Hoxc10 and Hoxd10 regulate mouse columnar, divisional and motor pool identity of lumbar motoneurons

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A central question in neural development is how the broad diversity of neurons is generated in the vertebrate CNS. We have investigated the function of Hoxc10 and Hoxd10 in mouse lumbar motoneuron development. We show that Hoxc10 and Hoxd10 are initially expressed in most newly generated lumbar motoneurons, but subsequently become restricted to the lateral division of the lateral motor column (ILMC). Disruption of Hoxc10 and Hoxd10 caused severe hindlimb locomotor defects. Motoneurons in rostral lumbar segments were found to adopt the phenotype of thoracic motoneurons. More caudally the ILMC and dorsal-projecting axons were missing, yet most hindlimb muscles were innervated. The loss of the ILMC was not due to decreased production of motoneuron precursors or increased apoptosis. Instead, presumptive ILMC neurons failed to migrate to their normal position, and did not differentiate into other motoneurons or interneurons. Together, these results show that Hoxc10 and Hoxd10 play key roles in establishing lumbar motoneuron columnar, divisional and motor pool identity.

KEY WORDS: Hoxc10, Hoxd10, Motoneuron specification, Spinal cord, Mouse

INTRODUCTION

A key question in neural development is how a seemingly uniform population of progenitor cells gives rise to the enormous diversity of neurons in the vertebrate CNS. One system that has been central to addressing this issue is the spinal cord motoneurons. Mature spinal cord contains a wide diversity of motoneuron populations that can be distinguished by their positions in the spinal cord, their peripheral targets, and the constellation of transcription factors and surface molecules they express (Jessell, 2000; Price et al., 2002), yet all are derived from a common progenitor pool.

Considerable progress has been made in defining the mechanisms that control specification and differentiation of spinal cord motoneurons. Early in development the spinal cord becomes patterned along both the dorsoventral and rostrocaudal axis, with motoneuron generation occurring in a restricted ventral domain (Jessell, 2000). Once generated, motoneurons become highly organized into lateral and medial motor columns (LMC and MMC, respectively), subdivisions (lateral and medial) of the columns, and motor pools (Fig. 1A), each with characteristic peripheral targets and a unique position in spinal cord (reviewed by Landmesser, 2001). Research carried out over the last decade, primarily at brachial spinal segments, motor pools are also clearly disrupted in mutant animals. Together, our results show that Hoxc10 and Hoxd10 are expressed in the right time and place to function in motoneuron patterning. In the absence of Hoxc10 and Hoxd10 function, motoneurons in rostral lumbar segments fail to establish an LMC and instead differentiate as thoracic neurons. Surprisingly, in more caudal segments the LMC consists almost entirely of medial LMC (mLMC) neurons with few, if any, motoneurons differentiating into ILMC neurons. Since nearly all thigh muscles become innervated by mLMC neurons from a reduced number of spinal segments, motor pools are also clearly disrupted in mutant animals. Together, our results show that Hoxc10 and Hoxd10 play major roles in specifying the columnar, divisional and motor pool identities of lumbar motoneurons. In addition, mutations in these genes have minor, but consistent, effects on hindlimb muscle development.

MATERIALS AND METHODS

Generation of Hoxc10+/–/Hoxd10+/– mutant mice

The Hoxc10 allele has been reported previously (Wahba et al., 2001). A Hoxc10+/– knockout allele was generated by replacing the first exon with the red fluorescent protein (RFP) gene and a Hoxc10+/– reporter allele was generated by replacing the first exon with the humanized Renilla green fluorescent protein (hrGFP) gene (Fig. 2A). To generate the Hoxd10+/– knockout allele, an 8.9 kb XhoI-SalI genomic fragment spanning the two Hox10 exons was used to construct the targeting vector. A reporter neo cassette containing the RFP, DsRed2 (Clontech) DNA and a self-excision neomycin resistance gene (neo), was used to replace the first exon of Hoxc10, the first five amino acids of Hoxc10 being left intact and in-frame.

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with RFP. To construct the Hoxd10 targeting vector, a 9.3 kb EcoRI-SpeI genomic fragment spanning the two Hoxd10 exons was used. A reporter-neo cassette containing an hrGFP (Stratagene) cDNA and a self-excision neo was used to replace the first exon of Hoxd10, the first three amino acids of Hoxd10 being left intact and in-frame with hrGFP. In addition, the thymidine kinase gene (Tk1) was included in the final targeting vectors of Hoxc10 and Hoxd10. To allow removal of the neo selection cassette subsequent to its use for isolation of targeted ES cell lines, the selectable gene neo, is embedded in a Cre/loxP-based self-excision cassette, in which Cre expression is mediated by a promoter, ACE, that is not expressed in ES cells, but is expressed in the male germline of mouse chimeras derived from these ES cells during spermatogenesis (Bunting et al., 1999).

The linearized targeting vectors were used for gene targeting in R1-45 embryonic stem (ES) cells. For Hoxc10, four out of 72 clones were confirmed by Southern blot to have undergone correct homologous recombination. For Hoxd10, two out of 144 clones were confirmed positive. One positive ES cell clone for Hoxc10 or Hoxd10 was injected into blastocysts to produce male chimeras, which were further backcrossed to C57BL/6j females. Among brown-colored offspring, heterozygotes were obtained for both Hox genes. The following PCR primers were used for genotyping: Hoxc10RFP (primer 1: 5’-AGATGTCAGCTCCCTCGCTGATG-3’; primer 2: 5’-GTCACCTTCAGGGTACGGT-3’; primer 3: 5’-AACAGGTGGGTCCAGCGG-3’; the mutant band is 248 bp and the wild-type band is 330 bp) and Hoxd10hrGFP (primer 1: 5’-CAATGCTTCGTTTGGCTGTTGACGGAT-3’; primer 2: 5’-CTCCAGGTTCACTTTGAGC-3’; primer 3: 5’-AAAGATCGTTCGTTTGGCTGTTGACGG-3’; the mutant band is 234 bp and the wild-type band is 385 bp). PCR conditions were: 94°C 30 seconds, 59.5°C 30 seconds and 72°C 30 seconds, for 31-35 cycles.

