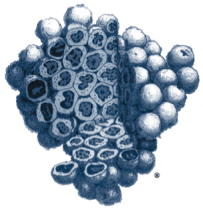

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Easy and Rapid Differentiation of Embryonic Stem Cells into Functional Motoneurons Using Sonic Hedgehog-Producing Cells

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TECHNOLOGY DEVELOPMENT

Easy and Rapid Differentiation of Embryonic Stem Cells into Functional Motoneurons Using Sonic Hedgehog-Producing Cells

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Key Words. Embryonic stem cells • Sonic hedgehog • Retinoic acid • Motoneurons • Lhx3

ABSTRACT

Directing embryonic stem (ES) cells to differentiate into functional motoneurons has proven to be a strong technique for studying neuronal development as well as being a potential source of tissue for cell replacement therapies involving spinal cord disorders. Unfortunately, one of the mitogenic factors (i.e., sonic hedgehog agonist) used for directed differentiation is not readily available, and thus this technique has not been widely accessible. Here, we present a novel and simple method to derive motoneurons from ES cells using readily attainable reagents. ES cells were derived from a mouse in which enhanced green fluorescent protein (eGFP) was linked to a motoneuron specific promoter. The cells were plated onto a monolayer of 293 EcR-Shh cells that carry an integrated construct for the expression of sonic hedgehog (Shh) under ecdysone-inducible control. To initiate motoneuron differentiation, 293 EcR-Shh:ES cell cocultures were treated with ponasterone A (PA) and retinoic acid for 5 days. PA induces ecdysone, and thus drives Shh expression. To assess differentiation, putative ES cell-derived motoneurons were studied immunocytochemically and cultured on chick myotubes for functional analysis. We found that ES cells differentiated into eGFP$^+$ cells that expressed transcription factors typical of motoneurons. Furthermore, ES cell-derived motoneurons were capable of forming functional connections with muscle fibers in vitro. Finally, when transplanted into the developing chick spinal cord, ES cell-derived motoneurons migrated to the ventral horn and projected axons to appropriate muscle targets. In summary, this simple treatment paradigm produces functional motoneurons that can be used for both developmental and preclinical studies. STEM CELLS *2007;25:1697–1706*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Spinal cord disorders such as amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy result in the dysfunction and eventual death of spinal motoneurons. Both neurodegenerative disorders have been targeted for cell replacement therapy using stem cells because they involve individual classes of neurons that are located within defined regions of the central nervous system [1–3]. To this end, several investigators have successfully directed embryonic stem (ES) cells to differentiate into motoneurons in vitro [4–6]. Furthermore, recent studies have shown that transplantation of ES cell-derived motoneurons dramatically attenuates abnormal locomotor behavior in an animal model of ALS [7]. However, despite these promising results, substantially more research is required before ES cells can be used for clinical therapies. Consequently, protocols directing ES cells to differentiate into motoneurons should use methods that are readily available to a wide range of investigators with varying scientific backgrounds and technical expertise.

The majority of protocols directing ES cells to differentiate into specific neuronal subtypes use a sequential combination of mitogens [8]. Unfortunately, some differentiation protocols use techniques or specific reagents that limit their widespread use. For example, a few laboratories have shown that ES cells readily differentiate into functional motoneurons when cultured with a sonic hedgehog (Shh) agonist (HhAg1.3) and retinoic acid (RA) [4, 5, 7, 9, 10]. Unfortunately, expansion of these studies by other investigators has been limited because the Shh agonist is not readily accessible. To overcome this limitation, we sought to determine whether Shh itself, or a recombinant Shh protein, could readily direct ES cells to differentiate into functional motoneurons in the presence of RA. For convenience, we used 293 EcR-Shh cells as a source of Shh in order to avoid tedious biochemical purification of the protein. The 293 EcR-Shh cells carry a stably integrated construct for the expression of murine Shh under ecdysone-inducible control [11].

Our results show that ES cells readily differentiate into motoneurons when cocultured with Shh producing cells, but not with a recombinant Shh protein. In ovo transplantation studies revealed that ES cell-derived motoneurons migrated to the ventral horn and projected axons to the epaxial muscle. Finally, physiological studies showed that the ES cell-derived motoneurons were capable of forming functional synapses with muscle fibers in vitro. In summary, this simple technique efficiently differentiates ES cells into functional motoneurons that can be used for developmental and preclinical studies.

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Materials and Methods

Cell Cultures

HBG3 ES cells were initially derived from a transgenic mouse (mHB9:GFP1b) where the expression of enhanced green fluorescent protein (eGFP) was linked to the promoter of the HB9 gene [4]. Because HB9 is expressed by all postmitotic somatic motoneurons [12], eGFP expression in these cells can be used to monitor motoneuron differentiation [4]. HBG3 ES cells were expanded and passaged using methods previously described [4, 10].

Two hundred and fifty thousand 293 EcR-Shh cells (number CRL-2782; American Type Culture Collection [ATCC], Manassas, VA, http://www.atcc.org) were plated in 6-well plates (number 3335; Corning Inc., Corning, NY, http://www.corning.com) containing 3 ml of KShh medium per well. KShh medium consisted of Dulbecco's modified Eagle's medium (DMEM) (number11995; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate (number 25080; Invitrogen), 4.5 g/l glucose, 0.4 mg/ml G-418 (Geneticin; number 11811; Invitrogen), 0.4 mg/ml Zeocin (number 45-0430; Invitrogen), and 10% fetal bovine serum (number 16000-044; Invitrogen). After 1 day in vitro (DIV), the cells were washed with DMEM, incubated in DFK10 medium, and then plated with 10–25 embryoid bodies. The 293 EcR-Shh cells were used in this study because they carry a stably integrated construct for the expression of murine sonic hedgehog under ecdysone-inducible control [11]. Ponasterone A (PA; number 45-0478; Invitrogen) induces ecdysone in 293 EcR-Shh cells and thus drives Shh expression. Consequently, PA (21.5 μM; Invitrogen) and RA (1 μM; number R2625; Sigma) were added to the medium immediately after plating the embryoid bodies to initiate motoneuron differentiation. The cells were fed every day with fresh DFK10 medium containing 1 μM RA. After 5 DIV, the eGFP$^+$ embryoid bodies were gently plucked from the 293 EcR-Shh cells using a blunt tungsten needle (0.125-mm tungsten wire; number TGW0515; World Precision Instruments, Sarasota, FL, http://www.wpiinc.com) and plated on growth-factor-reduced matrigel (number 35-4230; BD Biosciences, San Diego, http://www.bdbiosciences.com) coated coverslips or chick myotubes (see below) in wells containing DFK10 medium. The medium was supplemented with 20 ng/ml glial-derived neurotrophic factor (GDNF; number GF030; Chemicon) and 10 ng/ml ciliary neurotrophic factor (CNTF; number 01-195; Upstate, Charlottesville, VA, http://www.upstate.com) after 24 hours. In a separate series of experiments, 10–25 embryoid bodies were either cultured as free-floating cells in DFK10 medium containing recombinant mouse Shh protein (rmShh; 0.25 μg/ml; number 464-SH; R&D Systems Inc., Minneapolis, http://www.rndsystems.com) and 1 μM RA or in wells containing 250,000 human embryonic kidney (HEK) 293 cells (number CRL-1573; ATCC). The cells were fed every day with fresh DFK10 medium containing 1 μM RA. After 5 DIV, the embryoid bodies were plated on growth factor reduced matrigel-coated coverslips in wells containing DFK10 medium as described above.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 minutes, washed several times with phosphate-buffered saline (PBS), and incubated at 4°C for 12 hours with a primary antibody in a solution containing 0.3% Triton X and 10% goat serum. The following primary antibodies were used: Lim1/2 (1:2; Developmental Studies Hybridoma Bank [DSHB], Iowa City, IA, http://www.uiowa.edu/~dshbwww), Lhx3 (1:50, DSHB), Isl-1/2 (1:5, DSHB), β-III tubulin (1:5,000; Covance, Princeton, NJ, http://www.covance.com), and CD29 (Serotec; number MCA2028; Serotec Ltd., Oxford, U.K., http://www.serotec.com). After several washes with PBS, the cells were incubated for 2 hours at room temperature in a solution containing a goat anti-mouse secondary antibody (1:500; Cy3; Jackson Immunoresearch Laboratories, West Grove, PA, http://www.jacksonimmuno.com) and 10% goat serum. In some experiments, rhodamine conjugated α-bungarotoxin (1:100; Molecular Probes, Eugene, OR, http://probes.invitrogen.com) was applied during an additional 2-hour incubation period. To prevent fading, the sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) and stored at 4°C. Images were captured using a laser-scanning confocal microscope (Zeiss LSM 510; Carl Zeiss, Jena, Germany, http://www.zeiss.com) or an Axiovert microscope (Zeiss).

