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***Hoxa10* and *Hoxd10* coordinately regulate lumbar motor neuron
patterning**

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Running title: *Hox* genes pattern lumbar motor neurons

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Abstract

The paralogous *Hox* genes *Hoxa10* and *Hoxd10* are expressed in overlapping domains in the developing lumbar spinal cord and surrounding mesoderm. Independent inactivation of these two genes alters the trajectory of spinal nerves and decreases the complement of motor neurons present in the lumbar spinal cord, whereas dual inactivation of these two genes has been shown to alter peripheral nerve growth and development in the mouse hindlimb (Wahba et al., 2001). We have examined the organization and distribution of lumbar motor neurons in the spinal cords of *Hoxa10/Hoxd10* double mutant animals. Double mutant animals have decreased numbers of lumbar motor neurons in both the medial and lateral motor columns. The anteroposterior position of the lumbar motor column is shifted caudally in double mutant animals and the distribution of motor neurons is altered across individual spinal segments. Distinctions between classes of motor neurons based on positional specificity appear disrupted in double mutants. Double mutants also demonstrate abnormal spinal cord vasculature and altered kidney placement and size. Our observations suggest that *Hoxa10* and *Hoxd10* activity is required to specify the position of the lumbar motor column and to provide segmental specification and identity for the lumbar motor neurons.

Key words: spinal cord, motor neuron, lumbar motor column, patterning, *Hox*

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Introduction

Spinal cord segmental identity is determined by patterning along both the rostrocaudal and dorsoventral axes. Segmental identity may be visualized by the positions and projections of cells present in the different segments as well as by the relationship of the spinal segments to surrounding anatomical landmarks. In particular, the organization of the motor neurons reflects regional differences in identity along the length of the spinal cord. In the mouse, cervical, brachial, thoracic, lumbar, and sacral spinal cord all contain motor neurons of the medial motor column while the lateral motor columns are restricted to brachial and lumbar levels. The lumbar lateral motor column (LMC) occupies lumbar spinal segments (L)1-L5 in the adult mouse (McHanwell and Biscoe, 1981). The bulk of LMC neurons innervate the proximal and distal musculature of the hindlimb; these neurons are present in the L1-L4 spinal segments. A small component of cells innervating the inner pelvic and foot musculature is found in L5. LMC motor neurons may be distinguished based on their position within the motor column and by the muscle that they innervate. Anatomical studies using retrograde HRP tracing or Wallerian degeneration have mapped the position of lumbar motor pools in the chick (Hollyday, 1980), the mouse (McHanwell and Biscoe, 1981), and the cat (Romanes, 1951). These studies have demonstrated that in general, dorsolaterally positioned motor neurons innervate dorsal limb musculature, while ventromedially positioned motor neurons innervate ventral limb musculature.

Regional development in the mammalian spinal cord is governed by complex interactions between separate rostrocaudal and dorsoventral patterning systems. Dorsoventral patterning is mediated by complementary sonic hedgehog- and BMP-mediated signaling systems (Liem et al., 1995; Briscoe and Ericson, 2001), while rostrocaudal patterning appears in part regulated by the expression and activity of the *Hox* genes. Members of all four *Hox* gene clusters are expressed in broad domains along the rostrocaudal axis of the spinal cord. Within these domains, different *Hox* genes have distinct expression patterns along the dorsoventral axis, suggesting that these genes are expressed in multiple cell types (Awgulewitsch and Jacobs, 1990; Le Mouellic et al., 1992; Deschamps and Wijgerde, 1993; Peterson et al., 1994; Hostikka and Capecchi, 1998; reviewed in Carpenter et al., 2002). The expression of *HoxC* genes has been

demonstrated in motor neurons (Tiret et al., 1998; Liu et al., 2001) and regional expression of different *HoxC* genes may provide positional identity for motor neurons at different spinal cord levels (Liu et al., 2001).

