Nkx2.2+ Progenitors Generate Somatic Motoneurons in the Chick Spinal Cord

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Abstract
Heterogeneous classes of neurons are present in the spinal cord and are essential for its function. Expression patterns of transcription factors in neural progenitor cells determine neuron subtypes during development. Nkx2.2 is expressed in the progenitor cell pool located just ventrally to the Olig2-positive pool and is indispensable for V3 interneuron generation in the spinal cord and also for visceral motoneuron generation in the hindbrain. However, whether Nkx2.2-positive progenitor cells generate diverse classes of neuron is not fully understood. Using a chick lineage tracing method in a genetically-defined manner, we found that Nkx2.2-expressing progenitor cells differentiate into general visceral motoneurons as well as sim1-positive V3 interneurons. Surprisingly, we further observed that Nkx2.2-expressing progenitors differentiate into somatic motoneuron. Our findings suggest that the different classes of motoneurons are derived from more complex sources than were previously expected in the chick spinal cord.

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Introduction

The spinal motor circuitry that generates motor output consists of several types of motoneurons and interneurons. It is well known that the motoneuron forms a columnar structure along the rostro-caudal axis and each columnar neuron shows distinct muscle innervation patterns in both chicks and mice [1]. At limb levels, motoneurons form the medial motor column (MMC) which regulates trunk muscles and the lateral motor column (LMC) which regulates limb muscles, whereas the sympathetic preganglionic motor column (called the Column of Terni; CT in chicks) and MMC are formed in the thoracic spinal cord.

A gradient of morphogens, such as sonic hedgehog, induces expression of transcription factors that specify different types of neurons as well as glial cells. Progenitor cells of somatic motoneurons are located in the ventral neural tube and express Olig2, a basic helix-loop-helix transcription factor, thus forming the pMN domain. Olig2 initially specifies motoneurons and genetic deletion of olig2 causes loss of motoneurons in mice [2–4]. Nkx2.2 is a homeodomain transcription factor and is expressed just ventrally to the pMN domain, demarcating the p3 domain, and is required for V3 interneuron development [5]. These transcription factors show cross-repressive interactions [6], suggesting that their functional interaction is important for formation of the boundary between pMN and p3 domains. By using an in ovo electroporation technique, it was shown that forced expression of Nkx2.2 represses expression of Olig2 [7], thus Nkx2.2 itself is considered to be a negative regulator of motoneuron generation [8]. However, in the mouse hindbrain, Nkx2.2 positive cells differentiate into visceral motoneurons as well as serotonergic neurons [9]. Moreover, it was suggested that Nkx2.2-lineage cells contribute to visceral motoneurons in the spinal cord [10]. These reports raised the possibility that various types of neurons were generated from Nkx2.2 positive progenitors. However, whether a population of motoneurons derives from Nkx2.2-expressing progenitors is not fully understood in the chick spinal cord.

Here, we analyzed cell lineage from Nkx2.2-positive progenitors using the genetically-defined lineage tracing method in the chick spinal cord, which we developed recently [11]. In addition, we applied a new strategy for lineage tracing by electroporating floxed reporter plasmids and Cre expressing plasmids at quite low concentrations determined by limiting dilutions. The results show that Nkx2.2-expressing cells generate not only sim1-expressing V3 interneurons but also visceral motoneurons. Surprisingly, these progenitors also differentiate into somatic motoneurons in the spinal cord. Our results indicate that Nkx2.2-positive progenitor cells produce a highly diverse population of motoneurons as well as V3 interneurons in the chick spinal cord.
Materials and Methods

Animals and Gene Manipulation
Fertilized white leghorn eggs were obtained from the Glen Corporation (Gifu, Japan) or the Yamagishi Corporation (Mie, Japan) and were incubated at 38°C. Embryonic stages of chicks were determined according to Hamburger and Hamilton [12]. All experimental procedures were approved by the Animal Care Committee of the National Institute for Physiological Sciences and that of Kyoto Prefectural University of Medicine (No. M21–162).

