Reciprocal signaling between *Drosophila* epidermal muscle attachment cells and their corresponding muscles

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**SUMMARY**

Directed intercellular interactions between distinct cell types underlie the basis for organogenesis during embryonic development. This paper focuses on the establishment of the final somatic muscle pattern in *Drosophila*, and on the possible cross-talk between the myotubes and the epidermal muscle attachment cells, occurring while both cell types undergo distinct developmental programs.

Our findings suggest that the stripe gene is necessary and sufficient to initiate the developmental program of epidermal muscle attachment cells. In stripe mutant embryos, these cells do not differentiate correctly. Ectopic expression of Stripe in various epidermal cells transforms these cells into muscle-attachment cells expressing an array of epidermal muscle attachment cell-specific markers. Moreover, these ectopic epidermal muscle attachment cells are capable of attracting somatic myotubes from a limited distance, providing that the myotube has not yet been attached to or been influenced by a closer wild-type attachment cell.

Analysis of the relationships between muscle binding and differentiation of the epidermal muscle attachment cell was performed in mutant embryos in which loss of muscles, or ectopic muscles were induced. This analysis indicated that, although the initial expression of epidermal muscle-attachment cell-specific genes including stripe and groovin is muscle independent, their continuous expression is maintained only in epidermal muscle attachment cells that are connected to muscles. These results suggest that the binding of a somatic muscle to an epidermal muscle attachment cell triggers a signal affecting gene expression in the attachment cell.

Taken together, our results suggest the presence of a reciprocal signaling mechanism between the approaching muscles and the epidermal muscle attachment cells. First the epidermal muscle attachment cells signal the myotubes and induce myotube attraction and adhesion to their target cells. Following this binding, the muscle cells send a reciprocal signal to the epidermal muscle attachment cells inducing their terminal differentiation into tendon-like cells.

Key words: muscle, tendon, embryo, *Drosophila*, embryogenesis, stripe

**INTRODUCTION**

Instructive interactions between cells of different origins occur when a defined set of cells influence the differentiation of other nearby cell populations in order to form tissues and organs (Holtzer, 1968). Such interactions may occur in reciprocal directions, e.g. limb development is associated with an array of reciprocal interactions performed between the progress zone and the overlying ectoderm (Tabin, 1995; Martin and Tickle, 1996); synaptogenesis between a given motoneuron and its corresponding muscle involves the guidance of the neuronal growth cone towards its target and reciprocal signaling between the nerve ending and the muscle postsynaptic site (Kleiman and Reichardt, 1996).

Molecular information regarding axonal pathfinding has revealed that the guidance of an individual axon to innervate its target cell is the net result of attractive and repulsive, both secreted and membrane-bound cues, presented by the target cell (Tessier-Lavigne, 1994; Keynes and Cook, 1995; Goodman, 1996). The establishment of the vertebrates neuromuscular junction involves activation of the receptor tyrosine kinase, MuSK, at the myotube postsynaptic site, by Agrin released from the nerve terminal and clustering of postsynaptic proteins, including AChRs, erbB and others. Retrograde signals from the muscle to the presynaptic nerve terminal stop axonal growth and initiate presynaptic differentiation.

The differentiation of tendon cells and the formation of muscle-tendon interactions represents a non-neural model system to study the nature and hierarchy of molecular and cellular instructive interactions between distinct cell types, leading to the correct connections of body musculature and the skeleton. Here also, equivalent instructive interactions between distinct cell types, including muscles, tendon cells and bones may regulate the process.

Molecular dissection of myotube pathfinding and the formation of muscle-tendon cell interactions in *Drosophila* facilitate the understanding of parallel processes carried out in the vertebrate embryo and may demonstrate general principles dictating reciprocal cell-cell instructive interactions during development.

In *Drosophila*, the larval somatic muscle tissue develops from mesodermal cells, which express high levels of the bHLH...
protein, Twist (Baylies and Bate, 1996). Twist also mediates the initial determination of mesodermal fate (Leptin, 1991). The different somatic muscles comprise an array of 30 different types of myotubes which develop, through the second half of embryonic development, in close proximity to the basal surfaces of the epidermis (Bate, 1990). The identity of each of these somatic myotubes is thought to be determined by inductive patterning mechanisms that define a single founder cell with a given specificity (Bate, 1993; Rushton et al., 1995; Baker and Schubiger, 1995). The founder cell then fuses to predetermined somatic mesodermal cells, which, upon fusion, acquire the specificity of the primary founder cell. The specificity of the fused myotube, manifested by a distinct pattern of gene expression, determines the spatial and temporal development of a given myotube, the number of myoblasts to be fused and the polarity of the fused myotube (Bate, 1993; Abmayr et al., 1995).

