CHAPTER SIX

Hox Networks and the Origins of Motor Neuron Diversity

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Contents

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Abstract

Motor behaviors are the primary means by which animals interact with their environment, forming the final output of most central nervous system (CNS) activity. The neural circuits that govern basic locomotor functions appear to be genetically hard wired and are comprised of discrete groups of neurons residing within the spinal cord. These local microcircuits coordinate simple reflexive behaviors in response to sensory stimuli and underlie the generation of rhythmic patterns of neural activity necessary for walking. In recent years there have been significant advances in understanding the genetic and molecular programs that determine the specificity of neural connections within the spinal cord that are critical for the emergence of coordinate motor behaviors.

The assembly of circuits within the spinal cord requires the generation of diverse cell types to accommodate the intricate sets of interconnections between motor neurons, sensory neurons, interneurons, and muscle. The first and most critical aspect of this process is that motor neurons select their appropriate muscle targets in the periphery with fidelity and precision. All of the subsequent steps in motor neuron connectivity, such as their descending inputs from higher brain centers, their circuits with sensory neurons and interneurons are constrained by the early connections formed between motor neurons and their muscle targets. The actions of a single family of transcription factors, encoded by the chromosomally clustered *Hox* genes, appear to have a central role in defining the specificity of motor neuron-muscle connectivity. The emerging logic of Hox protein function in motor neuron specification may provide more general insights into the programs that determine synaptic specificity in other CNS regions.

1. Introduction

Much of the computational power of vertebrate nervous systems is dedicated to the goal of controlling movement (Sherrington, 1906), and motor systems have necessarily evolved flexibility and adaptability to respond to the biomechanical challenges imposed by the outside world. Among the most sophisticated motor programs are those executed by the limbs—from the orderly recruitment of flexor and extensor muscles during

locomotion to the higher level muscle synergies used in grasping and object manipulation. From a developmental perspective, the control of diverse motor behaviors presents a challenging problem in target specificity—demanding the coordinate activation of over a hundred distinct muscles, each by a dedicated set of spinal motor neurons. The selectivity with which motor neurons innervate their limb target muscles therefore represents an early and critical element in the assembly of vertebrate sensory-motor circuits.

The stereotypic nature of developing motor axonal projections within the limb led to the idea that motor neurons possess subtype identities that define their innervation patterns (Landmesser, 2001; Milner and Landmesser, 1999). The precision of nerve-muscle connectivity was argued to have its origins in the ability of motor neuron subtypes to send their axons into nerve branches that bring them to different muscle targets. The concept that motor neurons possess intrinsic features that direct selective patterns of axonal projection and target innervation received further support from studies showing that motor neurons are able to redirect their axons along new trajectories and find their correct muscle targets when forced to enter the limb from aberrant positions (Lance-Jones and Landmesser, 1980; Landmesser, 2001). Within the spinal cord, the cell bodies of motor neurons that project axons along a given peripheral pathway are grouped in discrete clusters—termed motor columns, divisions, and pools—and these neuronal subtypes occupy fixed positions within the spinal cord. These motor neuron subtypes appear to acquire an early identity that instructs their axons to grow along highly specific trajectories to their muscle targets.

Work over the past decade has begun to define the molecular programs that operate during embryonic development to determine motor neuron fate and associated patterns of connectivity. One key insight into these molecular programs is that core features of motor neuron identity that determine migratory routes, settling positions, patterns of axonal projections, and selection of synaptic targets are defined by the selective profile of transcription factor expression. The actions of one diverse family of transcription factors, encoded by the *Hox* genes, are central mediators of the intrinsic programs that shape motor neuron subtype identity and target muscle specificity.

The focus of this chapter is to outline recent studies that have helped to define the mechanisms by which a Hox-based transcriptional network controls motor neuron identity and connectivity in the developing spinal cord.

2. Spinal Motor Neuron Diversity

Terrestrial vertebrates possess hundreds of anatomically distinct muscle groups. The motor neurons that innervate these diverse targets are organized into discrete clusters within the spinal cord. The position that

these groups of motor neurons occupy within the spinal cord is relatively fixed from animal to animal, and thus cell body position is often predictive of target innervation pattern (Hollyday and Jacobson, 1990; Landmesser, 1978b). Understanding the genetic programs that contribute to the formation of motor innervation maps has been a major challenge in the field. In this section we describe studies that have helped to define the early programs which establish motor neurons as a class and the emergence of motor neuron topographic maps.

2.1. Generation of generic motor neuron identity

Motor neurons and several classes of interneurons are generated in response to graded extrinsic signals acting along the dorsoventral axis of the neural tube. These secreted signals include sonic hedgehog (Shh) from the notochord and floor plate, and fibroblast growth factors (FGFs) and retinoic acid (RA) by the paraxial mesoderm. The detailed mechanisms through which Shh, RA, and FGFs define progenitor identities will not be addressed here since this topic has been the subject of several review articles (Dessaud *et al.*, 2008; Jessell, 2000; Shirasaki and Pfaff, 2002). In brief, Shh, FGF, and RA signaling induce the expression of distinct combinations of transcription factors in neural progenitors. These initial patterns are subsequently refined through the selective transcriptional cross-repressive interactions between transcription factors expressed at the boundaries between progenitor domains (Fig. 6.1A). Each progenitor domain in the neural tube expresses a unique profile of transcription factors, and these combinatorial patterns define progenitor fates (Briscoe *et al.*, 2000). Progenitors that give to motor neurons depend on the activities of the bHLH protein Olig2 and the homeodomain factors Pax6, Nkx6.1, and Nkx6.2 (Novitch *et al.*, 2001; Vallstedt *et al.*, 2001; Zhou and Anderson, 2002). Thus a major output of the dorsoventral signaling system is to define the identity of motor neurons as opposed to ventral interneurons.

Soon after their generation, at about embryonic day (*e*) 9.5 in mouse, spinal motor neurons express a set of homeodomain transcription factors (notably Hb9, Lhx3, Isl1, and Isl2), that that control features common to all spinal motor neurons as well as those that are involved in later aspects of subtype diversification (Fig. 6.1A) (Arber *et al.*, 1999; Pfaff *et al.*, 1996; Sharma *et al.*, 1998; Thaler *et al.*, 1999, 2004). These generic motor neurons characteristics include the projections of axons outside the spinal cord and the release of acetylcholine as the primary neurotransmitter. Although patterning events mediated by Shh, RA, and FGF signaling define how motor neurons as a class are specified, additional signaling pathways are presumably necessary for the further diversification of motor neurons into distinct subtypes.

