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Functional Diversity of ESC-Derived Motor Neuron Subtypes Revealed through Intraspinal Transplantation

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SUMMARY

Cultured ESCs can form different classes of neurons, but whether these neurons can acquire specialized subtype features typical of neurons in vivo remains unclear. We show here that mouse ESCs can be directed to form highly specific motor neuron subtypes in the absence of added factors, through a differentiation program that relies on endogenous Wnts, FGFs, and Hh—mimicking the normal program of motor neuron subtype differentiation. Molecular markers that characterize motor neuron subtypes anticipate the functional properties of these neurons in vivo: ESC-derived motor neurons grafted isochronically into chick spinal cord settle in appropriate columnar domains and select axonal trajectories with a fidelity that matches that of their in vivo generated counterparts. ESC-derived motor neurons can therefore be programmed in a predictive manner to acquire molecular and functional properties that characterize one of the many dozens of specialized motor neuron subtypes that exist in vivo.

INTRODUCTION

During development, the emergence of specialized cell types is orchestrated by signaling events that progressively restrict the fates of progenitor cells. Most tissues contain a limited cellular repertoire, but even so, their generation from stem cells in vitro has remained a challenge, largely because of an incomplete understanding of relevant pathways of differentiation. The problem of cell specification is especially daunting in the mammalian central nervous system (CNS), where hundreds of primary neuronal classes are generated, many of which are further diversified into subtypes. The CNS contains, for example, a dozen or so dopaminergic neuronal classes, about two dozen retinal ganglion and amacrine neuronal subtypes, several dozen spinal motor neuron subtypes, and hundreds of receptor-specific

olfactory sensory neurons (Buck and Axel, 1991; Dasen and Jessell, 2009; Liss and Roeper, 2008; MacNeil and Masland, 1998; Rockhill et al., 2002). The diversity of CNS neurons contributes to the richness of central circuits and their encoded behaviors and can correlate with, or confer selective neuronal vulnerability in, neurodegenerative diseases.

Of many classes of neurons known to exhibit subtype diversity, programs of spinal motor neuron diversification have been characterized in particular detail (Dasen and Jessell, 2009; Jessell, 2000). The overall program of spinal motor neuron diversification can be deconstructed into a series of developmental steps, in which "generic" motor neurons progressively acquire subtype identities that match features of their muscle targets (Dasen et al., 2003, 2005; Jessell, 2000; Kania et al., 2000; Sockanathan et al., 2003). Initially, motor neurons acquire columnar identities—median (MMC), hypaxial (HMC), or lateral (LMC)—that dictate their settling positions in the ventral spinal cord as well as the selection of axial, body wall, or limb muscles as innervation targets. LMC neurons then acquire divisional identities that dictate the innervation of ventral or dorsal limb muscles, respectively (Kania et al., 2000). Finally, LMC neurons acquire diverse motor pool identities that direct their connections to specific muscles in the limb (Dasen et al., 2005). The existence of dozens of muscle groups in the limbs of most mammals demands an equivalent diversity of motor neuron pool subtypes.

The high degree of LMC diversification makes this a potentially informative population with which to resolve strategies of neuronal subtype specification from embryonic stem cells (ESCs). Prior studies have shown that mouse and human ESCs can be converted into spinal motor neurons of generic character, through a program of retinoid and Sonic hedgehog (Shh) exposure (Lee et al., 2007; Li et al., 2005; Wichterle et al., 2002). But ESC-derived motor neurons generated under these conditions exhibit a rostral cervical, MMC-like identity (Soundararajan et al., 2006; Wichterle et al., 2002), raising the issue of whether other columnar classes of neurons, and their inherent subtypes, can be generated. And if so, do these specialized motor neuron subtypes express molecular and functional characteristics that reflect those of their in vivo generated counterparts?

The emergence of LMC columnar, divisional, and motor pool identities is controlled by the interplay between retinoid and

FGF signals and a Hox transcriptional response network (Dasen et al., 2003, 2005; Liu et al., 2001). At forelimb levels, LMC columnar identity requires the induction of Hox6 expression by low-level FGF signaling (Dasen et al., 2003). The later emergence of divisional identity within the LMC is directed by paracrine sources of retinoids that promote lateral LMC fate (Sockanathan and Jessell, 1998). In contrast, the diversification of motor pools at a single segmental level has been suggested to depend on the cell-by-cell resolution of an intrinsic Hox repressor network (Dasen et al., 2005). Once established, these motor neuron transcriptional programs govern the settling position, axonal trajectory, and trophic factor sensitivity of LMC neuronal subsets.

With this developmental program as a guide, we have been able to define conditions under which ESCs can be differentiated into motor neurons with LMC columnar, divisional, and pool identities, in the absence of any added inductive factors. We also provide evidence that the emergence of LMC divisional and pool identity in individual neurons can occur independently of signals provided by other LMC neurons, and probably by any limb-level-specific signals. Most critically, we use isotopic and isochronic grafts of ESC-derived LMC neurons into host spinal cord to show that the transcriptional profile of ESC-derived LMC neuron subsets predicts their settling position within the lateral motor column, their axonal trajectory to the forelimb, and their sensitivity to target-derived inductive factors.

RESULTS

Generation of Motor Neurons with LMC Character from ESCs

Mouse ESCs exposed to retinoic acid (RA) and Hedgehog (Hh) receptor agonists generate motor neurons that exhibit rostral cervical identity (Wichterle et al., 2002). We therefore sought to define differentiation conditions that promote the generation of motor neurons of brachial LMC character. In vivo, brachial LMC identity is achieved by exposure of cells to FGF signals and evasion of the rostralizing influence of retinoids (Figure S1A available online; Dasen et al., 2003; Liu et al., 2001). Accordingly, we tested whether omission of RA from the culture medium would promote formation of brachial level motor neurons from ESCs.

We cultured ESCs at low density, to minimize the influence of BMP signals that suppress specification of neural fate (Figure S1K; Watanabe et al., 2005). By using this low-density, retinoid-free culture condition, we screened a series of basal media and supplements for their ability to promote the generation of GFP$^+$ motor neurons from *Hb9-GFP* (HBG3) ESCs (Wichterle et al., 2002). We found that Advanced D-MEM/F12/Neurobasal medium supplemented with 10% KnockOut Serum Replacement (referred to as caudalizing/ventralizing [CV] medium) resulted in the generation of spinal motor neurons of brachial LMC character. With this CV differentiation condition, motor neurons constituted 8% ± 1% (mean ± SEM; n = 3 independent experiments) of cells in embryoid bodies, an efficiency 4- to 5-fold lower than that typically obtained through the use of RA/Hh inductive signals (Table S1).

To define the rostrocaudal positional identity of CV differentiated Hb9:GFP$^+$ motor neurons, we analyzed their Hox protein expression status. 15% ± 2% of ESC-derived motor neurons expressed Hoxa5, 28% ± 8% expressed Hoxc6, 64% ± 2% expressed Hoxc8, and 25% ± 6% expressed Hoxc9 (Figures 1B and 1D; see Table S2 for quantification). The vast majority of CV-generated ESC-derived motor neurons exhibited mutually exclusive expression of Hoxa5 and Hoxc8, and of Hoxc6 with Hoxc9 (Figure 1D), consistent with the reciprocity of expression of these Hox protein pairs observed in vivo (Dasen et al., 2005). Moreover, the pattern of *Hox* gene expression achieved under RA/Hh and CV differentiation conditions was dramatically different: with RA/Hh exposure, 89% ± 2% of GFP$^+$ motor neurons expressed Hoxa5 and virtually none expressed Hoxc8 or Hoxc9 (Figures 1B and 1C). Thus, ESCs differentiated under CV, but not RA/Hh, conditions give rise to motor neurons of caudal brachial (Hoxc8$^+$) and thoracic (Hoxc9$^+$) positional character.

