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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 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 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 (Echttermeyer et al., 1996; Lluri and Jaworski, 2005; Lluri et al., 2008; Mylona et al., 2006; Ocalan et al., 1988; Olgun 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, ~1,000- 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 (AGTR1L, 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 myogenesis

| Gene  | 16 hours | 24 hours | 36 hours | Not expressed |
|-------|----------|----------|----------|--------------|
| Bmp10 | Agpr11   | Ccr8     | Gdf5     | Br1          |
| Ccl8  | Bdf5     | Ccr1     | Gpr2     | Bmp6         |
| Ccr5  | Bmp15    | Ccr2     | Gpr81    | Ccr2         |
| Ccr6  | Bmp18    | Ccr3     | Gpr13    | Lin2         |
| Ccr7  | Ccl1    | Cmtm2a   | Il18     | Cxcl12       |
| Ccr1  | Ccl19    | Cmtm5    | Il1a     | Cxcl15       |
| Ccr3  | Ccl20    | Cxcl1    | Il4      | Hfl3a        |
| Ccr9  | Ccl4    | Cxcl13   | Il8a     | Il3          |
| Cnmt3 | Cxcl15   | Cxcl1    | Il8b     | Inhb         |
| Cnmt6 | Cxcl6   | Cxcl2    | Ltb42    | Myd88        |
| Csf1  | Cxcl7   | Cxcl4    | Rgs3     | Nfkb1        |
| Csf2  | Cxcl8   | Cxcl5    | Slit2    | Tr4          |
| Cxcl1 | Cxcl9   | Cxcl7    | Tnf      |              |
| Cxcl11| Cxcl11  | Cxcl9    | Tnfrsf1a |              |
| Cxcl10| Cxcr4   | Cxcr3    | Tnfrsf14 |              |
| Cxcl11| Cxcr5   | Cxcr4    | Trem1    |              |
| Cxcr6 | Cxcr6   | Cxcr7    |          |              |

*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.
myocytes migration are unknown. Inhibitory factors secreted by cells into the medium during myogenesis; as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout 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; therefore, muscle cells are migratory throughout 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; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory throughout myogenesis, as most investigations have focused on myoblast migration, the majority of receptor–ligand pairs that regulate myogenesis; therefore, muscle cells are migratory through 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.

Myoocytes 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., 2005; Ratajczak et al., 2003; Vasyutina et al., 2005; Yusuf et al., 2007; Melchionna et al., 2010; Odemis et al., 2007). 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 ~two-fold 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+ CD31− CD45− myogenic cells was determined at day 3 and day 5 after injury (Fig. 5F,G). From day 3 to day 5, the percentage of myogenic CXCR4+ cells increased from ~45% to 77% (Fig. 5H). In addition, the amount of CXCR4 per muscle cell was increased ~2.5-fold at day 5 compared with day 3 (Fig. 5I), with no change in cell size (data not shown). The percentage of 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 by the regenerative process because we observe a 1.7-fold increase in the percentage of CXCR4+ myogenic cells from day 3 to day 7.

To validate expression of SDF1-α at the protein level, ELISA assays were performed using control DM and 24 hours CM; significant levels of SDF1-α were detected in CM (Fig. 5J). The levels of SDF1-α 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 SDF1-α compared with uninjured muscles or muscles at day 5 after injury (Fig. 5K). Therefore, SDF1-α might be released under certain conditions after injury. Together, these data demonstrate that CXCR4 and SDF1-α 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 myogenesis, 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 muscle types. For example, AGTRL1 has protective effects in ischemic heart disease (O’Donnell et al., 2007) and BMP10 regulates hypertrophic growth in heart muscle (Chen et al., 2006). Neither of these proteins has identified functions in skeletal muscle but might regulate skeletal muscle growth or repair given their role in smooth and cardiac muscle. Another gene that we found to be expressed during myogenesis, 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 in vitro. As muscle cells are heterogenous (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; Yusu 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-/- 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; Mitchel and Pavlath, 2001) and cultures were >99% myogenic as assessed by MyD 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 2×10^6 cells/well on dishes coated with entactin, collagen IV and laminin (E-C-L; Upstate Biotechnology) and switched from growth with growth medium (GM) to differentiation medium (DM) containing 5% horse serum, 1% insulin-transferrin-selenium-A supplement (Invitrogen), 100 U/ml penicillin G and 100 µg/ml streptomycin.

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
ana" "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 BaC1 injection (n=10 for each time point) and immunostained with antibodies to CD31-FITC (1:100; eBiosciences), CD45- FITC (1:100; BD Biosciences), α7-integrin-PE (1:200; a gift from Fabio Rossi, University of British Columbia, Vancouver, Canada) and CXCR4-APC (1:100; BD Pharmingen). CD31+CD45− cells were analyzed for α7-integrin-PE and 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 CD31-APC (1:100; eBiosciences), CD45-APC (1:100; BD Biosciences), α7-integrin-PE 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 α7-integrin and p21 expression (n=10 for each time point). For each sample, 10,000 cells were analyzed, and propidium iodide was used to remove dead cells. Isotype controls were used to determine gating. All data analysis was performed using FlowJo v. 6.2.1 (TreeStar).

Immunostaining

Myogenin and eMyHC immunostaining was 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% H2O2, biotin-streptavidin blocking kits (Vector), mouse IgG (M.O.M. kit, Vector) and then blocking buffer containing 4% BSA in PBS for 1 hour. Cells were then incubated overnight at 4°C with anti-myogenin (hybridoma supernatant, diluted 1:10 in blocking buffer, FSD; Developmental Studies Hybridoma Bank), anti-eMyHC (hybridoma supernatant, neat, F1.652; Developmental Studies Hybridoma Bank) or appropriate IgG (diluted 1:100 in blocking buffer, Genetex). Following successive washes in PBS with 0.1% BSA, cells were incubated with donkey anti-mouse IgG (Jackson ImmunoResearch) 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 diaminobenzidine (DAB). All immunostaining was performed at room temperature unless stated otherwise. Hybridoma cells were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, USA.

Real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Life Technologies). All RNA was treated with DNase (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 using gene-specific primers in a 36-well SABiosciences biochem kinase array (Chen, G. and Quinn, L. S. 2008). (Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/18/3052/DC1) 

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