

ORIGINAL ARTICLE

Characterization and transplantation of enteric neural crest cells from human induced pluripotent stem cells

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The enteric nervous system (ENS) is recognized as a second brain because of its complexity and its largely autonomic control of bowel function. Recent progress in studying the interactions between the ENS and the central nervous system (CNS) has implicated alterations of the gut/brain axis as a possible mechanism in the pathophysiology of autism spectrum disorders (ASDs), Parkinson's disease (PD) and other human CNS disorders, whereas the underlying mechanisms are largely unknown because of the lack of good model systems. Human induced pluripotent stem cells (hiPSCs) have the ability to proliferate indefinitely and differentiate into cells of all three germ layers, thus making iPSCs an ideal source of cells for disease modelling and cell therapy. Here, hiPSCs were induced to differentiate into neural crest stem cells (NCSCs) efficiently. When co-cultured with smooth muscle layers of ganglionic gut tissue, the NCSCs differentiated into different subtypes of mature enteric-like neurons expressing nitric oxide synthase (nNOS), vasoactive intestinal polypeptide (VIP), choline acetyltransferase (ChAT) or calretinin with typical electrophysiological characteristics of functional neurons. Furthermore, when they were transplanted into aneural or aganglionic chick, mouse or human gut tissues *in ovo*, *in vitro* or *in vivo*, hiPSC-derived NCSCs showed extensive migration and neural differentiation capacity, generating neurons and glial cells that expressed phenotypic markers characteristic of the enteric nervous system. Our results indicate that enteric NCSCs derived from hiPSCs supply a powerful tool for studying the pathogenesis of gastrointestinal disorders and brain/gut dysfunction and represent a potentially ideal cell source for enteric neural transplantation treatments.

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INTRODUCTION

The principal functions of the gut are to enhance nutrient absorption and transport food scraps, functions that are supported by motility and secretory activity directed mostly by the intramural enteric nervous system (ENS).^{1,2} The ENS is composed of numerous neurons (400–600 million in humans), glial cells and the neural connections between them that reside in the myenteric and submucosal plexus.³ Because its structural and chemical complexity resembles that of the central nervous system (CNS) and dominant autonomic control of bowel function, the ENS has been recognized as a second brain in our body.⁴

Congenital anomalies that interrupt ENS development or acquired diseases can lead to loss of enteric neurons/enteric glia or defects in their function, ultimately causing gastrointestinal (GI) motility disorders, including Hirschsprung's disease (HSCR), gastroparesis and irritable bowel syndrome.^{5,6} Recent progress in studying interactions between the CNS, ENS and GI tract has implicated alterations of the gut/brain axis as a possible

mechanism in the pathophysiology of autism spectrum disorders (ASDs), Parkinson's disease (PD) and other CNS disorders.^{7–10} Indeed, the ENS connects the CNS via the afferent and efferent pathways of the parasympathetic and sympathetic nervous systems, although it can function autonomously. Moreover, previous studies have shown that many of the synaptic genes associated with ASDs including the Shanks, neurexins and neuroligins are also expressed in the ENS.^{11–14} Therefore, the genetic defects underlying CNS disorders usually cause impairment of the ENS.^{4,15} McElhanon *et al.*¹⁶ found that gut disorders including abdominal pain, constipation and diarrhoea are the most common complications of ASD. In addition, recent studies demonstrated that the ENS is impaired during the pathological process of PD.^{17–19} The great majority of PD patients have GI motility disorders, and one of the pathological hallmarks of PD, Lewy bodies, are found primarily in vasoactive intestinal polypeptide (VIP)-immunoreactive neuronal cell bodies and processes within the colonic myenteric and submucosal plexus.^{20,21}

