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EphA4/ephrin-A5 interactions in muscle precursor cell migration in the avian forelimb

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SUMMARY

Limb muscles derive from muscle precursor cells that lie initially in the lateral portion of the somitic dermomyotome and subsequently migrate to their target limb regions, where muscle-specific gene transcription is initiated. Although several molecules that control the generation and delamination of muscle precursor cells have been identified, little is known about the mechanisms that guide muscle precursor cell migration in the limb. We have examined the distribution of members of the Eph family during muscle precursor cell development. The EphA4 receptor tyrosine kinase and its ligand, ephrin-A5, are expressed by muscle precursor cells and forelimb mesoderm in unique spatiotemporal patterns during the period when muscle precursors delaminate from the dermomyotome and migrate into the limb. To test the function of EphA4/ephrin-A5 interactions in muscle precursor migration, we used targeted in ovo electroporation to express ephrin-A5 ectopically specifically in the presumptive limb mesoderm. In the presence of ectopic ephrin-A5, Pax7-positive muscle precursor cells are significantly reduced in number in the proximal limb, compared with controls, and congregate abnormally near the lateral dermomyotome. In stripe assays, isolated muscle precursor cells avoid substrate-bound ephrin-A5 and this avoidance is abolished by addition of soluble ephrin-A5. These data suggest that ephrin-A5 normally restricts migrating, EphA4-positive muscle precursor cells to their appropriate territories in the forelimb, disallowing entry into abnormal embryonic regions.

Key words: Somite, Eph family, Receptor tyrosine kinase, In ovo electroporation, Mesoderm, Chick

INTRODUCTION

Skeletal muscle cells of the vertebrate limb are derived from the somites (Stockdale et al., 2000; Christ and Ordahl, 1995). Somites bud from the unsegmented paraxial mesoderm as epithelial balls of cells. Multiple signals emanating from adjacent tissues, such as sonic hedgehog, Wnt proteins, noggin and bone morphogenetic proteins (BMPs), then converge to establish the sclerotomal and dermomyotomal compartments of the somite (Stern et al., 1995; Pourquie et al., 1996; Hirsinger et al., 1997; Fan and Tessier-Lavigne, 1994; McMahon et al., 1998). Cells in the ventromedial somite undergo an epithelial to mesenchymal transition to form sclerotome, which generates the axial skeleton and ribs. In the dorsolateral somite, dermomyotomal cells retain their epithelial character, and form skeletal muscle and dermis.

Precursor cells for skeletal muscles of the limb lie at the lateral edge of the dermomyotome (Chevallier et al., 1977; Ordahl and Le Douarin, 1992; Christ and Ordahl, 1995). These muscle precursors undergo remarkable changes in their morphology and migratory behavior before myotube differentiation. First, they undergo an epithelial to mesenchymal transition that allows their delamination from the lateral dermomyotome and then migrate laterally into the limb bud. Myogenic cells begin to aggregate into dorsal and ventral premuscle masses in the limbs and undergo extensive cell proliferation (Hayashi and Ozawa, 1991). Shortly thereafter, muscle-specific gene transcription is initiated (Noakes et al., 1986; Williams and Ordahl, 1994).

Various transcription factors control specific steps in the development of limb muscle precursors (Blagden and Hughes, 1999; Dietrich, 1999; Birchmeier and Brohmann, 2000). The transcription factor Pax3 is expressed by premigratory and migratory muscle precursor cells; mice carrying mutations in Pax3 (e.g. splotch mice) lack limb and other hypaxial muscles (Franz et al., 1993; Bober et al., 1994; Goulding et al., 1994). Pax3 is required specifically for the establishment of muscle precursor cells in the dermomyotome and for their delamination at limb levels (Daston et al., 1996; Tremblay et al., 1998). Another transcription factor, Lbx1, is expressed strictly by muscle precursor cells at the lateral dermomyotome (Jagla et al., 1995). Lbx1 expression is maintained during muscle precursor cell migration and is downregulated shortly after muscle-specific gene expression is initiated in the limb. In mice that lack *Lbx1*, muscle precursor cells form and detach from the lateral edge of the dermomyotome at limb levels but their migration to the limb is compromised (Schafer and Braun, 1999; Brohmann et al., 2000; Gross et al., 2000). Muscle

precursor cells fail to move laterally towards the limbs but migrate ventrally instead. Defects in muscle differentiation or an overwhelming loss of cell motility do not contribute to the aberrant cell migration observed in the Lbx1 mutant; rather, the guidance of migration appears impaired.

Organized cell migration is an essential mechanism by which distinct populations of precursor cells navigate to their target regions. Neural crest cells, muscle precursor cells, cells of the subventricular zone in the brain, and primordial germ cells migrate extensively along stereotypical pathways to their final destinations (Le Douarin, 1982; Birchmeier and Brohmann, 2000; Conover et al., 2000; Montell, 1999). A combination of attractive and repulsive cues, either diffusible or cell-surface bound, is thought to guide these migrating cells (Wilkinson, 2001; Krull and Koblar, 2000). Previous results implicate both cell-surface and diffusible cues in the developing limb mesoderm in the migration of muscle precursor cells (Venkatasubramanian and Solursh, 1984; Solursh et al., 1987; Schramm et al., 1994; Hayashi and Ozawa, 1995). Two well-characterized factors with roles in muscle precursor migration are the receptor tyrosine kinase Met and its ligand, hepatocyte growth factor/scatter factor (HGF/SF). Met is expressed by muscle precursor cells in the dermomyotome and is regulated by Pax3, whereas HGF/SF localizes to the limb mesoderm (Sonnenberg et al., 1993; Bladt et al., 1995; Yang et al., 1996; Daston et al., 1996; Dietrich et al., 1999). Mice that lack either *Met* or *Hgf* possess muscle precursors that are correctly specified but they fail to delaminate and remain aggregated near the somite, instead of migrating laterally to colonize the limbs (Dietrich et al., 1999). Antibodies against N-cadherin or fibronectin inhibit muscle precursor cell migration, further supporting the idea that cell-cell interactions are important (Brand-Saberi et al., 1993; Brand-Saberi et al., 1996). However, the mechanisms that contribute to the guided migration of muscle precursor cells in the limb are poorly understood.

Members of the Eph family are excellent candidates to mediate the guidance and patterning of muscle precursor cells in the limbs (Davis et al., 1994; Gale et al., 1996). Eph receptor tyrosine kinases (RTKs) and their membrane-associated ligands, the ephrins, are thought to influence axon guidance, cell migration, and the formation of cellular compartments in the developing nervous system via contact-dependent mechanisms (Krull et al., 1997; Wang et al., 1997; Smith et al., 1997; Mellitzer et al., 1999; Kullander et al., 2001; Wilkinson, 2001). Our previous expression analysis during motor axon pathfinding in the hindlimb indicated that Eph RTKs and ephrins might also contribute to muscle development (Eberhart et al., 2000; Hirano et al., 1998) (C. E. K., unpublished). Interestingly, EphA4 RTK and ephrins were expressed in multiple cell types including motoneurons, somitic cells and limb mesoderm. Thus, members of the Eph family may have a more generalized role in cell guidance and morphogenesis. Previous results in zebrafish indicate a role for Eph signaling in early stages of somite segmentation (Durbin et al., 1998). In avians, EphA4 is strongly expressed in paraxial mesoderm that buds off to form epithelial somites (Hirano et al., 1998; Schmidt et al., 2001). The distribution and functional roles of Eph family members in muscle development have been largely unexplored.