Ectopic expression and inactivation studies also suggest that *Hox* genes play roles in spinal cord development and motor neuron patterning, both through the specification of regional spinal cord identity and through regulation of peripheral nerve development and pathfinding (Rijli et al., 1995; Carpenter et al., 1997; Tiret et al., 1998; de la Cruz et al., 1999). Inactivation of *Hoxc8* or *Hoxd10* alters the production and projection of motor neurons (Carpenter et al., 1997; Tiret et al., 1998) while inactivation of *Hoxb5* or *Hoxa10* transforms or shifts the trajectory of spinal nerves (Rijli et al., 1995; Rancourt et al., 1995). Ectopic peripheral expression of *Hoxc6* truncates cervical spinal nerves (Burke and Tabin, 1996), while alterations in *Hox8* gene expression patterns resulting from paraxial mesoderm transplantation accompany changes in motor neuron patterning in the spinal cord (Ensini et al., 1998). These studies suggest that *Hox* genes may be active in the intrinsic regulation of motor neuron identity in the spinal cord as well as in patterning the environment through which motor neurons grow.

The expression and activity of more than one *Hox* gene within an individual cell may be required to refine cellular identity. Motor neurons express more than one *HoxC* gene (Liu et al., 2001) and studies in which multiple *Hox* genes are inactivated produce more severe patterning defects (reviewed in Carpenter, 2002). We have recently examined the effects of a dual mutation in the *Hoxa10* and *Hoxd10* genes on peripheral nerve patterning in the developing hindlimb (Wahba et al., 2001). Inactivation of *Hoxa10* alone transforms the L1 spinal nerve to a thoracic morphology (Rijli et al., 1995), while inactivation of *Hoxd10* transforms projections from the L3 spinal segment to an L2 morphology (Carpenter et al., 1997). *Hoxa10* and *Hoxd10* are paralogous genes, and as such, have overlapping expression patterns along the rostrocaudal axis. In the hindlimb of *Hoxa10/Hoxd10* double mutant animals, tibial and peroneal nerves are truncated or deleted (Wahba et al., 2001), suggesting changes in the development and patterning of hindlimb innervation. To examine whether the peripheral alterations in hindlimb innervation observed in *Hoxa10/Hoxd10* double mutants might result from intrinsic changes to the motor neuron population in the spinal cord, our current study examines the placement and distribution of lumbar motor neurons in double mutant

animals. Our observations demonstrate reductions in the population of lumbar motor neurons along with changes in their distribution. In addition, our study documents changes in kidney placement and reveals abnormal vasculature, suggesting additional roles for *Hoxa10* and *Hoxd10* in regulating embryonic development.

Methods and Materials

Generation of double mutant animals

Hoxa10^{+/-}/*Hoxd10*^{+/-} dual heterozygous mice were intercrossed to produce animals carrying a full spectrum of genotypes from wild type to double mutant. Animals derived from these crosses were collected on postnatal day 0 (P0), sacrificed by CO₂ asphyxiation, skinned, and decapitated. Mice were fixed in Bouin's solution for 1 week at room temperature, dehydrated through graded ethanol and xylene and embedded in paraffin. 10 µm serial sections were collected and stained with hematoxylin and eosin as described (Carpenter et al., 1997). Newborn animals were genotyped using DNA extracted from tail biopsies collected prior to fixation using PCR amplification as previously described (Wahba et al., 2001). 87 mice were collected from 15 intercrosses. Two of these mice were wild type, 3 were double mutant and the rest were of intermediate genotypes, carrying 1 to 3 mutant alleles. An additional wild-type animal derived from a *Hoxd10* heterozygous intercross was included as a third control. Both wild type and double mutant animals were used for analysis of motor neuron position; motor neuron patterning in animals of intermediate genotype will be described elsewhere.

