Derivation of neural crest cells from human pluripotent stem cells

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Published online 18 March 2010; doi:10.1038/nprot.2010.35

Human pluripotent stem cell (hPSC)-derived neural crest (NC) cells present a valuable tool for modeling aspects of human NC development, including cell fate specification, multipotency and cell migration. hPSC-derived NC cells are also suitable for modeling human disease and as a renewable cell source for applications in regenerative medicine. Here we provide protocols for the step-wise differentiation of human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSCs) into neuroectodermal and NC cells using either the MS5 coculture system or a novel defined culture method based on pharmacological inhibition of bone morphogenetic protein and transforming growth factor-β signaling pathways. Furthermore, we present protocols for the purification and propagation of hPSC-NC cells using flow cytometry and defined *in vitro* culture conditions. Our protocol has been validated in multiple independent hESC and hiPSC lines. The average time required for generating purified hPSC-NC precursors using this protocol is 2–5 weeks.

INTRODUCTION

Human embryonic stem cells and neural crest differentiation

Since the first successful derivation of human embryonic stem cells (hESCs) by Thomson and colleagues[1], hESCs have become a valuable *in vitro* model of early human development. hESCs are characterized by their ability to differentiate into cell types of all three germ layers. Neural differentiation of hESCs is associated with the formation of neural rosettes, columnar epithelial cells representing the early developing neuroepithelium[2–6]. hESC-derived neuroepithelial cells are regarded as an *in vitro* source of neural (stem) cells capable of generating the various neural cell types that comprise the central nervous system (CNS)[6–8]. In addition to their CNS differentiation potential, neural rosettes can also give rise to cells of neural crest (NC) identity. Recently, we reported that a well-defined feeder-free hESC neural induction system employing Noggin and the small-molecule SB431542 (NSB) also efficiently yields cells of NC fate. The defined nature of the NSB protocol may be particularly suited to explore mechanisms of early human NC specification in hESCs[9]. Access to reliable protocols for the differentiation of hESCs and human induced pluripotent stem cells (hiPSCs) into NC lineages provides a model system to study normal development and also to interrogate the impact of genetic disease during NC development. Well-known disorders of human NC development include Hirschsprung's disease, DiGeorge syndrome, Waardenburg syndrome, Charcot-Marie-tooth disease, familial dysautonomia, CIPA (congenital insensitivity to pain with anhidrosis) and pediatric cancers, such as neuroblastoma[10–15]. Although several of these disorders have been modeled using genetically engineered mice[16,17], the phenotypes do not always reflect the human disease appropriately. Access to the different stages and lineages of human NC cells carrying the precise genetic makeup of the human disease offers a novel and unique *in vitro* model system that should contribute to a more detailed understanding of pathogenesis in NC disorders. Such a system may also be uniquely suited for discovery and validation of novel candidate therapeutic compounds. Finally, enriched populations of defined NC cell types may be used as a source for application in regenerative medicine such as the repair of peripheral neuropathy or cranial skeletal defects.

Aspect of NC development

In vertebrate development, it is thought that NC cells emerge at the interface between neural and non-neural ectoderm, and migrate extensively to form a variety of NC derivatives such as peripheral neurons, glia, melanocytes, endocrine cells and mesenchymal precursor cells[18,19] (**Fig. 1**). NC cells represent a somatic cell type with unique properties concerning their pluripotency and anterior–posterior organization, capable of undergoing cell fate decisions across multiple tissues and germ layers[20–23] (**Fig. 2**). These aspects of NC were studied in many organisms ranging from

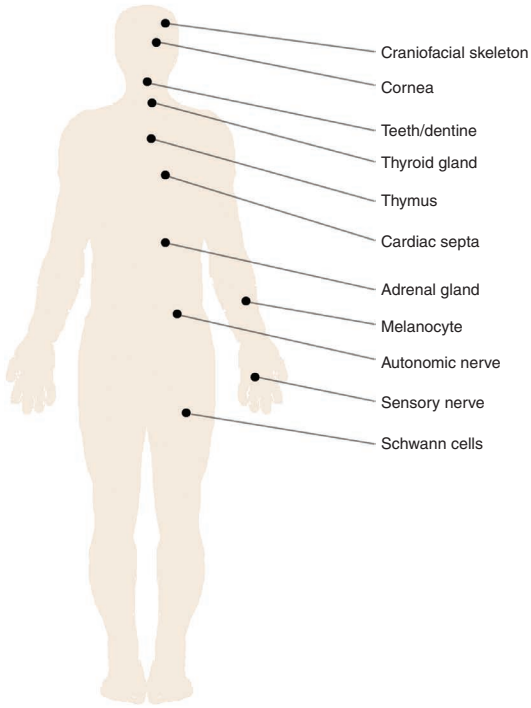

Figure 1 | Multipotentiality of NC cells in human body. The image was generated based on the publication of Crane JF and PA Trainor[19].

protochordate to higher vertebrates and using a broad range of embryology and molecular biology approaches. Rodent NC stem cell and/or precursor populations have been isolated from both fetal and adult tissues such as gut and sciatic nerve, and several studies have attempted the isolation of NC cells from mouse ESCs using transgenic approaches or antibody-based cell selection[24–27]. Such studies have shed light on the evolutionary history of NC development and defined conserved genetic regulatory systems[28,29]. However, despite these extensive studies across many species[25], our understanding of early human NC development has remained limited owing to the lack of an appropriate experimental system.

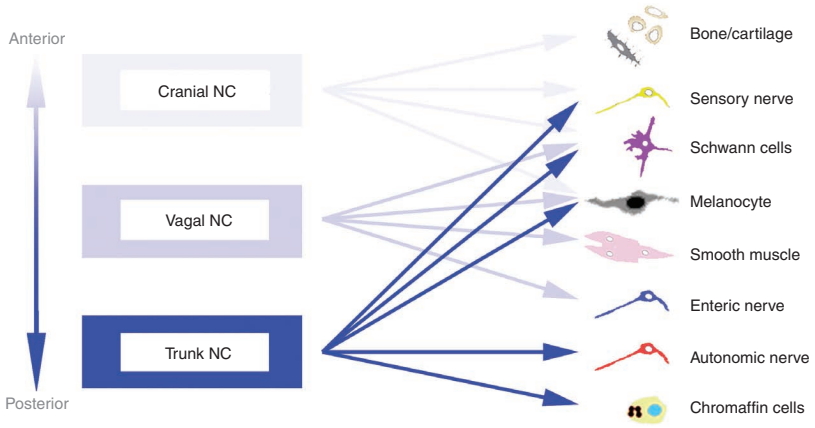

Figure 2 | Distinct NC precursor fates along the anterior–posterior axis of the embryo. The image was modified from Anderson *et al.*[41].

Derivation and isolation of NC cells from hESCs

Here we present protocols for the induction of NC cells derived from hESCs and for the isolation and further expansion/differentiation of hESC-derived NC cells (**Figs. 3** and **4**). Similar approaches based on enrichment of hESC-NC by flow cytometry were published by two independent laboratories after our initial report, although slightly different surface markers or antibodies were used in those studies[30,31]. Currently, it is unclear whether these alternative protocols offer any specific advantages as compared with our original protocol.

It is very difficult to obtain primary human embryonic or fetal NC cells as stage-matched control tissue. Therefore, it is critical to carefully validate NC identity of the differentiated cells to rule out alternative cell fates with potentially overlapping marker expression such as neuroblast- and cranial placode-derived cells. Other than hESCs, our protocols are also applicable to hiPSC lines derived from somatic cells such as normal fibroblasts, as well as fibroblasts[9,32] derived from patients with NC-related genetic disorders such as familial dysautonomia. We have recently shown the use of familial dysautonomia-induced pluripotent stem cells for modeling the pathogenesis and treatment of this rare genetic disorder[33]. The application of hiPSC technology in combination with NC differentiation protocol presented here should serve as a valuable tool for probing the pathogenesis and for validating candidate drugs[34] in many other NC-related disorders. Finally, our protocols can also serve as the basis for efforts aimed at further refining NC-directed differentiation protocols. Some of the key remaining issues are (1) precise manipulation of anterior–posterior identity of hESC-derived NC precursors (cranial versus trunk versus sacral NC lineages); (2) molecular control of cell fate decision along sensory versus autonomic neuron lineages and further sublineages (e.g., nociceptive neurons); (3) characterizing the lineage and stem cell potential of early versus late emerging NC populations from hESCs; (4) optimizing strategies for directed differentiation of other NC lineages, such as melanocytes, sympathoadrenal lineage, cardiac NC and enteric neurons not yet isolated using our protocol; (5) defining the lineage relationship of NC-associated mesenchymal lineages[35]; and (6) fully characterizing and comparing the lineage potential of NC precursors derived from NSB protocol[9] versus the MS5[6] and other alternative NC protocols.