During extension, the myotube sends elongated filopodia at its leading edge, which facilitate its pathfinding towards the epidermal attachment cells (Bate, 1990). The final targeting of the muscle towards its specific EMA cell and the genes regulating this process have been the subject of intense investigation.

Myotube pathfinding towards each specific epidermal muscle attachment (EMA) cell depends on the presence of correct target EMA cells; mutant embryos in which these cells are missing or disorganized exhibit an aberrant pattern of somatic muscles (Volk and Vijay-Raghavan, 1994). A key gene in the proper differentiation of the EMA cells is stripe (De la Pompa et al., 1989). Embryos that carry a stripe severe mutation exhibit aberrant somatic muscle pattern in which the myotubes often send filopodia in incorrect directions. In late stages of embryonic development, most of the myotubes are randomly scattered, do not attach to the epidermis and fusion is blocked (Frommer et al., 1996). The stripe gene encodes an EGR-like nuclear protein containing a triple zinc-finger putative DNA-binding domain in its C terminus and therefore may function as a transcription factor (Lee et al., 1995; Frommer et al., 1996).

The EMA cells in stripe mutant embryos fail to express the normal repertoire of genes, including groovin (Volk and Vijay-Raghavan, 1994; Strumpf and Volk, unpublished), delilah (Armand et al., 1994) and Btubulin (Buttgereit et al., 1991), all characteristic of these cells (Frommer et al., 1996).

An important question in understanding the mechanism regulating myotube pathfinding towards their attachment sites is whether the EMA cells are actively involved in the process by providing positional cues, which direct the myotube from a distance towards their specific target cells. In addition, it is not known to what extent the two developmental programs, namely muscle and tendon cell differentiation, depend on each other. Since both cell types originate from distinct germ layers, it appears that initial patterning and gene expression is influenced by intrinsic ectodermal (for tendon cells) or mesodermal (for the myotubes) positional information. However, it is possible that terminal differentiation of both cell types may be mediated by their mutual interactions. The expression of Btubulin, a structural protein characteristic of tendon cells during their terminal differentiation, depends on muscle insertion (Buttgereit, 1996), suggesting that the establishment of EMA-muscle interactions is necessary for gene expression in the tendon-like cells.

In this paper, we show that Stripe expression is sufficient to activate the expression of other EMA-specific genes and, therefore, its ectopic expression in the ectoderm generates ectopic EMA cells. Moreover, these ectopic EMA cells are capable of attracting myotubes from a distance to ectopic attachment sites. During late stages of embryonic development, gene expression within the tendon-like cells depends on their interactions with muscles. In mutant embryos, in which certain somatic muscles are missing, the corresponding EMA cells cease to express Stripe and Groovin. Furthermore, examination of mutant embryos containing ectopic muscles shows that the expression of Stripe and Groovin is maintained in the cells to which these muscles are connected. Thus, we propose that EMA cells guide the myotubes to interact specifically with these cells and, after attachment, the muscles signal the EMA cells to maintain specific gene expression, leading to their terminal differentiation into tendon-like cells.

**MATERIALS AND METHODS**

**Fly stocks**
The following Gal4 inducers were used: en-Gal4 (A. Brand, Wellcome/CRC Institute, Cambridge); ptc-Gal4 (J. Campos-Ortega, Instituto für Entwicklungbiologie, Universität of Köln, Köln); sim-Gal4 (C. Klambt, Instituto für Entwicklungbiologie, Universität of Köln, Köln); K25 2xsev hs-Gal4 (E. Hafen, University of Zurich, Switzerland). In addition the following strains were used: UAS-DN-Htl (B. Shilo, Weizmann Institute, Rehovot); hs-ap (J. B. Thomas, The Salk Institute, San Diego); Df(3)DG4 sr155, twi1D96 (Bloomingston Stock Center) and srG11 (H. Jäckle, MPI, Göttingen). To visualize the lateral transverse muscles, we used flies carrying apertus muscle enhancer (ME15) driving lacZ (J. Botas, Baylor College of Medicine, Houston, Texas).

pUAS-stripeB flies were constructed by ligating a 4.5 EcoRI fragment of stripeB cDNA with pUAST (Brand and Perrimon, 1993), digesting with EcoRI and introducing this construct into the fly germ line by a standard P-element transformation method.

