Ephrin-A5 promotes bovine muscle progenitor cell migration before mitotic activation

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ABSTRACT: Satellite cells are the resident stem cell population of adult skeletal muscle tissue that is responsible for growth and regeneration. The cells typically congregate near the tips of the muscle fibers and in close proximity to the neural muscular junction (NMJ). Ephrin-A5 is a chemotactic molecule that participates in the correct positioning and formation of the NMJ. The objective of the experiment was to examine the effects of ephrin-A5 signaling on bovine satellite cell (BSC) biology. Primary cultures of BSC demonstrate changes in velocity with time in culture that is unique to the Paired box protein 7 (Pax7):Myogenic factor 5 (Myf5) subpopulation. Treatment of the BSC with ephrin-A5 causes a reduction \((P < 0.05)\) in velocity with a concomitant increase \((P < 0.05)\) in directed migration. The chemotactant properties of ephrin-A5 occur before myogenic differentiation 1 (MyoD) expression in the myogenic precursors and are abrogated after their differentiation to committed myoblasts. Ephrin-A5 induced migration appears to require components of the Ras homolog gene family member A (RhoA) and Rho-associated protein kinase (ROCK) signaling machinery. Supplementation of culture media with a chemical ROCK inhibitor suppressed \((P < 0.05)\) ephrin-A5 initiated BSC migration. These results contrast with treatment of BSC with hepatocyte growth factor (HGF), a key modulator of myogenic and motogenic activity. Treatment of BSC with HGF had no effect on cell motility or migration immediately after culture establishment. Twenty-four hours after culture establishment, BSC demonstrated an increase \((P < 0.05)\) in transwell migration toward HGF. These results document that temporal and spatial gradients of chemokines and growth factors participate in the localization of BSC within the niche.

Key words: ephrin-A5, migration, motility, satellite cell

INTRODUCTION

Satellite cells are defined as a heterogeneous population of myogenic precursor cells positioned between the myofiber sarcolemma and basal lamina that are responsible for postnatal growth and repair of skeletal muscle. Normally quiescent in the adult animal, satellite cells respond to environmental cues to reenter the cell cycle (activate), proliferate, and ultimately differentiate into muscle fibers. Niche localized hepatocyte growth factor (HGF) is critical to activation of the cell (Allen et al., 1995; Sheehan et al., 2000; Tatsumi and Allen, 2004) and the HGF receptor is instrumental in directed migration of embryonic myoblasts into the developing limb buds (Bladt et al., 1995). The involvement of HGF during postnatal migration of satellite cells remains poorly understood.

Ephrins are a class of bidirectional signaling molecules found in multiple tissues that participate in migration and cellular motility (Pasquale, 2008). Skeletal muscle expresses many of the 7 ligands and 5 receptors of the A-class ephrin system (Siegel et al., 2009). Ephrin-A1 and -A5 ligands are present in myoblasts and engage in events defining the muscle and connective tissue boundaries of the embryonic limb (Swartz et al., 2001). Chemotactic signals mediated through the ephrin type-A receptor 4 (EphA4) receptor are critical to the correct positioning of the motor neuron and formation of the neuromuscular junction.
(Lai et al., 2001; Eberhart et al., 2002). The presence of the ligand:receptor system in muscle and satellite cells points toward their involvement in chemotactic actions during muscle growth and repair.

The objective of the research was to examine the motility parameters and characteristics of BCS as a function of time and in response to HGF and ephrin-A5. Results indicate that BCS migrate toward ephrin-A5 before expression of myogenic differentiation 1 (MyoD). By contrast, HGF does not alter the motogenic properties of satellite cells before activation and cell cycle progression.

MATERIALS AND METHODS

The University of Florida Animal Care and Use Committee approved the humane euthanasia and tissue collection protocol (201004212).