MATERIALS

REAGENTS

- Mitotically inactivated primary mouse fetal fibroblasts (PMEF, Chemicon, PMEF-CF or CF-1 MEF, GlobalStem)
- hESCs and hiPSCs **! CAUTION** Appropriate consent procedures and administrative regulations must be followed for work involving hESCs and hiPSCs. Please consult the institution to assure adherence with national and institutional guidelines and regulations.
- MS-5 (murine stroma cell line, DSMZ)
- Ca^{2+}- and Mg^{2+}-free Dulbecco's PBS
- Ca^{2}/Mg^{2}-free Hanks' balanced salt solution (HBSS, Invitrogen, cat. no. 14170-112)
- Accutase (Innovative Cell Technology, cat. no. AT104)
- Milli-Q water, or any cell culture grade water
- Dulbecco's modified Eagle's medium (DMEM, Invitrogen, cat. no. 11960-044)
- DMEM/F12 (Invitrogen, cat. no. 11330-032)
- Knockout DMEM (Invitrogen, cat. no. 10829-018)
- α-MEM (Invitrogen, cat. no. 32561-037)
- Knockout serum replacement (KSR, Invitrogen, cat. no. 10828-028) **! CAUTION** Each batch of KSR needs to be tested for its suitability for hESC culture
- Fetal bovine serum (FBS, Hyclone, cat. no. 16140-071). **▲ CRITICAL** Heat-inactivation is needed for culture of MEF and MS-5 feeder
- L-Glutamine (Invitrogen, cat. no. 21051-016)
- β-Mercaptoethanol, 1,000× solution (Invitrogen, cat. no. 21985-023) **! CAUTION** β-Mercaptoethanol is toxic; avoid inhalation, ingestion or contact with skin or mucous membranes.
- MEM nonessential amino acids, 100× solution (Invitrogen, cat. no. 11140-050)
- Penicillin/streptomycin (Pen/Strep), 100× solution (Invitrogen, cat. no. 15070-063)
- Basic fibroblast growth factor/FGF2 (R&D system, cat. no. 233-FB-001MG/CF)
- Recombinant mouse Noggin/Fc chimera (R&D system, cat. no. 719-NG)
- Fibroblast growth factor 8 (FGF8, R&D system, cat. no. 423-F8)
- Sonic hedgehog (Shh, R&D system, cat. no. 461-SH)
- Brain-derived neurotrophic factor (BDNF, R&D system, cat. no. 248-BD)
- Glial cell line-derived neurotrophic factor (R&D system, cat. no. 212-GD)
- Wnt3A (R&D system, cat. no. 1324-WN)
- Bone morphogenetic factor 4 (BMP4, R&D system, cat. no. 314-bp)
- Ascorbic acid (AA, Sigma, cat. no. A4034)
- Retinoic acid (RA, Sigma, cat. no. R2625)
- Epidermal growth factor (EGF, R&D system, cat. no. 236-EG)
- SB431542 (Tocris Bioscience, cat. no. 1614)
- Y-27632 dihydrochloride (ROCK inhibitor; Tocris Bioscience, cat. no. 1254)
- SU5402 (Calbiochem, cat. no. 572630)
- Dickkopf-1 (Dkk1, R&D system, cat. no. 1096-DK)
- Putrescine (Sigma, cat. no. P7630)

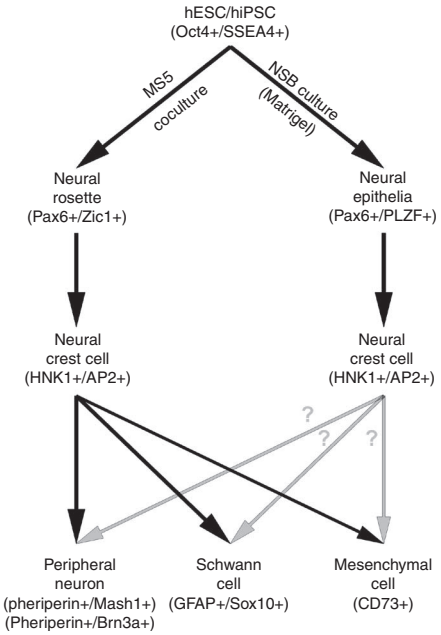

Figure 3 | Multistep differentiation of NC cells from hPSCs *in vitro*. Molecular or cell surface markers for each step are shown.

- Progesterone (Sigma, cat. no. P6149)
- Insulin (Sigma, cat. no. I6634)
- Human transferrin (APO) (Millipore, cat. no. 4452-01)
- Sodium selenite (Sigma, cat. no. S9133)
- IBMX (Isobutylxanthine, Sigma, cat.no. I7018-100MG)
- Dexamethasone (Sigma, cat.no. D2915-100mg)
- TGFβ-3 (R&D system, cat.no. 243-B3-010)
- β-Glycerol phosphate (Sigma, cat. no. 50020-100mg)
- 0.05% (wt/vol) trypsin/0.53 mM EDTA (Invitrogen, cat. no. 25300-054)
- Neutral protease (or Dispase) (6 IU ml^{-1}; Worthington Biochemical Corporation, cat. no. LS02104)
- Gelatin (Sigma, cat. no. G1890)
- Matrigel basement membrane matrix (BD, cat. no. 354234)
- Fibronectin (BD, cat. no. 356008)
- Ultra-pure laminin (BD, cat. no. 354239)
- Poly-L-ornithine hydrobromide (Sigma, cat. no. P3655)
- p75 antibody (Advanced targeting systems, cat. no. AB-N07)
- Pax-6 antibody (Covance, cat. no. PRB-278P)
- AP2 antibody (DSHB, cat. no. 3B5)
- HNK1 antibody (Sigma, cat. no. C6680)
- Alexa Fluor 633 goat anti-mouse IgG_1 (Molecular probes, cat. no. A21042)
- Alexa Fluor 488 goat anti-mouse IgM (Molecular probes, cat. no. A21126)
- 7-AAD (7-aminoactinomycin D, BD, cat. no. 559925)
- Syringe (1 ml; BD, cat. no. 309623)
- Cell scraper (Corning, cat. no. 3008)

EQUIPMENT

- Inverted microscope (i.e., Nikon TE or Olympus IX) with fluorescence equipment and digital imaging capture system
- Horizontal laminar flow hood or a biosafety cabinet with an embedded microscope
- Biosafety cabinet for cell culture
- CO_2 incubator with controlling and monitoring system for CO_2, humidity and temperature
- Cell culture centrifuge
- Cell sorting machinery (i.e., MoFlo, Cytomation; FACS Aria, Becton Dickinson; or similar)
- Glass hemocytometer
- *Cell culture disposables*: Petri dishes, multiwell plates, centrifuge tubes, fluorescence-activated cell sorting (FACS) tube, pipettes, pipette tips, etc.
- Gelatin-coated Petri dishes
- Matrigel-coated Petri dishes
- PO/Lam/FN-coated plate (poly-L-ornithine hydrobromide/laminin/fibronectin)

REAGENT SETUP

▲ **CRITICAL** Sterilize all media using 0.22 μm filtration.

For mesenchymal differentiation Prepare the following stock solutions: 0.5 M IBMX in DMSO (1,000× stock), 10 mM dexamethasone solution in ethanol (1,000× stock) and 1 M β-glycerol phosphate solution in distilled water (100× stock). Store stock solutions at 4 °C. Use within 1 month.

hESC medium for maintenance (1 liter, use within 2 weeks) Combine DMEM/F12 (800 ml), 200 ml of KSR, 5 ml of L-Glutamine, 5 ml of Pen/Strep, 10 ml of 10 mM MEM minimum nonessential amino acids solution, 1,000 μl of β-mercaptoethanol and FGF-2 (final concentration is 4 ng ml^{-1}).

KSR medium for hESC differentiation (1 liter, use within 2 weeks) Knockout DMEM (820 ml), 150 ml of KSR, 10 ml of L-Glutamine, 10 ml of Pen/Strep, 10 ml of 10 mM MEM and 1 ml of β-mercaptoethanol.

N2 medium for hESC differentiation (1 liter, use within 2 weeks) Dist. H_2O (985 ml) with DMEM/F12 powder, 1.55 g Glucose, 2.00 g $NaHCO_3$, 25 mg Insulin, 0.1 g apotransferrin, 30 nM sodium selenite, 100 μM putrescine and 20 nM progesterone.

DMEM with 10% FBS for preparing PMEF (1 liter, use within 2 weeks) DMEM (885 ml), 100 ml of FBS, 10 ml of Pen/Strep and 5 ml of L-Glutamine

α-MEM with 10% FBS for preparing MS-5 feeder (1 liter, use within 2 weeks) α-MEM (890 ml), 100 ml of FBS and 10 ml of Pen/Strep

Gelatin solution (500 ml) Dissolve 0.5 g of gelatin in 500 ml of warm (50–60 °C) Milli-Q water. Cool to room temperature (15–25 °C).

