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J Tissue Eng Regen Med. Author manuscript; available in PMC 2011 March 1.

Published in final edited form as:
J Tissue Eng Regen Med. 2010 March ; 4(3): 181–193. doi:10.1002/term.223.

Characterization of a Human Fetal Spinal Cord Stem Cell Line NSI-566RSC and Its Induction to Functional Motoneurons

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Abstract

Specific neuronal subtypes, especially motoneurons (MNs), derived from human stem cells provide a significant therapeutic potential for spinal cord diseases such as ALS and spinal cord injury. So far *in vitro*, MNs have only been successfully induced from embryonic stem cells (hESC) and human fetal cortical progenitors. Although neural progenitors from spinal cord would be a likely source for generating MNs, there has been no study reporting successful in *vitro* differentiation of MNs from spinal cord progenitors. This study first characterized a polyclonal spinal cord stem cell line isolated from an eight-week fetus. Then a paradigm was introduced to successfully induce MNs from this cell line, which was demonstrated by the immuostaining using the MN markers HB9, Islet1 and ChAT. The combination of HB9 and ChAT (Choline Acetyl Transferase) immunostainings indicated that ~20% of the cells were MNs after this induction protocol. The presence of other cell types in the differentiated culture was also analyzed. Finally, the electrophysiological properties of these differentiated MNs were characterized to confirm their functional integrity. The majority of these MNs fired repetitive action potentials (APs), which is an indicator of functional maturation. The recordings of spontaneous Excitatory Postsynaptic Currents (EPSCs) confirmed the formation of synapses onto these MNs. This study reports the first successful differentiation of MNs from human spinal cord stem cells *in vitro*, providing a novel approach for obtaining functional MNs when designing the therapeutic strategy for spinal cord diseases or injuries.

Keywords

stem cell; motoneurons; cell therapy; cell culture; *in vitro*; transplantation; regenerative medicine

1. Introduction

An *in vitro* source of human motoneurons (MNs) derived from human stem cells would be of enormous benefit, not only for building *in vitro* culture systems to study MN differentiation and function, perform drug screening, but also for cell replacement therapy to treat spinal cord injury (SCI) and human MN diseases, including Amyotrophic Lateral Sclerosis (ALS) and Spinal Muscular Atrophy.

MNs can be obtained either by direct purification from tissues, or by specific induction from stem cells. Isolating MNs from human tissues is not practical for basic research, and even more difficult for therapeutic purposes due to obvious source limitations. Conversely, MNs

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Author Disclosure Statement: No competing financial interests exist.

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isolated from animal sources suffer from the issue of species variation. Since transformed cell lines sometimes do not truly reproduce native biological properties, primary cell culture from animal model sources are a better choice for some basic research studies. However, for studies directly related to human health issues, especially for clinical applications, human cell lines derived from various stem cell sources are the best starting point for obtaining MNs.

Three classes of stem cells or progenitor cells have been of interest in the generation of specific neuronal subtypes of MNs: neural progenitors derived from fetal or adult nervous tissue, non-neural progenitor cells derived from other organs or tissues, and embryonic stem cells. To date, MNs have been successfully induced from embryonic stem cells (Li et al., 2005; Shin et al., 2005; Lim et al., 2006; Lee et al., 2007). Cholinergic neurons have also been successfully induced from fetal cortical neural stem cells (Wu et al., 2002), and these cells, when transplanted into rat spinal cord, innervated muscle and some level of recovery from spinal cord injury was observed (Gao et al., 2005). Fetal spinal cord stem cells are much closer in lineage to spinal MNs than either embryonic stem cells or fetal cortical cells. However, there has not yet been a report of successful *in vitro* differentiation of either neural stem cells or progenitors of spinal origin to MNs.

NSI-566RSC, isolated from an 8-week fetal spinal cord, is a novel human neural stem cell line with robust growth properties and neurogenic potential. When grafted into rat spinal cord, the cells differentiated extensively into neurons and glia, secreted neurotrophic factors and formed synapses with the host neural cells, but not muscles (Yan et al., 2007; Xu et al., 2006). Spinal grafting of the undifferentiated cells from this cell line into rats with ischemic paraplegia partially rescued their motor function deficits (Cizkova et al., 2007), and delayed the onset and progression of ALS in Superoxide Dismutase 1 (SOD1) rats (Xu et al., 2006). 50–60% of the grafted cells differentiated into Glutamic Acid Decarboxylase (GAD)-positive inhibitory neurons at the 6-month timepoint. However, the percentage of ChAT (Choline Acetyl Transferase)-positive neurons in the ventral horn was very small (Yan et al., 2007). Thus, results from this cell line suggests that without further induction the intrinsic differentiation of spinal neural stem cells is unlikely able to generate MNs for treating motoneuron diseases and further that the injured adult spinal tissue lacks endogenous cues to direct motoneuron differentiation of exogenously supplied spinal neural stem cells. Therefore, in this study, we sought to develop an *in vitro* method to induce motoneuron phenotype in this cell line.

Several factors have been identified as essential for MN generation during embryonic development. Retinoic Acid (RA) is required for proper induction of neuroectoderm from embryonic ectoderm and caudalization of neuroectoderm to specify spinal cord (Maden, 2002; Irioka et al., 2005; Muhr et al., 1999). Sonic Hedgehog (Shh), secreted from the notochord and floor plate, is the essential signaling molecule for dorsoventral patterning across the spinal cord, as well as for the specification of MNs (Chiang et al., 1996; Marti et al, 1995; Briscoe and Ericson, 2001). Basic Fibroblast Growth Factor (bFGF) has been found to be involved in MN induction during development, and that bFGF alone or combined with other inducing factors, can induce MN differentiation from human embryonic stem cells (Shin et al., 2005) and increase cholinergic neurons from fetal cortical neural stem cells (Wu et al., 2002). Vitronectin (VN) is an extracellular matrix (ECM) glycoprotein that fulfills multiple functions in serum, and in both adult and embryonic tissues (Preissner, 1991). VN expression in the ventral neural tube is induced by N-Shh at the time of MN differentiation. The treatment of embryos with anti-VN antibodies almost completely inhibits MN formation, without altering other aspects of the dorsoventral patterning in chick embryos (Martinez-Morales et al., 1997). VN can bind to Shh and enhance Shh's motoneuron induction synergistically, probably by mediating proper

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presentation of Shh to differentiating motoneurons (Pons and Marti, 2000). However, all these information are either from chicken (Pons and Marti, 2000), mice (Wichterle et al., 2002), human embryonic stem cells, (Li et al., 2005; Shin et al., 2005) or cortical stem cells (Wu et al., 2002). It would be of great interest to determine if these factors are also effective in inducing MNs from human spinal cord stem cells.

