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Efficient generation of trunk neural crest and sympathetic neurons from human pluripotent stem cells via a neuromesodermal axial progenitor intermediate

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Abstract

The neural crest (NC) is a multipotent embryonic cell population that generates various cell types in an axial position-dependent manner. Cranial NC cells, give rise to mesoectodermal derivatives, melanocytes, neurons and glia whereas the vagal NC generates the enteric nervous system and trunk NC cells produce sympathetic neurons and neuroendocrine cells. An attractive approach for studying human NC biology and modelling NC-associated developmental disorders (neurocristopathies) involves the in vitro production of NC cells from human pluripotent stem cells (hPSCs). However, most conventional differentiation protocols generate predominantly cranial NC but fail to induce trunk NC cells. Here we describe a detailed protocol for the efficient in vitro generation of trunk NC cells and their derivatives from hPSCs. This relies on the induction of an intermediate cell population that exhibits neural and mesodermal potential, resembling the embryonic neuromesodermal progenitors, which generate the postcranial body axis in vivo.

Keywords

Human pluripotent stem cells (hPSCs); neural crest; sympathetic neurons; neuromesodermal progenitors

Introduction

The neural crest (NC) is a multipotent embryonic cell population that arises in the dorsal neural plate/non-neural ectoderm border region during vertebrate embryogenesis. Following induction, NC cells undergo epithelial-to-mesenchymal transition, exit the neural tube, and migrate to generate various cell types. The identity of NC derivatives depends on their position along the anteroposterior (A-P) axis, which is reflected by the expression of HOX gene paralogous groups (PGs) (Le Douarin, Creuzet, Couly, & Dupin, 2004). The most anterior HOX-negative cranial NC cells, give rise to mesoectodermal derivatives (e.g. dermis, cartilage, bone), melanocytes, neurons and glia colonizing the head. Cardiac NC, marked by HOX PG(1-3) expression, contributes to various heart structures whereas HOX PG(3-5)+ vagal NC cells generate enteric neurons. At the thoracic level, HOX PG(5-9)+

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trunk NC produces sympathoadrenal cells, which in turn give rise to sympathetic neurons, neuroendocrine cells, and melanocytes. A wide spectrum of congenital conditions, known collectively as neurocristopathies, arise due to defects in NC development, often in an axial level-specific manner.

An attractive, tractable strategy to model human NC development and neurocristopathies involves the in vitro generation of NC cells from human pluripotent stem cells (hPSCs). However, most conventional hPSC differentiation protocols yield NC cells of an anterior cranial character but fail to induce a high number of NC cells of a HOX PG(5-9)+ trunk identity (Chambers et al., 2012; Hackland et al., 2017; Lee et al., 2007; Menendez, Yatskievych, Antin, & Dalton, 2011) even following addition of classic “posteriorisation” signals such as retinoic acid (Fattahi et al., 2016; Huang et al., 2016). Thus the production of trunk NC derivatives has relied on the isolation of small cell populations positive for lineage-specific fluorescent reporter (Oh et al., 2016) or cell surface markers (Abu-Bonsrah, Zhang, Bjorksten, Dottori, & Newgreen, 2018) through flow cytometry, a time-consuming and laborious approach.

We have recently described the efficient derivation of trunk NC cells and sympathetic neurons from hPSCs (Frith et al., 2018). Our method relies on the initial induction of a multipotent axial progenitor intermediate population, which is marked by the co-expression of mesodermal and neural markers such as T(BRACHYURY) and SOX2 and exhibits neuromesodermal potency (Frith et al., 2018; Gouti et al., 2014) (Fig. 1). These cells resemble the embryonic neuromesodermal progenitors (NMPs) which contribute to the generation of the postcranial body axis during embryonic development in various vertebrates (Brown & Storey, 2000; Henrique et al., 2015; Wymeersch et al., 2016; Tzouanacou et al 2009). Once plated in NC-inducing conditions hPSC-derived axial progenitors (hAPs) then give rise to trunk NC cells (Fig. 1). Subsequent culture of trunk NC cells in neurotrophic media yields a high percentage of sympathoadrenal progenitors and sympathetic neurons (Fig. 1). Here we provide a detailed description for the in vitro production of these cell types from hPSCs.

NOTE: Cells are cultured at 37°C under humidified atmosphere of 5% CO₂ in air. The described procedures using live cells are performed in sterile environment.