**Immunohemical reagents**

To visualize embryonic muscles, we used anti-myosin heavy chain polyclonal antibody, provided by P. Fisher (Stony Brook, NY). The serum was usually preadsorbed on 0- to 2-hour-old embryos and diluted 1:500 for staining. Stripe was visualized with anti-GST-StripeB fusion protein raised in guinea pig, and Groovin with monoclonal anti-Groovin hybridoma supernatant; both antibodies were developed in our laboratory. Anti-Alien antibody was obtained from A. Paululat (University of Marburg, Germany), delilah RNA was visualized by in situ hybridization with delilah cDNA (M. Cole, Princeton), and Btubulin expression was monitored using Btubulin-lacZ flies (D. Buttgereit, University of Marburg, Germany). Anti-β-gal antibodies were purchased from Cappel (USA). Secondary antibodies included HRP, fluorescein- or rhodamin-conjugated goat anti-rabbit IgG, anti-guinea pig IgG and anti-mouse IgM (Jackson).

**Whole-mount embryonic staining**

In addition to the HRP (see below) staining to determine expression of the appropriate markers, we routinely stained the embryos collected from the different mutant lines for β-galactosidase to identify homozygous mutant embryos.

Staining was performed essentially as described (Ashburner, 1989). In brief, embryos were collected and incubated as indicated, dechorionated and fixed with a mixture of 3% paraformaldehyde and heptane. Following two washes with PBT (PBS containing 0.1% Triton X-100), embryos were incubated in the X-gal staining solution, until blue staining was visible (15-30 minutes at 37°C), and then washed and devitellinized with a methanol-heptane mixture. Permeabilization was performed by incubation in PBT containing 10% BSA for 2-3
hours and incubation with primary antibody was usually performed for 16 hours at room temperature.

**Sections**

Embryos were fixed with 0.1% glutaraldehyde and 3% paraformaldehyde in PBS, stained for X-gal, dehydrated, washed in ethanol and infiltrated with JB-4 embedding media (Polysciences Inc, USA) according to the manufacturer’s instructions. Embryos were oriented in molds and the resin was allowed to harden in a desiccator. Sections (3-4 μm width) were obtained with a Sorvall MT2B microtome, stained with methylene blue and basic fuchsin by standard procedures and examined under a Zeiss Axioscope microscope.

**Flat preparation of embryos**

Flat preparations were prepared essentially according to Bate (1990); live embryos were dechorionated and the vitelline membrane was removed by hand. The embryos were opened and flattened on poly-L-lysine-covered coverslips, fixed with 3% paraformaldehyde in PBS and stained.

**Confocal microscopy**

Fluorescent labeled preparations were imaged using a BioRad MRC 1024 confocal microscope coupled to a Zeiss Axiovert 135M microscope. Bright-field and fluorescent digital images were processed using Photoshop (Adobe Systems Inc).

**RESULTS**

**stripe expression directs EMA cell fates**

Transcription factors that initiate an entire differentiation program, leading to the formation of a given cell type, are capable of regulating the transcription of a wide array of genes characteristic of that tissue, including the autoregulation of their own transcription.
Stripe is the earliest gene to be expressed in the future EMA cells. stripe mRNA is apparent at late stage 11, before and during germ band retraction (Volk and VijayRaghavan, 1994). In stripe loss-of-function mutant embryos, EMA-specific genes including groovin, delilah and B\(1\) tubulin are not expressed, indicating that stripe is required for their expression (Frommer et al., 1996). To further evaluate whether Stripe is sufficient to induce the expression of an array of EMA-specific genes, we produced embryos where Stripe was ectopically expressed in the ectoderm, utilizing the Gal4/UAS expression system (Brand and Perrimon, 1993) and examined the expression profile of the EMA-specific genes in the ectopic cells. The ectopic Stripe-expressing cells expressed EMA-specific genes, including Groovin, Alien, delilah (mRNA), the B\(1\) tubulin-lacZ construct and B-gal in stripe-enhancer trap flies (Fig. 1). These results indicate that Stripe is capable of inducing the expression of an array of EMA-specific genes, including autoregulating its own expression and, therefore, is a key factor in fate determination of the EMA cells.

