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Specification of motoneurons from human embryonic stem cells

Xue-Jun Li[1,2,4], Zhong-Wei Du[1,2,4], Ewa D Zarnowska[3], Matthew Pankratz[4], Lauren O Hansen[4], Robert A Pearce[3] & Su-Chun Zhang[1,2,4]

An understanding of how mammalian stem cells produce specific neuronal subtypes remains elusive. Here we show that human embryonic stem cells generated early neuroectodermal cells, which organized into rosettes and expressed Pax6 but not Sox1, and then late neuroectodermal cells, which formed neural tube–like structures and expressed both Pax6 and Sox1. Only the early, but not the late, neuroectodermal cells were efficiently posteriorized by retinoic acid and, in the presence of sonic hedgehog, differentiated into spinal motoneurons. The *in vitro*–generated motoneurons expressed HB9, HoxC8, choline acetyltransferase and vesicular acetylcholine transporter, induced clustering of acetylcholine receptors in myotubes, and were electrophysiologically active. These findings indicate that retinoic acid action is required during neuroectoderm induction for motoneuron specification and suggest that stem cells have restricted capacity to generate region-specific projection neurons even at an early developmental stage.

The generation of functional neuronal subtypes in the vertebrate central nervous system involves several steps, including the induction of the neuroectoderm from the embryonic ectoderm, patterning of the neural plate into complex regional compartments along rostrocaudal and dorsoventral axes, and differentiation of the regionalized progenitors into postmitotic neurons[1,2]. These developmental processes are orchestrated by interactions among prospective precursors and morphogens, such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), retinoic acid, sonic hedgehog (SHH) and Wnts, secreted from surrounding tissues in unique spatial and temporal orders[1–4]. On the basis of these developmental principles, mouse embryonic stem (ES) cells, isolated from the inner cell mass of a blastocyst embryo[5,6], have been induced *in vitro* to adopt a neuroectodermal fate[7–9], with subsequent differentiation into large projection neurons, such as midbrain dopaminergic neurons and spinal motoneurons, in response to specific sets of morphogens[10–12].

Human (h)ES cells can be maintained *in vitro* for a prolonged period with a stable genetic background and thus may provide a source of specialized human cells for biotechnological and clinical applications[13]. We differentiated hES cells (H1 and H9 lines) into neuroepithelial cells using our chemically defined adherent colony culture system[14]. Analyses of morphology and gene expression patterns revealed multiple stages in the differentiation to neuroectoderm. ES cells and those aggregated for 4 d did not express neuroectodermal transcription factors such as Pax6, Sox1 and Sox3. The first sign of neural differentiation was the appearance of columnar cells forming rosettes in the center of colonies 8–10 d after ES cells were removed from feeder cells for differentiation (**Fig. 1a**,**b**). The columnar cells in the rosettes, but not the flat cells in the outgrowth area, expressed Pax6 (**Fig. 1c**) but not Sox1 (**Fig. 1d**), which is the earliest neuroectodermal marker expressed during neural plate and tube formation[15]. With further culturing in the same medium for another 4–5 d, the columnar cells organized into neural tube–like rosettes with lumens (**Fig. 1e**) and expressed both Pax6 and Sox1 (**Fig. 1f–h**). Pax6 and Sox1 mRNA were expressed sequentially, as revealed by RT-PCR (**Supplementary Fig. 1** online). The same sequential expression of Pax6 and Sox1 was observed in the absence of FGF2 (**Supplementary Fig. 1** online). Cells at both stages expressed nestin[14], a common neural progenitor marker. Thus, differentiation of neuroectodermal cells from hES cells involves at least two easily identifiable stages, the $Pax6^+/Sox1^-$ columnar cells in the early rosettes 8–10 d after neural induction, and the $Pax6^+/Sox1^+$ cells forming neural tube–like late rosettes 14 d after induction.

Immunocytochemical analyses revealed that the rosette cells, which expressed Pax6 (**Fig. 1i**), Sox1 and nestin, were positive for Otx2 (**Fig. 1j**,**l**), a homeodomain protein expressed by fore- and midbrain cells, but negative for HoxC8 (**Fig. 1l**), a homeodomain protein produced by cells in the spinal cord. They were also negative for engrailed 1 (En1), which is expressed by midbrain cells (**Fig. 1k**). These results suggest that the neuroectodermal cells differentiated in the presence or absence of FGF2 possess a rostral character, similar to that initially acquired by neuroectodermal cells during early *in vivo* development[16].

Examination of dorsoventral markers indicated that the Pax6-expressing neuroepithelial cells did not express Olig2 and Nkx2.2, homeodomain proteins expressed in ventral neural progenitors[17,18]. Pax7, a dorsally expressed transcription factor, was uniformly expressed by cells surrounding and at the edge of the neural rosettes. At the late rosette stage, Pax7 was also expressed by some cells in the rosettes (**Supplementary Fig. 2** online). This intrinsic or default

[1]Departments of Anatomy, [2]Neurology and [3]Anesthesiology, School of Medicine, and [4]the Stem Cell Research Program, Waisman Center, 1500 Highland Avenue, University of Wisconsin-Madison, Wisconsin 53705, USA. Correspondence should be addressed to S.C.Z. (zhang@waisman.wisc.edu).