Motor neuron counting and analysis

Serial sections taken from posterior thoracic through caudal lumbar regions of newborn mice were initially scored for the position of dorsal root ganglia and spinal root entry zones to identify the spinal segments. The first lumbar spinal segment was identified as the segment that contained the rostral end of the lateral lumbar motor column; spinal segments were counted both rostrally and caudally from this reference point. The extent of the lumbar lateral motor LMC was identified based on the appearance and presence of motor neurons in the ventral horn. Once lumbar segmental

position was defined, motor neurons in each section were systematically counted. Cells were identified as motor neurons based on size and ventral position within the spinal cord; only motor neurons demonstrating a complete nuclear membrane and at least one nucleolus were counted in each section to minimize double counting. Motor neurons were divided into groups for counting based on position with the medial motor column and the dorsal and ventral components of the lateral motor column counted as described in the Results. Motor neurons were identified and counted in the first four lumbar spinal segments (L1-L4), segment boundaries were defined as opposite the caudal extent of the adjacent dorsal root ganglia, which was also the approximate midpoint between adjacent spinal nerves. These spinal segments contain the majority of the lumbar motor neurons (McHanwell and Biscoe, 1981) and encompass the spinal segmental levels previously reported to be affected in *Hoxa10*^{-/-}/*Hoxd10*^{-/-} animals (Wahba et al., 2001). Counting data were compiled using Microsoft Excel and subjected to statistical analysis. Comparisons between wild type and mutant cell counts were made using Student's two-sample *t* tests assuming unequal variances.

Kidney size and position

The position of the kidney was determined by comparison with the position of the adjacent vertebrae and axial musculature. The approximate cross-sectional area of the kidney and erector spinae musculature was determined by measuring the length and width of these structures in 18 sections at 100 μ m intervals encompassing the main body of the kidney. Measurements were scaled to the width of the dorsal aorta to reduce variation due to differences in animal size.

Results

The position of the lumbar motor column is altered in *Hoxa10/Hoxd10* double mutants

During embryonic development, the segmentation of the neural tube arises in register with the division of the paraxial mesoderm into the somites. Therefore, those neural tube segments that will develop the motor neurons of the lumbar motor column (LMC) arise adjacent to the somites that will generate the lumbar vertebrae. During later development, the position of the spinal segments loses its registration with the surrounding vertebrae due to unequal rates of growth in the spinal cord and vertebral column. The result of this growth pattern is that lumbar spinal segments are shifted rostrally with respect to the surrounding vertebrae.

We mapped the position of the LMC in wild-type and double mutant newborn mice with reference to the position of landmarks surrounding the motor column to determine if the LMC occupies the same relative position in double mutant mice. For these studies, we examined 10 µm serial transverse sections taken through posterior thoracic and lumbar levels in newborn mice and scored the position of the vertebrae, the kidneys and adrenal medulla, the dorsal root ganglia, the spinal nerves, and the lumbar motor column. These data were used to generate positional maps that illustrate the location of the lumbar motor column with respect to the position of the anatomical landmarks examined (Figure 1). As previously described (Wahba et al, 2001) and as seen in our current study, the wild-type mice examined had six lumbar vertebrae, suggesting a normal vertebral pattern consisting of 13 thoracic, 6 lumbar, and 4 sacral vertebrae (T13L6S4; Favier et al., 1996; Fromental-Ramain et al., 1996). The three double mutant mice examined showed alterations in vertebral morphology consistent with anterior transformation as previously described for adult animals (Wahba et al., 2001). One animal had 5 lumbar vertebrae while two animals had 6 lumbar vertebrae. In addition, the most posterior thoracic vertebra showed evidence of a full or partial transformation from a lumbar to thoracic phenotype. In the animal with 5 lumbar vertebrae, the next most rostral vertebra showed typical thoracic morphology including a thinner smoother vertebral body and the presence of a rib extending from the anterior part of the vertebra. This phenotype suggests a full lumbar to thoracic transformation to produce a 14th thoracic vertebra. The most caudal thoracic vertebrae in the other two animals showed

some features of lumbar vertebrae, such as angular lateral extensions from the ventrolateral face of the vertebral body and prominent ventral crests, along with the presence of ribs, suggesting that these vertebrae showed partial lumbar to thoracic transformation. These observations suggest that the animals used in this study had T14L5 or T14*L6 vertebral patterns, consistent with the vertebral patterns examined in adult animals of the same genotype (Wahba et al., 2001).