The EMA-specific genes induced by Stripe could be divided into two groups; genes that follow Stripe ectopic expression in all embryonic stages or genes that could not be detected in early (stage 10-11) or late (older than stage 14) developmental stages. groovin and alien represent the first group e.g. the en-Gal4 inducer could induce their expression at all stages from 10 to 16 of embryonic development. delilah mRNA and B\(1\) tubulin-lacZ represent the second group and were expressed only in stages 12-14. These differences may point to the requirement for additional factors for induction of the latter genes. Interestingly, in wild-type embryos, Groovin and Alien protein expression follows that of Stripe through all embryonic stages, while delilah and B\(1\) tubulin mRNA are observed only in later stages of embryonic development.

**Cells expressing ectopic Stripe are capable of attracting somatic myotubes**

The potential of a target tissue to direct specific axonal pathfinding has been demonstrated in various model systems, including innervation of embryonic muscle in Drosophila (Goodman, 1996). Similarly, the autonomous contribution of EMA cells for guidance of myotube pathfinding was analyzed. Previous experiments showed that the somatic muscle pattern of stripe mutant embryos is significantly disrupted (Frommer et al., 1996). This abnormal muscle pattern suggested that epidermal muscle attachment cells are required for the induction of the correct muscle pattern and that stripe is essential for this induction. The EMA cells could passively participate in the induction of muscle pattern by exhibiting one or several specific adhesion molecules on their surfaces, which trap the approaching myotubes. Alternatively, an attractive cue produced by the EMA cell may direct the myotubes to their target epidermal cells. To examine whether an ectopic EMA cell can actively attract the approaching myotube, we analyzed the muscle pattern in embryos where stripe expression was induced by various Gal4 lines.

When the ectopic Stripe-expressing cells were located within a distance of 2- to 3-cell diameters from the myotubes and were induced in embryos hemizygous for stripe, the myotubes changed their routes directing their leading edge towards these cells. This process often ended with the interaction of myotubes with incorrect cells. The most prominent effect was observed when Stripe expression was induced in the salivary glands. In that case, the somatic myotubes altered their direction internally towards the salivary gland epithelia and adhered to these cells (Fig. 2). To identify the hemizygous embryos, we double-labeled them with Stripe and myosin. The intensity of the ectopic Stripe was always higher than that of the endogenous protein. Other configurations, in which the myotubes ended in the ectopic patched-Gal4-expressing cells, or where elongated filopodia were extended ectopically towards the midline in sim-Gal4-expressing cells, were also observed (not shown).

When the ectopic Stripe-expressing cells were induced in wild-type embryos, only mild aberrations in the muscle pattern were found. Fig. 3 shows the relatively limited derangement of somatic muscle pattern in embryos expressing ectopic stripe in wild-type embryos utilizing en-Gal4 or ptc-Gal4 inducer lines. In several cases, some myotubes are missing, exhibit mistaken routes or are connected to the ectopic EMA cells. Interestingly, the lateral transverse muscles are often more sensitive and exhibit abnormal pattern (Fig. 3D,F). At later stages of embryonic development following muscle contraction, additional disruption of the muscle pattern is observed. The relative mild phenotype could result from the existence of wild-type EMA cells which express higher levels of Stripe relative to the ectopic cells at the time when the myotubes approach them.

To this end, the pattern of somatic muscles was further examined in embryos that, in addition to the en-Gal4;UAS-srB constructs, were homozygous for a weak sr allele (sr\(G^{11}\)). The somatic muscles in the sr\(G^{11}\) homozygous embryos is normal, and most of the embryos hatch, but do not develop into adult flies. In contrast, severe muscle abnormalities were observed in sr\(G^{11}\) embryos that included the en-Gal4;UAS-srB constructs. The lateral transverse muscles were hardly identified and often were removed from their normal positions (Fig. 4).

These results indicate that stripe expression in the ectopic cells induces attraction of the myotubes and their attachment to these cells, providing that the ectopic Stripe levels are higher than the endogenous levels. It is possible that a combination between attractive and adhesive mechanisms mediates the final pathfinding of the somatic myotubes towards their attachment cells.