Matrigel (1 ml aliquots) Thaw the frozen vial of matrigel on ice overnight at 4 °C. Prepare 1 ml aliquots in a 50 ml centrifuge tube using chilled pasture pipettes and freeze at −20 °C. ▲ **CRITICAL** Matrigel must be thawed slowly to prevent gelatinization. Chilled pasture pipettes and 50 ml centrifuge tubes should be used when making aliquots of the matrigel.

CF-1 MEF conditioned media (100 ml per d, use within 1 week) Thaw, rinse and plate two vials of MEFs (around 15 million cells) on a gelatin-coated T225 flask in DMEM with 10% FBS. The next day, aspirate the media, wash the Petri dishes with hESC media to remove residual serum and plate 100 ml of hESC media. After 24 h, collect the MEF conditioned media (CM), and replace with new hESC media. This can be repeated daily for no more than 2 weeks. The media may be stored at 4 °C for less than 1 month. Before using, CM can be sterilized by 0.22 μm filtration and supplemented with 10 ng ml^{-1} of FGF2.

EQUIPMENT SETUP

Gelatin-coated Petri dishes Coat culture Petri dishes or flasks with sterile gelatin solution (0.1% (wt/vol) in water) for about 5 min at room temperature. Aspirate the gelatin solution before plating the cells.

Matrigel-coated Petri dishes Dilute a 1 ml frozen aliquot of matrigel in 19 ml of DMEM:F12. A 45 μm cell strainer can be used to remove any insoluble clumps. Coat Petri dishes with the diluted matrigel solution and let them stand for 1 h. Aspirate the matrigel solution and rinse Petri dishes once with DMEM:F12 before plating the cells.

PO/Lam/FN-coated plate Coat 15 μg ml^{-1} PO in PBS in 60 mm Petri dish and keep it aside for overnight. Next day, wash the PO-coated Petri dish three times with PBS and coat 1 μg ml^{-1} laminin and10 μg ml^{-1} fibronectin in PBS for a period of at least 2 h (usually overnight). When cells are ready for plating, remove the solution and let the PO/Lam/FN-coat dry for a quick period (<5 min) before plating.

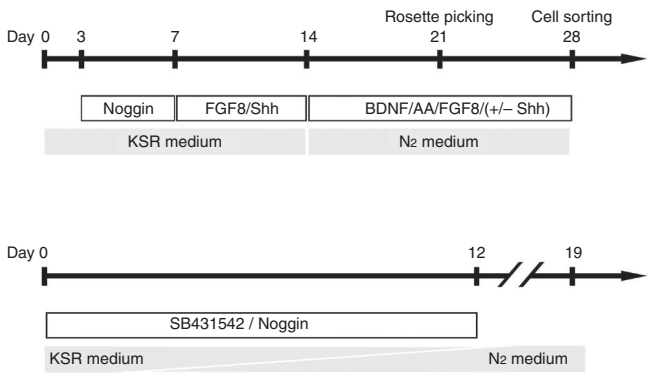

Figure 4 | Schematic of the two different NC induction methods ((**a**) MS5 coculture versus defined (**b**) "NSB" protocol).

PROCEDURE

Induction of NC cells

1| Maintain hESCs and hiPSCs as described in previous protocols[6,9,32,33].

2| Induce NC cells either using option A, through MS5 coculture, or option B, using defined system (NSB method) (**Fig. 4**).

(A) NC cells differentiation from hPSCs using MS5 coculture

(i) When hPSCs are confluent, discard hESC medium from Petri dish (60 mm Petri dish) and add dispase (2–3 ml).

(ii) Incubate at 37 °C for 5–10 min and check under the microscope. When the edges of the hPSC colony start to detach ("rolling up"), harvest the colonies in fresh hESC medium. Under these conditions, most of colonies should detach, whereas PMEFs should remain attached.

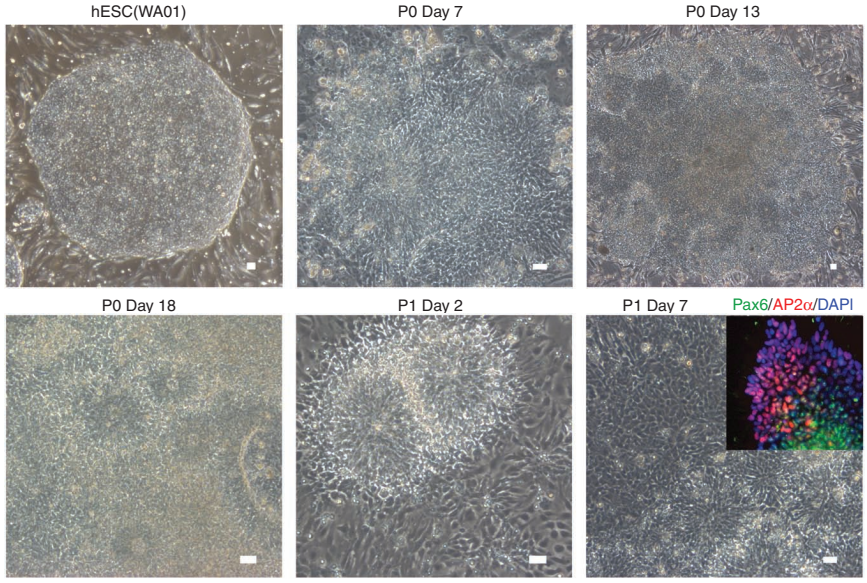

Figure 5 | Procedures for isolating hESC-derived neural crest cells with MS5 coculture. Undifferentiated (**a**) hESCs are induced to neural lineages (**b**–**f**, neural rosette) in the presence of appropriate morphogens/cytokines. (Inset in **f**) NC markers (AP2 and p75) are typically expressed at the periphery of neural rosette structure. Expression of the CNS marker Pax6 is observed in neural rosettes. Scale bars = 100 μm.

(iii) Centrifuge the harvested hPSCs for 5 min at 160*g* at room temperature and aspirate the supernatant.

(iv) Repeat Step (iii).

▲ **CRITICAL STEP** Repeated washing is necessary to dilute out dispase because dispase is not inactivated by serum. Incomplete washing out of dispase prevents hESC colonies from attachment and leads to high levels of cell death.

(v) Pipette and triturate the hPSC pellet with 1 ml of hESC medium using P1000 micropipette.

▲ **CRITICAL STEP** The number of repeats and the intensity of pipetting determine the size of hPSC colonies. Colonies that remain too large tend to spontaneously differentiate, whereas hPSCs triturated too harshly tend to die on replating.

(vi) For subculture (maintenance of undifferentiated hPSCs), in order to maintain hPSCs for longer time, plate hPSCs onto fresh PMEF plates at a ratio of 1:5 to 1:8. Incubate at 5% CO_2 and 37 °C. It is important to assure that hPSC colonies are dispersed evenly within the plate by carefully shaking Petri dishes.

(vii) For differentiation (day 0) (neural induction of hPSCs), in case of differentiation, plate small fraction of hPSCs (~ 5×10^3 to 30×10^3 cells per 60 mm Petri dish) onto mitotically inactivated MS-5 plate (gelatin-coated 60 mm Petri dish, ~1.2 million MS5 cells per Petri dish, plate them a day before).

▲ **CRITICAL STEP** The number of hPSCs in a MS-5 Petri dish (60 mm Petri dish) corresponds to ~2% of the undifferentiated hPSCs harvested from a 60 mm Petri dish before passage.

(viii) From days 3 to 7, change KSR medium supplemented with Noggin (500 ng ml^{-1}) every 2–3 d.

(ix) At day 7 and 12, change KSR medium supplemented with Shh (200 ng ml^{-1}) and FGF8 (100 ng ml^{-1}).

(x) From days 12 to 24, change N2 medium supplemented with AA (0.2 mM), Shh (200 ng ml^{-1}), FGF8 (100 ng ml^{-1}) and BDNF (20 ng ml^{-1}) every 2–3 d. The combination of cytokines/growth factors added at late P0 (passage 0) stage (after Step (x)) and P1 (passage 1) stage (Step (xii)) can be fine-tuned to increase NC yield. In our previous study, we observed that the number of NC (p75+) cells is significantly increased on exposure to FGF-2 or BMP4, although addition of extrinsic BMPs does not seem to be essential for NC induction in P1 stage. Antagonists of these pathways can reduce NC induction efficiency[6]. In addition, to influence the quantity of NC induction, different combinations of extrinsic factors also affect lineage predisposition. As observed in various model systems in the mouse and chick embryo, manipulations of the Wnt, BMP, FGF, RA and Shh pathways can greatly impact lineage decisions during NC development[36]. Concentrations of each extrinsic factor need to be individually optimized for each application aimed at biasing lineage choice. Examples of cytokines and compounds controlling efficiency of NC induction in hESCs that have been described previously include[36] Wnt3A (40 ng ml^{-1}), BMP4 (50 ng ml^{-1}), FGF8 (100 ng ml^{-1}), RA (0.5 μM), Shh (200 ng ml^{-1}), SU5402 (10 nM), Dickkopf-1 (100 ng ml^{-1}) and Noggin (500 ng ml^{-1}).