In wild-type mice, the rostral extent of the lumbar motor column, as determined by the appearance of large ventrolaterally positioned cell bodies appears midway through the L1 spinal segment (Figure 1; McHanwell and Biscoe, 1981). The L1 spinal segment is positioned adjacent to the body of the twelfth thoracic vertebra (T12) and as defined by our mapping criteria, extends to the posterior margin of the next most posterior dorsal root ganglion. This organization positions the spinal cord approximately two segments out of register with the vertebral column. Following this organization, the position of the L3 spinal segment was adjacent to the L1 vertebra. The identity of this spinal segment was confirmed by the appearance of the dLMC motor neurons near the rostral end of this segment as reported in adult mice (McHanwell and Biscoe, 1981).

Similar maps generated from double mutant animals demonstrate that the position of the lumbar lateral motor column is shifted posteriorly in mutant animals (Figure 1). The vLMC was first visible adjacent to the most caudal thoracic vertebra in two of three animals examined; in the remaining animal the lumbar LMC first appeared adjacent to the L1 vertebra. If, as suggested by our observations of vertebral morphology described above and by observations in adult animals (Wahba et al., 2001), double mutant animals have 14 thoracic vertebrae, then the position of the lumbar motor column is shifted two to three segments posteriorly in double mutant animals (Figure 1). Dorsolaterally positioned cells characteristic of the dLMC in the L3 segment appeared at least two segments posterior to the appearance of the vLMC, suggesting that the entire motor column is shifted posteriorly, but that spatial relationships among groups of cells are retained within the shifted motor column.

Motor neuron organization is disrupted in *Hoxa10/Hoxd10* double mutants

In addition to the gross differences in position of the LMC, patterning alterations within the lumbar motor columns are also evident in *Hoxa10/Hoxd10* double mutant

animals. In wild-type mice, the rostral extent of the vLMC appears just caudal to the L1 spinal segmental boundary. At this level, motor neurons innervating the quadriceps femoris and adductor muscles occupy the ventrolateral extent of the ventral horn (McHanwell and Biscoe, 1981). Caudally in the L3 spinal segment, the dLMC appears more dorsally within the ventral horn. At its rostral extent, this group of neurons provides innervation for the posterior crus musculature, distal calf musculature and more caudally, the hamstring and gluteal muscles (McHanwell and Biscoe, 1981). In wild-type animals, these two groups of neurons are quite distinct and spatially separated (Figure 2). However, in double mutant animals, the two groups of neurons are not widely separated and often are clustered into one large group (Figure 2).

Hoxa10/Hoxd10 double mutant animals have reduced numbers of motor neurons

The total number of lumbar motor neurons was counted in three wild-type and three double mutant newborn animals. For these studies, the appearance of ventrolaterally positioned cells in the lateral horn dictated the identity of the first lumbar spinal segment, regardless of the absolute position of this segment with respect to the adjacent vertebrae. Motor neurons were counted as three separate groups independently on both sides of the spinal cord (Table 1). Medial motor column motor neurons were identified by their medial position and counted throughout the four most rostral lumbar spinal segments in both wild type and double mutant animals. Lateral motor column motor neurons were counted in two different groups. The vLMC was counted beginning at the rostral extent of the lumbar LMC and continuing through the end of the L4 segment. The dLMC was counted from its rostral appearance in L3 through its caudal extent in L4, though a few scattered cells appeared in the dLMC position in more rostral segments. In double mutant animals, a distinction between the vLMC and dLMC could often not be clearly visualized, though the dorsal expansion of the ventral horn motor neurons served to indicate the rostral extent of the dorsal group of cells. In these animals, motor neurons of the ventral horn were divided into dorsal and ventral groups by bisecting the ventral horn midway between its dorsal and ventral extent and counting each group separately (Figure 2). Cell counts generated using this approach are presented in Table 1.