Immunohistochemistry and in situ hybridization

Immunostaining and in situ hybridization were performed as previously described (Boulet and Capecchi, 1996; Huber et al., 2005; Wang and Scott, 2007). The following primary antibodies were used: mouse anti-
Islet1 [1:50, 39.4D5, Developmental Studies Hybridoma Bank (DSHB)]; rabbit anti-Islet1 (1:2000, provided by Dr S. Pfaff, Salk Institute, San Diego, CA); rabbit anti-Hb9 (also known as Mnx1 – Mouse Genome Informatics; 1:8000, provided by Dr S. Pfaff); rabbit anti-Lim3 (also known as Lhx3 – Mouse Genome Informatics; 1:2000, provided by Dr S. Pfaff); rabbit anti-Lim1 (also known as Lhx1 – Mouse Genome Informatics; 1:2000, provided by Dr T. M. Jessell, Columbia University, New York); rabbit anti-nNOS (1:5000, ImmunoStar, Hudson, WI); rabbit anti-Olig2 (1:20.000, provided by Dr J. Albert, Harvard University, Boston, MA); sheep anti-Lim1 (also known as Lhx1 – Mouse Genome Informatics; 1:2000, provided by Dr S. Pfaff, Salk Institute, San Diego, CA); rabbit anti-Hb9 (also known as Mnx1 – Mouse Genome Informatics; 1:8000, provided by Dr T. M. Jessell, Columbia University, New York); rabbit anti-Islet1 (1:50, 39.4D5, Developmental Studies Hybridoma Bank (DSHB)); mouse anti-myosin (1:4000, my32, Sigma); rabbit anti-MyoD (1:50, Santa Cruz); mouse anti-neurofilament 165 (1:50, 2H3, DSHB); mouse anti-βIII-tubulin (1:1000, Sigma); mouse anti-Brdu antibody (1:5, G3G4, DSHB). Species-specific Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies (Invitrogen) were used at 1:1000. A Radlb2 (also known as Aldh1a2 – Mouse Genome Informatics) probe (provided by Dr Song Wang from our laboratory) was transcribed from a 1043 bp cDNA fragment (1199-2241 bp; NM_008263). Hoxa10 probe was transcribed from a 1043 bp cDNA fragment (1199-2241 bp; NM_008263). ER81 (also known as Er81 – Mouse Genome Informatics) and Paa3 (also known as Paa – Mouse Genome Informatics) plasmids were provided by Dr S. Arber, University of Basel, Switzerland; the Sema3F plasmid was provided by Dr T. M. Jessell. Other template plasmids (Hoxc10, Hoxd10, Hoxc9, Hoxd9, Hoxc11 and Hox11) were created in our laboratory (Hostikka and Capecchi, 1998).

To combine in situ hybridization and immunolabeling, sections were first processed for in situ hybridization with digoxigenin-labeled probes, visualized with NBT-BCIP, and then immunolabeled. In situ images were pseudocolored prior to combining with images of immunostaining.

Cell death in motoneurons was assessed by TUNEL (Cell Death Detection Kit; Roche) on Isl1- or Hb9-labeled sections. Mitotic cells were pseudocolored prior to combining with images of immunostaining. In situ images were processed for in situ hybridization with digoxigenin-labeled probes, created in our laboratory (Hostikka and Capecchi, 1998).

RESULTS

Hoxc10 and Hoxd10 are expressed exclusively in the lumbar spinal cord

To gain insight into the function of Hox10 genes during embryonic development, we analyzed their expression patterns in lumbar spinal cord from E10.0-E16.5. Mouse spinal cord has six lumbar segments, with hindlimb muscles being innervated by LMC motoneurons in segments L1-L5 (Lance-Jones, 1982; Lin and Carpenter, 2003; Tarchini et al., 2005). All three Hox10 transcripts were first detected in lumbar spinal cord at E10.5, and the rostrocaudal expression domain of each did not change in any of the ages examined (data not shown). By E13.5 Hoxc10 and Hoxd10 were expressed most intensely in segments L2-L4, with lower levels of expression in L1 and segments caudal to L4 (Fig. 1B). Whereas Hoxc10 and Hoxd10 were expressed only in lumbar regions, Hoxa10 expression extended from T10 to lumbar levels (Fig. 1B) (Choe et al., 2006).

Transverse serial sections confirmed the timing and rostrocaudal extent of expression of all three Hox10 transcripts (Fig. 1C-E). Furthermore, by staining these sections with antibodies to distinguish different motor columns (Fig. 1A), we were able to determine the cell types that expressed different Hox10 transcripts, and show that each transcript had a unique, highly dynamic pattern of expression. Postmitotic motoneurons, identified by Islet1 (Isl1) expression, first appeared in lumbar spinal cord around E10.0-E10.5 (data not shown), slightly later than reported for brachial spinal cord (Arber et al., 1999), and by E11 most, if not all, postmitotic motoneurons in segments L2-L4 expressed Hoxa10 and Hoxd10 (Fig. 1C). By contrast, Hoxc10 was expressed in a very focal ventral domain, overlapping the
characteristic position of the V3 interneuron domain (Fig. 1C) (Briscoe et al., 1999), and extending rostrally into thoracic spinal cord (data not shown).