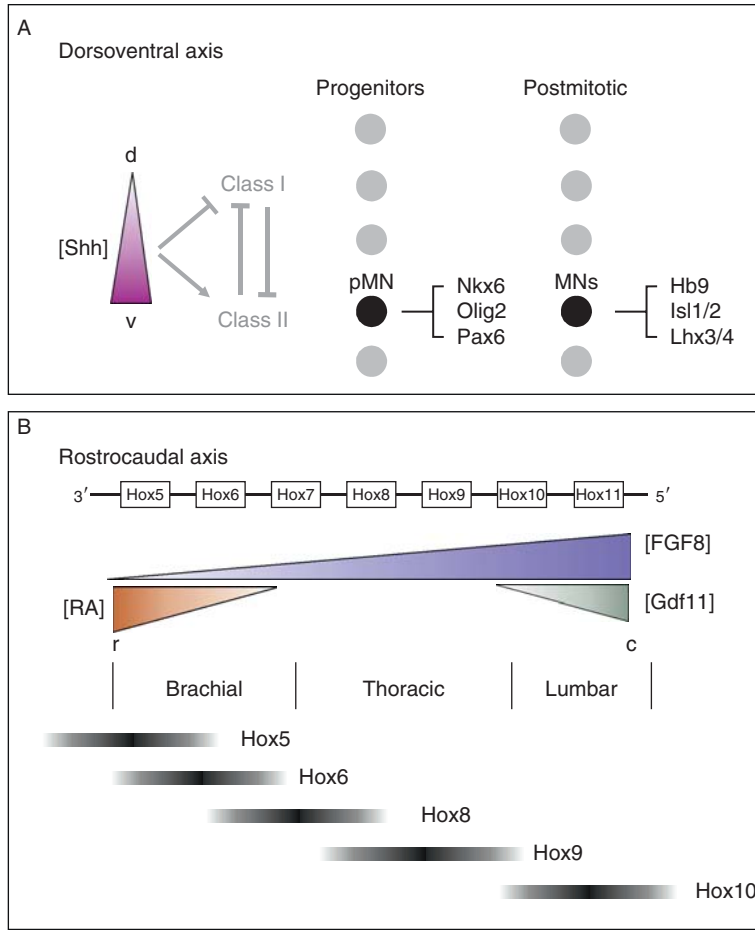

Figure 6.1 Patterning along the dorsoventral and rostrocaudal axes of the neural tube. (A) Motor neurons and ventral interneurons are generated along the dorsoventral (d-v) axis in response to the graded activities of sonic hedgehog (Shh) which induces the patterned expression of transcription factors in progenitor cells. Class I factors are induced by Shh while Class II transcription factors are repressed. Selective cross-repressive interactions between Class I and Class II transcription factor sharpen the boundaries between progenitor domains (Briscoe *et al.*, 2000). Retinoic acid (RA) from the paraxial mesoderm and fibroblast growth factor (FGF) signaling also influence the pattern of transcription factors in neural tube progenitors (not shown). (B) Along the rostrocaudal axis graded FGF signaling induces the expression of chromosomally linked *Hox* genes in the neural tube. *Hox* genes located at one end of the cluster are expressed more rostrally (r) while genes at the opposite end are expressed caudally (c) in response to higher levels of FGF. At more rostral levels *Hox* genes are regulated by graded RA signaling while at more caudal levels *Hox* genes are regulated by graded Gdf11.

2.2. Anatomical organization of spinal motor neuron subtypes

At the time of their birth, all motor neurons possess a set of core features that distinguish them from other classes of neurons, but rapidly diversify into subtype identities that allow them to form selective connections with target cells. Major distinctions in the identities of motor neurons have been defined through studies of their position, axon trajectory, and pattern of muscle innervation. One level of organization is the allocation of motor neurons to columnar groups, each column occupying a defined position along the rostrocaudal axis of the spinal cord. Four major columnar classes have been described, each innervating a unique set of peripheral target tissues (Fig. 6.2). The most prominent of these columnar groups are the lateral motor columns (LMCs) which are generated at limb levels of the spinal cord and innervate limb muscles. At thoracic levels visceral preganglionic column (PGC) motor neurons innervate sympathetic ganglia while hypaxial motor column (HMC) neurons innervate intercostal and abdominal wall musculature (Gutman *et al.*, 1993; Prasad and Hollyday, 1991). In contrast to these segmentally restricted motor columns, motor neurons in the median motor column (MMC) are present at all levels of the spinal cord and innervate dorsal axial musculature (Fetcho, 1987; Gutman *et al.*, 1993).

Additional layers of anatomical organization are present within these main columns, notably the segregation of the LMC into "divisions" and "pools." At both forelimb and hindlimb levels of the spinal cord, the LMC is split into two divisions: a medial division which contains neurons projecting axons ventrally within the limb mesenchyme and a lateral division which contains neurons that project dorsally (Fig. 6.2B) (Landmesser, 1978a; Tosney and Landmesser, 1985a,b). These two divisional subgroups have been linked to the particular mode of actions of their muscle targets: dorsally projecting lateral LMC axons frequently innervate extensor muscles, whereas medial LMC axons project ventrally and typically innervate flexor muscles. The functional relevance of this anatomical segregation is still unclear.

A third level of motor neuron diversity is evident in the segregation of motor neurons into motor pools (Romanes, 1942). Motor pools occupy distinct positions within the LMC, each pool innervating a dedicated target muscle (Hollyday and Jacobson, 1990; Landmesser, 1978b). Whereas forelimb and hindlimb motor neurons share similar columnar and divisional properties, the organization of motor pools between these two levels of the spinal cord is quite distinct, reflecting differences in the overall pattern of musculature between the forelimb and hindlimb. Nevertheless motor pools innervating forelimb and hindlimb form topographic maps which are similarly organized. At both levels of the spinal cord motor pools located more rostrally tend to innervate muscles located rostral and proximal within the limb, while caudal pools project to more caudal and distal regions (Hollyday and Jacobson, 1990; Landmesser, 1978b).

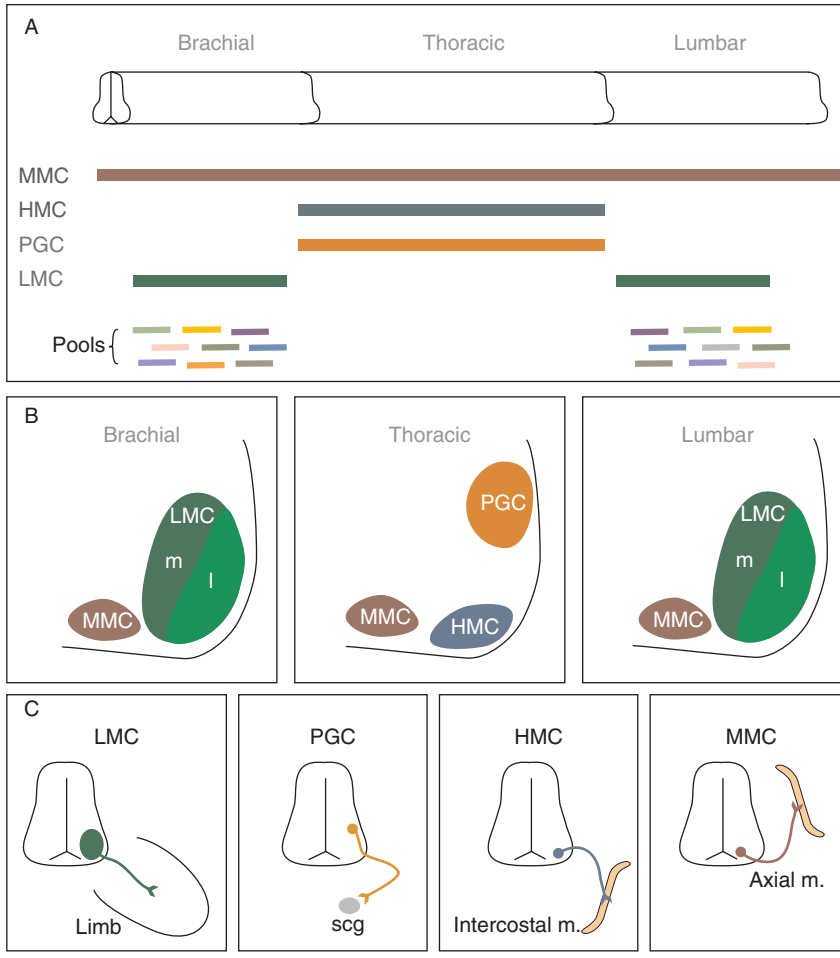

Figure 6.2 Motor neuron columnar, divisional and pool organization. (A) Motor columns and motor pools are generated at specific positions along the rostrocaudal axis. The cell bodies of motor neurons that send axons to the limb are contained within the lateral motor column (LMC) at brachial and lumbar levels of the spinal cord. Preganglionic column (PGC) motor neurons and hypaxial motor column (HMC) neurons are found at thoracic levels. Motor neurons within the medial motor column (MMC) are generated at all rostrocaudal levels of the spinal cord. Motor pools are generated at specific rostrocaudal positions within the LMC. (B) Schematic of cross sections of brachial, thoracic, and lumbar spinal cord showing the position of motor columns and divisions. Medial (m) and lateral (l) divisions of the LMC are indicated. (C) Projection patterns of motor neuron columnar subtypes. LMC neurons project to the limb, PGC neurons to sympathetic chain ganglia (scg), HMC neurons to intercostal and body wall muscles (m), MMC neurons to axial muscle.

3. Hox Expression in Developing Motor Neurons

The diversification of motor neurons into specific columnar, divisional, and pool subtypes relies on the acquisition of a positional identity along the rostrocaudal axis of the spinal cord. Like neuronal specification along the dorsoventral axis, motor neurons acquire positional information in response to graded signals which initiate transcriptional programs within progenitor and postmitotic cells. A critical output of the rostrocaudal signaling pathways acting on the neural tube is the establishment of selective patterns of *Hox* gene expression within specific motor neuron subtypes.