NOTE: We often observe marked differences in the performance of the same reagents obtained from different suppliers as well as batch-to-batch variation and therefore we recommend testing different concentrations if a different supplier is used or a new batch is purchased.

Basic Protocol 1

Neuromesodermal Axial Progenitor Differentiation

The first stage of the differentiation protocol entails culturing hPSCs in the presence of WNT and FGF signaling pathway agonists for three days to generate NM potent axial progenitors (hAPs) marked by co-expression of T and SOX2 (Fig. 2). This stage also

coincides with the acquisition of a posterior identity prior to commitment to a definitive NC state.

Materials

DMEM/F12 WITHOUT Glutamine (Sigma, St. Louis USA; D6421). *Store at 4°C*

Neurobasal™ Medium (ThermoFisher, Waltham USA; 21103049). *Store at 4°C*

N2 Supplement (ThermoFisher, Waltham USA; 17502048). *Store at 4°C*

B27 Supplement (ThermoFisher, Waltham USA; 17504001). *Store at 4°C*

Glutamax supplement (ThermoFisher, Waltham USA; 35050061). *Store at 4°C*

MEM Non-Essential Amino Acids Solution (ThermoFisher, Waltham USA; 11140050). *Store at 4°C*

2-mercaptoethanol (ThermoFisher, Waltham USA; 31350-010). *Store at 4°C*

CHIR99021 (Tocris, Minneapolis USA; #4423). Aliquot and store at -20°C. *Minimise freeze-thaw cycles.*

Recombinant Human FGF basic (146 aa) protein CF (R&D systems, Minneapolis USA; #233-FB/CF).

Aliquot and store at -20°C. Minimise freeze-thaw cycles.

Y-27632-dihydrochloride (Tocris, Minneapolis USA; #1254). Aliquot and store at -20°C. *Minimise freeze-thaw cycles.*

Vitronectin (VTN-N) (ThermoFisher, Waltham USA; A14700)- defrost as per manufacturer's instructions.

Make 60µl aliquots and store at -80°C.

Accutase (ThermoFisher, Waltham USA; A1110501). *Make 25ml Aliquots and store at -20°C. Once defrosted, store at 4°C*

Pre-Differentiation set up

- 1.** Before differentiation, coat plates with Vitronectin (0.5 µg/cm²) for the differentiation.
 - 1a.** Thaw an aliquot of frozen vitronectin at room temperature
 - 1b.** Dilute 1:100 in PBS (without Calcium and Magnesium) i.e. dilute 60µl of vitronectin into 5.94ml PBS for 6ml total.
 - 1c.** Coat plates (cover surface with 200µl of solution per cm²)

- 1d. Leave plates for 1 hour at room temperature or seal plates with parafilm and store at 4°C for up to one week. Leave plates at room temperature for one hour before use.
2. Prepare differentiation (N2B27) media (Table 1)
 - 2a. For 100ml of N2B27 media, combine the ingredients described at Table 1 at the stated volumes (Table 1).
 - 2b. Add GSK3 β antagonist CHIR99021 (final concentration 3 μ M) and recombinant basic FGF (final concentration 20ng/ml) to the required volume of N2B27.

Small molecules and recombinant proteins should be added in fresh.

For the first day (Day 0), (when hPSC are plated) the Rho-kinase inhibitor Y27632-dihydrochloride must be added at a final concentration of 10 μ M.

Differentiation set up

1. **DAY 0- plating hPSC for axial progenitor differentiation**
 - 1a. Grow human pluripotent stem cells to approximately 70% confluency.
 - 1b. Aspirate the media and replace with Accutase™ (250 μ l per cm²) and incubate the cells at 37°C/5%CO₂ for 7-10 minutes until a single cell suspension.