We next addressed whether CV differentiation conditions promote the generation of motor neurons through a program that resembles that operating in vivo. Because Wnt signals underlie the initial specification of spinal cord identity in vivo (Nordström et al., 2006), we examined the fate of CV-differentiated ESCs exposed to the Wnt antagonist Dickkopf-1 (Dkk1). In the presence of Dkk1, ESCs grown under CV conditions exhibited forebrain/midbrain rather than spinal positional character, revealed by the absence of Hox5 to Hox8 protein expression, the lack of Hb9::GFP$^+$ neurons, and the prevalence of Otx2$^+$ cells (Figure S1D). Within the context of spinal positional character, FGF signaling plays a role in assigning caudal positional identity (Liu et al., 2001). We therefore examined the Hox profile of CV-differentiated ESCs grown in the presence of a pan-FGF receptor antagonist (PD173074; Mohammadi et al., 1998). The resulting ESC-derived motor neurons exhibited a rostralized positional character—expressing Hoxa5 rather than Hoxc8 and Hoxd9 (Figure S1E). Thus, low-density retinoid-free conditions are conducive to embryoid body expression of endogenous Wnt and FGF factors that conspire to caudalize induced neural cells.

We next tested whether motor neuron differentiation under CV conditions reflects an endogenous supply of ventralizing factors (Jessell, 2000). Clusters of Shh$^+$, FoxA2$^+$, Brachyury$^+$ notochord as well as Shh$^+$, FoxA2$^+$, Brachyury^{off} floor plate cells were detected in embryoid bodies grown under CV conditions (Figures S1J–S1L). This endogenous source of hedgehog protein is functional, because exposure of ESCs to a potent Hh receptor antagonist (C61414; Williams et al., 2003) blocked the differentiation of ventral spinal progenitor cells and of motor neurons (Figures S1H and S1I). Thus ESCs grown under CV conditions are neuralized, caudalized, and ventralized solely through the actions of endogenous patterning signals, generating motor neurons of a caudal positional character. We note that our induction protocol differs markedly in outcome from prior FGF and Hh-free protocols that result in the generation of rostralized neural cells (Gaspard et al., 2008; Watanabe et al., 2005).

Do CV-generated ESC-derived motor neurons acquire definitive columnar and pool characters that conform to their Hox profiles? To assess the columnar character of Hb9:GFP$^+$ motor neurons, we monitored expression of Lhx3 and FoxP1, markers of MMC and LMC identity (Dasen et al., 2005). We inferred
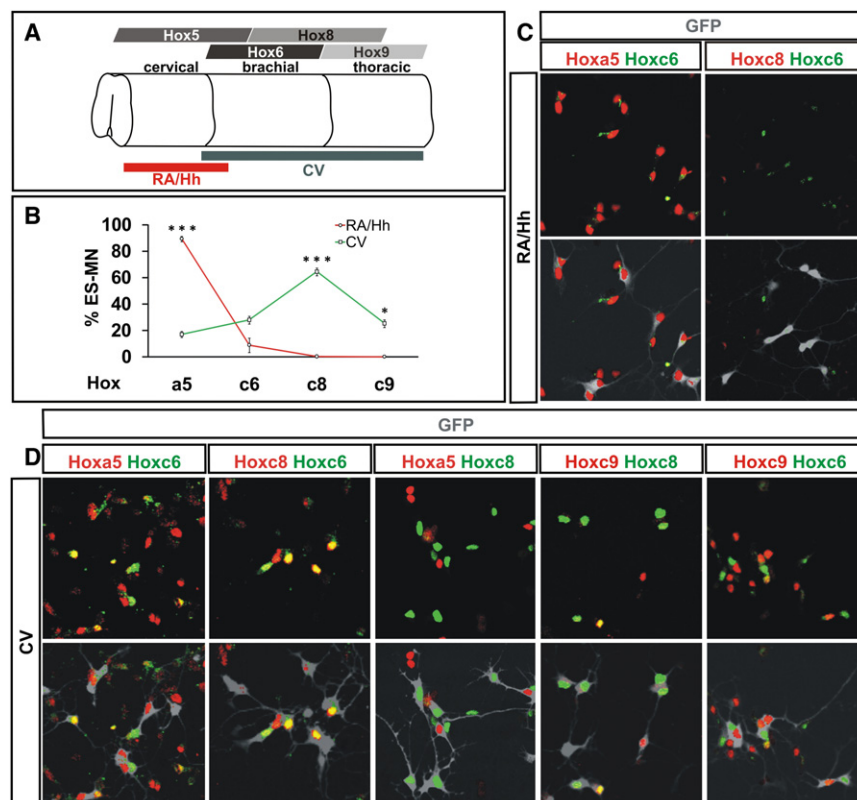
Figure 1. Characterization of Hox Protein Expression in ESC-Derived Motor Neurons

(A) Pattern of *Hox* gene expression in the developing spinal cord. Red and gray lines represent positional identity of ESC-derived motor neurons under RA/Hh and CV exposure, respectively.

(B) Quantification of Hox expression in ESC-derived motor neurons. Motor neurons derived under RA/Hh and CV conditions exhibited differences in Hoxa5 ($p < 0.001$), Hoxc8 ($p < 0.001$), and Hoxc9 ($p = 0.01$) expression. Data from three independent experiments (mean ± standard error of the mean, SEM).

(C) RA/Hh generated Hb9-GFP+ ESC-derived motor neurons (gray) expressed Hoxa5, low levels of Hoxc6, but not Hoxc8.

(D) Most CV-differentiated ESC-derived motor neurons (gray) expressed Hoxc8, although smaller subsets express Hoxa5, Hoxc6, and Hoxc9. Note mutually exclusive expression of Hoxa5/Hoxc8 and Hoxc6/Hoxc9.

HMC identity by the absence of Lhx3 and FoxP1 (Dasen et al., 2008). We found that 38% ± 2% of CV-differentiated motor neurons expressed FoxP1 in the absence of Lhx3, conversely 28% ± 2% expressed Lhx3 in the absence of FoxP1, and 34% ± 1% lacked both FoxP1 and Lhx3 (Figure S2). As in vivo (Dasen et al., 2008), >4-fold more Hoxc6+ motor neurons exhibited LMC than MMC character, and >4-fold more Hoxc9+ motor neurons exhibited HMC than MMC character (Figures 2C and 2D). In addition, we found that 19% ± 6% of motor neurons coexpressed Hoxc9+ and FoxP1, a molecular profile predictive of preganglionic motor column (PGC) neurons (Figures 2C and 2D; Dasen et al., 2008). The tight correlation between columnar identity and Hox status provides further evidence that CV differentiation conditions favor the formation of motor neurons of coherent caudal brachial or rostral thoracic character.

Position-Independent Programming of Motor Pool Identity

Soon after their generation, LMC motor neurons acquire specialized divisional and pool identities. Lateral divisional identity is imposed on late-born LMC neurons as they migrate through earlier-born medial LMC neurons that serve as a neuronal source of retinoid inductive signals (Sockanathan and Jessell, 1998). In parallel, the segment by segment diversification of LMC neurons into specific pool subtypes has been attributed to the probabilistic outcome of cross-repressive interactions between members of a Hox transcriptional network (Dasen et al., 2005). The scattered arrangement of LMC neurons within CV-differen-

tiated embryoid bodies provided us with an opportunity, not available in vivo, to test the requirement for close proximity between LMC neurons in the emergence of LMC divisional and pool character.

We first examined the emergence of lateral LMC divisional character. Very few CV differentiated motor neurons acquired lateral LMC divisional character, as assessed by expression of Lhx1 in GFP+ motor neurons (Figure 3A). However, exposure of embryoid bodies to RA (1 μM) on day 5 of differentiation elicited a 3-fold increase in the number of Lhx1+, GFP+ motor neurons ($p = 0.04$ versus control RA-free conditions; Figures 3A and 3B). This retinoid-mediated induction of Lhx1 occurred in postmitotic motor neurons, because BrdU addition at the time of retinoid exposure labeled few if any Lhx1+ motor neurons (Figures 3C–3E). We also verified that retinoid treatment did not alter rostrocaudal positional identity of motor neurons, assessed by the profile of Hox expression (Figures S3A and S3B). Thus, under CV differentiation conditions, the progression of LMC neurons to a lateral LMC fate cannot be driven by signals emanating from neighboring LMC neurons, and requires supplemental retinoid exposure.