We have examined the spatiotemporal distribution of EphA4 and ephrin-A5 on muscle precursor cells in the dermomyotome and during their delamination and migration, and in forelimb mesoderm. EphA4 is predominantly localized to delaminating and migrating Pax-7-positive muscle precursors whereas ephrin-A5 is primarily distributed in the forelimb mesoderm. The expression patterns of these factors suggests that they could play multiple roles in early muscle development. As a first step to investigate the function of EphA4/ephrin-A5 interactions, we have explored their roles in the migration of muscle precursors in the avian forelimb. Taking a gain-of-function approach, we have ectopically expressed ephrin-A5 in the developing forelimb mesoderm using in ovo electroporation. Subsequent quantitative analyses of muscle precursor cell migration in the presence of ectopically expressed ephrin-A5 reveal a significant reduction in the number of migrating muscle precursors in the proximal limb, compared with control limbs. These reductions in cell numbers are not accompanied by significant alterations in limb areas, suggesting that the defects are specific to ephrin-A5 and not related to negative effects on limb morphogenesis or growth. To ascertain whether the effects on muscle precursor cells in our transfected embryos were direct, we examined the behavior of surgically isolated muscle precursor cells on substrate-bound ephrin-A5 in vitro. Migrating Pax7- and EphA4-positive muscle precursors avoided substrates containing ephrin-A5; addition of soluble ephrin-A5 blocked this avoidance. These data suggest that EphA4/ephrin-A5 interactions contribute to the organized dispersal of early migrating muscle precursor cells in the avian forelimb.

MATERIALS AND METHODS

Embryos

Fertilized White Leghorn chicken eggs (Hy-Line International) were incubated at 38°C in a humidified incubator until stages 15-23 of development (Hamburger and Hamilton, 1951). Embryos were collected in Ringer's solution and fixed 2 hours at room temperature or overnight at 4°C in 4% paraformaldehyde in preparation for vibratome sectioning, followed by immunocytochemistry or in situ hybridization (Eberhart et al., 2000).

cDNA probes and in situ hybridization

EphA4 (Sajjadi and Pasquale, 1993) and ephrin-A5 (Cheng et al., 1995) digoxigenin-labeled probes were synthesized and used for in situ hybridization, as previously described (Eberhart et al., 2000).

Immunocytochemistry/membrane staining

Avian-specific EphA4, ephrin-A5 and ephrin-A6 antibodies were applied to vibratome sections collected from forelimb levels, as previously described (Eberhart et al., 2000; Menzel et al., 2001). Vibratome sections were labeled with Pax7 antibody (1 μg/ml) (Yamamoto et al., 1998) to mark dermomyotomal cells (Pax7 antibody obtained from the Developmental Studies Hybridoma Bank, under the auspices of the NICHD, and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242). Appropriate Alexa Fluor 488 and 568 secondary antibodies (4 μg/ml; Molecular Probes) were applied to detect primary antibody binding.

To examine whether EphA4 protein localized to the membranes of Pax7-positive muscle precursors, annexin was applied as a marker for lipids in the inner leaflet of the plasma membrane (Immunotech). Stage 17 embryos were collected and fixed in 4% paraformaldehyde for 2 hours, rinsed and vibratome sectioned at 50 μm. After permeabilization in 0.1% Triton-X/phosphate-buffered saline (PBS)

for 25 minutes, sections were blocked in annexin calcium buffer/3% bovine serum albumin (BSA) for 1 hour at room temperature. Sections were incubated in annexin V-biotin solution and Pax7 and EphA4 antibodies overnight at 4°C, followed by washing in Ca^{2+}/BSA buffer twice for 5 minutes each. After post-fixing in 4% paraformaldehyde for 15 minutes and washing twice for 5 minutes each in Ca^{2+}/BSA buffer, sections were incubated for 1 hour in fluorescein-avidin D (1:200; Vector), goat anti-rabbit rhodamine Alexa fluor antibody (1:500; Molecular Probes) and goat anti-mouse Cy5 antibody (1:200; Molecular Probes). After three 5 minute washes in PBS, staining was visualized and optical sections were collected using a BioRad Radiance 2000 laser scanning confocal system.

Stripe assays/dermomyotome isolations

Lanes or 'stripes' of alternating proteins were prepared as described previously (Krull et al., 1997; Vielmetter et al., 1990). In experimental dishes, one set of lanes contained ephrin-A5-Fc (5 μg/ml) preincubated with goat anti-IgG-Fc antibody and 50 μg/ml fibronectin; alternate lanes contained fibronectin alone. Three different sets of control dishes were prepared: (1) Fc (5 μg/ml)/fibronectin versus fibronectin lanes; (2) ephrin-A2-Fc (5 μg/ml)/fibronectin versus fibronectin lanes; and (3) soluble ephrin-A5-Fc (15 μg/ml) was added to the culture medium of some substrate-bound ephrin-A5 dishes.

Chicken embryos at stage 17 of development were collected in Ringer's solution. Somite stages were determined according to Christ and Ordahl (Christ and Ordahl, 1995). Somites X-XV (of a total 29 somites) at forelimb levels (Beresford, 1983) were surgically removed and placed in a watch glass containing a pancreatin/PBS solution to loosen the overlying ectoderm, which was removed with fine forceps (Auda-Boucher and Fontaine-Perus, 1994). To halt enzyme activity, somites were then placed in a watch glass containing fetal calf serum. Dermomyotomes were teased away from sclerotomes using a sharpened insect pin and a single whole dermomyotome or a lateral-half dermomyotome was applied per dish. After 24 hours, cultures were fixed and then stained with Pax7 antibody to verify their muscle precursor identity. Other cultures were stained live with EphA4 antibody to confirm receptor expression and their identity as migratory lateral dermomyotomal cells. Cultures were photographed using fluorescence and phase optics on an Olympus IX70 microscope equipped with Openlab software and an Optronics cooled CCD camera. Images were processed and compiled into figures using Adobe Photoshope 6.0.

Confocal imaging

Optical sections at 1 μm intervals were collected from vibratome sections previously stained with antibodies or ectopically expressing ephrin-A5/enhanced green fluorescent protein (EGFP) or EGFP alone in limb mesoderm using a BioRad Radiance 2000 laser scanning confocal microscope (Molecular Cytology Core, University of Missouri-Columbia). *z*-series stacks of 4, 8 or 21 μm were compiled from labeled, sectioned material. Each image in a Z series was viewed and analyzed individually to assure that antibody labeling was assigned to the correct cell type. Images were processed and compiled into figures using Adobe Photoshop 6.0.

In ovo electroporation

Experimental embryos were transfected via in ovo electroporation with the pMES construct to drive expression of ephrin-A5 and EGFP (n=10). Control embryos were transfected with the empty pMES construct to express EGFP alone (n=6) or with vehicle alone/no DNA (n=6). The pMES construct was made by placing the IRES-EGFP sequence from the pIRES2-EGFP construct (Clontech) into the pCAX construct which contains a chicken β-actin promoter/CMV-IE enchancer (Swartz et al., 2001; Osumi and Inoue, 2001). pIRES2 was cut with *Not*I, pCAX was cut with *Nhe*I and both cuts were made blunt with Klenow. Both plasmids were then cut with *Eco*RI. The IRES-EGFP sequence was then ligated into the MCS of pCAX, replacing the pCAX MCS with the 3′ region of the pIRES2-EGFP MCS. To synthesize full-length ephrin-A5, a cDNA fragment encoding the entire open reading frame of ephrin-A5 was PCR amplified using the primers 5′-GGA ATT CAT GGC GCA CGT GGA GAT G-3′ and 5′-TAA CCC GGG GGA GCA TAC TGT GCT ATA-3′ (Hornberger et al., 1999) and its DNA sequence was confirmed. The PCR fragment was directionally cloned into the *Eco*RI and *Sma*I sites of the pMES vector. In several embryos, ectopic expression of ephrin-A5 protein was confirmed post-electroporation, using ephrin-A5 antibody labeling, as described above. All cells expressing EGFP expressed ephrin-A5.

For electroporation of lateral plate mesoderm (i.e. future forelimb mesoderm), chicken embryos were incubated to stages 13-14 and windowed (Swartz et al., 2001). A solution of 3% India ink in Ringer's solution was injected below the blastoderm to enhance contrast, and the vitelline membrane overlying the forelimb lateral plate mesoderm was carefully removed. Plasmid DNA (3 μg/μl PBS) or vehicle alone, with a few Fast Green crystals added, was microinjected into the coelom, between the somatic and splanchnic mesoderm at forelimb levels. Approximately 0.5 ml Ringer's solution was then applied on top of the embryo. The cathode was placed in the Ringer's above, but not in direct contact with the future forelimb. The anode was inserted into the India ink injection site between the blastoderm and yolk, and positioned ventrally and parallel to, but not in direct contact with, the lateral plate mesoderm. Three 9 V pulses of 50 mseconds duration each were applied. After removal of the electrodes, each embryo was then sealed with tape, and re-incubated until stage 17 of development.

