

FGF as a Target-Derived Chemoattractant for Developing Motor Axons Genetically Programmed by the LIM Code

Ryuichi Shirasaki,^{1,2} Joseph W. Lewcock,¹
Karen Lettieri,¹ and Samuel L. Pfaff^{1,*}

¹Gene Expression Laboratory
The Salk Institute
10010 North Torrey Pines Road
La Jolla, California 92037

Summary

LIM transcription factors confer developing axons with specific navigational properties, but the downstream guidance receptors and ligands are not well defined. The dermomyotome, a transient structure from which axial muscles arise, is the source of a secreted long-range chemoattractant specific for medial-class spinal motor neuron axons (MMCm axons). We show that fibroblast growth factors (FGFs) produced by the dermomyotome selectively attract MMCm axons in vitro. FGF receptor 1 (FGFR1) expression is restricted to MMCm neurons, and conditional deletion of *FGFR1* causes motor axon guidance defects. Furthermore, reprogramming the identity of limb-innervating motor neurons to that of dermomyotome-innervating MMCm cells using the LIM factor *Lhx3* induces *FGFR1* expression and shifts an increased number of motor axons to an FGF-responsive state. These results point to a role for FGF signaling in axon guidance and further unravel how downstream effectors of LIM codes direct wiring of the developing nervous system.

Introduction

The correct wiring of the nervous system depends upon the ability of individual axons to recognize specific guidance cues as they grow toward their cellular targets (reviewed in Dickson, 2002; Tessier-Lavigne and Goodman, 1996). Axon pathfinding plays a fundamental role in this process through a stepwise series of phases in the neuronal differentiation program. Importantly, the topographic assembly and elaboration of neural circuits rely critically on the generation of functionally related but navigationally distinct neuronal subtypes (or subclasses). In general, the axonal trajectory of a neuron is composed of several discrete segments, some of which are shared by multiple subtypes and others of which are unique pathways selected by individual neuronal subclasses. Although the decisions that are made at specific axonal choice points are crucial for establishing the proper pattern of connectivity, the genetic programs that are employed to mediate this precisely orchestrated process are not well characterized.

A remarkable feature of axon pathfinding by spinal motor neurons, for example, is that after leaving the spinal cord, they initially follow a common ventral pathway, the ventral root, then subsequently diverge to select subtype-specific pathways to targets such as the limb, sympathetic ganglia, and axial musculature (reviewed in Jessell, 2000; Landmesser, 1992). Motor neurons that share these subtype-specific axon pathways coalesce into separate motor columns within the spinal cord, thus establishing a topographic relationship between their cell bodies and peripheral targets. Motor neurons located in the median portion of the medial motor column (MMCm) extend axons along the ventral root pathway and then turn dorsally to innervate the dermomyotome (Figure 1A), the embryonic precursor of dermis and epaxial (dorsal axial) musculature involved in stabilizing the back (Tosney and Landmesser, 1985). In contrast, axons from cells within the medial and lateral portions of the lateral motor column (LMCm and LMCI, respectively) ignore the choice point selected by MMCm axons and instead continue to grow ventrolaterally into the periphery toward the base of the limb (Figure 1A). Here, LMCm axons project exclusively into the ventral limb mesenchyme, whereas LMCI axons project only to the dorsal limb mesenchyme (Landmesser, 1992; Tosney and Landmesser, 1985). Classical embryological studies focusing on the topographic projections of spinal motor axons have shown that discrete motor neuron subtypes differ in their intrinsic properties for axon pathfinding (reviewed in Landmesser, 1992), suggesting that before motor axons reach their decision points they have already been genetically programmed to sense pathway-specific guidance cues.

Recent molecular and genetic studies have demonstrated that unique combinations of LIM homeodomain transcription factors, the LIM code, function in postmitotic motor neurons to define the target specificity of individual motor neuron subtypes (reviewed in Jessell, 2000; Shirasaki and Pfaff, 2002). For instance, MMCm motor neurons uniquely express *Lhx3* (Lim3), whereas other motor columns do not (Figure 1A) (Tsuchida et al., 1994; Sharma et al., 1998). In genetically engineered mice in which *Lhx3* is stably expressed in all motor neurons (*Lhx3-knockin*, or *Lhx3-ki*), the cell body settling pattern and gene expression profile of all motor neurons are converted to those of MMCm cells (Sharma et al., 2000). Strikingly, this reassignment of the LIM code results in a dramatic increase in the number of motor axon projections toward the dermomyotome at the expense of limb innervation (Sharma et al., 2000). Another line of evidence for a role of the LIM code in vertebrate motor axon guidance was provided by studies focusing on the selective expression of *Lhx1* (Lim1) in LMCI neurons (Figure 1A) (Kania and Jessell, 2003; Kania et al., 2000). In *Lhx1* mutant mice, LMCI axons project into the dorsal and ventral halves of the limb mesenchyme at equal incidence, whereas in wild-type embryos LMCI axons selectively enter only the dorsal limb. These genetic studies imply that the LIM code either directly or indirectly regulates the expression of

*Correspondence: pfaff@salk.edu

²Present address: Cellular and Molecular Neurobiology Laboratory, Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka 565-0871, Japan.

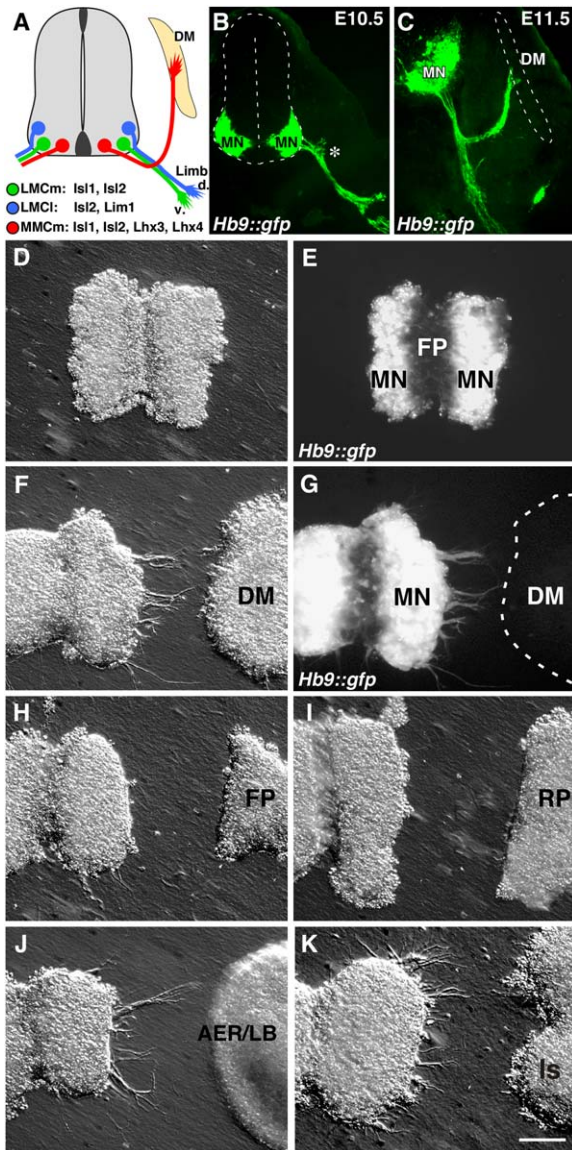


Figure 1. The Dermomyotome Secretes a Long-Range Chemoattractant for Developing Motor Axons In Vitro

(A) Motor axon projections and the LIM code for motor neuron (MN) columnar subtypes within the brachial spinal cord. After exiting the spinal cord, MMCm axons (red) break away from the ventral trajectory of the spinal nerve to form the dorsal ramus branch leading to the dermomyotome (DM). LMCm (green) and LMCI (blue) axons extend to the base of the limb where they turn ventrally (v.) or dorsally (d.), respectively.

(B) Motor axon projections are labeled by GFP expression in *Hb9::gfp* mice. At E10.5 brachial MMCm axons begin to form the dorsal ramus branch (asterisk).

(C) By E11.5 MMCm axons have reached the dermomyotome.

(D) MN column explants cultured alone for 15 hr ($n = 8$, see also Figure 4E). Ventral spinal cord explants taken from the brachial spinal cord of *Hb9::gfp* mice were cultured in 3D matrix gels.

(E) Fluorescent view of explant in (D), showing GFP-labeled motor neurons (MN) flanking the floor plate (FP).

(F) Coculture of MN column explants with the dermomyotome (DM) for 15 hr, showing long-range attraction of axons toward the DM ($n = 8$, see Figure 7M for quantification).

(G) Motor axons are attracted by the dermomyotome, as revealed by the GFP expression on responding axons from the explant in (F).

(H) Coculture of MN column explants with the floor plate (FP) ($n = 4$).

(I) Coculture of MN column explants with the roof plate (RP) ($n = 3$).

receptors for sensing guidance cues specific for individual subtypes of motor axons.

Because axonal navigation depends upon attractive and repulsive guidance cues operating both short- and long-range, the downstream axon guidance effectors of the LIM code could in principle include multiple receptor types. In the case of limb innervation, LMCI and LMCm axons segregate at the base of the limb and choose immediately to grow either dorsally or ventrally, suggesting that the peripheral signals and axonal receptors that control limb innervation are tightly regulated at the decision region (Tosney and Landmesser, 1985). Consistent with this, it has recently been shown that LMCm axons are directed ventrally by Npn-2-mediated repulsion from secreted Sema3F within the dorsal limb (Huber et al., 2005). Conversely, dorsally projecting LMCI axons are repelled from the ventral limb by ephrin-As (Eberhart et al., 2002; Helmbacher et al., 2000). This contact-mediated repulsion of LMCI neurons is under the control of the *Lhx1* transcription factor which regulates *EphA4* receptor expression in these cells (Kania and Jessell, 2003). Interestingly, in contrast to repulsive signaling for limb-innervating motor neurons, MMCm cells appear to be attracted to the dermomyotome. In the chick embryo, ablation of the dermomyotome prevents the dorsal deviation of MMCm axons toward the site of the missing dermomyotome, and instead axons grow toward adjacent intact dermomyotomes (Tosney, 1987, 1988). Similarly, *Xenopus* somitic myoblasts attract neurites from neural tube explants (McCaig, 1986). In the zebrafish mutant *spadetail*, which is deficient in trunk myotome development, the projection patterns of motor axons toward the myotome are abnormal (Eisen and Pike, 1991). Likewise, mouse embryos deficient for both *Myf-5* and *MyoD* lack myotomes and exhibit axial projection defects (Kablar and Rudnicki, 1999). However, direct proof of a dermomyotome-derived chemoattractant has not yet been shown and thus the nature of the signals that selectively guide MMCm axons remain unknown.