We next examined whether the emergence of motor pool character in an individual LMC neuron can be achieved in the absence of signals provided by neighboring LMC neurons. To address this issue, we focused on three pools generated within caudal brachial LMC: Scip+, Isl1+ motor neurons that innervate the flexor carpi ulnaris (FCU) muscle; Pea3+, Lhx1+ motor neurons that innervate latissimus dorsi (LD) muscle; and Pea3+, Isl1+ motor neurons that innervate the cutaneus maximus (CM) muscle (Figures 4A–4C). Pea3 expression is induced in CM and LD motor neurons as a consequence of Hox profiles that confer neuronal competence to a permissive limb-derived signal, glial cell line-derived neurotrophic factor (GDNF) (Dasen et al., 2005; Haase et al., 2002).
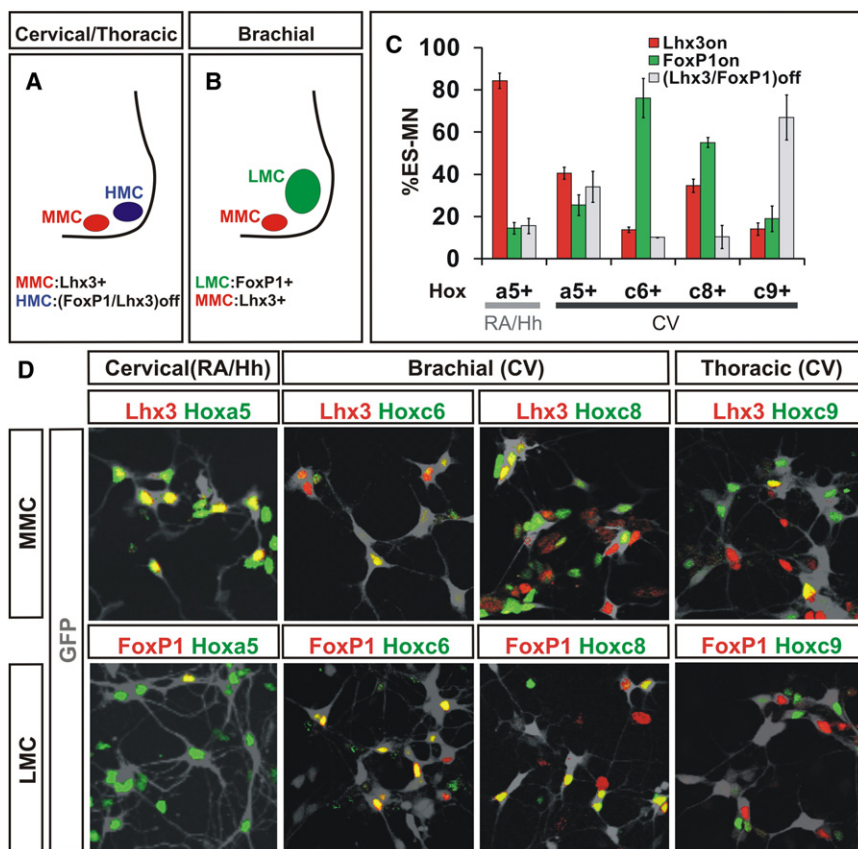
Figure 2. Rostro-Caudal and Columnar Identities of ESC-Derived Motor Neurons

(A) At cervical and thoracic levels, motor neurons are organized into median (MMC) and hypaxial (HMC) motor columns. MMC neurons express Lhx3. HMC neurons lack FoxP1 and Lhx3 expression.

(B) At brachial level, HMC is replaced by FoxP1^{+} lateral motor column (LMC).

(C) Quantification of Hox protein expression in the context of FoxP1^{+} and Lhx3^{+} ESC-derived motor neurons differentiated under RA/Hh or CV condition. ANOVA analysis of data presented in Table S2. Data from three independent experiments (mean ± SEM).

(D) FoxP1 and Lhx3 expression in Hoxa5-, Hoxc6-, Hoxc8-, or Hoxc9-expressing Hb9-GFP[+] ESC-derived motor neurons (gray) differentiated under RA/Hh or CV conditions.

a 5-fold excess of wild-type motor neuron progenitors generated under RA/Hh conditions and lacking GFP expression (Figures 5A–5C). Because under CV conditions, ~8% of cells differentiate into motor neurons, and under RA/Hh conditions ~40% become motor neurons, the resulting ratio of CV:RA/Hh-derived motor neurons in reaggregates is predicted to be ~1:25. In practice, we found that 2.6% ± 0.7% of all Hb9^{+} ESC-derived motor neurons gener-

To reflect this permissive target-derived influence, we supplemented basal CV differentiation medium with GDNF (10 ng/ml; from day 5 onward) and analyzed the transcriptional status of the resulting GFP[+] ESC-derived motor neurons. We found that 9% ± 1% of FoxP1^{+} ESC-derived LMC neurons coexpressed Scip with Hoxc8 and Isl1 (FCU-like; Figures 4D, 4E, and 4G). 17% ± 2% of FoxP1^{+} ESC-derived motor neurons coexpressed Pea3 with Hoxc8 in the absence of Scip (CM-like; Figures 4D–4G). Of these Pea3^{+} LMC neurons, 87% ± 6% coexpressed Isl1, and 8% ± 5% expressed Lhx1 (Figures 4F and 4H), indicating that most Pea3^{+} ESC-derived motor neurons acquired CM rather than LD character. Moreover, as in vivo, FoxP1^{+} neuronal coexpression of Pea3 but not Scip was dependent on GDNF (0.0% ± 0.0% of FoxP1^{+} ESC-derived motor neurons express Pea3 in the absence and 17% ± 2% in the presence of GDNF, p < 0.001; while 9% ± 1% express Scip in the absence and 8% ± 2% in the presence of GDNF, p = 0.70) (Figures 4D, 4E, and 4G). These results show that LMC neurons generated under CV conditions can acquire motor pool identities consistent with those generated in vivo.

To test the ability of individual ESC-derived LMC neurons to acquire authentic pool character in the absence of signals from their LMC neighbors, we devised a method of generating a few isolated LMC neurons within a vast majority of neighboring cervical non-LMC cells. To achieve this condition, we dissociated Hb9-GFP motor neuron progenitors generated under CV conditions on day 4 of differentiation and mixed them with

ated under this reaggregate condition expressed GFP (a 1:38 ratio) (Figure 5C, data not shown). And because motor neurons are surrounded by a majority of other cell types, the chances that any two LMC neurons occupy the same microdomain of the embryoid body are exceedingly low.

We therefore examined the pool subtype identities of individual CV GFP[+] Hoxc8^{+} ESC-derived motor neurons 3 days after reaggregation. We found that Scip and Pea3 were expressed in a mutually exclusive manner by GFP[+], FoxP1^{+} LMC neurons, and the proportional representation of these two pool subtypes was similar to that found in CV-differentiated conditions (Figures 5H–5N and 5Q). These findings indicate that in contrast to the rules of LMC divisional specification, authentic molecular motor pool identities can emerge in the absence of any influence from other LMC neurons.

ESC-Derived LMC Neurons Display Functional Character after Intraspinal Transplantation