3.1. Rostrocaudal positional information in spinal cord development

Early insights into the role of rostrocaudal positional information in locomotor circuit assembly came from embryological manipulations in the chick embryo. One of the most dramatic set of experiments was performed by Victor Hamburger and colleagues who demonstrated that if the region of the spinal cord responsible for synchronous activation of wing muscles is grafted to the level of the hindlimb, chickens will synchronously activate muscle in the legs (Narayanan and Hamburger, 1971). Conversely, grafting hindlimb-level spinal cord to wing levels causes the chick to alternate wing movements, in a pattern similar to walking. These studies provide evidence that the intrinsic properties of neurons generated at specific rostrocaudal levels have critical roles in establishing the local circuitries controlling basic behavioral outputs.

More recent studies on rostrocaudal programming have demonstrated that positional identities are regulated by signals derived from axial structures, such as the node and notochord, as well as from paraxial tissues, the presomitic mesoderm and somites (Ensini *et al.*, 1998; Lance-Jones *et al.*, 2001; Liu *et al.*, 2001). Grafting experiments in chick indicated that regionally restricted signals govern the specification of motor neuron columnar subtypes. Transposition of thoracic and brachial levels of the neural tube during a critical period of development respecifies columnar fates—neurons derived from the former thoracic region of the neural tube acquire LMC identity, and conversely, motor neurons from the former brachial level acquire a CT identity (Ensini *et al.*, 1998; Shieh, 1951). The signals for columnar respecification derive, in part, from the adjacent paraxial mesoderm, since a similar switching of motor neuron columnar identity can be elicited by transposition of brachial and thoracic paraxial mesoderm (Ensini *et al.*, 1998).

3.2. Regulation of Hox expression by FGF, Retinoid, Wnt, and TGFβ signaling

While grafting studies in chick indicated that the regional identities of motor neurons are controlled through mesodermal signals, the identity of these signals and the intrinsic mediators of their actions were not known. One class of transcription factors with an evolutionarily conserved role in establishing differences in cell identity along the rostrocaudal axis are members of the chromosomally arrayed *Hox* gene family. The expression of *Hox* genes within the spinal cord is closely aligned with their position within the *Hox* cluster: genes located at the 3′ end of the cluster are expressed more anteriorly than genes at the 5′ end (Fig. 6.1B) (Kmita and Duboule, 2003; Lemons and McGinnis, 2006). The precise mechanism by which spatial colinear expression of *Hox* genes emerges in the neural tube is unknown; although gradients of signaling molecules appear to impart the initial profiles of *Hox* expression in most tissues where colinearity has been examined.

The expression of *Hox* genes within the CNS is controlled by multiple signaling molecules including FGFs, retinoids, Wnts, and members of the transforming growth factor (TGF) β superfamily (Bel-Vialar *et al.*, 2002; Diez del Corral and Storey, 2004; Liu, 2006; Liu *et al.*, 2001; Nordstrom *et al.*, 2006). Graded FGF signaling is involved in establishing the initial induction of *Hox* gene expression at brachial, thoracic, and lumbar levels of the spinal cord (Bel-Vialar *et al.*, 2002; Dasen *et al.*, 2003; Liu *et al.*, 2001). At the caudal end of the chick embryo, an organizing region called Hensen's node and the presomitic mesoderm are primary sources of FGF signals. As the tail bud regresses caudally during axis extension, more posterior regions of the spinal cord are exposed to FGF in higher concentration and over longer periods of time (Dubrulle and Pourquie, 2004; Liu *et al.*, 2001). Both *in vitro* and *in vivo* studies have shown that *Hox* genes located at the 3′ end of a cluster are induced by low levels of FGF while those at the 5′ end are induced by progressively higher FGF levels (Bel-Vialar *et al.*, 2002; Dasen *et al.*, 2003; Liu *et al.*, 2001). As a consequence, *Hox4-Hox8* paralog genes are expressed at brachial levels, *Hox8-Hox9* genes at thoracic, and *Hox10-Hox13* genes at lumbar levels of the spinal cord (Fig. 6.1B).

While graded FGF signals contribute to initial *Hox* patterns, other signaling systems participate in regulating subsets of *Hox* genes within a cluster. At more anterior levels RA signaling provided by paraxial mesoderm and somites has been shown to regulate Hox expression at brachial levels (Liu *et al.*, 2001). The action of retinoids is in part to antagonize the FGF gradient (Diez del Corral and Storey, 2004); although the mechanisms underlying this process are not known. At more caudal levels, the TGFβ family member Gdf11 has been shown to be essential both *in vitro* and *in vivo* in the regulation of *Hox8-Hox10* paralogs at thoracic and lumbar levels of the

spinal cord (Liu, 2006; McPherron *et al.*, 1999), and this inductive factor appears to act in concert with high levels of FGF signaling (Liu *et al.*, 2001). Thus Hox expression in motor neurons relies on the convergent actions of multiple signaling pathways (Fig. 6.1B).

3.3. The emergence of definitive Hox patterns in motor neurons

Although graded signals are necessary to establish the initial pattern of *Hox* gene expression in motor neurons, the links between extrinsic signals and Hox protein expression in motor neurons are still unclear. While RA response elements have been characterized in the *Hox* genes controlling regional identity in the hindbrain (Glover *et al.*, 2006; Trainor and Krumlauf, 2000), similar control elements have yet to be described for *Hox* genes expressed within the spinal cord. How the neural tube interprets the FGF gradient is also unknown; although a class of homeodomain proteins related to the *Drosophila* gene *caudal* has been implicated. Manipulation of vertebrate *caudal* homeobox (*Cdx*) activities produces phenotypes which are similar to manipulations of FGF signaling. Misexpression of activated forms of Cdx can shift the patterns of Hox expression in the spinal cord (Bel-Vialar *et al.*, 2002), while in Zebrafish loss of Cdx activities deregulates Hox expression and the spinal cord acquires a hindbrain-like character (Shimizu *et al.*, 2006; Skromne *et al.*, 2007). The mechanisms by which Cdx proteins regulate *Hox* expression are not known, but may involve direct Cdx binding to individual *Hox* regulatory elements or interaction with locus control regions within the *Hox* clusters.

Another unresolved issue is the inordinate temporal delay between the time of neural tube exposure to graded FGF signals and the emergence of Hox expression in postmitotic motor neurons. FGF signaling appears to act on neural progenitors, and manipulation of FGF signals can rapidly switch *Hox* transcriptional profiles at early stages of development (Bel-Vialar *et al.*, 2002; Dasen *et al.*, 2003). However the expression of some Hox proteins by motor neurons is detected only in postmitotic cells (Dasen *et al.*, 2003). The mechanisms which introduce this apparent delay between Hox RNA and protein expression are not known; although *Hox* genes are under the control of several layers of posttranscriptional regulation, including silencing by microRNAs (Chopra and Mishra, 2006). Another possibility is that expression of *HoxB* cluster genes in progenitors, which are generally not detected in postmitotic motor neurons (Dasen *et al.*, 2005), prefigures progenitors to express *HoxA*, *HoxC*, and *HoxD* genes.

4. Hox Proteins Determine Motor Neuron Columnar Identity and Connectivity

Early insights into the role of *Hox* genes in CNS development came from studies in the vertebrate hindbrain. In the hindbrain the metameric organization of the rhombomeres provides anatomical landmarks for defining global aspects of Hox function, and several *Hox* gene mutants are characterized by homeotic transformation of rhombomeres identities and changes in neuronal identity (reviewed in Guthrie (2007)). In contrast, the relative morphological homogeneity and lack of columnar and pool specific molecular markers in the spinal cord created a challenge in linking Hox function to the specification of motor neuron subtypes. Although it had been recognized that different levels of the spinal cord express distinct *Hox* genes (Carpenter, 2002), the link to motor neuron columnar, divisional, and pool identities were unclear.

4.1. Specification of segmentally restricted columnar subtypes by *Hox* genes

Certain motor neuron populations can be defined by the combinatorial expression of LIM-homeodomain proteins (Tsuchida *et al.*, 1994); although no single LIM-homeodomain protein is specific for a single neuronal class in the spinal cord. The identification of molecular markers that are selectively expressed in two segmentally restricted motor columns, LMC and PGC neurons, provided a means to explore the signaling pathways that specify motor neuron subtypes. PGC neurons can be distinguished from other thoracic-level spinal motor neurons by expression of *BMP5*, a TGFβ family member (William *et al.*, 2003) as well as nuclear phospho-(p)-Smad1/5/8 (Dasen *et al.*, 2008). At limb levels LMC neurons can be defined by their selective expression of retinaldehyde dehydrogenase-2 (RALDH2), a key enzyme in retinoic acid synthesis (Sockanathan and Jessell, 1998).