Cell Culture

Primary BCS were isolated from young Holstein bulls (<5 d of age) by trypsin digestion and differential centrifugation, as described (Li et al., 2011). Satellite cells were cultured on entactin-laminin-collagen (ECL; BD Biosciences, Franklin Lakes, NJ)-coated plasticware in low glucose Dulbecco’s modified Eagle media (DMEM) supplemented with 10% horse serum, 1% penicillin-streptomycin, and 0.2% gentamicin (Invitrogen, Grand Island, NY). Mouse myoblasts (23A2) were cultured on ECL-coated plasticware in high glucose DMEM supplemented with 15% fetal bovine serum, 1% penicillin-streptomycin, and 0.2% gentamicin (Invitrogen). Recombinant human ephrin-A5 (R&D Systems, Minneapolis, MN) was preclustered with anti-human IgG (Jackson Immuno-Research, West Grove, PA) for 20 min at room temperature. Cells were incubated at 4°C overnight in PBS for 10 min at room temperature. Cultures were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Nonspecific antigen sites were blocked with 5% horse serum containing ephrin-A5 (5 μg/mL; Invitrogen) was included for the visualization of nuclei. After extensive washing with PBS, immune complexes were visualized with a Nikon TE2000 equipped with an epifluorescent light source and representative images were captured with a charge-couple device (CCD) camera (CoolSnap K4, Photometrics, Tucson, AZ) using NIS Elements imaging software (Nikon, Melville, NY).

Western Blot

Cells were lysed into SDS-PAGE sample buffer, sonicated, and heated at 95°C for 5 min. Protein content was measured (BCA Assay; ThermoFisher Scientific, Waltham, MA) and an equal amount of protein was separated through 12% polyacrylamide gels. After transfer to nitrocellulose, the blots were incubated with 5% nonfat dry milk in PBS containing 0.1% Tween 20 for 30 min to block nonspecific antigen sites. Blots were further incubated with anti-EphA4, anti-extracellular signal-related kinase 1 and 2, anti-phospho-extracellular signal-related kinase 1 and 2, and anti-α-tubulin overnight at 4°C. All antibodies were purchased from Cell Signaling Technology (Beverly, MA) and diluted 1:1,000 in blocking solution. After incubation with appropriate peroxidase-conjugated second antibody, immune complexes were visualized with chemiluminescence (Enhanced Chemiluminescence kit, GE Healthcare Biosciences, Piscataway, NJ) and exposure to X-ray film.

Time-Lapse Videography

Satellite cells were seeded on gridded, glass-bottomed plates coated with ECL in complete growth media. After 18 h, the cells were washed to remove debris and cultured in low glucose DMEM supplemented with 5% heat-inactivated horse serum containing ephrin-A5 (5 μg/mL), HGF (10 ng/mL), or an equivalent amount of vehicle only. Cells were placed in a stage-mounted miniculture chamber (LiveCell, Pathology Devices, Westminster, MD) with temperature maintenance at 37°C and 5% CO₂ in air. Images were captured at 5 min intervals for 24 or 48 h with a CCD camera (CoolSNAP, Photometrics, Tucson, AZ) using a Lambda SC-SMART shutter control (Sutter Instruments, Novato, CA) integrated with NIS Elements software (Nikon). Upon completion of the experiment, cultures were fixed and immunostained for Paired box protein 7 (Pax7) and Myogenic factor 5 (Myf5) for post hoc identification of
the analyzed cells. Motion kinetics were measured with NIH ImageJ freeware (http://rsbweb.nih.gov).

**Transwell Migration**

Satellite cells were seeded atop ECL-coated 8 μm pore size transwell membrane inserts (Corning, Lowell, MA) and the membrane was placed over lower chambers containing low glucose DMEM supplemented with 5% heat inactivated horse serum or the aforementioned media supplemented with 5 μg/mL ephrin-A5 or 10 ng/mL HGF. After 4 or 24 h, stationary cells were scraped from the upper side of the membrane and the membrane placed in 4% paraformaldehyde in PBS. Fixed cells were further incubated in Hoechst 33245 (1 μg/mL) before enumeration. Migration percent was calculated as the number of migrating cells divided by the total number of cells placed in the upper chamber multiplied by 100.