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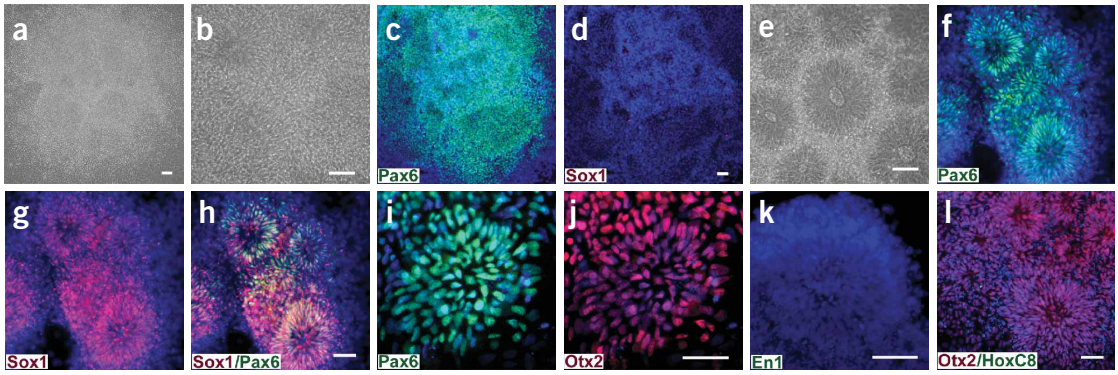

Figure 1 hES cell–derived neuroectodermal cells display rostral phenotypes. (**a**,**b**) ES cells, differentiated in FGF2 for 10 d, displayed small, columnar morphology in the colony center, and organized into rosette formations. (**c**,**d**) The columnar cells in the rosettes, but not the surrounding flat cells were positive for Pax6 (**c**) and negative for Sox1 (**d**). (**e**–**h**) By 14 d, the columnar cells formed neural tube–like rosettes (**e**) and were positive for both Pax6 and Sox1 (**f**–**h**). (**i**–**k**) The Pax6$^+$ cells (**i**) in the rosettes were also Otx2$^+$ (**j**) but were En1$^-$ (**k**). (**l**) Cells in the neural tube–like rosettes were positive for Otx2 and negative for HoxC8. Blue indicates Hoechst-stained nuclei. Bar, 50 μm.

dorsoventral patterning, together with the rostral identity of the neuroectoderm, indicates a need for caudalization and ventralization to generate spinal motoneurons.

We isolated hES cell–generated, Sox1$^+$ neuroectodermal cells in the rosettes through enzymatic treatment[14] and differentiated them on a laminin substrate in the presence of retinoic acid (0.001–1 μM) and SHH (50–500 ng/ml). Cells migrated out from the rosette cluster and extended neurites as early as 24–48 h after plating. By 14 d after plating, a large number of cells in the outgrowth area formed networks through their processes (**Fig. 2a**). Immunostaining analyses indicated that the majority of differentiated cells were positive for βIII-tubulin and MAP2. A large proportion (>50%) of them were also positive for Islet1 (**Fig. 2a**) and Lim3 (data not shown), transcription factors that are associated with motoneuron development[1,19]. However, very few cells in cultures plated from 1–3 weeks expressed HB9, a motoneuron-specific transcription factor[20] (**Fig. 2a**). A similar result was obtained when the neuroectodermal cells generated in the absence of FGF2 were used or when we used the suspension differentiation culture, as described for mouse ES cell differentiation[12].

The above observation suggests that the Sox1$^+$ neuroectodermal cells may be refractory for motoneuron induction. The cells expressing Sox1 may correspond to neuroectodermal cells in the neural plate or neural tube that are regionally specified[21]. This consideration led us to hypothesize that retinoic acid may promote caudalization and/or motoneuron specification before neuroectodermal cells express Sox1. We thus treated the neuroectodermal cells with retinoic acid (0.001–1 μM) at an earlier stage, that is, when columnar cells began to organize into rosettes and expressed Pax6. Cells treated in this way for 6–7 d displayed neural tube–like rosettes and expressed Sox1, indistinguishable from FGF2-treated cultures. We did not observe cells with