Expression of Hox proteins is closely aligned with the position in which molecularly defined columnar subtypes are generated: expression of Hox6 proteins segregates with brachial (forelimb) LMC neurons, Hox9 proteins with thoracic PGC neurons, and Hox10 proteins with lumbar (hindlimb) LMC neurons (Fig. 6.3A) (Choe *et al.*, 2006; Dasen *et al.*, 2003; Lance-Jones *et al.*, 2001; Liu *et al.*, 2001). Consistent with the model where Hox protein expression is controlled by graded FGF signaling, elevation of FGF levels at brachial levels of the spinal cord induces the expression of Hoxc9, a *Hox* gene normally restricted to thoracic levels (Dasen *et al.*, 2003).

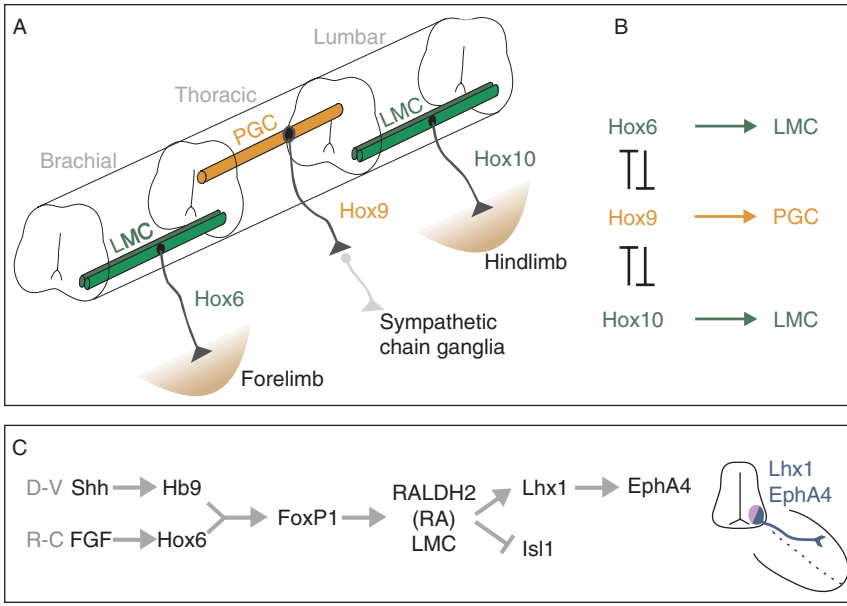

Figure 6.3 *Hox* genes and the specification of segmentally restricted motor columns. (A) Hox6, Hox9, and Hoxd10 are expressed in motor neurons at distinct rostrocaudal levels of the spinal cord and direct motor neuron identity and peripheral target connectivity. Hox6 activities control brachial LMC identity, Hox9 control PGC identity, and Hox10 lumbar LMC identity. (B) Regulatory interactions between *Hox* genes in motor neuron columnar fates. Cross-repressive interactions between Hox6, Hox9, and Hox10 proteins refine Hox profiles and Hox activator functions define LMC and PGC identities. (C) Role of LMC *Hox* genes in the program controlling motor neuron divisional identities and axonal projections along the dorsoventral axis of the limb. The convergent activities of motor neuron specific transcription factors (Hb9) and limb-level *Hox* genes (e.g., Hoxc6) direct expression of FoxP1 and RALDH2 in LMC neurons. RALDH2 creates a neuronal source of RA which leads to the induction of Lhx1 expression by lateral LMC neurons. Lhx1 directs expression of the guidance receptor EphA4, and EphA4 directs motor axons toward the dorsal limb.

This switch in Hox patterns is accompanied by the loss of brachial LMC neurons, characterized by the abolishment of Hoxc6 and RALDH2 expression. Motor neurons are instead converted to a PGC cell fate, defined by expression of *BMP5* and pSmad. The effects of elevated FGF are not restricted to changes in marker gene expression, but extend to multiple aspects of columnar identity including switches in the patterns of migration, peripheral connectivity, and the number of motor neurons generated at limb and thoracic levels (Dasen *et al.*, 2003).

Are the effects of elevating FGF signaling on motor neuron columnar identity mediated by changes in *Hox* gene expression? Consistent with a direct role in columnar specification, misexpression of Hoxc9 at brachial

levels is sufficient to convert LMC to PGC motor neurons, while expression of Hoxc6 or Hoxd10 at thoracic levels can convert PGC and HMC to LMC neurons (Dasen *et al.*, 2003; Shah *et al.*, 2004). These actions of Hox proteins rely on their ability to cross-repress each others expression: Hox6 and Hox10 protein can repress Hox9 expression while Hox9 can repress Hox6 expression (Fig. 6.3B). Moreover, the actions of Hox proteins in inducing columnar fates and establishing Hox boundaries appear to be separable, as expression of a constitutive repressor forms block columnar inducing activities but retain their cross-repressive functions (Dasen *et al.*, 2003). Thus like many transcription factors Hox proteins possess intrinsic activator and repressor functions, and these functional differences serve distinct but coherent roles in motor neuron subtype specification (Fig. 6.3B).

The principles of Hox protein function along the rostrocaudal axis parallel those that operate along the dorsoventral axis which specify motor neurons as a class. Along both axes, the initial graded activity of a secreted signaling factor establishes broad domains of homeodomain protein expression that are subsequently refined through selective cross-repressive interactions. However, these two programs of transcriptional cross-repression appear to operate at different stages of neuronal specification. Dorsoventrally, homeodomain cross-repressive interactions are evident within neural progenitor cells (Briscoe *et al.*, 2000), whereas along the rostrocaudal axis Hox cross-repression occurs within postmitotic neurons (Dasen *et al.*, 2003). Nevertheless, the convergence of these two patterning programs ensures that Hox-directed features of columnar differentiation are confined to postmitotic motor neurons.

Aspects in the logic of Hox function in spinal motor neuron diversification are distinct from that used in rostrocaudal patterning of *Drosophila* larvae and in the vertebrate hindbrain. In these two systems, the actions of posteriorly expressed *Hox* genes typically dominate over those of more anteriorly expressed genes—a phenomenon termed posterior dominance (Duboule and Morata, 1994). The findings in the spinal cord argue against posterior prevalence of Hox function in postmitotic motor neuron specification, since ectopic caudal expression of Hoxc6 is as effective as ectopic rostral expression of Hoxc9. Several exceptions to the posterior prevalence rule of Hox function have been reported in both fly and vertebrate embryos (Duboule and Morata, 1994; Jegalian and De Robertis, 1992). Recent genetic analysis of axial skeleton patterning in *Hox* mutants provides further evidence against a global dominance of posterior *Hox* genes in somite derivatives (McIntyre *et al.*, 2007). Thus tissue context may influence functional dominances between *Hox* genes. In neural progenitors *Hox* genes are expressed in overlapping and nested patterns, one possibility is that posterior dominance operates at early stages of spinal cord development to help determine the final Hox pattern in postmitotic neurons.

4.2. Mechanisms of columnar Hox function in motor neuron connectivity

In addition to regulating the expression of columnar-specific genes, Hox proteins can also direct the peripheral connectivity of LMC and PGC neurons. At forelimb levels of the spinal cord the switching of LMC neurons to a PGC fate forces motor neurons to project their axons to the normal PGC targets, sympathetic chain ganglia. Conversely, expression of Hoxc6 or Hoxd10 at thoracic levels induces LMC fate and these neurons project into the limb (Dasen *et al.*, 2005; Shah *et al.*, 2004). Thus Hox proteins not only influence columnar identity at the level of molecular marker expression but also contribute to the initial specificity of motor axon projections in the periphery.

How might the activities of Hox proteins determine columnar-specific patterns of axonal innervation? Markers of newly generated LMC and PGC neurons, RALDH2 and BMP5/pSmad, are intimately involved in inductive signaling. Although the function of BMP5/pSmad signaling in PGC neurons is not known, RALDH2 activity appears to be required for the initial specificity in which LMC neurons project into the limb (Fig. 6.3C). The RALDH2-dependent synthesis of retinoids by LMC neurons is necessary for the specification of lateral LMC neuronal identity, in order to induce expression of Lhx1, a LIM-homeodomain protein (Kania *et al.*, 2000). Lhx1 expression has been shown to direct the dorsal projection of LMC motor axons in the developing limb through its ability to regulate EphA4 expression, a guidance receptor required for axons to avoid the ventral limb mesenchyme (Eberhart *et al.*, 2002; Kania and Jessell, 2003).