AFTER ADDING ACCUTASE, DO NOT ASPIRATE AS CELLS ARE NOW IN SUSPENSION.
 - 1c. Add media to the accutase and transfer the cell suspension to a 15ml falcon tube.
 - 1d. Take 10 μ l of cell suspension to count using a haemocytometer
 - 1e. Spin the remaining cell suspension at 200 x g for 4 minutes in a tissue culture centrifuge.
 - 1f. After centrifugation, the cell pellet should be clearly visible at the bottom of the tube. Aspirate the supernatant being careful not to aspirate the cell pellet.
 - 1g. Resuspend the cell pellet in N2B27 supplemented with 3 μ M CHIR99021 and 20ng/ml bFGF at a ratio of 1 million cells per millilitre.
 - 1h. Plate cells at a ratio of 50,000 cells per cm²

i.e 50 μ l of cell suspension per cm²- for example a 12 well plate is 4cm² so add 200 μ l of cell suspension to one well
 - 1i. Add 200 μ l per cm² of N2B27 supplemented with 3 μ M CHIR99021 and 20ng/ml bFGF and 10 μ M of Y-27632-dihydrochloride

- 1j. rock plate gently in a 'North, South, East, West' fashion to disperse cells evenly across the surface area and incubate at 37°C/5% CO₂ overnight.
2. **Day 2**
 - 2a. The next day (day 1), change the media (250µl per cm²) to N2B27 media supplemented with 3µM CHIR99021 and 20ng/ml bFGF and leave until day three (Fig. 2A).
For a representative image of desired density, see fig. 2B
3. **Day 3**
 - 3a. Differentiation to hAPs is complete. Cells should appear as a confluent monolayer of cells (See Fig. 2B)
 - 3b. Cells are now ready to be replated for further differentiation to trunk neural crest.

Anticipated results

By day three, the cells should be almost 100% confluent (Fig. 2B). The cultures are marked by the co-expression of classic NMP markers such as T and SOX2 together with CDX2 and HOXC9 both at the protein (70-90% depending on cell line) and transcript level. Early neural plate border and dorsal neural tube/neural crest markers (e.g. PAX3, MSX1, SOX9) should also be detectable by both immunostaining and quantitative real time PCR (qPCR) (Tables 2, 3; Fig. 2C, D).

Basic Protocol 2

Trunk Neural Crest Differentiation

This step involves the directed differentiation of hAPs toward definitive trunk NC cells marked by expression of HOX PG(5-9) genes. A scheme outlining this stage of differentiation is shown in Fig. 3A. During the neural crest induction step, we employ an intermediate level of BMP signaling stimulation by using a "Top-down inhibition" approach involving the parallel addition of recombinant BMP4 and the BMP inhibitor DMH-1 (Hackland et al 2017).

Materials

DMEM/F12 WITHOUT Glutamine (Sigma, St. Louis USA; D6421). *Store at 4°C*

N2 Supplement (ThermoFisher, Waltham USA; 17502048). *Store at 4°C*

Glutamax supplement (ThermoFisher, Waltham USA; 35050061). *Store at 4°C*

MEM Non-Essential Amino Acids Solution (ThermoFisher, Waltham USA; 11140050). *Store at 4°C*

SB 431542 (Tocris, Minneapolis USA; #1614). *Aliquot and store at -20°C. Minimise freeze-thaw cycles.*

CHIR99021 (Tocris, Minneapolis USA; #4423). *Aliquot and store at -20°C. Minimise freeze-thaw cycles.*

Y-27632-dihydrochloride (Tocris, Minneapolis USA; #1254). *Aliquot and store at -20°C. Minimise freeze-thaw cycles.*

DMH1 (Tocris, Minneapolis USA; #4126) *Aliquot and store at -20°C. Minimise freeze-thaw cycles.*

Recombinant Human BMP4 (Thermofisher, Waltham USA; PHC9534). *Aliquot and store at -20°C. Minimise freeze-thaw cycles.*

Geltrex™ (Thermofisher, Waltham USA; A1413202)- store at -20°C

Accutase (Thermofisher, Waltham USA; A1110501). *Make 25ml Aliquots and store at -20°C. Once defrosted, store at 4°C*

Pre-Differentiation set up

1. hAPs are replated onto Geltrex™ coated plates. Before the differentiation begins, prepare Geltrex™ plates as per the instructions below.
 - 1a. Thaw a 1ml aliquot of 1:10 diluted Geltrex™ (i.e. 1ml of stock Geltrex originally diluted in 9 ml of DMEM/F12 and stored at -80°C) on ice until liquid (approximately one hour)
 - 1b. Add 9ml ice cold DMEM/F12 to give 10ml of 1:100 dilution Geltrex™ solution
 - 1c. Add Geltrex™ to plates (200µl per cm²) and incubate at 37°C for one hour.
 - 1d. Alternatively, plates can be sealed with laboratory film and kept at 4°C for one week. Before use, place in the incubator at 37°C for one hour.