At later stages, from E11.5 onward, Hoxa10 is expressed in motoneurons (data not shown). Although expression of all three paralogs expanded to cells throughout much of the ventral two-thirds of the spinal cord, Hoxc10 and Hoxd10 transcripts are lost from many motoneurons with each becoming restricted to specific populations of motoneurons (Fig. 1D,E). For example, at E13.5, the stage when LMC and MMC neurons have segregated into distinct motor columns, both genes are weakly expressed in Is11+ mMMC neurons in caudal L1 and throughout segment L2, but are almost undetectable in the LMC. At L3, although both Hoxc10 and Hoxd10 are expressed in Lim1+ lLMC motoneurons (in addition to the mMMC), they appear to be expressed in different subpopulations of motoneurons, most likely in different motor pools. At L4 and L5, whereas Hoxc10 or Hoxd10 are expressed throughout the ILMC and mMMC, a small number of Is11+ mLMC neurons also express Hoxc10 and Hoxd10 (Fig. 1D,E).

The posterior expression limits of Hox10 genes are more easily defined in section in situ hybridizations than in whole-mount preparations. Hoxc10 and Hoxd10 expression in motoneurons extends only through L5 (data not shown). These genes are also expressed at extremely low levels in intermediolateral regions in more caudal spinal cord, which could account for the caudal expression of Hox10 genes observed in whole-mount preparations (Carpenter, 2002) (Fig. 1B). Taken together, our data show that both Hoxc10 and Hoxd10 are initially expressed in almost all newly generated motoneurons but later become restricted to subpopulations of motoneurons, primarily to ILMC and mMMC neurons, suggesting they may play sequential roles in specifying or maintaining motoneuron identity at different stages. Conversely, the absence of Hoxa10 from motoneurons at early stages suggests that this paralog may have relatively little influence on motoneuron specification. Moreover, because Hoxc10 and Hoxd10 have similar, although not identical, expression patterns in the developing spinal cord, they may share redundant functions in motoneuron development.

**Generation of Hoxc10 and Hoxd10 double mutants**

To examine the roles of Hox10 genes in the development of hindlimb motoneurons, we analyzed different combinations of Hox10 double and triple-mutant mice. The Hoxa10 allele has been reported previously (Wahba et al., 2001). Here we describe mice carrying new alleles of Hoxc10 and Hoxd10 that lacked the neo cassette (Fig. 2), which we created because the presence of neo can affect the mouse phenotype by altering the expression of nearby genes (Greer and Capecchi, 2002; Manley et al., 2001). This is
particularly a problem with Hox genes, since the density of genes is high within this complex. Homozygous mutant mice were found to lack Hoxc10 or Hoxd10 transcripts when examined by in situ hybridization (data not shown), and thus appear to be null mutants. Both single and double-mutant animals were viable and had an apparently normal lifespan, although the double mutants were sterile. Double heterozygotes and single homozygous mutants did not have any obvious alteration in gait, and therefore heterozygotes were sometimes used with wild-type (WT) animals as controls in this study. The lack of an aberrant phenotype in double heterozygous and single homozygous mutants differs from the phenotypes of previously generated Hoxd10 null mutants, which had obvious defects in locomotor behavior (Carpenter et al., 1997; Tarchini et al., 2005). The more normal behavior observed in our single mutants most likely results from the lack of the neo gene in our alleles. The lack of apparent locomotor phenotypes associated with mutations in either Hoxc10 or Hoxd10 alone also emphasizes the redundant functions of these Hox genes.

Animals carrying three mutant alleles had obvious locomotor defects, which varied in severity among different animals. Hoxc10–/–/Hoxd10–/– double-mutant animals had even more severe defects in locomotion. Hindlimbs in these animals were held crossed in a rigid, extended position, and were not used to support body weight or for walking in an alternating right-left fashion. This phenotype was nearly invariant among Hoxc10–/–/Hoxd10–/– mutant animals and was 100% penetrant (n>20; Fig. 2C).

We next compared Hoxc10–/–/Hoxd10–/– double-mutant animals with Hoxa10–/–/Hoxc10–/– and Hoxa10–/–/Hoxd10–/– double mutants. Locomotor defects in Hoxc10–/–/Hoxd10–/– mutants were significantly more severe than in the latter two groups. Surprisingly, locomotor defects in the Hox10 triple-mutant animals (n=3) seemed to be less severe than in Hoxc10–/–/Hoxd10–/– double mutants, although the triple-mutant mice died around weaning, as a result of kidney defects. Thus, it appears that the loss of Hoxa10 does not significantly contribute to defects in locomotor behavior, most likely because Hoxa10 is not expressed within the motoneuron domain at early stages (Fig. 1C). Given the less prominent expression pattern of Hox10 in the lumbar motor column, and the negligible additional contribution of the Hoxa10 mutation to the Hoxc10–/–/Hoxd10–/– mutant phenotype, we focused further analysis primarily on Hoxc10–/–/Hoxd10–/– double-mutant animals.
Hindlimb muscle morphology and innervation patterns in Hox10 mutants

Severe locomotion defects could result from either motoneuron projection errors and/or altered muscle patterning. Analysis of the overall pattern of muscle (Greene, 1935) and nerve innervation in cross-sections through the hindlimbs of E14.5-E15.0 mutant and WT embryos revealed that the anterior head of the biceps was missing from the thigh in four out of seven Hoxc10<sup>−/−</sup>/Hoxd10<sup>−/−</sup> mutant embryos. In the shank, the extensor hallucis longus was missing from the anterior group in four out of four double-mutant embryos, and two more muscles were missing from the lateral group in three out of four double-mutant embryos (Fig. 3A and see Fig. S1 in the supplementary material). The loss of these muscles was confirmed by dissecting P0 hindlimb muscles stained with AP-conjugated anti-myosin (data not shown). To our knowledge, this is the first report of muscle defects associated with Hox10 mutant animals.