? TROUBLESHOOTING

(xi) At ~12–20 d of differentiation, numerous neural rosette structures emerge from the differentiating hPSCs (**Fig. 5**). At the latest by day 28 of differentiation, harvest rosette structures mechanically by aspirating colonies using a 1 ml

syringe with a fine (27 G) needle (P0) and gently replate colonies on 15 μg ml^{-1} PO/1 μg ml^{-1} Lam/10 μg ml^{-1} FN-coated culture Petri dishes in N2 medium supplemented with AA (0.2 mM), Shh (200 ng ml^{-1}), FGF8 (100 ng ml^{-1}) and BDNF (20 ng ml^{-1}) (P1). Change medium every 2–3 d.

▲ **CRITICAL STEP** For preparing PO/Lam/FN-coated plate, coat 15 μg ml^{-1} PO in PBS in a 60 mm Petri dish and keep it aside overnight. Next day, wash the PO-coated Petri dish with PBS and coat 1 μg ml^{-1} laminin and10 μg ml^{-1} fibronectin in PBS for more than 2 h (usually overnight). Just before plating rosette structure, dry the PO/Lam/FN-coated Petri dish.

? TROUBLESHOOTING

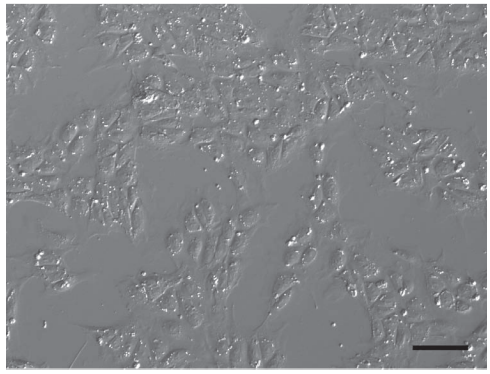

Figure 6 | Target hESC/hiPSC density for NSB induction toward NC cells. The initial density of pluripotent cells will impact the amount of NC cells formed. Shown is the ideal density for starting the NSB induction to achieve NC cell formation. Scale bar=100 μm.

(B) NC differentiation from hPSC using defined NSB culture system

(i) When hPSCs are confluent, aspirate the media and add a minimal amount of accutase solution needed to cover the surface of the Petri dish.

(ii) Incubate the Petri dish at 37 °C for 15–30 min and check under the microscope. When all cells are rendered to single cells, harvest the colonies in fresh hESC medium.

(iii) Avoiding air bubbles, triturate the cells in the Petri dish using a pasteur pipette with additional hESC media until the cells are in single cell suspension and filter using a 40 μm cell strainer to remove debris and cell clumps.

(iv) Wash and centrifuge cells twice for 5 min at 160*g* at room temperature in hESC media to remove any remaining accutase solution.

(v) Resuspend the cells in hESC media containing ROCK inhibitor and plate on a gelatin-coated Petri dish at a density of <200,000 cells per cm^2.

(vi) Incubate the Petri dish for 30 min at 37 °C in a cell incubator in the presence of hESC media containing ROCK inhibitor. Prepare matrigel-coated Petri dishes (see EQUIPMENT SETUP) while the cells are incubating.

(vii) Collect the nonadherent cells, wash the Petri dish with hESC media containing ROCK inhibitor and centrifuge the cells.

(viii) Resuspend the cells in CM containing ROCK inhibitor.

(ix) Determine the cell concentration using a hemocytometer and add CM containing ROCK inhibitor to the appropriate cell concentration.

(x) Plate cells on matrigel-coated Petri dishes at 10,000 cells per cm^2.

(xi) Grow cells in CM containing 10 ng ml^{-1} FGF2, feed daily. In addition, for the first 2 d, CM should contain ROCK inhibitor.

(xii) When the cells are 50–70% confluent, differentiation should be initiated (**Fig. 6**). To initiate differentiation, replace the media with KSR medium containing 10 μM SB431542 and 500 ng ml^{-1} Noggin. Replace the media and slowly switch from KSR to N2 on days 2, 3, 5, 7, 9 and 11, and as detailed below.

▲ **CRITICAL STEP** Cell density at the time of initiation of differentiation determines the relative amounts of CNS versus NC cells produced, with lower confluency biasing toward increased numbers of NC cells and high confluency biasing toward CNS lineage. However, at very low confluencies (<50% confluent), cell viability diminishes. Initial seeding densities ('plating efficiency') can vary between pluripotent cell lines and should be empirically determined to reach ideal starting conditions.

(xiii) On day 2 of differentiation, aspirate the KSR using a sterile glass pipette and add KSR containing 10 μM SB431542 and 500 ng ml^{-1} Noggin.

(xiv) On day 3 of differentiation, aspirate the KSR using a sterile glass pipette and add KSR containing 10 μM SB431542 and 500 ng ml^{-1} Noggin.

(xv) On day 5 of differentiation, aspirate the KSR using a sterile glass pipette and add media (75% KSR, 25% N2) containing 10 μM SB431542 and 500 ng ml^{-1} Noggin.

(xvi) On day 7 of differentiation, aspirate the KSR using a sterile glass pipette and add media (50% KSR, 50% N2) containing 10 μM SB431542 and 500 ng ml^{-1} Noggin.

(xvii) On day 9 of differentiation, aspirate the KSR using a sterile glass pipette and add media (25% KSR, 75% N2) containing 10 μM SB431542 and 500 ng ml^{-1} Noggin.

(xviii) On day 11 of differentiation, aspirate the KSR using a sterile glass pipette and add N2 media containing 10 μM SB431542 and 500 ng ml^{-1} Noggin.

(xix) NC cells can be observed by day 11 of differentiation based on AP2a, p75 and HNK1 staining (**Fig. 7**). Render the cells to single cells using accutase followed by replating and maintenance of the cells in N2 medium or under alterative culture conditions detailed in Step 4. If desired, cells at day 11 may be further purified for NC lineage using flow cytometric sorting for p75 and HNK1, as described below.

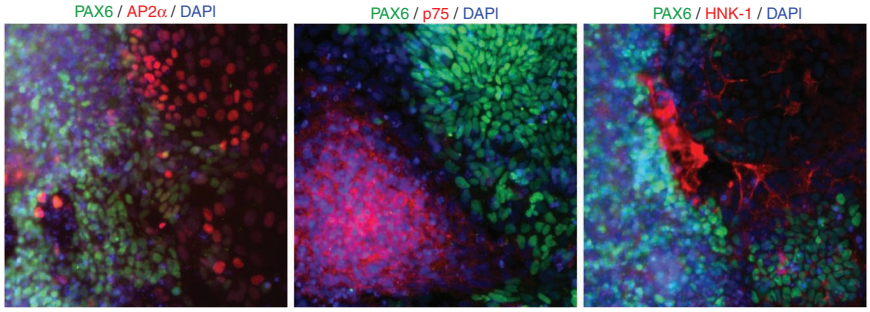

Figure 7 | NC cell marker expression using NSB induction. By day 11 in cultures, the NC markers AP2, p75 and HNK1 (all red) can all be found expressed in the non-PAX6 (green)-expressing cells.

Isolation of hPSC-derived NC cells using antibody-mediated flow cytometry sorting

3| On the day 6 or 7 in P1 culture in the MS5 protocol (see Step 2A), change medium with accutase (or HBSS) for 20–30 min and harvest the P1 culture with cell scraper.

▲ **CRITICAL STEP** Note that we describe this step in detail for cells generated using the MS5 protocol (Step 2A). However, the same strategy can be adapted for cells generated using the NSB protocol (Step 2B).

▲ **CRITICAL STEP** To determine the presence of NC populations at P1, parallel cultures can be pre-screened by immunocytochemistry. Neuroectodermal marker (Pax6)-positive cells should be interspersed with NC marker (AP2)-positive cells (**Figs. 5f** and **7**). If the goal is to prepare a NC-depleted population, it is possible to manually harvest rosette structures and remove any surrounding cells using a fine needle (27–30 G) attached to 1 ml syringe.

4| Centrifuge the harvested cells from P1 culture stage for 5 min at 160*g* at room temperature and aspirate the supernatant. Mechanically triturate cells in PBS containing 2% serum (FBS).

5| Label triturated P1 cells (10 million cells per ml) with antibodies (p75, 5–10 μl ml^{-1}; HNK1, 10–20 μl ml^{-1}) for flow cytometry for 20 min on ice in the dark. Centrifuge for 5 min at 160*g* at room temperature and aspirate the supernatant.

▲ **CRITICAL STEP** The concentration of each lot of antibody may need to be optimized. FACS analysis with appropriate controls is essential to identify optimal antibody dilutions.