Quantitative analyses of muscle precursor cell migration

A straight line was drawn from dorsal to ventral at the proximal base of the limb, immediately lateral to the dermomyotome, across optically sectioned (21 μm *z*-series stack) control and electroporated forelimbs from stage 17 embryos. Pax7-positive muscle precursor cells that were distal to the line and had delaminated from the dermomyotome were counted. Ratios of the numbers of Pax7-positive cells on the electroporated versus the non-electroporated (control) sides were calculated for each of the ephrin-A5/EGFP (n=10) and control EGFP (n=6) embryos. A ratio of 1 would indicate that Pax7-positive cell numbers were identical in electroporated and non-electroporated limbs. Total limb area (μm^2) was calculated for each of the ephrin-A5/EGFP (n=10), and control EGFP (n=6) embryos using Metamorph software and limb area ratios were calculated, by once again comparing electroporated versus non-electroporated limbs. Ratios were analyzed with the Statistical Analysis System (SAS, version 6.12) general linear models procedure (PROC GLM; SAS, 1995).

RESULTS

Muscle precursor cells and limb mesoderm exhibit dynamic patterns of expression of EphA4 and ephrin-A5

To determine if Eph family members exhibit a spatiotemporal distribution that suggests potential roles in the development of muscle precursors, we examined the localization of particular Eph receptors and ephrins at the level of the forelimb using in situ hybridization and avian-specific antibodies on vibratome sections (Sajjadi and Pasquale, 1993; Eberhart et al., 2000). Eph and ephrin antibody-labeled sections were also stained with a Pax7 antibody, a definitive marker of muscle precursor cells (Yamamoto et al., 1998; Heanue et al., 1999). Using confocal microscopy, we collected optical sections at 1 μm intervals through labeled sections and compiled 4 or 8 μm *z*-

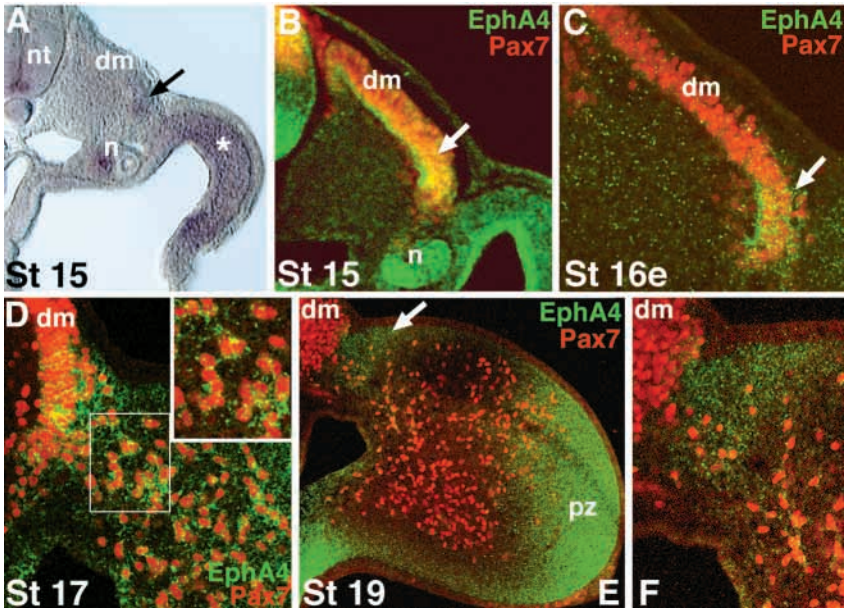

Fig. 1. EphA4 RTK is expressed during muscle precursor migration. (A) Cross-section through stage 15 embryo at forelimb levels, hybridized with probe for EphA4 mRNA. EphA4 mRNA is present in lateral dermomyotomal cells (arrow), in the lateral plate mesoderm (*) and the developing nephric system (n). dm, dermomyotome; nt, neural tube. (B-F) Confocal *z*-series stacks (8 μm) from vibratome sections stained with EphA4 and Pax7 antibodies. (B) At stage 15, EphA4 appears diffusely distributed in the dermomyotome, localizing primarily to its lateral (arrow) and ventral edges, and at low levels in the lateral plate mesoderm. (C) At early stage 16, EphA4 is restricted to the lateral edge of the dermomyotome (arrow). (D) At stage 17, EphA4 is prominent in the lateral dermomyotome and encircles migrating muscle precursors in the proximal limb. Boxed area is high magnification view in inset. (E) EphA4 protein appears absent from muscle precursor cells at stage 19 but is strongly expressed in the progress zone (pz) and dorsoproximal limb mesenchyme (arrow). (F) High-magnification view of E, the lateral edge of the dermomyotome and adjacent dorsoproximal mesenchyme at stage 19.

series stacks. We focused our inquiry on the EphA4 RTK and one of its ligands, ephrin-A5 because our previous analyses suggested that these molecules were present at limb levels during the process of muscle precursor cell migration (Eberhart et al., 2000). We screened for other relevant ligands, including ephrin-A2 and the newly-identified ephrin-A6, but found that their expression coincided with motor axon patterning in the limb or was absent, respectively (data not shown) (Menzel et al., 2001).

At stage 15, before the emigration of muscle precursor cells from the dermomyotome, EphA4 mRNA and protein appear diffusely associated with the dermomyotome but most intense concentrated at its lateral edge (Fig. 1A,B). In addition, EphA4 protein localizes to the ventral surface of the dermomyotome (Fig. 1B). At early stage 16, EphA4 labeling is more strictly associated with Pax-7-positive muscle precursor cells that are located at the lateral dermomyotome, but appears downregulated in the medial dermomyotome compared with stage 15 (Fig. 1C). At stage 17, EphA4 protein is present on cells in the lateral dermomyotome, and marks delaminating and migrating Pax7-positive muscle precursor cells in the proximal limb in a punctate manner (Fig. 1D). EphA4 protein could define muscle precursors migrating from the somitic mesoderm or alternatively, EphA4 expression may mark limb mesodermal cells. To distinguish between these possibilities, we used annexin, a marker for lipids localized on the inner leaflet of the cell membrane, combined with EphA4 and Pax7 antibody labeling. We found that EphA4 protein localizes to the surfaces of Pax7-positive muscle precursors at stage 17, indicating that EphA4 protein is indeed expressed by muscle precursors (Fig. 2).

We examined single optical sections from stage 19 embryos at forelimb levels to determine the distribution of EphA4 protein on Pax7-positive muscle precursors. EphA4 protein is negligible or expressed at very low levels on the most lateral cells in the dermomyotome and on migratory cells in the proximal limb, respectively (Fig. 1E,F). Muscle precursor cells that have migrated into more distal EphA4-rich aspects of the limb appear to lack EphA4 protein. In a striking manner, EphA4 protein is also associated with the developing nephric system (Fig. 1A,B).