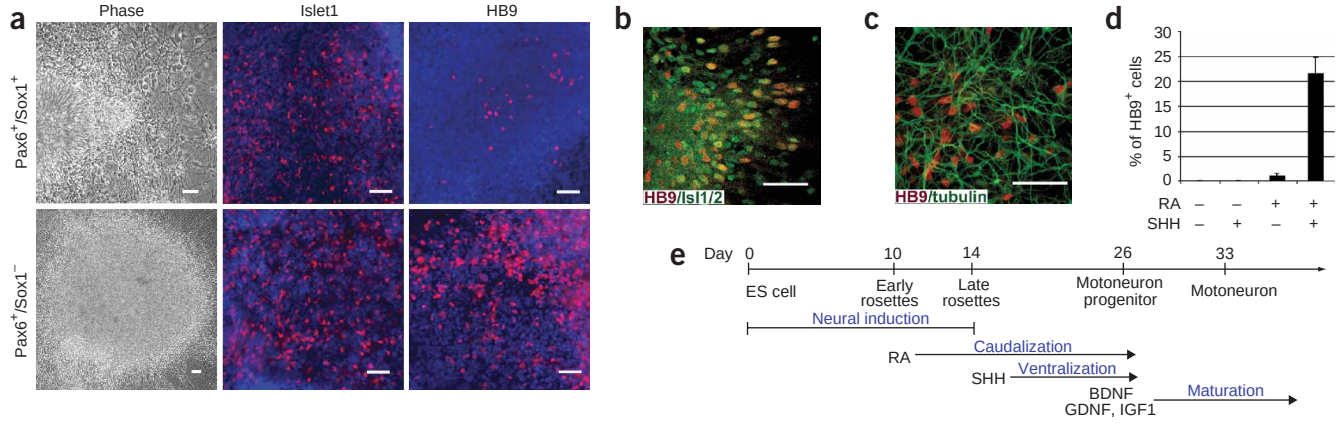

Figure 2 Generation of motoneurons from neuroectodermal cells. (**a**) Differentiation of Sox1$^+$ neuroectodermal cells for 2 weeks (1st row) revealed extensive neuronal generation in the outgrowth area, expression of Islet1, but few HB9$^+$ cells. Treatment of Pax6$^+$/Sox1$^-$ neuroectodermal cells (2nd row) resulted in extensive neurite outgrowth with few migrating cells, expression of Islet1 and a large proportion of HB9$^+$ cells. (**b**) About 50% of Islet1/2$^+$ cells differentiated from early neuroectodermal cells were also HB9$^+$. (**c**) HB9$^+$ cells were also positive for βIII-tubulin. (**d**) About 21% of the cells in the cluster were HB9$^+$ when the cultures were differentiated in the presence of both RA and SHH, whereas few HB9$^+$ cells were observed when cultured in either RA alone, SHH alone or neither. (**e**) Schematic procedures for motoneuron differentiation. hESCs were differentiated to early neuroectodermal cells in the form of early rosettes in 10 d. They were then treated with RA for 1 week and the neural tube–like rosettes were isolated through 3–5 d of differential adhesion and then adhered to the laminin substrate (around day 20) in the presence of RA and SHH for neuronal differentiation. Olig2$^+$ motoneuron progenitors appeared after 3 weeks, and HB9$^+$ motoneurons around 4–5 weeks after differentiation from ES cells. Trophic factors were added in the subsequent maturation process of motoneuron cultures. Blue indicates Hoechst stained nuclei. Bar, 50 μm.

neuronal morphology and expression of neuronal markers such as βIII-tubulin. After the rosette clusters were isolated through 3–5 d of differential adhesion and then adhered to the laminin substrate (2–4 clusters/coverslip), numerous neurites extended from the cluster as early as 24–48 h after plating, with fewer cells migrating out of the rosette cluster. By 14 d after plating, the neurite outgrowth area covered almost the entire (11-mm diameter) coverslip, although there were limited numbers of neuronal cell bodies in the outgrowth area (**Fig. 2a**). Immunocytochemical analyses indicated that the differentiated cells were positive for βIII-tubulin and MAP2. The majority of neurons were positive for Islet1/2 (**Fig. 2a**,**b**). Among the Islet1/2-expressing cells, about 50% were also HB9$^+$ (**Fig. 2b**), suggesting that these double-positive cells are motoneurons. The Islet1/2$^+$ and HB9$^-$ cells were likely interneurons.

HB9-expressing cells were first observed 6 d after the neuroepithelial cells had adhered to the laminin substrate for differentiation or about 4 weeks after hES cells were differentiated (**Fig. 2e**). The highest population of HB9$^+$ cells was observed a week later. The HB9$^+$ cells were largely localized to the cluster area, representing about 21% of the total cells in the cluster (**Fig. 2a**,**d**). A few cells in the outgrowth area, often close to the cluster, expressed HB9. In the absence of retinoic acid, or SHH, or both, there were few HB9$^+$ cells in the cluster (**Fig. 2d**). Almost all the HB9-expressing cells were stained with βIII-tubulin, a neuronal marker (**Fig. 2c**); cells in all the control groups (without retinoic acid and SHH, or with retinoic acid or SHH alone) were also positive for βIII-tubulin. Thus, treatment with retinoic acid at an early stage of neuroectodermal development is required for an efficient induction of spinal motoneurons.

To understand why retinoic acid induces the early but not the late neuroectodermal cells to differentiate into motoneurons, we first examined the effect of retinoic acid on caudalization of neuroectodermal cells. Treatment of early rosette cells (Pax6$^+$/Sox1$^-$) with retinoic acid (0.001–1 μM) or FGF2 (1–100 ng/ml) for 7 d resulted in the maturation of rosettes, that is, formation of multilayered rosette cells and expression of Sox1. RT-PCR analyses indicated that retinoic acid treatment resulted in decreased expression of *OTX2* and increased expression of HOX genes such as *HOXB1*, *HOXB6*, *HOXC5* and *HOXC8* in a dose-dependent manner (**Fig. 3a**). Genes expressed by more caudal cells were induced by higher doses of retinoic acid. FGF2 induced a similar set of caudal genes (**Fig. 3a**) although another caudal gene, *HOXC10*, was also induced by FGF2 but not by retinoic acid. However, treatment with FGF2 did not eliminate OTX2 expression (**Fig. 3a**), suggesting that FGF2 elicits a wide range of rostrocaudal gene expression. Treatment of late rosette cells (Pax6$^+$/Sox1$^+$) with retinoic acid for 1 week did not alter the HOX gene expression pattern induced by FGF2.

Immunostaining revealed that neuroectodermal cells that were treated with FGF2 or retinoic acid for 1 week from the early rosette stage were still Otx2 positive and HoxC8 negative. After treatment with retinoic acid for 1 week from the early rosette stage followed by culture in the neuronal differentiation medium, neuroectodermal cells stopped expressing Otx2 (**Fig. 3c**). They began to express HoxC8 protein after 6 d, with the majority of cells expressing it at 10–12 d after differentiation (**Fig. 3d**). Almost all the HoxC8$^+$ cells were βIII-tubulin$^+$ neurons (**Fig. 3e**). In contrast, late rosette cells treated with retinoic acid for 1 week and then allowed to differentiate for 2 weeks, yielded few HoxC8$^+$ cells, although Otx2-expressing cells were decreased (data not shown). Thus, treatment of early but not late neuroectodermal cells with retinoic acid results in efficient caudalization with expression of HoxC proteins, which are associated with spinal motoneurons[22].