In Ovo Transplantation

Fertile eggs (Truro, Nova Scotia, Canada) from white Leghorn chickens (*Gallus gallus*) were incubated at 37°C in a humidified incubator. E2.5 (HH17–18) [13] chicken embryos were selected, and a window (15 × 15 mm) was made in the shell using a dental drill. Under sterile conditions, the vitelline membranes were opened, and an incision spanning one somite was made in the T7-LS1 region of the neural tube using a flame-sharpened tungsten needle (0.077-mm wire; World Precision Instruments, Sarasota, FL, http://www.wpiinc.com). The ependymal layer on the ventral right side of the embryo was disrupted, and a single embryoid body containing ~120 eGFP$^+$ motoneurons was transplanted into the ventral region of the neural tube lumen. The eggs were sealed with a coverslip and returned to the incubator for an additional 5 days.

Tissue Preparation and Immunohistochemistry

Embryos were killed at stage (St.) 13 (E7), eviscerated, ventral laminectomized, fixed in 4% formaldehyde for 1 hour, washed several times in PBS, and then left in PBS overnight at 4°C. The embryos were then incubated in 30% sucrose/PBS for 2 hours at 4°C, mounted in optimal cutting temperature compound (Tissue Tek), and stored at −80°C. Coronal sections (30 μm) were washed for 20 minutes in PBS and then incubated overnight at 4°C in 0.3% Triton X/PBS, 10% goat serum, and a primary antibody. The antibodies used were: Isl-1/2 (1:5), Lim1/2 (1:2), Lhx3 (1:50), and β-III tubulin (1:5,000). The sections were washed in PBS and incubated in a goat anti-mouse secondary antibody (1:500) at room temperature for 2 hours and mounted with Vectashield. Confocal images were captured using a laser-scanning confocal microscope (Zeiss LSM 510), and confocal orthogonal images were rendered using Zeiss LSM image browser software (Zeiss).

Intracellular Recordings of Muscle Fibers

Chick myotube cultures were generated as previously described [14]. Briefly, pectoralis muscles were extracted from E12 (St. 38) chick embryos and triturated several times in Hanks' balanced salt solution (HBSS) using a fired polished Pasteur pipette. The dissociated myoblasts were plated on type I rat tail collagen (3.66 mg/ml; number 354236; BD Biosciences) coated coverslips in 24-well plates (50,000 cells per well) containing Ham's F-10 medium (number 11,550; Invitrogen) supplemented with 1.26 mM $CaCl_2$, 10% horse serum (number 16050-122; Invitrogen) and 5% chicken serum (number 16110-082; Invitrogen). Multinucleated myotubes typically formed after 3 DIV, at which time 5–10 eGFP$^+$ embryoid bodies were added to the cultures. To promote neuronal survival, the Ham's F-10 medium was replaced with DFK10 medium that was supplemented with $CaCl_2$ (1.36 mM) and sodium pyruvate (1 mM; number 11360; Invitrogen). Twenty-four hours later, the medium was further supplemented with GDNF (20 ng/ml) and CNTF (10 ng/ml).

Sharp electrode recording techniques were used to characterize neuromuscular activity in the cocultures 3 days after plating the eGFP$^+$ embryoid bodies. To improve electrode accessibility, the coverslips containing the myotubes/embryoid bodies were placed in 60-mm Petri dishes containing artificial CSF (127 mM NaCl, 3 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 26 mM NaHCO3, 1.25 mM NaH2PO4, 10 mM D-glucose). Single myotubes near the differentiated motoneurons were visualized using a stereomicroscope and impaled with sharp electrodes (20–40 MΩ) that were filled with 3 M KCl. Only cells with stable resting membrane potentials (*V*m) between 50 and 60 mV were included in the analysis. Postsynaptic end-plate potentials (EPPs) were amplified via an intracellular amplifier (World Precision Instruments) coupled to a differential amplifier (Dagan, Minneapolis, http://www.dagan.com) and acquired at 10 kHz using a Digi-Data 1322A A/D board

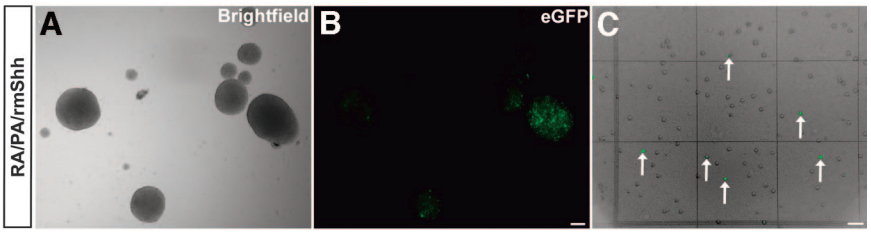

Figure 1. Very few HBG3 embryonic stem cells differentiate into eGFP$^+$ motoneurons when cultured for 5 days in medium containing rmShh, RA, and PA. **(A):** Bright-field illumination shows several embryoid bodies cultured for 5 days in medium containing rmShh, RA, and PA. **(B):** Corresponding fluorescent image shows very few eGFP$^+$ cells within the embryoid bodies. **(C):** Merged bright-field/fluorescent image of dissociated cells in a hemocytometer. Approximately 5% of the cells differentiated into eGFP$^+$ motoneurons (arrows). Scale bar **(A–C)** = 100 μm. Abbreviations: eGFP, enhanced green fluorescent protein; PA, ponasterone A; RA, retinoic acid; rmShh, recombinant mouse sonic hedgehog.

(Columbia, MD, http://digidata.com) and AxoScope software (Axon Instruments/Molecular Devices Corp., Union City, CA, http://www.moleculardevices.com). Signals were analyzed off-line using the Mini Analysis Program (Synaptosoft, Decatur, GA, http://www.synaptosoft.com).

Isolation of Motoneurons Derived from 293 EcR-Shh:ES Cell Cocultures

To prepare the 293 EcR-Shh:ES cells into a single cell suspension after 5 DIV, the cocultures were gently triturated with a Pasteur pipette and transferred to a 15-ml tube where they were washed twice with HBSS. The HBSS was replaced with papain/DNase (20 units/ml papain and 0.005% DNase; number LK003150; Worthington Biochemical Corp., Lakewood, NJ, http://www.worthington biochem.com), and the cells were gently agitated for 10 minutes at 37°C. The dissociated cells were transferred and triturated in a DNase/albumin inhibitor solution to inactivate the papain. Following centrifugation at 1,000 rpm for 3 minutes, the supernatant was discarded, and eGFP$^+$ motoneurons were isolated from the 293 EcR-Shh cells using either fluorescence-activated cell sorting (FACS) or an immunoisolation technique (see below).

Isolation of Motoneurons Derived from ES Cells Using FACS. eGFP$^+$ motoneurons were isolated from 293 EcR-Shh cells using a FACSAria machine (BD Biosciences). Briefly, the sorting rate was up to 1,000 cells per second with a purity of up to 97%. Linear measurements of forward and side light scatter were made using a 488 nM blue laser. A primary gate based on the physical parameters, forward and side light scatter, was set to exclude dead cells or debris. The FACS analysis was performed using FACSDIVA software. Sorted cells were collected in 10-ml tubes coated with 4% bovine serum albumin (BSA). To assess the viability of the cells, 1 μg/ml propidium iodide (a dye taken up by only dead cells) was added to a sample volume of cells and analyzed with the cell sorter. Subsequently, sorted eGFP$^+$ motoneurons were plated on matrigel-coated coverslips for further analysis.

Isolation of Motoneurons Derived from ES Cells Using Dynabeads. Cells were incubated in PBS containing mouse anti-human CD29 (1:25; Serotec) and 0.1% BSA (AlbuMAX I; number 11020; Invitrogen) for 10 minutes at 4°C. After two washes with PBS/0.1% BSA, the cells were incubated in a 1.5-ml Eppendorf tube containing magnetic Dynabeads coated with a secondary Pan Mouse IgG antibody (number 110-41; Dynal Biotech LLC, Burlington, ON, http://www.dynalbiotech.com) for 30 minutes at 4°C. The Eppendorfs containing the cells were placed in a magnet (Dynal) for 2 minutes, after which time the supernatant containing the cells that were not attached to the magnetic beads was removed. The cells were centrifuged for 3 minutes at 1,000 rpm and then plated on matrigel-coated coverslips or chick myotubes in DFK10 medium for further analysis.