Although a few muscles failed to form normally, this minor disruption in muscle patterning is unlikely to account for the very severe gait abnormalities observed in Hoxc10<sup>−/−</sup>/Hoxd10<sup>−/−</sup> mutant animals. By contrast, nerve patterning was markedly abnormal in these double mutants. Axons were clearly detected in all muscles in cross-sections through the thigh of Hoxc10<sup>−/−</sup>/Hoxd10<sup>−/−</sup> mutants. In the shank, however, muscles in both the anterior and lateral groups received no, or greatly reduced, innervation (Fig. 3A and see Fig. S1 in the supplementary material). In contrast to the Hoxc10<sup>−/−</sup>/Hoxd10<sup>−/−</sup> mutants, there were no apparent muscle patterning or innervation defects in mice homozygous for the single, new Hoxc10<sup>−/−</sup> or Hoxd10<sup>−/−</sup> mutant alleles (see Fig. S1 in the supplementary material), consistent with the observation that these single-mutant mice show no obvious locomotor defects.

The lack of innervation to anterior and lateral shank muscles in the double mutants was confirmed in whole-mount embryos stained with neurofilament antibody at E12. Importantly, neurofilament

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**Fig. 5. Expression patterns of Raldh2, Pea3 and ER81 are altered in Hoxc10<sup>−/−</sup>/Hoxd10<sup>−/−</sup> double-mutant embryos. (A-C) In situ hybridization of Raldh2, Pea3 and ER81 in (A-C) whole-mount spinal cord (ventral view, rostral is to the top) and (A’-C’) cross-sections through ventral spinal cord (outlined in black), as indicated in the drawing; lateral is to the left in each panel. D, dorsal root ganglion, outlined in white. Note that in Hoxc10<sup>−/−</sup>/Hoxd10<sup>−/−</sup> mutant embryos, Raldh2 and Pea3 expression was restricted to segments L3-L5, being absent from segments L1 and L2. By contrast, ER81 expression extends from thoracic segments into segments L1 and L2, but is lost from segments L3 and L4 in mutants. Scale bar: 100 μm for A’-C’.
staining also revealed striking abnormalities in the contribution of spinal nerves to hindlimb innervation. In WT mice, segments L1-L3 contribute axons to the rostral lumbar plexus and segments L3-L5 contribute to the caudal sacral plexus (Fig. 3B). In the Hoxc10^{-/-}/Hoxd10^{-/-} mutant mice, however, L1 and L2 did not project to the hindlimb, and instead appeared to innervate the body wall, and L3 and L4 contributed axons to the lumbar plexus and L4 and L5 contributed to the sacral plexus (n=6; 100% penetrance; Fig. 3B). At the level of the lumbar plexus, both the dorsal and ventral branches were present but significantly reduced in size, most likely because of the reduced number of segments projecting to this plexus. By contrast, the dorsal branch of the sacral plexus (the peroneal nerve), which normally supplies the anterior and lateral groups of muscles in the shank, was totally absent, whereas the ventral branch (the tibial nerve) was only slightly smaller than in WT embryos (Fig. 3B,C). The near total lack of innervation of anterior and lateral shank muscles, which normally extend and abduct the limb, could be a major reason for the crossed-limb phenotype observed in Hoxc10^{-/-}/Hoxd10^{-/-} double-mutant mice.

In Hox10 triple-mutant embryos, the limb was innervated by even more caudal segments, with L4 and L5 contributing to the lumbar plexus and L5 and L6 contributing to the sacral plexus (data not shown). Surprisingly, the peroneal nerve was present in the triple mutants, although it was significantly smaller than normal (n=3).

This may explain why the Hox10 triple-mutant animals seemed to have less severe locomotor defects than Hoxc10^{-/-}/Hoxd10^{-/-} double mutants.

**L1 and L2 motoneurons become thoracic motoneurons in Hoxc10^{-/-}/Hoxd10^{-/-} double mutants**

The above observations indicated that the L1 and L2 spinal nerves projected to the body wall rather than to the limb in Hoxc10^{-/-}/Hoxd10^{-/-} mutants. This was confirmed by DiI injections into spinal cord segments L1 and L2 in double mutants (Fig. 4A). In addition, we observed many features consistent with the hypothesis that motoneurons in segments L1 and L2 actually differentiated into thoracic motoneurons in Hoxc10^{-/-}/Hoxd10^{-/-} mutants, and never acquired characteristics of LMC neurons. For example, the columnar organization of motoneurons in the ventral horn of segments L1 and L2 resembled that of thoracic rather than lumbar spinal cord in double mutants (Fig. 4B). Normally at thoracic levels in WT animals, there are two motor columns in the spinal cord: the MMC in ventral cord, which has a medial and lateral division (mMMC and lMMC), and the visceral sympathetic preganglionic motor column (PGC) in the intermediolateral cord (Fig. 1A). By contrast, at lumbar levels motoneurons occupy the large LMC and smaller mMMC. In
Hoxc10−/−/Hoxd10−/− mutants, however, staining of spinal cord sections for Isl1 and Lim3 showed that the arrangement of motor columns characteristic of thoracic cord extended caudally to the rostral part of L3 (Fig. 4B), with an apparent LMC first appearing only at L3. Furthermore, neuronal form of nitric oxide synthase (nNOS)+/Isl1+ staining in intermediolateral cord, which is characteristic of visceral motoneurons (Thaler et al., 2004), extended caudally into rostral L3 in the double mutants (Fig. 4C). These ectopic nNOS+/Isl1+ motoneurons behave like those in the thoracic region, projecting their axons to sympathetic ganglia (Fig. 4D). Thus, in Hoxc10−/−/Hoxd10−/− mutants, motoneurons characteristic of thoracic cord were present in segments L1 and L2.