Differentiation set up

1. **Day Three-** re-plating hAPs for neural crest differentiation
 - 1a. Aspirate the media from axial progenitors and replace with Accutase™ (250µl per cm²) and incubate the cells at 37°C/5%CO₂ for 7-10 minutes until a single cell suspension.
 - 1b. Add media to the accutase and transfer the cell suspension to a 15ml falcon tube.
 - 1c. Take 10µl of cell suspension to count using a haemocytometer
 - 1d. Spin the remaining cell suspension at 200 x g for 4 minutes in a tissue culture centrifuge.

- 1e. After centrifugation, the cell pellet should be clearly visible at the bottom of the tube. Aspirate the supernatant being careful not to aspirate the cell pellet.
- 1f. Resuspend the cell pellet in Neural crest media (Table 4) supplemented with 10 μ M Y27632-dihydrochloride at a ratio of 1 million cells per millilitre.
50ml of Neural crest media can be made and kept at 4°C for one week.
- 1g. Plate cells at a ratio of 30,000 cells per cm². (i.e 30 μ l of cell suspension per cm²- for example a 12 well plate is 4cm² so add 120 μ l of cell suspension to one well.
- 1h. Add 200 μ l per cm² of Neural Crest media supplemented with 10 μ M of Y-27632-dihydrochloride
- 1i. rock plate gently in a 'North, South, East, West' fashion to disperse cells evenly across the surface area and incubate at 37°C/5% CO₂ overnight.
2. **Day 4**-Cells should form small well-defined colonies (Fig. 3B).
3. **Day 5**-Aspirate neural crest media from cells and replace with 200 μ l per cm² of neural crest media without Y27632-dihydrochloride.
4. **Day 7**-Aspirate neural crest media from cells and replace with 200 μ l per cm² of neural crest media
Cells should be approaching confluence (for representative image, see Fig. 3B)
Confluency between days 6 and 7 is a common indicator of differentiation success. (See troubleshooting section)
5. **Day 8-final day of differentiation**
Cells should form a confluent monolayer at this stage
Cells can be analysed for gene expression or replated into conditions for sympathetic neuronal differentiation.

Anticipated results

Trunk neural crest cells (40-60% of the cultures) are defined by the co-expression of neural crest markers such as SOX10 together with trunk HOX genes such as HOXC9 (Tables 5, 6; Fig. 3C, D).

Basic Protocol 3

Sympathetic Neuron Differentiation

The final stage of this differentiation involves re-plating day 8 trunk neural crest cells in media with BMP and Sonic Hedgehog (SHH) agonists. Over the course of four days, cells transit from a SOX10 positive neural crest identity to an ASCL1+, PHOX2B+ identity that is

consistent with sympathoadrenal precursors. After four days in BMP and SHH stimulating conditions, the media is changed to contain the neurotrophins BDNF, NGF and GDNF that stimulate the formation of functional catecholaminergic sympathetic neurons (See Fig. 4A).

Materials

BrainPhys (Stem Cell Technologies, Vancouver Canada; #05790). *Store at 4°C.*

N2 Supplement (Thermofisher, Waltham USA; 17502048). *Store at 4°C*

B27 Supplement (Thermofisher, Waltham USA; 17504001). *Store at 4°C*

Glutamax supplement (Thermofisher, Waltham USA; 35050061). *Store at 4°C*

MEM Non-Essential Amino Acids Solution (ThermoFisher, Waltham USA; 11140050). *Store at 4°C*

Recombinant Sonic Hedgehog C24II N-Terminus (R&D systems, Minneapolis USA; #1845-SH). *Aliquot and store at -20°C. Minimise freeze-thaw cycles. We have found other non-modified recombinant Sonic Hedgehog proteins are less effective in the differentiation.*

Purmorphamine (Sigma Aldrich, St. Louis USA; SML0868). *Aliquot and store at -20°C. Minimise freeze-thaw cycles.*

Recombinant Human BMP4 (Thermofisher, Waltham USA; PHC9534). *Aliquot and store at -20°C. Minimise freeze-thaw cycles.*

Accutase (Thermofisher, Waltham USA; A1110501). *Make 25ml Aliquots and store at -20°C. Once defrosted, store at 4°C*

Recombinant Human NGF (Peprotech, Rocky Hill USA; 450-01)

Recombinant Human/Murine/Rat BDNF (Peprotech, Rocky Hill USA; 450-02)

Recombinant Human GDNF (Peprotech, Rocky Hill USA; 450-10)

Pre-Differentiation

This stage of differentiation is done on Geltrex coated plates. For preparation instructions see above.