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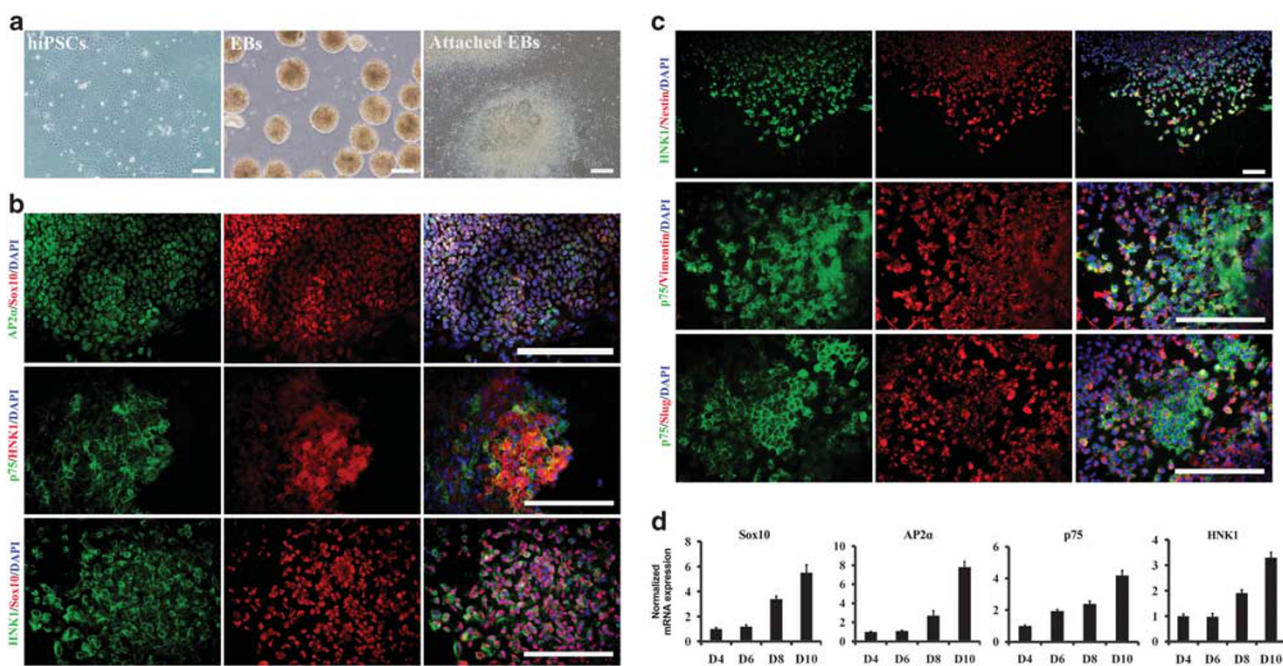


Figure 1. Neural crest differentiation of human induced pluripotent stem cells (hiPSCs). (a) Human iPSCs were cultured in mTeSR1 medium and plated on Matrigel-coated plates. After culturing in N2B27 medium for 5 days, dissociated cells formed uniform embryoid bodies (EBs) in AggreWell plates. After replating onto PO/LN-coated plates, multiple rosette structures formed in the centre of the attached EBs. (b) Immunofluorescence analysis showed that cells migrating out from the rosette structures co-expressed the neural crest-specific genes Sox10, Ap2 α , p75 and HNK1. (c) Most migratory cells also expressed Nestin, Vimentin and Slug, as shown by immunostaining. (d) quantitative PCR (qPCR) demonstrated that mRNAs for the neural crest stem cell (NCSC)-specific markers Sox10, Ap2 α , p75 and HNK1 were highly upregulated after differentiation (normalized to day 4 mRNA expression). Scale bar, 200 μ m.

However, the underlying molecular mechanisms that lead to ENS defects and GI disorders and their roles in CNS disorders remain largely unknown.

The *in vitro* disease modelling using patient-derived stem cells will be of great value in uncovering the mechanisms of disease pathogenesis. Reprogramming human somatic cells to a pluripotent state allows the generation of human induced pluripotent stem cells (hiPSCs).²² The hiPSCs share characteristics with human embryonic stem cells with respect to their self-renewal capacity and pluripotency. Consequently, iPS technology offers a powerful tool for modelling human disease in the culture dish.^{23–25} Throughout early embryonic development in vertebrates, vagal neural crest stem cells (NCSCs) enter the foregut and migrate through the developing GI tract, giving rise to the majority of neurons and glial cells in the ENS.^{26,27} Thus, the generation of functional enteric neurons from hiPSCs via neural crest specification will provide a valuable tool for modelling human disease and for cell replacement therapies.^{28–31}

In this study, we successfully induced the differentiation of hiPSCs into NCSCs. When co-cultured with tissues from normal human gut in neural differentiation medium *in vitro*, hiPSC-derived NCSCs (hiPSC-NCSCs) efficiently differentiated into functional enteric-like neurons. When transplanted into chicken, mouse or human aneural or aganglionic gut tissues, hiPSC-NCSCs showed extensive migration and neural differentiation capacity.

MATERIALS AND METHODS

Cell culture

HDF-hiPSCs (derived from human adult dermal fibroblasts), HEF-hiPSCs (derived from human embryonic dermal fibroblasts), humanized *Renilla reniformis*-derived green fluorescent protein (hrGFP)-labelled HDF-hiPSCs and human embryonic stem cell lines (H1, H9) were cultured as reported previously.^{32,33} Briefly, cells were attached to Matrigel-coated culture plates

and were maintained in mTeSR1 medium (Stemcell Technologies, Vancouver, BC, Canada). The medium was changed every day, and cells were routinely passaged in small clumps every 4–5 days by treating with 0.5 mM EDTA (pH 8.0) (Life Technologies, Carlsbad, CA, USA).