Isolation of Motoneurons Derived from ES Cells Using Inserts. Transwell collagen-coated membrane inserts (3-μm pore size; number 3494; Corning) were inverted so that 293 EcR-Shh cells could be plated on the external bottom surface. This inversion places the basket-shaped insert upside down. A small volume of KShh medium containing ~300,000 293 EcR-Shh cells was placed on the external bottom surface and then placed in the incubator for 1 hour in order for the cells to adhere to the collagen. The inserts were then placed upright in wells containing KShh medium for 2 days. Following washes, the inserts were transferred to wells containing DFK10 medium with PA (21.5 μM) and RA (1 μM). We placed 5–10 embryoid bodies inside the inserts, where they attached to the internal bottom surface. The medium inside the wells was replaced every day. After 5 DIV, the embryoid bodies were extracted with a blunt tungsten needle and plated on matrigel-coated coverslips or chick myotubes in DFK10 medium for further analysis.

Quantification of eGFP$^+$ Cells in Differentiated Embryoid Bodies

Differentiated embryoid bodies were collected and gently agitated in a solution containing papain/DNase (20 units/ml papain and 0.005% DNase) for 10 minutes at 37°C. The dissociated cells were transferred into a DNase/albumin inhibitor solution to inactivate the papain. Ten microliters of the cell suspension was placed in a hemocytometer, where the proportion of eGFP$^+$/eGFP$^-$ cells was quantified.

RESULTS

eGFP is expressed under the control of a motoneuron specific promoter (HB9) in HBG3 ES cells [4]. Consequently, eGFP expression can be used as an initial screen for motoneuron differentiation [4, 5, 7, 9, 10]. Previous studies have stated that approximately 23% of HBG3 ES cells differentiate into eGFP$^+$ motoneurons when cultured with a Shh agonist (Hh-Ag1.3; 2.2 μM) and RA (1 μM) for 5 days [4]. Because the Shh agonist is not readily available, we sought to determine whether Shh recombinant mouse protein (250 ng/ml) could substitute for the agonist. To test this ability, we first cultured free-floating HBG3 ES cells in the presence of leukemia inhibitory factor for 2 days, as previously described for the agonist [4, 5, 10]. During this time, the dissociated cells formed spherical embryoid bodies containing 30–400 undifferentiated ES cells. The embryoid bodies were then cultured in DFK10 medium containing 250 ng/ml rmShh, RA (1 μM), and PA (21.5 μM; rationale for including the PA is discussed below) for an additional 5 days. This concentration of rmShh was chosen because previous studies have shown that 250 ng/ml is sufficient to induce motoneuron differentiation in chick neural tube explants [15].

Figure 1 shows that very few HBG3 ES cells within embryoid bodies differentiated into eGFP$^+$ motoneurons after 5 DIV when cultured in medium containing rmShh, RA, and PA. Similar results were obtained when the cells were cultured in rmShh and RA (data not shown). Further analysis (see Materials and Methods for details) indicated that only 5.43% $\pm$ 0.205% (n = 4 separate experiments) of the cells differentiated into eGFP$^+$ motoneurons after 5 DIV (Fig. 1C). Thus, rmShh does not have the same capacity to induce ES cells to differentiate into motoneurons as the Shh agonist.

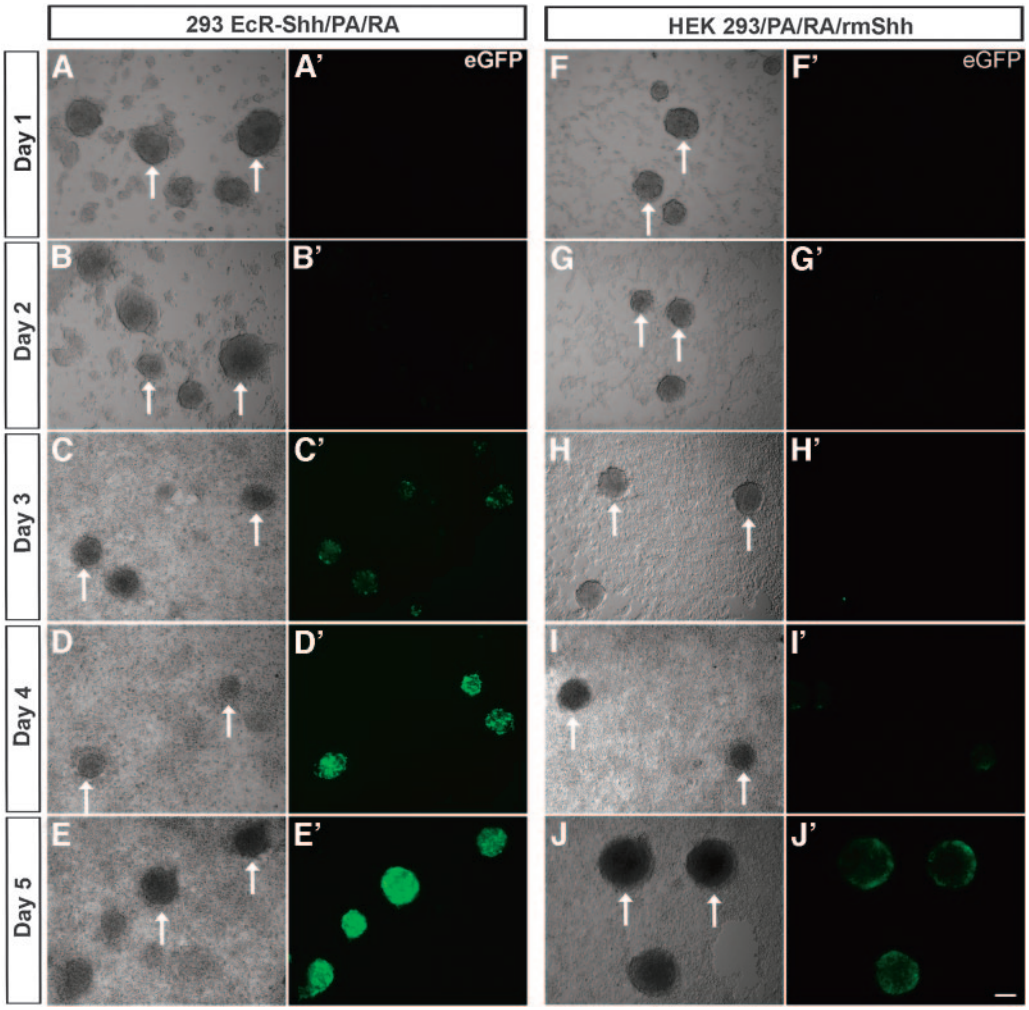

Figure 2. Embryonic stem (ES) cells differentiate into eGFP$^+$ motoneurons when cultured with 293 EcR-Shh cells, RA, and PA but not when cultured with HEK 293 cells and rmShh and RA. **(A–E):** Bright-field illumination shows ES cells, in the form of embryoid bodies (arrows), plated on a layer of 293 EcR-Shh cells in medium containing RA and PA. **(A'–D'):** The corresponding fluorescent images show that the expression of eGFP is first observed in HBG3 ES cells after 3 days in vitro (DIV). This expression gradually increased over the next 2 DIV. **(F–J):** Bright-field illumination shows ES cells (arrows) plated on a layer of HEK 293 cells in medium containing RA and PA. **(F'–J'):** The corresponding fluorescent images show that very few HBG3 ES cells express eGFP in medium containing rmShh and RA. Scale bar = 100 μm. Abbreviations: eGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; PA, ponasterone A; RA, retinoic acid; rmShh, recombinant mouse sonic hedgehog.

ES Cells Differentiate into eGFP$^+$ Motoneurons when Cultured with 293 EcR-Shh Cells and RA

To determine whether fully modified Shh protein can direct ES cells to differentiate into motoneurons, we cultured embryoid bodies containing HBG3 ES cells on 250,000 293 EcR-Shh cells or 250,000 HEK 293 cells in the presence of RA. PA was added to the medium containing the 293 EcR-Shh cells to induce Shh expression (see Materials and Methods for details), and rmShh (250 ng/ml) and PA were added to the tissue culture plates containing the HEK 293 cells as a control. Bright-field photomicrographs in Figure 2 show several representative embryoid bodies (arrows) 1–5 days after they were plated on either 293 EcR-Shh cells or HEK 293 cells. Fluorescent microscopy of the same cells showed that all of the embryoid bodies plated on the 293 EcR-Shh cells contained some eGFP$^+$ cells 3 days after plating (Fig. 2C'). Furthermore, the number of eGFP$^+$ cells within the embryoid bodies increased substantially over the next 2 days (Fig. 2D', 2E'). This time course, and extent of eGFP expression, is remarkably similar to that observed when HBG3 cells are cultured with HhAg1.3 and RA [4, 5, 10]. In striking contrast, only a few eGFP$^+$ cells were observed in the cultures containing HEK 293 cells and rmShh/RA/PA, even though the integrity of the embryoid bodies appeared to be well preserved (e.g., Fig. 2J, 2J', arrows). To rule out the possibility that the concentration of rmShh was not high enough to induce motoneuron differentiation, we increased its concentration to 2.5 μg/ml in the HEK 293:ES cell cocultures. However, despite the higher concentration, the number of eGFP$^+$ cells did not increase (data not shown). Taken together, these results indicate that HBG3 ES cells readily differentiate into eGFP$^+$ motoneurons when cultured on Shh expressing cells, but not when they are exposed to the rmShh protein.