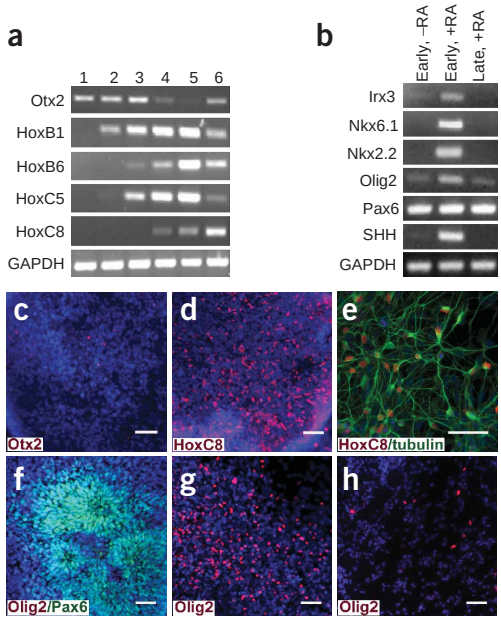

Figure 3 Effect of RA, FGF2 and SHH on neuroectodermal cells. (**a**) RT-PCR analyses indicated changes of rostrocaudal genes from early rosette cells that were cultured with retinoic acid (μM) or 20 ng/ml of FGF2 for 1 week in the neural induction medium. Lanes 1–5, retinoic acid (μM), 0, 0.001, 0.01, 0.1, 1.0, respectively; lane 6, FGF2. (**b**) Comparison of homeobox gene expression in early and late neuroectodermal cells treated with RA 0.1 μM for 1 week. (**c**,**d**) The early neuroectodermal cells, treated with retinoic acid and then allowed to differentiate for 12 d, became mostly negative for Otx2 (**c**) but positive for HoxC8 (**d**). (**e**) All the HoxC8$^+$ cells were βIII-tubulin$^+$. (**f**) The Pax6-expressing neuroectodermal cells were negative for Olig2. (**g**) After treatment with retinoic acid for 1 week and differentiation for 2 weeks in the presence of SHH (100 ng/ml), many cells expressed Olig2. (**h**) Few Olig2$^+$ cells were observed when late neuroectodermal cells were treated with retinoic acid and then allowed to differentiate under the same culture conditions. Blue, Hoechst stained nuclei. Scale bar, 50 μm.

Motoneurons are specified from progenitors in the ventral neural tube, and SHH is a key molecule for inducing ventral neural cell types[1,2]. The hES cell–derived neuroectodermal cells, whether they were Pax6$^+$ or Sox1$^+$, did not express Olig2 (**Fig. 3f**) or Nkx2.2 (data not shown). When the Pax6$^+$/Sox1$^-$ neuroectodermal cells were cultured in the presence of retinoic acid for 1 week, then isolated and allowed to differentiate for another 2 weeks in the absence of SHH, very few cells expressed Olig2. However, in the presence of SHH (50–500 ng/ml), many cells expressed Olig2 in the nuclei (**Fig. 3g**). In contrast, Pax6$^+$/Sox1$^+$ neuroectodermal cells, differentiated for 2 weeks under the same conditions, generated few Olig2$^+$ cells (**Fig. 3h**). Thus, neuroectodermal cells, treated with retinoic acid at an early but not a late stage, can be efficiently induced to a ventral neural progenitor fate in response to SHH.

To further discern why early retinoic acid treatment is required for motoneuron specification (**Fig. 3a**), we examined the expression of class I (Irx3, Pax6) and class II (Olig2, Nkx2.2, Nkx6.1) homeodomain molecules that are important in refining progenitor domains in the ventral neural tube[1,2]. Retinoic acid induced a much more robust expression of SHH and class II molecules, particularly Olig2 and Nkx6.1, in the early than in the late neuroectodermal cells (**Fig. 3b**). This suggests that early neuroectodermal cells are more responsive to retinoic acid in upregulating the expression of SHH and class II factors, which are essential for motoneuron specification.