6| Resuspend cells in PBS containing 2% serum (FBS) and label it with appropriate fluorochrome-labelled secondary antibody (1:1000 to 1:500 dilution) for each primary antibody.

7| Perform cell sorting by flow cytometry. Collect p75+ and HNK1+ cells (double-positive cells) for further culture (**Fig. 8**).

▲ **CRITICAL STEP** It is essential to have controls of hESC-derived NC cells that are unstained and stained with appropriate secondary antibodies only for defining sorting gates in the FACS machine. As most cells under these conditions are either double positive (p75+ and HNK1+) or double negative, it may be possible to use sorting for single markers (HNK1 or p75) without a major loss of specificity. However, using single markers outside the narrow differentiation window (P1 stage) may result in contamination with other cell types such as CNS neuroblast, cells of placode origin or early mesodermal cells. Dead cells can be excluded for cell sorting by using 7-AAD or DAPI.

? TROUBLESHOOTING

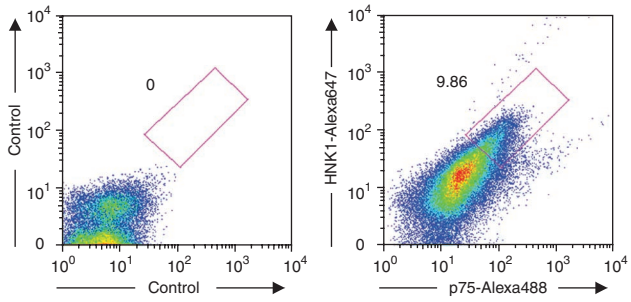

Figure 8 | Representative image of FACS isolation for NC cells. For flow cytometric isolation, dissociated cells from neural rosettes are sorted for expression of p75 and HNK1.

8| Collect double-positive cells in PBS containing 2% serum.

9| Centrifuge the sorted cells for 5 min at 160*g* at room temperature and aspirate the supernatant.

Propagation of isolated hPSC-derived NC cells

10| Propagate isolated hPSC-derived NC cells using option A with attached culture or option B using sphere formation.

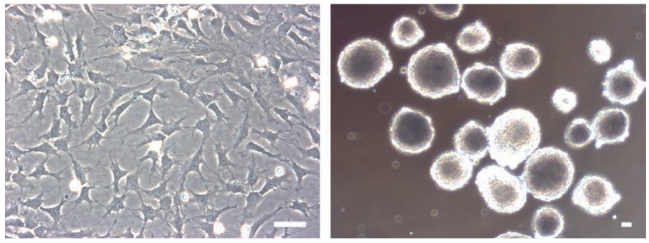

Figure 9 | Propagation of sorted NC cells. hESC-derived NC cells are highly enriched in the double-positive (p75+ and HNK1+) population and can be further cultured on PO/Lam/FN-coated plate under serum-free conditions or as sphere on ultra-low attachment plate. Scale bars = 100 μm.

(A) Proliferation of sorted NC cells in conventional attaching culture

(i) Resuspend the pellet by tapping the tube (not pipetting) and plate on culture Petri dishes pre-coated with PO/Lam/FN (**Fig. 9**, left panel).
▲ **CRITICAL STEP** Minimum plating number is 50,000–100,000 cells per cm^2 to ensure survival of sorted cells. Viability of sorted cells is high when special precautions are taken to minimize pipetting and other mechanical stress.
▲ **CRITICAL STEP** Supplementing the medium with serum or chick embryo extract (as commonly used in primary NC cultures) did not enhance the survival or proliferation of hESC-NC cell cultures under these conditions. For preparing pre-conditioned medium, collect medium from P1 culture (Step 16) and sterilize by 0.22 μm filtration. The pre-conditioned medium can be used by mixing at ratios of 1:1 to 1:6 with fresh N2 medium to further enhance survival of sorted cells after plating.
? TROUBLESHOOTING

(ii) Culture the hPSC-NC cells with N2 medium supplemented with 10 ng ml^{-1} of FGF2 and 10 ng ml^{-1} of EGF. Change the media every 2–3 d and passage every 7–8 d. Accutase or HBSS treatment can be used for dissociation of NC cells for splitting.

(B) Culturing of sorted NC cells in sphere formation

(i) Resuspend the pellet by tapping the tube (not pipetting) and plate on ultra-low attachment six-well plate in 1,000–10,000 cells per cm^2 with 2 ml of N2 medium supplemented with 10 ng ml^{-1} of FGF2 and 10 ng ml^{-1} of EGF. The sphere is typically observed by 24 h after plating (**Fig. 9**, right panel).

(ii) After every 7 d of culture, dissociate spheres with accutase treatment for 20 min. Filter triturated cells using a 40 μm cell strainer and obtain NC cells. After cell counting using hemocytometer, plate at 1,000–10,000 cells per cm^2 into new ultra-low attachment plates in N2 medium supplemented with 10 ng ml^{-1} of FGF2 and 10 ng ml^{-1} of EGF.

Differentiation of hPSC-derived NC cells

11| Differentiate hPSC-derived NC cells using option A for peripheral neurons, option B for Schwann cells or option C for myofibroblast cells.

(A) Peripheral neuronal differentiation with hPSC-derived NC cells

(i) For neuronal differentiation, resuspend dissociated NC cells with N2 medium and plate on culture Petri dishes pre-coated with PO/Lam/FN (20,000 cells per 10 μl drop on pre-dried plate, corresponding to a local density of ~100,000 cells per cm^2).

(ii) For the first 2 d, culture hESC-NC cells with N2 medium supplemented with 10 ng ml^{-1} of FGF2 and 10 ng ml^{-1} of EGF.

(iii) Afterward, culture cells in N2 medium supplemented with BDNF (10 ng ml^{-1}), AA (200 μM), glial cell line-derived neurotrophic factor (10 ng ml^{-1}), nerve growth factor (10 ng ml^{-1}), Neurotrophin-3 (10 ng ml^{-1}) and cyclic AMP (0.5 mM) for 2 weeks. Change the medium including growth factors every 3–4 d (**Fig. 10**). For improving selective subtype specification toward either sensory or autonomic neuron fate, optimizing growth factor composition, in particular Wnt and BMP signaling factors, is of further consideration.

(B) Schwann cells specification with hPSC-derived NC cells

(i) After flow cytometric purification at the end of P1 stage (Step 3), culture hESC-NC cells in N2 supplemented with 10 ng ml^{-1} of FGF2 and 10 ng ml^{-1} of EGF for more than 30 d (Step 4). Depending on the hPSC line used, the optimal time period to maintain cells under FGF2/EGF conditions (Step 4) can vary. The average period is ~60 d. After FGF2/EGF culture, differentiate the 'aged' hESC-NC cells along the Schwann cell lineage in N2 medium supplemented with ciliary neurotrophic factor (10 ng ml^{-1}), neuregulin (20 ng ml^{-1}), FGF2 (10 ng ml^{-1}) and cyclic AMP (0.5 mM) for 3 weeks (**Fig. 11**).
▲ **CRITICAL STEP** As shown in our previous report, Schwann cell differentiation is not observed during early

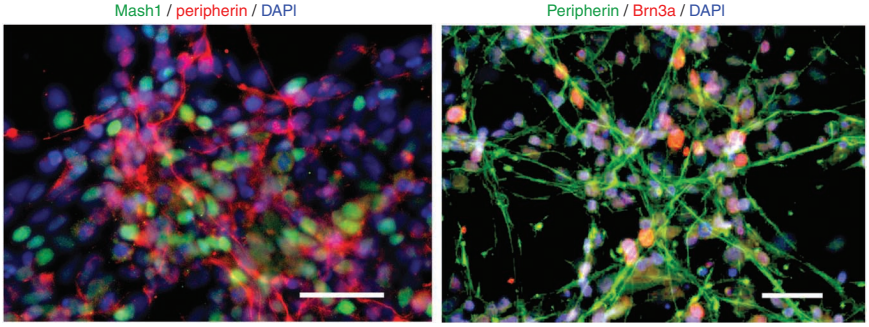

Figure 10 | Specification of hESC-NC cells toward peripheral neurons. hPSC-derived NC cells differentiated toward autonomic neurons (MASH1+/peripherin+) and sensory neurons (Brn3a+/peripherin+). Scale bars = 100 μm.

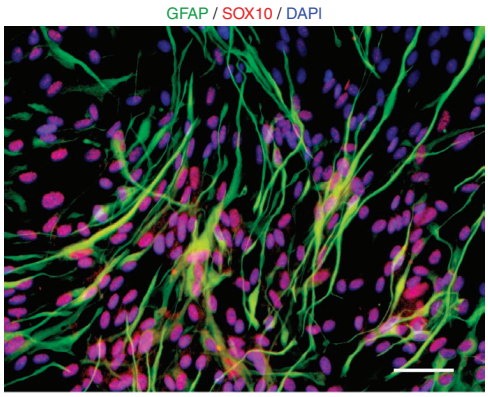

Figure 11 | Schwann cell differentiation from hESC-NC cells. Schwann cell fates (GFA, green; Sox10+, red) are observed from hPSC-derived NC cells. Scale bars = 100 μm.