We next turned to the issue of whether CV-generated LMC neurons exhibit functional attributes of their in vivo generated counterparts. Adhering to the principle of "in vivo veritas," we examined the behavior of CV generated LMC neurons after isochronic grafting into embryonic chick spinal cord, focusing on the intraspinal settling position and peripheral axonal trajectories of grafted neurons (Wichterle et al., 2002, 2009). In addition, we devised a method to compare directly the behavior of CV and RA/Hh ESC-derived motor neurons within a single host embryo.
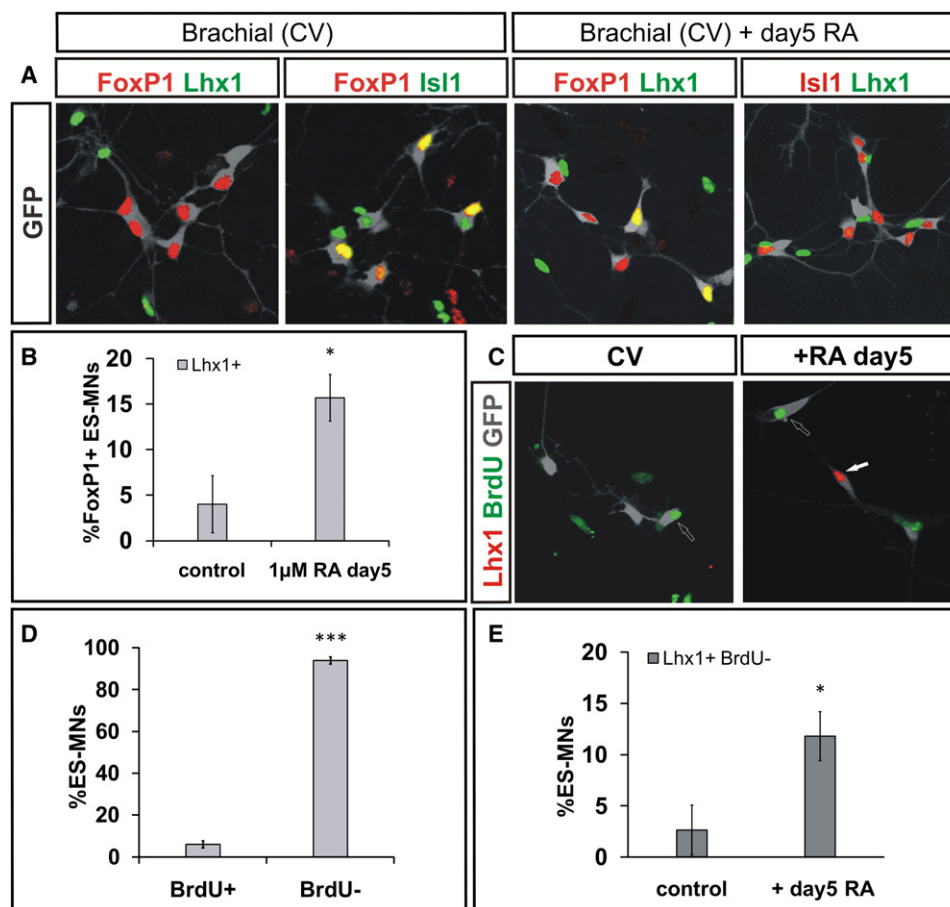
Figure 3. Retinoid Signaling Specifies Lateral LMC Divisional Identity in Postmitotic ESC-Derived Motor Neurons

(A) Expression of Lhx1 and Isl1 in CV-generated GFP[+] ESC-derived motor neurons (gray) on day 7 of differentiation under control conditions and after 1μM RA treatment on day 5. Note mutually exclusive expression of Isl1 and Lhx1.

(B) Increase in Lhx1^{+} FoxP1^{+} ESC-derived motor neurons after retinoid treatment (p = 0.044). Results from three independent experiments (mean ± SEM).

(C) Labeling of motor neurons born after day 5 in cultures by BrdU treatment on days 5–7.

(D) A majority of ESC-derived motor neurons (94% ± 2%) do not incorporate BrdU supplemented between day 5 and 7 of differentiation. Results from three independent experiments (mean ± SEM).

(E) RA treatment on day 5 significantly increases the fraction of postmitotic, BrdU[−] ESC-derived motor neurons that express Lhx1 (p = 0.028). Results from three independent experiments (mean ± SEM).

We first generated a new ESC line (HBR) that carries a motor neuron-specific *Hb9-RFP* transgene, and we used this line to generate rostral cervical RFP[+] ESC-derived motor neurons under RA/Hh differentiation conditions and the classic HBG3 GFP line to generate caudal brachial GFP^{+} motor neurons under CV differentiation conditions. RFP[+] and GFP^{+} motor neurons were dissociated and mixed in equal numbers (Figures 6A and 6B; Figure S6C). Aggregates were harvested on day 6 and transplanted into the brachial or thoracic spinal cord of Hamburger-Hamilton (HH) stages 15–17 chick embryos, at the time of endogenous motor neuron generation. Under both differentiation conditions, the vast majority (94% ± 2%) of ESC-derived motor neurons were postmitotic at the time of transplantation, as revealed by the lack of BrdU incorporation, when added from day 5 of differentiation (Figure 3D). Chick embryos were harvested 3 days after grafting and motor neuron settling preference and axon pathfinding analyzed. We estimate that 30%–50% of transplanted ESC-derived motor neurons survive

at 3 days after transplantation (Supplemental Experimental Procedures).

We first examined whether transplanted motor neurons retain their subtype-specific molecular character in the chick spinal cord. Three days after transplantation, 50% ± 3% of GFP[+] ESC-derived motor neurons expressed FoxP1 and 29% ± 6% expressed Lhx3 (Figures 6C–6E). In addition, 52% ± 8% of RFP[+] ESC-derived motor neurons expressed Lhx3 and 5.6% ± 0.4% FoxP1 (Figures 6C–6E). These values are consistent with the proportional allocation of columnar subtypes in culture (Figure S2), providing evidence for maintenance of columnar character after transplantation.

We did detect a decrease in the representation of Lhx3^{+} neurons (52% ± 8% in vivo compared to 82.0% ± 0.3% in vitro, p = 0.022) among RFP[+] rostral cervical cells (Figure 6E; Figure S2). As a consequence, 42% ± 5% of grafted RFP[+] ESC-derived motor neurons exhibited a transcriptional profile (Lhx3[off]/FoxP1[off]) indicative of HMC columnar character
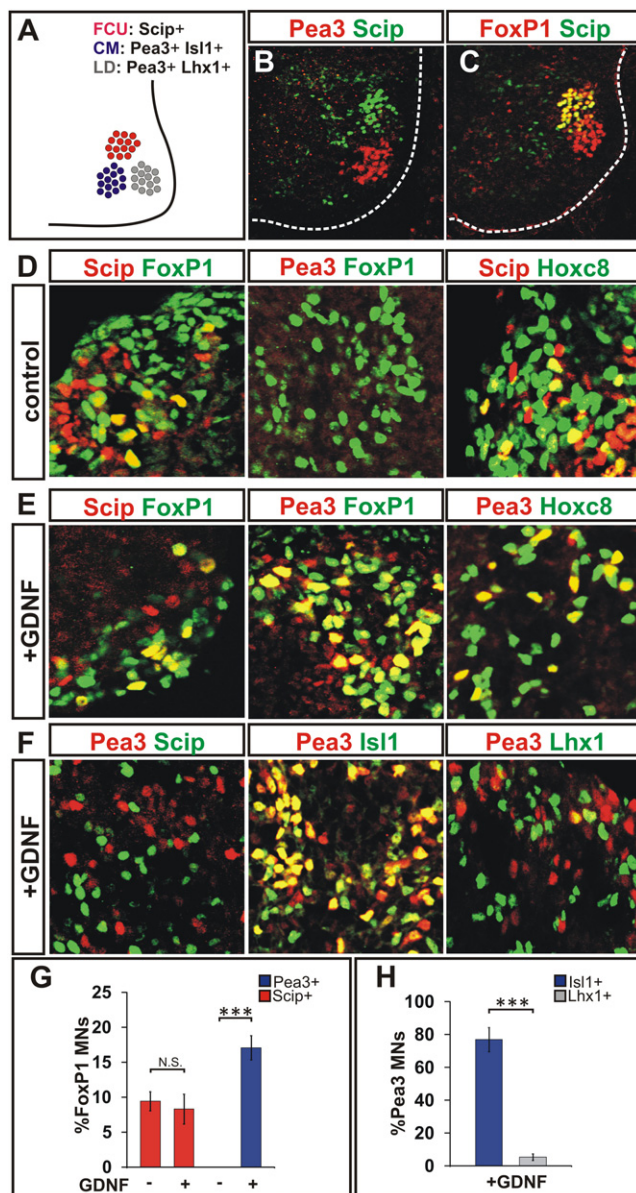
Figure 4. ESC-Derived Motor Neurons Acquire Defined Caudal Brachial Motor Pool Identities

(A) Molecularly defined LMC motor pools in caudal brachial spinal cord: Scip$^+$ pool innervates flexor carpi ulnaris (FCU), Pea3$^+$/Isl1$^+$ pool innervates cutaneous maximus (CM), and Pea3$^+$/Lhx1$^+$ motor pool innervates latissimus dorsi (LD) muscles.