In the previous studies on the generation of human MNs from either embryonic stem cells or neural progenitors, only a few have reported on the electrophysiological properties of the differentiated MNs. In one report, ChAT-positive cells induced from fetal cortical neural stem cells fired single Action Potentials (AP) during prolonged depolarization (Wu et al., 2002). Also, human MNs derived from embryonic stem cells fired single APs, decrementing APs (Li et al., 2005), or repetitive firings (Lee et al., 2007) during prolonged depolarization. However, the information about the basic electrophysiological properties of MNs derived from stem cells or neuroprogenitor cells is still very limited and has not been shown for MNs differentiated from fetal spinal stem cells.

In this study, we performed extensive immunocytochemical characterization of the spinal cord stem cell lines NSI-566RSC. The results indicated that most of the cells were at an early neural progenitor stage and could be readily propagated. A paradigm was then developed to generate MNs from this spinal cord stem cell line which was analyzed by immunocytochemistry. The electrophysiological properties of the differentiated human MNs were analyzed and the majority of these MNs fired repetitive Action Potentials (APs), an indicator of functional maturation. Synapse formation onto these MNs was also confirmed by recordings of spontaneous Excitatory Postsynaptic Currents (EPSCs). This is the first demonstration that MNs can be induced from spinal cord stem cells *in vitro*, and that they can be fully electrophysiologically active even *in vitro*.

2. Materials and Methods

2.1. Derivation and Culture of Human Spinal Cord Stem Cells

Human spinal cord stem cells were prepared from the cervical to upper thoracic region of spinal cord tissue obtained from a single 8-week human fetus after an elective termination. The fetal tissue was donated by the mother in a manner fully compliant with the guidelines of the National Institute of Health (NIH) and the Food and Drug Administration (FDA) and approved by an outside independent review board. The spinal cord tissue was removed of meninges and dorsal root ganglia and dissociated into a single cell suspension by mechanical trituration in serum-free, modified N2 media (N2B, Neurostem, Inc., Rockville, MD, USA). The initial culture was expanded as a monolayer culture in PDL (poly-D-Lysine)/ fibronectin-precoated flasks (T175, Nunc, Rochester, NY, USA) or plates (150 mm, Nunc) in N2 media with bFGF (10 ng/ml) as the sole mitogen (Johe et al., 1996). The first passage was conducted 16 days after plating. At this point, the culture was composed mostly of post-mitotic neurons and mitotic human spinal cord stem cells. The mitotic cells were harvested at approximately 75% confluence and passaged serially with trypsin (0.05% + 0.53 mM Ethylenediaminetetraacetic Acid (EDTA)). At various passages, the cells were frozen in the growth medium plus 10% Dimethyl Sulfoxide (DMSO) at $5\sim10\times10^6$ cells/ml using a programmable freezer. The frozen cells were stored in liquid nitrogen. The resulting cell line, produced by epigenetic means only and by using bFGF as the sole mitogen, was coded “566RSC”.

Karyotype analysis was conducted by Applied Genetics Laboratories, INC (Melbourne, FL, USA).

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2.2. Induction of Motoneurons

Passage 9 cells were used in this study. The induction procedure consists of three steps (Fig 2A). First for proliferation, one aliquot of cryopreserved cells (~3×10^6 cells) was seeded into a T175 flask precoated with PDL in N2B medium with bFGF (10 ng/ml) in the presence of fibronectin (1.5 ug/ml). The medium was changed every other day, while bFGF (10 ng/ml) was added on the days when media was not changed. The cells were proliferated for 6~8 days to 70~90% confluent. Next, they were trypsinized, replated onto chamberslides (d = 15 mm, Nunc) precoated with PDL and fibronectin at a density of $2\sim5 \times 10^5$/well. For motoneuron induction, the stem cells were first treated in priming media (Table 1) for 4 days. They were then switched into MN differentiation media (Table 1) for 6 days, or longer for complete differentiation. For priming and differentiation, the cells were fed every 2 days by changing half of the media.

2.3. DETA Surface Modification

For electrophysiological recordings, the cells were plated and differentiated on glass coverslips coated with DETA (a self-assembled monolayer (SAM) of N-1(3-(trimethoxysilyl) propyl) diethylenetriamine). Previous studies have proved that DETA surface supports neuronal growth as well as biological surfaces if not better (Das et al., 2003), and it has been shown to support the growth of both embryonic and adult MNs (Das et al., 2005; 2007). The differentiation pattern of the human spinal stem cells on DETA coverslips was similar to that on PDL/fibronectin coated chamber slides as to the numbers and morphology of MNs induced.

For making DETA coverslips, glass coverslips (6661F52, 22×22 mm No. 1; Thomas Scientific, Swedesboro, NJ, USA) were cleaned using HCl/methanol (1:1) for at least 2 hours, rinsed with water, soaked in concentrated H_2SO_4 for at least 2 hours and rinsed with water. Coverslips were boiled in nanopure water and then oven dried. The trimethoxysilylpropyldiethylenetri-amine (DETA, T2910KG; United Chemical Technologies Inc., Bristol, PA, USA) film was formed by the reaction of cleaned surfaces with 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (T2904; Fisher, Suwanne, GA, USA). The DETA coated coverslips were heated to ~80°C, then cooled to room temperature (RT), rinsed with toluene, reheated to approximately the same temperature, and then cured for at least 2 hours at 110°C. Surfaces were characterized by contact angle and X-ray photoelectron spectroscopy as described previously (Das et al., 2003, 2005, 2007).

2.4. Immunocytochemistry and Microscopy

Cells in chamberslides were fixed in freshly prepared 4% paraformaldehyde for 15 min. Cells were washed twice in Phosphate Buffered Saline (PBS) (pH 7.2, w/o Mg^{2+}, Ca^{2+}) for 10 min each at room temperature, and permeabilized with 0.1% triton X-100/PBS for 15 min. Non-specific binding sites were blocked with 5% Donkey serum plus 0.5% BSA in PBS for 45 min at room temperature. Cells were then incubated with primary antibodies overnight at 4°C. After being washed with PBS 3×10 min, the cells were incubated with secondary antibodies for 2.5 hours at room temperature. The cells were then washed with PBS 3×10 min and mounted with Vectorshield with 4′-6-Diamidino-2-Phenylindole (dapi) (Vector laboratories, Inc.). Primary antibodies used in this study includes: Rabbit-anti-Nestin (Chemicon, 1:200), Rabbit-anti-β III Tubulin (Sigma, 1:1000), Rabbit-anti-SOX1 (Chemicon, Millipore, Billerica, MA, USA, 1:100), Rabbit-anti-Olig2 (Chemicon, 1:500), Mouse-anti-GFAP (Chemicon, 1:150), Mouse-anti-O1 (Chemicon, 1:200), Goat-anti-ChAT (Chemicon, 1:100), Mouse-anti-MAP2 (Chemicon, 1:200), Mouse-anti-O4 (Chemicon, 1:100), Mouse-anti-GAD67 (Chemicon, 1:800). The monoclonal antibody against HB9 (81.5C10, 1:30), Nkx2.2 (74.5A5, 1:10), Islet1 (39.4D5, 1:10) developed by Thomas Jessell