In Ovo Lineage Tracing Analysis
Approximately 0.1 μl of the mixed solution containing 400 ng/μl of pNkx2.2-mCAT1-myc [11] and a 1/10 volume of 0.5% fast green was injected into the neural tube of HH stage 13 to 14 chicken embryos. Needle type electrodes were placed near the lumbar neural tube of the embryo and a 20 V, 30 ms pulse was applied three times using an electronic stimulator (SEN-3310; Nihon Kohden, Japan). For the retroviral injection, approximately 0.1 μl of virus solution (titer of retrovirus was 1×10^7 cfu/ml) was injected into the neural tube 24 h after electroporation. EGFP-expressing high-titer retroviral particles were prepared and concentrated as previously described [13]. For Cre-loxP lineage tracing, mCAT1 was excised from pNkx2.2-mCAT1-myc and replaced by Cre (pNkx2.2-Cre). Approximately 0.1 μl of the mixed solution containing 1 ng/μl of pNkx2.2-Cre, 1 μg/μl of cAct-xstopx-lacZ [14], and 0.05% fast green was electroporated to the neural tube. For fluorescent labeling by Cre-loxP system, the same volume of mixed solution containing 0.25 ng/μl of pNkx2.2-Cre, and 0.25 to 0.5 μg/μl of CMV-brainbow-1.0L [15] (obtained from Addgene, Boston, USA) was electroporated.

In Situ Hybridization
Chick embryos were harvested and fixed in 4% paraformaldehyde/PBS at 4°C for 16 h, followed by incubation with DEPC-treated 20% sucrose/PBS for 24 h. For lacZ staining, chick embryos were fixed in 2% paraformaldehyde/PBS at 4°C for 1 h, followed by incubation with DEPC-treated 20% sucrose/PBS for 12 h. Embryos were embedded in OCT compound (Sakura Finetek Japan, Japan) and sections were prepared using a cryostat. Two days after FG injection, embryos were fixed with 4% paraformaldehyde/PBS as above-mentioned. After X-gal staining or GFP immunostaining, recombined cells were examined whether they were labeled with FG.

Immunohistochemistry
Procedures of in situ hybridization and immunohistochemistry were as previously described (Gotoh et al., 2011). For immunohistochemical staining after in situ hybridization, sections were treated with heat by microwave for 5 min in 10 mM citrate buffer (pH 6.0) and were cooled to room temperature before incubation with primary antibodies. The primary antibodies used in this study were as follows; mouse anti-Hb9, mouse anti-Nkx2.2, mouse anti-Lim3 (DSHB, University of Iowa, USA), rabbit anti-GFP (Invitrogen, USA), rabbit anti-Olig2, goat anti-ChAT (Millipore, USA), chiken anti-LacZ (Abcam, USA), and rabbit anti-Myc (MBL, Japan). Sections were observed under a fluorescence microscope (BX51; Olympus, Japan) or confocal microscope (FV-1000; Olympus, Japan).

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In Ovo Lineage Tracing Analysis
Approximately 0.1 μl of the mixed solution containing 400 ng/μl of pNkx2.2-mCAT1-myc [11] and a 1/10 volume of 0.5% fast green was injected into the neural tube of HH stage 13 to 14 chicken embryos. Needle type electrodes were placed near the lumbar neural tube of the embryo and a 20 V, 30 ms pulse was applied three times using an electronic stimulator (SEN-3310; Nihon Kohden, Japan). For the retroviral injection, approximately 0.1 μl of virus solution (titer of retrovirus was 1×10^7 cfu/ml) was injected into the neural tube 24 h after electroporation. EGFP-expressing high-titer retroviral particles were prepared and concentrated as previously described [13]. For Cre-loxP lineage tracing, mCAT1 was excised from pNkx2.2-mCAT1-myc and replaced by Cre (pNkx2.2-Cre). Approximately 0.1 μl of the mixed solution containing 1 ng/μl of pNkx2.2-Cre, 1 μg/μl of cAct-xstopx-lacZ [14], and 0.05% fast green was electroporated to the neural tube. For fluorescent labeling by Cre-loxP system, the same volume of mixed solution containing 0.25 ng/μl of pNkx2.2-Cre, and 0.25 to 0.5 μg/μl of CMV-brainbow-1.0L [15] (obtained from Addgene, Boston, USA) was electroporated.