ES cells differentiate into neurons when cultured with RA for 5 days [16]. Therefore, to exclude the possibility that HEK 293 cells prevent neuronal differentiation, we harvested the embryoid bodies from the RA/PA/rmShh treated HEK 293:ES cell cocultures after 5 DIV and plated them on matrigel for an additional 2 days. For comparison, we also cultured HBG3 ES cells in the presence of PA and RA. The vast majority of the ES cells in both culture conditions were β-III tubulin$^+$, indicating

that the lack of motoneurons in the HEK 293:ES cell cocultures was not due to inhibition of neuronal differentiation (supplemental online Fig. 1A). Interestingly, RA treated embryoid bodies contained a few eGFP$^+$ cells (supplemental online Figure 1), presumably because the differentiating cells express some Shh (Discussion and [4]).

HBG3 ES Cell-Derived Motoneurons from HEK EcR-Shh/RA:ES Cell Cocultures Express Lhx3 and Isl1/2 In Vitro

Previous studies showed that 23% of HBG3 cells differentiate into eGFP$^+$ motoneurons when treated with the Shh agonist HhAg1.3 and RA. The other 77% differentiated into presumptive interneurons [4]. Interestingly, the treated HBG3 ES cells selectively differentiated into a subset of motoneurons that continually expressed the transcription factor Lhx3 [4, 10]. In order to determine whether the same proteins were expressed by ES cell-derived motoneurons that were differentiated on 293 EcR-Shh cells, we plucked the eGFP$^+$ cells from the cells 5 days after plating and cultured them on matrigel for an additional 3 days. As observed with the Shh agonist, the eGFP$^+$ cells were β-III tubulin$^+$ (Fig. 3A). The vast majority of them also expressed Lhx3 (97.2% $\pm$ 1.10%, $n = 3$; Fig. 3B) and Isl1/2 (96.4% $\pm$ 0.638%, $n = 3$; Fig. 3C) but not Lim1 (1.33% $\pm$ 0.176%, $n = 3$; Fig. 3D). The Lhx3$^+$/eGFP$^-$ and Lim1$^+$/eGFP$^-$ cells in Figure 3 are most likely interneurons [4, 5]. Taken together, these results indicate that ES cell-derived motoneurons that were differentiated on 293 EcR-Shh cells selectively express Lhx3 and Isl1/2 in a manner that is very similar to HhAg1.3/RA treated ES cells [4, 5, 9, 10].

Transplanted HBG3 ES Cell-Derived Motoneurons from HEK EcR-Shh/RA:ES Cell Cocultures Migrate to the Medial Motor Column and Continually Express Lhx3

Although Lhx3 is transiently expressed by all motoneurons during early neuromuscular development [17], it is continuously expressed by motoneurons that reside in the medial aspect of the ventral horn within the medial motor column (MMC$_m$). During development, continuously expressing Lhx3$^+$ motoneurons migrate to the MMC$_m$, extend axons out of the spinal cord through the ventral root, project axons around the dorsal root ganglia (DRG), and ultimately innervate the epaxial muscles lining the vertebral column [18]. Soundararajan et al. [10] recently showed that motoneurons from HhAg1.3/RA treated ES cells develop the same phenotype as endogenous Lhx3$^+$ MMC$_m$ neurons. Specifically, they showed that HhAg1.3/RA treated ES cell-derived motoneurons remained Lhx3$^+$, migrated to the MMC$_m$, and exclusively innervated axial muscles when transplanted into the spinal cord of developing chick embryo. To determine whether the ES cell-derived motoneurons in the present study develop the same phenotypic fate, we plucked eGFP$^+$ motoneurons from the 293 EcR-Shh cells and transplanted them into the neural tube lumen of St. 17–18 ($\sim$E2.5) chick embryos at the level of somite 26. Care was taken to transplant approximately 120 eGFP$^+$ motoneurons in the ventral aspect of the neural tube where motoneurogenesis normally occurs [19]. The embryos were allowed to develop until St. 31 ($\sim$E7). Cross-sections through the developing spinal cords at LS1 showed that the transplanted eGFP$^+$ motoneurons translocated from the lumen to the medial aspect of the ventral horn (Fig. 4A and 4C, boxes) ($n = 3$). Higher magnification confocal microscopy, with orthogonal views, showed that the transplanted eGFP$^+$ motoneurons continued to express Lhx3 5 days after transplantation (Fig. 4B, arrow and arrowhead). It should be noted that one of the two

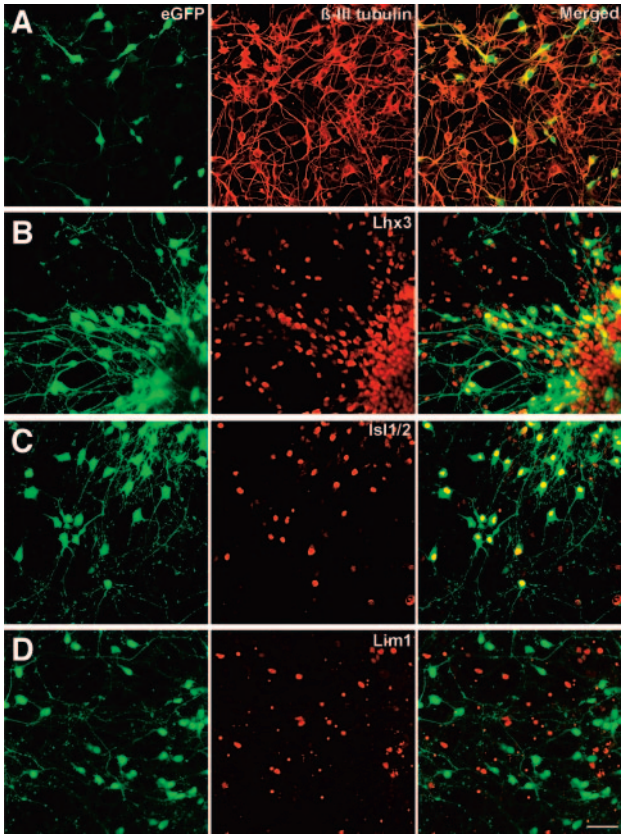

Figure 3. Embryonic stem (ES) cell-derived motoneurons express proteins and transcription factors typical of motoneurons from the medial motor column. **(A):** Both eGFP$^+$ and eGFP$^-$ cells from the 293 EcR-Shh/RA:ES cell cocultures extended neurites that expressed β-III tubulin after 3 days in vitro (DIV) when plating on matrigel. **(B, C):** The vast majority of the eGFP$^+$ cells were Lhx3$^+$ **(B)** and Isl1/2$^+$ **(C)** after 3 DIV. **(D):** In contrast, very few eGFP$^+$ ES cell-derived motoneurons expressed Lim1, although some eGFP$^-$/Lim1$^+$ cells (presumably interneurons) were present. Scale bar **(A–D)** = 50 μm. Abbreviation: eGFP, enhanced green fluorescent protein.

eGFP$^+$ motoneurons in this single optical section appeared weakly stained with Lhx3 (Fig. 4B, arrowhead) because Lhx3 is a nuclear protein, whereas eGFP is found throughout the cell. The neighboring Lhx3$^+$/eGFP$^-$ neurons in the composite image of the Z-stack (Fig. 4B) are likely endogenous chick MMC$_m$ motoneurons, chick interneurons or Lhx3$^+$ cells derived from the embryoid bodies [20]. As observed with the Shh agonist [10], the vast majority of the eGFP$^+$ neurons expressed Lhx3 (97.2% $\pm$ 0.281%, $n = 3$). None of the transplanted eGFP$^+$ motoneurons expressed Lim1, a homeobox gene expressed by motoneurons in the lateral aspect of the lateral motor column (arrowhead) (Fig. 4C) ($n = 3$).