We show that MMCm axons are guided by a long-range chemoattractant secreted from the dermomyotome. Fibroblast growth factors (FGFs) expressed in the dermomyotome are responsible for the selective chemoattractive activity for MMCm axons, thereby demonstrating a role for this family of signaling proteins in axon navigation. Importantly, among receptors for FGFs (FGFRs), *FGFR1* is expressed in MMCm cells as they extend axons toward the dermomyotome, while other motor neuron subclasses lack FGF receptors. We converted LMC motor neurons into MMCm cells by reconfiguring the LIM code in *Lhx3-ki* mice and found that this was accompanied by an expansion in the *FGFR1* expression domain and an increase in the number of motor axons that respond to the dermomyotome-derived chemoattractant. Conversely, Cre-mediated

(J) Coculture of MN column explants with a portion of the limb bud (LB) containing the apical ectodermal ridge (AER) ($n = 4$).

(K) Coculture of MN column explants with the isthmus (Is) ($n = 3$). Neither the floor plate nor the roof plate possesses chemoattractive activity for motor axons, whereas limb bud and isthmus tissue can attract motor axons.

Scale bars, 125 μm in (B) and (C), and 100 μm in (D)–(K).

deletion of *FGFR1^{fl/fl}* alleles resulted in epaxial motor axon guidance defects in vivo. Thus, in this study we provide direct evidence for a long-range chemoattractant specific for medial-class spinal motor neurons genetically defined by the LIM code.

Results

Detection of a Motor Axon Chemoattractive Activity Secreted by the Dermomyotome

To begin to identify the guidance programs employed by MMCm cells, we monitored the development of the segmentally repeated dorsal ramus nerve in the mouse (Nakao and Ishizawa, 1994), comprised of MMCm axons projecting toward the dermomyotome (Figure 1A). To visualize motor axons, we used transgenic mice in which GFP is expressed under the control of a promoter derived from the motor neuron-specific transcription factor Hb9. *Hb9::gfp* transgenic mice have labeled motor neuron cell bodies and axons (Lee et al., 2004) (Figures 1B and 1C). Postmitotic spinal motor neurons appear around E9.5 in the ventral spinal cord and extend axons laterally away from the midline to exit the spinal cord together through the ventral root. We found that at E10.5, a subset of GFP⁺ motor axons begin to turn from the main spinal nerve trunk and grow dorsally toward the dermomyotome, while limb-innervating motor axons continue to grow ventrally (Figure 1B). At E11.5, MMCm motor axons which have diverged from the ventral root continue to grow dorsally to reach the dermomyotome (Figure 1C; see also Figure 6). These observations indicate that motor axon projections toward the dermomyotome begin to form around E10.5 to E11.5, thus establishing a time window in which MMCm cells are expected to express guidance receptors for this choice point.

We next tested whether the dermomyotome secretes a long-range chemoattractant for developing motor axons using mouse E9.75 to E10.0 lower cervical (brachial) level explants dissected from the ventral third of the neural tube (MN column explants). A mixture of motor neuron subtypes is present in these explants, including MMCm, LMCm, and LMCI cells (Figure 1A). MN column explants were cultured for 15 hr in 3D collagen/matrigel matrices, since this method is well established for detection of long-range chemotropic activity for developing axons (Brose et al., 1999; Lumsden and Davies, 1983; Serafini et al., 1994). When MN column explants were cultured alone, motor axon outgrowth from the explants was minimal (Figures 1D and 1E). In contrast, when MN column explants were cocultured with the dermomyotome, we found that axon outgrowth toward the dermomyotome was dramatically induced (Figure 1F). In vivo, the ventral root is separated from the dermomyotome by ~250 μm, and in vitro, chemoattractive activity was detected at a comparable distance. Next, we examined whether the dermomyotome induces general outgrowth of axons or is specific for motor neuron axons. Using *Hb9::gfp* MN column explants, we found that attracted axons were all GFP⁺ (Figure 1G). These results indicate that the dermomyotome, the target of MMCm motor neurons, secretes a diffusible molecule that selectively attracts developing motor axons from a distance.

FGFs Expressed in the Dermomyotome Possess Chemoattractive Activity

To identify the molecule responsible for the dermomyotome-derived chemoattractive activity, we assayed a variety of embryonic tissues that have been shown to secrete diffusible chemotropic signals. The floor plate and roof plate are known to secrete several types of axon guidance molecules, including netrins, BMPs, slits, Shh, and wnts (Augsburger et al., 1999; Brose et al., 1999; Charron et al., 2003; Lyuksyutova et al., 2003; Serafini et al., 1996). However, when we cocultured MN column explants with the floor plate or the roof plate, these tissues failed to mimic the activity of the dermomyotome (Figures 1H and 1I). In contrast, tissues from both the limb bud/apical ectodermal ridge (AER) and the mid-brain/hindbrain isthmus region were found to promote axon outgrowth from MN explants (Figures 1J and 1K).

The dermomyotome, the isthmus, and the limb bud are related to one another by virtue of each expressing multiple FGFs (Colvin et al., 1999; Crossley and Martin, 1995; Niswander and Martin, 1992; Thisse and Thisse, 2005 and references therein), suggesting that this growth factor might represent a guidance factor for spinal motor neurons. As a control to establish an assay for bath-applied growth factors, we first monitored motor axon outgrowth triggered by hepatocyte growth factor (HGF) because it has been shown to function as a chemoattractant for spinal motor axons in vitro (Ebens et al., 1996). As expected, HGF induced robust GFP⁺ motor axon outgrowth when bath applied to *Hb9::gfp* MN column explants (Figures 2A and 2B). Since HGF is expressed by limb tissue, but not by the dermomyotome, other signals are likely to be responsible for MMCm chemoattraction (Ebens et al., 1996). Therefore, based on the finding that FGF⁺ tissues, including the isthmus, limb bud, and dermomyotome, promote axon outgrowth, we tested whether any of the known FGF family members expressed by the dermomyotome, which included FGF2, FGF4, FGF8, and FGF9 (Colvin et al., 1999; Crossley and Martin, 1995; Niswander and Martin, 1992; Thisse and Thisse, 2005 and references therein), are sufficient to trigger axon outgrowth. Each FGF was added separately to the explant culture bath, and we found that FGF2, FGF4, FGF8, and FGF9 all triggered GFP⁺ motor axon outgrowth (Figures 2C and 2D; data not shown). In contrast, FGF10, which is expressed in the limb mesenchyme but not the dermomyotome, lacked activity (Figures 2E and 2F).

To present FGF ligand as a point source which would more accurately reflect the in vivo context of the signal, we used heparin-coated beads for generating FGF gradients. We focused on FGF8 because its heparin binding is well characterized (Loo and Salmivirta, 2002) and it is expressed by the dermomyotome during the period when MMCm axons navigate toward this target (Crossley and Martin, 1995; Mahmood et al., 1995). Heparin-only control beads had no effect on axon outgrowth, whereas FGF8-loaded beads elicited outgrowth of motor axons toward the FGF source (Figures 2G and 2H). In principle, FGFs could promote axon extension without influencing the directionality of growth cone navigation. Therefore, to determine if FGF8 could redirect axon growth, we positioned an FGF8 bead orthogonal to the normal axis of motor axon growth from the explants.

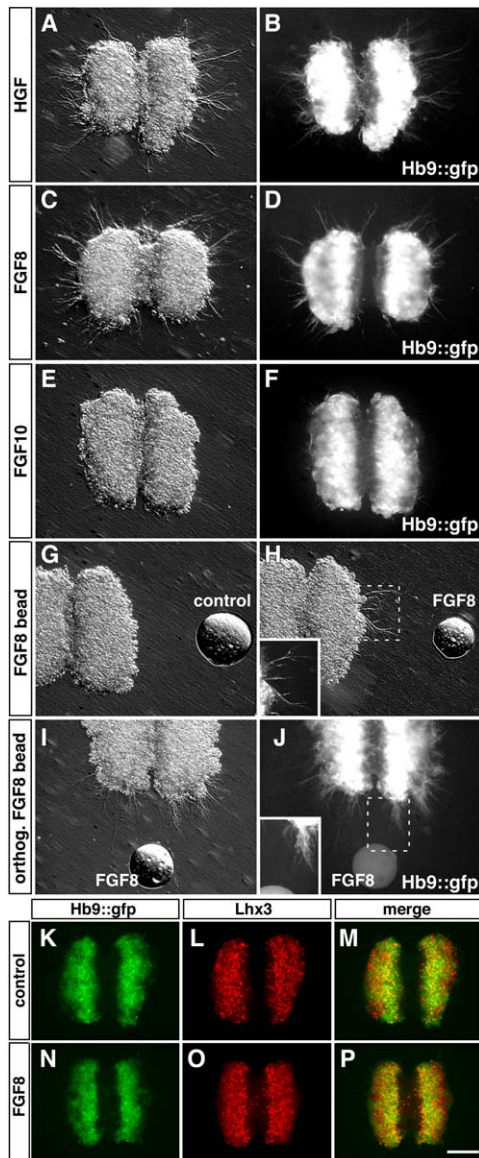


Figure 2. Long-Range Chemoattraction of Motor Axons by FGF
(A–J) Bright field and GFP labeling of motor axons from MN column explants exposed to growth factors. (A and B) HGF induces motor axon outgrowth ($n = 7$, see Figure 4H for quantification). (C and D) Likewise, FGF8 triggers GFP⁺ motor axon outgrowth from *Hb9::gfp* explants ($n = 8$, see Figure 7N for quantification). (E and F) FGF10 lacks axon outgrowth promoting activity ($n = 8$). (G) Coculture of MN column explants with a heparin-coated control bead ($n = 9$). (H) Coculture of MN column explants with an FGF8-loaded heparin bead ($n = 8$). A point source of FGF can attract GFP⁺ motor axons from *Hb9::gfp* explants (inset). (I and J) An FGF8-coated bead placed orthogonal to MN explants alters the normal lateral pattern of axons, redirecting growth toward the bead ($n = 5$). (K–M) GFP and Lhx3 labeling in control MN column explants dissected from *Hb9::GFP* embryos. (N–P) GFP and Lhx3 labeling in MN column explants treated with FGF8. (K–P) Expression of Lhx3 in the MN column explants was revealed by whole-mount immunohistochemistry of the explants using anti-Lhx3 antibody. MN column explants ($n > 3$) were dissected from the brachial spinal cord and cultured for 15 hr. Scale bars, 100 μm in (A)–(J), 150 μm in (K)–(P), and 70 μm (insets in [H] and [J]).