Thus an early step in the Hox-dependent specification of LMC identity is to direct RALDH2 expression, and trigger a series of downstream signaling events within postmitotic motor neurons that govern the pattern of motor neuron connectivity in the developing limb. More generally these findings suggest that a key step in the organization of columnar differentiation at different segmental levels of the spinal cord is the induction of signaling factors in different columnar subtypes of motor neurons, which in turn directs a molecular program for motor neuron connectivity.

5. Hox Transcriptional Networks and the Specification of Motor Pool Identities

Within LMC divisions, motor neurons are further subdivided into motor pools, each destined to innervate a single muscle target in the limb. A typical vertebrate limb contains over 50 muscle groups, requiring the generation of a diverse array of motor pool subtypes. Like columns, a motor

pools occupy stereotypic rostrocaudal positions within the spinal cord and multiple pools can occupy a single segmental level (Fig. 6.4). As with the specification of segmentally restricted motor columns, Hox transcription factors appear to be critical determinants of pool identity and muscle target specificity.

The proposal that intrinsic motor pool identities direct target muscle connectivity emerged first through embryological manipulations which revealed that the axons of specific LMC neurons project to their limb muscle targets with high precision (Landmesser, 1978a), even when forced to enter the limb from innapropriate positions (Landmesser, 2001). Classical embryological studies have provided evidence that motor neurons within

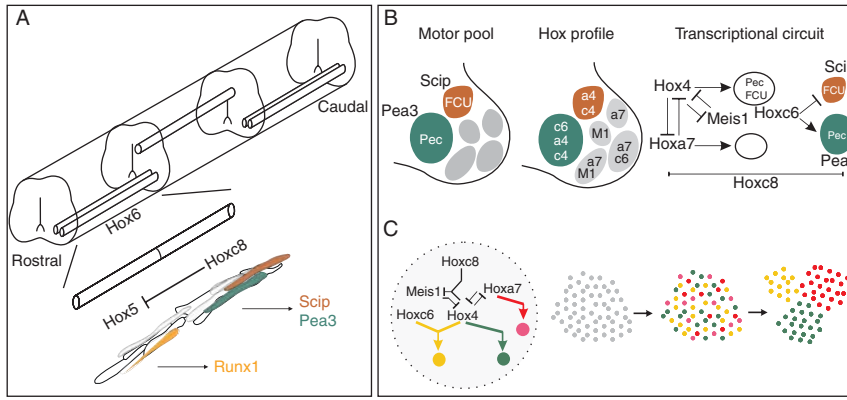

Figure 6.4 A *Hox* transcriptional network controls motor pool identity and connectivity. (A) *Hox* genes determine the rostrocaudal position of motor pools within the LMC. At brachial levels of the spinal cord cross-repressive interactions between Hox5 proteins and Hoxc8 establish the boundary between molecularly defined motor pools. Hox5 proteins (Hoxa5 and Hoxc5) are required to generate the motor pool that expresses the transcription factor Runx1 in rostral LMC neurons. Hoxc8 is required in caudal LMC neurons to generate the motor pools that express the transcription factors Pea3 and Scip. (B) Intrasegmental specification of motor pool identity. At a single segmental level of the spinal cord ~6-10 pools are generated. Motor pools projecting to the pectoralis (Pec) and flexor carpi ulnaris (FCU) can be molecularly defined by expression of the transcription factors Pea3 and Scip, respectively. Both Pec and FCU pools express unique profiles of Hox expression (M1:Meis1, Hoxc6 shown as c6, etc.). The patterns of Hox expression in the Pec and FCU pools are established through a transcriptional network which appears to be driven largely by Hox cross-repressive interactions. (C) Model for a Hox repressilator network in motor pool specification. Individual motor neurons initially inherit expression of multiple *Hox* genes as a function of their position along the rostrocaudal axis. These patterns are refined through repressive interactions on a cell by cell basis, giving rise to motor neurons with a specific Hox pattern (yellow, red, and green cells) that are scattered throughout the column. Subsequently, motor neurons cluster into discrete pools. Biases in the strength of repression may favor expression of one Hox protein over another giving rise to pools of different sizes.

the LMC acquire aspects of their pool identity as their axons first invade the limb mesenchyme, well before approaching muscle targets (Hollyday, 1980a,b, 1995; Landmesser, 1978a,b). As we discuss below *Hox* genes appear to impart the initial selectivity of motor neuron connectivity during this early intrinsic phase of motor neuron differentiation and regulate a diverse repertoire of downstream transcriptional programs that control multiple characteristics of motor pool identity.

5.1. Assignment of motor pool identities by *Hox* genes

The establishment of diverse motor pool subtypes presumably requires the activities of a large number of transcriptional regulators. Systematic analysis of the expression of *Hox* gene expression in chick spinal cord revealed that nearly two dozen are expressed by motor neurons, in a manner consistent with a role in motor pool specification (Dasen *et al.*, 2005). Roles for Hox protein activities in pool specification have been most thoroughly investigated in the brachial level motor neurons that innervate forelimb musculature. Specific brachial motor pools can be molecularly defined by transcription factor expression (e.g., Runx1, Pea3, Scip, and Nkx6 proteins, see Fig. 6.4), and the combinatorial expression of Hox4, Hox5, Hox6, Hox7, and Hox8 proteins appear to define motor neuron pool fate. Experimental manipulation of the pattern of Hox expression in motor neurons leads to changes in motor pool identity, defined by a switch in the molecular profile of pool-specific transcription factors and a change in the pattern of peripheral connectivity of motor axons (Dasen *et al.*, 2005).

Two Hox-dependent programs appear to operate within LMC neurons to control pool fates, one assigning rostrocaudal motor pool position, and a second directing intrasegmental motor pool diversity (Fig. 6.4). The mechanisms by which the rostrocaudal positioning of motor pools is established by Hox proteins largely follow the strategy deployed in columnar specification. Graded FGF and RA signaling determines the initial pattern of *Hox3-Hox8* expression by brachial LMC pools, and rostrocaudal motor pool boundaries are established through selective cross-repressive interactions between pairs of Hox protein. One set of Hox interactions, exemplified by the activities of the Hox5 and Hox8 proteins, constrains motor pool specification to specific rostrocaudal levels of the LMC. At rostral levels of the brachial LMC two *Hox5* paralogs (*Hoxa5* and *Hoxc5*) define the position and identity of rostral motor pools whereas Hoxc8 defines caudal pools (Fig. 6.4A). Thus, the patterns of Hox expression that determine motor pool identity along the rostrocaudal axis of the LMC appear to be set by the same extrinsic signals that establish columnar identities, and are subsequently reinforced through selective cross-repressive interactions.

A single segmental level of the spinal cord contains as many as 10 motor pools (Fig. 6.4B). The neurons that populate these pools derive from progenitors that presumably have been exposed to the same level of rostrocaudal patterning signals, raising the issue of how this aspect of pool diversity is achieved. One model proposed for the intrasegmental diversification posits that all LMC neurons generated at a specific segmental level of the LMC initially express the same set of Hox proteins, as a reflection of their rostrocaudal position. But within this cohort of neurons, the expression of certain Hox proteins is favored over others, by virtue of their mutual repressive interactions (Fig. 6.4C). As a consequence, minor fluctuations in starting Hox conditions within individual motor neurons will result in the gradual extinction of expression of one or other of two opponent Hox proteins. The final complement of Hox proteins expressed within any given LMC neuron will therefore represent only a small subset of the starting repertoire. Consistent with this model expression of Hox4, Hox6, Hox7 proteins and a Hox cofactor called Meis1 are initially co-expressed by most motor neurons at caudal levels of the brachial LMC but eventually segregate in patterns that align with the expression of a set of pool specific transcription factors (Dasen *et al.*, 2005).

The existence of mechanisms that impart a bias to the outcome of Hox cross-regulatory interactions could account for the observation that motor neurons are allocated to distinct pools in different numbers, in anticipation of the size of their muscle target (Lin *et al.*, 1998). Mechanistically this process may involve asymmetries in the strength of Hox repression, or initial differences in the level or onset of Hox expression within individual neurons. This view shares elements in common with the workings of transcriptional repressor networks that have been engineered *de novo* in bacterial model systems (Elowitz and Leibler, 2000).