EphA4 protein is also present in the developing forelimb mesoderm during the process of muscle precursor migration. At stages 15 and 16, EphA4 mRNA and protein are diffusely distributed at low levels in the lateral plate mesoderm that will form the forelimb (Fig. 1A-C). At stage 17, EphA4 protein is

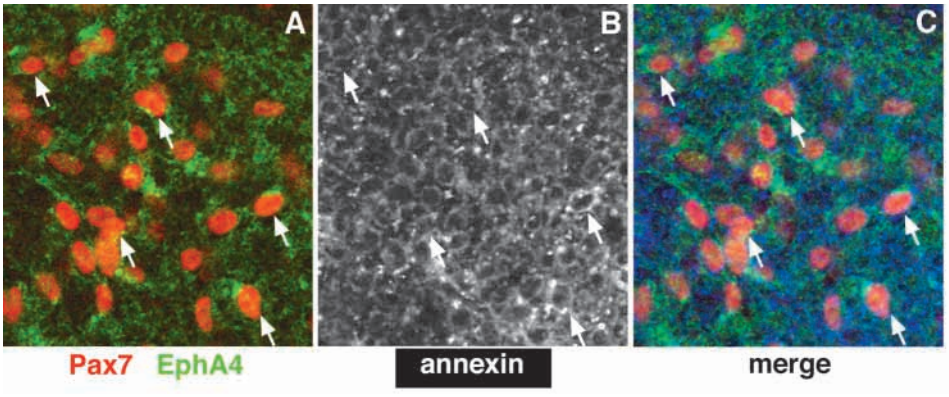

Fig. 2. EphA4 protein marks the surfaces of muscle precursors. High magnification views of proximal limb mesoderm from early stage 17 embryo, stained with annexin, EphA4 (green) and Pax7 (red) antibodies. (A) Many muscle precursors possess EphA4 protein on their surfaces (arrows in A-C). (B) Annexin labeling reveals cell surfaces of all cells in forelimb. (C) Merged image of A,B, with annexin labeling in blue.

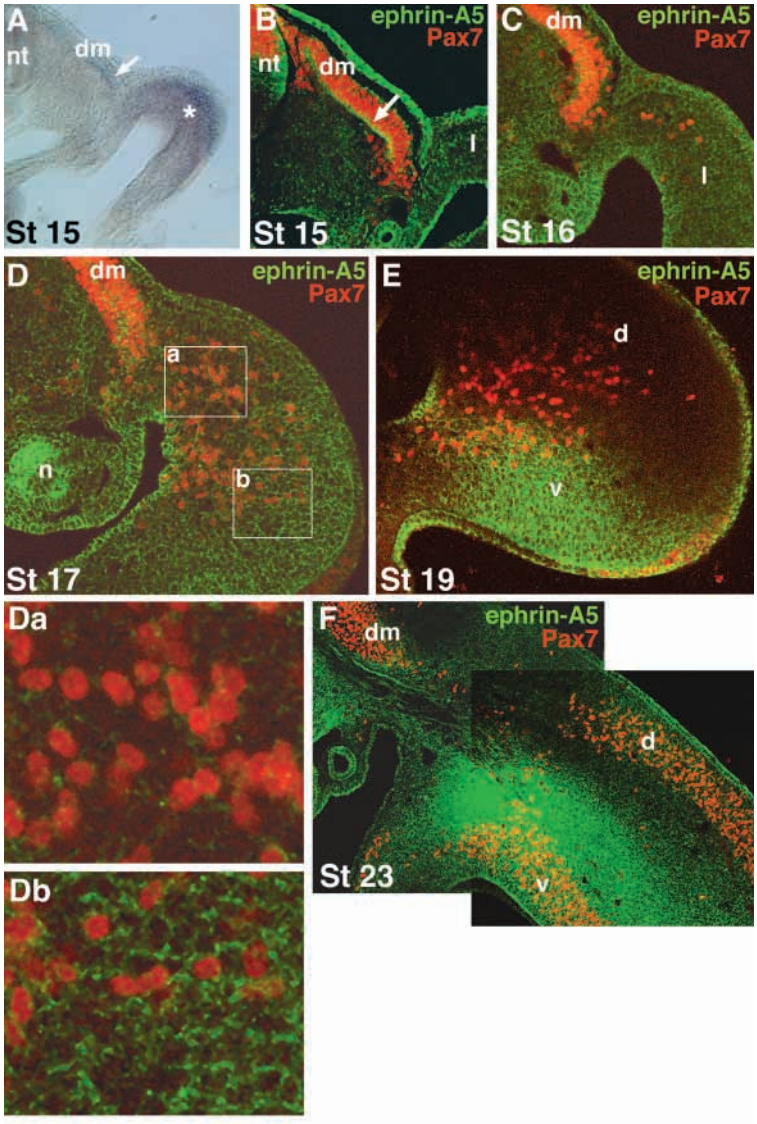

Fig. 3. Ephrin-A5 is primarily localized to forelimb mesoderm. (A) Cross-section through stage 15 embryo at forelimb levels, hybridized with probe for ephrin-A5 mRNA. Ephrin-A5 mRNA is present at apparently low levels in the dermomyotome (arrow) and in the limb mesoderm (*). nt, neural tube. (B-F) Confocal *z*-series stacks (8 μm) from vibratome sections stained with ephrin-A5 and Pax7 antibodies. (B) At stage 15, ephrin-A5 protein is found in the ventral dermomyotome (dm, arrow), in the dorsal neural tube (nt) and at very low levels in the limb mesoderm (l). (C) At stage 16, ephrin-A5 is found at low levels in the lateral dermomyotome (dm) and in the limb (l). (D) At stage 17, ephrin-A5 localizes to the surfaces of many limb mesoderm cells (b, boxed area) but is expressed at very low levels in proximal territory containing Pax7 muscle precursor cells (a, boxed area). Boxed areas are shown at higher magnification in Da and Db. Prominent ephrin-A5 expression is present in the developing nephric system (n). (E,F) Ephrin-A5 is strictly associated with the ventral portion of the limb at stage 19 and 23. v, ventral; d, dorsal.

expressed in the presumptive progress zone that underlies the AER (data not shown). At stage 19, EphA4 is expressed in a dorsoproximal part of the limb mesoderm, a region fated to become shoulder girdle (Saunders, 1948), in addition to continued high levels of EphA4 protein in the progress zone (Fig. 1E,F) (Patel et al., 1996).

The EphA4 ligand, ephrin-A5, also exhibits a dynamic, spatially and temporally restricted pattern of expression during muscle precursor cell migration. Ephrin-A5 is distributed on the ventral surface of the dermomyotome at stage 15, coincident with EphA4 protein (Fig. 3B). At stage 16, ephrin-A5 is expressed at lower levels in the dermomyotome, at the time myotome formation is initiated and muscle precursors initiate their delamination (Fig. 3C). At stage 17, ephrin-A5 protein remains weakly expressed in the dermomyotome but a close inspection of single optical sections reveals that ephrin-A5 is not present on delaminating or migrating muscle precursor cells at this stage or later (Fig. 3D-F). However, ephrin-A5 protein is found in the developing nephric system (Fig. 3D), similar to the distribution of EphA4.

At stages 15-16, ephrin-A5 is expressed at low levels and in a broad manner across the forelimb mesoderm (Fig. 3A-C). Strikingly, ephrin-A5 is distributed in an uneven manner in the forelimb at stage 17 (Fig. 3D). Ephrin-A5 protein clearly marks the surfaces of many limb mesodermal cells with strong expression localized to territory that borders Pax7-positive cells in the limb (Fig. 3Db). However, ephrin-A5 protein is reduced in more proximal limb territory that contains Pax7-labeled muscle precursor cells (compare Fig. 3Da with 3Db). At stages 19 and 23, ephrin-A5 sharply defines the ventral portion of the forelimb mesoderm (Fig. 3E,F) and is absent in the dorsal region.

The discrete expression of EphA4 and ephrin-A5 on muscle precursor cells and in the limb mesoderm suggests that these factors are involved in several aspects of muscle precursor

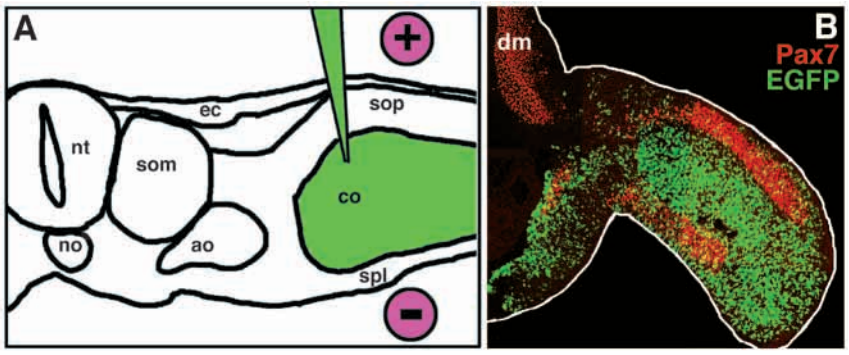

Fig. 4. In ovo electroporation targets gene expression to the forelimb mesoderm. (A) The microinjection of plasmid DNA into the coelom (co) and electrode placement, dorsal (+) and ventral (–) to the embryo. (B) Confocal *z*-series stack (16 μm) from a vibratome section through a stage 23 embryo that was electroporated at forelimb levels with plasmid DNA encoding EGFP. Red, Pax7-positive muscle precursor cells; green, EGFP expression in limb mesodermal cells. ao, aorta; dm, dermomyotome; ec, ectoderm; no, notochord; nt, neural tube; som, somite; sop, somatopleure; spl, splanchnic mesoderm.

