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Directed differentiation of neural-stem cells and subtype-specific neurons from hESCs

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Abstract

We describe a chemically defined protocol for efficient differentiation of human embryonic stem cells (hESCs) to neural epithelial cells and then to functional spinal motor neurons. This protocol comprises four major steps. Human ESCs are differentiated without morphogens into neuroepithelial cells that form neural tube-like rosettes in the first two weeks. The neuroepithelial cells are then specified to Olig2-expressing motoneuron progenitors in the presence of retinoic acid (RA) and sonic hedgehog (SHH) in the following 2 weeks. These OLIG2 progenitors generate post-mitotic, HB9 expressing motoneurons at the 5th week and mature to functional motor neurons thereafter. The protein factor SHH can be replaced by a small molecule purmorphamine in the entire process, which may facilitate potential clinical applications. This protocol has been shown equally effective in generating motor neurons from human induced pluripotent stem (iPS) cells.

Keywords

stem cells; motor neuron; spinal cord; neural differentiation; motor neuron disease; neuro-muscular junction

1. Introduction

Directing human embryonic stem cells (hESCs) to specific lineages is prerequisite for using hESCs to model early human development and for applying the hESCs derived lineages in clinic. In the past decade various protocols have been presented to differentiate hESCs to neuroectodermal cells (1,2) including the spinal motor neurons (3–5). These differentiation protocols vary considerably in the starting hESCs, feeder cells, unknown factors (e.g., sera and conditioned media), efficiency, and cell purity (6). We developed a series of neural differentiation protocols, including the one described here, for two objectives: modeling the early human brain development; and producing enriched/pure populations of functional neural cells for therapeutics.

The protocol was devised based on the developmental principle underlying motoneuron development. Spinal motor neurons are differentiated from neuroepithelial (NE) cells in a very narrow band of the ventral neural tube called the pMN domain, where the progenitors express the helix-loop-helix transcription factor Olig2. These Olig2-expressing progenitors are specified in the presence of a particular amount of sonic hedgehog (Shh) that is released from the notochord and subsequently the floor plate (7,8). Through interaction of Olig2 and neurogenic transcription factors including Ngn2 and Pax6, the Olig2-expressing progenitors

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differentiate to post-mitotic motor neurons during the neurogenesis phase and express motoneuron-specific transcription factors such as HB9 and Isl1 while downregulating Olig2 (9–12). Thereafter, HB9-expressing motoneurons mature and express choline acetyltransferase (ChAT), an enzyme that catalyzes the synthesis of the transmitter acetylcholine for transmitting signals through the neuromuscular junctions.

Generation of spinal motoneurons from hESCs follows the same basic steps of neuroectoderm induction, motoneuron progenitor specification, differentiation and maturation of post-mitotic motoneurons (Fig. 1). hESCs are removed from the mouse embryonic fibroblast feeder to initiate differentiation. In the serum-free culture condition, these hESCs differentiate to the neuroectoderm fate in two weeks (13). During the neural induction phase, the hESC aggregates are reseeded from day 7 onto a culture surface free of feeder to form individual monolayer colonies, allowing an even exposure to morphogens and a synchronized differentiation of the neuroepithelia. By the end of the 2nd week (day 14–17), NE cells, in the readily identifiable neural tube-like rosettes (1), develop. They express a panel of neuroectoderm transcription factors including PAX6 and SOX1.

NE cells generated in this way bear an anterior phenotype by expressing OTX2 (13). Hence, it is necessary to caudalize and ventralize the NE cells in order to generate spinal motor neurons. We found that early NE cells at day 10, also referred to as primitive NE cells (13), present higher competence to respond to morphogens including RA (3,14). Therefore, the anterior NE cells are patterned with retinoic acid (RA) and SHH for the subsequent 2 weeks. This treatment results in the induction of OLIG2-expressing ventral spinal progenitors in the 4th week. These OLIG2 cells become post-mitotic in the 5th week and express MN transcription factors like HB9 and ISL1. The MNs, when growing on substrate, extend substantial projections and express distinctive ChAT, indicating gradual maturation. When co-cultured with myoblasts, these hESC-derived MNs form characteristic neuro-muscular junctions. The 5-week in vitro differentiation process coincides with the appearance of motor neurons in the ventral horn of the developing human spinal cord at the 5–6th week.