**Two waves of gene expression in EMA cells**

The experiments described above indicate the central role of Stripe within the EMA cells to induce the proper guidance of the somatic muscles. We wished to further analyze possible muscle-dependent mechanisms controlling Stripe expression in EMA cells. Stripe wild-type expression precedes muscle interaction with the EMA cells (Frommer et al., 1996 and Fig. 5A,B). It appears therefore that initial determination of the EMA cells is muscle-independent and may be induced by general patterning mechanisms of the ectoderm. Additional support for this notion is that Stripe is expressed in twist mutant embryos, which do not have mesoderm, although the pattern is significantly abnormal, presumably due to defective development of this embryo (Fig. 6A). We noticed, however, that stripe expression during late stages of embryonic development is refined relative to earlier stages. For example, during stage 14 of embryonic development, the number of stripe-expressing cells at the ends of lateral transverse muscles is around 8-10 cells (Fig. 5D). At stage 16-17, this number is reduced to three
cells, those that are attached to muscles LT1-3 (muscles 21,22,23) (see Fig. 5C,E). Thus, the expression of Stripe in the other cells in this cluster is significantly reduced. This observation raises the possibility that the maintenance of Stripe expression within the subset of EMA cells that established muscles connections is regulated by mechanisms related to the interaction of the EMA cells with the muscles. Interestingly, the expression of β1 tubulin is restricted to the final stage of gene expression in tendon-like cells (Buttgereit, 1996), supporting the idea of a distinct mechanism regulating gene expression within the tendon cells as a result of muscle interactions.

**Reduction of Stripe and Grovin levels in EMA cells that do not interact with muscles**

To test the idea raised above, namely, that only the EMA cells that are bound to muscles maintain the characteristic EMA cell-specific gene expression, we examined the expression of Grovin and Stripe proteins in mutant embryos in which some of the somatic muscles were missing. We chose to examine the expression of Grovin and Stripe in the background of a dominant-negative allele of heartless, *htl* (a mutation in the mesoderm-specific *Drosophila* FGF receptor. Activation of the dominant-negative construct at 3.5 hours AEL by Heat-Shock-Gal4 results in the random deletion of a number of dorsal, lateral and ventral muscles, but the differentiation and pattern of the rest of the muscles appear to be normal (Beiman et al., 1996). Since Htl is expressed only in the mesoderm, any alteration in gene expression in the EMA cells of the mutant embryos must result from an indirect effect of the muscles on these cells. The unaffected somatic muscles in the mutant embryos served as an internal control.

We examined embryos exposed to a single heat shock (3.5 hours AEL) and allowed the embryos to develop for an additional 11 hours. The cuticle of such embryos is normal (not shown); thus, it is assumed that the pattern of the ectoderm in these embryos is not affected. Three levels of Stripe and Grovin protein expression were observed in the EMA cells of the mutant embryos (shown in Fig. 6). Normal intensity of Stripe and Grovin in the EMA cells were always accompanied by the connection of these cells to wild-type somatic muscles, while the absence of expression was observed in areas of muscle loss. Moreover, intermediate levels of Stripe or Grovin were detected in areas associated with abnormal muscles. This result suggests that the maintenance of Grovin and Stripe expression in the EMA cells depends on their connection to muscles.

**Ectopic muscles induce maintenance of Stripe and Grovin expression**

The above experiment indicated the contribution of muscles to the maintenance of EMA-specific gene expression in EMA cells. The ability of muscles to maintain EMA-specific genes in various types of ectodermal cells was further examined in mutant embryos with ectopic muscles. To this end, we used embryos carrying an *apterous* construct controlled by heat-shock promoter (hs- ap). Apterous is a Lim-domain-containing homeobox protein, which functions to establish dorsoventral pattern of the wing imaginal disc. In the embryo, *apterous* mediates differentiation of the lateral transverse muscles (LT1-4) and the ventral acute muscles, VA2 and VA3 (Bourgouin et al., 1992). Following heat shock at 6 hours AEL, hs- ap embryos develop a variable number of ectopic lateral transverse muscles (Bourgouin et al., 1992). To identify the segments that carry ectopic lateral transverse muscles, we introduced to the hs-ap embryos an *apterous-lacZ* construct specific for the lateral transverse muscles (LT1-4, obtained from J. Botas). The lateral transverse muscles were visualized by anti-β-galactosidase antibody and the EMA cells were immunostained for Stripe. Comparison between the EMA cells in embryos that were treated and not treated by heat shock revealed that, in segments where the ectopic lateral transverse muscles ended in the domain of the lateral cluster (cells that may be competent to be EMA cells and express Stripe at this stage), they were properly connected to Stripe-expressing cells. This led to increased number of Stripe-expressing cells in the lateral transverse cluster (Fig. 7) However, in segments where the ectopic muscles developed into long or abnormal muscles which terminated far from the lateral EMA cluster, they were not attached to any epidermal cells and did not induce Stripe at their ends (Fig. 7).