By contrast, markers characteristic of LMC neurons were absent from L1 and L2 in these mutant embryos. Retinaldehyde dehydrogenase 2 (Raldh2), a generic marker for LMC motoneurons, is expressed in LMC motoneurons throughout all lumbar segments in WT animals (Sockanathan and Jessell, 1998). In Hoxc10−/−/Hoxd10−/− mutant animals, however, Raldh2 expression was absent from segments L1 and L2 (Fig. 5A,A′), and was reduced in more caudal lumbar segments. Further evidence that LMC neurons were missing is that hindlimb muscles normally innervated by LMC neurons in these segments, such as the adductor (not shown) and quadriceps (Fig. 6E), were innervated by motoneurons in segment L3. Finally, several markers characteristic of specific LMC motor pools were not expressed in segments L1 and L2 in double mutants. For example, Pea3 (Arber et al., 2000) and Sema3E (Livet et al., 2002; Messersmith et al., 1995) are normally expressed in several LMC motor pools at lumbar levels from L1 to L5, but not in thoracic regions. In double-mutant embryos, neither gene was expressed in segments L1 and L2, but both were still expressed in more caudal segments (Fig. 5B,B′ and data not shown). In mice, ER81 is normally expressed in both thoracic (Fig. 5C,C′) and lumbar spinal cord (Arber et al., 2000), with a clear gap between the thoracic domain and the two lumbar pools (Fig. 5C,C′, Control). By contrast, in the double mutants, the thoracic domain of ER81 expression extended into segments L1 and L2, and expression of ER81 was lost in segments L3 and L4 (Fig. 5C,C′).

Because Hox9 genes, especially Hoxc9, are suggested to be thoracic motoneuron determinants in chick (Dasen et al., 2003; Dasen et al., 2005), we asked whether altered expression of Hoxc9 and Hoxd9 in Hoxc10−/−/Hoxd10−/− mutants could account for the switch of L1 and L2 motoneurons to a thoracic identity. In WT embryos, Hoxc9 and Hoxd9 were expressed in thoracic and lumbar spinal cord, with expression terminating around lumbar segment L5. There was no obvious change in expression of either Hoxc9 or Hoxd9 in the double knockouts (see Fig. S2 in the supplementary material; data not shown) (Carpenter et al., 1997). Hoxc11 and Hoxd11 transcripts were observed from L3 through the sacral segments in control embryos, and expression of these Hox genes was also not obviously altered in the double mutants (see Fig. S3 in the supplementary material; data not shown) (Tarchini et al., 2005).

Taken together these data show that motoneurons in segments L1 and L2 in Hoxc10−/−/Hoxd10−/− double mutants differentiate as thoracic, rather than lumbar, motoneurons. Moreover, these findings identify Hoxc10 and Hoxd10 as having important roles both in establishing the border between thoracic and lumbar segments of spinal cord, and in defining the identity of motoneurons in these segments.

**Lim1+ lateral LMC motoneurons are absent in segments L3-L5 of double mutants**

Whereas motoneurons from L1 and L2 were converted to thoracic phenotypes, motoneurons from L3-L5 maintained their LMC identity, defined by Raldh2 expression, and innervated the hindlimb in double mutants. However, neurons in segments L3-L5 are clearly impacted by deletion of Hoxc10 and Hoxd10 function. Since most hindlimb muscles received some innervation (Fig. 3A and see Fig. S1 in the supplementary material), motoneurons in segments L3-L5 must have taken over some functions normally mediated by neurons in L1 and L2. Despite this, there appeared to be fewer LMC neurons in L3-L5 in double mutants; Raldh2 (Fig. 5A′) and Hb9 (Fig. 6C) expression were greatly reduced, and the peroneal nerve was missing entirely (Fig. 3B,C). To resolve these discrepancies, with an eye toward elucidating additional functions of Hox10 genes in motoneuron differentiation, we examined the subtype identity of LMC neurons in double mutants. Surprisingly, ILMC motoneurons, as defined by Lim1 staining, were severely reduced or absent in eight out of ten double-mutant embryos at E13.5, and noticeably reduced in the other two embryos (Fig. 6A). Lim1+ ILMC neurons were also not observed in lumbar segments at earlier stages (see Fig. S5 in the supplementary material), indicating that Lim1+ LMC neurons failed to differentiate in double mutants, rather than having differentiated and died. Instead, neurons in ventrolateral spinal cord, the usual location of the ILMC, expressed Isl1, characteristic of mLMC, and Pea3 was restricted to Isl1+ neurons, instead of being expressed in both Lim1+ and Isl1+ motoneurons (Fig. 5B′ and data not shown), as in WT mice (Arber et al., 2000; Wang and Scott, 2007).