**Statistical Analysis**

Differences between groups were determined by ANOVA using the general linear model program (SAS Inst. Inc., Cary, NC) and the PDIFF function. Results are presented as means ± SEM. Statistical significance was reached at \( P < 0.05 \).

**RESULTS**

**Satellite Cells Exhibit a Range of Motility that is Reflective of Their Lineage Identity**

Satellite cells exit quiescence and migrate along the periphery of the muscle fibers to sites of damage. The motion of bovine satellite cells (BSC) was measured in vitro by time-lapse videography to establish velocity as a function of mitotic activation. Cells were cultured for 48 h and a 4-h block of time (20 to 24 h and 44 to 48 h, respectively) was analyzed for the total distance traveled and the subsequent calculation of velocity. Upon completion of the videography, cells were fixed and immunostained with anti-Pax7 and anti-Myf5 for post hoc identification of the cells. Results demonstrate a wide range of individual BSC velocity (Fig. 1A). During the first 24 h in culture, the BSC exhibit speeds ranging from 1 to 10 μm/h. An increase in the numbers of BSC traveling at a speed greater than 10 μm/h is evident during the 44 to 48 h timeframe. Satellite cells are classified as muscle progenitor cells by co-expression of Pax7 and Myf5 or as muscle stem cells by expression of Pax7 only (Kuang et al., 2007). Post hoc identification of the individual cells indicated that the majority of the rapidly moving cells were Pax7:Myf5 progenitors (Fig. 1B). The progenitor cells nearly double in speed with time in culture. By contrast, the Pax7 muscle stem cells remained relatively inert. Changes in the morphology of the myogenic stem cell occurred over time, thus demonstrating metabolic activity (Fig. 1C). However, little movement across the substratum surface was noted. The striking difference in velocity as a function of lineage suggests that Pax7 stem cells remain largely stationary on the muscle fiber with the progenitor cell serving as the motile damage-amending cell.
Ephrin-A5 Alters the Motility Patterns of Satellite Cells

Ephrin ligands and their cognate receptors act as bidirectional signaling molecules between adjacent cells to initiate chemoattractant and repulsive actions. Although skeletal muscle expresses a host of ephrin ligands and receptors, EphA4 signals are requisite for proper positioning and formation of the neuromuscular junction (NMJ). Because satellite cells often cluster near the NMJ and ends of fibers (Allouh et al., 2008; Kirkpatrick et al., 2008), we examined the effects of ephrin signaling on motility parameters in myoblasts and progenitor cells in vitro. Mouse 23A2 myoblasts were used as antibody and motility controls. Both BSC and mouse myoblasts express EphA4 receptors, as noted by Western blot (Fig. 2A). No apparent difference in the amount of receptor between the 2 cell types was noted. Ephrin-A5, a common ligand for EphA4, was incubated with cultures of BCS and myoblasts for 24 h with image capture at 5-min intervals. Results indicate that ephrin-A5 represses ($P < 0.05$) BSC movement across the substratum by approximately 30% but 23A2 myoblasts are unaffected by the ligand (Fig. 2B). Interestingly, the reduction in BSC velocity is accompanied by an increase ($P < 0.05$) in directed migration. Satellite cells or 23A2 myoblasts were seeded on ECL-coated transwell filters atop preclustered ephrin-A5. After 4 h, the membranes were fixed and the numbers of migratory cells enumerated. Myoblasts remained refractile to the chemotactic properties of ephrin-A5 (Fig. 2C). An increase ($P < 0.05$) in the number of BCS emerging through the ECL-coated membrane in response to ephrin-A5 was apparent. These results indicate that myogenic progenitor cells are highly responsive to the chemoattractant properties of ephrin-A5.