The analysis of motor pool specification reinforces the view that repressive interactions between Hox proteins direct motor neuron diversification within the spinal cord. But there are important distinctions in the interactions between Hox protein pairs. The Hox6/Hox9 protein pair exhibits mutual repressive interactions during motor neuron columnar specification (Dasen *et al.*, 2003), whereas the Hox5 and Hox8 interaction that occurs during motor pool specification is asymmetric (Dasen *et al.*, 2005). And in chick the repressive interactions between Hox4 and Hoxa7 proteins that occur during the intrasegmental diversification of pools appears not to be absolute, but rather unfolds gradually over several days of development. This reliance on Hox repressive interactions to allocate identities to spinal motor neurons is in apparent contrast with the Hox circuitry involved in hindbrain patterning, where positive autoregulatory interactions have been observed (Gavalas *et al.*, 1997; Nonchev *et al.*, 1997).

5.2. *Hox* genes control the specificity of motor neuron-muscle connectivity

How might Hox activities in motor pools coordinate motor axon trajectory to specific muscle targets in the developing limb? On arriving at the base of the limb, the axons of LMC neurons select a ventral or dorsal trajectory in the limb mesenchyme, and then establish specific anteroposterior and proximo-distal trajectories which take them to the position of newly cleaved muscle masses (Tosney and Landmesser, 1985a). As developing axons project into the limb they navigate through a series of choice points en route to their synaptic targets. A major output of the Hox network in motor neurons is the control of downstream transcription factor expression, some of which are necessary for motor axon guidance decisions (Fig. 6.4). To what extent are the patterns of motor axon innervation driven through Hox-regulated intermediate transcription factors and to what extent might they be controlled directly by Hox targets?

As described earlier, aspects of limb innervation pattern can be linked to the program of columnar specification. The Hox6/10-activated program of LMC specification appears to direct axons toward the limb and determine a pattern of LIM-homeodomain protein expression that controls the dorsoventral trajectory of motor axons, through regulation of EphA4 expression (Kania and Jessell, 2003). The selection of certain muscle-specific nerve trajectories appears to be determined through activities of the Hox-induced transcription factors. Nkx6 homeodomain proteins are expressed by subsets of LMC neurons in a pool-specific pattern that is controlled by Hox proteins (Dasen *et al.*, 2003; De Marco Garcia and Jessell, 2008). In *Nkx6.1* mutant mice these motor neuron pools fail to innervate their normal target and invade foreign muscle targets (De Marco Garcia and Jessell, 2008). Another pool-specific Hox target, Pea3, is required for distinct aspects of motor neuron differentiation, including the clustering of neurons into pools, muscle-specific axonal arborization, and synaptic input onto motor neurons from sensory neurons (Livet *et al.*, 2002; Vrieseling and Arber, 2006). Thus multiple facets of the Hox programming of motor pool identities are regulated through intermediate transcription factors.

Could Hox proteins exert a more direct role in the control of axon guidance decisions within the limb? After making their initial dorsoventral choice at the base of the limb, motor axons follow cues that guide them along the anteroposterior and proximo-distal axes (Stirling and Summerbell, 1988). The basic pattern of muscle nerve branches is preserved in the absence of the target muscle itself (Lewis *et al.*, 1981; Phelan and Hollyday, 1990, 1991), implicating the limb mesenchyme as a source of cues that specify motor axon trajectories. Hox proteins are also expressed by the limb mesenchyme (Izpisua-Belmonte and Duboule, 1992) and could contribute to the establishment of axonal trajectory by positioning guidance cues at

specialized decision regions (Tosney and Landmesser, 1985a). The existence of a topographic relationship between cell body position and projections along these axes raises the possibility that Hox proteins also exert direct roles in the control of specific guidance receptors, or regulate other determinants that direct innervation specificity within the limb.

5.3. Extrinsic and intrinsic programming of motor pool identities

While many aspects of motor pool identity appear to be programmed through cell-intrinsic Hox transcriptional networks, the expression of certain pool specific transcription factors relies on the presence extrinsic signals from the periphery. Expression of the ETS transcription factors Pea3 and Er81 in pools depends on target-derived neurotrophic signals provided by the limb mesoderm and muscle targets (Haase *et al.*, 2002; Lin *et al.*, 1998). These signals appear to be permissive rather than instructive and not all motor neurons are competent in their ability to respond to neurotrophins. In explants of spinal cord treated with glial-derived neurotrophic factor (GDNF), Pea3 is induced in a pattern approximating the normal number in vivo and confined to the level of the spinal cord which normally expresses Pea3 (Haase *et al.*, 2002). Thus not all motor neurons are equivalent in their ability to respond to GDNF.

The competence of motor neurons to activate ETS genes appears to be constrained by their pattern of Hox expression. Ectopic expression of Hoxc8 in LMC neurons is sufficient to expand the domain of Pea3 expression (Dasen *et al.*, 2005), suggesting the normal domain of Hoxc8 expression defines the region of GDNF competence. Consistent with this hypothesis, in *Hoxc8* mutants motor neurons fail to fully activate Pea3 expression (Vermot *et al.*, 2005), likely as a consequence of the inability of motor neurons to respond to GDNF. Hox proteins may therefore control the expression of targets that endow motor neurons with the ability to respond to peripheral cues. Hox targets may include receptors for neurotrophic factors or other components necessary for activation of the GDNF pathway.

These observations suggest that despite the importance of Hox-dependent steps in motor neuron specification, target-derived cues also contribute to the transcriptional programming of motor pools. Expression of the target-induced factor Pea3 is critical for later aspects of motor pool differentiation such as clustering of motor neurons into pools, muscle-specific patterns of axonal innervation, and sensory-motor connectivity (Livet *et al.*, 2002; Vrieseling and Arber, 2006). Thus, motor pool specification appears to unfold in two main phases: an early phase that confers aspects of motor neuron identity involved in the selection of target muscle connectivity (Landmesser, 2001; Milner and Landmesser, 1999), and a later phase, operating after motor axons have reached their muscle targets, that is

associated with ETS gene expression and the clustering of motor neurons within the LMC (Livet *et al.*, 2002; Price *et al.*, 2002).

6. Restriction and Refinement of Hox Activities During Motor Neuron Differentiation

Although Hox protein activities appear to be critical in the generation of diverse motor neuron subtypes, several lines of evidence suggest that additional factors are necessary to restrict their functions. First, Hox proteins are broadly expressed throughout the embryo, and within the CNS they are expressed by multiple classes of neurons including interneurons and sensory neurons (Belting *et al.*, 1998; Dasen *et al.*, 2005; Ensini *et al.*, 1998). Second, within a given spinal segment the same Hox protein can be expressed by multiple columnar subtypes (Dasen *et al.*, 2008). These observations suggest the requirement for additional mechanisms to gate the actions of Hox proteins in motor neurons. This gating function may be controlled through transcriptional cofactors or through motor neuron-specific targets of Hox proteins.

Hox functions generally rely on interactions with a conserved family of DNA binding cofactors that refine and constrain their activities (Mann and Affolter, 1998). Two classes of canonical Hox cofactors, Meis and Pbx/Prep proteins (vertebrates homologs of the *Drosophila* Extradenticle and Homothorax proteins), have pervasive roles as regulators of Hox activity (Mann and Affolter, 1998; and Selleri, 2006). Within the spinal cord Meis and Pbx/Prep proteins display broad patterns of expression (Dasen *et al.*, 2005), and thus they are unlikely candidates as cell-type specific regulators of motor neuron Hox target specificities. Studies in *Drosophila* indicate that a distinct group of Hox accessory factors, the homeodomain protein Engrailed and the forkhead homeodomain proteins Sloppy-paired 1/2, functions with Extradenticle and Homothorax to modify Hox activities (Gebelein *et al.*, 2004). These factors have more restricted domains of expression and activity: Engrailed regulates Hox activity in posterior compartment cells whereas Slp1/2 regulate Hox activity in anterior cells (Gebelein *et al.*, 2004).