These cell counting studies demonstrate a substantial reduction in the total number of motor neurons in *Hoxa10/Hoxd10* double mutant animals, with an average of 2098 motor neurons per side counted in wild-type animals and 1345 counted in double mutants. This suggests a 36% reduction in the number of motor neurons in double mutant animals. The average number of LMC motor neurons was 1717 in wild-type animals and 1134 in double mutants, while the average number of MMC motor neurons was 381 in wild-types animals and 211 in double mutants. Therefore, the reduction occurs within both the MMC and the LMC, with the number of motor neurons in the MMC reduced by 39% and in the LMC reduced by 35%, suggesting that both populations of cells are reduced to approximately the same extent.

The segmental distribution of motor neurons was examined by plotting the number of motor neurons in each segment versus segmental position (Figure 3). The profile of motor neuron distribution in the MMC is similar between wild-type and double mutant animal; however, significantly more neurons ($p < 0.05$, t-test) are present in wild-type animals, particularly in the L2 segment. The distribution of motor neurons in the LMC was altered in double mutant animals. The vLMC showed a peak in the number of cells in the L2 segment in wild-type animals, but a peak in the L3 segment in mutant animals. Similarly, the dLMC reached its highest value in the L3 segment in wild-type animals, while more motor neurons were seen in this position in L4 than L3 in double mutant animals. Significant differences among these populations were seen for the vLMC in the L2 segment ($p < 0.05$) and for the vLMC in the L3 segment ($p < 0.05$). These results suggest that the LMC motor neurons are redistributed in double mutant animals.

Abnormal vasculature in *Hoxa10/Hoxd10* double mutant animals

Spinal cord vasculature normally consists of dorsally and ventrally positioned marginal blood vessels that issue branches traversing the interior of the spinal cord. In all three mutant animals examined, ectopic blood vessels were seen around the margin of the lumbar spinal cord. In addition, one double mutant animal had a large blood vessel traversing dorsoventrally through the body of the spinal cord (Figure 4). Similar ectopic vasculature was never observed in wild-type animals, nor was it seen outside of the lumbar region of the spinal cord in double mutant animals.

Kidney placement in *Hoxa10/Hoxd10* double mutant animals

The rostrocaudal position of the kidney appeared roughly the same in wild-type and double mutant animals, with the rostral extent of the kidney visible near the L1 or L2 vertebra. The position of the adrenal medulla overlapped the anterior end of the kidney and extended up to 350 μ m more rostrally. However, in one double mutant animal, the kidney was shifted more dorsally (Figure 5), extending above the position of the adjacent vertebra. This dorsal shift positioned the kidney adjacent to the dorsal body wall, which was characterized by a single layer of abdominal wall musculature, as opposed to the three layers formed by the transverse abdominus, internal oblique, and external oblique muscles visible more ventrally. In addition, the average cross-sectional area of the double mutant kidney is increased approximately 21%, while the cross-sectional area of the adjacent erector spinae musculature is decreased approximately 20%.

Discussion

The paralogous *Hox* genes *Hoxa10* and *Hoxd10* appear to coordinately regulate the position and organization of the mouse lumbar motor column. *Hoxa10/Hoxd10* double mutant animals show several distinct changes in spinal cord organization that support a role for these genes in spinal cord patterning. First, the position of the lumbar motor column appears shifted posteriorly by 2 to 3 segments with respect to the rest of the developing body. Previous observations on *Hoxd10* single mutants suggests that LMC motor neurons were displaced posteriorly by approximately half a segment (Carpenter et al., 1997), while LMC position in *Hoxa10* mutants has not been described. Our current observations suggest that the activity of both *Hoxa10* and *Hoxd10* may coordinately regulate the position of the LMC. This alteration may be explained by postulating an anterior transformation in the spinal cord, such that segments that should be lumbar in identity based on absolute position along the length of the spinal cord adopt a thoracic morphology in response to changes in the expression of *Hoxa10* and *Hoxd10* during spinal cord development. However, dual inactivation of *Hoxa10* and *Hoxd10* also produces anterior vertebral transformation such that the most rostral lumbar vertebra is partially or completely transformed to a thoracic identity (Wahba et al., 2001) and alters