The protocol is the modification of our previous reports (3,14). Major modifications include streamlined procedure, simplified media, the use of more potent recombinant SHH (resulting from a mutation at the N-terminus), and application of small molecules capable of activating SHH signaling in human cells. The optimized protocol typically generates about 50% of HB9 expressing motoneurons among the total hESC progenies. This protocol has recently been tested effective for differentiating human iPS cells to spinal motor neurons.

2. Materials

2.1. Stock solutions

1. **ACCUTASE** (Innovative Cell Technology, San Diego, CA, cat. no. AT104): ready to use.
2. **Ascorbic acid (200ug/ml)**: Dissolve 2 mg ascorbic acid in 10mL PBS. Aliquot and store at –80°C.
3. **B27 supplement without vitamin A**: 50× (Invitrogen, cat. no. 12587-010).
4. **BDNF, GDNF, IGF1 (100 µg/ml)**: Dissolve 100 µg of growth factor in 1 ml sterilize distilled water, aliquot and store at –80 °C.
5. **beta-Mercaptoethanol (14.3 M)**: ready to use.
6. **Boric acid buffer (pH 8.4)**: In 100 ml distilled water add 0.927 g H₃BO₃ and 0.6 g NaOH. Adjust pH to 8.4 by adding HCl.

7. **Bovine serum albumin (BSA):** Dissolve 50 ug BSA powder in 50 ml PBS, filter and store at -80°C .
8. **Cyclic AMP (1 mM):** Dissolve 4.914 mg cyclic AMP in 10 ml sterilized water. Aliquot and store at -80°C .
9. **Dispace (1 U/ml):** Dissolve 50U Dispace (Invitrogen; cat. no. 17105-041) in 50 ml F12/DMEM. Warm at 37°C for 15 min. Filter with a 50 ml-Steri-flip. Be aware that the right amount of 50 U dispace varies among lots.
10. **Dulbecco's modified eagle medium: Nutrient mixture F-12 1: 1** (DMEM/F12, Invitrogen, cat. no. 11330). Ready to use.
11. **FGF2 (100 $\mu\text{g/ml}$):** Dissolve 100 μg bFGF in 1 ml sterilized PBS with 0.1% bovine serum albumin (BSA)
12. **Heparin (1mg/ml):** Dissolve 10mg heparin in 10 mL DMEM medium, aliquot and store at -80°C .
13. **Knockout serum replacer** (Invitrogen; cat. no. 10828). Store stock in -80°C . Make aliquots of 50 mL and store at -20°C if it cannot be used up in a week after thaw.
14. **Laminin from human placenta:** Ready to use.
15. **L-Glutamine solution** (200 mM). Ready to use. Make aliquots of 5 ml and store at -20°C .
16. **MEM non-essential amino acids solution 100 $\times$** (Invitrogen, cat. no. 11140); ready to use.
17. **N2 supplement 100 $\times$** (Invitrogen, cat. no. 17502-048).
18. **Poly-L-Ornithine 10 $\times$ (1 mg/ml):** Add 0.1 g poly-L-ornithine to 100 ml pH 8.4 boric acid buffer. Filter through a 0.22 μm teflon filter.
19. **Purmorphamine (10 mM):** Dissolve 5mg purmorphamine in 480 μl ethanol and 480 μl DMSO, aliquot and store at -20°C . The working concentration range of purmorphamine is very narrow. Prepare the stock solution as accurately as possible. When adding stock solution into the culture medium, use the smallest tip and a well-calibrated pipette.
20. **Retinoic Acid (RA, 100 mM):** Dissolve 50 mg RA in 1.67 ml DMSO. Aliquot 50 μl into brown microtubes and store at -80°C . RA is extremely sensitive to UV light, air, and oxidizing agents, especially in solution. It is recommended to use all the powder immediately after opening the ampule. Dilute each aliquot with 4.95 ml ethanol and store at -20°C as working stock solution. Try not to use working stock solution older than two weeks.
21. **SHH (100 $\mu\text{g/ml}$):** Dissolve 100 μg of SHH in 1 ml sterilized PBS with 0.1% BSA. Aliquot 100 μl into sterilized tubes and store at -80°C .
22. **Trypsin inhibitor (1 mg/ml):** Dissolve 50 mg trypsin inhibitor in 50 ml DMEM/F12 and filter through 50 ml-Steri-flip.