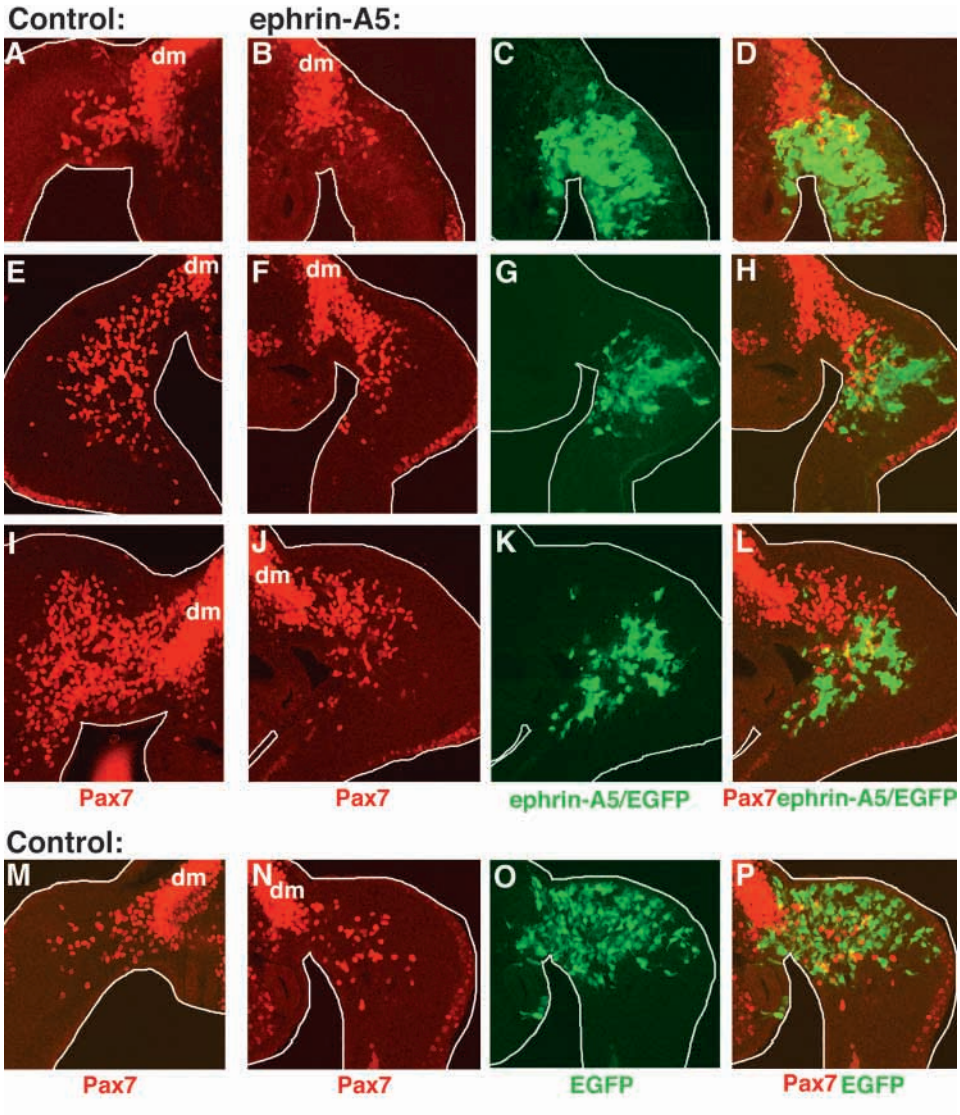

Fig. 5. Ectopic expression of ephrin-A5 results in a reduction of muscle precursor cells in the proximal forelimb. All images are confocal *z*-series (21 μm) from vibratome sections at forelimb levels. (A-D,E-H,I-L) Forelimbs from three different stage 17 embryos in which ephrin-A5/EGFP was targeted to the lateral plate mesoderm via in ovo electroporation at stage 13. For the top three rows, the contralateral, unelectroporated (control) limb is on the left (A,E,I). (B,F,J) Distribution of Pax7 muscle precursor cells in limbs ectopically expressing ephrin-A5. (C,G,K) Same field, EGFP images. (D,H,L) Merged Pax7 and EGFP images. Note that Pax7-positive cells cluster near the lateral dermomyotome in B,F, compared with controls (A,E). (M-P) Forelimbs from control embryos electroporated with pMES-EGFP. (M) Distribution of Pax7 cells in the contralateral, unelectroporated limb. (N-P) The distribution of Pax7 cells in the presence of EGFP alone is similar to the contralateral limb (M).

cell development. Because of the expression of EphA4 localized to migrating muscle precursor cells and the uneven distribution of ephrin-A5 in the limb mesoderm at stage 17, we focused our functional analysis on the role of EphA4-ephrin interactions in the migration and dispersal of muscle precursor cells in the forelimb.

Ectopic expression of ephrin-A5 in limb mesoderm significantly reduces the number of muscle precursor cells in the proximal limb

To evaluate the function of EphA4-ephrin-A5 interactions in muscle precursor cell migration, we ectopically expressed full-length ephrin-A5 and EGFP in a targeted manner in the lateral plate mesoderm (presumptive forelimb mesoderm) of stage 13-14 embryos using in ovo electroporation (Swartz et al., 2001). Plasmid DNA encoding full-length ephrin-A5 driven by a chick β-actin promoter/CMV-IE enhancer (Swartz et al., 2001; Osumi and Inoue, 2001) and containing an IRES-EGFP was microinjected into the coelom at the level of somites adjacent to the developing forelimb (Fig. 4). Electrodes were placed to avoid direct tissue contact (see Materials and Methods), and three 9 V pulses of 50 mseconds duration each were applied, driving DNA into lateral plate mesoderm cells. Embryos were sealed and reincubated until approximately stage 17 of development, when they were sectioned and stained with Pax7 antibody to mark muscle precursor cells. Within single embryos, the forelimb where ephrin-A5 was ectopically expressed served as the experimental side; the contralateral limb served as a control (n=10).

Ectopic ephrin-A5 expression in the forelimb mesoderm resulted in a dramatic reduction of Pax7-positive muscle precursor cells in the proximal portion of that limb, compared with the contralateral limb of the same embryo (Fig. 5A,B,E,F,I,J). Delamination of muscle precursor cells from the lateral dermomyotome appeared to proceed normally in all forelimbs ectopically expressing ephrin-A5. However, in the majority of embryos (7/10), we observed an abnormal accumulation of muscle precursor cells near the lateral dermomyotome with a simultaneous reduction of muscle precursor cells in the proximal limb (Fig. 5B,F,J). Some variability was present in the numbers of cells ectopically expressing ephrin-A5 and their location at 24 hours post-electroporation in the forelimb mesoderm (Fig. 5C,G,K). Moreover, the timing of our electroporations resulted in consistent transfection of mesodermal cells in more proximal regions of the forelimb; ectopic expression of ephrin-A5 was never observed in muscle precursor cells. We examined the position of muscle precursor cells at later stages of development and on a gross morphological level, muscle masses in the forelimb appeared normal (data not shown).