the position and size of the kidney and adjacent musculature (this study). This complicates assignment of the absolute position of the LMC, because the landmarks used to measure the position of the LMC are also altered, suggesting more global changes in body patterning. If the position of the LMC were not affected by the dual mutation, then the LMC might be expected in a more anterior position, adjacent to thoracic vertebrae, similar to the position observed in wild-type animals. However, the LMC appears adjacent to vertebrae with a partial or complete lumbar morphology in double mutant animals, suggesting a posterior shift in the position of the LMC possibly induced by anterior transformation of rostral lumbar spinal segments.

A second observation supporting a dual role for *Hoxa10* and *Hoxd10* in spinal cord patterning is that the organization of the LMC is altered in double mutant animals. Clearly defined dorsal and ventral components of the LMC are not present in double mutant animals, though a relatively normal distribution of LMC motor neurons is observed in *Hoxd10* single mutants (Carpenter et al., 1997). *Hox* gene involvement in the specification of neuronal identity has been observed in the developing hindbrain (Carpenter et al., 1993; Goddard et al., 1996; Gavalas et al., 1998) where changes in *Hox* gene expression are accompanied by alterations in the position and projection of motor neurons from the different hindbrain segments. Motor neuron projections into the hindlimb are clearly disrupted in *Hoxa10/Hoxd10* double mutants (Wahba et al., 2001) and our current observations demonstrate changes in motor neuron position and distribution in the spinal cord. These two observations suggest that motor neuron identity is likely disrupted in the lumbar spinal cord in the absence of *Hoxa10* and *Hoxd10* activity. The observed changes may stem from a respecification in the cellular identity of the lumbar motor neurons themselves or from environmental changes disrupting the migration and position of the motor neurons during development.

Third, motor neuron numbers are decreased in *Hoxa10/Hoxd10* double mutant animals. Our counts of LMC motor neurons in wild-type animals ranged from 1457 to 1989, with an average of 1717. This average excludes MMC motor neurons. Previous studies have suggested 1500-1700 motor neurons in the LMC (McHanwell and Biscoe, 1981; Lance-Jones, 1982). However both previous studies used retrograde HRP tracing to identify motor neurons whereas our study used morphological criteria to identify cells visible in histological sections. This suggests that HRP tracing may potentially

underrepresent the total number of LMC motor neurons, or alternatively, that counting cells in histological sections may slightly overrepresent the number of motor neurons. In addition, our studies also examined MMC motor neurons and demonstrated similar reductions in this population of neurons in double mutant animals. These observations suggest that both populations of motor neurons are susceptible to regulation by the expression of *Hoxa10* and *Hoxd10*. These changes may stem from increased cell death in postmitotic motor neurons, or alternatively, alterations in cell proliferation and/or specification at early stages of embryonic development. We think the second possibility is more likely, because defects in peripheral innervation have been observed as early as E12.5 in double mutant embryos (Wahba et al., 2001), preceding the major period of cell death in the lumbar spinal cord at E14 (Lance-Jones, 1982). In addition, no changes were observed in limb musculature during development or soon after birth (Carpenter, unpublished observations) suggesting that a decrease in target size is not responsible for the concomitant loss of motor neurons.

One additional alteration in double mutant spinal cords is the appearance of abnormal vasculature. In its most extreme form, this is manifested by the appearance of a major blood vessel traversing the spinal cord, but vascular changes are visible to some degree at lumbar levels in all double mutants examined as ectopic marginal blood vessels. Previous observations have demonstrated the involvement of *Hoxb3* and *Hoxd3* in angiogenesis (Myers et al., 2000; Boudreau et al., 1997) and have suggested complementary activities for these paralogous *Hox* genes in regulating endothelial cell migration, adhesion, and invasion (*Hoxd3*) and promoting capillary morphogenesis (*Hoxb3*). Neither *Hoxa10* nor *Hoxd10* has been individually implicated in angiogenesis; however, our observations suggest a role for these two genes together in regulating vascular development in and around the spinal cord. Preliminary observations using microarray screening suggest that *Hoxd10* may regulate several genes involved in angiogenesis (Hedlund and Carpenter, in preparation), further supporting a role for this gene in regulating the development of spinal cord vasculature.