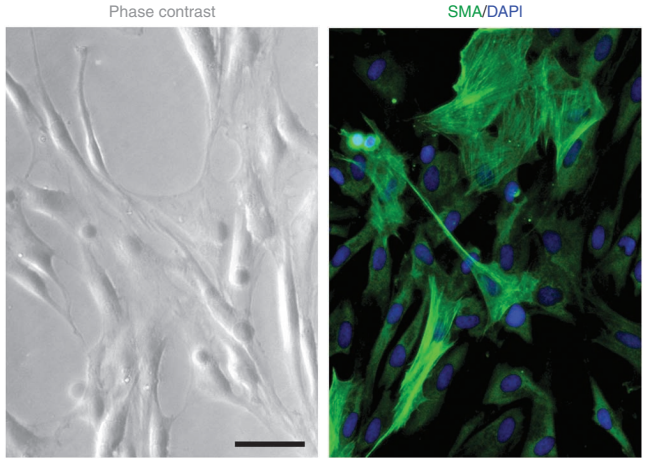

Figure 12 | Representative morphology of myofibroblast cells derived from hESC-NC cells. Scale bars = 100 μm.

stages of NC cell culture and we observed glial fibrillary acidic protein-positive (GFAP+) cells only in the aged NC cells cultured for more than 60 d. We propose that the *in vitro* proliferation period reflect an *in vitro* maturation ('aging') similar to developmental progression *in vivo*. The developmental window for Schwann cell specification in response to various culture conditions and for individual hPSC lines requires further future studies.

It may be important for some of the experiments to formally distinguish GFAP+ Schwann cell precursors generated using this protocol from GFAP+ CNS astrocytes. This can be achieved by double labeling of GFAP+ cells with p75 and/or O4 that are coexpressed in Schwann cells but not in astrocytes. Other Schwann cell makers that can be used for this purpose are Sox10 or myelin basic protein among others.

(C) Myofibroblast differentiation derived from hPSC-derived NC cells

(i) Culture the cells for 2–3 weeks in α-MEM medium containing 10% FBS. This should yield hPSC-NC cultures expressing myofibroblast markers (**Fig. 12**). These cells express a series of cell surface marker such as CD73, Stro-1 and CD146, typically associated with mesenchymal stem cell identity[37]. Indeed, our previous study showed that cells expressing myofibroblast markers in hPSC-NC cultures can readily give rise to adipocytes, chondrocytes, osteocytes and smooth muscle cells, as shown previously for primary cranial NC precursors (**Fig. 13**)[35,38,39].

(ii) For adipogenic differentiation, grow hESC-NC-MPCs to confluence, followed by exposure to 1 mM dexamethasone, 10 μg ml^{-1} Insulin and 0.5 mM IBMX in α-MEM medium containing 10% FBS for >3 weeks.

(iii) For chondrogenic differentiation, pellet hESC-NC-MPCs for 5 min at 160*g* at room temperature and take out supernatant without disruption of pellet structure. Then pellet culture can be performed with 10 ng ml^{-1} TGFβ-3 and 200 μM AA in α-MEM medium containing 10% FBS for >4 weeks.

(iv) For osteogenic differentiation, plate hESC-NC-MPCs at low density (1 × 10^3 cells per cm^2) on tissue culture-treated Petri dishes in the presence of 10 mM β-glycerol phosphate, 0.1 μM dexamethasone and 200 μM AA in α-MEM medium containing 10% FBS for 3–4 weeks.

● TIMING

The NC cell differentiation takes ~30 d using the MS5 coculture approach (Step 2A) and about 12 d using the NSB protocol (Step 2B). For the isolation and propagation of NC precursor cells (Steps 3–10), an additional period of 1–2 weeks is required. Cultures of cells at the NC precursor stage can be maintained for very extensive time periods (> 4 months) if required. Neuron, Schwann cell and myofibroblast differentiation takes around 3 weeks starting from the appropriate NC precursor cell stage (Step 11). Further

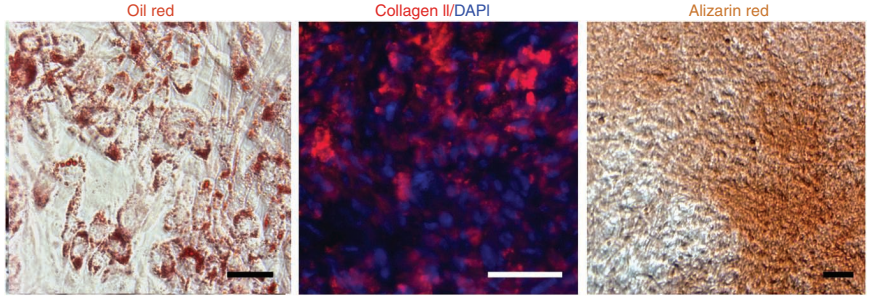

Figure 13 | Adipogenic, chondrogenic and osteogenic differentiation of myofibroblast cells derived from hESC-NC cells. Oil red staining method was used to detect adipocyte. Collagen II staining shows chondrogenic differentiation and alizarin red shows osteogenic cells from hPSC-derived NC cells. Scale bars = 50 μm.

differentiation of myofibroblast lineages toward adipogenic, osteogenic and chondrogenic cells will take additional 3–4 weeks (Step 11C).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
2A(xi)	No formation of neural rosettes during hESC differentiation	Check the concentration or activity of each cytokines
		Change the batch of MS-5
		Check hESC line for genomic changes or use alternative hESC line
		Change (reduce) the seeding density of hESCs in MS-5 culture
		Check for contamination with *Mycoplasma* species in hESCs/hiPSCs or MS-5 cells
	Low survival of P1 cells	Check the concentration or activity of each cytokines
		Minimize mechanical stress for rosettes before seeding on PO/Lam-coated dish
7	Small percentages of p75+/HNK1+ cells in cell sorting	Check the dilution of antibodies
		Check settings for cell sorting machine
		Stain P0 or P1 culture before or after cell sorting with antibodies for Pax6, Ap2, p75 and HNK1
		The percentage of NC cells in P1 culture can vary among different hESC lines
		Harvest P1 cells with cell scraper to ensure the detachment of all cells in P1 culture dish
7, 10A(i)	Low survival of sorted hESC-NC cells	Use pre-conditioned medium for initial culturing of sorted cells
		Minimize mechanical stress after cell sorting
		Increase plating density after sorting (plate cells at densities ≥ 50,000 to 100,000 cells per cm^2)

ANTICIPATED RESULTS

This protocol should reliably yield enriched populations of hPSC-NC cells after cell sorting. Final yield depends on the specific hPSC line used, the efficiency of rosette formation and the specific cytokines added during P1 culture (**Fig. 5**). The protocol allows purification of hPSC-NC cells without the need for genetic manipulation or drug selection using a simple antibody-mediated cell sorting strategy (**Fig. 8**). Approximately 30% (32.4% ± 5%) of total cells can be considered NC precursors based on coexpression of p75 and HNK1 at day 7 of P1 culture (MS5 protocol). Approximately 15–40% of total cells coexpress p75/HNK1 at day 12 of the NSB protocol. Flow cytometric analysis of purified NC precursors should show coexpression with CD29 (97.1% ± 2.5%); CD133 (65.3% ± 15%); CD49d (75.3% ± 5%); CD49b (67.4% ± 19%); and CXCR4 (67.1% ± 12%). When plated at clonal density, approximately half of the single cell-derived NC precursor cultures should yield multipotent differentiation (Tuj1+ neuronal cells, GFAP+ Schwann cells and SMA+ myofibroblast). We have previously shown that unbiased microarray gene expression analysis identifies the majority of highly enriched transcripts as established NC precursors markers such as AP2b, Sox10 and Erbb3. Accordingly, more than 90% of the purified NC precursors should coexpress Sox10 and Ap2. The yield of p75+/HNK1+ precursors (15–40%) using the NSB protocol is comparable with the yield using the MS5 protocol. However, the efficiency of generating specific NC sublineages using the NSB protocol remains to be determined. At the current stage, it is also unclear whether there are distinct properties of NC precursors derived through the NSB versus MS5 protocol, such as a possible bias toward pre- versus post-migratory or NC populations (**Figs. 2** and **3**).

Another powerful technique to identify and purify neural cell lineages derived from hPSCs includes the use of stable transgenic reporter lines. We have recently established the use of bacterial artifical chromosome (BAC)-based transgenic reporters in hESCs[40]. BAC transgenic reporter hESC lines can be used in a complementary manner with the protocols presented here such as for the purification of peripheral neurons derived from p75+/HNK1+ hESC-NC precursors cells (e.g., using the

Table 2 | List of antibodies used in this study.