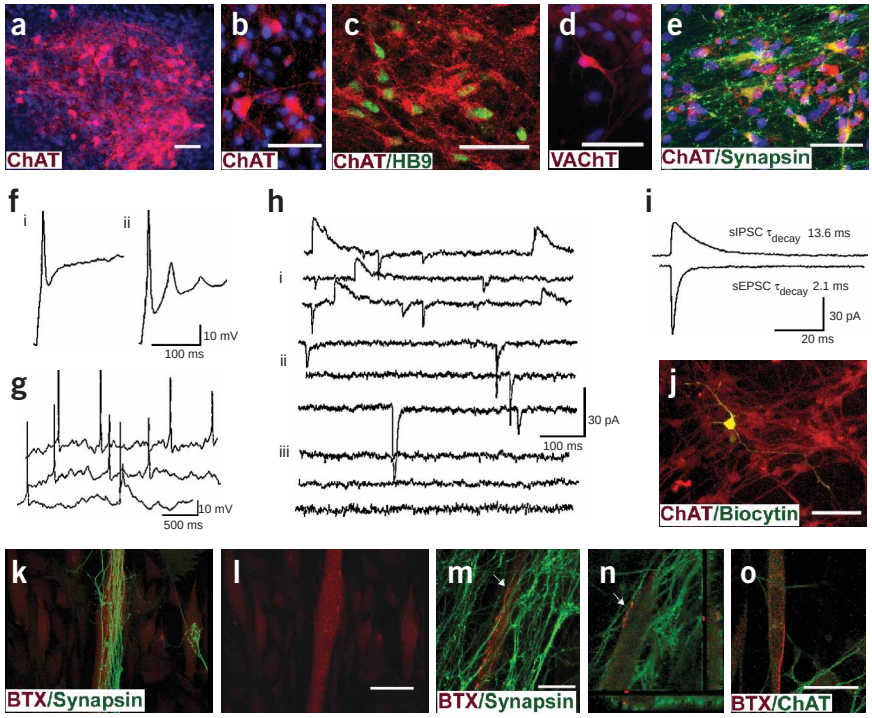

Figure 4 Maturation and functional properties of *in vitro*–generated motoneurons. (**a**,**b**) ChAT-expressing cells were localized mainly in the cluster (**a**), and were large multipolar cells (**b**). (**c**) Confocal analyses showed colocalization of ChAT in the soma and processes and HB9 in the nuclei in a 3-week culture. (**d**) Most cells in the cluster expressed VAChT. (**e**) Many ChAT$^+$ cells were also positive for synapsin in somas and processes after 5 weeks in culture. (**f**–**j**) Electrophysiological characterization of *in vitro*–generated motoneurons was evaluated by whole-cell, patch-clamp recording. Action potentials evoked by depolarizing current steps (0.15 nA) in neurons maintained for 42 DIV. Resting membrane potential (Vm) –59 mV (**fi**) and –70 mV (**fii**). (**g**) Spontaneous action potentials in a neuron maintained for 42 DIV, Vm –50 mV. (**h**) Spontaneous inward and outward synaptic currents at –40 mV using K-gluconate–based pipette solution under control conditions (**hi**). Bath application of bicuculline (20 μM) and strychnine (5 μM) blocked outward currents (inhibitory postsynaptic currents (IPSCs) **hii**). Subsequent application of AP-5 (40 μM) and CNQX (20 μM) blocked the remaining inward currents (excitatory postsynaptic currents (EPSCs) **hiii**). (**i**) Averaged sIPSCs and sEPSCs from the cell illustrated in panel **h**. (**j**) Double immunostaining for biocytin (from the recording electrode) and ChAT. (**k**–**o**) Further functional analysis was assessed using motoneuron-C2C12 myoblast cocultures. After 4 d of coculture, synapsin-expressing neurites made contact with myoblasts and myotubes, and patched α-BTX staining was observed on the myotubes (**k**,**l**). (**m**) After 2 weeks of coculture, synapsin positive neurites were very extensive and the α-BTX clusters were more obvious. A 0.3-μm confocal section derived from a Z-stack (arrows in **m** and **n**) showed colocalization of synapsin$^+$ axons and α-BTX stained acetylcholine receptors (**n**). (**o**) The myotubes with patched α-BTX staining were innervated by ChAT$^+$ fibers. Blue indicates Hoechst stained nuclei. Bar, 50 (**a**–**e**, and **j**) and 30 μm (**k**–**o**).

After treatment with retinoic acid at the early stage, neuroectodermal cells were enriched[14] and then allowed to differentiate into motoneurons by culture in the presence of SHH (100 ng/ml) for 6 d and then in neuronal differentiation medium with the addition of brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), insulin-like growth factor-1 (IGF1) and a low concentration of SHH (50 ng/ml) (**Fig. 2e**). Under such culture conditions, HB9$^+$ cells were present for at least 5 weeks, although the number began to decrease after 3 weeks. Cells that expressed choline acetyltransferase (ChAT) appeared 3 weeks after the neuroectodermal cells were plated for motoneuron differentiation, and the number of ChAT$^+$ cells increased steadily for up to 7 weeks, the longest culture period analyzed in this study (**Fig. 4a**,**b**). All ChAT$^+$ cells were βIII-tubulin$^+$ neurons (**Supplementary Fig. 3** online). The ChAT$^+$ cells were largely localized to the cluster (**Fig. 4a**), corresponding to the localization of the HB9$^+$ cells. These cells were mainly multipolar cells with large somas. Most of the ChAT$^+$ cells were 15–20 μm in diameter, with some as large as 30 μm (**Fig. 4a**–**c**). We observed coexpression of HB9 in the nuclei and ChAT in the soma and processes after 3 weeks of culture (**Fig. 4c**). By 7 weeks in culture, most of the ChAT$^+$ cells became negative for HB9 as assessed by immunohistochemistry. Most of the neurons were also positively stained for vesicular acetylcholine transporter (VAChT) (**Fig. 4d**), a functional molecule for packaging acetylcholine in the presynaptic vesicles. Many ChAT$^+$ cells, especially after 5 weeks in culture, were positively labeled for synapsin on cell bodies and processes (**Fig. 4e**). GFAP$^+$ glia were rare within 3 weeks after the neural progenitors were differentiated. They gradually increased in number and the glial fibers lined up with neurites over time, as shown previously[14].

We assessed functional maturation using electrophysiological techniques ($n = 28$ cells). The mean resting potential was -36.9 ± 2.6 mV, and input resistance was 920 ± 57 MΩ. Single action potentials (**Fig. 4f**,**i**) or decrementing trains (**Fig. 4fii**) were elicited by depolarizing current steps (0.15–0.2 nA × 1 s) in 11 of 13 neurons tested. Spontaneous action potentials triggered by spontaneous depolarizing synaptic inputs were also observed (**Fig. 4g**). Although not all cells survived recording and subsequent immunohistochemical analysis, double immunostaining with biocytin and ChAT demonstrated that many of the cells from which we recorded were motoneurons (**Fig. 4j**).