The vertebrate counterparts of these ancillary Hox cofactors, Engrailed and Fox proteins are expressed by subsets of spinal neurons, and a vertebrate FoxP protein, FoxP1, is selectively expressed by spinal motor neurons (Saueressig *et al.*, 1999; Shu *et al.*, 2001; Tamura *et al.*, 2003). Consistent with a role in gating Hox activities, expression of FoxP1 is restricted to two Hox sensitive columns (PGC and LMC neurons) while it is excluded from Hox-independent populations (HMC and MMC neurons). This exclusion from HMC and MMC motor neurons appears to be a function of the presence of inhibitory factors within these subtypes that prevent FoxP1

expression shortly after motor neurons are born (Dasen *et al.*, 2008). As described below, recent studies indicate that FoxP1 is an essential cofactor in the Hox-dependent program of differentiation in LMC and PGC neurons (Dasen *et al.*, 2008; Rousso *et al.*, 2008)

6.1. FoxP1: An accessory factor for Hox proteins in motor columns and pools

Although FoxP1 is expressed by LMC and PGC neurons, the levels of FoxP1 protein in these two columns differ dramatically: PGC neurons express low levels of FoxP1 and LMC neurons express high levels. These differences in FoxP1 levels appear to be set by the pattern of Hox expression as misexpression of Hox determinants of LMC identities such as Hoxc6 and Hoxd10 can switch HMC and PGC neurons to FoxP1 high LMC motor neurons at thoracic levels (Dasen *et al.*, 2008). In turn, the levels of FoxP1 protein appear to be critical in the specification of columnar fates as misexpression of FoxP1 in PGC and HMC neurons can convert these columns to LMC identities. These actions of FoxP1 appear to require continuous Hox function, since forced expression of FoxP1 is unable to induce LMC fates under conditions in which Hox activity is repressed (Dasen *et al.*, 2008). Thus FoxP1 appears to act jointly with Hox proteins rather than as a linear intermediary in the pathway of columnar specification (Fig. 6.5).

Consistent with a central role in the Hox-dependent steps of motor neuron differentiation, genetic inactivation of *Foxp1* does not effect the generation of motor neurons, or Hox expression, but nevertheless the number of LMC and PGC neurons is dramatically reduced (Dasen *et al.*, 2008; Rousso *et al.*, 2008). Instead prospective LMC and PGC motor neurons acquire molecular features of HMC neurons and the spinal cord consists almost entirely of two continuous columns (Fig. 6.5C). Strikingly all Hox-dependent steps of motor neuron differentiation, included the columnar, divisional and pool identities, are effectively erased. Molecular features of LMC divisional and pool identities are eroded in *Foxp1* mutants including the loss of specific LMC transcription factors (e.g., *Lhx1*, *Pea3*, *Nkx6.1*) as well as connectivity and synaptic specificity determinants (*EphA4*, *Sema3E*, *Cad20*) (Dasen *et al.*, 2008).

Although FoxP1 activities are essential for the transcriptional output of Hox proteins in LMC and PGC neurons, not all motor neuron Hox functions depend on FoxP1. Expression of FoxP1 is induced by Hox proteins, and thus this aspect of Hox function must be FoxP1 independent. In addition the repressive interactions between Hox paralogs are evident in MMC and HMC neurons (Dasen *et al.*, 2003), even though they lack FoxP1 expression. The activities of *Hox* genes involved in hindbrain motor neuron specification (Trainor and Krumlauf, 2000) also do not require FoxP1, since FoxP proteins are not expressed in motor neurons at

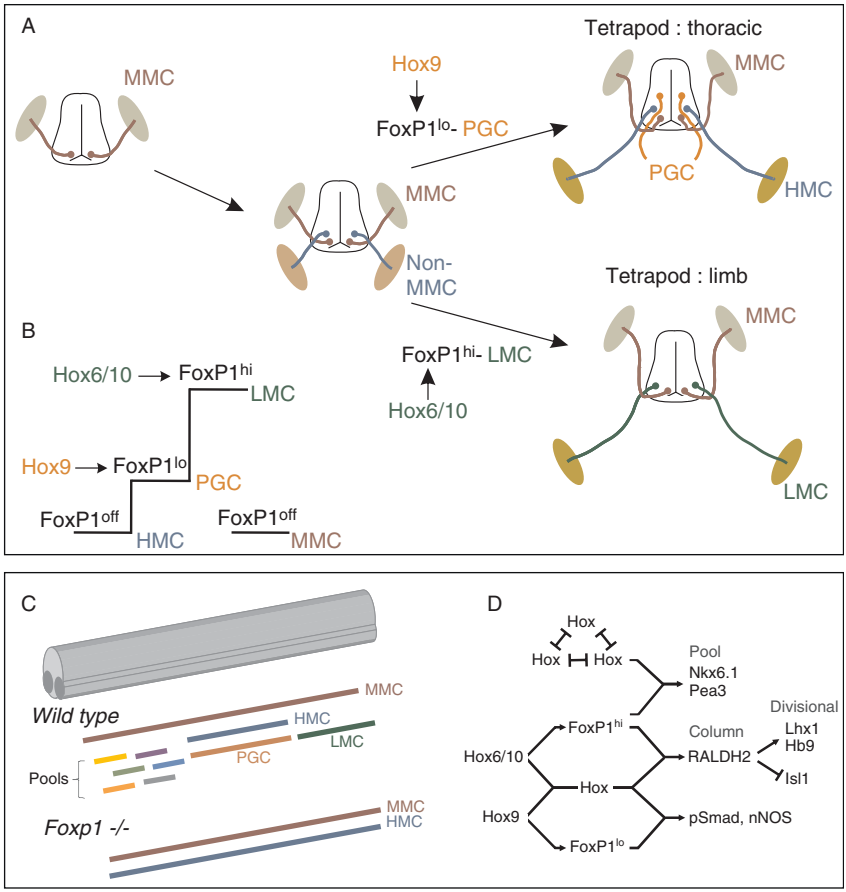

Figure 6.5 The *Hox/FoxP1* gene network and the emergence of motor neuron diversity. (A) Model for the emergence of motor neuron columnar subtypes. The presumed ancestral state of motor neurons is that of MMC neurons. Early aquatic vertebrates appear to possess two columnar subtypes, dorsally projecting MMC and ventrally projecting HMC neurons. In tetrapod vertebrates expression of FoxP1 at thoracic levels is under the control of Hox9 proteins, allowing for the generation of PGC neurons. At limb levels expression of FoxP1 is under the control of Hox6 and Hox10 proteins, allowing for the generation of LMC neurons and its resident motor pools. (B) The levels of FoxP1 are controlled by Hox proteins in PGC and LMC neurons. HMC but not MMC neurons are competent to respond the patterning activities of *Hox* genes, which control the level of FoxP1 expression and columnar fates. (C) Motor neuron columnar differentiation in wild type and *Foxp1* mutant mice, showing that FoxP1 controls the formation of PGC and LMC columns, as well as the diversification of motor pools within the LMC. In the absence of FoxP1, two continuous columns (MMC and HMC) are generated. (D) Interactions of FoxP1 and Hox proteins during the transcriptional programming of motor neuron columnar and pool fates. FoxP1 gates the activities of the two Hox networks involved in the assignment of columnar and pool identities. The Hox/FoxP1 network controls a repertoire of downstream genes including transcription factors, signaling molecules, and guidance receptors.

this level of the neuraxis. Meis and Pbx/Prep cofactors are expressed in the hindbrain, and Pbx proteins regulate cranial motor neuron identity (Moens and Selleri, 2006). Thus, the activities of FoxP1 as a mediator of Hox output during motor neuron differentiation may be exerted in a broader context of Meis and Pbx/Prep activities. Meis and Pbx/Prep cofactors may impart a first level of Hox specificity (Joshi *et al.*, 2007), with FoxP1 providing additional filters on target gene activation.

6.2. Coordinate control of motor axon targeting by FoxP1 and Hox factors

How does FoxP1/Hox network activity control the decisions taken by motor axons as they project to their targets? At thoracic levels of the spinal cord, the switch from PGC to HMC fate in *Foxp1* mutants is accompanied by a redirection of motor axons from sympathetic ganglia to body wall targets (Dasen *et al.*, 2008). In contrast, HMC-like motor neurons generated at brachial and lumbar levels in *Foxp1* mutants still project into the limb, following a path similar to that of LMC axons. This observation is consistent with studies showing that after transplantation of thoracic spinal cord to limb levels, motor axons are capable of innervating the limb (O'Brien *et al.*, 1990; O'Brien and Oppenheim, 1990). Further evidence that this is the predicted trajectory of HMC neurons generated at limb levels comes from more recent studies showing that ectopic limbs induced adjacent to thoracic spinal cord are innervated selectively by axons of HMC neurons (Turney *et al.*, 2003). Thus, HMC and LMC neurons appear to be similar in their initial pursuit of a distal trajectory that takes them to body wall or limb targets.