These results complement the findings described in the previous section indicating that, in the absence of muscles the expression of Stripe and Grovin is reduced in EMA cells. Conversely, the binding of ectopic myotubes can stabilize Stripe and Grovin expression in competent EMA cells. Thus, our findings indicate that muscle binding to an EMA cell triggers maintenance of Stripe and Grovin expression within the EMA cell, but not in non-related epidermal cells.

**DISCUSSION**

Elucidation of the process of myotube pathfinding and the reciprocal interrelationships between the EMA and myotube cells in *Drosophila* may serve as a general model to study the regulation of directed instructive interactions between different cell types during organogenesis. Our findings suggest that reciprocal interactions between EMA cells and muscles are essential for the establishment of the correct muscle connections to the various epidermal attachment sites and for the development of mature tendon-like cells.

In vertebrates, not much information is available on the process of the final patterning of the muscles and the processes regulating muscle-specific pathfinding towards their attachment sites along the bone. Some indications suggest that tendons can be formed without muscles, although tendons do require muscle for survival, and without muscle attachment they will degenerate (Shellswell and Wolpert, 1977; Kieny and Chevallier, 1979).

The present results support the notion that Stripe expression in a subset of epidermal cells initiates a dynamic developmental program, at the end of which a subset of the Stripe-expressing cells develop into mature tendon-like cells. While the initial expression of Stripe is presumably induced by general patterning mechanisms in the ectoderm, its further expression appears to be positively autoregulated. During late stages of embryonic development, Stripe autoregulation is not sufficient to maintain expression and additional muscle-dependent external signals are required.

**The attractive potential of the EMA cells**

Previous studies (Bate, 1990) indicated morphological simi-
Fig. 3. Muscle abnormalities are detected in embryos carrying ectopic StripeB in a wild-type background. Whole-mount embryos (A,B,C,E,F) or flat opened embryo (D) are shown. Embryos in A,C,E,F are wild type while embryos in B and D carry en-Gal4;UAS-srB, and embryo in F carries ptc-Gal4;UAS-srB. Whole embryos in A,B,F were double-labeled for Stripe (black) and Myosin (brown), while embryos in C and D were double labeled for Groovin (red) and Myosin (green). The embryos carrying ectopic stripe (B,D,F) show mild muscle abnormalities in which the muscle pattern is deranged. In some cases, the muscles are missing (arrowheads in B). Note that the lateral transverse muscles (LT) often show abnormal morphology. In some cases, they are turned towards the ectopic Stripe-expressing cells (arrowhead in D) and, in other cases, they are not connected properly (arrowhead in F).

Fig. 4. Enhancement of the effect of ectopic Stripe in embryos homozygous for weak stripe allele. Embryo homozygous for srG11 stained for myosin, indicating that the somatic muscle pattern in this embryo is close to that of wild type (A). The embryo in B contains both the en-Gal4 and UAS-SrB constructs and is homozygous for srG11. In contrast to the embryo in A, this embryo shows a severe disruption of the muscle pattern, especially that of the lateral transverse muscles.

Fig. 5. Stripe expression is refined during development into muscle-bound cells. Whole-mount embryos double labeled for Stripe (red) and Even-skipped (green) in stage 12 embryo (A), or for Stripe (red) and Myosin (green) in embryos at stages 14 (B) or 16 (C) are shown. Stripe expression at stages 12-14 is observed in many epidermal cells irrespective of muscle binding. At stage 16, Stripe expression is confined only to cells that are connected to muscles. The lateral cluster stained for Stripe (green) at stage 14 includes 8-10 cells (arrow in D) while, at stage 16, Stripe expression is reduced to three cells (arrow in E).