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Quantitative Analysis
For quantitative analysis, at least three independent experiments were performed. Sections were collected approximately every 300 μm and all sections that were positive for GFF or lacZ were counted. All quantitative data are shown as mean±SEM.

Results
Somatomotor Neuron Generation from Nkx2.2+ Progenitors at HH 19
In our previous study [11], we showed that Nkx2.2-expressing progenitor cells in the gliogenic phase differentiate into mature oligodendrocytes in the chick spinal cord. To analyze whether Nkx2.2-lineage cells generate diverse classes of neurons in the chick spinal cord, we employed the same strategy (Fig. 1A). First, the expression pattern of Olig2 and Nkx2.2 was examined within the ventricular zone. Three thoracic sections were analyzed in each embryo and four embryos were used for this analysis. In the early embryonic stage (HH stage 14), a small population of Nkx2.2/Olig2 double-positive cells were present only at the boundary of p3 and pMN domains (16.5±1.64%, percentage of Nkx2.2/Olig2 positive cells/total Nkx2.2 positive cells, n=4; Fig. 1B-D) and double-positive cells decreased in number at HH 17 (4.14±0.69%, n=4; Fig. 1E-G), indicating that the border became sharper as embryos developed. pNkx2.2-mCAT1-myc, which express mCAT1 (receptor for murine retrovirus) under the regulation of the enhancer region of nks2.2 [17], was introduced into the chick embryonic neural tube at HH 14 by electroporation. mCAT1-Myc was expressed exclusively in Nkx2.2-expressing cells 24 h after electroporation (HH 17-19; Fig. 1H-I; n=4) as previously demonstrated in later stages [11]. This was also confirmed by in situ hybridization that showed mCAT1 mRNA was present in Nkx2.2-expressing cells, but not in Olig2-expressing cells, just dorsally to the p3 domain within the ventricular zone (Fig. 1K and L). These observations suggest that electroporated mCAT1 was expressed only in the Nkx2.2-expressing cells in our experimental condition, thus the reliability of this system is also confined to neurogenic stages.

To trace the lineage of p3 domain cells, an EGFP-expressing retroviral solution was injected into the neural tube 24 h after electroporation of pNkx2.2-mCAT1-myc (HH 19). We analyzed embryos 24 h after retroviral transduction and found that EGFP positive cells were present in the ventral neural tube (Fig. 2A; Nkx2.2+ Progenitors Generate Somatic Motoneurons

LacZ Staining
For lacZ staining, chick embryos were fixed in 2% paraformaldehyde/PBS at 4°C for 1 h, followed by incubation with DEPC-treated 20% sucrose/PBS for 12 h. Embryos were embedded in OCT compound (Sakura Finetek Japan, Japan) and sections were prepared using a cryostat. LacZ staining was performed as previously described [16]. For lacZ/immunohistochemistry or in situ hybridization double staining, lacZ-stained sections were fixed in 4% paraformaldehyde/PBS at room temperature for 30 min, followed by staining procedures as described above.
Nkx2.2+ Progenitors Generate Somatic Motoneurons

A

pNkx2.2-mCAT1 -myc

electric pulses

EGFP expressing retrovirus

24 hrs

HH14 → HH19

HH25 or 35

B

Olig2

HH14

C

Nkx2.2

D

merge

E

Olig2

HH17

F

Nkx2.2

G

merge

H

mCAT1 -myc

HH14 E.P. → HH19

I

Nkx2.2

J

merge

K

mCAT1

Nkx2.2

L

mCAT1

Olig2
brown). EGFP-positive cells that were observed in the most ventral area expressed sim1 mRNA, which is a marker for V3 interneurons (Fig. 2A; arrow).