To verify that our effects on muscle precursor cells in the forelimbs of embryos ectopically expressing ephrin-A5 were

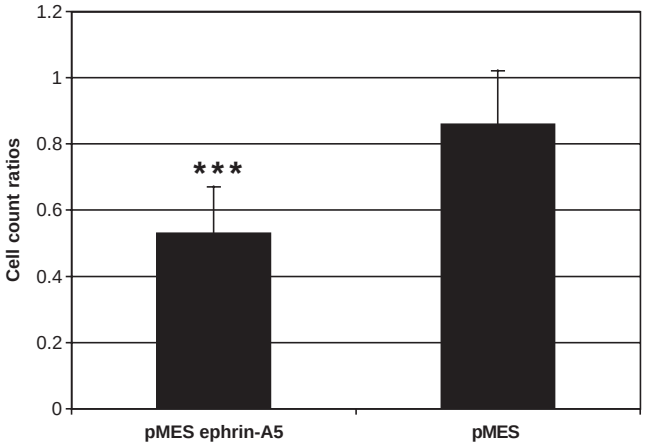

Fig. 6. Quantitative analysis of the effects of ectopic ephrin-A5 on muscle precursor cell numbers. Pax7-positive cells were counted, as described in the Materials and Methods. Ratios of cell numbers in ectopic ephrin-A5 limbs versus the contralateral limbs (pMES ephrin-A5) were compared with limbs expressing EGFP alone versus the contralateral limbs (pMES). Ratios were analyzed with the Statistical Analysis System general linear models procedure. The mean ratio for pMES ephrin-A5 is 0.53, whereas the mean ratio for pMES is 0.86. Ectopic expression of ephrin-A5 using pMES results in significant reductions in the mean numbers of muscle precursor cells in the forelimb (P=0.0008).

specific, several embryos were electroporated with the empty pMES DNA construct, which encodes EGFP (n=6) or with vehicle alone (n=6). The numbers of Pax7-positive muscle precursor cells in EGFP-expressing and non-expressing limbs in these control embryos were comparable (see Fig. 5M-P), suggesting that the reductions in the numbers of Pax7-positive muscle precursor cells in experimental embryos were due to the ectopic expression of ephrin-A5.

Although our visual observations suggested reductions in muscle precursor cell numbers in the limb in the presence of ectopic ephrin-A5, we considered the possibility that nonspecific effects of the electroporation procedure on limb morphogenesis or growth might account for these defects. Therefore, we counted the numbers of Pax7-positive muscle precursor cells in optical sections through forelimbs from ephrin-A5/EGFP (n=10) and control EGFP (n=6) embryos. The ratios of Pax7-positive cells on the electroporated versus unelectroporated (contralateral) sides were then calculated for each of the ephrin-A5/EGFP and control EGFP embryos. Total limb areas (μm^2) of ectopic ephrin-A5 limbs, contralateral limbs and control-EGFP limbs were calculated using Metamorph software and limb area ratios were calculated. Ratios were analyzed with the Statistical Analysis System general linear models procedure (PROC GLM; SAS, version 6.12, 1995). Statistical analyses were carried out initially on the ratios of Pax7-positive cells alone using limb area ratios as a covariant. Ectopic expression of ephrin-A5 results in significant reductions in the mean numbers of muscle precursor cells in the forelimb (P=0.0008) compared with control embryos (Fig. 6). When using limb area ratios as a covariate, differences in the ratios of Pax7-positive cells in experimental and control embryos remain significant (P=0.0035). These data indicate that the presence of fewer muscle precursor cells in forelimbs ectopically expressing ephrin-A5 cannot be attributed to alterations in forelimb size generated by the electroporation procedure. Rather, these results suggest that

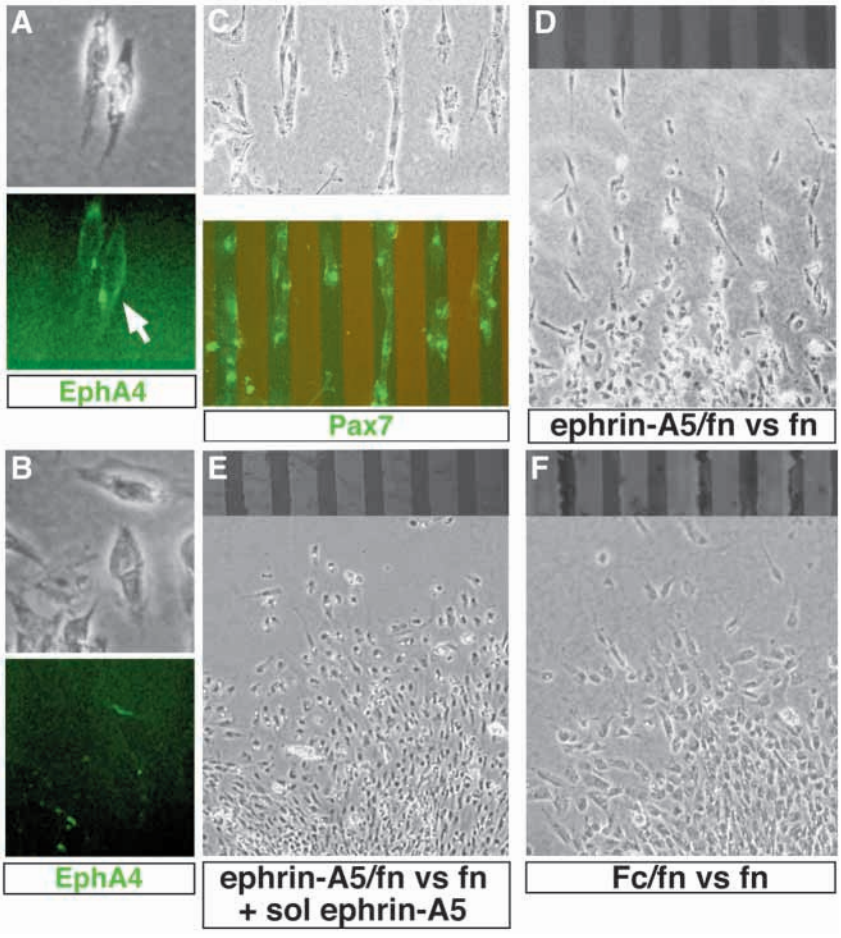

Fig. 7. The effects of ephrin-A5 on muscle precursor cells are direct and specific. Surgically isolated muscle precursor cells from somites at forelimb levels were applied to tissue culture dishes upon which alternating lanes of proteins had been applied. Twenty-four hours later, muscle precursor cells and the striped substrates were visualized and photographed. (A) EphA4 protein (arrow) is distributed in a fine manner on the surfaces of migrating dermomyotomal cells; phase contrast (top) and fluorescence (bottom) microscopy. (B) EphA4 protein is absent on a presumed non-migratory subset of muscle precursors; phase contrast (top) and fluorescence (bottom) microscopy. (C) Dermomyotomal cells on ephrin-A5/fibronectin (fn) versus fn lanes express Pax7, indicating they are muscle precursors. (D) Muscle precursors avoid substrate-bound ephrin-A5/fn (light lanes) and migrate instead on fn alone (dark lanes). (E) Addition of soluble ephrin-A5 blocks the repulsive effects of substrate-bound ephrin-A5 on muscle precursor cells. (F) Muscle precursors grow uniformly on substrates coated with Fc/fn versus fn.

ephrin-A5 directly affects the distribution of muscle precursor cells.

Ephrin-A5 directly affects muscle precursor cell behavior in vitro

To further determine if the effects of ephrin-A5 on muscle precursor cells in vivo were direct, we examined the responses of muscle precursor cells to ephrin-A5 substrates in vitro (Vielmetter et al., 1990; Krull et al., 1997). Whole dermomyotomes or lateral-half dermomyotomes were surgically isolated from stage 17 somites that contribute muscle precursor cells to the forelimb (Auda-Boucher and Fontaine-Perus, 1994) and placed on individual culture dishes containing lanes of substrate-bound proteins. After 24 hours, some cultures containing whole dermomyotomes were stained live with EphA4 antibody to confirm receptor distribution and lateral dermomyotomal identity (Fig. 7A,B); other cultures were fixed and post-stained with Pax7 antibody to verify that muscle precursor cells were present in the dishes (Fig. 7C). Migrating muscle precursors exhibited a fine, even distribution of EphA4 protein on their surfaces (Fig. 7A) whereas other muscle precursors, presumably non-migratory (Ordahl and Le Douarin, 1992), possessed little if any EphA4 protein (Fig. 7B).