Committed Myoblasts are Unresponsive to the Chemoattractant Properties of Ephrin-A5

The experimental timeframe encompasses a period during which the BCS exist as myogenic progenitors (MyoD null) distinct from committed myoblasts (MyoD immunopositive; Li et al., 2011). Using the established MyoD expression kinetics, BSC cultured for 24 or 96 h were manually removed from the substratum surface and reseeded in transwell chambers. After 4 h, the membranes were fixed and the numbers of migratory cells enumerated. Myoblasts remained refractile to the chemotactic properties of ephrin-A5 (Fig. 2C). An increase ($P < 0.05$) in the number of BCS emerging through the ECL-coated membrane in response to ephrin-A5 was apparent. These results indicate that myogenic progenitor cells are highly responsive to the chemoattractant properties of ephrin-A5.

Satellite Cell Activation and Migration Are Distinct Events

Ephrin-A5 rapidly alters the motogenic actions of BSC during the apparent activation period before mitosis. Hepatocyte growth factor, a requisite activation factor for satellite cells, also possesses chemotactic activities (Lee et al., 1999). To determine if the 2 ligands have overlapping functions, the effect of HGF on BSC motility was examined. Primary BSC were seeded on ECL-coated plates and cultured for 24 h in the presence or absence of 10 ng/mL HGF. Images were captured at 5 min intervals, as described above. During the first 24 h of treatment, no changes in BSC velocity were noted in response to HGF (Fig. 4A). A reduction ($P < 0.05$) in average cell velocity was apparent; however, after 48 h of HGF supplementation. By contrast to the results found with ephrin-A5, BCS did not migrate toward HGF until 24 h in culture ($P < 0.05$; Fig. 4B). The inability of HGF to initiate the early chemotactic response is not due to a faulty signaling system. Satellite cells were cultured for...
24 h, treated with HGF, and lysed for Western analysis of extracellular signal-related kinase 1 and 2 (ERK1/2) activity. As shown in Fig. 4C, HGF initiated a rapid and transient increase in phospho-ERK1/2 abundance. No changes in the amounts of total ERK1/2 or tubulin were noted. These results indicate that satellite cells prefer to use an ephrin signaling mechanism to direct their migration during the early stage of culture that is synonymous with the activation period.

Ephrin-A5 Affects Satellite Cell Migration via Intracellular Signal Emanating from Ras-Related C3 Botulinum Toxin Substrate 1 and Rho-Associated Protein Kinase Proteins

The EphA4 receptor elicits responses mediated by the small guanosine triphosphate hydrolases, Rac1, and Ras homolog gene family member A (RhoA; Ogita et al., 2003; Sahin et al., 2005). Rho-associated protein kinase serves as a direct effector of RhoA signal transmission (Castellani et al., 2006). The importance of Rac1 and ROCK activity to ephrin-A5 mediated migration was investigated in BSC. Primary BSC were seeded on ECL-membranes positioned over chambers containing 5 μg/mL ephrin-A5 with or without inhibitors for Rac1 or ROCK. After 4 h, the numbers of migratory cells were quantified. Similar to previous results, BSC readily traverse through the membrane toward the ephrin-A5 ligand (Fig. 5). Movement in response to the chemottractant is blunted (P < 0.05) by inclusion of Y27632, a ROCK inhibitor. Disruption of either Rac1 or ROCK signals did not affect the basal migration rate. These results provide evidence that ROCK specifically mediates ephrin-A5 migratory kinetics in BSC.