EGFP-positive cells were also observed dorsally to sim1-positive cells (Fig. 2A; arrow heads). Approximately 10% of total EGFP-positive cells extended their axons into the ventral roots at HH 25.
positive cells in the ventral horn were Isl1/2-positive and HB9 strongly expressed Nkx2.2 in all embryos (n = 4). Some LacZ-positive cells in both the ventricular zone and ventral horn (Fig. 3L). LacZ-positive cells were observed in the Olig2+ ventricular zone, adjacent sections were triple labeled using anti-LacZ and anti-ChAT antibodies, which is consistent with previous reports [18]. GFP-positive axons were also observed in the ventral root at HH 35 (E9; Fig. 2C). In addition, a small number of EGFP-positive neurons expressed HB9 at HH 35 in the chick spinal cord (E9; Fig. 2D-F). These data suggest that some progenitor cells in the p3 domain differentiate into HB9 positive somatic motoneurons.

Diverse Motoneuron Generation from Nkx2.2+ Progenitors at HH14

We next analyzed the distribution of Nkx2.2-lineage motoneurons, and their contribution to the columnar structure. Because motoneuron generation in LMC or CT starts around HH 15 [19], it is conceivable that labeling by a retrovirus (HH 17 to 19) could not label all the progenitors that generate motoneurons. In addition, labeled cells by retroviral injection at HH 19 rarely differentiate into CT cells located near the central canal. In order to avoid this bias, we employed a Cre-loxP mediated lineage-tracing method; a floxed-nlacZ reporter plasmid was co-electroporated with the pNkx2.2-Cre plasmid, which is regulated by the same nks2.2-enhancer, into HH 14 chick spinal cords. The minimum concentration of the pNkx2.2-Cre plasmid was determined by limiting dilutions to avoid nonspecific labeling. After 36 h, the initial LacZ expression in the ventricular zone was restricted to the Nkx2.2-positive cells at HH 21 (E3.5, Fig. 3A), which was similar to that observed in retroviral labeling. As some LacZ-positive cells were located close to Olig2-positive cells in the ventricular zone, adjacent sections were triple labeled using anti-LacZ, anti-Nkx2.2, and anti-Olig2 antibodies. Some LacZ-positive cells were present at the domain boundary and few LacZ-positive cells were observed in the Olig2+/Nkx2.2- ventricular zone whereas most cells were located in Nkx2.2+/Olig2- region (Fig. 3B-F). We counted cells in the Olig2+/Nkx2.2 boundary and 15.3 ± 8.16% (mean ± SEM; n = 4) of total labeled cells within the ventricular zone cells were located at the domain boundary. In later stages (HH 32 or E7), LacZ-positive cells were observed in both the ventricular zone and ventral horn (Fig. 3L). LacZ-positive cells within the ventricular zone expressed Nkx2.2 (Fig. 3 G; inset and H-J) but not Olig2 (Fig. 3 K-M). At this stage, no LacZ-positive cells expressed Olig2 at detectable levels, whereas they strongly expressed Nkx2.2 in all embryos (n = 4). Some LacZ-positive cells in the ventral horn were Isl1/2-positive and HB9-positive (Fig. 3N, O), indicating that they differentiated into somatic motoneurons.

To define the distribution of Nkx2.2-lineage cells, we stained brachial or thoracic spinal cord sections by LacZ histochemistry. Fig. 4A and 4B show schematic diagrams of the distribution of LacZ-positive cells in the brachial or thoracic spinal cord of three independent chick embryos. At HH 32 (E7), LacZ-positive cells were mostly present in the gray matter and the ventricular zone. In two of three embryos (Fig. 4A, left and right), there were LacZ-positive cells in the ventral horn in the brachial spinal cord. In the thoracic spinal cord, LacZ-positive cells were observed more dorsally near the ventricular zone, suggesting that they were preganglionic motoneurons (Fig. 4B). In order to confirm that recombined cells included motoneurons that send axons outside the spinal cord, retrograde tracer (fluorogold; FG) was used to label motoneurons. Injected FG was spread not only to the wing bud but also widely to the body cavity including peritoneal and thoracic ones, and therefore, somites and other mesenchymal tissues showed FG fluorescence bilaterally in the coronal sections. Within the spinal cord, somatic motoneurons in the ventral horn and preganglionic cells in the intermediate region showed yellowish white FG fluorescence bilaterally. In cases with FG injection, a LacZ-positive cell was located in the ventral horn where most of cells were retrogradely labeled with FG so that this LacZ-positive cell was highly likely labeled with FG also (Fig. 4C, D). To further analyze this, we used the brainbow plasmid which expresses RFP, and GFP after Cre mediated recombination. GFP-positive cells observed in the ventral part of the ventral horn showed GFP immunoreactivity, one of which was retrogradely labeled with FG (Fig. 4E, F, and G arrowheads). Therefore, some of the recombined cells were apparently motoneurons that sent axons outside the spinal cord (Fig. 4F arrows). At later stages (HH 42 or E16), some LacZ-positive cells expressed choline acetyltransferase (ChAT), an enzyme for acetylcholine generation and, thus, often used as a marker for mature motoneurons, in the ventral horn and around the central canal (Fig. 4H and 4L). Expression of ChAT in recombined cells were also confirmed by double-immunohistochemistry using anti-LacZ and anti-ChAT antibodies (Fig. 4I K, 4M O; arrowheads). We collected 20 μm sections in each 300 μm section from electroporated spinal cord. The ratio of LacZ/ChAT-double positive cells per total LacZ-positive cells was calculated from five embryos. A large number of motoneurons were derived from Nkx2.2-positive cells (Fig. 4P; average = 31.3 ± 8.4%). ChAT-negative cells might represent interneuron other than motoneurons. These data suggest that a large population of Nkx2.2-progenitors differentiated into functional motoneurons.