While environmental factors may influence the position of *Hox* gene expression domains (Ensini et al., 1998), recent evidence also suggests that the patterning of the posterior spinal cord is established early in development, possibly in response to posterior patterning factors (Lance-Jones et al., 2001). This suggests that early changes in *Hox*

gene expression are likely to have a direct effect on the development of the nervous system, and not secondary effects mediated through the surrounding environment. Therefore, by changing gene expression patterns through inactivation of *Hoxa10* and *Hoxd10*, we are likely directly affecting the patterning of the central nervous system. These changes are revealed through changes in motor neuron organization and reflected by alterations in peripheral projection of nerves arising from affected segments.

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Table 1: Motor neuron counts

	Left Side				Right Side			
	MMC	vLMC	dLMC	Total	MMC	vLMC	dLMC	Total
+/, +/+	283	1034	585	1902	280	1098	617	1995
+/, +/+	363	1364	625	2352	362	1364	554	2280
+/, +/+*	385	1058	549	1992	611	1051	406	2068
-/-, -/-	219	754	392	1365	262	884	452	1598
-/-, -/-	163	776	320	1259	157	826	372	1355
-/-, -/-	245	823	167	1235	220	884	151	1255

*counts were proportionally adjusted to reflect loss of sections in this animal

Figure Legends

Figure 1: Schematic representation of the position of the lumbar motor column in wild-type and *Hoxa10*^{-/-}/*Hoxd10*^{-/-} double mutant mice. The equivalent rostrocaudal position of the vertebrae in wild-type and double mutant animals is given on the vertical axis. The LMC in wild-type animals is positioned opposite the T12-L3 vertebrae. In mutant animals, the lumbar motor column is positioned 1-2 segments posteriorly, opposite the T14*/L1 vertebra and continuing caudally to opposite the L4 or L5 vertebra.

Figure 2: Transverse sections taken through the lumbar (L) 1-L4 spinal segments in wild-type and *Hoxa10/Hoxd10* double mutant newborn mice stained with hematoxylin and eosin. Motor neuron positions are circled in all panels. At the L1 level, motor neurons are evident in vLMC and MMC in both wild-type and mutant animals. At the L2 level, vLMC is larger and more well-defined in wild-type animals. At the L3 level, dLMC is visible in wild-type animals and continues posteriorly into the L4 segment. There is no clear distinction between dLMC and vLMC cell groups in double mutant animals at either the L3 or L4 level; arbitrary distinctions made for cell counting studies are indicated by the dotted lines.

Figure 3: Motor neuron distribution in wild-type and double mutant animals. The average number of motor neurons for each segment is plotted vs. lumbar segment. Filled circles designate wild-type animals while open circles indicate double mutants. Segments with significantly different numbers of motor neurons ($p < 0.05$, Student's t-test) in wild-type and double mutant animals are indicated with asterisks.

Figure 4: Abnormal vasculature in *Hoxa10/Hoxd10* double mutant spinal cords. An ectopic blood vessel (arrows) traverses the L4 spinal segment in this double mutant animal. Motor neurons are present despite this vascular abnormality. Some motor neurons occupy normal positions (circles) while others are displaced by the ectopic blood vessel (arrowheads).

Figure 5: Kidney position in wild-type (A, B) and *Hoxa10/Hoxd10* double mutants (C, D). In wild-type animals, the kidney lies ventral to the L3 vertebra (V) and is covered by the three layers of abdominal wall musculature (arrows, A and B). In double mutant animals, the kidney is shifted dorsally with respect to the position of the vertebra and is covered by a single layer of abdominal wall muscle (arrows, C and D), characteristic of more dorsal body wall. The normal three-layered abdominal wall is present more ventrally (paired arrows, D).

Figure 1

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