Typically, motor axons extend away from the floor plate (included within the explant) because the midline is the source of motor neuron repellents (Brose et al., 1999; Guthrie and Pini, 1995; Tzarfati-Majar et al., 2001), which creates a lateral bias in axon growth. In this culture configuration, we found that motor axons were reoriented to grow toward the bead (Figures 2I and 2J). These results indicate that FGF proteins function as long-range chemoattractants for developing motor axons.

FGFs have also been shown to regulate neuroepithelial cell fate specification at early developmental stages (reviewed in Diez del Corral and Storey, 2004). Although this signaling occurs within dividing progenitors prior to motor neuron generation, we examined whether FGFs might have an indirect effect on postmitotic motor axon growth by altering gene expression. To assess this, we assayed the expression profiles of several motor neuron-specific transcription factors, including *Hb9*, *Isl1*, and *Lhx3*, using whole-mount immunohistochemistry on MN column explants cultured in the presence or absence of FGF. We detected no apparent change in the expression patterns of these molecular markers in the presence of FGF8 compared to controls (Figures 2K–2P; see Figure S1 in the Supplemental Data). These results suggest that FGF elicits motor axon outgrowth from the explants without changing the columnar subtype identity of postmitotic motor neurons.

Expression of FGF Receptors in the Developing Mouse Spinal Cord

Although expression of FGF ligands in the dermomyotome has been well documented, the temporal and spatial expression of the four known FGF receptors have not been well characterized in the mouse neural tube during the period of motor axon pathfinding. We therefore examined whether the sites and timing of expression of *FGFR* mRNAs were consistent with a role in motor axon guidance, focusing on stages from E10.5 to E11.5 when MMCm axons project toward the dermomyotome. *FGFR4* mRNA was not detected in the neural tube at these stages (Figures 3D and 3I), whereas *FGFR1–3* were present. Specifically, *FGFR2* and *FGFR3* were expressed by progenitor cells within the ventricular zone spanning much of the dorsoventral axis of the neural tube, with *FGFR2* levels appearing higher in the dorsal region and *FGFR3* more abundant ventrally (Figures 3B, 3C, 3G, and 3H). Although *FGFR1* mRNA levels appeared to be low, expression was detected in a subset of motor neurons located in the ventral-medial region of the neural tube (Figures 3A and 3F; Figure S2).

To determine whether *FGFR1*⁺ cells in the ventral spinal cord are MMCm neurons, we characterized the settling pattern of MMCm neurons at E10.5 and E11.5 using *Lhx3* as a marker in combination with GFP labeling in *Hb9::gfp* embryos. At E10.5, *Lhx3*⁺ MMCm cells have just begun to settle laterally (Figures 3K–3M), and at E11.5 they represent a subset of the total GFP⁺ motor neuron population and are situated in a compact ventral-medial position (Figures 3N–3P). Thus, the position of MMCm cells at E10.5 and E11.5 overlaps with the pattern of *FGFR1* expression. On the other hand, mRNA for *c-Met*, a receptor for HGF, was expressed broadly in

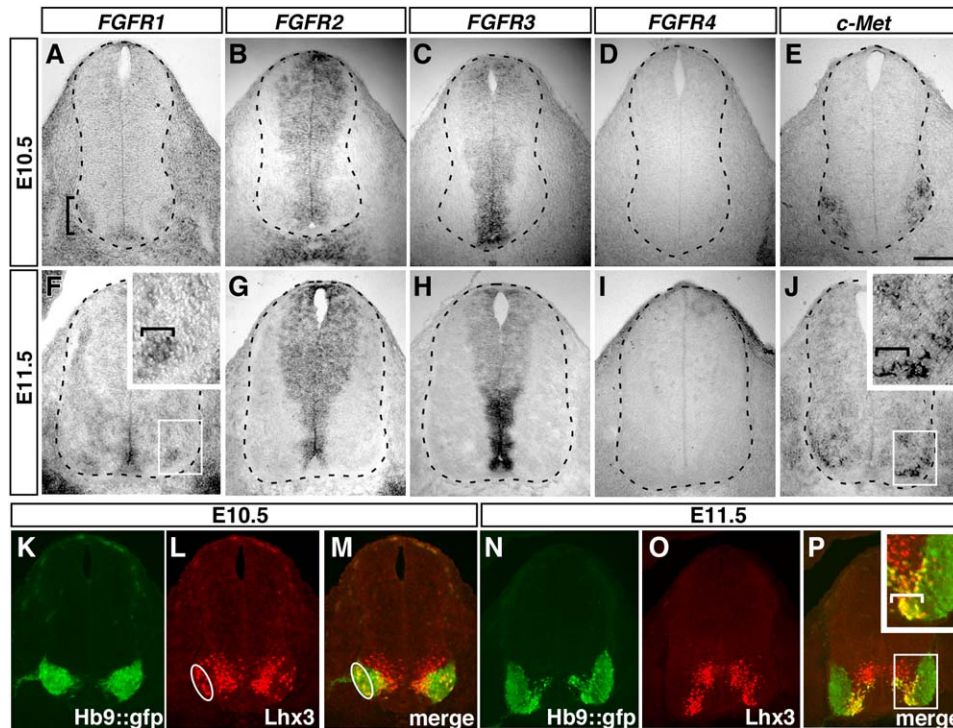


Figure 3. Expression of *FGFRs* and *c-Met* during the Period of MMCm Motor Axon Pathfinding

Detection of FGF and HGF receptors by in situ hybridization in the brachial spinal cord of E10.5 (A–E) and E11.5 (F–J) embryos. *FGFR2* and *FGFR3* are expressed in the ventricular zone. *FGFR1* is detected in a subset of ventrally located postmitotic motor neurons, whereas *c-Met* has a broader distribution in motor neurons. Brackets in (A), (F), (J) and (P) indicate location of MMCm cells. (K–M) GFP and Lhx3 coexpression define the location of MMCm motor neurons (oval) at E10.5 in the brachial neural tube of *Hb9::gfp* embryos. (N–P) Similarly, brachial E11.5 MMCm motor neurons (bracket, [P]) are defined by GFP/Lhx3 colabeling. Scale bars, 100 μm in (A)–(E), 140 μm in (F)–(J), 105 μm in (K)–(M), and 150 μm in (N)–(P).

several subsets of motor neurons, including both LMC (Lhx3-negative) and MMCm (Lhx3-positive) cells (Figures 3E and 3J). Together, these expression data are consistent with the possibility that FGFs, acting via *FGFR1*, are responsible for the long-range chemoattraction of MMCm motor axons to the dermomyotome.

Involvement of *FGFR* Signaling in Dermomyotome-Derived Chemoattraction of Motor Axons

To examine whether the dermomyotome-derived chemoattractive activity is mediated by *FGFR* signaling, we tested whether attraction of motor axons to the dermomyotome is blocked in the presence of SU5402, a selective inhibitor of FGF receptor signaling. SU5402 functions by interacting with the catalytic domain of the FGF receptors, thereby suppressing their tyrosine kinase activity and disrupting their ability to signal (Mohammadi et al., 1997). To assess the specificity of this FGF receptor tyrosine kinase inhibitor, HGF/*c-Met* signaling was utilized as a control. We found that, whereas SU5402 had no effect on HGF-induced motor axon outgrowth (Figures 4G and 4H), it inhibited dermomyotome-triggered chemoattraction of motor axons (Figures 4A, 4B, and 4E). To eliminate the possibility that SU5402 acts indirectly on the dermomyotome rather than the motor neurons in the explants, we next tested the effects of recombinant FGF8 in this explant assay. We found that SU5402 blocked axon outgrowth induced by the FGF8 protein (Figures 4C, 4D, and 4F). Thus, these results

indicate that *FGFR* signaling in neurons is required for the long-range chemoattraction of motor axons elicited by the dermomyotome.

Although multiple FGF receptors are expressed within the embryonic spinal cord (Figure 3), only *FGFR1* appeared to be expressed by MMCm cells when their axons navigate to the dermomyotome. To determine whether *FGFR1* is necessary for proper innervation of epaxial musculature, we examined motor neuron projections using a conditional allele of *FGFR1* (*FGFR1^{fl/fl}*) (Ohkubo et al., 2004; Pirvola et al., 2002) since null mutants of the receptor fail to develop beyond gastrulation (Deng et al., 1994; Yamaguchi et al., 1994). *Nestin::Cre* mice and *Hb9::gfp* mice were crossed with the conditional *FGFR1^{fl/fl}* line to generate embryos with GFP-labeled motor axons in which *FGFR1* was selectively deleted from neural progenitor cells. The dorsal ramus was present in *FGFR1^{fl/fl}; Nestin::Cre; Hb9::gfp* embryos, but motor axon guidance defects were frequently observed in the area where MMCm axons turn from the spinal nerve toward the dermomyotome. Motor axons were less fasciculated in conditional *FGFR1* mutants when compared to spinal nerves in *FGFR1^{fl/wt}* littermate controls (Figures 5A–5D). In some segments, a large portion of the axons projecting to the dermomyotome first turn toward the dorsal root ganglion (DRG) before correcting their trajectory, leading to a wavy-appearing dorsal ramus branch (compare Figures 5F to 5H). The most obvious phenotypes, however, were inappropriate