2.2. Media

1. **Human ESC growth medium** (500 ml): Sterilely combine 392.5 ml DMEM-F12, 100 ml Knockout serum replacer, 5 ml MEM non-essential amino acids solution, 2.5 ml of 200 mM L-glutamine solution (final concentration of 1 mM), and 3.5 μl 14.3 M 2-Mercaptoethanol (final concentration of 0.1 mM). The medium can be stored at 4°C for up to 7–10 d.

2. **Neural differentiation medium (DMEM/F12/N2, 500ml)** Sterilely combine: 489 ml of DMEM/F12, 5 ml N2 supplement, 5 ml MEM non-essential amino acids solution, and 1 ml of 1 mg/ml Heparin. The medium can be stored at 4°C for up to 2 w. For neuronal differentiation, add cAMP (1:10000), ascorbic acid (1:1000), BDNF (1:10000), GDNF (1:10000) and IGF-1 (1:10000) before use.

2.3. Antibodies

2.3.1 For neuroepithelial cell identity

1. Pax6 (monoclonal, Developmental Studies Hybridoma Bank-DSHB): use at 1:5000 for immunostaining on cultured cells.
2. Sox2 (monoclonal, R&D systems MAB2018): use at 1:1000.
3. Sox1 (goat IgG, R&D AF3366): use at 1:1000.

2.3.2 For regional identity

1. Otx2 (goat IgG, R&D AF1979): use at 1:2000.
2. Bf1 (FoxG1, Rabbit IgG) : use at 1:1500.
3. HoxB4 (rat IgG, DSHB 112): use at 1:50.

2.3.3 For neurons and progenitors

1. β III-tubulin (Rabbit IgG, Covance PRB-435P): use at 1:5000.
2. Synapsin (Rabbit IgG, CALBIOCHEM 574777): use at 1:250.

2.3.4 For motor neurons and progenitors

1. Olig2 (goat IgG, Santa Cruz SC-19969): use at 1:500.
2. MNR2 (HB9, monoclonal antibody, DSHB 81.5C10): use 1:50.
3. ChAT (goat IgG, Chemicon AB144P): use at 1:500.

2.4. Culture substrate preparation

1. Poly-L-ornithine coated coverslips: In a sterile hood, put one sterilized coverslip in each well of a 24-well plate. Add 75 μ l of 0.1 mg/ml Poly-ornithine onto each coverslip. Incubate plates at 37 °C overnight. The next day, aspirate Poly-ornithine off and let the coverslips dry for approximately 30 minutes. Wash 3 times with 1 ml sterile water for each well. Leave the plate open in the hood until completely dry. Cover plates, wrap in foil, label with date and store at -20°.
2. Laminin coated 6-well plate: Dilute laminin with fresh neural differentiation medium at final concentration of 20 μ g/ml. Put 300 μ l of laminin solution into each well of a 6-well plate. Let the medium hold as a big drop and spread within the central area of the well. Do not let the medium drain to the edge. Incubate the plate at 37 °C for 1 h. Laminin is very easy to be absorbed by plastic and tends to form aggregates in room temperature. Store laminin at -80 °C and thaw at 4 °C before using. Try not aliquot laminin to plastic tubes from the original glass vial.

3. Methods

The undifferentiated state of the starting hESCs is a prerequisite for efficient differentiation of neuroepithelial cells and subsequent functional motor neurons. Presence of partially

differentiated hESCs or contamination of differentiated hESC colonies will result in unsynchronized neural differentiation and reduce the differentiation efficiency.