Differentiation set up

1. DAY 8-Sympathoadrenal priming

- 1a.** Aspirate the media from axial progenitors and replace with Accutase™ (250µl per cm²) and incubate the cells at 37°C/5%CO₂ for 7-10 minutes until a single cell suspension.
- 1b.** Add media to the accutase and transfer the cell suspension to a 15ml falcon tube.

- 1c. Take 10µl of cell suspension to count using a haemocytometer
 - 1d. Spin the remaining cell suspension at 200 x g for 4 minutes in a tissue culture centrifuge.
 - 1e. After centrifugation, the cell pellet should be clearly visible at the bottom of the tube. Aspirate the supernatant being careful not to aspirate the cell pellet.
 - 1f. Resuspend the cell pellet in sympathoadrenal media A media (Table 7) supplemented with 10µM Y27632-dihydrochloride at a ratio of 1 million cells per millilitre.
 - 1g. Plate cells at a ratio of 200,000 cells per cm². (i.e 150µl of cell suspension per cm²- for example a 12 well plate is 4cm² so add 600µl of cell suspension to one well).

It is strongly recommended that initial attempts at this differentiation optimise for cell density. Cell death is observed throughout the course of the priming stage and hinders differentiation if too many cells are lost.
 - 1h. Add 200µl per cm² of Sympathoadrenal media A supplemented with 10µM of Y-27632 dihydrochloride
 - 1i. Rock plate gently in a 'North, South, East, West' fashion to disperse cells evenly across the surface area and incubate at 37°C/5% CO₂ overnight.
2. **Day 9**-Cells should have acquired a neuronal morphology (See Fig 4B)
 - 2a. Aspirate media from cells
 - 2b. Add 250µl per cm² to Sympathoadrenal media A without Y-27632 dihydrochloride
 - 2c. Incubate at 37°C/5% CO₂
 3. **Day 10**
 - 3a. Aspirate media from cells
 - 3b. Replace with 250µl per cm² to Sympathoadrenal media A
 - 3c. Incubate at 37°C/5% CO₂
 4. **Day 11**
 - 4a. Aspirate media from cells
 - 4b. Replace with 250µl per cm² to Sympathoadrenal media A
 - 4c. Incubate at 37°C/5% CO₂
 5. **Day 12- End of priming stage.**
 - 5a. Aspirate media from cells

- 5b.** Replace with 250µl per cm² to Sympathoadrenal media B (Table 8)
- 5c.** Incubate at 37°C/5% CO₂
 - *replenish Sympathoadrenal media B every two to three days until desired timepoint*
 - *If cells begin to lift, supplement vitronectin to the media to replenish the matrix*
 - *PHOX2B positive cells are present from day 12 onwards (see Figure 4B/C).*

6. Day 12 onwards- maturation of Sympathetic neurons.

- 6a.** Change media from Sympathoadrenal media A to Sympathoadrenal media B
- 6b.** Change media every 2-3 days until desired timepoint.
Cells can be cultured in these media for extended periods. To date, we have kept cells in this media up to day 30 (18 days).

Anticipated results-comments

- During the priming stage (days 8 to 12 of differentiation), there is a large amount of cell death. This is normal for the differentiation, although if too many cells die, the differentiation will fail.
- By day 9, cells will begin to show a neuronal morphology
- We found that by day 12, a large proportion (50-80%) of cells express the sympathoadrenal markers ASCL1 and PHOX2b (Table 9; Fig. 4C, D).
- Following a week of culture in Sympathoadrenal media B, cell bodies will converge, and neuronal projections will extend.
- At day 19, we observe expression of sympathetic neuronal markers such as GATA3, TH and DBH consistent with catecholaminergic sympathetic neurons (Table 9; Fig. 4C, D).