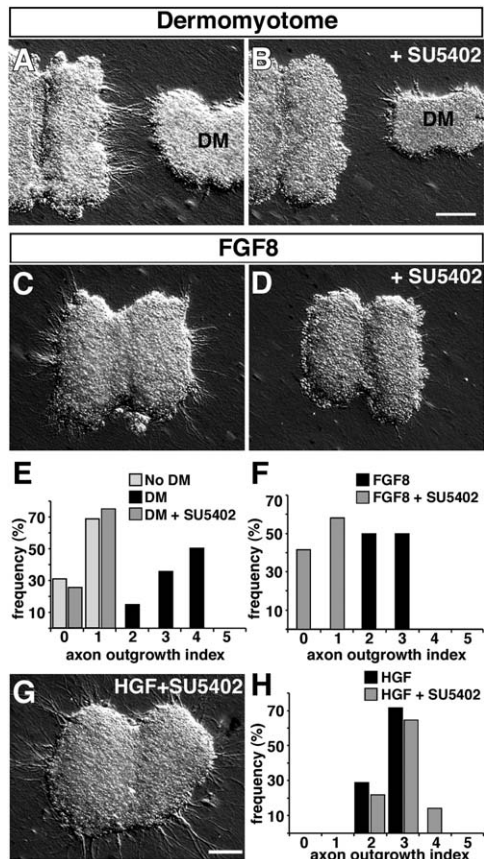


Figure 4. Blocking of FGFR Signaling Inhibits Motor Axon Responsiveness to the Dermomyotome-Derived Chemoattractant
 (A) Coculture of MN column explants with the dermomyotome (DM) mock-treated with DMSO ($n = 7$). (B) In the presence of the FGFR signaling inhibitor SU5402 ($12.5 \mu\text{M}$), motor axon responsiveness to the dermomyotome-derived chemoattractant is abolished ($n = 8$). (C) MN column explants cultured in the presence of FGF8 mock-treated with DMSO vehicle ($n = 8$). (D) FGF8-mediated axon outgrowth from the explants is blocked by SU5402 ($n = 6$). (E, F, H) Motor axon outgrowth was scored blindly on a 0–5 scale (see Figure S3) by two independent observers. The frequency (percentage) of explants with axonal outgrowth at each index level from 0–5 is shown as a histogram for each of the indicated culture conditions. (E) Histogram comparing responses elicited by the dermomyotome in the presence or absence of SU5402 ($12.5 \mu\text{M}$). “No DM” represents MN column explants cultured alone. DM-mediated outgrowth in the presence of SU5402 was significantly reduced compared with DM controls cultured with vehicle alone (DMSO) ($p < 0.001$, Mann-Whitney U test). Axonal growth from SU5402-treated explants did not statistically differ from MN column explants cultured alone (No DM) ($p = 0.78$, Mann-Whitney U test). (F) Histograms comparing responses elicited by FGF8 in the presence of SU5402 ($12.5 \mu\text{M}$) or DMSO. FGF8-mediated axon outgrowth was significantly reduced in the presence of SU5402 ($p < 0.001$, Mann-Whitney U test). (G) Unlike FGF8, HGF-mediated axon outgrowth from MN explants is not blocked by $12.5 \mu\text{M}$ SU5402 ($n = 7$). (H) Histograms comparing responses elicited by HGF in the presence or absence of SU5402 ($12.5 \mu\text{M}$). HGF-induced outgrowth in the presence of SU5402 was not statistically different from that observed from MN column explants cultured with HGF in the absence of SU5402 ($p = 0.45$, Mann-Whitney U test). Scale bars, $100 \mu\text{m}$ in (A–D) and (G).

motor axons that were found to grow far into the DRG (Figures 5A–5H). The conditional deletion of *FGFR1*^{fl/fl} using *Nestin::Cre* was not found to alter the normal pattern of expression of *Isl1/2* and *Lhx3* in motor, sensory, and spinal interneurons (Figures 5E, 5G, and 5I–5L). Taken together, our findings suggest that FGFR1 signaling is required in MMCm motor neurons for their proper navigation to the dermomyotome. The finding that the dorsal ramus nerve forms in conditional *FGFR1* mutants, however, suggests that the timing and/or efficiency of Cre-mediated recombination was not complete. In addition, it is possible that MMCm axons are guided by multiple classes of guidance cues, of which FGFs/FGFR1 may participate with other signals.

The Dermomyotome and FGF Selectively Attract MMCm Axons

Although the above results support the view that the dermomyotome secretes a chemoattractant for MMCm axons, they do not directly address whether the action of the dermomyotome-derived chemoattractant is specific for MMCm cells. For example, the dermomyotome could be the source of a general motor axon outgrowth factor or possibly promote the growth of axons from non-MMCm neurons, such as LMC cells. To address these possibilities, we first took advantage of an anatomical feature of the spinal cord. Namely, since MMCm cells reside along the entire rostrocaudal length of the spinal cord, whereas LMC cells are only located at brachial and lumbar levels, we isolated explants from thoracic levels devoid of LMC motor neurons. We found that the dermomyotome, as well as FGF, attracted motor axons from the thoracic MN column explants (Figures 6A–6D), thereby excluding the possibility that the dermomyotome attracts only LMC axons.

Next, we directly tested whether the dermomyotome-derived attraction of motor axons is specific for MMCm cells. To accomplish this we used an evolutionarily conserved MMCm-specific enhancer (*SE1*, *CREST1*) identified 3' to the *Isl1* gene (Uemura et al., 2005) to generate a transgenic mouse line in which MMCm axons are labeled by GFP (Figure 6N). *SE1::gfp* embryos have GFP-labeled axons within the dorsal ramus leading to the dermomyotome as well as *Hb9⁺/Lhx3⁺/GFP⁺* MMCm cell bodies (Figures 6E–6I, 6K, and 6M). By comparison, whole-mount preparations of *Hb9::gfp* embryos revealed labeling of all somatic motor axon pathways, including branches into the limb, body wall, and dorsal ramus (Figures 6J and 6L). Thus, GFP selectively labels MMCm cell bodies and axons in *SE1::gfp* embryos.

We next dissected MN column explants from *SE1::gfp* embryos to directly monitor MMCm axon growth in response to the dermomyotome-derived factor in explant culture. We found that the axons attracted by the dermomyotome as well as by FGF8-loaded beads were labeled with GFP (Figures 6O–6R). These results therefore indicate that MMCm cells respond to the dermomyotome-derived chemoattractant, whereas other motor neuron subclasses appear to lack responsiveness.

The LIM Code Programs Motor Axon Responsiveness to the Dermomyotome

Non-MMCm motor neurons, such as the LMC, express *Hb9* but lack *Lhx3*, whereas MMCm cells express both

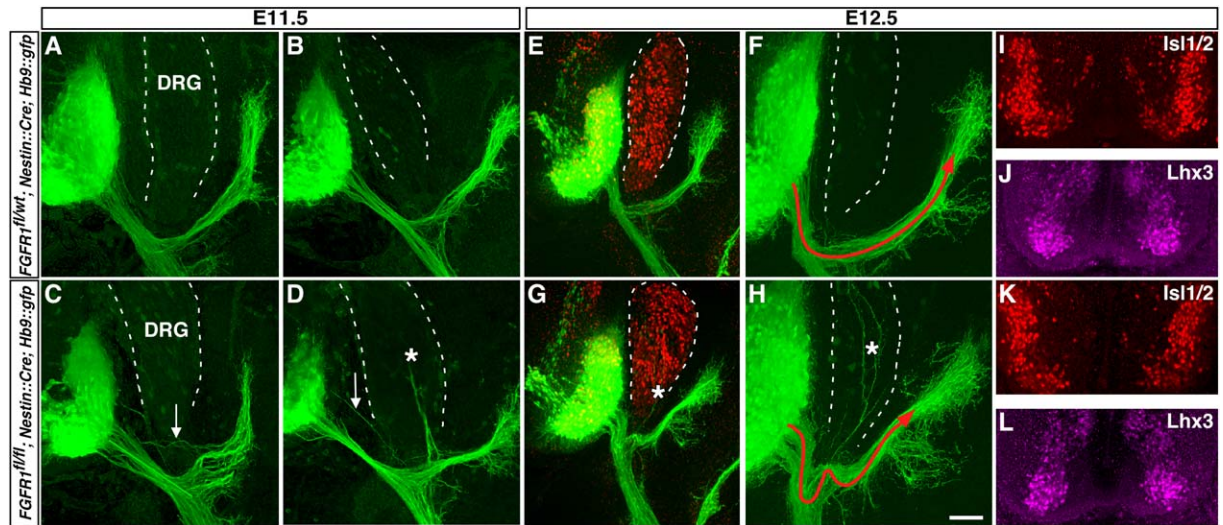


Figure 5. Proximal Motor Neuron Guidance Defects Arise in *FGFR1* Mutants

(A–D) GFP-labeled motor axons in E11.5 control embryos (*FGFR1^{fl/wt}; Nestin::Cre; Hb9::gfp*) and neural tube-specific *FGFR1* mutant embryos (*FGFR1^{fl/fl}; Nestin::Cre; Hb9::gfp*) at the brachial level. Axons in *FGFR1^{fl/fl}* animals (C and D) are less fasciculated than in *FGFR1^{fl/wt}* animals (A and B) as they exit the spinal cord, and often deviate from the spinal nerve (arrows) or errantly project into the dorsal root ganglion (DRG) (asterisk).

(E–H) Defects in axon guidance to the dermomyotome are also apparent in *FGFR1^{fl/fl}* animals at E12.5. Motor axons (green) normally avoid the *Isl1/2⁺* DRG (red nuclei in [E] and [G]) in *FGFR1^{fl/wt}* controls, whereas motor axons are inappropriately found within the DRG in *FGFR1^{fl/fl}* animals (asterisks).

(I–L) Expression of motor neuron markers *Isl1/2* and *Lhx3* appear normal in E12.5 *FGFR1^{fl/fl}* mice compared to *FGFR1^{fl/wt}* controls. At least 3 embryos were assayed for each age and genotype.

Scale bars, 50 μm in (A)–(D), 115 μm in (E) and (G), 65 μm in (F) and (H), and 70 μm in (I)–(L).

Hb9 and *Lhx3* (Sharma et al., 1998, 2000). Forced expression of *Lhx3* in Hb9-positive motor neurons has been shown to switch their subclass identity to that of MMCm cells (Sharma et al., 2000). This conversion is accompanied by an increase in the number of motor axons projecting toward the dermomyotome, leading to the prediction that the number of motor axons which respond to the dermomyotome-derived chemoattractant should increase in *Lhx3-ki* mice. To test this, we first examined whether the number of motor axons responding to the dermomyotome-derived chemoattractant is increased when using MN column explants derived from *Lhx3-ki* mice. As predicted, explants from *Lhx3-ki* mice exhibited a dramatic increase in the number of axons attracted by the dermomyotome compared with explants from wild-type embryos (Figures 7A, 7B, and 7M; also compare Figure 1F to Figure 7B). Furthermore, crossing the *Hb9::gfp* transgene into the *Lhx3-ki* mouse background revealed that the increased response was due to an enhancement in the number of GFP⁺ motor axons rather than axons from other neuronal types (Figures 7D–7H). Thus, the subtype interconversion of non-MMCm motor neurons into MMCm cells is accompanied by an increase in projections to the dermomyotome in vivo (Sharma et al., 2000) as well as an increase in the number of cells that respond to the dermomyotome-derived chemoattractant in vitro.