In order to define the motoneuron subtype from Nkx2.2-positive progenitors, we stained LacZ, together with subtype-specific markers [20], [21] at HH 32 in brachial or thoracic spinal cords using Lim3 for MMC, raldh2 for LMC, and foxP1 for CT. Nkx2.2-derived cells differentiated into Lim3 positive (Fig. 5A) and raldh2-positive motoneurons (Fig. 5B) in the brachial spinal cord. In the thoracic spinal cord, we observed LacZ/Lim3 double positive MMC (data not shown) as well as LacZ/foxP1 double positive preganglionic neurons (Fig. 5C). These observations suggest that Nkx2.2-positive progenitors differentiate into all subtypes of motoneurons.

Discussion

In this study, we used genetically-defined lineage tracing analysis for Nkx2.2-expressing progenitors using a murine retrovirus [11]. Furthermore, we applied a new electroporation of Cre-loxP mediated lineage tracing strategy and showed the relevance of this strategy. We used Cre at the concentration of end-point dilution, together with floxed lacZ plasmids. Initial LacZ-positive cells that were recombined by Nkx2.2-Cre were always positioned in the Nkx2.2-positive cell population, but not in Olig2+/Nkx2.2-ventricular zone cells (Fig. 3). In addition, LacZ-positive cells were not observed when the Cre-driver plasmid was diluted to 0.1 ng/μl (1/10 dilution of our labeling condition). Finally, the distribution of LacZ-positive cells was similar to that of retroviral labeling. These observations support the relevance of our Cre-loxP lineage strategy with limiting dilutions of the Cre plasmid.

It has been reported that overexpression of Olig2 or HB9 [7], [22], [23] suggests that Nkx2.2 has a negative effect on motoneuron lineage. However, whether there is a direct lineage relationship between Nkx2.2-positive progenitors and motoneurons remains to be resolved due to the lack of lineage tracing analysis from Nkx2.2-positive progenitors. We used the above mentioned methods for analyzing Nkx2.2-lineage cells in the chick spinal cord. Our finding first shows that Nkx2.2-positive progenitor cells in chick spinal cords generate not only V3 interneurons but also Terni column cells (Fig. 4), the avian
visceral preganglionic motoneurons located near the central canal. In the mouse hindbrain, Nkx2.2 has been reported to be expressed in progenitor cells that give rise to both visceral motoneurons and serotonergic neurons [9], which supports our observation that Nkx2.2-positive progenitors contributed to different classes of neurons in the chick spinal cord. Surprisingly, we found that