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and PAX6 (1:10), PAX7 (1:10) developed by Atsushi Kawakami, were obtained from the Developmental Studies Hybridoma Bank which is under the auspices of the NICHD and maintained by the University of Iowa (Department of Biological Sciences, Iowa city, IA 52242). Secondary antibodies include: Donkey-anti-Goat-568 (Invitrogen, 1:250), Donkey-anti-Mouse-488 (Invitrogen, 1:250) and Donkey-anti-Rabbit-594 (Invitrogen, 1:250). All antibodies were diluted in Blocking Buffer. For HB9 and Islet1 immunostaining, 0.1% Triton was included in the Blocking Buffer and in the PBS for washing.

2.5. Electrophysiological Recording

Electrophysiological properties of spinal cord stem cell-derived motoneurons were investigated in cultures differentitated for 3–6 weeks using whole-cell patch-clamp recording techniques (Das et al., 2003). The recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope (Gao and Ziskind-Conhaim, 1998).

Motoneurons were identified visually under an infrared DIC-videomicroscope. The largest multipolar or round cells (15–25 μm diam) with bright illuminance in the culture were tentatively identified as motoneurons (Gao BX and Ziskind-Conhaim, 1995; Takahasi, 1978). Patch pipettes with a resistance of 6–10 MΩ were made from borosilicate glass (BF 150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller (Sutter Instrument Company). Current-clamp and voltage-clamp recordings were made utilizing a Multiclamp 700A amplifier (Axon, Union City, CA). The pipette (intracellular) solution contained (in mM) K-gluconate 140, $MgCl_2$ 2, Na_2ATP 2, Phosphocreatine 5, Phosphocreatine kinase 2.4 mg, Hepes 10; pH 7.2. For certain experiments, 1% Alexa-488 was added to the intracellular solution to label the recorded cells for post experimental identity confirmation. After the formation of a gigaohm seal and the membrane puncture, the cell capacitance was compensated. The series resistance was typically < 23 MΩ, and it was compensated > 60% using the amplifier circuitry. Signals were filtered at 3 kHz and sampled at 20 k Hz using a Digidata 1322A interface (Axon instrument). Data recording and analysis were performed with pClamp8 software (Axon instrument). Membrane potentials were corrected by subtraction of a 15 mV tip potential, which was calculated using Axon's pClamp8 program. Membrane resistance and capacitance were calculated using 50 ms voltage steps from −85 to −95 mV without any whole-cell or series resistance compensation. The resting membrane potential and depolarization-evoked action potentials were recorded in current-clamp mode. Spontaneous and depolarization-evoked inward and outward currents were examined in voltage-clamp mode. Spontaneous synaptic currents were acquired at a holding membrane potential of −70 mV.

2.6. Quantification

For morphological and immunocytochemical characterization of the undifferentiated cell line, the populations of cells expressing certain differential markers were quantified in two different ways based on their approximate number. In an average view under 200× magnification, if the numbers of marker-positive cells were consistently less than 10, all the maker-positive cells on the whole coverslip were counted. The total number of cells on the coverslip was estimated by multiplication of the cell-plating number with a propagation factor, which is 2 in this case, since the cells were fixed approximately 48 hours after plating. If the number of marker-positive cells was consistently more than 10 in one view under the microscope, at least 10 views were randomly chosen. All the marker-positive cells were counted as well as the total number of cells in these views. In the differentiated cultures, the quantification of motoneurons was only performed for unclustered cells. For each coverslip, at least 10 pictures were taken from randomly chosen unclustered areas, and the ratio of HB9-positive cells and ChAT-positive cells to those positive for dapi were

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quantified. For an approximate quantification of astrocytes and neuroprogenitors, the culture was first trypsinized to dissociate cell clusters, replated and counted the next day, since the Glial Fibrillary Acidic Protein (GFAP) and Nestin staining overlapped significantly which made it very difficult to identify individual cells.

At least three coverslips in each group were quantified and data were expressed as an average +/− st. dev. Statistical differences between different experimental groups were analyzed by Student's t-test.

3. Results

3.1. Cell Line Characterization

To establish the baseline characteristics for the cell line NSI-566RSC, expression of various spinal cord-relevant antigens was analyzed in the cultured cells 48 hours after plating and prior to overt differentiation or induction.

The cells were first stained for the expression of Nestin and β III Tubulin. Nestin is generally considered as a marker for neural stem cells in the developing central nervous system (Lendahl et al., 1990; Frederiksen et al., 1998). β III Tubulin is a marker for neuronal cells. As indicated in Fig 1A and Table 2, most of the cells (93.8%) expressed Nestin, while few (8%) expressed β III Tubulin. Nearly all those Nestin-negative cells were positive for β III Tubulin, suggesting that they have already differentiated into neurons since they have lost the progenitor marker Nestin. A few cells were positive for both Nestin and β III Tubulin, implying that they were in the process of this transition. Therefore, prior to induction, the vast majority of cells are still uncommitted to either neuron or glia lineage, which is also supported by the extremely low percentage of cells expressing GFAP or O1 which recognizes Galactosylcerebroside, markers of differentiated astrocytes or oligodendrocytes, respectively.

Cells were also examined for the expression of Sox1, one of the earliest transcription factors expressed in ectodermal cells committed to a neural fate. During development, its expression coincides with the induction of the neuroectoderm and ceases when cells exit mitosis (Pevny et al., 1998). All of the cells were positive for Sox1, confirming that they were of early neuroectodermal origin.

Since these cells were isolated from developing spinal cord, it was also of interest to determine how many of the markers normally found in the dorso-ventral gradient during development were present in this cell line. Thus, the cells were analyzed for the expression of several LIM homeodomain transcription factors: Pax6, which is usually expressed in the majority of cells located in the middle to dorsal regions of the spinal cord; Pax 7, a dorsally expressed transcription factor; and Olig2 and Nkx2.2, ventrally expressed transcription factors (Ericson et al., 1997). 64% of the cells expressed Pax6, while none expressed Pax7 or Nkx2.2. Olig2 is a marker for MN and oligodendrocyte progenitors, and HB9 is a hallmark of postmitotic MNs. Very few cells in the uninduced cell line expressed either Olig2 (0.75%) or HB9 (0.018%).