These findings raised the possibility that the LIM code regulates the expression of the guidance receptors in MMCm cells. Since FGF receptors are required to mediate the responsiveness to the dermomyotome-derived activity, we next asked whether expression of *FGFR1*

is shifted to include more motor neurons due to the reprogramming of motor neuron identity in *Lhx3-ki* mice. We found that the expression of *FGFR1* is expanded to include motor neurons in which *Lhx3* expression has been forced (Figures 7I–7L) without affecting expression of other *FGFRs* (Figure S2). This prompted us to test whether the MN column explants from *Lhx3-ki* mice exhibit increased responsiveness to FGF8. As expected, we detected a significant increase in motor axon chemoattraction in response to FGF8 using explants from *Lhx3-ki* mice compared with explants from wild-type control mice (Figures 7C and 7N; compare Figures 2C to 7C). Together, these results demonstrate that *FGFR1* signaling in MMCm cells constitutes one of the downstream components regulated by the LIM code when MMCm axons project to the dermomyotome.

Discussion

The genetic programs that underlie motor neuron differentiation and subtype diversification are regulated sequentially by unique combinations of LIM homeodomain factors. Importantly, LIM factors also regulate genetic programs that specify where axons grow, presumably by controlling the expression of axon guidance molecules (reviewed in Jessell, 2000; Shirasaki and Pfaff, 2002). In this study, we examined how the MMCm subclass of motor neurons targets the axial musculature embryologically derived from the dermomyotome. We found that FGFs are long-range chemoattractants secreted by the dermomyotome and that the LIM code programs MMCm cells to express *FGFR1*.

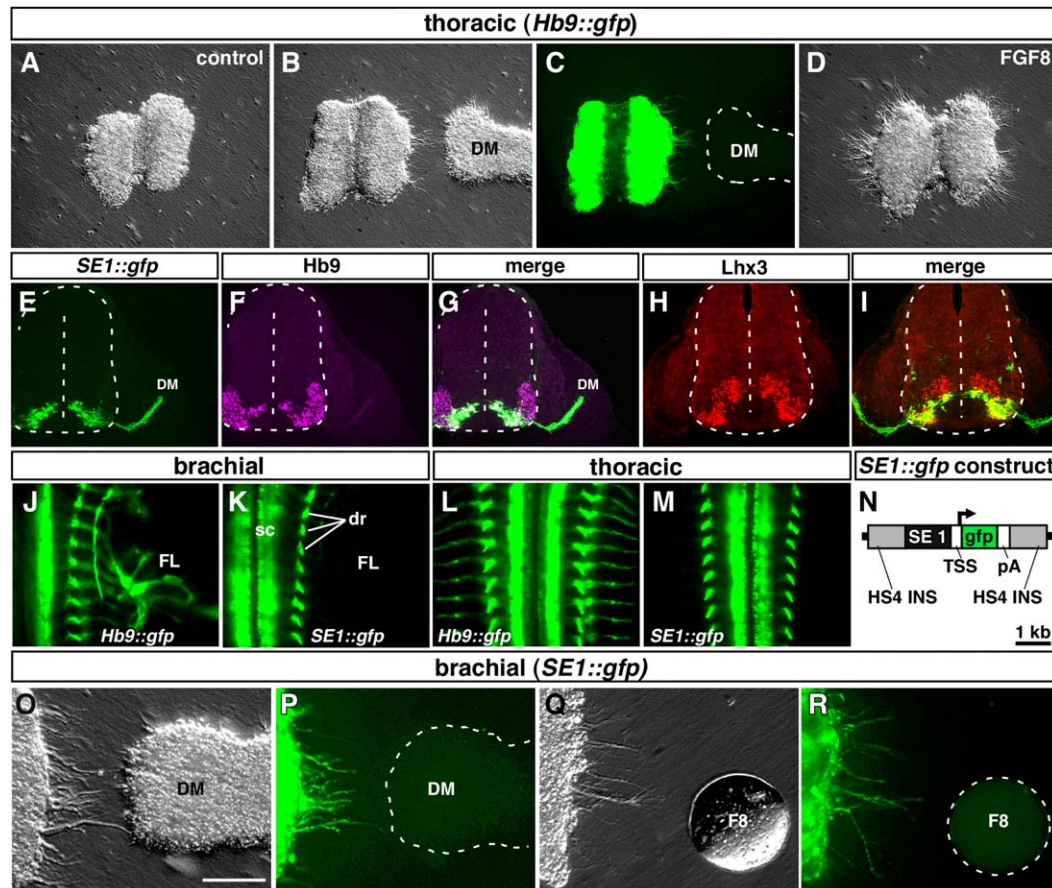


Figure 6. Selective Chemoattraction of MMCm Motor Axons by the Dermomyotome and FGF

(A–D) Thoracic MN column explants from *Hb9::gfp* embryos cultured for 15 hr. (A) Axon outgrowth is minimal from MN explants cultured alone ($n = 3$). (B) The dermomyotome (DM) attracts axons from MN explants ($n = 8$). (C) Fluorescent view of (B) reveals GFP-labeling of motor axons. (D) FGF8 induces motor axon outgrowth from thoracic MN explants ($n = 7$). (E–N) *SE1::gfp* reporter mice display selective labeling of MMCm motor neurons. (E–G) GFP labels a subset of ventro-medial $Hb9^+$ motor neurons (magenta) and axons growing toward the dermomyotome (DM) at E11.5. (H and I) The MMCm marker *Lhx3* (red) is expressed in GFP-labeled cells within the brachial spinal cord of *SE1::gfp* mice at E11.5. (J) Fluorescent whole-mount image of *Hb9::gfp* mice at E11.5. Dorsal view of the right half of the brachial level is shown. Limb-innervating GFP⁺ motor axons have entered the forelimb (FL). (K) Dorsal view of brachial level in *SE1::gfp* mice at E11.5. Dermomyotome-projecting GFP⁺ MMCm axons can be selectively visualized in the dorsal ramus (dr). (L) Fluorescent image of dorsal whole-mount view of E11.5 *Hb9::gfp* embryo. At the thoracic level, MMCm axons projecting to the dermomyotome as well as non-MMCm axons projecting to muscles of the ventral body wall are GFP labeled. (M) Dorsal thoracic view of *SE1::gfp* embryo at E11.5. GFP labeling is restricted to MMCm axons. (N) DNA construct used to generate *SE1::gfp* transgenic mice (see Experimental Procedures). (O and P) Coculture of brachial *SE1::gfp* MN column explants with the dermomyotome ($n = 6$). (P) Fluorescent view of (O) reveals DM attraction of MMCm GFP⁺ axons. (Q and R) Coculture of brachial *SE1::gfp* MN column explants with an FGF8-loaded bead ($n = 4$). As is the case with the DM coculture, the FGF8-bead (F8) attracts GFP⁺ axons. By contrast, HGF attracts many motor axons lacking GFP from *SE1::gfp*-derived MN explants (see Figure S4). Scale bars, 200 μm in (A)–(D), 190 μm in (E)–(I), 400 μm in (J)–(M), and 95 μm (O)–(R).

Downstream Effectors of the LIM Code: Axon Guidance Molecules

MMCm motor neuron identity is regulated by the combinatorial actions of the LIM factors *Isl1* and *Lhx3* (Sharma et al., 2000). Mice genetically engineered to artificially create the MMCm-LIM code in all motor neuron subtypes develop with limb-innervating LMC cells converted into MMCm cells. These converted LMC cells share many properties with the native MMCm motor neurons, including axon growth toward the dermomyotome. In some instances, the artificially generated MMCm cells overshoot the normal dorsal ramus choice point, but correct themselves by arching back to their axial targets as if they are responding to a long-range, target-derived signal (Sharma et al., 2000). Nevertheless,

direct evidence for a dermomyotome-derived chemoattractant has not been established. Here we show that FGF mediates selective attraction of MMCm axons. Furthermore, we show that MMCm cells are programmed to respond to FGF chemoattraction by the LIM code through selective expression of FGFR1 in this motor neuron subtype.

In addition to their role in MMCm cells, LIM factors regulate guidance receptors in a variety of other neuronal types. At the base of the limb, LMCI motor axons selectively enter the dorsal compartment of the limb bud. The selective guidance of LMCI cells is dependent on *Lhx1*, a LIM homeodomain factor selectively expressed by this motor neuron subclass (Kania et al., 2000). Interestingly, the expression of the EphA4 receptor tyrosine

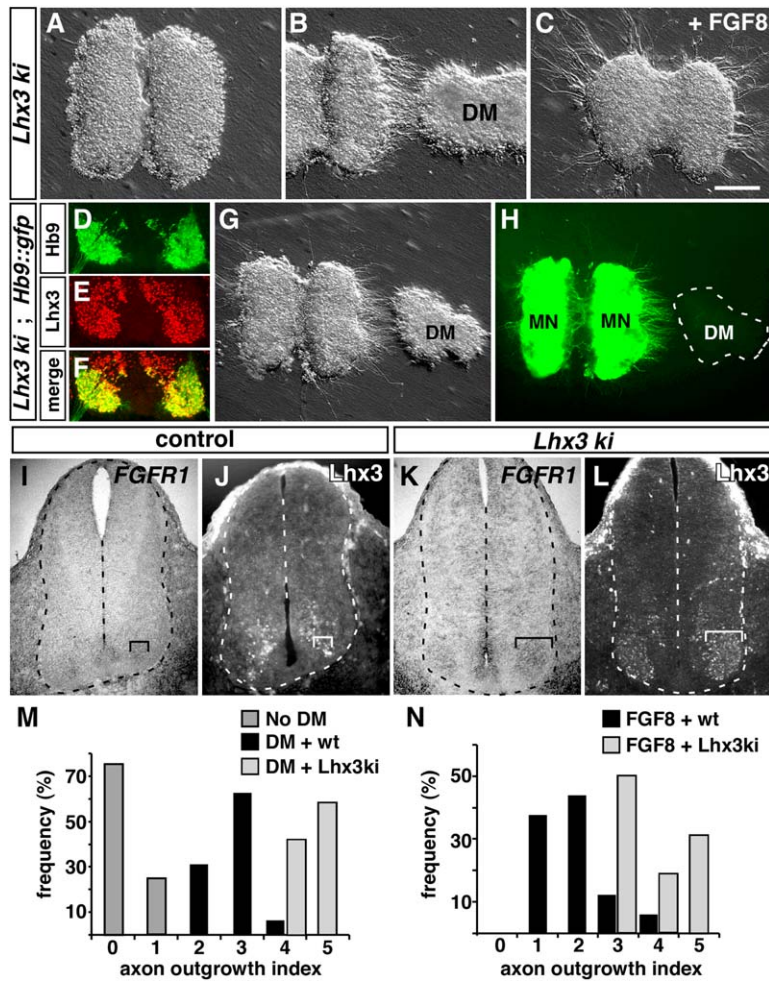


Figure 7. The LIM Code Is Capable of Regulating FGF Responsiveness in Motor Neurons

(A–C) Brachial MN column explants from *Lhx3-knockin (ki)* embryos. (A) Like MN explants from wild-type embryos, few axons extend from *Lhx3-ki* explants when cultured alone ($n = 8$). (B) Coculture of *Lhx3-ki* explants with the dermomyotome (DM) induces robust axon outgrowth toward the DM ($n = 6$). (C) FGF8 induces many axons to extend from *Lhx3-ki* explants ($n = 8$).