Pax7- and EphA4-positive muscle precursor cells avoided lanes containing ephrin-A5 protein and preferred to extend on lanes containing fibronectin alone (Fig. 7D; n=11 dishes). Addition of soluble ephrin-A5-Fc to the culture medium blocked the avoidance of ephrin-A5 by muscle precursors (Fig. 7E; n=6), demonstrating the specificity of the ephrin-A5 effects. In other control dishes, muscle precursor cells extended uniformly on lanes containing Fc protein versus fibronectin (Fig. 7F; n=5 dishes). Interestingly, when ephrin-A2-Fc protein was applied to the lanes instead of ephrin-A5-Fc, muscle precursor cells again demonstrated no preference (n=14 dishes; data not shown), suggesting that ephrin-A2 and ephrin-A5 have distinct effects on muscle precursors. In combination, these data show that the effects of ephrin-A5 on muscle precursor cells are specific and direct. Furthermore, these data suggest that ephrin-A5 acts as a repulsive factor in vivo for muscle precursor cells during their migration in the forelimb.

DISCUSSION

Muscles of the limb derive from migratory muscle precursor cells that emanate from the lateral edge of the dermomyotome at limb levels. Although several of the molecular mechanisms that control the generation and delamination of these cells from the dermomyotome have been characterized, little is known about the factors that guide the migration of these cells to their target regions in the limb (Birchmeier and Brohmann, 2000). Results of previous experiments indicate that cues in the developing limb mesoderm influence muscle precursor migration (Venkatasubramanian and Solursh, 1984; Solursh et al., 1987; Schram et al., 1994; Hayashi and Ozawa, 1995). However, the identity of these limb-localized factors is not well understood.

The aim of the studies reported here was to assess the distribution of EphA4 and its ligand, ephrin-A5, during early development of limb muscle and to examine the potential function of these cell-surface proteins in muscle precursor migration in the forelimb. Our main results are as follows. First, Pax7-positive muscle precursor cells express the EphA4 RTK on their surfaces before emigration from the lateral dermomyotome, and during their delamination and migration in the limb mesoderm. Later-migrating muscle precursor cells appear to lack EphA4 protein on their surfaces. Second, ephrin-A5, a ligand for EphA4, is associated at early stages with premigratory muscle precursors. However, ephrin-A5 is expressed predominantly in the limb mesoderm when the process of muscle precursor migration is well-under way. Interestingly, ephrin-A5 is diminished in proximal territories occupied by migrating muscle precursor cells at this time. At later stages, ephrin-A5 is restricted to the ventral domain of the limb mesoderm. Third, selective ectopic expression of ephrin-A5 in the proximal limb mesoderm markedly and specifically reduces the numbers of muscle precursor cells in this region, when compared with controls. Fourth, isolated Pax7/EphA4-positive muscle precursor cells specifically avoid substrate-bound ephrin-A5 in vitro. Taken together, these results support the hypothesis that EphA4-ephrin-A5 interactions regulate the migration of muscle precursor cells in the limb.

Expression of EphA4 and ephrin-A5 suggests multiple roles during development of limb muscle precursors

EphA4 and ephrin-A5 exhibit complicated spatiotemporal patterns of expression during muscle precursor development. The most striking expression of EphA4 in developing muscle is apparent at stage 17, when EphA4 marks delaminating Pax-7-positive cells in the lateral dermomyotome and migrating muscle precursors in the proximal limb. Our analysis using annexin indicates that this prominent EphA4 expression localizes primarily to the surfaces of muscle precursor cells. However, we cannot rule out that EphA4 is also weakly expressed in the limb mesoderm at stage 17. Furthermore, we cannot exclude the possibility that Pax7 and EphA4 antibodies label a population of angioblastic precursors derived from the somitic dermomyotome (Noden, 1989; Pardanaud et al., 1989; Pardanaud and Dieterlen-Lievre, 1995; Wilting et al., 1995; Pardanaud et al., 1996; Cox and Poole, 2000). Elegant studies using chick-quail chimeras and QH-1 antibody have shown that the somitic mesoderm generates endothelial cells in the limb mesoderm. However, the precise contribution of the somitic dermomyotome to endothelial lineages is not well understood and requires further lineage analysis.

At stage 17, ephrin-A5 possesses an uneven distribution in the developing limb: it is chiefly associated with limb mesenchyme that borders the collection of Pax7-positive muscle precursors in the limb. By contrast, ephrin-A5 expression is weak in limb mesoderm occupied by Pax7-positive muscle precursors in more proximal limb regions. These remarkable expression patterns suggested to us that EphA4-positive muscle precursor interactions with ephrin-A5-positive limb mesoderm facilitate the organized dispersal and migration of muscle precursors in the developing forelimb.

The patterns of expression of EphA4 and ephrin-A5 suggest they could indeed function in multiple steps of muscle precursor cell development, including the formation of muscle precursors in the dermomyotome, and their delamination (Birchmeier and Brohmann, 2000). EphA4 and ephrin-A5 are

found at early stages in the dermomyotome, before muscle precursor migration has commenced. EphA4 is expressed diffusely at first and then becomes more restricted to the lateral dermomyotome. Ephs and ephrins have been implicated in the formation of distinct cellular compartments (Wilkinson, 2001). Thus, the expression of these molecules suggests potential roles in cell-cell interactions that organize the lateral dermomyotomal epithelium. We found that EphA4 is strongly expressed by delaminating muscle precursor cells. Increases in EphA4 protein may alter cellular affinities, thereby allowing detachment from neighboring cells.

EphA4 protein localizes to two distinct regions in the limb bud mesoderm from stage 19 onwards: the progress zone or distal mesenchyme underlying the AER and a dorsoproximal region (Patel et al., 1996). A similar pattern of expression has been noted for EphA4 in mouse (Helmbacher et al., 2000). Removal of the AER results in a downregulation of EphA4 in the distal mesenchyme, suggesting that EphA4 expression is influenced by AER signals, including FGF (Patel et al., 1996). The potential function of EphA4 in the dorsoproximal region of the limb mesoderm is unknown. However, fate mapping studies have shown that cells in this region give rise to the shoulder girdle (Saunders, 1948; Bowen et al., 1989; Vargesson et al., 1997). In chick and mouse limbs, cells in the EphA4 expression domain also express Pax1. Furthermore, mice that are mutant or null for Pax1 show shoulder girdle malformations (Timmons et al., 1994; Dietrich and Gruss, 1995; Wilm et al., 1998). Therefore, EphA4 may contribute to the construction of shoulder girdle skeletal elements, perhaps in collaboration with Pax1.

In a striking manner, ephrin-A5 is restricted to a ventral domain of the limb at stages 19-23. Muscle precursors migrating to this area appear to lack ephrin-A5 protein on their surfaces; however, we cannot exclude the possibility that these cells may upregulate ephrin-A5, upon their arrival in the ventral limb. This discrete expression of ephrin-A5 prompts us to speculate that it may have functions later in the formation of ventral muscle masses or in myotube differentiation. Perhaps ephrin-A5 segregates ventral muscle from central chondrogenic regions in the limb (Schramm and Solursh, 1990). Recent results indicate ephrin-A5 marks rostral, but not caudal, muscles, and is required for the topographic innervation of muscle by motor axons (Donoghue et al., 1996; Feng et al., 2000).