In the multiple-step process, we use adherent culture mode except the suspension culture steps in the initial separation of hESCs from MEF and in the purification of neuroepithelial cells. The adherent culture allows direct visualization of neural differentiation, including the neural tube-like rosettes during neuroepithelial induction and neural progenitor migration and neurite outgrowth in the neuronal differentiation phase.

In the neuroepithelial induction phase, we employ a colony culture. Almost all the colonies possess neural tube-like rosettes or at least 90% of the total differentiated cells represent neuroepithelial cells that express PAX6 and SOX1. The colony culture permits readily removal of non-neural colonies. Once non-neural colonies are scraped from the culture, 95–99% among the total population should be PAX6+ cells. This ensures subsequent neural differentiation efficiently.

Motoneuron progenitor population reaches a peak in the 4th week. If purmorphamine replaces SHH in the protocol, it increases the proportion of OLIG2-expressing cells from 50% to 60–80% of the total cells.

Differentiation of OLIG2-expressing motoneuron progenitors to HB9-expressing post-mitotic motor neurons takes another week. By the end of the 5th week, the HB9-expressing cells account for half of the total population. The HB9-expressing cells rarely migrate away from the cluster; rather, they stay in the cluster or immediate periphery of the cluster and extend extremely long processes (axons) that often travel throughout the entire 11-mm diameter coverslip. Dissociating the OLIG2-expressing progenitor spheres often results in a significant motor neuron loss, thus we use small clusters of MNs for final differentiation.

After the 5th week, the motor neurons can be further cultured for several weeks or months depending on the applications. Other mature motoneuron markers, e.g., ChAT and VaChAT, will appear over time.

3.1. Induction of Neuroepithelial Cells

3.1.1. Lift hESC colonies from MEF feeder cells

1. Culture hESCs in a 6-well plate with mouse embryonic fibroblast (MEF) feeder cells and feed the cultures daily for 5–7 days. These hESCs grow as colonies and express OCT4 uniformly (Fig. 2A).
2. Remove the old medium and rinse each well of hESCs with 2 ml warm DMEM/F12 for 2 min. Remove DMEM/F12.
3. Add 1 ml freshly-made dispase (1 U/ml) to each well of a 6-well plate, incubate the cultures at 37 °C for 3–5 min. Carefully observe the cells under a microscope every 3 min (see Note ¹). When the edge of hESC colonies starts to curl, aspirate the Dispase off.
4. Gently rinse the well with 2 ml DMEM/F12. The hESC colonies are now loosely attached and very easy to dislodge. Remove the medium carefully without disturbing the colonies.
5. Add 2 ml of fresh hESC medium to each well. Lift the colonies off by gently swirling the plate and/or pipetting. This usually leaves the MEF attaching to the well. If there

¹Do not leave hESCs in dispase longer than 15 min. Longer incubation in dispase may result in poor survival of the lifted hESCs.

are substantial numbers of hESC colonies remain attached, blow off the colonies by pipetting. Pipette the hESC colonies to the size of 50–100 μm . Limit the times of pipetting to no more than 5.

6. Gently collect the colonies with a 5-mL serological pipette or a 1000 μL -pipette tip into a 15-ml conical tube. Spin down the cells at 50g for 1 min.; alternatively, let the hESC colonies sink by standing the tube for 3 min.
7. Aspirate the medium without disturbing the pellet or colonies. Re-suspend the hESC colonies with fresh hESC medium, and wash the cells. Remove the medium carefully.
8. Re-suspend the hESC colonies with 5 ml hESC medium without FGF2.
9. Transfer the cells to a T25 or T75 culture flask. Cultures from one 6-well plate go to one T75-flask with 40 ml medium or three T25-flasks in 12 ml medium (see Note ²). Record the date when hESC colonies are lifted off from feeder cells as day 0 of differentiation.