(D–F) Ventral brachial spinal cord of *Lhx3-ki* embryo in an *Hb9::gfp* background at E11.5. *Lhx3* expression (red) is expanded to include all GFP⁺ motor neurons (see also Sharma et al., 2000).

(G) Coculture of *Lhx3-ki* explants (*Hb9::gfp* background) with the DM.

(H) Fluorescent image of (G), showing that GFP⁺ motor axons respond to the DM-chemoattractant.

(I–L) Brachial spinal cord of wild-type and *Lhx3-ki* embryos at E11.5. (I and J) In situ hybridization detects *FGFR1* transcript in *Lhx3*⁺ MMCm motor neurons in control embryos (littermates to *Lhx3-ki* embryos). (K and L) Expression of *FGFR1* is expanded in *Lhx3-ki* embryos to include ectopic *Lhx3*⁺ motor neurons. (I) and (K) are adjacent sections to (J) and (L), respectively.

(M) Histogram comparing the frequency of explants with axonal outgrowth similar to the index shown in Supplemental Figure S3 based on three independent experiments for each condition. Wild-type and *Lhx3-ki* explants were cultured with the DM. Axon outgrowth from *Lhx3-ki* explants (DM+*Lhx3ki*) was significantly greater than that observed from wild-type mouse explants (DM+wt) ($p < 0.001$, Mann-Whitney U test). “No DM” represents *Lhx3-ki*-derived explants cultured alone.

(N) Like the DM response, axon outgrowth induced by FGF8 from *Lhx3-ki* explants (FGF8+*Lhx3ki*) was significantly greater than that observed from wild-type mouse MN explants (FGF8+wt) ($p < 0.001$, Mann-Whitney U test).

Scale bars, 100 μm in (A)–(C), 120 μm in (D)–(F), 130 μm in (G and H), and 175 μm in (I)–(L).

kinase is under the control of *Lhx1* (Kania and Jessell, 2003), thus contributing, at least in part, to the proper dorsal trajectory of these axons as they enter the limb (Eberhart et al., 2002; Helmbacher et al., 2000; Kania and Jessell, 2003). In the retina, *Isl2* controls ganglion cell axon crossing at the optic chiasm and has been implicated in repressing, either directly or indirectly, the expression of *Zic2* and *EphB1* (Pak et al., 2004). Thus, expression of *Eph*/*ephrin* guidance receptors on growing axons is regulated in several cases by LIM factors in neuronal subpopulations.

In *Drosophila* embryos, a combinatorial code of *Islet* (*Isl1/2* homolog) and *Lhx3* specifies axon target selection of a subset of motor neurons that innervate ventral muscles (Thor et al., 1999). Interestingly, a recent study has shown a strong genetic interaction between *Islet*, *Lhx3*, and *Beaten path* (*Beat*) *Ic* (Certel and Thor, 2004), a member of a multigene family encoding immunoglobulin-superfamily cell adhesion molecules (IgSF CAM) related to the *Beat Ia* axon guidance molecule. This suggests that *Beat Ic* may be a downstream transcriptional target of the LIM code involved in *Drosophila*

axon pathway selection. In *C. elegans*, a regulatory link between the LIM homeodomain transcription factors *ceh-14* (*Lhx3* homolog) and *lim-6* (*Lmx1* homolog) has been made to the IgSF *zig* genes (Aurelio et al., 2003). These genetic studies in flies and worms therefore suggest that LIM factors could likewise control the expression of IgSF genes in the vertebrate CNS.

It should be noted that semaphorin-mediated signaling may also be under the control of LIM transcription factors. In zebrafish embryos, *Isl2* is required for the proper outgrowth and peripheral axon branching of sensory neurons (Segawa et al., 2001). Notably, it has been shown that class III secreted semaphorins are recognized by *PlexinA4*, a transmembrane protein which forms a coreceptor complex with *neuropilin* (reviewed in Fujisawa, 2004). Since this signaling pathway regulates branching of peripheral sensory axons (Miyashita et al., 2004), *Isl2* may control neuronal responses to secreted semaphorins. Consistent with this, motor neurons that innervate the ventral portion of the limbs (*Isl1/2*⁺ LMCm cells) are guided by *Npn2*/*Sema3F*-mediated signaling (Huber et al., 2005).

Identification of a Guidance Cue that Selectively Attracts a Subset of Motor Neurons

HGF can function as a long-range chemoattractant for developing spinal motor axons in vitro (Ebens et al., 1996). Many embryonic tissues encountered by growing motor axons express HGF, such as cells at the base of the limb (Ebens et al., 1996). Indeed, the expression of the receptor for HGF, c-Met, is not confined to a specific motor neuron subtype such as the LMCI and LMCm (Figure 3; see also Ebens et al., 1996). Consistent with this, our preliminary in vitro assays using explants derived from mice with GFP labels in specific neuronal populations (*SE1::gfp* mice) indicated that HGF triggers axon outgrowth from multiple motor neuron subtypes (Figure S4). Therefore, HGF exhibits long-range chemoattractive activity, but seems to function in a general way to stimulate the growth of multiple subclasses of motor neurons.

The dermomyotome expresses several FGFs, such as FGF4, FGF8, and FGF9 during the period when MMCm axons grow toward this target (Colvin et al., 1999; Crossley and Martin, 1995; Niswander and Martin, 1992; Thisse and Thisse, 2005, and references therein). We found that *FGFR1* expression is restricted to MMCm motor neurons during axonogenesis, which likely accounts for the selective attraction of MMCm axons by FGF. Our axon outgrowth assays were based on brachial- (lower cervical) and thoracic-level spinal cord explants. MMCm motor neurons form a continuous column of cells extending the length of the spinal cord. Although we did not test MN explants derived from lumbosacral levels, we did find that *FGFR1* is expressed within motor neurons located at caudal levels of the spinal cord (data not shown). Thus, it is likely that MMCm motor neurons at all spinal cord levels are attracted by FGFs.

Despite the finding that FGF can attract MMCm axons, what evidence suggests that the dermomyotome factor is FGF? Axon outgrowth from MN explants in response to the dermomyotome is blocked by SU5402, an FGF receptor antagonist. In addition, direct evidence for FGF-mediated attraction of MMCm axons comes from the finding that motor axon guidance defects occur when the *FGFR1* gene is selectively deleted in neural tube cells. It should be noted, however, that a previous study was unable to detect motor axon chemoattractive activity from the dermomyotome using explant cultures and also observed no effect when testing FGF2 (Ebens et al., 1996). Possibly the dermomyotome failed to differentiate properly in culture and/or motor axons lost FGF responsiveness in vitro. An important consideration with FGFs, however, is their dependence on interactions with heparan sulfate proteoglycans (HSPGs) for proper biological activity (reviewed in Ornitz, 2000). In our cultures we included HSPGs (present in matrigel), which likely facilitated the detection of FGF activity.

Role of FGFR Signaling in Axon Guidance

FGFRs comprise a gene family of four membrane-bound receptor tyrosine kinases that interact with at least 22 FGF ligands. FGFR signaling is critical for many biological processes, including regulation of cell proliferation, cell migration and differentiation, mesoderm induction and patterning, and organ formation and regeneration

(reviewed in Thisse and Thisse, 2005). Recent studies have also begun to identify functions for this receptor family in wiring of the nervous system. For example, in the developing *Xenopus* visual system, FGFR1 signaling contributes to the initial target recognition of retinal ganglion cell axons in the optic tectum (McFarlane et al., 1996). In the developing cerebral cortex, FGF2 has the ability to induce interstitial axon branching of cortical pyramidal neurons in vitro (Szebenyi et al., 2001), which is thought to contribute to the formation of collateral branching of layer five projection neurons toward their target cells. Furthermore, FGFR2 activation by FGF22 and its closely related family members, FGF7 and FGF10, contributes to synaptogenesis by inducing vesicle aggregation in nerve terminals of cerebellar mossy fibers (Umemori et al., 2004).

Evidence for FGF signaling in axon guidance also came from a study focusing on the isthmus, an FGF8-releasing organizing center at the segmental boundary between the midbrain and hindbrain (Irving et al., 2002). Trochlear motor axons in the rostral hindbrain grow dorsally, away from the floor plate along the isthmus to exit the neural tube. Although isthmus tissue and FGF8 lack neurite outgrowth promoting activity for trochlear motor axons, they can redirect the growth of these axons in vitro (Irving et al., 2002). It therefore seems likely that FGF8 expressed in the path of these axons plays a role in constraining their trajectory along the segmental boundary at the midbrain-hindbrain junction. Interestingly, the action of FGF on trochlear axons is somewhat different from that of classical chemoattractants such as netrins, HGF, and neurotrophins in that they also possess neurite outgrowth-promoting activity (Ebens et al., 1996; O'Connor and Tessier-Lavigne, 1999; Serafini et al., 1994). We detected a strong axon outgrowth-promoting activity in our in vitro assays, indicating that, unlike the case of trochlear axons, the action of FGF on MMCm axons is similar to that of other classical chemoattractants.

Among the four FGFRs, we found that *FGFR1* is specifically expressed in MMCm cells at the stage when their axons grow toward the dermomyotome. Consistent with this observation, chemoattraction was observed with FGF2, FGF4, FGF8, and FGF9, all of which bind FGFR1 (reviewed in Ornitz et al., 1996), whereas the FGFR2 ligand FGF10 lacked outgrowth activity. Furthermore, in vitro studies using PC12 cells have revealed that FGFR1 signaling induces more potent and persistent neurite outgrowth than other members of the FGFR family (Lin et al., 1996; Raffioni et al., 1999). We examined motor axon projections in conditional mutants of *FGFR1* using *Nestin::Cre* to delete the gene within developing motor neurons and found that axonal navigation in the region of the dorsal ramus choice point was abnormal. In contrast, when we examined *FGFR3* null embryos (Colvin et al., 1996) we did not detect motor axon guidance defects (data not shown), consistent with the observation that only *FGFR1* appears to be expressed by embryonic MMCm cells.