(B) Scip and Pea3 expression in E13.5 mouse spinal cord.

(C) A subset of FoxP1$^+$ LMC neurons expresses Scip at caudal brachial E13.5 mouse spinal cord.

(D) A subset of FoxP1$^+$ LMC neurons expresses Scip but not Pea3. Scip$^+$ motor neurons coexpress Hoxc8.

(E) FoxP1$^+$ ESC-derived motor neurons acquire Pea3 expression in response to GDNF treatment on day 5–6. Pea3$^+$ motor neurons express Hoxc8.

(F) Mutually exclusive expression of Pea3 and Scip in ESC-derived motor neurons. A majority of Pea3$^+$ ESC-derived motor neurons express Isl1 but not Lhx1.

(G) Quantification of Pea3 and Scip expressing FoxP1$^+$ ESC-derived motor neurons in the presence and absence of GDNF. The fraction of Pea3$^+$ but not Scip$^+$ LMC neurons was increased upon GDNF treatment (p < 0.001). Results from three independent experiments (mean ± SEM).
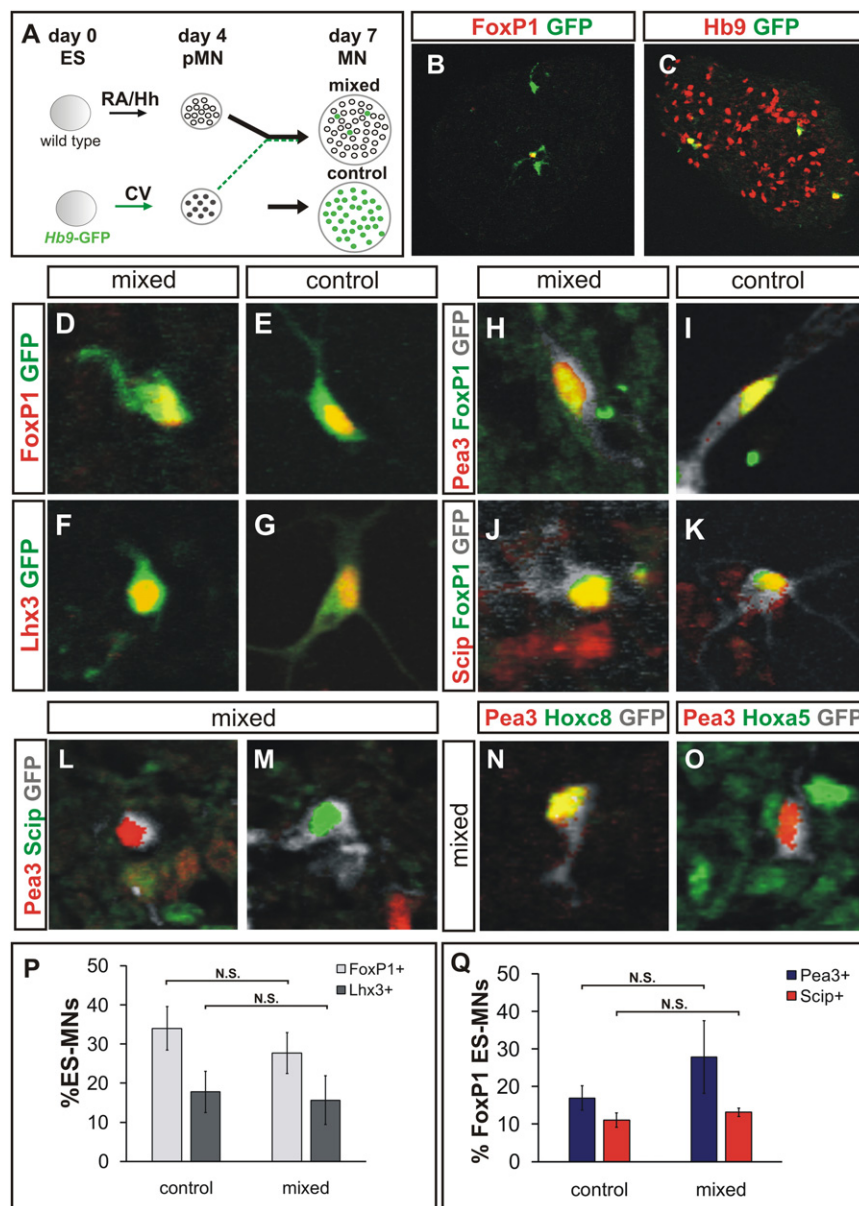
(Figures 6C–6E). The loss of Lhx3 expression resembles the normal developmental program of Lhx3 extinction during the maturation of spinal motor neurons (Agalliu et al., 2009; Sharma et al., 1998). Thus, ESC-derived motor neurons largely maintain their columnar identities after transplantation, permitting analysis of their settling behavior and axonal trajectory.

In the ventral spinal cord in vivo, MMC neurons are positioned medially whereas LMC neurons settle laterally (Figure 6F). To examine whether grafted ESC-derived motor neurons settle in distinct domains according to their columnar identities, we monitored the segregation of CV differentiated Lhx3$^+$ and FoxP1$^+$ ESC-derived motor neurons 72 hr after transplantation into host chick embryos (equivalent to HH stage 28), by which time endogenous MMC and LMC columns are well separated (Figures 6F–6K). The position of individual transplanted and endogenous motor neurons was assigned a mediolateral (m-l) positional value between 0.0 (medial) and 1.0 (lateral) (Figure 6L). CV generated Lhx3$^+$ ESC-derived motor neurons settled medially (m-l value 0.28 ± 0.07) close to endogenous MMC neurons (m-l value 0.33 ± 0.06) (Figures 6I, 6K, and 6M; Figures S5F–S5H), whereas FoxP1$^+$ ESC-derived motor neurons settled laterally (m-l value 0.68 ± 0.08) close to endogenous LMC neurons (m-l value 0.77 ± 0.04) (Figures 6J, 6K, and 6M; Figures S5I–S5K). Thus, CV-generated ESC-derived motor neurons are found in distinct ventral domains, appropriate for their columnar subtype identities.

We considered whether differential survival of ESC-derived motor neurons within columnar "niches" might underlie the differential distribution of Lhx3$^+$ and FoxP1$^+$ ESC-derived motor neurons. Against this idea, we observed that FoxP1$^+$ ESC-derived motor neurons survived when transplanted into the thoracic spinal cord (Figures 6O and 6P), a region that lacks an endogenous LMC niche. Moreover, these neurons settled laterally (Figures 6O and 6P). Thus, the survival and molecular character of LMC neurons is not contingent on the presence of endogenous LMC, or a specialized limb-level niche. Similarly, heterotopic transplantation of RA/Hh-induced cervical ESC-derived motor neurons into the brachial spinal cord did not increase the incidence of FoxP1$^+$ neurons (Figures 6D and 6N). The differential segregation and settling pattern of ESC-derived motor neurons with distinct columnar identities is likely therefore to reflect the recognition of intraspinal cues that normally establish the columnar architecture of the spinal motor system.

We next examined whether ESC-derived LMC neurons pursue a characteristic axonal trajectory to the limb. Aggregates containing an equal mixture of RFP$^+$ RA/Hh-induced cervical and GFP$^+$ CV-induced brachio-thoracic motor neurons were transplanted into brachial-level chick spinal cord. Three days later, embryos were harvested and the contribution of RFP$^+$ and GFP$^+$ ESC-derived motor neuron axons to axial- and limb-directed nerve branches was examined (Figures 7A and 7B; Figure S6D). If the axons of ESC-derived motor neuron columnar subtypes pursued a trajectory that conforms to that of their

(H) Quantification of Lhx1 and Isl1 expression in Pea3$^+$ motor neurons. Significantly greater fraction of Pea3$^+$ ESC-derived motor neurons expressed Isl1 compared to Lhx1 (p < 0.001). Results from three independent experiments (mean ± SEM).

Figure 5. Microenvironment-Independent Acquisition of Motor Pool Identities

(A) CV differentiated Hb9-GFP ESC-derived motor neuron progenitors were dissociated on day 4 and mixed with a 5-fold excess of dissociated RA/Hh-differentiated wild-type (WT) ESC-derived motor neuron progenitors, reaggregated, and cultured until day 7.