DISCUSSION

Skeletal muscle growth in cattle is attributed to changes in protein synthesis and degradation such that a net gain in protein content (accretion) occurs (Lobley et al., 1980). With increasing size of the muscle fiber, myonuclei are added to meet the increased demands for mRNA templates for protein synthesis. Satellite cells supply said myonuclei during fiber hypertrophy by fusing with the sarcolemma membrane. Feed additives and synthetic hormones that promote fiber growth likely rely on satellite cell contribution to myonuclear pool (Chung and Johnson, 2008). Although our understanding of growth factor and hormonal control of proliferation and differentiation of BSC is becoming clearer (Kamanga-Sollo et al., 2010, 2011), the correct positioning of...
Placement of BSC in regions of myoblastic proliferation is required for patterning and correct placement of muscle within the embryo with chemotactic signals (Mauro, 1961). As these cells progress into myogenesis, they become associated with the target mesoderm. Over the past decade, attention has focused on redefining the population using a variety of surface markers and transcription factors. The simplest model of satellite cell identity defines the myogenic stem cell as a Pax7-only cell and the progenitor subpopulation as one expressing Pax7 and Myf5 (Kuang et al., 2006, 2007). However, additional layers of heterogeneity lie within these 2 groups including divergent extracellular matrix protein composition, growth kinetics, fusogenic capacity, and transcription factor profiles (Bierecci and Rando, 2010; Boldrin et al., 2010). Our results further extend the diverse nature of satellite cells to include motogenic properties. Initial observations indicated that satellite cells gain velocity as a function of time in culture. The doubling in speed that occurs between 24 and 48 h in culture is restricted to the Pax7:Myf5 progenitor population. These cells represent approximately 85% of the total cells and often are larger in size than their myogenic stem counterparts. An occasional small, light-refractile cell with a post hoc muscle stem cell lineage profile was observed to rapidly move across the substratum similar to a progenitor cell, thus disproving the hypothesis that size and motility are absolutely correlated. A word of caution regarding individual cell identity is imparted, as we are unable to assess variation in marker protein expression as a function of time. Previous work from our group detected no difference in the percentage of Pax7-only and Pax7:Myf5 cells during the 72 h window that precedes cell division (Li et al., 2011). Although subtle fluctuations in absolute amounts of the nuclear markers may occur, it is unlikely that a satellite cell would gain, lose, and reestablish expression of Pax7 or Myf5 during the 48 h videography period. No patterns of movement were found in the progenitor cells that allowed for their further segregation into subclasses. The time-lapse videography did provide a wealth of information regarding cell-to-cell contact, repulsive activity, membrane blebbing, and lamellipodia formation and retraction. Many of the observations reported herein are documented by others using mouse satellite cells (Siegel et al., 2009; Otto et al., 2011) arguing that satellite cell motility parameters likely are conserved across species.

Satellite cells express a multitude of genes coding for chemokines and their receptors, adhesion molecules and guidance molecules (Siegel et al., 2009). Four of the 10 A-subclass of ephrin receptors (ephrin type-A receptors 1 through 4) and 4 of the 5 ligands (ephrin-A1, -A3, -A4, and -A5) are expressed by mouse satellite cells. The activities of the ephrin-A signaling systems are repulsive within the confines of the developing chick limb bud (Swartz et al., 2001). Ectopic misexpression of ephrin-A5 causes Pax7 immunopositive myogenic cells to accumulate in regions distant from the targeted mesoderm injection site. Moreover, avian muscle cells evaded contact with ephrin-A5 in stripe assays. Our results run counter to these reports as exposure to ephrin-A5 acts as an attractant to BSC. Several possibilities may underlie the disparate results including species and donor age. Epigenetic programming within the satellite cell as a function of animal age exists. Ablation of Pax7 during gestation or in the early neonate leads to lethality but genetic removal of the transcription factor in juvenile or adult mice does not alter muscle function (Lepper et al., 2009). By extrapolation, the developmental profile of the neonatal BSC likely differs from that of its embryonic counterpart. Repulsive ephrin-A5 signals may be required for patterning and correct placement of the muscle within the embryo with chemoattractant signals acting as a guidance cue for muscle precursors in the postnatal animal. It should be noted, however, that only a fraction of the total BSC migrate toward ephrin-A5. As these cells progress into myogenesis, they become refractive to ephrin signaling. It remains to be determined if the ephrin-responsive BSC are fated for fusion or if these progenitors are early responders that detect areas of minor damage.