Figure 3. LacZ-labeled cells by electroporation with pNkx2.2-Cre;cAct-xStopx-nLucZ strongly express Nkx2.2 in the ventricular zone. pNkx2.2-Cre and cAct-xStopx-nLucZ were electroporated at HH 14 in the chick neural tube and an embryo was grown to be HH 21 (E3.5; A–F) or HH 32 (E7; G–O). A, LacZ-staining followed by immunohistochemistry using Nkx2.2 antibody. Sections were arranged in rostral (left) to caudal (right) order. B–F, Triple immunostaining with anti-Nkx2.2, anti-Olig2, and anti-LacZ antibodies. Arrowheads indicate recombined cells. G, LacZ-staining followed by immunohistochemistry using Nkx2.2 antibody. Sections were arranged in rostral (left) to caudal (right) order. Insets show higher magnification pictures of LacZ-positive cells in the Nkx2.2-positive ventricular zone. H–M, Sections at HH 32 were double-immunolabeled using anti-LacZ and anti-Nkx2.2 (H–J), or anti-LacZ and anti-Olig2 (K–M), respectively. Arrowheads indicate recombined cells. N, LacZ-positive cells with Islet1/2 immunoreactivity. O, LacZ-positive cells with HB9 immunoreactivity. Scale bars in A, G, M, O = 100 μm; in F = 50 μm.
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visceral preganglionic motoneurons located near the central canal. In the mouse hindbrain, Nkx2.2 has been reported to be expressed in progenitor cells that give rise to both visceral motoneurons and serotonergic neurons [9], which supports our observation that Nkx2.2-positive progenitors contributed to different classes of neurons in the chick spinal cord. Surprisingly, we found that
Figure 4. Distribution of Nkx2.2 lineage motoneurons in the spinal cord. pNkx2.2-Cre and cAct-xStop-xnLacZ were electroporated at HH 14 in the chick neural tube. A and B. LacZ-positive cells of one chick spinal cord at HH 32 (E7) were superimposed and marked with dots on a schematic diagram. Three independent experiments were performed in the brachial spinal cord (A) or thoracic spinal cord (B). C and D. A LacZ-positive cell in the ventral horn in which most of the motoneurons were retrogradely labeled with fluorogold (FG). E-G. GFP-positive recombined cells with brainbow system in the ventral horn were retrogradely labeled with FG (G; arrowheads). GFP-positive axons were extending toward the ventral root (arrows). H and L. Sections of HH 42 spinal cord were subjected to LacZ-staining followed by immunohistochemistry using ChAT antibody. LacZ-positive cells in the ventral horn (H; somatomotor neuron) and close to the central canal (L; preganglionic CT neuron) are shown. Arrowheads indicate double positive cells and the asterisk shows the location of the central canal. I-K and M-O. Expression of ChAT in LacZ-positive cells was also analyzed by double fluorescent labeling (I-K, M-O). P. LacZ/ChAT-positive cells of one chick spinal cord were counted and divided by the total number of LacZ-positive cells and were shown as percentages (P). Five chick embryos were examined in this study. Scale bars in A and L = 200 μm; D, F and O = 50 μm.

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Nkx2.2-positive progenitors also gave rise to somatic motoneurons. It was reported that preganglionic motoneurons have a close lineage relationship with somatic motoneurons as demonstrated by the tritium thymidine labeling method [24]. It is well-known that motoneurons are arranged into columnar structures and their arrangement is dependent on the combinatorial expression of transcription factors [1], [25], thus we analyzed the subtype of Nkx2.2-lineage motoneurons. Since a recent report suggested that the pMN domain can be subdivided into a ventral part and a dorsal part and that the ventral part of the pMN domain contributes to MMC motoneurons [26] and cells derived from Nkx2.2-positive progenitors were present in the most dorsal part of the p3 domain, it is possible that the dorsal part of the p3 domain would contribute to MMC motoneurons. However, we found that Nkx2.2-positive progenitors gave rise to preganglionic neurons as well as somatic motoneurons in LMC and MMC within the ventral horn (see Fig. 4), thus no preferential differentiation into MMC motoneurons was observed, suggesting different regulatory mechanisms of motoneuron development from Nkx2.2-positive progenitors. Although clonal analysis would help us to analyze whether GFP-labeled motoneurons and V3 interneurons are derived from the same progenitor cells, it is difficult to perform clonal analysis using our systems because expression levels of mCAT1 are dependent on the concentration of plasmids or the electroporated area in the spinal cord as previously described [11].