Name	Expression	Company	Cat. no.	Dilution for staining
Brn3a	Peripheral sensory neuron	Millipore	AB5945	1:200
GFAP	Schwann cell	MP biomedical	8691102	1:300
HNK1	Neural crest	Sigma	C6608	1:300
MASH1	Peripheral autonomic neuron	BD Pharmingen	556604	1:200
p75	Neural crest	Advanced targeting system	AB-N07	1:200
Pax6	Neuroepithelium	DSHB	pax6	1:200
Peripherin	Peripheral neuron	Santa Cruz	sc-7604	1:200
AP2 alpha	Neural crest	DSHB	3B5	1:200

DSHB, developmental studies hybridoma bank; GFAP, glial fibrillary acidic protein.

recently published *Dll1::eGFP* line[40], marking neuronal committed cells after hESC-NC precursor cell isolation or the isolation and propagation of putative hESC-derived NC stem cells using the recently established *Sox10::eGFP* hESC line (unpublished data)). A brief outline for generating stable BAC transgenic reporter lines is provided including retrofitting of the BAC construct for mammalian selection (**Box 1**) and the specific conditions for introducing the BAC construct into hESC lines and selecting for stable BAC transgenic reporter lines (**Box 2**).

Table 3 | List of growth factors and chemicals used in this study.

Recombinant protein	Stock concentration	Dilution	Final concentration	Company	Cat. no.
AA	100 mM	500	200 μM	Sigma	A4034
BDNF	10 μg ml^{-1}	500	20 ng ml^{-1}	R&D systems	248-BD
BMP4	50 μg ml^{-1}	1,000	50 ng ml^{-1}	R&D systems	314-bp
cAMP	100 mM	200	0.5 mM	Sigma	D0260
Dkk-1	100 μg ml^{-1}	1,000	100 ng ml^{-1}	R&D systems	1096-DK
EGF	10 μg ml^{-1}	500	20 ng ml^{-1}	R&D systems	236-EG
FGF2 (hESC)	10 μg ml^{-1}	2,500	4 ng ml^{-1}	R&D systems	233-FB-001MG
FGF2 (Diff.)	10 μg ml^{-1}	500	20 ng ml^{-1}	R&D systems	233-FB-001MG
FGF8	50 μg ml^{-1}	500	100 ng ml^{-1}	R&D systems	423-F8
Fibronectin	1 mg ml^{-1}	1,000	1 μg ml^{-1}	BD	356008
GDNF	10 μg ml^{-1}	500	20 ng ml^{-1}	R&D systems	212-GD
Laminin	0.5 mg ml^{-1}	500	1 μg ml^{-1}	BD	354239
Noggin	50 μg ml^{-1}	100	500 ng ml^{-1}	R&D systems	719-NG
PO	15 mg ml^{-1}	1,000	15 μg ml^{-1}	Sigma	P3655
Retinoic acid	1 mM	2,000	0.5 μM	Sigma	R2625
SB431542	10 mM	1,000	10 nM	Tocris	1614
Shh (C25II substitution)	100 μg ml^{-1}	5,000	20 ng ml^{-1}	R&D systems	464-SH
SU5402	10 uM	1,000	10 nM	Calbiochem	572630
Wnt3A	40 μg ml^{-1}	1,000	40 ng ml^{-1}	R&D systems	1324-WN
NGF	10 μg ml^{-1}	1,000	10 ng ml^{-1}	R&D systems	256-GF
NT-3	10 μg ml^{-1}	1,000	10 ng ml^{-1}	R&D systems	267-N3
CNTF	10 μg ml^{-1}	1,000	10 ng ml^{-1}	R&D systems	257-NT
Neuregulin	20 μg ml^{-1}	1,000	20 ng ml^{-1}	R&D systems	396-HB

AA, ascorbic acid; BDNF, brain-derived neurotrophic factor; BMP4, bone morphogenetic factor 4; CNTF, ciliary neurotrophic factor; Dkk, dickkopf-1; EGF, epidermal growth factor; FGF2, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; hESC, human embryonic stem cell; NGF, nerve growth factor; NT-3, neurotrophin-3; PO, poly-L-ornithine hydrobromide; Shh, sonic hedgehog.

BOX 1 | RETROFITTING BACS

Reagent setup

***E.coli* strains:** EL350 and SW106 strains can be obtained from NCI (http://recombineering.ncifcrf.gov).

10% L(+)Arabinose: Mix 1 g of L(+)arabinose in 10 ml of sterile water and filter sterilize.

Solution 1: No lysozyme is needed and do not add RNase as it is used as a carrier. Combine 10 ml of 1 M glucose (50 mM glucose final), 5 ml 1 M Tris (pH 8.0; 25 mM Tris final), 4 ml of 0.5 M EDTA (10 mM EDTA final) and 181 ml water. This will give a total of 200 ml solution.

Solution 2: Combine 20 ml of 10% SDS (1.0% SDS final), 8 ml of 5 N NaOH (0.2 N NaOH) and 172 ml water to give a total of 200 ml solution.

Solution 3: Dissolve 58.8 g potassium acetate in 100–160 ml water. Add 23 ml glacial acetic acid and make up to 200 ml of final volume with water.

Procedure

BAC miniprep

1. Grow 5 ml overnight culture of DH10B *E. coli* containing BAC of interest in LB broth with 12.5–20 μg ml^{-1} chloramphenicol.
2. Transfer 1.5 ml of saturated overnight culture to a microfuge tube and spin at 16,000*g* for 15–20 s at room temperature.
3. Aspirate supernatant and resuspend pellet in 250 μl of solution 1.
4. Add 250 μl of solution 2 and invert the tube gently a few times.

▲ **CRITICAL STEP** Vigorous mixing can shear the bacterial genome, contaminating the BAC preparation.

5. Add 350 μl of solution 3 and invert the tube gently a few times.
6. Microfuge at 16,000*g* for 4 min at 4 °C.
7. Transfer supernatant to a fresh tube by carefully avoiding the precipitated debris.
8. Microfuge at 16,000*g* for 4 min at 4 °C.
9. Carefully transfer the supernatant to a new microtube containing 750 μl of isopropanol.
10. Mix gently a few times and incubate for 10 min at room temperature.
11. Microfuge at 16,000*g* for 10 min at 4 °C.
12. Carefully aspirate the supernatant with a p1000 or an aspirating pipette.
13. Gently add 1 ml of 70% ethanol to the tube trying not to disturb the pellet.
14. Microfuge at 16,000*g* for 3 min at 4 °C.
15. Carefully aspirate the supernatant. Remove residual moisture with a cotton-tipped applicator while avoiding the pellet.
16. Air dry the tube inverted with the lid bent back for 5 min.

▲ **CRITICAL STEP** Overdrying the pellet will make it difficult or impossible to resuspend the DNA.

17. Gently resuspend the pellet in 16 μl of sterile water. Place on ice until one is ready for the electroporation. It is good practice to use genomic DNA pipette tips at any stage after lysis to avoid shearing the large BAC DNA.

Transferring BACs into Cre-expressing *E.coli* strains

18. Inoculate 5 ml of LB with EL350 or SW106 *E. coli*.
19. Pellet the bacteria by centrifuging at 6,000*g* for 5 min at 4 °C.
20. Remove the supernatant, resuspend cells in 1 ml ice-cold sterile water and transfer to a cold microtube.
21. Microfuge at 16,000*g* for 15–20 s at room temperature.
22. Place the tubes on ice and carefully aspirate the supernatant.
23. Repeat wash three more times as above.
24. Resuspend the final cell pellet in 50 μm of ice-cold water and add 1–2 μl of BAC miniprep DNA.
25. Transfer *E. coli* and DNA into ice-cold 0.1 cm electroporation cuvette.
26. Electroporate using 1.75 kV, 25 μF with the pulse controller set at 200 Ω. Time constant should be around 4 ms.
27. Add 1 ml LB to the electroporated cuvette and transfer its contents to a round-bottomed 15 ml snap-cap tube, leaving the lid loose for aeration.
28. Shake at 225 r.p.m. for 1 h at 30–32 °C.
29. Plate on LB agar with 12.5–20 μg ml^{-1} chloramphenicol.

Retrofitting BAC with pRetroNeo

30. Inoculate 5 ml of LB with EL350 or SW106 *E.coli* containing BAC of interest and grow overnight at 30–32 °C.
31. The next day, add 1 ml of overnight culture to 20 ml of LB and shake at 225 r.p.m. for 1 h, at 30–32 °C.
32. Split the culture into two flasks containing 10 ml each. In one flask, add 100 μl of 10% L(+)arabinose and shake for another hour. The second culture receives no arabinose: it is the "no Cre" control.
33. Put the cells on wet ice and shake manually to drop the temperature of the culture as quickly as possible.
34. Leave the flask in ice for another 5 min, shaking occasionally.
35. Transfer to a cold 50 ml conical tube and spin in a swinging bucket rotor at 4,650*g* for 5 min at 4 °C.
36. Resuspend the cells in 1 ml ice-cold water and transfer to a cold microfuge tube.
37. Microfuge at 16,000*g* for 15–20 s at room temperature and then place the tubes on ice.
38. Carefully aspirate the supernatant.
39. Repeat wash three more times as above.