(B) One of three GFP+ ESC-derived motor neurons expresses FoxP1.

(C) ESC-derived motor neurons derived from WT ESCs (Hb9+/GFPoff) under RA/Hh condition surround scattered brachial GFP+ ESC-derived motor neurons.

(D–G) ESC-derived motor neurons in control and mixed conditions express FoxP1 (D, E) and Lhx3 (F, G).

(H–K) Expression of Pea3 (H, I) and Scip (J, K) LMC ESC-derived motor neurons (gray) treated with GDNF on day 5.

(L, M) Expression of Pea3 and Scip in ESC-derived motor neurons (gray) does not overlap in mixed aggregates.

(N, O) Hoxc8-expressing Pea3+ ESC-derived motor neurons (N) are surrounded by Hoxa5+ cervical cells (O).

(P) Similar fractions of FoxP1+ and Lhx3+ ESC-derived motor neurons in control and mixed cultures (p = 0.29 and p = 0.81, respectively). Results from three independent experiments (mean ± SEM).

(Q) The fractions of FoxP1+ ESC-derived motor neurons expressing Pea3 and Scip were not significantly different in control or mixed cultures (p = 0.40 and p = 0.44, respectively). Results from three independent experiments (mean ± SEM).

endogenously generated counterparts, the subtype composition of grafted motor neurons (RFP+: 52% ± 8% MMC, 42% ± 5% HMC; GFP+: 50% ± 3% LMC, 20% ± 5% HMC, 29% ± 6% MMC) predicts that greater fraction of RFP+ axons (∼50%) than GFP+ axons (∼30%) will pursue the axial motor trajectory (Figures S6A and S6B; Dasen et al., 2008). Our analysis of GFP axonal projections revealed that 65% ± 7% of GFP+ motor axons were found in the limb-directed nerve branch and only 35% ± 7% within the axially directed branch (Figures 7B and 7C). Conversely, 59% ± 5% of RFP+ motor axons projected axially and 41% ± 5% projected into the limb (Figures S6D and S6E). The axonal trajectories of ESC-derived motor neurons of cervical and brachio-thoracic character are therefore distinct, and moreover they conform to the expected trajectories of their in vivo generated motor neuron counterparts.

To provide a more rigorous assessment of the axonal trajectory of grafted CV differentiated ESC-derived motor neurons, we compared directly transcriptional status (Lhx3 and FoxP1 expression) and retrograde tracer accumulation, after injection of tetramethylrhodamine-dextran (RhD) into axial or limb motor nerve branches (Figures 7D and 7J). We found that 97% ± 2% of transplanted ESC-derived motor neurons retrogradely labeled from axial nerve expressed Lhx3 (Figures 7N–7P) and that 80% ± 10% of ESC-derived motor neurons retrogradely labeled from the limb expressed FoxP1 (Figures 7E–7G and 7P). Fewer than 5% of ESC-derived motor neurons retrogradely labeled from the axial nerve expressed FoxP1 and fewer than 5% of ESC-derived motor neurons retrogradely labeled from the limb nerve expressed Lhx3 (Figures 7H, 7I, 7K–7M, and 7P). The ∼20% of ESC-derived motor neurons that possessed HMC molecular character (Lhx3off, FoxP1off) were retrogradely labeled after limb tracer injection (Figure 7P), consistent with the trajectory of ectopic HMC neurons induced to differentiate at limb levels of mouse spinal cord (Dasen et al., 2008). Thus, LMC and MMC neurons derived from ESCs extended axons along
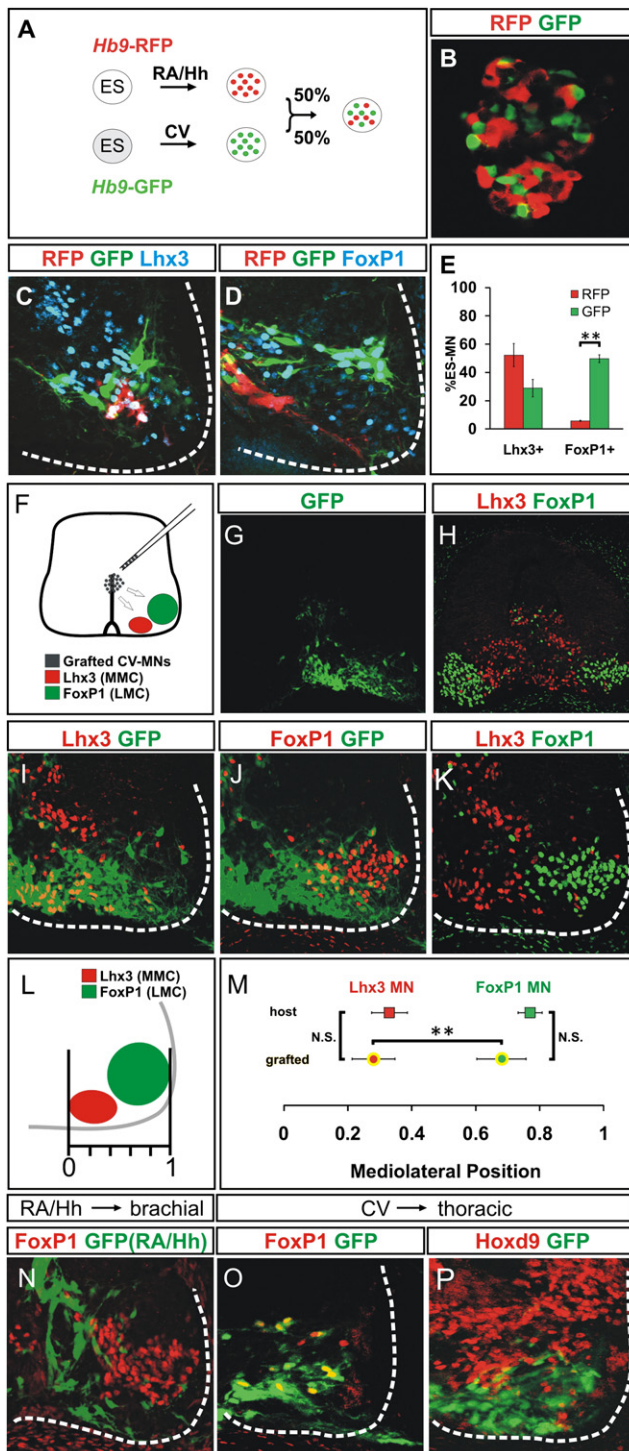
Figure 6. Column-Specific Settling of Transplanted ESC-Derived Motor Neurons

(A) Hb9-RFP and Hb9-GFP ESC lines differentiated under RA/Hh and CV conditions, respectively. RFP$^+$ and GFP$^+$ motor neuron aggregates were transplanted into HH stage 15–17 brachial spinal cord and analyzed 3 days later.

(B) Intermixing of RFP$^+$ and GFP$^+$ ESC-derived motor neurons in aggregates.

(C and D) ES-derived neurons in host ventral spinal cord (white outline) express Lhx3 (C) and FoxP1 (D).

divergent peripheral trajectories. These findings indicate that ESC-derived motor neurons acquire functional subtype characters that reflect those of corresponding motor neurons generated in vivo.

DISCUSSION

Studies on the directed differentiation of ESCs to nerve cells have typically relied on transgene expression and/or exposure to exogenous signaling factors (e.g., Andersson et al., 2006; Bain et al., 1995; Wichterle et al., 2002). In this study we have considered whether ESCs might be coaxed into the formation of endogenous signaling centers that direct the generation of specialized cell subtypes with properties that match those of counterpart cells generated in vivo. To address this issue, we devised a differentiation protocol for generation of functional motor neuron columnar, divisional, and pool subtypes without the need for added factors. With this trick, we have been able to distinguish the relative contributions of late-stage inductive signals and position-independent programs to the specification of motor neuron subtypes, providing insights into the mechanisms of motor neuron differentiation. Moreover, we have used intraspinal transplantation of ESC-derived motor neurons to demonstrate a tight link between the molecular character of motor neuron subtypes and two of their critical functional and developmental attributes: neuronal settling position and axonal trajectory.