The molecular mechanisms which regulate motoneuron development from the p3 domain are unknown whereas Nkx2.2 is reported to have inhibitory functions in motoneuron development [8]. It is known that Olig2 is essential for motoneuron development as revealed by a gain-of-function study using chick embryos [7] as well as loss-of-function studies using olig2-deficient mice [2–4]. We observed that LacZ-positive cells were located at the pMN/p3 domain boundary with strong Nkx2.2 expression. Although initially mCAT1 or LacZ-positive cells in the ventricular zone were restricted to the p3 (Nkx2.2-positive) domain, a small population of labeled cells was present at the Olig2/Nkx2.2 boundary. Our data showed that 13.3% of the labeled cells were present at the domain boundary at HH21 (E3.5) and no labeled cell was located at Olig2-positive area at HH32 (E7). The percentage of mature ChAT-positive neurons to ChAT-negative...
neurons (31.3%) at more later stage (HH 42, E16) suggest that early Nkx2.2 progenitors in the domain boundary might express Olig2 transiently in a short time window and differentiate into motoneurons (Fig. 5D). Using labeling techniques including retroviral clonal analysis, it was reported that chick neural progenitor cells show extensive migration along the dorso-ventral axis inside the ventricular zone [27–29]. These reports raised the possibility that p3 progenitor cells acquire Olig2 expression during the dorso-ventral migration of progenitor cells, and then differentiate into motoneurons. It was suggested that motoneurons have a lineage relationship with oligodendrocytes with respect to the requirement of Olig2 [16]. Mouse oligodendrocytes are generated from Olig2-positive cells during glial cell development, whereas oligodendrocytes are developed from Nkx2.2-positive p3 domain cells in the chick spinal cord [11], [30], [31]. Therefore, there may be subtle species differences in the developmental origin of oligodendrocytes [32] as well as motoneurons in the chick spinal cord, as suggested by the present study. It is interesting to examine whether or not Nkx2.2-lineage cells differentiate into motoneurons in the mouse spinal cord.

In conclusion, we found that p3 domain progenitor cells contributed to all kinds of motoneuron subtypes in the chick spinal cord, suggesting that diverse embryonic origins contribute to diverse mature motoneurons.

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Author Contributions
Conceived and designed the experiments: HG KO KI. Performed the experiments: HG. Analyzed the data: HG KO KI. Contributed reagents/materials/analysis tools: HH HN. Wrote the paper: HG KO TN HT KI.