In the limb, LMC axons establish stereotypic projections to individual muscles (Landmesser, 2001). The establishment of these precise patterns of connectivity requires the actions of a multitude of Hox-dependent transcription factors, recognition molecules and guidance receptors. Despite the loss of expression of these factors in *Foxp1* mutants, nerve branching and limb muscle innervation patterns are remarkably well preserved (Dasen *et al.*, 2008; Rousso *et al.*, 2008). These findings fit best with the view that the overall pattern of motor nerve branching within the limb is determined by preestablished permissive or inhibitory domains within the limb mesenchyme, with the FoxP1/Hox program providing LMC neurons with identities that enable axons to respond to local cues that promote the selection of just one of many available conduits. In this view, the trajectory of motor neurons deprived of FoxP1 activity will still be constrained by the existence of preordained paths, but without Hox-dependent intrinsic molecular programming, individual axons are reduced to choosing haphazardly among their potential options. Consistent with this model, in *Foxp1* mutants the topographic map of motor projections is lost, as motor neurons no longer project into the limb in an organized manner (Dasen *et al.*, 2008).

6.3. Hox/FoxP1 interactions and the origins of motor neuron diversity

Analysis of the Hox/FoxP1 transcriptional network has provided clues into the possible mechanisms by which the tetrapod motor system emerged during evolution. Aquatic vertebrates with behaviors driven by axial and hypaxial muscles lack PGC and LMC neurons but possess neurons that are similar to MMC and HMC neurons in character (Fetcho, 1992; Kusakabe and Kuratani, 2005). The appearance of LMC and PGC neurons is linked to the formation of paired appendages (lateral fins and limbs) and a sympathetic nervous system—structures that emerged later in vertebrate evolution (Fetcho, 1992; Freitas *et al.*, 2006; Funakoshi and Nakano, 2007). The Hox/FoxP1 dependent program of motor neuron diversification may therefore have evolved to meet the demands of a new and diverse set of peripheral target tissues, and to generate more elaborate set of motor behaviors.

How did the Hox/FoxP1 transcriptional network emerge within motor neurons? The ancestral state of spinal motor neurons appears to be marked by expression of Lhx3, Isl1/2, and Hb9, a set of transcription factors conserved in the motor neurons of invertebrates (Landgraf and Thor, 2006). This transcriptional profile also defines early-born primary motor neurons of Zebrafish and Xenopus embryos (Appel *et al.*, 1995; Borodinsky *et al.*, 2004), presumed counterparts of the motor neurons of jawless vertebrates (Fetcho, 1992), as well as the MMC neurons of birds and mammals (Jessell, 2000). The conserved transcriptional profile of this ancestral set of motor neurons may reflect common patterns of connectivity—the innervation of segmentally arrayed muscles involved in undulatory locomotor behaviors. The diversification of columnar subtypes from this ancestral group requires relief from the confining influence of the LIM-homeodomain factor Lhx3, as Lhx3 exerts a dominant activity in specifying MMC fates over other columnar subtypes (Sharma *et al.*, 2000; William *et al.*, 2003). This evasive step may have involved a decrease in the strength of the Wnt signaling component of the dorsoventral inductive pathway, since reducing Wnt4/5 activity in mice promotes the generation of HMC neurons at the expense of MMC neurons (Agalliu *et al.*, 2009). Thus the basic spinal motor system induced by the dorsoventral signaling pathway comprises MMC and HMC neurons, arrayed in coextensive columns.

The induction of *Foxp1* expression under the regulatory control of Hox proteins may have permitted the twinned columnar organization of limbless vertebrates to diversify and generate PGC and LMC neurons. The formation of HMC neurons from an ancestral MMC-like group was a crucial step in this diversification program—creating a malleable population of Lhx3-negative motor neurons to serve as the substrate for the FoxP1/Hox program of columnar and pool specification. Prospective HMC neurons, freed from

the dominant actions of Lhx3, became competent to respond to the rostrocaudal patterning activities of Hox proteins, allowing them to acquire PGC or LMC fates. FoxP1, through its dose-dependent inductive activity, is a key mediator of the Hox program of columnar specification (Figure 6.5). Under the influence of Hox9 activity, newly generated Lhx3-negative thoracic motor neurons acquire the capacity for low-level FoxP1 expression and appear to resolve their bi-potential HMC or PGC fate through mutual repressive interactions between FoxP1 and Hb9 (Dasen *et al.*, 2008). In the limb-level domains of Hox6/10 expression, FoxP1 is induced at high levels which allow motor neurons to acquire an LMC fate.

The mechanisms by which the FoxP1/Hox network was recruited to the task of motor neuron columnar diversification remains unclear. The absence of PGC and LMC neurons from early vertebrates could have its basis in changes in the cis-regulatory elements that control *Foxp1* expression (Prud'homme *et al.*, 2007; Shubin *et al.*, 1997), such that Hox-sensitive elements responsible for expression in spinal motor neurons were configured only at the time of formation of the sympathetic nervous system and paired appendages. Alternatively, the rostrocaudal pattern of expression of *Hox* genes in the spinal cord of jawless vertebrates may differ from that in birds and mammals (Force *et al.*, 2002; Takio *et al.*, 2007), and thus may fail to produce a productive Hox code capable of activating FoxP1 expression. Further investigation into the regulatory elements controlling the *Foxp1* and *Hox* genes and their targets in motor neurons may provide insights into how columnar subtypes arose in diverse vertebrate species.

6.4. Coordinate regulation of neuronal and mesodermal Hox programs

The same extrinsic signaling systems which pattern Hox profiles in the CNS also control Hox expression within the mesoderm, raising the possibility that regulation of Hox expression in these distinct tissues may be involved in coordinating the position of motor neuron subtypes with the location and identity of their peripheral target fields. One aspect of this program is the establishment of a register between the rostrocaudal positions motor columns and their targets. Application of FGFs to thoracic mesoderm has been shown to induce the formation of an ectopic limb in place of body wall mesoderm (Cohn *et al.*, 1995), and the regulation of *Hox9* gene paralog expression in lateral plate mesoderm in response to FGFs has been implicated to determine limb bud position (Cohn *et al.*, 1997). These observations support the idea that exposure of neural and lateral plate mesodermal cells to a common source of FGFs establishes distinct profiles of *Hox* gene expression in these two tissues, profiles that in turn control the alignment of LMC and limb position.

Similarly, the muscle targets of LMC motor neurons within the limb appear to acquire aspects of their identity through Hox-dependent programs.

Limb musculature is derived from a population of migratory muscle precursors that is generated selectively at limb levels through the actions of the LIM-homeodomain protein Lbx1 (Brohmann *et al.*, 2000; Gross *et al.*, 2000). Interestingly, a Hox determinant of LMC identity, Hoxa10, has been shown to be sufficient to induce Lbx1 expression in muscle precursors at thoracic levels and to reprogram nonmigratory myoblasts into migratory muscle cells (Alvares *et al.*, 2003). Moreover, *Hox* genes control patterning within the limb mesenchyme and may therefore exert additional roles in the guidance of axons along the anterior-posterior and proximal-distal axes of the limb (Izpisua-Belmonte and Duboule, 1992), prior to establishment of the mature pattern of musculature. Thus, the coordinate regulation of Hox expression patterns during vertebrate evolution may provide a plausible basis for linking the formation and diversification of LMC motor neuron subtypes to the appearance and patterning of paired appendages.

7. Conclusions

The task of specifying hundreds distinct motor neuron subtypes, each projecting to a specific target cell group, appears to have been met by deploying a regulatory network of nearly two dozen *Hox* genes. The selective connections formed between motor neurons and muscle are however just one aspect of circuit assembly in the spinal cord. Additional components of motor neuron connectivity, such as the specificity of synaptic inputs from sensory neurons and interneurons, may also rely on the actions of the FoxP1/Hox transcriptional network. Hox and Fox proteins are expressed by other classes of spinal neurons, suggesting these transcription factor families could have a more extensive role in the assembly of locomotor circuits. The self-organizing features inherent in the FoxP1/Hox network may have therefore helped endow developing motor circuits with their apparent high degree of genetic determination.

Studies on the transcriptional programs that control diversity and synaptic specificity in the spinal cord have provided a valuable model system for understanding the mechanisms of neuronal specification throughout the CNS. Emerging work on retinal and cortical neurons suggest an equivalent high degree of subtype diversity, even within a single class of neurons (Klausberger and Somogyi, 2008; Kong *et al.*, 2005). Although retinal and cortical neurons are devoid of chromosomally clustered *Hox* gene expression, it is likely that similar cell-intrinsic transcriptional networks control neuronal specification throughout the CNS. Whether other CNS circuits utilize coherent gene families to generate highly diverse neuronal subtypes remains to be determined.

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