Transplanted HBG3 ES Cell-Derived Motoneurons from the 293 EcR-Shh/RA:ES Cell Cocultures Project Axons to the Epaxial Muscle

Using Neurolucida software, we anatomically quantified the location of all of the eGFP$^+$ cells and axons in embryos to determine if the transplanted ES cells selectively targeted the epaxial muscles. A representative Neurolucida reconstruction from a single embryo (Fig. 5A) shows that the transplanted eGFP$^+$ motoneurons migrated to the medial aspect of the ventral horn (green dots), extended axons through the ventral nerve root (broad arrow), formed part of the dorsal ramus (open

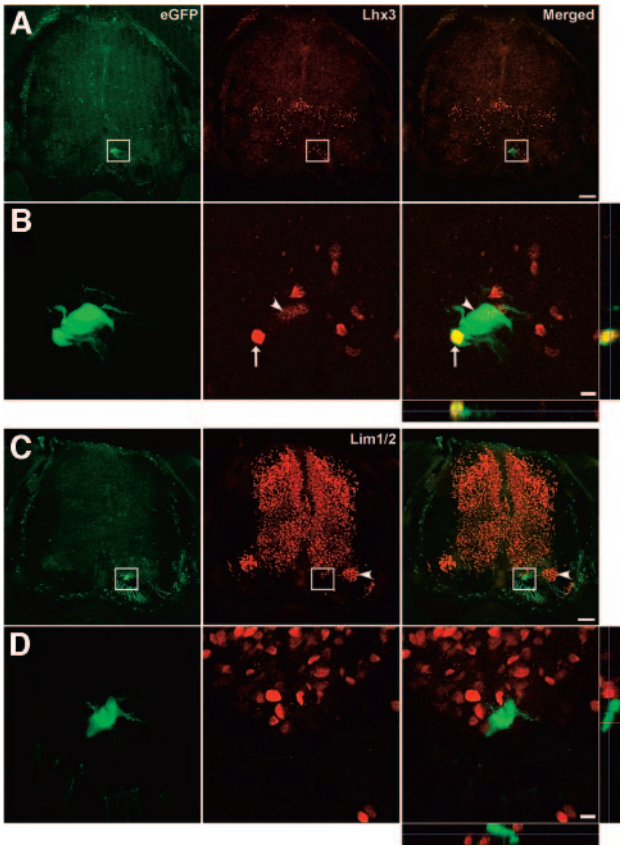

Figure 4. Embryonic stem (ES) cell-derived motoneurons from 293 EcR-Shh/RA:ES cell cocultures migrate to the medial motor column and continue to express Lhx3 when transplanted into the neural tube of stage (St.) 17 chick embryos. **(A, C):** Cross-sections of St. 31 chick embryo spinal cords showed that the majority of the transplanted ES cell-derived $eGFP^+$ motoneurons migrated to the medial/ventral aspect of the developing neural tube (box). **(A, B):** Immunolabeling and confocal images show two $Lhx3^+$ transplanted motoneurons. **(B):** Both the composite image of the Z-stack at higher magnification and the orthogonal planes confirm colocalization. In this section, one of the $eGFP^+$ cells contained high levels of Lhx3 (arrow), whereas the other contained low levels (arrowhead) because Lhx3 is only located in the nucleus and eGFP is expressed throughout the cell. **(C, D):** None of the transplanted ES cell-derived $eGFP^+$ motoneurons express Lim1. Endogenous chick $Lim1^+$ motoneurons are indicated with an arrowhead. **(D):** Higher magnification and orthogonal planes confirm the lack of colocalization. In all sections, dorsal is up. Scale bar: **(A, C)** = 100 μm; **(B, D)** = 10 μm. Abbreviation: eGFP, enhanced green fluorescent protein.

arrowhead), and finally projected axons to the epaxial muscle (thin arrow; epaxial muscle outlined with white dots) or the skin (small arrowhead). For comparison, endogenous chick axons are shown in red on the contralateral side (Fig. 5A; large arrow indicates axons innervating the developing limb). Figure 5B is a survey photomicrograph showing $eGFP^+$ axons from the transplanted ES cell-derived motoneurons extending axons around the DRG to form part of the dorsal ramus (Fig. 5B, arrow). Similarly, Figure 5C shows $eGFP^+$ axons extending along the ventral border of the longissimus muscle (an epaxial muscle; outlined by white dots). These results are basically indistinguishable from a similar in ovo study where the transplanted motoneurons were derived from HBG3 ES cells using HhAg1.3 and RA [10]. Taken together, these results indicate that ES cell-derived motoneurons, which were differentiated on 293 EcR-Shh cells, develop phenotypic characteristics of MMC_m motoneurons.

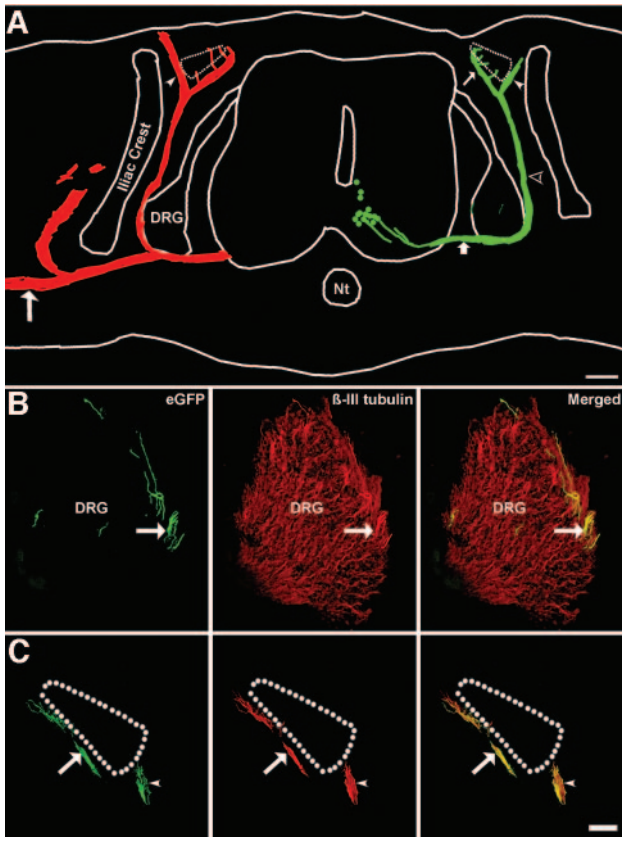

Figure 5. Transplanted embryonic stem (ES) cell-derived motoneurons from the 293 EcR-Shh/RA:ES cell cocultures selectively project axons to epaxial muscles. **(A):** Neurolucida reconstruction shows that all of the transplanted ES cell-derived motoneurons in a representative stage (St.) 31 chick embryo (green tracings) extended axons out of the neural tube through the ventral root (broad arrow) and projected dorsally around the DRG as part of the dorsal ramus (open arrowhead). The $eGFP^+$ axons in the dorsal ramus eventually bifurcated and either correctly innervated the longissimus muscle (long arrow) or incorrectly innervated the skin (arrowhead). Each green dot represents a single $eGFP^+$ motoneuron. For comparison, the projection patterns of the endogenous β-III $tubulin^+$ chick neurons are shown in red on the contralateral side. The large arrow indicates axons projecting into the developing limb. **(B, C):** Single representative β-III tubulin immunolabeled cross-sections through a St. 31 chick embryo show $eGFP^+$ axons projecting around the DRG (**[B]**, arrow) and extending along the ventral border of the longissimus muscle (**[C]**, arrow). As reported by Soundararajan et al., some transplanted ES cell-derived motoneurons incorrectly projected axons to the skin (arrowheads in **[A, C]**). The longissimus muscle is indicated with dotted lines **(A, C)**. In all sections, dorsal is up. Scale bar: **(A)** = 100 μm; **(B, C)** = 50 μm. Abbreviations: DRG, dorsal root ganglion; eGFP, enhanced green fluorescent protein; Nt, notochord.

ES Cell-Derived Motoneurons from the 293 EcR-Shh/RA:ES Cell Cocultures Induce Acetylcholine Receptor Clustering and Form Functional Connections with Muscle Fibers In Vitro

The primary function of motoneurons is to cause muscle contraction by releasing acetylcholine (ACh) at the neuromuscular junction (NMJ). NMJs are easily identifiable because they contain a very high concentration of ACh receptors (AChRs). This high concentration of AChRs arises during development because the innervating motoneurons induce both AChR clustering and local protein synthesis on the region of the muscle fibers that is destined to become the motor endplate (reviewed in [21]).