Voltage clamp analysis revealed time- and voltage-dependent inward and outward currents consistent with sodium and delayed rectifier potassium currents. Inward currents and action potentials were blocked by 1 μM tetrodotoxin (TTX, $n = 3$), confirming the presence of voltage-activated sodium channels. Outward currents were not further characterized. We also observed spontaneous synaptic currents (**Fig. 4h**, $n = 21$ of 23 cells tested). These were reduced in frequency but not eliminated by 1 μM TTX, demonstrating the existence of functionally intact synaptic neurotransmission. With a cesium gluconate–based pipette solution, outward (inhibitory) currents decayed slowly (13.6 ms, $n = 10$ events) and were blocked by a combination of strychnine and bicuculline, whereas the remaining inward (excitatory) currents decayed rapidly (2.1 ms, $n = 17$ events) and were blocked by a combination of D-AP5 and CNQX (**Fig. 4h**,**i**), demonstrating that inhibitory (GABA/glycine) and excitatory (glutamate) neurotransmission was occurring, as in the intact spinal cord[23]. Thus, the *in vitro*–generated motoneurons were electrophysiologically active and formed functional synapses with neighboring neurons.

We performed further functional analysis using motoneuron-myotube cocultures. Four days after the ventralized neural progenitors were plated onto C2C12 myocytes, synapsin-expressing neurites made contact with forming myotubes. We observed acetylcholine receptor clustering[24], indicated by patched α-bungarotoxin (BTX) staining, on the myotubes immediately adjacent to the axons (**Fig. 4k,l**). By 2 weeks of coculture, neurites were very extensive and the receptor clustering was more obvious (**Fig. 4m,n**). Confocal analysis confirmed the apposition of synapsin$^+$ axons and the α-BTX–labeled acetylcholine receptors (**Fig. 4n**). Dual staining indicated that the myotubes with clustered receptors were innervated by ChAT$^+$ fibers (**Fig. 4o**). Myocytes or myotubes that did not contact or come close to axons in cocultures (**Fig. 4k**) or in myocyte culture alone (data not shown) did not show obvious receptor clustering. After 3 weeks of coculture, contraction of the myotubes was observed in the coculture group but not in the cultures of C2C12 myocytes alone. These results indicate that the hES cell–generated motoneurons induce acetylcholine receptor clustering and establish functional neuromuscular transmission.

The present study demonstrates that functional motoneurons can be efficiently generated from hES cells through induction of neuroectoderm, specification and/or caudalization by retinoic acid during the late phase of neuralization, and subsequent differentiation to postmitotic motoneurons in the presence of the ventralizing morphogen SHH. By dissecting the process of neuroectodermal differentiation, we have discovered that specification of early-born projection neurons such as spinal motoneurons requires treatment with morphogens like retinoic acid before precursors become Sox1-expressing neuroectodermal cells.

Although the roles of Pax6 and Sox1 in human neuroectodermal specification are still not completely understood, the functional significance of the two stages of neuralization is becoming clearer. Only the Pax6$^+$ early neuroectodermal cells can be efficiently respecified to a more caudal fate in response to retinoic acid and subsequently differentiated to motoneurons. Considering that Sox1 is expressed by neural plate epithelia during neural tube closure[15], the fact that the forebrain Sox1$^+$ neuroepithelial cells cannot be respecified agrees with the process of *in vivo* neuronal differentiation from progenitors in the neural tube, which are regionally specified[21].

From the standpoint of stem cell biology, ES cells need to be neuralized and then regionalized to progenitors before differentiation into specialized neurons, as demonstrated by the stepwise differentiation of midbrain dopaminergic neurons from mouse ES cells[10,11]. However, these processes are not simply linear but perhaps overlapping. Both the Pax6- and Sox1-expressing neuroectodermal cells can differentiate into large neurons and express transcription factors, such as Islet1 and Lim3, that are associated with motoneuron development[1,19]. But only neuroectodermal cells treated with retinoic acid at an early not a late stage express a balanced level of class I and class II homeodomain genes, leading to the specification of spinal motoneurons. Consequently, the *in vitro*–produced motoneurons possess the key motoneuron transcription factor HB9 and the correct neurotransmitter, transmitter transporter, electrophysiological properties and neuromuscular transmission. Thus, it is necessary to couple the intrinsic program of precursor cells with appropriate morphogens to generate neurons with correct positional identity, and other phenotypes. From the standpoint of both stem cell biology and developmental biology, it is not difficult to understand why brain-derived neuroepithelial cells do not normally generate projection neurons of a different regional identity, particularly after expansion[25].

FGF2 is the most commonly used mitogen for expanding neural precursors. We exposed the hES cell–derived precursors to FGF2 for biotechnological purposes, to increase the population of neuroepithelia, even though exogenous FGF2 is not necessary for neuroepithelial induction. However, continued exposure to FGF2 suppressed the expression of SHH and skewed the expression of class I and class II genes, and subsequently inhibited motoneuron differentiation. Similarly, prolonged exposure of mouse neural progenitor cells to FGF2 disturbed regulation of normal dorsoventral identity[26]. This may explain why FGF2-expanded brain stem cells do not generate large projection neurons, apart from changes in their intrinsic program.