3.1.2. Formation of hESC aggregates (or embryoid bodies)

1. Next day (day 1), the lifted hESCs colonies generally round up as individual spheres with some dead cells and cell debris floating in the medium. Briefly pipette the clusters with a 5-ml serological pipette to strip the attached debris off from the cell cluster. Stand the flask and let the aggregates sink for 5 min. A simple standing of the flask but no centrifugation will allow separation of the hESC colonies from debris.
2. Remove the old medium and resuspend the hESCs aggregates with fresh hESC medium. Transfer the culture to a new flask, if there are substantial carry-over MEF adhering to the flask, and culture the cells in suspension at 37 °C.
3. In the next few days, hESCs aggregates (also termed embryoid bodies) become brighter overtime (Fig. 2B). They should be floating in the medium without attaching to the flask (see Note ³). Observe and feed the cells daily with fresh hESC medium using the procedure described above.

3.1.3. Differentiation of primitive neuroepithelial cells

1. From day 4, switch the culture medium to the neural differentiation medium and feed the cells in the same way every other day.
2. On day 7, collect the hESC aggregates to a 15-ml conical tube, centrifuge at 50g for 2 min. An additional wash with DMEM/F12 is optional to remove the dead cells and facilitate the attachment of the clusters to the culture surface.
3. Aspirate off the medium and re-suspend the hESC aggregates with 5 ml neural differentiation medium. Transfer the cells to a 60-mm Petri-dish.
4. Seed 20–25 clusters evenly to each well of a laminin coated 6-well plate in 300 μL medium (see Note ⁴).

²The cell density significantly affects the neural differentiation. High density significantly compromises the efficiency of neural specification.

³hESC aggregates (embryoid bodies) free of feeder cells usually float and do not attach to the culture surface. Feeder fibroblasts around the hESCs may re-form feeder, which results in EB attachment. Gently tapping the bottom of the flask will release the loosely attached EBs. Briefly pipette the EBs to remove the dead cells and feeder cells, and then transfer the EBs to a new flask.

⁴Do not seed the colonies in a high density. The ideal density is that after 7 days of growth attached clusters remain as individual colonies without merging to each other. Incubate the culture at 37 °C overnight.

5. Clusters usually attach to the culture surface within 12 hours (see Note ⁵). Add 2 ml of fresh neural differentiation medium to each well. Continue culturing by feeding the cells with 2 ml neural differentiation medium every other day.
6. 3–4 d after attachment (around 10 days from hESC differentiation), carefully examine the morphology of the attached clusters. Columnar epithelial cells appear in the colony center and radially line up (Fig. 2C). This time point represents a critical step toward the specified fates upon presence of appropriate morphogen (see Note ⁶).
7. On day 14, check the morphology again under a microscope. The columnar epithelial cells proliferate quickly and form multiple layers, forming neural tube-like rosettes (Fig. 2D) (see Note ⁷).

3.2. Specification of Olig2-expressing motoneuron progenitors

1. From day 10 of differentiation, feed the primitive neural epithelial cells with fresh neural differentiation medium supplemented with RA at the final concentration of 0.1 μ M (1:10,000 of the stock solution). RA patterns the cells to the upper spinal cord phenotype which expresses HOXB4. Feed the culture every other day for another 5 days.
2. On day 15, carefully observe the cells. Evaluate the quality of differentiation based on the rosette formation (see Note ⁷).
3. Remove the old medium, add 2 ml of fresh neural differentiation medium to each well of the 6-well plate.
4. Gently blow the clusters with a 1-ml pipette to detach the neural tube-like rosettes in the colonies. Keep the pipette tip within the medium to avoid bubbles when pipetting. The rosettes detach easily while the flat cells at the peripheral part of the colony should remain attached.
5. Collect the rosette clumps into a 15-ml conical tube, briefly triturate the clumps with a 5- or 10-ml serological pipette up and down twice. It is not necessary to break up the clumps too much.
6. Centrifuge at 50g for 2 min at room temperature. Remove the medium, re-suspend the clusters in 5 ml fresh neural differentiation medium. Transfer the culture to a T25 or T75 flask (cells from 3 wells may be added to one T25 flask). Culture the cells at 37 °C.
7. Add SHH at 100 ng/ml and RA at 0.1 μ M. In the optimized protocol, we use a small molecule purmorphamine as a replacement of SHH. Purmorphamine at 1 μ M ventralize the neural epithelial cells similarly as SHH at 100 ng/ml.