Motor Neuron Differentiation under Retinoid-Free Conditions

The generation of motor neurons from ESCs requires directed neural differentiation. Early exposure of ESCs to retinoids directs cells along a neurogenic lineage (Bain et al., 1995). The application of retinoids in neural differentiation protocols has therefore become a prevalent practice, despite the fact that retinoids have not been implicated in early stages of neural programming within the intact embryo (Niederreither et al., 1999). But retinoid exposure also consigns progenitor cells in the neural tube to caudal hindbrain and rostral cervical identity (Liu et al., 2001).

To uncover conditions for LMC neuron differentiation from ESCs, we developed a differentiation protocol that more closely

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(E) FoxP1 and Lhx3 expression in RFP$^+$ and GFP$^+$ motor neurons 3 days after transplantation. More transplanted GFP$^+$ neurons express FoxP1 (p = 0.004). Results from three transplanted embryos (mean ± SEM).

(F) CV-differentiated ESC-derived motor neurons transplanted into HH stage 15–17 brachial spinal cord.

(G) Ventrally localized GFP$^+$ motor neurons 3 days after transplantation.

(H–K) Spinal cord section (from G) triple labeled for Lhx3, FoxP1, and GFP. White dashed line delineates spinal cord margin.

(L) Measuring the settling position of transplanted cells.

(M) Settling preferences of transplanted and endogenous motor neurons expressing Lhx3 or FoxP1. The settling positions of host and grafted FoxP1$^+$ or Lhx3$^+$ motor neurons are not significantly (N.S.) different (p = 0.85, resp. p = 0.92). Settling position of transplanted Lhx3$^+$ ESC-derived motor neurons is different (p < 0.01) from that of FoxP1$^+$ ESC-derived motor neurons. Results from four transplanted embryos (mean ± SEM).

(N) Lack of FoxP1 expression in RA/Hh-differentiated ESC-derived motor neurons grafted into brachial spinal cord.

(O and P) FoxP1 expression by CV ESC-derived motor neurons (O) transplanted into Hoxd9$^+$ thoracic spinal cord (P).
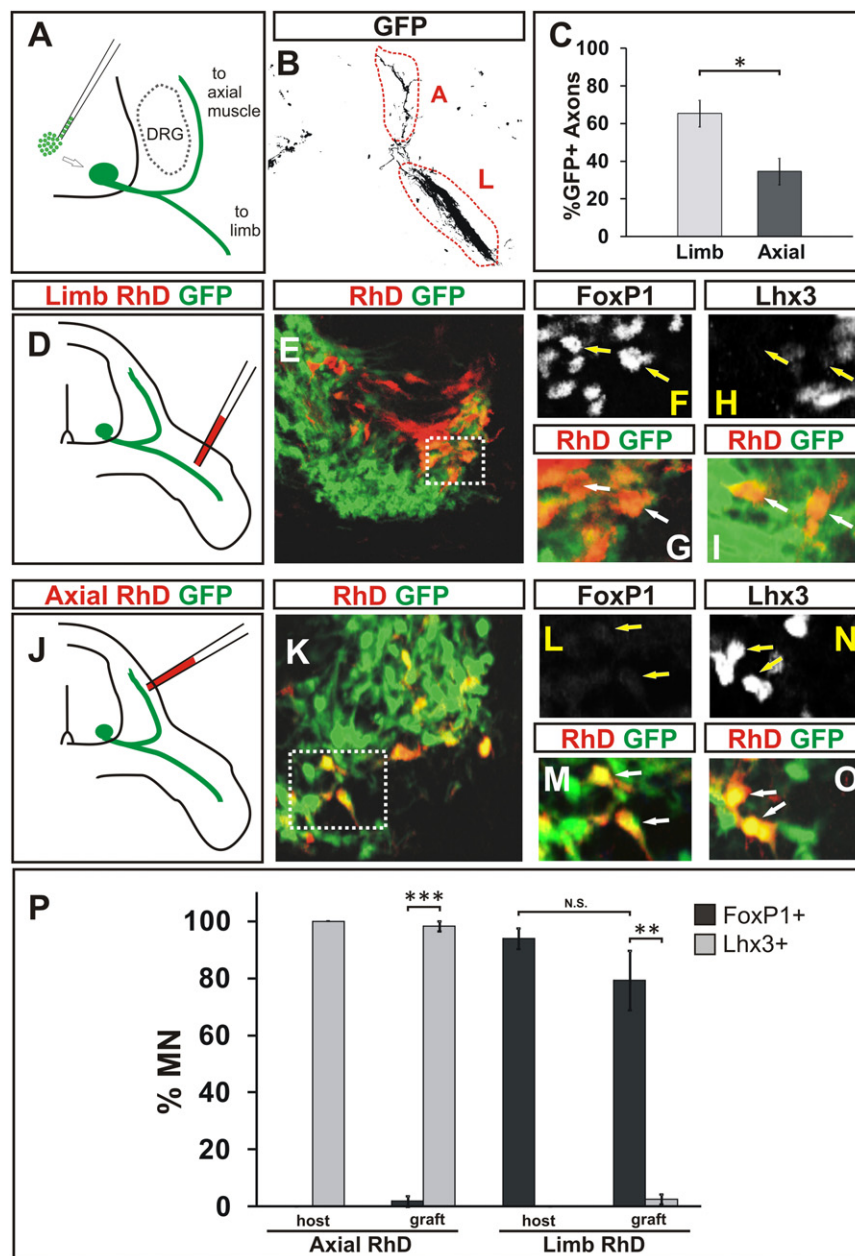
Figure 7. Axons of LMC and MMC ESC-Derived Motor Neurons Project to Limb and Axial Muscles

(A) Strategy to examine axon pathfinding preference of GFP$^+$ ESC-derived motor neurons differentiated under CV condition 3 days after grafting into brachial spinal cord (HH stage 15–17).

(B and C) A greater fraction of transplanted GFP$^+$ (black) axons is detected in the limb (L) nerve compared to axial (A) nerve branch 3 days after grafting (p = 0.011). Results from six transplanted embryos (mean ± SEM). See also Figures S6D and S6E.

(D) CV-differentiated transplanted motor neurons were backfilled with tetramethylrhodamine-dextran (RhD) from the limb nerve branch.

(E) Section of a ventral spinal cord containing GFP$^+$ transplanted and RhD$^+$ limb innervating motor neurons (boxed area corresponds to F and G).

(F–I) Transplanted motor neurons retrogradely labeled from the limb (RhD$^+$/GFP$^+$) express FoxP1 (arrows, F, G) but not Lhx3 (arrows, H, I).

(J and K) Transplanted ESC-derived motor neurons backfilled with RhD from the axial nerve branch (boxed area in K corresponds to L and M).

(L–O) ESC-derived motor neurons retrogradely labeled from the axial musculature (RhD$^+$/GFP$^+$) expressed Lhx3 (arrows, N, O) but not FoxP1 (arrows, L, M).

(P) More axially projecting grafted ESC-derived motor neurons expressed Lhx3 than FoxP1 (p < 0.001). More limb projecting ESC-derived motor neurons expressed FoxP1 than Lhx3 (p = 0.002). The fractions of endogenous and grafted FoxP1$^+$ motor neurons labeled from the limb, or Lhx3$^+$ motor neurons labeled from the axial muscles are not different (p = 0.26, resp. p = 0.37). Results from three embryos each for limb and axial backfills (mean ± SEM).

2008), neural cells generated under CV conditions exhibit caudal and ventral neural progenitor character and give rise to motor neuron columnar and pool subtypes normally located at brachial and thoracic levels of the spinal cord. These caudal fates depend on embryoid body-derived Wnt, FGF, and Hh signals

mimics the patterning events that operate during vertebrate neural differentiation. Local interactions between early embryonic cells are thought to engage a BMP signaling pathway that suppresses the potential for neural differentiation (Finley et al., 1999; Ying et al., 2003), such that dissociation and maintenance of cells at low density avoids paracrine BMP exposure and permits neural differentiation (Watanabe et al., 2005). Our findings indicate that ESCs grown initially under low-density retinoid-free conditions give rise to neural cells of caudal brachial character.