Ectopic ephrin-A5 inhibits the migration of EphA4-positive muscle precursor cells

Although our expression analysis suggests multiple potential functions for these factors, we focused our functional investigation on the role of EphA4 and ephrin-A5 in the dispersal and migration of muscle precursors in the forelimb. Using in ovo electroporation, we ectopically expressed ephrin-A5 in the presumptive forelimb mesoderm, independent of the somitic mesoderm and before the emigration of muscle precursors from the lateral dermomyotome. Ectopic ephrin-A5 inhibited the migration of muscle precursor cells into the forelimb mesoderm in vivo, with a significant reduction of muscle precursor cell numbers in ectopic ephrin-A5 limbs compared with controls. In the majority of embryos, Pax7-positive muscle precursors were found abnormally congregated near the lateral dermomyotome. Our visual inspection of ectopic ephrin-A5 limbs suggests that the delamination of muscle precursors from the lateral dermomyotome proceeds normally. However, muscle precursors are prevented from entering the proximal limb by the ectopic presence of ephrin-A5.

Ectopic expression of ephrin-A5 via in ovo electroporation could be altering some aspect of limb morphogenesis that indirectly affects the migration of muscle precursor cells. However, our statistical analysis indicates that limb area measurements do not vary significantly among limbs that ectopically express ephrin-A5 and control limbs. Furthermore, limbs from embryos electroporated with vehicle alone exhibit no significant differences in the numbers of Pax7-positive muscle precursors, compared with their contralateral limbs. The gross morphology of limbs in which ephrin-A5 was ectopically expressed appeared indistinguishable from control limbs at later stages (data not shown). Our results, taken together, suggest that ephrin-A5 has direct effects on muscle precursor cells that are independent of limb morphology or area, the electroporation procedures or the expression of EGFP. Moreover, results of our stripe assays lend additional support to the idea that ephrin-A5 directly affects this cell population. The avoidance by EphA4-positive muscle precursor cells of ephrin-A5 is specific, as addition of soluble ephrin-A5 abrogates the avoidance response. There do appear to be alterations in the morphology of limb mesodermal cells that ectopically express ephrin-A5/EGFP, compared with cells in control limbs that express EGFP alone. Ephrin-A5-expressing cells appear highly aggregated, suggesting alterations in their adhesive properties (Davy and Robbins, 2000). Whether these changes in cell behavior involve integrins or other cell-surface proteins including cadherins is unknown.

Reductions in the numbers of muscle precursor cells in ectopic ephrin-A5 limbs could indicate that ephrin-A5 affects cell proliferation or cell death. Although we have not ruled out this possibility completely, our analyses thus far suggest that ephrin-A5 does not exert these effects at early stages. First, we have analyzed the effects of ephrin-A5 on muscle precursor cells prior to their normal period of cell proliferation that occurs at stage 23. Second, staining with a marker for programmed cell death, shows no increased numbers of dying cells in ectopic ephrin-A5 limbs compared with controls (data not shown). We do presume that cell proliferation later compensates for our initial reductions in cell numbers, as older ectopic ephrin-A5 limbs appear to have normal muscle masses.

Molecular control of muscle precursor migration in the forelimb

The results of our expression and functional analyses directly implicate EphA4 and ephrin-A5 in the organized migration of muscle precursors in the developing forelimb. We propose that ephrin-A5 controls the entry and dispersal of EphA4-positive muscle precursor cells into certain limb territories. In the proximal limb, where ephrin-A5 is low at stage 17, EphA4-positive muscle precursors enter unimpeded. In presumptive distal regions of the limb, ephrin-A5 is more prevalent and prevents muscle precursors from advancing. Ephrin-A5 may restrict the entry of muscle precursors to allow limb maturation to proceed or prevent the migration of muscle precursors beyond limb borders. Our in vivo and in vitro functional

analyses suggest that ephrin-A5 guides muscle precursors by acting as a repulsive factor.

The results of our stripe assays reveal unique responses of muscle precursors to ephrin-A2 and ephrin-A5. Although both ligands activate the EphA4 receptor when presented in clustered forms (Davis et al., 1994), ephrin-A5 elicits an avoidance response by muscle precursors, whereas cells migrate uniformly on ephrin-A2. Previous studies have shown that EphA4 binds poorly to ephrin-A2 and possesses a higher affinity for ephrin-A5 in vitro (Gale et al., 1996). Alternately, these distinct responses suggest that the downstream signaling effectors in EphA4-expressing muscle precursors activated by ephrin-A5 and ephrin-A2 are unique. The signal transduction cascades triggered by activation of Eph receptors are not well understood (Kullander et al., 2001). These differential cell responses will require additional analyses that examine the signaling components that are altered upon exposure to ephrins and that follow muscle precursor cell movements over time (Krull et al., 1997).

It is interesting to speculate about possible interactions of EphA4 and ephrin-A5 with other known factors involved in early muscle development, including Pax3, Lbx1, HGF/SF and Met (Birchmeier and Brohmann, 2000). Based on its expression, EphA4 may interact with Pax3 or Met to define migratory muscle precursors at the lateral edge of the dermomyotome and regulate their delamination. *Lbx1* mutants exhibit impaired guidance of muscle precursors in the limb; EphA4 may cooperate with Lbx1 to achieve the proper dispersal of migratory muscle precursors in the proximal limb. HGF/SF is expressed in the limb mesoderm at later stages of development (stages 18/19), compared with ephrin-A5 (stages 15/16) (Scaal et al., 1999). At stage 17, ephrin-A5 appears to control the entry and dispersal of muscle precursor cells in the proximal limb when HGF/SF is apparently absent. At stage 19, ephrin-A5 strongly demarcates the ventral portion of the limb mesoderm, whereas HGF/SF appears more uniformly expressed throughout the limb mesoderm and only later becomes localized to anterior regions. Clearly, it will be important to analyze EphA4 and ephrin-A5 expression in mutant mice that lack these factors to determine where EphA4 and ephrin-A5 act in muscle precursor development.

Analyses of EphA4 and ephrin knockout mice should provide additional insights into the functions of these factors during muscle precursor cell development. Mice that lack EphA4 and ephrin-A5/ephrin-A2 have been generated (Helmbacher et al., 2000; Dottori et al., 1998; Feldheim et al., 2000); however, analyses thus far have focused on the developing nervous system. Both *Epha4* knockout mouse lines that have been generated exhibit locomotor defects, with bilaterally symmetrical hopping gaits, suggesting neural and/or muscle defects. Although gross anatomical analyses indicated muscles were present in the correct place in these mice, other as yet unknown defects in muscle development and function may be present that contribute directly to locomotor deficits. EphA4 is expressed in multiple tissues in mice, similar to its distribution in avians, suggesting that deficits in neural innervation in the absence of EphA4 may not be cell autonomous (Helmbacher et al., 2000). Experiments are in progress to examine potential defects in muscle development/function in mice that lack the genes for EphA4 and ephrin-A2/A5.

These results and previous studies on the functions of Ephs and ephrins in limb innervation and vascular morphogenesis (Araujo et al., 1998; Adams et al., 1999; Helmbacher et al., 2000; Feng et al., 2000) (J. E. and C. E. K., unpublished) suggest that Eph family members play a more generalized role in configuring multiple cell types in the limb. EphA4 in particular is required for the innervation of a subset of dorsal muscles in the murine limb (Helmbacher et al., 2000). Furthermore, the topographic mapping of motor axons onto muscle is impaired in mutant mice lacking ephrin-A5 and ephrin-A2 (Feng et al., 2000). Ephrin-Eph signaling between endothelial cells and adjacent mesenchymal cells is required for proper vascularization (Adams et al., 1999). The present findings suggest that EphA4 and ephrin-A5 interactions are another element in the molecular control of muscle precursor migration. Together, these data implicate Eph family members in building the proper architecture of vessels, muscle, and neural innervation in the limb.

We have taken advantage of a newly developed technique, in ovo electroporation, to dissect the roles of EphA-4 and ephrin-A5 in specific steps of muscle precursor cell development (Swartz et al., 2001; Itasaki et al., 1999). The location of the plasmid DNA injection and the orientation of the electrodes allow substantial spatial control of the region electroporated. Thus, we were able to perturb the expression of ephrin-A5 in the limb mesoderm, independent of neural tissue or somitic mesoderm, at restricted stages of development, and assess the subsequent effects on muscle precursor cells. Our targeted approach provides an enormously powerful tool with which to examine the functions of distinct signaling molecules in specific cell types during embryogenesis.

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