⁵Well differentiated hESC aggregates tend to attach to plastic surface after a week in suspension. Dead cells around the clusters may interfere with the attachment of the aggregates. Wash the aggregates with neural differentiation medium and plate them again onto a new plate coated with laminin (20 μ g/ml). Alternatively, addition of 10% FBS into the culture overnight will promote the attachment of the aggregates. The FBS should be removed right after the aggregate attachment. Presence of FBS will inhibit neural differentiation.

⁶We refer to these columnar epithelial cells as early neural rosettes. Surrounding the neural epithelial rosettes are flat round cells which are likely of the neural crest lineage. The neural epithelial cells at this state express Pax6 and many other neural transcription factors but not Sox1. We refer to cells at this stage as primitive neural epithelial cells. These primitive neural epithelial cells are responsive to morphogens like RA and SHH for regional patterning. Therefore, we will start the process of motoneuron specification at day 10.

⁷Within the clusters, multiple neural tube-like rosettes appear. Immunostaining will indicate that these cells express both PAX6 and SOX1. The cells in the form of neural tube-like rosettes attach to the substrate loosely whereas the flat cells in the surrounding attach more tightly. We refer to these cells as definitive neural epithelial cells. Thus, it takes about 2 weeks for hESCs to differentiate to neural epithelial cells. The readily identifiable rosettes formation is a valuable parameter to judge the quality of differentiation. Partially differentiated hESCs, overly damaged EBs or early RA-treated culture may result in poor rosette formation. If there are colonies that do not possess rosettes, these are usually non-neural colonies. Scrap those colonies with a pipette tip after marking them using an objective marker that is mounted in a phase contrast scope. This step will minimize, if not eliminate, the contamination of non-neural cells.

8. Feed the cultures by replacing 2/3 of the medium every other day using the same medium. Simply stand the flask for 2–3 minutes and aspirate the supernate, and then add the fresh medium. Clusters of neuroepithelia tend to become spherical within two days, typically 100–200 μm in diameter (Fig. 2E; see Note ⁸).
9. If the spheres grow bigger than 300 μm , break them with a fire polished Pasteur pipette. Alternatively, incubate the big clusters with accutase at 37 °C for 3 min followed by gentle pipetting.
10. On day 23, Olig2-expressing progenitors should be detected. The highest population of Olig2-expressing motoneuron progenitors appears at the end of the 4th week (day 28). This can be done by either plating the cells onto a coverslip for immunostaining, or FACS analysis after immunostaining on dissociated cells.

3.3. Generation of Spinal motor neurons

1. From the 5th week (day 29), the motoneuron progenitors are differentiating to post-mitotic motoneurons. Plate the progenitor spheres onto glass coverslips that are coated with polyornithine and laminin (2–4 clusters/coverslip in a 24-well plate) in the presence of 50 μl of the neural differentiation medium. The medium is supplemented with BDNF, GDNF, IGF1, cAMP (1 μM), ascorbic acid (AA, 200ng/ml) whereas RA and SHH are reduced to 50 nM and 50ng/ml, respectively. Incubate at 37 °C for at least 2 hours or overnight until attachment.
2. The next day, feed the attached spheres with 0.5 ml of fresh neural differentiation medium with the above supplements. Feed the cells every other day for long term differentiation (see Note ⁹).
3. 2 days after plating, the spheres flatten and some cells migrate out of the spheres. Long neurites start to extend from the sphere.
4. By the end of the 5th week, extensive neurites grow out of the sphere (Fig 2F). Immunostaining will reveal HB9-expressing cells in the sphere whereas Olig2-expressing progenitors decrease (Fig 2G). The HB9+ neurons also express tubulin (Fig. 2H). With the optimized protocol above half of the total cells are positive for HB9 staining (see Note ¹⁰). (you just said 30–40% in the beginning of method)