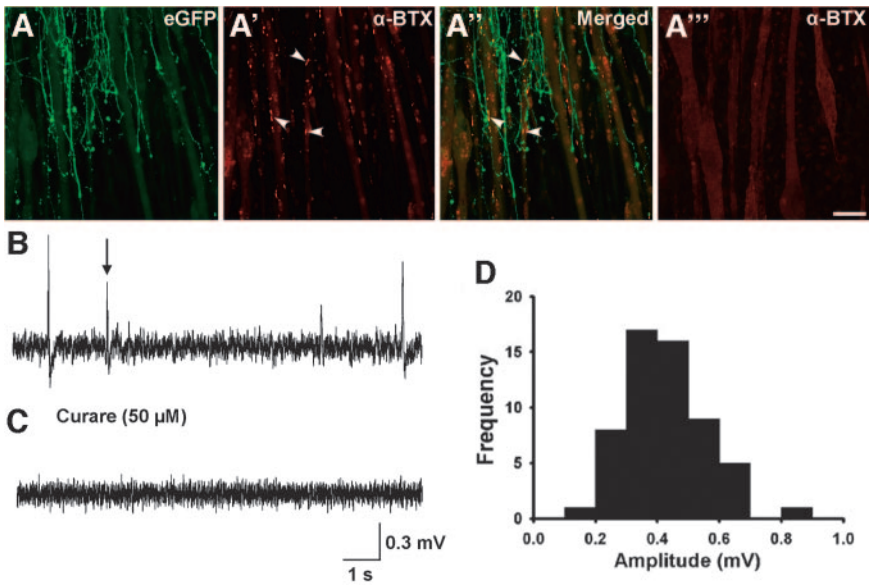

Figure 6. Embryonic stem (ES) cell-derived motoneurons from 293 EcR-Shh/RA:ES cell cocultures induce acetylcholine receptor (AchR) clustering and form functional connections with muscle fibers in vitro. **(A, A'')**: ES cell-derived motoneurons from 293 EcR-Shh/RA:ES cell cocultures extended neurites along embryonic chick myotubes after 2 days in vitro. **(A', A'')**: α-BTX staining revealed clustering of AChRs in close proximity to the eGFP$^+$ neurites (arrowheads). **(A''')**: AChR clustering was absent on myotubes several millimeters away from the embryoid body. **(B)**: End-plate potentials (EPPs) were recorded from chick myotubes that were in close proximity to the ES cell-derived motoneurons. **(C)**: EPPs were blocked from the same cell after application of 50 μM curare. **(D)**: The amplitudes of the EPPs were similar is size, suggesting that they were spontaneously occurring miniature EPPs. Scale bar: **(A)** = 50 μm. Abbreviations: α-BTX, α-bungarotoxin; eGFP, enhanced green fluorescent protein.

In order to determine whether ES cell-derived motoneurons, which were differentiated on 293 EcR-Shh cells, induce AChR clustering, we cultured them with chick myotubes for several days. After 2 DIV, eGFP$^+$ motoneurons were observed to extend neurites along individual myotubes (Fig. 6A, myotubes are oriented vertically in the photomicrograph). Furthermore, discrete areas containing a high concentration of AChRs could be detected in close proximity to the eGFP$^+$ neurites using rhodamine conjugated α-bungarotoxin (Fig. 6A' & 6A'', arrowheads). AChR clustering preferentially occurred near eGFP$^+$ axons because very few clusters occurred in regions that were not adjacent to the differentiated motoneurons (Fig. 6A'''). Interestingly, the diffuse clustering of AChRs around the neurites parallels what is observed during neuromuscular development in the chick [22] and mouse [23], where receptor clustering does not always occur in direct anatomical contact with the innervating motor axons. These results indicate that ES cell-derived motoneurons induce AChR clustering, a prerequisite for neuromuscular neurotransmission.

In order to determine whether ES cell-derived motoneurons are capable of establishing functional connections with chick muscle fibers, we performed sharp electrode recordings from individual myotubes that were in close proximity to the eGFP$^+$ neurites ($n = 4$). Figure 6B shows a recording of four EPPs from an individual myotube 6 days after plating (a single EPP is indicated with an arrow). EPPs were completely blocked from the same cell shortly after bath application of D-tubocurarine (50 μM; Fig. 6C), indicating that the potentials were due to cholinergic transmission. Quantification of the EPPs revealed that their amplitudes were unimodally distributed and similar in size with a mean value of 0.443 $\pm$ 0.0198 mV (Fig. 6D). Both the size and distribution of the recorded EPPs suggested that they were miniature end-plate potentials resulting from the spontaneous release of ACh from the presynaptic terminal. Taken together, these results indicate that ES-cell derived motoneurons, which were differentiated on 293 EcR-Shh cells, are physiologically functional and capable of forming connections with muscle fibers in vitro.

Isolation of Motoneurons Derived from 293 EcR-Shh:ES Cell Cocultures

The above results indicate that functional motoneurons can be derived from ES cells when plated on 293 EcR-Shh cells in the presence of RA. Furthermore, the phenotype of the differentiated motoneurons is the same as Shh agonist- and RA-treated ES cell-derived motoneurons [4, 5, 10]. However, in order for this method to be widely used for developmental and preclinical studies, it is imperative that the differentiated motoneurons can be isolated from the 293 EcR-Shh cells. Consequently, we used and/or developed three methods to isolate the differentiated ES cells.

The first isolation technique involved dissociating the cocultures into a single cell suspension and then sorting them with FACS. Linear measurements of forward and side light scatter were made using a 488-nM blue laser, and then a primary gate was set to exclude dead cells or debris (Fig. 7Ai). FACS histogram analysis showed that the nonfluorescent 293 EcR-Shh cells (Fig. 7Aii, Box 1) could easily be distinguished from the eGFP$^+$ cells (Fig. 7Aii, Box 2 and Fig. 7Aiii). Using this stringent FACS protocol, 100% of the sorted cells were eGFP$^+$. Furthermore, the FACS-isolated eGFP$^+$ motoneurons were viable and extended neurites when cultured for 3 days in medium supplemented with CNTF and GDNF (Fig. 7B).

Although isolation of eGFP$^+$ motoneurons from nonfluorescent cells by FACS results in a pure culture of motoneurons, it is an impractical technique if the ES cells are not fluorescent or if the researchers do not have access to FACS. In order to overcome these limitations, we developed two alternative methods to isolate differentiated ES cells from 293 EcR-Shh cells. The first method takes advantage of the fact that 293 cells express human β_1 integrin (CD29; [24]), whereas mice ES cells do not. In this technique, we dissociated the cocultures and then incubated the cell suspension with an antibody that selectively recognizes the extracellular component of human β_1 integrin. The cells were then incubated with magnetic Dynabeads (Dynal) coated with a secondary mouse IgG antibody. The 293 EcR-Shh cells were subsequently removed from the cell suspension with a magnet. Figures 7C shows several eGFP$^+$ ES cell-derived motoneurons in the cell suspension (arrows). eGFP$^+$ cells were not found in the cell fraction containing the magnetic beads (Fig. 7D, 7F). Using fluorescent/bright-field illumination (Fig. 7C, 7C'), we determined that 29% $\pm$ 4.78% ($n = 6$) of the cells in the suspension were eGFP$^+$ motoneurons. This percentage is greater than the 23% previously reported using the Shh agonist [4]. The eGFP$^-$ cells (Fig. 7C', arrowheads) were presumably interneurons because the dissociated cells yielded β-III tubulin$^+$ neurons 24 hours after plating on