The present study, together with a previous study on motoneuron differentiation from mouse ES cells[12], suggest that fundamental biological principles learned from intact animal studies may be recapitulated *in vitro*, reinforcing the notion that hES cells offer a way to dissect mechanisms of early development in humans, an otherwise inaccessible system[27]. Our study, however, also reveals some unique aspects of neural differentiation from hES cells. The uniform expression of Pax6 in human neuroepithelial cells before Sox1 contrasts with the patterned expression of Pax6 after Sox1 expression in other animals, including mouse. Species differences have been reported in the original derivation of hES cells in terms of growth requirements and expression of cell surface markers[13] and are corroborated by a recent comparison of gene expression profiles in mouse and human ES cells[28]. Hence, one should not assume that information gained from animal studies and protocols established for murine ES cell differentiation can be directly translated to hES cells. hES cells also appear to be more sensitive to morphogens in motoneuron differentiation than are mouse ES cells, as much lower concentrations of morphogens are required for hES cells (0.001–0.1 μM retinoic acid and 50–500 ng/ml SHH) than for mouse ES cells (0.1–2 μM retinoic acid and 6,000 ng/ml SHH)[12]. This, however, could be due to differences in the culture conditions[29]. Technical differences may also account for other differences observed in motoneuron phenotypes. Motoneurons generated in this study express homeodomain protein HoxC8, which is expressed by cells in thoracic regions, whereas motoneurons generated from mouse ES cells[12] have a relatively restricted cervical phenotype. One possible explanation is that hES cells are neuralized by treatment with FGF2, a cytokine that itself possesses caudalizing activity[30], and then treated with retinoic acid. In the earlier study, mouse ES cells were neuralized and caudalized by retinoic acid alone[12]. It is possible that FGF and retinoic acid collaborate in promoting posteriorization.

Functional motoneurons generated from a renewable source of hES cells may be useful for screening pharmaceuticals targeting motoneuron-related disorders, such as amyotrophic lateral sclerosis. These cells may also one day provide replacement motoneurons for applications in patients with motoneuron diseases or spinal cord injury.

METHODS

Culture of hES cells. hES cells (lines H1 and H9, passages 19 to 42) were cultured and passaged weekly on a feeder layer of irradiated embryonic mouse fibroblasts as described[13]. Differentiated colonies were physically removed before passaging and the undifferentiated state of ES cells was confirmed by typical morphology and expression of Oct4 and SSEA4.

Culture of neuroectodermal cells. The procedure for generating neuroectodermal cells from hES cells was described previously[14]. Briefly, ES cell colonies were detached from the feeder layer and grown in the ES cell growth medium in suspension as cell aggregates for 4–6 d. The ES cell aggregates were then adhered to a substrate in a neural induction medium consisting of F12/DMEM, N2 supplement and heparin (2 μg/ml) with or without FGF2 (20 ng/ml). By about 10 d after ES cell differentiation, cells in the center of each colony differentiated into neuroectodermal cells, displaying small columnar

morphology followed by organization of the columnar cells into neural tube–like rosettes after an additional 4–5 d. These neuroectodermal cells were isolated from surrounding nonneural cells through differential response to dispase treatment and a 3–5 d differential adhesion/expansion in the same neural induction medium[14].

Culture of motoneurons. For motoneuron induction, hES cell–derived neuroectodermal cells were first treated with retinoic acid (0.001–1 μM) and/or FGF2 (1–100 ng/ml) at different developmental stages for caudalization (see results). The posteriorized neuroectodermal cells were then differentiated into motoneurons on ornithine/laminin-coated coverslips in a neuronal differentiation medium, which consisted of neurobasal medium (Gibco), N2 supplement, and cAMP (Sigma, 1 μM) in the presence of retinoic acid (0.1 μM) and SHH (50–500 ng/ml, R&D) for one week. After that, BDNF, GDNF and IGF1 (10 ng/ml, PeproTech Inc.) were added to the medium and the concentration of SHH was reduced to 50 ng/ml.

Coculture of motoneurons and myocytes. C2C12 myoblasts were purchased from American Type Culture Collection (ATCC), cultured with DMEM (ATCC) containing 4.5 g/l glucose, 4 mM L-glutamine, 1.0 mM sodium pyruvate and supplemented with 10% fetal bovine serum. After 3–5 d differentiation, multinucleated myotubes began to form.

For coculture of motoneurons and myocytes, the posteriorized neuroectodermal cells were differentiated for 1–2 weeks in the presence of retinoic acid and SHH. Either these ventral progenitor cells were plated on top of myotubes or the dissociated C2C12 myoblasts were plated onto the neuronal progenitor cells in the neural differentiation medium consisting of neurobasal medium, N2 supplement, cAMP, BDNF, GDNF and IGF1.

Immunocytochemistry and microscopy. Immunohistochemical staining was performed as previously described[14]. Primary antibodies used in this study included polyclonal antibodies against βIII-tubulin (Covance Research Products, 1:2000), nestin (Chemicon, 1:750), Sox1 (Chemicon, 1:1000), synapsin I (Calbiochem, 1:500), ChAT (Chemicon, 1:100), VAChT (Chemicon, 1:1000), Islet1/2 (S. Pfaff), Otx2 (F. Vaccarino) and Olig2 (M. Nakafuku). Antibodies against MNR2 or HB9 (81.5C10), Islet1 (40.2D6), Lim3 (67.4E12), Pax6, Pax7 and Nkx2.2, were purchased from Developmental Studies Hybridoma Bank (DSHB), and anti-HoxC8 from Covance Research Products (1:200). For identification of electrophysiologically recorded cells, cells filled with biocytin (Molecular Probes) were labeled with streptavidin-FITC (Sigma, 1:200) and stained for ChAT. For labeling acetylcholine receptors, coverslip cultures were incubated with Alexa Fluor 594 conjugated α-BTX (Molecular Probes, 1:500) at 20 °C for 30 min. Images were collected using a Spot digital camera mounted onto a Nikon fluorescent microscope 600 (Fryer Inc.) or a confocal microscope (Nikon). Orthogonal confocal images were rendered via Nikon-C1 image software. The specificity of antibodies against motoneuron transcription factors and homeodomain proteins, which were originally developed against non-primate tissues, were verified in embryonic (E34 or E36) rhesus monkey spinal cord and brain tissues (provided by the Wisconsin Primate Research Center). In each set of experiments, positive controls were set up using embryonic mouse (E13) and monkey (E34 or E36) spinal cord and brain tissues and negative controls lacking primary or secondary antibodies were included.