The number of Hb9+ neurons was reduced (Fig. 6C and see Fig. S6 in the supplementary material), demonstrating a paucity of LMC neurons. However, the number of mLMC neurons in L3-L5 was the same in control and Hoxc10−/−/Hoxd10−/− mutants, based on counts of Isl1+ cells in serial sections of seven embryos of each genotype (Fig. 6B). Similarly, the number of Lim3+ neurons was not obviously affected in double mutants (data not shown). Thus, the missing Lim1+ motoneurons do not appear to have become Isl1+ or Lim3+ neurons. Instead, the ILMC appears to be missing entirely, with Isl1+ mLMC neurons being displaced to the most lateral part of the spinal cord in its absence. By contrast, the ILMC appeared to form normally in embryos homozygous for the individual new Hox10 or Hoxd10 mutant alleles (see Fig. S4 in the supplementary material).

Because Lim1+ ILMC motoneurons normally project their axons to dorsally derived hindlimb muscles in WT animals, the loss of the Lim1+ LMC neurons is most likely responsible for the absence of the peroneal nerve (Fig. 3B,C). This differs from the loss of the peroneal nerve in EphA4 mutants, which results from misrouting of ILMC neurons into ventral branches (Helmbracher et al., 2000). We verified that neurons in segments L3-L5 did not project in any dorsal nerve by injecting DiI into motoneurons in these segments (Fig. 6D). Thus, the loss of the Lim1+ ILMC neurons explains the lack of innervation in anterior or lateral shank muscles, which are normally innervated by axons in the peroneal nerve (Fig. 3A and see Fig. S1 in the supplementary material).

Intriguingly, however, dorsal thigh muscles, which are also normally innervated by Lim1+ ILMC neurons projecting in a dorsal nerve, clearly received some innervation (Fig. 3A) even though their usual motor pools appeared to be missing. To determine which neurons supplied dorsal thigh muscles, we retrogradely labeled quadriceps motoneurons with tetramethylrhodamine dextran. As expected (McHanwell and Biscoe, 1981), the quadriceps in WT

**DEVELOPMENT**
animals was innervated by Lim1+/Isl1− ILMC motoneurons in segment L2, with a smaller contribution from L1. By contrast, quadriceps muscles in double-mutant embryos were innervated by Lim1+/Isl1− mLMC motoneurons in segment L3 (Fig. 6E). Thus, in the absence of Hox10 and Hoxd10 function some mLMC motoneurons became misrouted and innervated dorsal-derived thigh muscles. The aberrant innervation of extensor muscles in WT animals most likely contributed significantly to the locomotor defects in double mutants. If motoneurons receive their usual complement of central connections, as occurs when they innervate inappropriate muscles following surgical manipulations (Landmesser and O’Donovan, 1984; Vogel, 1987), then both extensors and flexors would be activated at the same time, leading to the rigid extended posture of limbs in mutant animals.

**Motoneurons are generated on schedule and in normal numbers in the double mutants**

There are a number of possible explanations for the absence of ILMC neurons in Hox10+/−/Hoxd10+/− double mutants. To elucidate the functions of Hoxc10 and Hoxd10 during normal development, it was important to determine whether these gene products affect the initial generation of motoneurons, or are required for subsequent steps in their differentiation, migration and/or survival. To this end, we examined whether the earliest stages of motoneuron generation were compromised in Hox10+/−/Hoxd10+/− mutants. Both the specification of motoneuron precursors and generation of postmitotic motoneurons appeared normal in double-mutant embryos. There was no obvious change in expression of Olig2, a marker for motoneuron progenitors, at E10.0-E10.5 at any segmental level in the double mutants (Fig. 7A). Moreover, there were no differences in Ngn2 and Nkx6.1 expression, two additional markers for ventral progenitors, between control and mutant embryos (data not shown). Isl1 is initially expressed by all newly generated postmitotic motoneurons in WT animals as they emerge from the ventricular zone, although its expression is subsequently lost in ILMC motoneurons (Arber et al., 1999; Ericson et al., 1992; Thaler et al., 2004). As with motoneuron progenitors, the numbers of newly generated Isl1+ postmitotic motoneurons were similar in control and double knockout embryos at early stages (E10.0-E11.0; Fig. 7B). These observations suggest that Hoxc10 and Hoxd10 function are dispensable for the generation and initial specification of motoneurons during early embryogenesis.

**Late-born motoneurons survive, but are misplaced in double mutants**

The striking reduction in ILMC neurons in double mutants was not brought about by increased apoptosis. TUNEL staining of serial sections of lumbar spinal cord was nearly identical in controls and double mutants at all ages examined (E10.5-E14.0; see Fig. S6 in the supplementary material). This differs from a previous study in which increased apoptosis of neurons was suggested as a reason for forelimb locomotor defects in Hoxc8 mutants (Tiret et al., 1998).

Motoneurons were initially generated in normal numbers and did not die in excess of normal, yet the entire Lim1+ ILMC was absent in double mutants. Where are these missing neurons? To address this question, we compared the fate of Hoxd10-expressing cells, the cells we expected to be most directly affected by loss of the Hoxd10 gene product, in control and double-mutant animals. Because the Hoxd10 mutant allele was generated by replacing the first exon of the Hoxd10 gene with the hrGFP gene, we could follow the fate of cells in the double mutants by analyzing hrGFP expression in sections of heterozygous and double-mutant embryos. At E13.5 in Hoxc10+/+/Hox10+/− heterozygous control embryos, hrGFP+ motoneurons were located in the most lateral part of ventral horn (Fig. 8A), closely resembling the pattern of endogenous Hoxd10 expression in the ILMC in WT embryos (Fig. 1E). By contrast, in the double-mutant mice, hrGFP+ cells were no longer tightly clustered laterally, but instead were scattered throughout the entire ventral horn area (Fig. 8A). This finding suggests that the inactivation of Hoxc10 and Hoxd10 alters migration of Hoxd10-expressing cells in lumbar spinal cord.