(continued)

BOX 1 | CONTINUED

40. Resuspend the final cell pellet in 50 µl ice-cold water and add 100 ng of pRetroNeo to one induced and one uninduced sample.
41. Transfer DNA/*E.coli* to an ice-cold 0.1 cm electroporation cuvette.
42. Electroporate using 1.75 kV, 25 µF with the pulse controller set at 200 Ω.
43. Add 1 ml of LB to the electroporated cuvette and transfer its contents to a round-bottomed snap-cap tube, leaving the lid loose for aeration.
44. Shake at 225 r.p.m. for 1 h at 30–32 °C.
45. Plate onto LB agar with 12.5–20 µg ml^{-1} chloramphenicol and 50 µg ml^{-1} spectinomycin.
46. Incubate plates overnight (possibly two nights) at 30–32 °C.
47. Pick isolated colonies, make glycerol stocks and perform restriction enzyme digests to verify BAC structure.
48. Correct BAC clones should be moved into DH10B *E.coli* to ensure that there is no leaky Cre expression. Follow the first protocol for synthesizing BAC DNA, and either make DH10B electrocompetent as above or purchase it from Invitrogen (e.g., cat. no. 6400-03).

In addition to the isolation of NC precursor cells, we also present conditions that allow hESC-NC cell proliferation and further differentiation toward peripheral neuron fates (**Fig. 10**), Schwann cells (**Fig. 11**) and myofibroblasts (**Fig. 12**). During peripheral neuronal differentiation, we could observe 40–60% of Tuj1+/peripherin+ cells from total cell populations. During

BOX 2 | NUCLEOFECTING BACS INTO HUMAN ESCS

Reagent setup
hESC media: Combine 780 ml DMEM:F12, 200 ml Knockout Serum Replacement, 5 ml L-glutamine, 10 ml MEM-NEAA and 1 ml β-mercaptoethanol. Filter sterilize the media before adding FGF2 at a final concentration of 6 ng ml^{-1}.
Conditioned media: Plate MEFs at 50,000 cells per cm^2 in DMEM+10% FBS medium. The next day, switch the media to hESC before overnight incubation. The following day, collect the conditioned media (CM) and add fresh media onto the MEFs (this can continue for at least 2–3 weeks with one batch of MEFs). Spike the CM with 10 ng ml^{-1} fresh FGF2 before use.
Y-27632 (Tocris): Make a 10 mM stock in water, filter sterilze and store the aliquots at −80 °C before use.
Accutase (Innovative Cell Technologies)
Neo-resistant MEFs (GlobalStem, cat. no. GSC-6101M): Plate feeders at two different densities depending on the application. For hESC plating, we use ~12,000 cells per cm^2, whereas CM requires ~50,000 cells per cm^2. It is important to note that MEFs from different manufacturers (and to some extent from different lots) adhere to the Petri dish with different efficiencies. We always coat the tissue culture plastic with 0.1% gelatin and keep it for at least 15 min before plating.
Dispase (neutral protease) (Worthington Bioscience): Prepare dispase based on its activity (6 U ml^{-1}) and make up the solution using hESC media. Filter sterilize before use.
Matrigel and matrigel-coated Petri dishes (BD Bioscience): We only use Matrigel with high protein content, 10 mg ml^{-1} or higher. To coat a Petri dish, add 1 ml Matrigel to 19 ml of DMEM:F12. Pipette the mixture a few times to mix before placing in culture Petri dish. Leave the Petri dish in the hood for 1 h at room temperature. Before using, aspirate the Matrigel off the surface and plate cells directly onto the coated Petri dish without a wash.
Preparation of hESCs
1. Expand hESCs on MEFs until they are ready to passage (~7 d).
2. Passage hESCs after digestion using 6 U ml^{-1} dispase for ~7 to 9 min at 37 °C tissue culture incubator. Use 10 ml dispase per 15 cm Petri dish.
3. Carefully detach colonies from MEFs by lightly pipetting dispase over the surface of the Petri dish.
4. Split 10 ml dispase/hESCs into two 15 ml conical tubes, 5 ml in each.
5. Gently add 10 ml hESC media to each conical tube, making up the total volume to 15 ml in each tube.
6. Centrifuge at 200*g* for 5 min at room temperature.
7. Carefully aspirate the supernatant.
8. Add 10 ml hESC media and gently break up the pellet.
▲ **CRITICAL STEP** Do not break up the colonies. Excessive trituration reduces viability, hence pipette up and down gently 2–3 times.
9. Centrifuge at 200*g* for 5 min at room temperature.
10. Carefully aspirate the supernatant.
11. Carefully resuspend the pellet into MEF CM and replate hESCs in the ratio of 1:1 to 1:2 onto Matrigel-coated Petri dishes.
▲ **CRITICAL STEP** Residual dispase can cause poor adhesion to the Matrigel. Perform a third wash if adhesion is a problem.
12. Expand hESCs on Matrigel from 3 to 5 d, feeding with fresh MEF-conditioned hESC media containing 10 ng ml^{-1} FGF2 daily.
Nucleofection
13. The day before nucleofection, plate a 15 cm Petri dish of Neomycin-resistant MEFs for each nucleofection sample.
14. On the morning of nucleofection, prepare MEFs by aspirating the plating media, washing once with PBS and adding hESC media containing 10 µM Y-27632. Place it in tissue culture incubator (37 °C).

(continued)

BOX 2 | CONTINUED

15. Remove nucleofection solution V from the refrigerator (4 °C) and allow it to warm to room temperature. (Amaxa Cell Line Nucleofector Kit V, Lonza Bioscience cat. no. VCA-1003).
16. Aspirate the CM from hESCs and add 10 ml accutase per 15 cm Petri dish.
17. Place the Petri dishes in the tissue culture incubator (37 °C) for 20 min.
18. Pipette accutase/hESCs into two 15 ml conical tubes, 5 ml in each tube.
19. Add 10 ml hESC media to each conical tube, making up the total volume to 15 ml in each tube.
20. Centrifuge at 200*g* for 5 min at room temperature.
21. Aspirate the supernatant.
22. Resuspend the pellet into 10 ml hESC media with 10 μM Y-27632. Pipette robustly enough to make a monodisperse cell suspension.
23. Count the number of cells using a hemocytometer.
24. Add enough volume of cell suspension to obtain 4–5 million cells in a 15 ml conical tube. Prepare enough conical tubes to hold all desired samples, a 'no DNA' control and the GFP plasmid control (included in the kit).
25. Centrifuge at 200*g* for 5 min at room temperature.
26. Aspirate the supernatant and add 100 μl of solution V maintained at room temperature.

▲ **CRITICAL STEP** Transfer quickly into a sterile Eppendorf tube once cells are in solution V because the viability of cells drops after ~15 min in solution V.

27. Add 5–100 μg BAC or plasmid DNA to the hESCs in solution V (do not exceed 10 μl).
28. Transfer the mixture to a cuvette and nucleofect using program B-16.
29. Use prewarmed hESC media from 15 cm Petri dish taken in an eyedropper (included in kit) to help remove the cell mixture from the cuvette.
30. Add the cell mixture dropwise into the Petri dish with MEFs, taking care to spread the cells across the entire Petri dish.

Selection

31. Feed the cells daily, including 10 μM Y-27632 on the d after nucleofection only.
32. Begin the selection on day 4 with 25 μM G418.
33. Increase the selection on day 14 with 40–50 μM G418.
34. Colonies should be visible after 2 weeks, and are ready for picking between 2 and 3 weeks.

Schwann cell differentiation, around 30% of total cells expressed GFAP after 4 weeks differentiation. During myofibroblast differentiation in the presence of serum, most of the cells (over 90%) express CD73 antigen by FACS analysis. Myofibroblast cells can be further directed into various mesenchymal cell lineages such as smooth muscle, osteogenic, chondrogenic and adipogenic cells[6,33]. Our method can also be used to enrich for CNS lineages by negatively sorting for p75+/HNK1+ cells and/or positive selection of Forse1+ cells at the P1 stage[8].

Finally, the use of our NC differentiation protocols should be an important tool for modeling human disease. A recent example is the successful modeling of the pathogenesis and treatment of Familial Dysautonomia in patient-specific iPSCs[33].

ACKNOWLEDGMENTS We thank all the members of Studer, Tabar and Tomishima labs for technical advice and helpful discussions on the protocol. Our work was supported by grants from the Starr Foundation, NYSTEM and NINDS. Additional support was provided by the New York Stem Cell Foundation (NYCSF, Druckenmiller fellowships to G.L.) and by the Starr Scholar fellowship to S.M.C.

AUTHOR CONTRIBUTIONS All authors contributed to the development of the method and the writing of the manuscript.

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