3.4. Maturation of spinal motor neurons

1. For longer cultures of mature motoneurons, continue feeding the cultures with the same neural differentiation medium every other day.
2. Mature, ChAT positive motor neurons begin to appear at around the 6th week (day 40–42) and increase overtime (see Note ¹¹). Meanwhile, HB9-expressing cells decrease. During the 6th and 7th week, some cells co-express HB9 in the nucleus and ChAT in the cytoplasm and neurites.

⁸Clusters of non-neural lineage may be present in the culture if the non-neural colonies are not scraped before lifting. Instead of forming bright round spheres, those clusters are usually grey or dark with irregular shapes. Should there be any non-neural cell contamination in culture, the partially differentiated hESCs are inevitably the source. These partially differentiated hESCs usually generate “bad colonies” which can be easily recognized by direct observation. Mark the “bad colonies” and manually remove them in the step of “rosettes” formation.

⁹Motor neuron progenitors represent a vulnerable population in culture. Enzymatic disaggregation of neuroepithelial spheres can damage the population thus resulting in very few motor neurons. Mild dissociation of the progenitor clusters with accutase (for 3–5 min) can facilitate monolayer formation after attachment. Plating cells at a higher density (30,000 cells/11-mm coverslip), or seeding small clusters (100–200 μm) will help cell survival. Addition of B27 and low concentration of SHH/RA in culture will also help minimize cell death.

¹⁰We’ve noticed that the HB9 antibodies from different sources vary significantly in terms of specificity. The MNR2 (HB9, monoclonal antibody, DSHB 81.5C10) is a reliable antibody for staining motoneurons from various species including human. The Chemicon Inc is releasing a new polyclonal anti-HB9 that is developed in goat to replace its previous less-specific rabbit HB9 antibody.

3. The motor neurons survive for several weeks on coverslips. They usually die in an environment without target cells. When co-cultured with myocytes, the motoneuron axons induce clustering of acetylcholine receptors, which can be visualized by bungarotoxin staining.

3.5. Passaging neuroepithelial spheres

The cell clusters in suspension grow big over time. When the spheres are larger than 300 μm in diameter, they should get dissociated to smaller ones for continued growth and expansion. We usually split the big clusters using two simple procedures.

3.5.1. Passage cells using polished Pasteur pipette

1. Feed the cells with fresh neural differentiation medium containing SHH and RA the day before splitting.
2. Prepare the pipette for dissociating the cells before taking the cells from incubator for dissociation. Choose the cotton plugged 9"-Pasteur pipettes. In the hood, fire polishes the Pasteur pipette tip and adjusts the diameter of the inner lumen to around 200 μm . Carefully check the tip and the lumen every 3–5 seconds.
3. Heat the pipette at 2 cm from the tip. Gently bend the pipette to 145–150 degree. The curve helps shearing the spheres when they pass through the pipette.
4. Cool down the pipette to room temperature. Prepare extra pipettes of different lumen sizes. Keep the pipette sterile in hood.
5. Take out the cells from incubator, lean the flask at 45 degree to let the clusters sink to a corner.
6. Rinse the pipette with the supernate three times. Cells will stick on glass pipette if not well rinsed before using.
7. Take all the clusters into the glass pipette using the pipette aid. If clusters are too big to be sucked into the pipette, change to another one with a bigger lumen size. Blow out the spheres into the medium in flask. The shearing force breaks the clusters to smaller pieces without completely dissociating to single cells.
8. If necessary, triturate the remaining large spheres one more time but do not triturate the spheres for more than twice. If the spheres are not broken, it indicates that the Pasteur pipette is not appropriately narrowed and bended.
9. Culture the cells at 37 °C.
10. Feed the cells with fresh medium containing the supplements needed from the 3rd day. Since the spheres are small, in the first two feedings simply add fresh medium.