Commentary

Background Information

Conventional protocols aiming to generate NC from hPSCs are based on the production of a neuroectodermal intermediate, via TGFβ signalling inhibition, which is subsequently steered toward a NC fate, usually through stimulation of WNT activity combined with the appropriate levels of BMP signalling (Chambers et al., 2012; Hackland et al., 2017; Lee et al., 2007). These strategies yield NC cells of an anterior cranial character and the generation of more posterior HOX+ NC subtypes typically depends on the addition of retinoic acid (RA) and/or further WNT signalling stimulation (Fattahi et al., 2016; Huang et al., 2016; Oh et al., 2016). More recent approaches have relied on the isolation of small cell populations positive for lineage-specific fluorescent reporter (Oh et al., 2016) or cell surface markers

(Abu-Bonsrah et al., 2018) through flow cytometry, a time-consuming and laborious approach.

We have recently developed a robust protocol for the in vitro production of trunk NC cells and sympathoadrenal progenitors/sympathetic neurons (Frith et al., 2018). Our approach utilizes findings from embryo studies indicating that at least a fraction of posterior (trunk/lumbosacral) NC cells arise independently from their anterior counterparts, within a pool of axial progenitors localised near the primitive streak and the tailbud during axis elongation (Schoenwolf & Nichols, 1984) (Schoenwolf, Chandler, & Smith, 1985) (Catala, Teillet, & Le Douarin, 1995). Thus our differentiation protocol relies on the induction of an axial NM-potent progenitor population, marked by T expression, which is then employed as the optimal starting point for the efficient in vitro derivation of trunk NC, sympathoadrenal progenitors and functional sympathetic neurons without the use of FACS sorting. This strategy represents a considerable improvement over current approaches and is in line with a recent study reporting the successful production of chromaffin-like cells through the use of an NC-induction protocol which transiently produces T+SOX2+ cells (Abu-Bonsrah et al., 2018).

Critical Parameters

Quality of starting hPSC cultures.

Culture of hPSC should be free of spontaneously differentiated cells as this will reduce the efficiency of differentiation through the secretion of paracrine factors and a lesser number of pluripotent cells going into the differentiation. Manual clearing of cultures using a plastic pipette is one approach that may assist in the removal of unwanted cells. In addition, contamination of cells by yeast or bacteria should also be monitored, the addition of antibiotics (such as PenStrep) during routine culture is one approach to prevent contamination. Testing for Mycoplasma should be performed to ensure that they do not contaminate cultures. hPSCs should also express pluripotency associated markers such as NANOG, OCT4 and SOX2 and display immunoreactivity to SSEA3 and Tra-1-81 which can be determined by immunofluorescence or flow cytometry. We routinely culture hPSCs in mTESR or Essential 8 media on GELTREX or vitronectin. Differences in the hPSC maintenance conditions may also have an impact on the efficiency of the protocols described. Cell lines should be monitored regularly for genetic abnormalities by karyotyping.

Cell density during differentiation

We have found that the plating density for each stage of differentiation should be optimized. We observed variation of efficiency based on cell line and stage of differentiation. We recommend that the optimal density for each stage of differentiation be determined for each end user.

Troubleshooting

See table 10

Time Considerations

See individual sections and Figure 1.

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Significance Statement

The neural crest (NC) is an important embryonic cell population that gives rise to a variety of cell types depending on their position along the anteroposterior axis. A wide spectrum of developmental defects arise due to mutations in NC cells. An attractive approach to model these defects involves the generation of NC from human pluripotent stem cells (hPSCs). However, conventional differentiation strategies yield low numbers of NC cells corresponding to the trunk/thoracic level of the axis. This limits the study of disorders arising in trunk NC such as neuroblastoma. Here we describe a highly efficient protocol for the production of trunk NC cells and their derivatives from hPSCs.

Figure 1. Overview of stages of differentiation protocol.

The first stage of differentiation involves the generation of neuromesodermal axial progenitors which are induced from hPSCs within the first three days of the protocol. Axial progenitors are replated into neural crest inducing conditions and over the course of five days, acquire a trunk neural crest identity. Trunk neural crest cells give rise to sympathoadrenal precursors after 4 days of priming in BMP and Sonic Hedgehog and with further neural maturation from day 12 onwards in neurotrophin containing media.

Figure 2. In vitro generation of neuromesodermal axial progenitors from hPSCs
(A) hPSCs are replated into N2B27 media containing the WNT agonist CHIR99021 and recombinant bFGF supplemented with Y-27632-dihydrochloride for the first day. During days 2 and 3 of differentiation, cells are exposed to CHIR99021 and bFGF alone. **(B)** Representative phase contrast images (Top: low magnification, Bottom: High magnification) of cells at day one after plating and at day three at the end of the Axial Progenitor differentiation stage. **(C)** Immunofluorescence images showing the heterogeneous co-expression of the axial progenitor markers BRACHYURY and SOX2. **(D)** qPCR data

showing the induction of indicated axial progenitor marker genes. Note the downregulation of the pluripotency associated gene *NANOG*.