Ephrin type-A receptor 2 (EphA2) and EphA4 signaling is context dependent with matrix adhesion and migration a reflection of tissue metabolism and homeostasis. After ligand docking, EphA4 phosphorylates focal adhesion kinase, Rac1, and RhoA that cause cytoskeletal reorganization and changes in cellular morphology.
(Miao and Wang, 2012). In neurons, activation of EphA4 and downstream RhoA stabilizes the cytoskeletal membrane and inhibits axon outgrowth and migration (Shi et al., 2007; Takeuchi et al., 2009). Thus, BSC migration toward ephrin-A5 contradicts that found in nerve cells. The intracellular signaling axis involves RhoA and ROCK as chemical inhibitors to ROCK eliminate the migratory effect. Multiple ephrin type-A ligands exist in satellite cells and both EphA2 and EphA4 respond to ephrin-A5. It remains possible that ephrin-A5 promotes migration of a subset of satellite cells through a specific A-receptor and inhibits chemotaxis through a second receptor. Alternatively, it remains to be demonstrated that all satellite cells within the cultures are capable of generating an intracellular signal. In data not shown, immunocytochemistry indicates that all BSC express both EphA2 and EphA4. The limited response of the muscle progenitors is suggestive of a differential reaction at the level of intracellular signaling.

Hepatocyte growth factor is an established modulator of satellite cell myogenesis and is the requisite growth factor for activation (Anderson and Wozniak, 2004; Tatsumi et al., 2006). Moreover, HGF serves as a chemotactic molecule for myoblasts and satellite cells in vivo and in vitro (Brand-Saberi et al., 1996; Bischoff, 1997; Corti et al., 2001). Results presented herein describe a similar migratory action of HGF on BSC. However, the effects occur later in myogenesis than the chemotactic responses to ephrin-A5. The delay in response is not a product of insufficient receptor activation as treatment of the satellite cells with HGF leads to robust phosphorylation of ERK1/2. Although this points to the existence of a functional HGF receptor, it does not demonstrate full activation of the plethora of signaling systems within the satellite cell. For example, HGF stimulates the phosphatidylinositil 3-kinase (PI3K) and protein kinase B signaling axis in avian myoblasts (Haley and Cantley, 2004); a similar event was not found in our hands (data not shown). It is unlikely that a failure to activate the PI3K signaling system is the culprit behind the delayed migratory response as others report that the mitogen activated protein kinase/extracellular signal-related kinase intracellular signal underlies the chemotactic properties of HGF (Haley and Cantley, 2004; Leloup et al., 2007). Therefore, the downstream events emanating from ERK1/2 may be incomplete or disconnected. It is possible that HGF preferentially targets genes required for activation in the immediate timeframe, which may include the transcription of genes necessary for guidance and motion. After G0 exit, the satellite cell commits to developmental progression of myogenesis, which allows the cell to migrate in response to HGF. Migration toward HGF may represent a means of positioning a satellite cell adjacent to a fiber as well as allow for reengagement of the quiescent state. Chronic exposure of muscle progenitors to HGF results in phospho-ERK1/2 activity that leads to myostatin production and G0 entry (Reed et al., 2007; Yamada et al., 2010). Although an attractive scenario, the existence of localized pockets of HGF within the fiber niche remains unresolved.

In summary, BSC display variable motility kinetics and migratory properties. The ability to respond to the chemotactic signals supplied by ephrin-A5 is dependent on the developmental status of the muscle progenitor. Expression of MyoD in satellite cells or myoblasts is directly correlated with an impaired migration toward ephrin-A5. Ephrin-A5 directed guidance involves downstream activation of RhoA and ROCK but not Rac1. Moreover, the response of satellite cells to ephrin-A5 occurs before that of HGF, suggesting that HGF preferentially acts as a chemoattractant for muscle cells after withdrawal from G0 and reentry into the cell cycle. The results presented herein provide the foundation for future efforts uncovering the divergent motility parameters displayed by satellite cells. Manipulation of motility with feed additives and/or synthetic hormones may promote regionalized placement of satellite cells, thereby improving the efficient accumulation of myonuclei within the fiber during calf growth.

**LITERATURE CITED**


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