3.5.2. Splitting big neuroepithelial spheres using ACCUTASE—Alternatively, the bigger clusters are dissociated with ACCUTASE. The enzymatic effect of ACCUTASE is not as powerful as trypsin and no enzyme inhibitor is needed after dissociation. Store the ACCUTASE in aliquots of 5 ml or 10 ml at -20°C . Thaw the frozen aliquot in a refrigerator overnight before using.

1. Collect the big clusters into a 15-ml or 50-ml conical tube.

¹¹When using antibodies against ChAT to label mature motor neurons, the available ChAT antibody may present strong background in cultured cells (though it stains ChAT-expressing cells in vivo very well). This is usually because of an inappropriate fixation of the enzyme. Using picric acid buffer for fixation and diluting the antibody will reduce the background. Try to use this antibody against ChAT at 1:500 dilution (goat IgG, Chemicon AB144P).

2. Centrifuge at 50g for 2 min to pellet the cells.
3. Remove the medium from the tube.
4. Add 1 ml of ACCUTASE to each tube. Re-suspend the pellet by gentle shaking or tapping.
5. Incubate the clusters in ACCUTASE at 37 °C for 3 to 5 min. Inspect and gently shake the tubes every 2 min.
6. When the clusters look loose and/or the solution looks foggy, add 9 ml medium to the tube and centrifuge at 50 g for 2 min.
7. Remove the medium containing ACCUTASE as clean as possible without disturbing the palette.
8. Add 800 ul medium back to the tube. Pipette the clusters with a 1-ml tip up and down gently for less than 5 times.
9. Let the cells stand for 2 min. Transfer the medium containing single cells and small clusters to the flask prefilled with the fresh medium. Leave the big clusters in the tube.
10. Repeat step 8 and 9 to further break the rest large clusters.
11. The cells aggregate to small clusters within hours. Feed the cells with fresh medium the next day.

3.6. Plate cells on coverslips that are coated with Polyornithine and laminin

1. Leave the frozen 24-well plate with pre-coated coverslips in room temperature for 20 min.
2. Dilute the laminin with neural differentiation medium at a final concentration of 20 ug/ml.
3. Add 50 ul of medium containing laminin and spread evenly on top of the coverslip pre-coated with poly-ornithine. Leave the plate in incubator for an hour.
4. Remove the medium.
5. Transfer the motoneuron progenitor clusters to a Petri-dish, pick up 3–5 small clusters and seed them in 50 ul medium onto the pre-coated coverslips.
6. Incubate at 37 °C for 2 hours. Once the cells have attached, add 500 ul of medium to each well.
7. The cells on coverslips can be fixed for staining once they have attached. For long-term culture, feed the cells every other day.

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Figure 1.

Scheme of differentiation of spinal cord motoneurons from hESCs. The hESCs are directed to neuroepithelial cells in the first 2 weeks. These neuroepithelial cells are patterned to OLIG2-expressing motoneuron progenitors in the subsequent 2 weeks in response to RA and SHH (or purmorphamine). Finally, the progenitors differentiate to post-mitotic motoneurons in the presence of neurotrophic factors. The process employs a simple serum-free neural differentiation medium for motoneuron differentiation. The adherent culture during the neural induction phase is uniquely designed for directly observing neuroepithelial differentiation and for purifying the neuroepithelial cells by removing the non-neural colonies.

Figure 2.

Differentiation of motor neurons from hESCs. (A) hESCs growing on MEF feeder as a uniform colonies. (B) After lifting the hESCs from the MEF and growing in suspension, the hESCs aggregate to spheres. (C) From day 10, columnar epithelial cells appear within the hESC colonies. The columnar cells are starting to organize into rosettes in the colony. (D) At day 15, neural tube-like rosettes are obvious. (E) The motoneuron progenitors are cultured in suspension. (F) Extensive axonal projections come out from the clusters a week after adherent culture. (G) At the 4th week, HB9-expressing motoneurons present among the OLIG2-expressing progenitors. (H) HB9 motoneurons are also positively stained for neuronal marker Tuj1+ at the 5th week.