Karyotype analysis was performed to determine if the genetic integrity of this cell line has been maintained through passage 9. As indicated in Fig 1B, the cells examined have 22 pairs of autosomes and 1 pair of sex chromosomes (XY). The analysis of 100 cells did not find any chromosomal aberrations.

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3.2. Induction of Motoneurons

To determine the baseline condition, the proliferating cells were harvested and replated into NSDM media in the absence of any MN induction. NSDM media is a DMEM-based, N2-like defined media without glutamate. Morphological observation indicated that cell proliferation stopped immediately and differentiation was initiated. On both day 2 and day 7 after replating, ~50% of the cells were identified as neurons based on β III Tubulin staining, and very few were HB9-positive (< 0.1%). In a previous study, when the undifferentiated cells were implanted into the rat ventral spinal cord, >= 75% of the cells were positive for β III Tubulin 3 months later, but cells identified as MNs were less than 1% (Yan et al., 2007). Therefore intrinsically, or without any specific MN induction, very few cells demonstrated the capability to become MNs.

Experiments were then initiated to determine if this fetal stem cell line could be induced to generate MNs. The cells were treated with combinations of trophic factors and/or chemicals that had previously been shown to be important in the development of MNs including: Shh (Chiang et al., 1996; Marti et al, 1995; Briscoe and Ericson, 2001), RA (Maden, 2002; Irioka et al., 2005; Muhr et al., 1999), bFGF (Shin et al., 2005; Wu et al., 2002), and vitronectin (Martinez-Morales et al., 1997; Pons and Marti, 2000). Each experiment was conducted in three phases: proliferation, priming and differentiation (Fig 2A). In differentiation media, slight proliferation of cells was still observed in the initial 1~2 weeks. After this time frame, the cultures were stable and cell differentiation was clearly observed even by morphological observation alone (Fig 2B). To analyze the MN number after differentiation, the cells were fixed and examined for the expression of MN markers HB9, Islet1, and ChAT 6 days after switching to differentiation media, unless otherwise specified.

Shh, RA, vitronectin and bFGF were first tested individually for their ability to affect MN induction by inclusion in the priming media, and the results were determined utilizing HB9 immunostaining (Fig 2C–F). Each individual induction factor showed significant enhancement of MN induction in the cultures (Fig 2F) when added to NSDM during priming. In addition, different combinations of these factors were tested to make sure that they do not antagonize each other during MN induction. Then all the effective factors were combined and added to the priming media to determine their collective ability to direct the cells to a MN fate.

For each of the tested formulations of factors added to the priming media the number of neurons was analyzed by immunostaining for both β III Tubulin and Microtubule Associated Protein 2 (MAP2). Both indicate that ~50% of the cells became neurons, similar to the number obtained under intrinsic differentiation condition (Table 4).

MN induction was first examined with HB9 immunostaining (Fig 2). Co-immunostaining with β III Tubulin indicated that all HB9 signals were located in the nuclei of neurons (Fig 2E). Intrinsically, or without any MN induction, very few cells became HB9-positive (< 0.1%). If cells were primed with NSDM alone but differentiated in the differentiation media as described above, 1.83 +/− 0.75% of the cells became HB9-positive. In the presence of all the inducing factors, followed by the differentiation as above, 14.51 +/− 6.12% of the cells were HB9-positive on day 10 ($P<0.0005$, Fig 2F, Table 3).

In addition to HB9, Islet1 is also used as a common MN marker during development. As in Fig 3A, the number of Islet1-expressing cells was also greatly increased after induction. Increased Islet1 staining was observed in both clustered and non-clustered cells. Co-immunostaining with β III Tubulin confirmed the nuclear localization of Islet1.

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MN induction was also examined using ChAT immunostaining (Fig 3B, C). The expression of ChAT suggests the synthesis of acetyl choline, the neurotransmitter synthesized by MNs and released at the neuro-muscular junction. Very few cells expressed ChAT intrinsically. After induction, 7.39 +/− 0.8% of the cells were ChAT positive on day 10. Although only non-clustered cells were quantified, increased ChAT-positive cells were observed in both clustered and non-clustered cell populations. Optical sections from confocal microscope indicated that ChAT staining is specifically located inside the soma as expected. Colocalization of ChAT and HB9 was found in some cells when ChAT-HB9 coimmunostaining was employed (Fig 3D), but not in all the cells, suggesting that the expression window of these two MN markers do not completely overlap.

The temporal expressions of HB9 and ChAT in the culture were then examined. On day 10, ~14.51% of the cells were HB9-positive, while ~7.39% of the cells were ChAT-positive. On day 20, both HB9-positve and ChAT-positive cells increased slightly to ~17% and ~10.96% respectively. On day 35, HB9-positive cells decreased to ~4.78%, while ChAT-positive cells increased to ~16.07%. This agrees with a previous study which observed that HB9 expression was initiated earlier than ChAT expression but gradually disappeared later in the culture (Li et al., 2005). Combining the percentage of HB9-positive cells with that of ChAT-positive cells that were not co-stained with HB9, the total number of MNs in our culture was approximately 20%. In agreement of this, one culture that survived to day 70 showed ~30% of the cells as ChAT-positive, although the contribution of selective survival could not be excluded in this case.

In addition to the analysis for MN induction, immunocytochemical analysis was performed to determine the number and type of other cells present in the culture after induction. Based on the analysis on day 10 after induction the induced culture also contained astrocytes as determined by GFAP immunostaining (Fig 4A, ~25%), oligodendrocytes, based on O4 immunostaining (Fig 4B, < 1%) and undifferentiated neuroprogenitors, based on Nestin immunostaining (Fig 4C, ~25%). Nestin-positive cells gradually disappeared as the culture matured.

It has also been shown previously in an *in vivo* study that a significant number of cells became GABAergic neurons after being implanted into rat spinal cord (Yan et al., 2007; Xu et al., 2006; Cizkova et al., 2007). Also, that when these cells were cocultured on a rat astrocyte monolayer, in the absence of any specific induction, 45 +/− 6% of neurons became GABAergic (Cizkova et al., 2007). However, under the conditions we have outlined for *in vitro* MN differentiation, only a few GABAergic neurons were found (<0.1%), based on GAD immunostaining (Fig 4D).