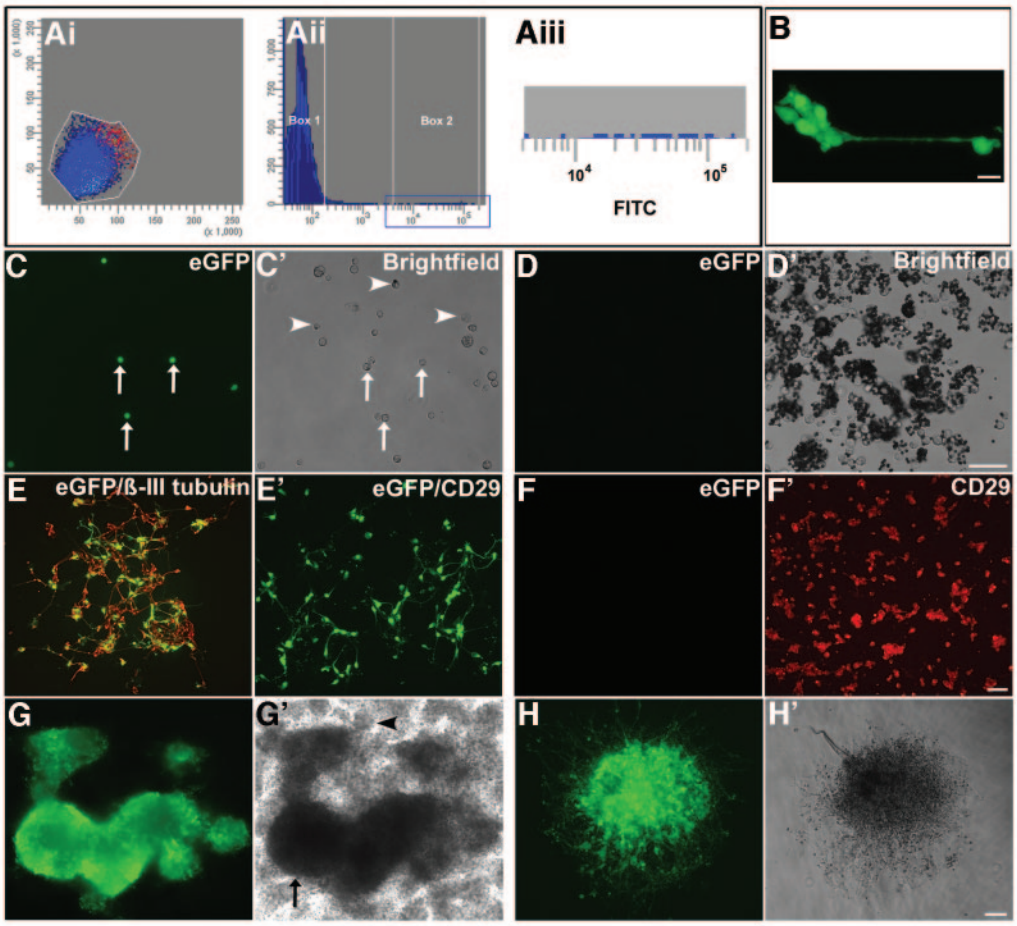

Figure 7. Three different techniques were used to isolate embryonic stem (ES)-cell derived motoneurons from the cocultured 293 EcR-Shh cells. **(Ai):** Density plots of dissociated eGFP$^+$ motoneurons and 293 EcR-Shh cells from cocultures. Blue and red dots represent nonfluorescent and eGFP$^+$ cells, respectively. Line indicates gate used for sorting eGFP$^+$ motoneurons from the 293 EcR-Shh cells (*y*-axis = side scatter, *x*-axis = forward scatter). **(Aii):** Fluorescence-activated cell sorting (FACS) histogram analysis showed that the nonfluorescent 293 EcR-Shh cells (Box 1) could easily be distinguished from the eGFP$^+$ cells (Box 2) (*y*-axis = count; *x*-axis = fluorescein isothiocyanate). **(Aiii):** Enlargement of blue box in **(Aii)**. **(B):** Confocal image shows that eGFP$^+$ motoneurons extend neurites and survive for at least 3 days in vitro when isolated with FACS and cultured with ciliary neurotrophic factor and glial-derived neurotrophic factor. **(C):** Fluorescent image shows several dissociated eGFP$^+$ motoneurons (arrows) after immunoisolation with Dynabeads. **(C'):** Bright-field photomicrograph of the cells in **(C)** shows that ~30% of the immunoisolated cells were eGFP$^+$ (arrows). eGFP$^-$ cells (arrowheads) likely represent ES cells that differentiated into interneurons. **(D):** Fluorescent image shows that none of the cells bound to the Dynabeads expressed eGFP. **(D'):** Bright-field photomicrograph of the cells in **(D)** shows large clusters of 293 EcR-Shh cells attached to small opaque Dynabeads. **(E):** Merged fluorescent images showing eGFP$^+$ (green) and β-III tubulin$^+$ (red) cells 24 hours after immunoisolation. **(E'):** None of the immunoisolated cells, from the same experiment described in **(E)**, immunostained with CD29 (red; eGFP$^+$ cells appear green). **(F):** Fluorescent image showing that the cells attached to the Dynabeads are not eGFP$^+$. **(F'):** Same cells as in **(F)** immunostained with CD29. **(G):** Fluorescent image shows that ES cells differentiate into eGFP$^+$ motoneurons when plated on the internal bottom surface of inserts with 293 EcR-Shh cells plated on the external bottom surface. **(G'):** Bright-field image shows ES cell-derived motoneurons (arrow) and 293 EcR-Shh cells (arrowhead) on the internal and external bottom surfaces, respectively, of semitransparent collagen-coated inserts. **(H)**: ES cell-derived motoneurons that were harvested from the inserts extend neurites when plated on matrigel for 24 hours. **(H'):** Bright-field image of the cells shown in **(H)**. Scale bar: **(B)** = 20 μm; **(C, D, G)** = 200 μm; **(E, F, H)** = 100 μm. Abbreviations: eGFP, enhanced green fluorescent protein; FITC, fluorescein isothiocyanate.

matrigel (Fig. 7E). None of the plated cells expressed CD29 (Fig. 7E'). Finally, EPPs were recorded from individual muscle fibers 3 days after plating the cell suspension on chick myotubes (supplemental online Fig. 2B), indicating that the purification procedure did not attenuate the motoneurons' capacity to form functional synaptic connections.

It has often been advantageous for developmental biologists to maintain the differentiated motoneurons in a spherical cluster of cells [4, 10]. In this way, the cluster behaves like an explant of tissue, and explants are routinely used in experiments such as axon guidance studies. Consequently, we developed a method to isolate a cluster of differentiated ES cells from 293 EcR-Shh cells. In this technique, we initially attached 293 EcR-Shh cells to the external bottom surface of transwell collagen-coated membrane inserts (see Materials and Methods for details). After 2 DIV, RA was added to the medium, and several embryoid bodies were placed inside the inserts, where they attached to the internal bottom surface. The inserts prevented the two cell types from mixing together yet allowed signaling to occur between the ES cells and 293 EcR-Shh cells. Figure 7G shows that the ES cells in the inserts differentiated into eGFP$^+$ motoneurons after 5 DIV in a manner that was comparable to when they were plated directly onto the 293 EcR-Shh cells (e.g., Fig. 2E'). The cluster of differentiated ES cells could easily be removed from the inserts with blunt tungsten needles and extended neurites within 24 hours when plated on matrigel (Fig. 7H). The cluster of ES cells also extended neurites and induced AChR clustering when plated on myotubes (supplemental online Fig. 2A). Fi-

nally, EPPs were recorded from individual muscle fibers 3 days after plating the cluster on chick myotubes (supplemental online Fig. 2C), indicating that this purification procedure did not reduce their capacity to form functional synaptic connections.

DISCUSSION

It is well established that ES cells differentiate into functional motoneurons when treated with a Shh agonist and RA [4, 5, 9, 10]. Our present results extend these findings to show that ES cells rapidly and easily differentiate into functional motoneurons when cocultured with Shh producing cells and RA. Furthermore, we demonstrated that the ES cell-derived motoneurons were virtually indistinguishable from those generated with the Shh agonist. Several lines of evidence support this conclusion. First, as shown with the agonist [4, 10], virtually all ES cell-derived motoneurons in the current study were $Hb9^+$ and $Lhx3^+$. Second, both differentiation protocols generated ES cell-derived motoneurons that migrated to the MMC_m and selectively extended neurites to epaxial muscles when transplanted into the neural tube of developing chick embryos [10]. Finally, motoneurons derived from either Shh agonist/RA treatment [5] or 293 EcR-Shh:ES cell/RA cocultures induced AChR clustering and formed functional synaptic connections with muscle fibers in vitro.

Motoneuron Induction by Recombinant Shh Versus the Modified Shh Protein

A striking finding in the present study was the low level of motoneuron differentiation in the presence of RA and rmShh (Figs. 1, 2). This result was particularly surprising in light of the fact that recombinant Shh (250 ng/ml) induces motoneuron differentiation in neural plate explant cultures [15]. Although the reason for this discrepancy is not entirely clear, it could be due to the fact that the Shh expressed in 293 EcR-Shh cells is efficiently processed into a mature signaling molecule that is covalently modified with the addition of both cholesterol and fatty acids [25]. Previous studies indicate that post-translational modification by cholesterol [26] and/or fatty acids [27, 28] increases its potency by 15- to 160-fold over unmodified Shh. Recombinant Shh protein is not cholesterol or palmitate modified, and thus it is likely far less potent than native Shh protein. Several studies have also shown that ES cells and neural progenitor cells express low levels of Shh [4, 29]. This endogenous source of Shh causes some cells within embryoid bodies to differentiate into motoneurons when they are exposed to only RA ([4, 6], supplemental online Fig. 1B). Consequently, it is possible that neural plate explant cultures contain some Shh, and that this endogenous source induces motoneuron differentiation when combined with exogenous rmShh.