The altered distribution of Hoxd10-expressing cells in the ventral horn raised the possibility that the missing ILMC motoneurons had changed their identity, but the numbers of motoneurons in the mLMC and mMMC had not increased in double mutants (Fig. 6B) and the numbers of Hb9+ cells had decreased (Fig. 6C). Further, there was no obvious change in the expression of Chx10, a V2 interneuron marker (Arber et al., 1999; Ericson et al., 1997) in double mutants (data not shown). Instead, it appeared that cells fated to be ILMC neurons were born in normal numbers, but failed to acquire or retain markers characteristic of other populations of mature motoneurons or interneurons.

Motoneuron generation starts at E10.0 at the hindlimb level and is mostly completed by E11.0. Prospective ILMC motoneurons exit the cell cycle later than prospective mLMC motoneurons. These late-born motoneurons emerge from the ventricular zone, migrate laterally past the earlier-born mLMC motoneurons, acquiring their ILMC identity during the migration process, and eventually settle in the lateral part of the ventral horn (Sockanathan and Jessell, 1998). The difference in timing of generation of mLMC and ILMC neurons allowed us to investigate the fate of the late-born motoneurons in more detail. We labeled late-born cells by injecting BrdU into pregnant females at E10.5, a time by which the early-born motoneurons in WT embryos have already exited cell cycle and no longer incorporate BrdU, and analyzed motoneuron identity and distribution at E12.0. In the control embryos, most BrdU+ cells in the ventral horn settled laterally and were Lim1+/Isl1+, suggesting that late-born cells were indeed ILMC motoneurons (Fig. 8B). By contrast, in double-mutant embryos, BrdU+ cells were scattered throughout the ventral horn and intermingled with Isl1+ cells.
Importantly, most BrdU+ cells expressed neither Isl1 nor Lim1. Thus, the loss of Hoxc10 and Hoxd10 appears to cause late-born motoneurons to differentiate incompletely. These neurons downregulated expression of Isl1 on schedule, but failed to migrate to their appropriate location or acquire other markers characteristic of mature motoneurons, consistent with the observed decrease in HB9+ cells. It is possible that some of the late-born motoneurons in double mutants differentiated into interneurons, but investigation of this possibility must await discovery of additional markers of mature interneurons.

**DISCUSSION**

During embryonic development, motoneurons become patterned with respect to their columnar, divisional and pool identities, which enables them to establish connections with the appropriate peripheral target muscles with remarkable precision (Jessell, 2000; Landmesser, 2001). Here we show that Hoxc10, which had not been previously studied, and Hoxd10 together play essential roles in patterning lumbar motoneurons at all three levels of organization, and elucidate some of the cellular and molecular processes governed by these genes. We also show that these genes have minor, but consistent, effects on patterning hindlimb muscles.

**Hoxc10 and Hoxd10 determine the rostral boundary of lumbar motor columns**

Our analysis of Hoxc10+/–/Hoxd10+/– double-mutant embryos showed conclusively that Hox10 gene products govern the patterning of lumbar versus thoracic motor columns. Motoneurons in segments L1 and L2 differentiated as thoracic motoneurons in double mutants, expressing nNOS, but failing to express markers of LMC neurons, such as Raldh2. The remaining LMC in segments L3-L5 innervated the entire hindlimb, indicating that the LMC in Hoxc10+/–/Hoxd10+/– mutants was compressed from five to three segments, rather than simply being shifted posteriorly, as suggested previously (Lin and Carpenter, 2003).

Several lines of evidence suggest that Hoxc10 and Hoxd10 play primary roles in the patterning of thoracic versus lumbar motor columns within the spinal cord, with Hoxc10 playing a lesser role. For example, Hoxa10, unlike Hoxc10 and Hoxd10, is not expressed in prospective motoneurons during their early genesis, and therefore is unlikely to be involved in the early steps of their specification. Furthermore, locomotor defects were more severe in Hoxc10+/–/Hoxd10+/– mutants than in Hoxc10+/–/Hoxc10+/– or Hoxa10+/–/Hoxd10+/– mutants. Importantly, ectopic expression of Hoxd10 in thoracic motoneurons in chick is sufficient to convert them into lumbar-like motoneurons (Shah et al., 2004). The conversion of prospective LMC neurons in L1 and L2 to thoracic motoneurons in Hoxc10+/–/Hoxd10+/– mutants may result from the persistence of Hox9 gene function in these neurons in the absence of normal Hoxc10 and Hoxd10 expression [i.e. a case of elimination of posterior prevalence (Duboule and Morata, 1994)].

Thus, it appears that Hoxc10 and Hoxd10, together with more rostrally expressed Hox genes, determine the rostral border between thoracic and lumbar motor columns in the spinal cord. The failure to convert more caudal segments to thoracic cord as well as the persistence of an LMC in more caudal segments in Hoxc10+/–/Hoxd10+/– mutants is most likely due to the presence of Hox11 genes, which are expressed in the caudal spinal cord from segment L3 in both WT (Carpenter, 2002) and Hoxc10+/–/Hoxd10+/– mutant embryos (see Fig. S3 in the supplementary material). Interestingly, the LMC in caudal lumbar segments normally consists of elimination of posterior prevalence (Duboule and Morata, 1994).
predominantly of Isl1+ mLMC neurons in both chick and mouse (data not shown), similar to the LMC in Hoxc10+/+Hoxd10+/– mutants. Hox11 genes appear to be important in generating these motoneurons, since misexpression of Hox11 in rostral lumbar motoneurons induces an overabundance of Isl1+ mLMC neurons relative to Lim1+ lLMC neurons (Misra et al., 2005). In summary, Hox10 and Hox11 are required for proper columnar specification of the lumbar motoneurons.