Although motor axon guidance defects occur within *FGFR1*^{fl/fl} embryos, many MMCm axons enter the epaxial musculature correctly despite the loss of FGFR1. We cannot eliminate the possibility that the timing and/or efficiency of Cre-dependent deletion of the *FGFR1*^{fl/fl}

allele is incomplete when MMCm cells are pathfinding; however, it seems more likely that these motor neurons will rely on multiple signals for proper axon pathfinding. For example, commissural axons are guided ventrally within the neural tube by the chemoattractant netrin-1 acting in collaboration with BMPs and Shh (Butler and Dodd, 2003; Charron et al., 2003; Serafini et al., 1996). Intriguingly, it has recently been shown that EphA4 binds directly and specifically to the juxtamembrane region of FGFR1, which leads to receptor transactivation (Yokote et al., 2005). Because MMCm motor neurons express both EphA4 (Eberhart et al., 2004) and FGFR1 (Figure 3) when they grow to the dermomyotome, it is possible that these receptors function coordinately to regulate MMCm axon guidance. Recently, cooperation between GDNF/Ret and ephrinA/EphA4 signaling has been reported for limb-innervating motor neurons (Kramer et al., 2006). By extension, FGF/FGFR1 and ephrinA/EphA may cooperate in a similar manner to guide MMCm motor neurons to epaxial muscle targets.

In summary, our observations identify FGF as a target-derived chemoattractant specific for cells within the MMCm motor column. FGFR1 is selectively expressed by MMCm cells and becomes ectopically expressed when motor neuron identity is reprogrammed using the LIM homeodomain transcription factor *Lhx3*, which is normally restricted to MMCm cells. Thus, the collective weight of the results presented in this study further unravel how downstream axon guidance effectors of the LIM code direct correct wiring of the nervous system during development. Moreover, our study further strengthens the notion that, like other embryonic patterning molecules such as Shh, BMPs, and wnts, FGF also represents a multitasking molecule that acts as a critical coordinator in many locations throughout development.

Experimental Procedures

Mice

The generation of Tg (*Hb9::gfp*), *Lhx3-ki*, and *FGFR3* null mice have been previously described (Colvin et al., 1996; Lee et al., 2004; Sharma et al., 2000). Tg (*SE1::gfp*) utilized an MMCm specific enhancer (SE1) isolated from the mouse *Isl1* gene. This enhancer was based on the CREST1 regulatory element reported previously (Uemura et al., 2005). SE1 was cloned from mouse genomic DNA by PCR and refined through deletion studies to be described elsewhere (S.K. Lee, B. Lee, and S.L.P.). The Tg (*SE1::gfp*) construct was generated by fusing the SE1 element to a minimal CMV promoter to drive the expression of eGFP (BD Biosciences). The construct utilized an SV40 polyadenylation sequence and was flanked by 1.2 kb insulator sequences isolated from the chicken β -globin locus (West et al., 2002). Motor axon projection phenotypes were examined in conditional *FGFR1* null embryos (Ohkubo et al., 2004; Pirovola et al., 2002) by crossing *FGFR1^{fl/fl}* animals to mice expressing Cre recombinase from a rat Nestin promoter and enhancer (Tronche et al., 1999) (available from Jackson Labs) in a transgenic *Hb9::gfp* background.

Explant Cultures

Neural tube, dermomyotome, and isthmus tissues from E9.75 to E10.0 mouse embryos (E0.5 denotes noon of the day of vaginal plug) were dissected in ice-cold DMEM/F12 (Invitrogen) with 25% fetal bovine serum after treatment with 0.6% trypsin in Ca²⁺/Mg²⁺ free Hanks' solution for 60 min on ice. Limb bud tissues were obtained from E10.5 embryos. Following complete removal of surrounding connective tissues, the ventral one-third of the brachial (forelimb) or thoracic level neural tube was cultured in an "open-book"

configuration. These MN column explants and cocultured tissues were embedded in three-dimensional matrix gels and cultured for 15 hr (Lumsden and Davies, 1983; Serafini et al., 1994). The matrix was a 1:1 mixture of rat tail collagen and Matrigel (BD Biosciences). Culture medium conditions for MN explants were as previously described (Shirasaki et al., 1998). When indicated, motor axon outgrowth was elicited by adding 100 ng/ml of recombinant FGF8b and 10 ng/ml of HGF proteins to the culture media. Recombinant proteins were all purchased from R&D systems. FGF2 (10 ng/ml), FGF4 (10 ng/ml), FGF9 (100 ng/ml), and FGF10 (100 ng/ml) were used for motor axon outgrowth assays. FGF10 was also tested up to 250 ng/ml for the brachial MN column explant assay, which also failed to induce motor axon outgrowth in the assay (data not shown). Where indicated, FGFR tyrosine kinase inhibitor SU5402 (Calbiochem) was added to the culture media (12.5 μ M). DMSO, the vehicle used for SU5402, was used in controls. For bead assays, heparin-coated acrylic beads (Sigma) were incubated in a solution of recombinant FGF8b proteins (5 μ g/ml) for 3 hr at room temperature. The beads were then washed several times in DMEM/F12 prior to use as a focal source of FGF in cocultures.

Immunohistochemistry and In Situ Hybridization

After explants were fixed with 4% paraformaldehyde in PB for 2 hr at 4°C, whole-mount immunohistochemistry was performed on cultured explants in the gels. Staining of explants with rabbit anti-Hb9, rabbit anti-*Isl1* (K5), and guinea pig anti-*Lhx3* was performed as described (Sharma et al., 1998, 2000; Shirasaki et al., 1998), except that 0.5% Triton X-100 was used for washes and antibody incubations. Immunohistochemistry on sections of embryos and in situ hybridization (Schaeren-Wiemers and Gerfin-Moser, 1993) were carried out as described (Lee et al., 2004; Sharma et al., 1998). In situ probes for *FGFR1*, *FGFR2*, and *FGFR3* were generated by amplification by RT-PCR from RNA derived from E11.5 mouse embryos. Probes for *HGF* and *c-Met* were gifts from Flavio Maina and *FGFR4* was obtained from Image Clone. To assay motor axon projections in conditional *FGFR1* null embryos, a series of images were obtained with an Olympus Fluoview 1000 confocal microscope from 100 μ m sections in the z plane and flattened.

Supplemental Data

The Supplemental Data for this article can be found online at <http://www.neuron.org/cgi/content/full/50/6/841/DC1/>.

Acknowledgments

We thank Flavio Maina for providing us with in situ probes for *c-Met* and *Hgf*; and the Developmental Studies Hybridoma Bank for antibodies. We are also grateful to Yasuhiko Kawakami for advice on limb development and to other members of the Pfaff lab for helpful discussions and encouragement. We thank Soo-Kyung Lee and Bora Lee for helping to generate the *SE1::gfp* mice, Flora Vaccarino for providing *FGFR1^{fl/fl}* mice, and David Ornitz and Jeff Esko for *FGFR3* mutant mice. R.S. was supported by fellowships from the JSPS Fellowships for Research Abroad, Uehara Memorial Foundation, the Pioneer Foundation, and the Maximilian & Marion Hoffman Foundation. J.W.L. is supported by a fellowship from NRSA. This work is supported by the NINDS.

Received: July 20, 2005

Revised: March 15, 2006

Accepted: April 19, 2006

Published: June 14, 2006

References

- Augsburger, A., Schuchardt, A., Hoskins, S., Dodd, J., and Butler, S. (1999). BMPs as mediators of roof plate repulsion of commissural neurons. *Neuron* 24, 127–141.
- Aurelio, O., Boulin, T., and Hobert, O. (2003). Identification of spatial and temporal cues that regulate postembryonic expression of axon maintenance factors in the *C. elegans* ventral nerve cord. *Development* 130, 599–610.