In contrast to previously reported RA-free differentiation conditions that rostralize neural cells (Eiraku et al., 2008; Gaspard et al., 2008; Watanabe et al., 2005; Wataya et al.,

that mimic the signaling milieu of the intact embryo. Several Wnts (*Wnt3, Wnt5b, Wnt8a*), *FGFs* (*FGF4, FGF5*, and *FGF15*), and Shh are expressed by cells in embryoid bodies over the period that neural cells first appear (Lako et al., 2001; Stavridis et al., 2007). Moreover, blockade of Wnt, FGF, or Hh signaling in embryoid bodies prevents the acquisition of caudal neural character. Thus, the generation of LMC neurons from ESCs is driven solely by the actions of endogenously supplied inductive factors. It remains to be seen whether rational application of Wnt and Fgf signals to differentiating ESCs might increase motor neuron yields without sacrificing the columnar and motor pool subtype diversity achieved under CV differentiation conditions.

Neighbor-Dependent and -Independent Programs of LMC Subtype Specification

Motor neurons within the LMC acquire divisional and pool identities that can be defined by expression of LIM, POU, and ETS transcription factors (Arber et al., 2000; Dasen et al., 2005; Jessell, 2000; Livet et al., 2002; Sockanathan and Jessell, 1998). The acquisition of divisional character within an initially generic set of LMC neurons is driven by a retinoid-mediated paracrine signal from early-born LMC neurons (Sockanathan and Jessell, 1998). Furthermore, assignment of the rostrocaudal position of motor pools within the span of the brachial LMC reflects the ambient balance of retinoid and FGF signaling (Dasen et al., 2003; Liu et al., 2001).

How LMC neurons acquire distinct pool identities remains less clear. Analysis of the segregation of Hox protein expression patterns and of switches in motor pool fate after manipulation of spinal *Hox* gene activity has led to a model in which the segment by segment diversification of LMC neurons into motor pools is driven by cell-intrinsic cross-repressive interactions between the cohort of Hox proteins that is initially expressed by an LMC equivalence group (Dasen et al., 2005). To date, however, there has been no direct experimental test of this "intrinsic" view, and thus the possibility that signals supplied by neighboring LMC neurons drive intrasegmental pool diversity has not been falsified. One telling prediction of the intrinsic model is that individual LMC neurons should be able to progress to discrete pool identities when isolated from LMC neurons or other limb-level cells that could serve as potential sources of pool-inducing signals. Systematic manipulation of the local position of individual LMC neurons in vivo is a formidable and, so far, unmet challenge.

The identification of conditions that permit the generation of LMC neurons from ESCs provided us with an opportunity to examine motor pool specification under conditions in which individual LMC neurons are forced to differentiate in the virtual absence of neighboring LMC neurons and other limb-level neural cells. Our analysis reveals that ESC-derived brachial LMC neurons grown at limitingly low density in a sea of rostral cervical cells can still progress to molecularly authentic CM and FCU fates. These findings are difficult to reconcile with the view that local signals provided by LMC neurons (or other limb level cells) are needed to assign motor pool identity. By extension, they lend experimental support for the idea that the Hox profile inherited by postmitotic LMC neurons is the primary determinant of the subsequent intrasegmental diversification of motor pools.

ESC-Derived Motor Neuron Subtype Identity Validated by Cellular Behavior In Vivo

The segregation of motor neuron cell bodies into distinct intraspinal columnar domains and the projection of motor axons along different peripheral trajectories are two prominent features of motor neuron subtypes linked to distinct transcriptional profiles (Kania and Jessell, 2003; Kania et al., 2000; Sharma et al., 1998; Sockanathan and Jessell, 1998). Our studies have addressed whether the molecular character of motor neuron subtypes predicts distinct cellular behaviors in the developing embryo. Isochronic grafting of ESC-derived motor neurons into embryonic spinal cord reveals that neurons with the transcriptional character of MMC and LMC columnar subtypes segregate into different domains of the ventral spinal cord and settle close to endogenously generated motor neurons of the same columnar character. Thus, ESC-derived motor neurons can sense their position within the host spinal cord and migrate appropriately in response to column-specific settling signals. Close inspection of settling position, however, reveals that ESC-derived motor neurons of MMC and LMC character failed to mix with their endogenously generated columnar counterparts, possibly because of mismatches in the surface properties of mouse and chick LMC neurons. In contrast to the striking homing behavior of ESC-derived motor neurons when introduced in vivo, columnar segregation is not evident in embryoid bodies (Figure S3C), presumably because relevant migratory substrates and guidance cues are inadequately organized under these in vitro conditions.

Grafted ESC-derived motor neurons of LMC and MMC character establish peripheral axonal trajectories appropriate for their columnar subtype—selecting axial and limb nerve branches with a fidelity that approaches that of endogenous motor neurons. A substantial majority of motor neurons of caudal brachial character projected axons into the limb mesenchyme, consistent with the predominance of FoxP1$^+$ LMC neurons. More persuasively, retrograde labeling studies revealed that individual Lhx3$^+$ and FoxP1$^+$ ESC-derived motor neurons established peripheral axonal trajectories appropriate for their transcriptional identity with high fidelity. The specificity of the link between molecular identity and axonal trajectory implies that the transcriptional status of ESC-derived motor neurons directs expression of guidance receptors that underlie the selection of distinct peripheral trajectories (Marquardt et al., 2005).

We have not resolved whether ESC-derived motor neurons with distinct pool characters direct axonal projections to specific target muscle groups in the limb. The divergence in anatomical and functional organization of mouse forelimb and chick wing musculature (Ferns and Hollyday, 1995; Jones, 1979; Ryan et al., 1998) confounds analysis of innervation patterns in the mouse-to-chick transplantation assays. Nevertheless, our studies argue that the programming of motor neuron columnar subtypes from naive ESCs generates neuronal subsets that express cell-specific transcriptional markers and, in parallel, exhibit specialized cellular behaviors in vivo.

The ability to generate defined motor neuron subtypes without transgene expression or exogenous factor exposure may prove beneficial for studies of neuronal diversification in other regions of the mammalian CNS, as well as for disease modeling. Motor neuron subtypes exhibit differential susceptibility to neurodegeneration in two prominent motor neuron diseases, amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (Kanning et al., 2010). The ability to drive the differentiation of ESCs into disease-sensitive and -resistant motor neuron subtypes could help to uncover new strategies for therapy in motor neuron disorders.

EXPERIMENTAL PROCEDURES

Differentiation of ESCs

Differentiation of ESCs under RA/Hh conditions was performed as previously described (Wichterle et al., 2002). For CV differentiation, ESCs (~20,000 cells/ml) were plated in ADFNK medium (see Supplemental Experimental Procedures).

Medium was changed on days 1, 2, and 5 of differentiation and embryoid bodies were split 1:4 on day 2 of differentiation.

Transplantation of ESC-Derived Motor Neurons into Chick Neural Tube

Transplantation of ESC-derived motor neurons into chick developing neural tube was performed as described (Wichterle et al., 2002). Embryoid bodies were harvested on day 5 or day 6 of differentiation and transplanted into lesioned chick spinal cord. Three days later transplanted embryos were fixed with 4% paraformaldehyde (PFA), cryosectioned, and processed for immunohistochemistry.

Retrograde Labeling of ESC-Derived Motor Neurons

Retrograde labeling was performed as described (Dasen et al., 2005). Embryos were dissected 3 days after transplantation and axial or limb GFP$^+$ nerve branches were retrogradely labeled with 3000 MW lysine-fixable tetramethylrhodamine-dextran (RhD, Molecular Probes) under a fluorescence dissection microscope. Embryos were incubated in an oxygenated bath for 3–5 hr at 37°C, fixed with 4% PFA (1 hr at 4°C), and processed for immunohistochemistry.

Statistical Analysis

Two-tail Student's t test was used for statistical analysis. Relevant p values (*p = 0.01–0.05, **p = 0.001–0.01, ***p < 0.001) are listed in the Table S2. We used one-way ANOVA to test for a difference between groups and a two-tailed t test for all pairwise comparisons with a Bonferroni correction for multiple testing.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at doi:10.1016/j.stem.2010.07.013.

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