**Hoxc10 and Hoxd10 determine divisional specification in the LMC**

In addition to establishing the boundary between thoracic and lumbar motor columns, our results reveal a novel role for Hox10 genes in the divisional specification of LMC. Hoxc10 and Hoxd10 are essential for development of the lateral division of the LMC in lumbar cord. The ILMC was nearly eliminated in Hoxc10+/+Hoxd10+/– mutants as evidenced by the reduction or loss of lateral Lim1+ neurons and the dorsal nerve branches of the lumbar and sacral plexii. Our results differ from the reported milder phenotype for Hoxa10+/+Hoxd10+/– mice, in which both divisions of LMC neurons were present in neonates but reduced in size; although the spatial relationships between the two groups of cells were retained, the groups were clustered together (Lin and Carpenter, 2003).

Perturbation of any number of developmental processes could produce a lack of ILMC neurons in Hoxc10+/+Hoxd10+/– double mutants. We show here that the loss of the ILMC did not result from a decreased production of motoneuron precursors or from the increased apoptosis of Lim1+ motoneurons. Instead, presumptive ILMC neurons failed to migrate to their normal position and never acquired markers characteristic of known populations of motoneurons or interneurons. The observed migratory defect resembles the effects of perturbing cadherin expression on motoneuron sorting (Price et al., 2002), suggesting a mechanism by which Hox genes could govern migration of prospective ILMC neurons. Thus, in the absence of Hox10 and Hoxd10, motoneuron precursors appear to be generated normally, but late-born neurons fail to differentiate into ILMC neurons or into any other clearly recognizable neuron population.

**Hoxc10 and Hoxd10 affect motor pool specification and limb muscle development**

Motor pools represent specific groups of motoneurons in the LMC that establish functional connections with individual muscles in the limb. Our findings show that Hoxc10 and Hoxd10 influence lumbar motor pool formation, although these effects may be indirect as a consequence of Hox10 function in columnar and divisional specification. Motor pools were clearly aberrant in Hoxc10+/+Hoxd10+/– mutants. There was no LMC in segments L1 and L2 and no ILMC in more caudal segments in double-mutant embryos, yet most hindlimb muscles were innervated. The remaining Isl1+ mLMC neurons in segments L3-L5 must, therefore, have distributed themselves among many more muscles than normal, clearly altering motor pools. Retrograde labeling showed that at least one muscle, the quadriceps, was innervated inappropriately by Isl1+ neurons in the absence of Lim1+ neurons. We expect that other dorsal muscles in the thigh were similarly innervated by Isl1+ neurons, which normally innervate ventral muscles.

Further evidence that Hox10 genes influence motor pool formation is that the normal expression patterns of the ETS transcription factor genes, ER81 and Pea3, which are restricted to specific motor pools in WT animals (Arber et al., 2000), were altered in Hoxc10+/+Hoxd10+/– mutant embryos (Fig. 5B, B′ and 5C, C′). Some change in ETS expression was expected, since some motoneurons that usually express ETS factors were missing in double mutants. In addition, innervation of inappropriate muscles by the remaining motoneurons may also contribute to altered expression of ER81 and Pea3, since ETS expression is normally initiated and shaped by signals from the periphery (Lin et al., 1998; Wang and Scott, 2004). In addition, the peripheral signals themselves may be perturbed in double mutants.

Hox10 genes are expressed in the developing hindlimb (Morgan and Tabin, 1994; Nelson et al., 1996; Welik and Capecci, 2003) as well as in the lumbar spinal cord. Thus, the disruption of Hox10 genes in the periphery may have contributed to the observed perturbations in muscle innervation. For example, we have previously shown that restricted inactivation of Hoxb1 in the periphery resulted in the failure of these motoneuron axons to innervate the facial muscles (Arenkiel et al., 2004). Therefore, the locomotor and innervation mutant phenotypes in the Hoxc10+/+Hoxd10+/– double mutants reported here are likely to have resulted from contributions of Hox function in both motoneuron specification and in motoneuron targeting in the periphery. These potential contributions should be separable through the use of conditional mutagenesis. Importantly however, the motoneuron specification defects discussed in this paper are not likely to have been affected by the functions of Hoxc10 and Hoxd10 in the periphery, since this specification occurs before the axons grow into the limb and indeed before the motoneurons are born (Matise and Lance-Jones, 1996).

In conclusion, we have elucidated novel functions of Hoxc10 and Hoxd10 in the patterning of lumbar motoneurons. We showed that disruption of Hoxc10 and Hoxd10 causes rostral lumbar motoneurons to adopt a thoracic phenotype, and prevents the differentiation of Lim1+ lateral LMC neurons. Most hindlimb muscles in double mutants become innervated by the remaining medial LMC neurons. Together, these results show that Hoxc10 and Hoxd10 are important in establishing the columnar, divisional and motor pool identity of lumbar motoneurons. The downstream cascades of genes activated and repressed by Hoxc10 and Hoxd10, which ultimately govern the differentiation of lumbar motoneurons, remain to be determined.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/1/171/DC1

**References**