- Brose, K., Bland, K.S., Wang, K.H., Arnott, D., Henzel, W., Goodman, C.S., Tessier-Lavigne, M., and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* 96, 795–806.
- Butler, S.J., and Dodd, J. (2003). A role for BMP heterodimers in floor plate-mediated repulsion of commissural axons. *Neuron* 38, 389–401.
- Certel, S.J., and Thor, S. (2004). Specification of *Drosophila* motoneuron identity by the combinatorial action of POU and LIM-HD factors. *Development* 131, 5429–5439.
- Charron, F., Stein, E., Jeong, J., McMahon, A.P., and Tessier-Lavigne, M. (2003). The morphogen sonic hedgehog is an axonal chemoattractant that collaborates with netrin-1 in midline axon guidance. *Cell* 113, 11–23.
- Colvin, J.S., Bohne, B.A., Harding, G.W., McEwen, D.G., and Ornitz, D.M. (1996). Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat. Genet.* 12, 390–397.
- Colvin, J.S., Feldman, B., Nadeau, J.H., Goldfarb, M., and Ornitz, D.M. (1999). Genomic organization and embryonic expression of the mouse fibroblast growth factor 9 gene. *Dev. Dyn.* 216, 72–88.
- Crossley, P.H., and Martin, G.R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* 121, 439–451.
- Deng, C.X., Wynshaw-Boris, A., Shen, M.M., Daugherty, C., Ornitz, D.M., and Leder, P. (1994). Murine FGFR-1 is required for early post-implantation growth and axial organization. *Genes Dev.* 8, 3045–3057.
- Dickson, B.J. (2002). Molecular mechanisms of axon guidance. *Science* 298, 1959–1964.
- Diez del Corral, R., and Storey, K.G. (2004). Opposing FGF and retinoid pathways: a signalling switch that controls differentiation and patterning onset in the extending vertebrate body axis. *Bioessays* 26, 857–869.
- Ebens, A., Brose, K., Leonardo, E.D., Hanson, M.G., Jr., Bladt, F., Birchmeier, C., Barres, B.A., and Tessier-Lavigne, M. (1996). Hepatocyte growth factor/scatter factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. *Neuron* 17, 1157–1172.
- Eberhart, J., Swartz, M.E., Koblar, S.A., Pasquale, E.B., and Krull, C.E. (2002). EphA4 constitutes a population-specific guidance cue for motor neurons. *Dev. Biol.* 247, 89–101.
- Eberhart, J., Barr, J., O'Connell, S., Flagg, A., Swartz, M.E., Cramer, K.S., Tosney, K.W., Pasquale, E.B., and Krull, C.E. (2004). Ephrin-A5 exerts positive or inhibitory effects on distinct subsets of EphA4-positive motor neurons. *J. Neurosci.* 24, 1070–1078.
- Eisen, J.S., and Pike, S.H. (1991). The *spt-1* mutation alters segmental arrangement and axonal development of identified neurons in the spinal cord of the embryonic zebrafish. *Neuron* 6, 767–776.
- Fujisawa, H. (2004). Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. *J. Neurobiol.* 59, 24–33.
- Guthrie, S., and Pini, A. (1995). Chemorepulsion of developing motor axons by the floor plate. *Neuron* 14, 1117–1130.
- Helmbacher, F., Schneider-Maunoury, S., Topilko, P., Tietz, L., and Charnay, P. (2000). Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. *Development* 127, 3313–3324.
- Huber, A.B., Kania, A., Tran, T.S., Gu, C., De Marco Garcia, N., Lieberam, I., Johnson, D., Jessell, T.M., Ginty, D.D., and Kolodkin, A.L. (2005). Distinct roles for secreted semaphorin signaling in spinal motor axon guidance. *Neuron* 48, 949–964.
- Irving, C., Malhas, A., Guthrie, S., and Mason, I. (2002). Establishing the trochlear motor axon trajectory: role of the isthmic organizer and *Fgf8*. *Development* 129, 5389–5398.
- Jessell, T.M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet.* 1, 20–29.
- Kablar, B., and Rudnicki, M.A. (1999). Development in the absence of skeletal muscle results in the sequential ablation of motor neurons from the spinal cord to the brain. *Dev. Biol.* 208, 93–109.
- Kania, A., and Jessell, T.M. (2003). Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A:EphA interactions. *Neuron* 38, 581–596.
- Kania, A., Johnson, R.L., and Jessell, T.M. (2000). Coordinate roles for LIM homeobox genes in directing the dorsoventral trajectory of motor axons in the vertebrate limb. *Cell* 102, 161–173.
- Kramer, E.R., Knott, L., Su, F., Dessaud, E., Krull, C.E., Helmbacher, F., and Klein, R. (2006). Cooperation between GDNF/Ret and ephrinA/EphA4 Signals for Motor-Axon Pathway Selection in the Limb. *Neuron* 50, 35–47.
- Landmesser, L.T. (1992). Growth Cone Guidance in the Avian Limb: A Search for Cellular and Molecular Mechanisms (New York: Raven Press).
- Lee, S.K., Jurata, L.W., Funahashi, J., Ruiz, E.C., and Pfaff, S.L. (2004). Analysis of embryonic motoneuron gene regulation: derepression of general activators function in concert with enhancer factors. *Development* 131, 3295–3306.
- Lin, H.Y., Xu, J., Ornitz, D.M., Halegoua, S., and Hayman, M.J. (1996). The fibroblast growth factor receptor-1 is necessary for the induction of neurite outgrowth in PC12 cells by aFGF. *J. Neurosci.* 16, 4579–4587.
- Loo, B.M., and Salmivirta, M. (2002). Heparin/Heparan sulfate domains in binding and signaling of fibroblast growth factor 8b. *J. Biol. Chem.* 277, 32616–32623.
- Lumsden, A.G., and Davies, A.M. (1983). Earliest sensory nerve fibres are guided to peripheral targets by attractants other than nerve growth factor. *Nature* 306, 786–788.
- Lyuksytova, A.I., Lu, C.C., Milanese, N., King, L.A., Guo, N., Wang, Y., Nathans, J., Tessier-Lavigne, M., and Zou, Y. (2003). Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. *Science* 302, 1984–1988.
- Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colquhoun, K., Martin, P., Lumsden, A., Dickson, C., and Mason, I. (1995). A role for FGF-8 in the initiation and maintenance of vertebrate limb bud outgrowth. *Curr. Biol.* 5, 797–806.
- McCaig, C.D. (1986). Myoblasts and myoblast-conditioned medium attract the earliest spinal neurites from frog embryos. *J. Physiol.* 375, 39–54.
- McFarlane, S., Cornel, E., Amaya, E., and Holt, C.E. (1996). Inhibition of FGF receptor activity in retinal ganglion cell axons causes errors in target recognition. *Neuron* 17, 245–254.
- Miyashita, T., Yeo, S.Y., Hirate, Y., Segawa, H., Wada, H., Little, M.H., Yamada, T., Takahashi, N., and Okamoto, H. (2004). PlexinA4 is necessary as a downstream target of *Islet2* to mediate Slit signaling for promotion of sensory axon branching. *Development* 131, 3705–3715.
- Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B.K., Hubbard, S.R., and Schlessinger, J. (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* 276, 955–960.
- Nakao, T., and Ishizawa, A. (1994). Development of the spinal nerves in the mouse with special reference to innervation of the axial musculature. *Anat. Embryol. (Berl.)* 189, 115–138.
- Niswander, L., and Martin, G.R. (1992). *Fgf-4* expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* 114, 755–768.
- O'Connor, R., and Tessier-Lavigne, M. (1999). Identification of maxillary factor, a maxillary process-derived chemoattractant for developing trigeminal sensory axons. *Neuron* 24, 165–178.
- Ohkubo, Y., Uchida, A.O., Shin, D., Partanen, J., and Vaccarino, F.M. (2004). Fibroblast growth factor receptor 1 is required for the proliferation of hippocampal progenitor cells and for hippocampal growth in mouse. *J. Neurosci.* 24, 6057–6069.
- Ornitz, D.M. (2000). FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays* 22, 108–112.
- Ornitz, D.M., Xu, J., Colvin, J.S., McEwen, D.G., MacArthur, C.A., Coulier, F., Gao, G., and Goldfarb, M. (1996). Receptor specificity of the fibroblast growth factor family. *J. Biol. Chem.* 271, 15292–15297.

- Pak, W., Hindges, R., Lim, Y.S., Pfaff, S.L., and O'Leary, D.D. (2004). Magnitude of binocular vision controlled by islet-2 repression of a genetic program that specifies laterality of retinal axon pathfinding. *Cell* 119, 567–578.
- Pirvola, U., Ylikoski, J., Trokovic, R., Hebert, J.M., McConnell, S.K., and Partanen, J. (2002). FGFR1 is required for the development of the auditory sensory epithelium. *Neuron* 35, 671–680.
- Raffioni, S., Thomas, D., Foehr, E.D., Thompson, L.M., and Bradshaw, R.A. (1999). Comparison of the intracellular signaling responses by three chimeric fibroblast growth factor receptors in PC12 cells. *Proc. Natl. Acad. Sci. USA* 96, 7178–7183.
- Schaeren-Wiemers, N., and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* 100, 431–440.
- Segawa, H., Miyashita, T., Hirate, Y., Higashijima, S., Chino, N., Uye-mura, K., Kikuchi, Y., and Okamoto, H. (2001). Functional repression of Islet-2 by disruption of complex with Ldb impairs peripheral axonal outgrowth in embryonic zebrafish. *Neuron* 30, 423–436.
- Serafini, T., Kennedy, T.E., Galko, M.J., Mirzayan, C., Jessell, T.M., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to *C. elegans* UNC-6. *Cell* 78, 409–424.
- Serafini, T., Colamarino, S.A., Leonardo, E.D., Wang, H., Bedington, R., Skarnes, W.C., and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* 87, 1001–1014.
- Sharma, K., Sheng, H.Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H., and Pfaff, S.L. (1998). LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. *Cell* 95, 817–828.
- Sharma, K., Leonard, A.E., Lettieri, K., and Pfaff, S.L. (2000). Genetic and epigenetic mechanisms contribute to motor neuron pathfinding. *Nature* 406, 515–519.
- Shirasaki, R., and Pfaff, S.L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* 25, 251–281.
- Shirasaki, R., Katsumata, R., and Murakami, F. (1998). Change in chemoattractant responsiveness of developing axons at an intermediate target. *Science* 279, 105–107.
- Szebenyi, G., Dent, E.W., Callaway, J.L., Seys, C., Lueth, H., and Kalil, K. (2001). Fibroblast growth factor-2 promotes axon branching of cortical neurons by influencing morphology and behavior of the primary growth cone. *J. Neurosci.* 21, 3932–3941.
- Tessier-Lavigne, M., and Goodman, C.S. (1996). The molecular biology of axon guidance. *Science* 274, 1123–1133.
- Thisse, B., and Thisse, C. (2005). Functions and regulations of fibroblast growth factor signaling during embryonic development. *Dev. Biol.* 287, 390–402.
- Thor, S., Andersson, S.G., Tomlinson, A., and Thomas, J.B. (1999). A LIM-homeodomain combinatorial code for motor-neuron pathway selection. *Nature* 397, 76–80.
- Tosney, K.W. (1987). Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: deletion of the dermamyotome. *Dev. Biol.* 122, 540–558.
- Tosney, K.W. (1988). Proximal tissues and patterned neurite outgrowth at the lumbosacral level of the chick embryo: partial and complete deletion of the somite. *Dev. Biol.* 127, 266–286.
- Tosney, K.W., and Landmesser, L.T. (1985). Development of the major pathways for neurite outgrowth in the chick hindlimb. *Dev. Biol.* 109, 193–214.
- Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., and Schutz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat. Genet.* 23, 99–103.
- Tsuchida, T., Ensini, M., Morton, S.B., Baldassare, M., Edlund, T., Jessell, T.M., and Pfaff, S.L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* 79, 957–970.
- Tzarfati-Majar, V., Burstyn-Cohen, T., and Klar, A. (2001). F-spondin is a contact-repellent molecule for embryonic motor neurons. *Proc. Natl. Acad. Sci. USA* 98, 4722–4727.
- Uemura, O., Okada, Y., Ando, H., Guedj, M., Higashijima, S., Shimazaki, T., Chino, N., Okano, H., and Okamoto, H. (2005). Comparative functional genomics revealed conservation and diversification of three enhancers of the *isl1* gene for motor and sensory neuron-specific expression. *Dev. Biol.* 278, 587–606.
- Umemori, H., Linhoff, M.W., Ornitz, D.M., and Sanes, J.R. (2004). FGF22 and its close relatives are presynaptic organizing molecules in the mammalian brain. *Cell* 118, 257–270.
- West, A.G., Gaszner, M., and Felsenfeld, G. (2002). Insulators: many functions, many mechanisms. *Genes Dev.* 16, 271–288.
- Yamaguchi, T.P., Harpal, K., Henkemeyer, M., and Rossant, J. (1994). *fgfr-1* is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev.* 8, 3032–3044.
- Yokote, H., Fujita, K., Jing, X., Sawada, T., Liang, S., Yao, L., Yan, X., Zhang, Y., Schlessinger, J., and Sakaguchi, K. (2005). Trans-activation of EphA4 and FGF receptors mediated by direct interactions between their cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* 102, 18866–18871.