3.3. Electrophysiology Evaluation of MNs Differentiated from NSI-566RSC

The functional maturation of these differentiated MNs was assessed by electrophysiological evaluation. Single voltage clamp analysis revealed that voltage-dependent currents could be invoked (Fig 5A). The delay and dynamics of the inward and outward currents were consistent with sodium and delayed rectifier potassium currents. The maximum inward and outward currents reached values of 4967pA and 1057 pA, respectively. Similar recordings from undifferentiated stem cells showed much smaller inward and outward currents (Fig 5B).

Prolonged stepped current clamp recordings (0–100 pA x 1s) indicated that 17/17 of recorded cells had elicited APs, among which, 4/17 of cells recorded fired single APs and 13/17 recorded cells displayed repetitive firing, while recordings from undifferentiated stem cells displayed no APs (Fig 5C, D). The maximum firing frequency was 17 Hz. Single APs elicited by a brief saturating depolarization current usually had an amplitude of ~150 mV

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(Table 4, Fig 5F). For a positive control, it was difficult to compare to other human-derived MNs, due to the unavailability of primary human MNs and the absence in the literature of electrophysiological characterization for MNs derived from human embryonic stem cells. Therefore, the electrophysiological analysis from rat MNs isolated from E14 embryonic spinal cord was utilized for comparison in Table 4 (Das et al., 2003). These MNs were cultured and analyzed by electrophysiology under comparable conditions. The actual recording profile was similar to human MNs described in this study. Most parameters between rat MNs and human MNs showed no significant difference, except for the outward K^+ current, which may be due to a species difference. Average repetitive firing frequency and AP amplitude were not listed for rat eMNs because the recording and analysis criteria were different in the cited study.

Spontaneous inward and/or outward synaptic currents were recorded at −70 mV using K-gluconate-based intracellular solution. Miniature inward currents were recorded with an amplitude of ~18 pA and a duration of ~10 ms (Fig 5G). The recorded spontaneous EPSCs indicated that functional synapses were formed between other neurons and the MNs in the culture. To confirm the MN identity of the recorded cells, the dye Alexa-488 was included in the intracellular solution during the recording from certain patched cells, which was followed immediately by ChAT immunocytological analysis (Fig 5H). Although not all the patched cells remained on the coverslip after the recording and subsequent immunostaining procedures, ChAT staining analysis demonstrated that all the cells that survived this process were MNs in agreement with our morphological identification protocols.

4. Discussion

This study reports the first *in vitro* induction of MNs from a polyclonal human fetal spinal cord stem cell line. The cell line NSI-566RSC is a stem cell line isolated from the spinal cord of an eight-week fetus. Our result indicates that intrinsically this cell line generates almost no MNs *in vitro. In vivo* studies have also demonstrated that these cells, without any *ex vivo* induction, when implanted into rat spinal cord, predominately did not differentiate into MNs, but tended to become GABAergic or Glutamergic neurons (Yan et al., 2007; Xu et al., 2006; Cizkova et al., 2007). However, with this induction procedure, 20% of the cells can become MNs, with very few GABAergic neurons observed. These results suggest that the protocol described here is very effective in tuning the differentiation of the neural stem cells isolated from fetal human spinal cord towards MNs.

The finding that 93.8% of the undifferentiated cells are Nestin-positive indicates that this stem cell line mainly consists of neural progenitor cells before the induction procedure. Also, these cells are proliferation-active based on the fact that all the cells were Sox1-positive. Both of these results indicated that this cell line mainly consists of neuroepithelial cells at a very early stage of CNS development, which may be pluripotent for multiple cell types. During development, MNs are produced in a ventrally restricted region of the spinal cord and are generated earlier than more dorsal neuronal subtypes (Price and Briscoe, 2004). Since MNs can be effectively induced from this cell line, it is reasonable to speculate that this cell line could also give rise to other cell types as well under proper induction procedures.

The protocol used to induce MN differentiation in this study contained Shh, RA, VN and bFGF, which had been reported to be important for MN differentiation in both non-human and human embryonic stem cells as well as human cortical stem cells. This study demonstrates for the first time that the combination of these factors is also effective in inducing MN differentiation from neural stem cells of developing human spinal cord. Moreover, the involvement of VN in MN induction during development has been mostly

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studied in chicken embryos (Martinez-Morales et al., 1997; Pons and Marti, 2000). Our study for the first time demonstrated its effectiveness in MN induction in human stem cells.

Since this is a polyclonal stem cell line and to ensure that cells at different differentiation stages have an equal opportunity of being induced, Shh and RA were included throughout the differentiation stage, but kept at a lower level. To support the long-term differentiation and survival of the MNs, the differentiation media also included GDNF, BDNF, CNTF, NT-3/4, cAMP, IGF-1 and VN. All of these factors have been reported to be important for MN differentiation, by increasing MN number, enhancing ChAT expression, promoting MN survival, and/or neurite growth either *in vitro* or *in vivo* (Dutton et al., 1999; Zurn et al., 1996; Hughes et al., 1993; Oorschot and McLennan, 1998; Hanson et al., 1998; Henderson et al., 1994). The effectiveness of the differentiation media composition was confirmed by two experimental results in this study. First, and most important, was the ability of the differentiated MNs to exhibit repetitive firing during prolonged depolarization. Repetitive APs have only been observed in MNs of postnatal rats but not from embryonic MNs (Gao and Ziskind-Conhaim, 1998). The appearance of repetitive APs in the MNs differentiated from NSI-566RSC suggests that the density of multiple voltage-gated ion channels (Na^+, K^+, Ca^{2+}), especially L-type Ca^{2+} channels on these cells, has already reached levels comparable to postnatal stage (Miles et al., 2004; Johnson et al., 2007; Gao and Ziskind-Conhaim, 1998), and that these induced MNs may be physiologically mature. Second is that the differentiation media could support the culture for up to 70 days, which allowed sufficient neuronal maturation for investigation of the functionality of these differentiated MNs.

The MN differentiation paradigm was developed based on the literature from both findings concerning MN lineage differentiation during native development (Briscoe and Ericson, 2001; Maden, 2002; Wichterle et al., 2002; Zurn et al., 1996; Price and Briscoe, 2004) and reports for MN *in vitro* differentiation from embryonic stem cells (Li et al., 2005; Shin et al., 2005; Lim et al., 2006; Lee et al., 2007). In principle, this paradigm should work for any stem cell line isolated from human fetal spinal cord at a similar age, which has been confirmed by our testing of another fetal spinal stem cell line, NSI-G044MSC, which exhibited the same properties as the differentiated NSI-566RSC line (data not shown). For stem cell lines isolated at earlier developmental stages, the procedures for caudalization and/or neural induction could be added depending on the fetal stage. For those isolated at later developmental stages, some factors in the priming media may be eliminated as those genes affected may not be active. As for stem cells isolated from other sources such as mesenchymal or even adult tissues, since their differentiation lineage is different compared to fetal spinal cells, their differentiation pathway towards MNs (if there are any) could be dramatically different, which may require a new set of induction factors. However, if MN progenators are able to be induced from these cell lines, it could be worthwhile to utilize the differentiation media developed in this study, which was designed for physiologically maturation of MNs and their long term maintenance *in vitro*.