Figure 3. In vitro differentiation of axial progenitors toward trunk neural crest cells
(A) Day three axial progenitors are replated into neural crest-inducing conditions for five days (from day three until day eight). **(B)** Representative phase contrast images at 10x and 40x showing cell density and morphology after axial progenitor replating at day 4. Cells should proliferate and be confluent at day 7. **(C)** Immunostaining analysis showing SOX10 and HOXC9 co-expressing cells which comprise the bulk of the cultures at day 8 of differentiation **(D)** qPCR analysis showing a gain of neural crest identity over the course of days three to eight as indicated by expression of *SOX10*, *TFAP2α*, *PAX3* and *PAX7*.

Figure 4. In vitro differentiation of trunk neural crest toward sympathetic neurons

(A) Day 8 trunk neural crest cells are plated into sympathoadrenal priming conditions containing Recombinant BMP4 and Sonic Hedgehog and the small molecule agonist of Sonic Hedgehog pathway Purmorphamine. At day 12, media is changed to contain neurotrophins to promote further differentiation toward a sympathetic neuronal identity. (B) Phase contrast images showing the acquisition of a neuronal morphology at day 9 after trunk neural crest cells have been replated. (C) Flow cytometry analysis of PHOX2b:GFP expression showing high yield of PHOX2b positive putative sympathoadrenal precursors at

d12. **(D)** Immunofluorescence analysis of cells at day 19 after neuronal maturation showing the expression of sympathetic neuron markers GATA3, ISLET1/2, ASCL1 and the catecholamine synthesizing enzyme Tyrosine Hydroxylase.

Table 1
Preparation of N2B27 media

Component	Stock Concentration	Volume for 100ml	Final Concentration
DMEM/F12 (Sigma)	1x	46ml	
Neurobasal (ThermoFisher)	1x	46ml	
B27 Supplement (ThermoFisher)	50x	2ml	1x
N2 Supplement (ThermoFisher)	100x	1ml	1x
Glutamax (ThermoFisher)	100x	1ml	1x
Non-Essential Amino Acids (ThermoFisher)	100x	1ml	1x
β -mercaptoethanol (ThermoFisher)	50mM	0.2ml	100 μ M

Table 2
Antibodies employed for characterization of hAPs

Antibody	Supplier	Dilution	Catalogue no/RRID
T(BRACHYURY)	R&D	1:200	AF2085/ AB_2200235
T(BRACHYURY)	Abcam	1:1000	ab209665/ AB_2750925
SOX2	Abcam	1:200	ab92494/AB_10585428
CDX2	Abcam	1:200	ab76541/AB_1523334
HOXC9	Abcam	1:50	ab50839/AB_880494
PAX3	DSHB	1:100	NA/AB_528426

Table 3
Primers employed for characterization of hAPs by qPCR.

Gene	Forward	Reverse
CDX2	atcaccatccggaggaaag	tccggttctgaaaccagatt
SOX2	atgggttcgggtgtcaagt	ggaggaagaggtaaccacagg
T	gctgtgacaggtaccaacc	catgcaggtgagttgtcag
HOXC9	gcagcaagcacaagagga	cgtctgttacttgggttaggg
WNT3A	ctgagggtgggcttttcc	gaggagtactgccccgttta
FGF8	agctcgcttccctgttcc	attaggtgaggactgaacagttacc
NANOG	agatgcctcacacggagact	ggactggtggaagaatcagg
NKX1-2	gtcgaagcgggaaagat	gatcctccgcctcctct

Table 4
Recipe for Neural crest media.

