclei per condition were analyzed for each assay.

Transfection of primary myoblasts

Stable RNAi (Invitrogen) was used to knockdown Cxcr4 expression in primary myoblasts. Myoblasts were plated in growth medium (GM; F10, 20% fetal bovine serum, 100 U/ml penicillin G and 100 µg/ml streptomycin) at a density of 8–105 cells/cm² on collagen-coated 100 mm plates and after 6 hours, duplexed siRNAs at a final concentration of 27 nM each were used to transfect cells using Lipofectamine 2000 (Invitorgen) in GM according to the manufacturer’s instructions. Cells were transfected with either scrambled control or a mixture of three Cxcr4 siRNAs (Invitrogen, ACGAGUGAAGGAGCAUGAAAUAGCAUGAAGGUAGUAUCCUGU; ACAGGUACAUUGACAGCCCCCUUUA; CAGUCAUC- CCUAUCCUAUGUCCUU). After 6 hours of incubation, medium containing transfection complexes was replaced by fresh GM. Twenty-four hours after the start of transfection, cells were trypsinized, plated on six-well dishes and differentiated for 24 hours and 48 hours as described for differentiation and fusion assays above. CXCR4 knockdown was assessed by immunoblotting using anti-CXCR4 (Abcam) after 24 hours of transfection. Results represent data from three independent isolates.

Flow cytometry

To analyze CXCR4 expression in vitro by flow cytometry, primary myoblasts were immunostained with anti-CXCR4-APC antibody (1:100; BD Pharmingen) 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 BaCl2 injection (n=10 for each time point) and immunostained with antibodies to CD31-FITC (1:100; eBioscience), CD45-APC (1:100; BD Pharmingen). CD31+ CD45- cells were analyzed for CXCR4 expression. For analysis of p21 expression during regeneration, mononucleated cells were dissociated from gastrocnemius muscles of mice at the indicated times after BaCl2 injection (n=10 for each time point), fixed with cold 70% ethanol overnight at −20°C and immunostained with antibodies to CD1-A1-APC (1:100; eBioscience), CD45-APC (1:100; BD Biosciences), CD45+CD31- and p21 (1:100; LifeSpan Biosciences). To detect p21, cells were incubated with biotin-conjugated donkey anti-goat (1:100; Jackson Immunoresearch) for 20 minutes, then FITC-conjugated streptavidin (1:100; Jackson Immunoresearch Lab., Inc.) for 20 minutes. CD31− CD45− cells were analyzed for p21 and p21 expression (n=10 for each time point).

Cell-migration assays

Migration of muscle cells was quantified using time-lapse microscopy as described (Jansen and Pavlath, 2006). Briefly, cells were seeded at 2×10^5 cells per 35 mm dish, and switched to DM for the indicated times before imaging. Images were recorded (Q Imaging Camera and OpenLab 3.1.4 software) every 5 minutes for 3 hours. Cell velocities were calculated in μm/hour using ImageJ software by tracking the paths of mononucleated cells. Approximately 20 mononucleated cells were tracked for each experiment.

Boyden chamber assays were performed as described (Mylona et al., 2006). Primary myoblasts were seeded on 150-mm plates at low density (9×10^5 cells/plate) and switched to DM for 24 hours to generate myocytes in the absence of myotube formation. (7.5×10^5 cells in 200 μl DM) were loaded in the upper wells of the Boyden chamber and incubated at 37°C for 5 hours. Migrated cells were fixed, stained and counted. HGF and PDGF were used at 100 ng/ml in DMEM with 1% BSA, SDF1α at 10−20 ng/ml in DM (Sigma). To prepare conditioned medium (CM), myoblasts were incubated in DM for 24 hours; the medium, which had been conditioned with secreted factors, was then collected, filtered (0.45 μm), flash frozen, and stored at −80°C until use.

**References**


BETA-chemokine receptors SDF-1 and 3 are expressed on the head region of human spermatozoa. FASEB J. 19, 2048-2050.