To develop therapeutic strategies for spinal cord diseases or injuries, having a readily available human MN source is essential whether for *in vitro* or *in vivo* applications. This is especially true now that the drug discovery process is moving more toward functional human based *in vitro* systems. Examples of *in vitro* model culture systems would be the co-culture of MNs with myotubes for neuromuscular junction model systems and the co-culture of MNs with oligodendrocytes or Schwann cells for myelination model systems. Human cell based *in vitro* systems would be powerful tools to study the regulation of MN survival, function and regeneration for use in high-throughput drug screen systems. For clinical applications, human derived MNs are essential as they represent the major integrators of synaptic inputs in the spinal cord, and the central area of focus in neurodegenerative diseases

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or spinal cord injuries. Unless the cells are derived from the patient however, some type of immunosuppression will be necessary for the clinical application irregardless of whether the stem cells are from embryonic, fetal, adult or some other source (Bongso and Fong, 2009). However, the use of fetal stem cells or other partially differentiated cells may be better for clinical use than embryonic stem cells due to a much reduced threat of teratoma formation. Conversely, fetal and other committed lines would be limited in the number of passages possible while embryonic stem cells would not have this limitation.

Increasing the efficiency of *in vivo* MN differentiation could improve the therapeutic potential of the cell supplementation approaches. Many spinal grafting studies in animal models have utilized implanted stem cells without *ex vivo* induction, instead depending on *in vivo* environment cues to drive pluripotent stem cells towards a MN fate. Studies with the cell line NSI566RSC have demonstrated that *in vivo*, these cells give rise to very few MNs and this finding is independent of whether the spinal cord is normal, diseased or subjected to traumatic injury (Yan et al., 2007; Xu et al., 2006; Cizkova et al., 2007). The lack of MN differentiation from these cells after implantation strongly suggests that spinal tissues from adult rat lack the environmental cues necessary for MN induction from human spinal stem cells. This in turn implies the necessity of *ex vivo* induction in spinal repair strategies. This study may help to further improve the *in vivo* quantity of MNs by differentiating the cells to MNs before grafting or inducing MNs during or after grafting by supplementing with the necessary factors such as Shh and RA, and therefore obtain better therapeutic effect for future human clinical trials.

The generation of MNs has been well explored with embryonic stem cells (Li et al., 2005; Shin et al., 2005; Lim et al., 2006; Lee et al., 2007). In our study, ~20% of the cells become MNs and this is comparable to similar studies with embryonic stem cells, in which the percentage of MNs achieved was ~ 21%, and 20–30% respectively for human and mouse embryonic stem cell studies (Li et al., 2005; Wichterle et al., 2002). However, with the fetal stem cell line utilized here, the differentiation period is much shorter (10 days compared to 40 days in Li et al., 2005). We speculate that the major reason for this difference could be that this is a spinal cord derived stem cell line, which is closer to spinal MNs in lineage compared to embryonic stem cells. Therefore, the induction procedure is simpler, in which only ventralization is the focus, whereas for the induction to MNs from embryonic stem cells, both neural induction and caudalization need to be considered. The differentiation period (~10 days) is comparable to the timeframe for ventralization in the previous study with embryonic stem cells (Li et al., 2005).

In conclusion, this study reports the first paradigm for inducing MNs from human fetal derived spinal cord stem cells. The fast and simplified paradigm could facilitate a new avenue for obtaining MNs for *in vitro* and *in vivo* applications. The protocols and media compositions developed in this study are all essential elements for building defined, serum-free *in vitro* culture or engineered systems to study the physiology of MNs and can be useful for developing therapeutic strategies for spinal cord injuries or diseases. This work could also be especially important for future cell transplantation studies for various MN diseases. Also, the protocol and media composition developed in this work provides valuable guidance for differentiating MNs from other cell lines along the same differentiation pathway. The electrophysiological characterization of differentiated MNs also provides a reference for baseline comparison for the function of human MNs in other future *in vitro* studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments

We appreciate Dr. Stephen Lambert for his continual support and for constructively appraising the manuscript. Funding support for this research was provided by NIH grant number 5R01NS050452.

This research was funded by NIH grant 5R01NS050452.

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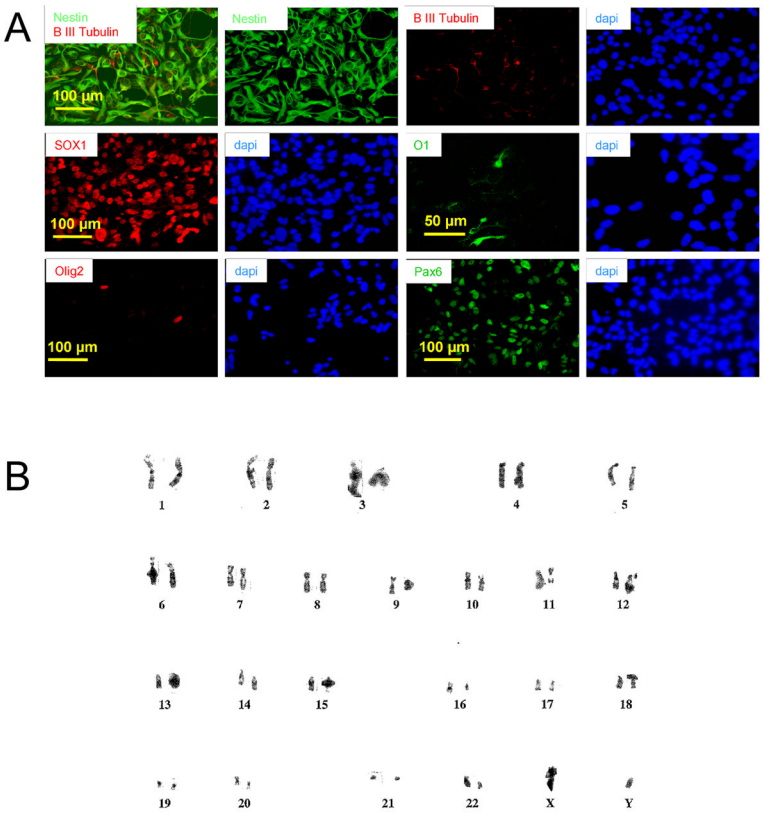

Figure 1. Characterization of fetal spinal cord stem cell line NSI-566RSC

A. Immunostaining with various developmental markers. The expression of developmental markers was demonstrated for cells grown after 48 hours in culture without any induction for cell differentiation.