The filopodia extended from the leading edge of the myotube may be chemotactically attracted by the EMA cells and then stopped by specific adhesive signals exhibited by these cells. Our results favor the possibility that the EMA cells produce an attractive activity, in addition to their potential to induce myotube adhesion as a result of Groovin activity (Strumpf and Volk, unpublished). The nature of this attractive activity is yet to be elucidated. The ectopic EMA cells produced by the induction of Stripe expression do not exhibit strong attraction. This could be due to a relatively low concentration of the putative attractant produced by the ectopic EMA cells, or to a relative short range of attractant action.

In embryos where the thoracic muscles were partially trans-
formed into abdominal muscles as a result of ectopic expression of AbdA or Ubx in the whole mesoderm (Gréig and Akam, 1993), some of the ectopic muscles form connections with EMA cells, while other muscles remain unconnected to the epidermis (Michelson, 1994). It is possible that the muscles that do find their way to the EMA cells are only the muscles that are close enough to a given EMA cell. For example, the ectopic ventral oblique muscle VO6 (no. 17) in the A1 segment is close to ventral EMA cells, and therefore binds to them, while ectopic ventral acute muscle VA1 (no. 26) is too far from any attractive cues, and thus does not extend and remains unattached.

The only known gene involved in myotube pathfinding in Drosophila is derailed, a receptor tyrosine kinase. derailed is expressed by the lateral transverse muscles LT1-3, and by a group of epidermal cells surrounding these muscles, corresponding to epidermal attachment sites, in addition to its expression in the CNS. In derailed mutant embryos, LT1-3 muscles continue to extend their leading edge ignoring their attachment sites, although these EMA cells do express Stripe normally (Callahan et al., 1996). Derailed is not sufficient to induce attractive activity, since when expressed in ectopic muscles, the muscles are not attracted to the corresponding muscle attachment cells of LT1-3. Although loss of derailed did not affect the expression of Stripe, it may be involved in modulation of the putative attractant produced by the Stripe-expressing cells, thereby, facilitating the recognition between the lateral transverse muscles and their attachment cells.

**Correlation between myotube-tendon cells and neuromuscular interactions**

Our results suggest an intriguing similarity between directed muscle-tendon reciprocal interactions and the formation of neuromuscular junctions. In a manner similar to that described here for muscle pathfinding, the nerve growth cone is also attracted by distinct positional mechanisms directing its binding to a specific muscle. Moreover, during formation of the neuromuscular junction in vertebrates, reciprocal signaling between both cell types induces postsynaptic differentiation exhibited by the specific transcription and clustering of the acetylcholine receptors.

It appears that muscle-tendon junction formation affects the expression of at least three genes, groovin, stripe and β1 tubulin. This last stage of regulation of EMA cell differentiation into a tendon-like cell is controlled by the proper interaction between the muscle and tendon cells, and may prevent nonessential differentiation of potential extraneous tendon cells.

The molecular nature of the signal activated as a result of EMA cell-myotube interaction is as yet, unknown. Two candidate
signaling mechanisms may be involved: integrin-mediated signaling, or DER (Drosophila EGF-like receptor)-mediated signaling. The EMA cells express a specific integrin heterodimer, αPS1βPS (Leptin et al., 1989). The embryonic function of this integrin heterodimer is not clear, since in newt, an αPS1 null mutation, the muscles appear to be properly attached to the EMA cells, though the embryo hatches and dies at the first instar larval stage (Brower et al., 1995). It is possible that the αPS1βPS integrin mediates signaling from the muscle to the EMA cell to maintain Stripe and Groovin expression only in muscle-bound cells. A second possibility, which is not mutually exclusive, is activation of the Drosophila EGF receptor homologue DER (reported to be expressed by the EMA cells (Zak and Shilo, 1992)), which may be initiated as a result of muscle binding. This may be analogous to signaling mechanisms mediating vertebrates neuromuscular synaptic sites. Differentiation of post synaptic site is accompanied by the activation of erbB at this site, triggered by the nerve presynaptic signaling. This activates the ras-mediated signaling cascade leading to acetyl choline receptor transcription and clustering at the postsynaptic site (Kleiman and Reichardt, 1996; DeChiara et al., 1996; Sangmee et al., 1995).

Further characterization of the molecular events affecting myotube attraction by the tendon-like cell, and mutual inductive interactions between the myotube and tendon cell during organogenesis, elucidates general principles underlying the formation of intercellular inductive interactions during development.

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