Component	Stock Concentration	Volume for 50ml	Final Concentration
DMEM/F12 (Sigma)	1x	48.5ml	
N2 Supplement (ThermoFisher)	100x	0.5ml	1x
Glutamax (ThermoFisher)	100x	0.5ml	1x
Non-Essential Amino Acids (ThermoFisher)	100x	0.5ml	1x
SB431542 (Tocris)	10mM	10µl	2µM
CHIR99021 (Tocris)	10mM	5µl	1µM
DMH1 (Tocris)	10mM	5µl	1µM
Recombinant BMP4 (ThermoFisher)	50ng/ml	15µl	15ng/ml

Table 5
Antibodies employed for characterization of trunk NC cells

Antibody	Supplier	Dilution	Catalogue no/RRID
SOX10	Cell Signalling Technology	1:400	89356/Unknown
HOXC9	Abcam	1:50	ab50839/AB_880494

Table 6
Primers employed for characterization of trunk NC cells by qPCR.

Gene	Forward	Reverse
SOX10	ggctcccccagtcagat	ctgtcttcgggggtggtg
TFAP2A	acatgctcctggctacaaac	aggggagatcggctcctga
PAX3	aggaggccgactggaga	cttcactgattgggtgct
PAX7	gaaaaccaggcatgttcag	gcggctaatacgaactactaa

Table 7
Recipe for Sympathoadrenal media A

Component	Stock Concentration	Volume for 50ml	Final Concentration
BrainPhys (Stem Cell Technologies)	1x	47.5ml	
B27 Supplement (ThermoFisher)	50x	1ml	1x
N2 Supplement (ThermoFisher)	100x	0.5ml	1x
Glutamax (ThermoFisher)	100x	0.5ml	1x
Non-Essential Amino Acids (ThermoFisher)	100x	0.5ml	1x
Sonic Hedgehog C24II (R&D systems)	50µg/ml	50µl	50ng/ml
Purmorphamine * (Sigma Aldrich)	10mM	7.5µl	1.5µM
BMP4 (ThermoFisher)	50µg/ml	50µl	50ng/ml

* A titration of Purmorphamine concentration is recommended for optimal differentiation. We have found between 1.25 and 1.5µM work well. If the concentration is too high, then there will be excessive cell death. Conversely, if the concentration is too low, differentiation efficiency is low (based on PHOX2b expression at day 12)

Table 8
Recipe for Sympathoadrenal media B

Component	Stock Concentration	Volume for 50ml	Final Concentration
BrainPhys (Stem Cell Technologies)	1x	47.5ml	
B27 Supplement (ThermoFisher)	50x	1ml	1x
N2 Supplement (ThermoFisher)	100x	0.5ml	1x
Glutamax (ThermoFisher)	100x	0.5ml	1x
Non-Essential Amino Acids (ThermoFisher)	100x	0.5ml	1x
BDNF (Peprotech)	10µg/ml	50µl	10ng/ml
GDNF (Peprotech)	10µg/ml	50µl	10ng/ml
NGF (Peprotech)	10µg/ml	50µl	10ng/ml

Table 9
Antibodies employed for characterization of sympathetic neurons

Antibody	Supplier	Dilution	Catalogue no/RRID
Phox2B	Santa Cruz	1:100	sc-376997/unknown
GATA3	Santa Cruz	1:100	sc-269/ AB_627666
ASCL1	BD Biosciences	1:100	556604/AB_396479
ISL1/2	DSHB	1:100	NA/AB_2314683
TH	Sigma	1:1000	T1299/ AB_477560

Table 10
Troubleshooting

Problems	Solution
Poor hPSC cultures	<ul style="list-style-type: none"> -Passage cells at higher density (i.e a 1 in 3 split) -Regular karyotyping to monitor genetic stability. -Regular mycoplasma testing
Contaminated cells	-Add antibiotics; ensure strict routine to clean incubators and tissue culture hoods
Poor cell plating	<ul style="list-style-type: none"> -Increase the concentration of Geltrex or Vitronectin. -Prepare plates fresh each time. -Ensure that part of plate does not dry out when kept in the fridge. Wrap with laboratory film to prevent loss of liquid.
Low T/SOX2 induction from axial progenitor differentiation	<ul style="list-style-type: none"> -Titrate the concentration of CHIR99021 -Minimise freeze thaw cycles of all recombinant proteins and small molecule inhibitors. -Test plating of different cell numbers at day 0 of differentiation
Poor SOX10/HOXC9 induction	<ul style="list-style-type: none"> -Ensure quality of axial progenitor differentiation by assessing gene expression of cells at day 3 -Optimise density when replating cells at day 3 -Optimise the concentrations of BMP4 and DMH-1 in order to determine optimal BMP signaling activity for neural crest induction.