In contrast to the present study, Li et al. [6] showed that recombinant Shh protein induces human ES (hES) cells to differentiate into functional $Hb9^+$ motoneurons in the presence of RA. Although these results appear to contradict ours (Figs. 1, 2), there are several differences between the two studies that could account for the discrepancy. First, the ES cells were derived from entirely different species (human vs. mouse). Second, the hES cells were induced to differentiate into neuroectodermal cells with fibroblast growth factor 2 before they were treated with RA and recombinant Shh. Finally, $Hb9^+$ motoneurons did not appear until 4 weeks after the onset of the ES cell differentiation protocol (the highest population of $HB9^+$ cells did not occur until the 5th week). Consequently, it is possible that hES and murine ES cells respond differently to recombinant Shh. Alternatively, neuroectodermal cells may express some Shh, and it is possible that this endogenous source induces motoneuron differentiation when combined with recombinant Shh. It is also possible that hES cells would differentiate into motoneurons more rapidly if cultured using the techniques described in the present study.

293 EcR-Shh Cells and ES Cell-Derived Dopaminergic Neurons

Apart from its role in generating various classes of motoneurons and interneurons within the ventral spinal cord [30], Shh also induces ventral neurons to become dopaminergic neurons in the developing forebrain [31] and midbrain [32]. Based on these developmental studies, several investigators have included recombinant Shh in their protocols to differentiate ES cells into dopaminergic neurons [33–35]. Interestingly, only 10%–20% of the cells differentiate into tyrosine hydroxylase-positive neurons in the presence of recombinant Shh [33, 34]. Furthermore, several weeks of differential culturing conditions are required to derive dopaminergic neurons from ES cells. Based on the present results, it would be tempting to determine whether dopaminergic neurons would be produced more rapidly, and at a higher yield, if the mature Shh protein was substituted for the recombinant Shh protein in the differentiation protocols.

ES Cell-Derived Motoneurons as a Tool to Study Motoneuron Development and Disease

Purification of distinct neuronal subtypes is often required to study channel physiology, cell survival, axonal guidance, gene expression, and apoptosis. Isolation protocols are also essential for screening the effects of pharmacological reagents on specific cell types. To this end, Camu and Henderson [36] developed a protocol to purify embryonic rat motoneurons from spinal cord tissue using differential centrifugation and immunopanning. This technique results in the isolation of approximately 100,000 putative motoneurons per E15 spinal cord. The differentiation protocol in the present study provides an alternative method to study factors affecting motoneuron survival and development. In addition, millions of motoneurons can be reproducibly derived with our protocol, making this an ideal technique for transplantation studies or high throughput screening of pharmacological reagents. Indeed, studies showing improved locomotion in paralyzed rats after transplantation of ES cell-derived motoneurons [7, 9] have ignited the quest to develop a cell replacement therapy for patients who have ALS or a spinal cord injury. The availability of easily obtainable ES-cell derived motoneurons will likely aid in this quest.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

1 Silani V, Fogh I, Ratti A et al. Stem cells in the treatment of amyotrophic lateral sclerosis (ALS). Amyotroph Lateral Scler Other Motor Neuron Disord 2002;3:173–181.
2 Boillee S, Vande Velde C, Cleveland DW. ALS: A disease of motor neurons and their nonneuronal neighbors. Neuron 2006;52:39–59.
3 Nayak MS, Kim YS, Goldman M et al. Cellular therapies in motor neuron diseases. Biochim Biophys Acta 2006;1762:1128–1138.
4 Wichterle H, Lieberam I, Porter JA et al. Directed differentiation of embryonic stem cells into motor neurons. Cell 2002;110:385–397.
5 Miles GB, Yohn DC, Wichterle H et al. Functional properties of motoneurons derived from mouse embryonic stem cells. J Neurosci 2004; 24:7848–7858.
6 Li XJ, Du ZW, Zarnowska ED et al. Specification of motoneurons from human embryonic stem cells. Nat Biotechnol 2005;23:215–221.
7 Deshpande DM, Kim YS, Martinez T et al. Recovery from paralysis in adult rats using embryonic stem cells. Ann Neurol 2006;60:32–44.
8 Keller G. Embryonic stem cell differentiation: Emergence of a new era in biology and medicine. Genes Dev 2005;19:1129–1155.
9 Harper JM, Krishnan C, Darman JS et al. Axonal growth of embryonic stem cell-derived motoneurons in vitro and in motoneuron-injured adult rats. Proc Natl Acad Sci U S A 2004;101:7123–7128.
10 Soundararajan P, Miles GB, Rubin LL et al. Motoneurons derived from embryonic stem cells express transcription factors and develop phenotypes characteristic of medial motor column neurons. J Neurosci 2006; 26:3256–3268.
11 Cooper MK, Porter JA, Young KE et al. Teratogen-mediated inhibition of target tissue response to Shh signaling. Science 1998;280:1603–1607.
12 Arber S, Han B, Mendelsohn M et al. Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron 1999; 23:659–674.
13 Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. J Morphol 1951;88:49–92.
14 Rafuse VF, Landmesser LT. Contractile activity regulates isoform expression and polysialylation of NCAM in cultured myotubes: Involvement of Ca^{2+} and protein kinase C. J Cell Biol 1996;132:969–983.
15 Ericson J, Morton S, Kawakami A et al. Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell 1996;87:661–673.
16 Sockanathan S, Jessell TM. Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. Cell 1998;94: 503–514.
17 Sharma K, Sheng HZ, Lettieri K et al. LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell 1998;95:817–828.
18 Tosney KW, Hageman MS. Different subsets of axonal guidance cues are essential for sensory neurite outgrowth to cutaneous and muscle targets in the dorsal ramus of the embryonic chick. J Exp Zool 1989; 251:232–244.
19 Hollyday M, Hamburger V. An autoradiographic study of the formation of the lateral motor column in the chick embryo. Brain Res 1977;132: 197–208.
20 Tsuchida T, Ensini M, Morton SB et al. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell 1994;79:957–970.
21 Sanes JR, Lichtman JW. Development of the vertebrate neuromuscular junction. Annu Rev Neurosci 1999;22:389–442.
22 Dahm LM, Landmesser LT. The regulation of synaptogenesis during normal development and following activity blockade. J Neurosci 1991; 11:238–255.
23 Lupa MT, Hall ZW. Progressive restriction of synaptic vesicle protein to the nerve terminal during development of the neuromuscular junction. J Neurosci 1989;9:3937–3945.
24 Bodary SC, McLean JW. The integrin beta 1 subunit associates with the vitronectin receptor alpha v subunit to form a novel vitronectin receptor in a human embryonic kidney cell line. J Biol Chem 1990; 265:5938–5941.
25 Taipale J, Chen JK, Cooper MK et al. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature 2000; 406:1005–1009.
26 Zeng X, Goetz JA, Suber LM et al. A freely diffusible form of Sonic hedgehog mediates long-range signalling. Nature 2001;411:716–720.
27 Pepinsky RB, Zeng C, Wen D et al. Identification of a palmitic acid-modified form of human Sonic hedgehog. J Biol Chem 1998; 273:14037–14045.
28 Taylor FR, Wen D, Garber EA et al. Enhanced potency of human Sonic hedgehog by hydrophobic modification. Biochemistry 2001;40: 4359–4371.
29 Rafuse VF, Soundararajan P, Leopold C et al. Neuroprotective properties of cultured neural progenitor cells are associated with the production of sonic hedgehog. Neuroscience 2005;131:899–916.
30 Ericson J, Briscoe J, Rashbass P et al. Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. Cold Spring Harb Symp Quant Biol 1997;62:451–466.
31 Ericson J, Muhr J, Placzek M et al. Sonic hedgehog induces the differentiation of ventral forebrain neurons: A common signal for ventral patterning within the neural tube. Cell 1995;81:747–756.
32 Hynes M, Porter JA, Chiang C et al. Induction of midbrain dopaminergic neurons by Sonic hedgehog. Neuron 1995;15:35–44.
33 Lee S-H, Lumelsky N, Studer L et al. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotech 2000;18:675–679.
34 Chung S, Shin BS, Hwang M et al. Neural precursors derived from embryonic stem cells, but not those from fetal ventral mesencephalon, maintain the potential to differentiate into dopaminergic neurons after expansion in vitro. STEM CELLS 2006;24:1583–1593.
35 Lau T, Adam S, Schloss P. Rapid and efficient differentiation of dopaminergic neurons from mouse embryonic stem cells. Neuroreport 2006; 17:975–979.
36 Camu W, Henderson CE. Purification of embryonic rat motoneurons by panning on a monoclonal antibody to the low-affinity NGF receptor. J Neurosci Methods 1992;44:59–70.

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Easy and Rapid Differentiation of Embryonic Stem Cells into Functional Motoneurons Using Sonic Hedgehog-Producing Cells

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