Quantification. The population of HB9-expressing cells among total differentiated cells (Hoechst labeled) were counted in two ways. One was to capture images using a digital camera and individual, positively stained cell nuclei were counted by a person who was blind to experimental groups using the Metamorph software (Universal Imaging Corporation). The second method was stereological measurement using an automated stage movement operated by Stereo Investigator software (MicroBrightField Inc.). In this method, an area to be measured was outlined by a tracer, with the number of counting frames preset so that the scope sampled the measuring sites randomly. For counting areas with overlapping cells, the microscope was preset to move up and down to focus on the positive cells in different layers and the total cell number in the cluster was estimated by the software. We quantified the HB9 positive cells in the cluster and the outgrowth area separately. Both methods produced very similar results. Three to four coverslips in each group were counted and data were expressed as mean ± s.d.

RT-PCR assays. Total RNA was extracted from hES cell–derived neuroectodermal cells at different stages and motoneuron differentiation cultures using RNA STAT-60 (TEL-TEST, INC.). cDNA was synthesized using SuperScript III first-strand synthesis system (Invitrogen) according to the supplier's protocol and was used as templates for the PCR reaction. PCR reactions were performed in 15-μl mixture containing cDNA, primers and 1× PCR Master mix (Promega). The following primers were used: HoxC8, 5′-TTTATGGGGCTCAG CAAGAGG-3′, 5′-TCCACTTCATCCTTCGGTTCTG-3′, 318 bp; HoxC5, 5′-TC GGGGTGCTTCCTTGTAGC-3′, 5′-TTCGTGGCAGGGACTATGGG-3′, 290 bp; HoxB6, 5′-AACTCCACCTTCCCCGTCAC-3′, 5′-CTTCTGTCTCGCCG AACACG-3′, 340 bp; Otx2, 5′-CAACAGCAGAATGGAGGTCA-3′, 5′-CTGGGT GGAAAGAGAAGCTG-3′, 429 bp; HoxB1, 5′-TCAGAAGGAGACGGAGGC TA-3′, 5′-GTGGGGGTGTTAGGTTCTGA-3′, 218 bp; GAPDH, 5′-ACCACAGT CCATGCCATCAC-3′, 5′-TCCACCACCCTGTTGCTGTA-3′, 450 bp; Olig2, 5′-AAGGAGGCAGTGGCTTCAAGTC-3′, 5′-CGCTCACCAGTCGCTTCATC-3′, 315 bp; Nkx2.2, 5′-TGCCTCTCCTTCTGAACCTTGG-3′, 5′-GCGAAATCTGC CACCAGTTG-3′, 337 bp. Irx3, 5′-AAGAACGCCACCAGGGAGAG-3′, 5′-TTG GAGTCCGAAATGGGTCC-3′, 473 bp; Pax6, 5′-GGCAACCTACGCAAGATG GC-3′, 5′-TGAGGGCTGTGTCTGTTCGG-3′, 459 bp; SHH, 5′-CCAATTACAA CCCCGACATC-3′, 5′-CCGAGTTCTCTGCTTTCACC-3′, 339 bp; Nkx6.1, 5′-ACACGAGACCCACTTTTTCCG-3′, 5′-TGCTGGACTTGTGCTTCTTCAA C-3′, 335 bp; Sox1, 5′-CAATGCGGGGAGGAGAAGTC-3′, 5′-CTCTGGACCA AACTGTGGCG-3′, 464 bp.

Electrophysiology recording. Electrophysiological properties of hES cell–derived motoneurons were investigated in cultures differentiated for 5–6 weeks using whole-cell, patch-clamp recording techniques[23]. Tetrodotoxin (TTX, 1 μM; Sigma), bicuculline (20 μM; Sigma), strychnine (5 μM; Sigma), D-2-amino-5-phosphonovaleric acid (AP-5, 40 μM; Sigma) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM; RBI) were applied in the bath solution to confirm the identity of voltage-activated or synaptic currents. For some experiments, 1% biocytin was added to the recording solution to fill the cells after recording. Current-clamp and voltage-clamp recordings were made using a MultiClamp 700A amplifier (Axon Instruments). Signals were filtered at 4 kHz, sampled at 10 kHz using a Digidata 1322A analog-digital converter (Axon Instruments), and acquired and stored on a computer hard disk using commercially available software (pClamp9, Axon Instruments). Access resistance was typically 8–15 MΩ and was compensated by 50–80% using amplifier circuitry. The resting membrane potential and spontaneous and depolarization-evoked action potential properties were examined in current-clamp mode. Spontaneous excitatory (inward) and inhibitory (outward) synaptic currents were characterized in voltage-clamp mode. For analysis, events were detected using a template detection algorithm (Mini Analysis Program 5.6.28; Synaptosoft). Synaptic current decays were fitted to a monoexponential function using the Levenberg-Marquardt algorithm. Data are presented as mean ± s.e.m.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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