B. Karyotype analysis of the cell line NSI-566RSC.

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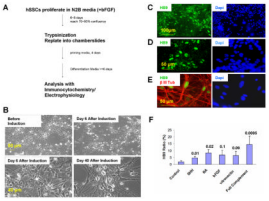

Figure 2. MN differentiation from spinal cord stem cell line NSI566RSC

A. This schematic outlines the procedures of MN induction from the stem cells.

B. Phase contrast pictures of the cultures before and after induction of MN differentiation. After induction, the cells gradually changed from a uniform shape into a variety of soma and dendritic morphology during a differentiation process.

C and D. HB9 immunostaining. Views under two different magnifications were presented to give a better impression of induced MNs in the culture. The total number of cells was indicated by dapi staining.

E. Double immunostaining of HB9 (green) and β-III Tubulin (red) in a culture after 12 days differentiation confirmed the neuronal identity of HB9-positive cells and nuclear localization HB9 signals.

F. Histogram of the ratio of HB9-positive cells after different priming conditions. Cells were primed for 4 days in NSDM containing different induction factors or combination of them, followed by a 6 day time period in differentiation media. All factor conditions induced significant increase of MN ratio compared to control. Correspondent t-test P-value was listed on the top of each bar (one-tailed, unequal variance).

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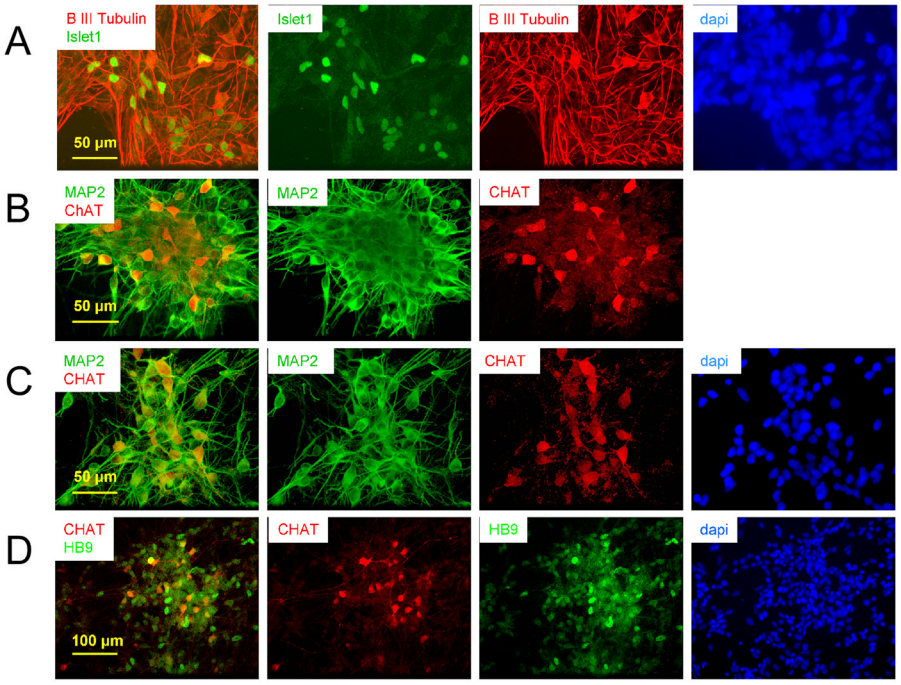

Figure 3. Differentiated MNs demonstrated by Islet1 and ChAT immunostaining. All cultures were analyzed on day 10 after induction unless specified

A. Islet1-positive cells in differentiated cultures. Islet1 increase was observed after differentiation. Double immunostaining of Islet1 (green) and β-III Tubulin (red) confirmed the neuronal identity of HB9-positive cells and nuclear localization Islet1 signals.

B and C. Coimmunostaining of ChAT and MAP2 in differentiated cultures. The cells were co-stained with MAP2 Ab to indentify neurons vs. glia. Abundant ChAT-positive cells can be observed both in clusters (B) and in discrete cells (C).

D. Differentiated cultures are immunostained for ChAT (red) and HB9 (green). Some of the ChAT-positive cells and HB9-positive cells were colocalized.

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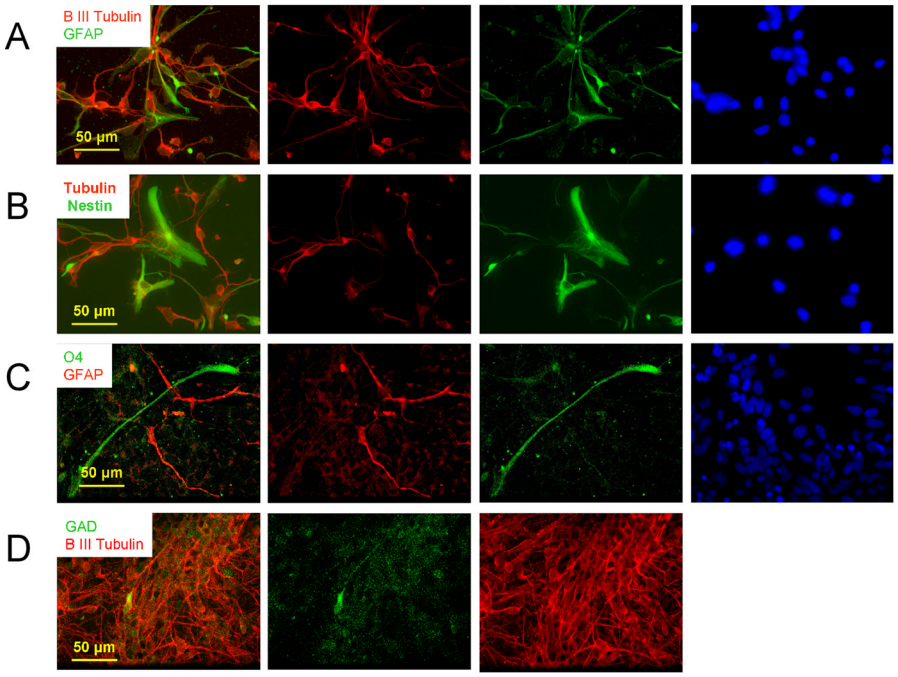

Figure 4.