References
1. Dalla Torre di Sanguinetto SA, Dasen JS, Arber S (2008) Transcriptional control mechanisms controlling motor neuron diversity and connectivity. Curr Opin Neurobiol 18: 36–43.
2. Lu QR, Sun T, Zhu Z, Ma N, Garcia M, et al. (2002) Common developmental requirement for Olig function indicates a motor neuron/oligodendrocyte subtype specification. Cell 109: 61–73.
3. Takebayashi H, Nabeshima Y, Yoshida S, Chisaka O, Bernaka K, et al. (2002) The basic helix-loop-helix factor olig2 is essential for the development of motoneuron and oligodendrocyte lineages. Curr Biol 12: 1157–1163.
4. Zhou Q, Anderson DJ (2002) The bHLH transcription factors Olig2 and Olig1 couple neuronal and glial subtype specification. Cell 109: 61–73.
5. Briscoe J, Susel I, Serup P, Hartigan-O'Connor D, Jessell TM, et al. (1999) Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. Nature 398: 622–627.
6. Sun T, Dong H, Wu L, Kane M, Rowitch DH, et al. (2003) Cross-repressive interaction of the Olig2 and Nkx2.2 transcription factors in developing neural tube associated with formation of a specific physical complex. J Neurosci 23: 9547–9556.
7. Novitch BG, Chen AI, Jessell TM (2001) Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH effector Olig2. Neuron 31: 773–789.
8. Briscoe J, Irie I, Jessell TM, Ericson J (2000) A homeodomain protein code specifies progenitor cell identity and neuronal fate in the ventral neural tube. Cell 101: 433–445.
9. Pattyn A, Vollbrecht A, Dias JM, Samad OA, Krumlauf R, et al. (2003) Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. Genes Dev 17: 729–737.
10. Richardson WD, Smith HK, Sun T, Pringle NP, Hall A, et al. (2000) Oligodendrocyte lineage and the motor neuron connection. Glia 29: 136–142.
11. Gotro H, Ono K, Takebayashi H, Harada H, Nakamura H, et al. (2011) Genetically-defined lineage tracing of Nkx2.2-expressing cells in chick spinal cord. Dev Biol 349: 504–511.
12. Hamburger V, Hamilton HL, (1951) A series of normal stages in the development of the chick embryo. Journal of Morphology 88: 49–92.
13. Namboku K, Kawano M, Iwasaki Y, Bernaka K (2003) Highly efficient gene transduction into the brain using high-titer retroviral vectors. Dev Neurosci 25: 152–161.
14. Zinyk DL, Mercer EH, Harris E, Anderson DJ, Joyner AL (1998) Fate mapping of the mouse midbrain-hindbrain constriction using a site-specific recombination system. Curr Biol 8: 665–668.
15. Litov J, Weissman TA, Kang H, Draft RW, Lu J, et al. (2007) Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature 450: 56–62.
16. Masahira N, Takebayashi H, Ono K, Watanabe K, Ding L, et al. (2006) Olig2-positive progenitors in the embryonic spinal cord give rise not only to motoneurons and oligodendrocytes, but also to a subset of astrocytes and ependymal cells. Dev Biol 295: 358–369.
17. Lei Q, Jeong Y, Misra K, Li S, Zelman AK, et al. (2006) Wnt signaling inhibitors regulate the transcriptional response to morphogenetic Shh-Gli signaling in the neural tube. Dev Cell 11: 325–337.
18. Cajal Ramon y S (1911) Histology of the nervous system. Vol. I Oxford Univ Press, 1995 translation, Oxford.
19. Haldyddy M, Hamburger V (1977) An autoradiographic study of the formation of the lateral motor column in the chick embryo. Brain Res 132: 197–206.
20. Dasen JS, De Camilli A, Wang R, Tucker PW, Jessell TM (2008) Hox repertoire for motor neuron diversity and connectivity gated by a single accessory factor, FoxP1. Cell 134: 304–316.
21. Kousco DL, Gaber ZB, Wellik D, Morrisset EE, Novitch BG (2008) Coordinated actions of the forkhead protein FoxP1 and Hox proteins in the columnar organization of spinal motor neurons. Neuron 59: 226–240.
22. Mizuguchi R, Sagimoto M, Takebayashi H, Kosako H, Nagao M, et al. (2001) Combinatorial roles of ollig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. Neuron 15: 757–771.
23. Lee SK, Jurata LW, Funahashi J, Ruiz EC, Pfaff SL (2004) Analysis of embryonic motoneuron gene regulation: derepression of general activators function in concert with enhancer factors. Development 131: 3295–3306.
24. Prasad A, Haldyddy M (1991) Development and migration of avian sympathetic preganglionic neurons. J Comp Neurol 307: 237–250.
25. Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nature Reviews Genetics 1: 29–39.
26. Agulliu D, Takada S, Agulliu I, McMahon AP, Jessell TM (2009) Motor neurons with axial muscle projection trajectories specified by Wnt4/5 signaling. Neuron 61: 708–720.
27. Clarke JD, Erskine L, Lumsden A (1998) Differential progenitor dispersal and the spatial origin of early neurons can explain the predominance of single-phenotype clones in the chick hindbrain. Dev Dyn 212: 14–26.
28. Erickse I, Patel K, Clarke JD (1998) Progenitor dispersal and the origin of early neuronal phenotypes in the chick embryo spinal cord. Dev Biol 199: 26–41.
29. Leber SM, Sanes JR (1995) Migratory paths of neurons and glia in the embryonic chick spinal cord. J Neurosci 15: 1236–1248.
30. Fu H, Qi Y, Tan M, Cai J, Takebayashi H, et al. (2002) Dual origin of spinal oligodendrocyte progenitors and evidence for the cooperative role of Olig2 and Nkx2.2 in the control of oligodendrocyte differentiation. Development 129: 691–693.