The analysis of cell types other than MNs in differentiated cultures by immunostaining with specific cellular markers. The cultures were analyzed 10 days after differentiation for the presence of astrocytes by GFAP staining (A), neuroprogenitors by Nestin staining (B), oligodendrocytes by O4 staining (C), and GABAergic neurons by GAD staining (D). The cells that are positive for GFAP, Nestin are not positive for β-III Tubulin. The cells that are O4-positive are not positive for GFAP. All GAD-positive cells are also positive for β-III Tubulin. All these observations confirmed that these stainings are cell-type specific.

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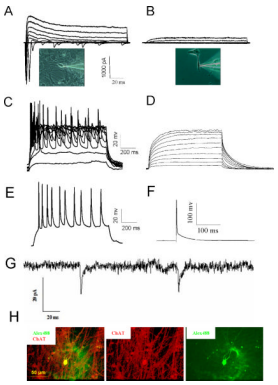

Figure 5. Electrophysiological recordings from differentiated MNs and undifferentiated stem cells

A. An example trace of active Na^+ and K^+ currents from a voltage clamp recording of a differentiated neuron. The insert picture indicates the recorded neuron.

B. An example trace of active Na^+ and K^+ currents from a voltage clamp recording of an undifferentiated cell (at the same scale as in A). The insert picture indicates the recorded cell.

C. An example of a repetitive firing action potential from a current clamp recording of a differentiated neuron.

D. An example of a current clamp recording from an undifferentiated cell (at the same scale as in C).

E. An example trace of repetitive firing action potentials from figure 5C.

F. An example trace of an Action Potential (AP) elicited when the cell received a saturated stimulus (2 msec 500pA inward current).

G. Spontaneous EPSCs from an induced MN that indicates it has been synapsed by other neuron(s) in the culture.

H. ChAT staining to confirm the identity of a recorded cell as a MN, which was labeled with Alexa 488 during recording from the intracellular solution.

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Table 1

Composition of Priming Media and Differentiation Media.

Component	Full Name	Concentration	Company	Catalog Number
Priming Media				
NSDM	Neuralstem Differentiation Media		Neuralstem Inc.	
Shh	Sonic Hedgehog, N-terminal peptide	100 ng/ml	R&D	1845-SH-025
RA	Retinoic Acid	0.01-1 uM	Sigma	R2625
bFGF	Fibroblast Growth Factor-basic	10 ng/ml	Cell Sciences	CRF001A
cAMP	Adenosine 3′ 5′-cyclic monophosphate	1 uM	Sigma	A9501
vitronectin		100 ng/ml	Sigma	V8379
Differentiation Media				
Neurobasal/Neurobasal A			Invitrogen	10888/21103
B27 (50X)		1X	Invitrogen	17504-044
Glutamax (100X)		1X	Invitrogen	35050
GDNF	Glial-derived Neurotrophic factor	10 ng/ml	Cell Sciences	CRG400B
BDNF	Brain-derived Neurotrophic factor	20 ng/ml	Cell Sciences	CRB600B
Shh	Sonic Hedgehog, N-terminal peptide	50 ng/ml	R&D	1845-SH-025
RA	Retinoic acid	0.1 uM	Sigma	R2625
IGF-1	Insulin-like Growth Factor -I	10 ng/ml	PeproTech	100-11
cAMP	Adenosine 3′,5′-cyclic monophosphate	1 uM	Sigma	A9501
CNTF	Ciliary Neurotrophic factor	5 ng/ml	Cell Sciences	CRC400A
NT-3	Neurotrophin-3	20 ng/ml	Cell Sciences	CRN500B
NT-4	Neurotrophin-4	20 ng/ml	Cell Sciences	CRN501B
Vitronectin		100 ng/ml	Sigma	V8379

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Table 2

Percentage of cells with different developmental markers in undifferentiated cells.

Developmental Markers (Number of Coverslips)	Percentage (%)	STDEV
Nestin (4)	93.8	0.82
β III Tubulin (4)	8.03	1.58
Sox1 (5)	100	0
Pax6 (3)	64.25	7.99
Pax7 (2)	0	
Nkx2.2 (2)	0	
Olig2 (4)	0.75	1.07
HB9 (3)	0.018	0.016
Islet1 (3)	0.203	0.198%
GFAP (3)	0.0017	0.0019
O1 (2)	0.055	

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Table 3

The percentage of cells positive for a range of neural cell lineage markers.

	Undifferentiated	Differentiated NSDM only	Differentiated: NSDM ↓ differentiation media	Differentiated: priming media ↓ differentiation media
β III Tubulin	8.03+/−1.58%	50%	50%	50.65+/−2.04% *
HB9	0.018+/−0.016%	<0.1%	1.83 +/− 0.75%	14.51 +/− 6.12% *
ChAT	0			7.39 +/− 0.76% *
Islet1	0.203+/−0.198%	0.4% (2)		18.21+/−8.51 % *
GFAP	0.0017 +/− 0.0019%			24.9% (1)
Nestin	93.8 +/− 0.82%			24.7% (1)
O4	0			<0.01% (2)
GAD	0			<0.01% (2)

Data show mean+/−STDEV for three coverslips unless specified in brackets.

* indicates significant difference between differentiated and undifferentiated cells at P<0.001 by Student's t-test (one-tailed distribution, unequal variance).

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Table 4

Electrophysiological analysis of the differentiated MNs.

	Resting Membrane Potential (mV)	Membrane Resistance (MΩ)	Membrane Capacitance (pF)	Number of Repetitive Action Potentials	Inward Current (pA)	Outward Current (pA)	Action Potential Amplitude (mV)
Undifferentiated	−50.9+/− 9.0 (10)	1082+/− 399 (9)	19.74+/− 14.07 (10)	0+/−0 (9)	−45.1+/−36 (10)	184.3+/− 80.9 (10)	0+/−0 (10)
After MN Differentiation	−43.75+/− 8.9 (14)	639.75+/− 287.07 (16) *	16.63+/− 9.24 (12)	5.94+/− 5.08 (17) *	− 1879.09+/−1136.01 (17) *	347.04+/ − 257.58 (17)	151.7+/− 18.76 (10) *
Rat eMNs (E14)	−49.7+/− 7.5	419+/−213	18.5+/− 20.1		−3541+/− 1718	4536+/− 2572	

Data show mean+/−STDEV (No. of cells analyzed). Student's t-test was performed to analyze the significant difference between differentiated and undifferentiated cells (two tailed distribution, unequal variance).

*indicates the significant difference at P<0.01. The data from last row about rat embryonic MNs (eMNs) are cited from Das et al (2003).