Additional experimental procedures

For more detailed and additional information on experimental procedures, please see Supplementary Materials and Methods and Supplementary Tables 1 and 2.

RESULTS

Generation of NCSCs from hiPSCs

Two human iPS cell lines that were generated previously (HDF-hiPSCs and HEF-hiPSCs)^{32,33} and the human ES cell lines H1 and H9 were used in this study. Both *in vivo* and *in vitro* analyses revealed that these human iPS cells exhibited the essential characteristics of human ES cells, particularly the capacities for self-renewal and differentiation (Supplementary Figures 1A–C). Previous studies have demonstrated that human pluripotent stem cells—human embryonic stem cells and hiPSCs—can differentiate into NCSCs via neural rosette formation.^{28,34} Here, dissociated HDF-hiPSCs cultured in suspension in N2B27- and Y27632-containing medium for 5 days formed uniform-sized embryoid bodies (EBs) with defined edges in AggreWell plates (STEMCELL Technologies, Vancouver, BC, Canada). The EBs were then allowed to attach to PO/LN-coated culture plates and cultured in neural crest culture medium (NCCM) for ~5–7 days before fluorescence-activated cell sorting (FACS) enrichment of p75+/HNK1+ NCSCs (Supplementary Figure 2A). Multiple rosette structures emerged in the centre of the attached EBs, and cells migrated out from the rosette structures to the periphery of the attached EBs (Figure 1a). Immunofluorescence analysis of the migrated cells for neural crest

lineage marker expression showed that most of these cells co-expressed neural crest-specific transcription factors, including Sox10, AP2 α , Brn3a, Isl1 and Mash1, and some of the differentiated cells expressed the vagal neural crest markers Hoxb2 and Hoxb3 (Figure 1b; Supplementary Figure 2B) that have been shown to play essential roles in the multipotency, delamination, differentiation and migration capacity of NCSCs.³⁵ The cell clusters surrounding the rosettes also co-expressed cell surface markers of NCSCs including p75 and HNK1 (Figure 1b). Moreover, the intermediate filaments Nestin and Vimentin, and the epithelial-mesenchymal transition regulatory factor Slug, were widely expressed by these cells (Figure 1c), consistent with previous findings.²⁸ In accord with these immunocytochemistry data, quantitative PCR (qPCR) analysis showed that mRNAs for the NCSC-specific markers Sox10, AP2 α , p75, HNK1 (Figure 1d), Brn3a, Isl1, Mash1, Hoxb2 and Hoxb3 were highly upregulated, whereas the expression of endogenous pluripotency markers was down-regulated rapidly (Supplementary Figure 2C) in a time-dependent manner during the neural crest differentiation of hiPSCs.

HNK1 and p75 are widely used to enrich human NCSCs from pluripotent stem cells by FACS.^{28,29,34} Here, we isolated p75 and HNK1 double-positive (HNK1+/p75+) cells by FACS and further characterized these hiPSC-derived NCSCs. This analysis showed that the proportion of HNK1+/p75+ cells was $90.45 \pm 3.41\%$ among the differentiated HDF-hiPSCs and the differentiation efficiency of this hiPSC line was consistent between individual differentiations ($n=4$; Figure 2a and Supplementary Figure 3A). Comparable differentiation efficiency was also obtained in other pluripotent stem cell lines (H1, H9) and HEF-hiPSCs (Supplementary Figure 3B). These freshly isolated NCSCs were then cultured on PO/LN-coated dishes in NCCM for adherent culture (Figure 2b). Attached NCSCs maintained their typical cellular morphology and their NCSC identity as evidenced by robust expression of p75 ($88.12 \pm 1.46\%$), HNK1 ($92.26 \pm 1.01\%$), Sox10 ($94.75 \pm 1.10\%$) and AP2 α ($91.72 \pm 1.66\%$) detected by immunostaining (Figures 2c and d). Isolated NCSCs could be propagated *in vitro* for up to 15–20 passages with characteristic gene expression (Supplementary Figure 4A), normal karyotype (Supplementary Figure 4B) and self-renewal ability (Supplementary Figure 4C). qPCR analysis further showed that the transcription of the neural crest-specific genes p75, Sox10, Zic1, Pax3 and AP2 α was 5- to 10-fold higher in enriched NCSCs than in undifferentiated hiPSCs (Figure 2e).

Extensive migration ability *in vivo* is one of the characteristic features of NCSCs, and it has been reported that NCSCs migrate caudally along the entire length of the gut at speeds of $35\text{--}40 \mu\text{m h}^{-1}$ ($5.83\text{--}6.67 \mu\text{m per 10 min}$) in both birds and explants from embryonic mice.^{36,37} Here, freshly isolated HNK1+/p75+ cells were replated on PO/LN-coated plates for migration assays. Time-lapse microscopy revealed that these NCSCs possessed considerable migration ability, exhibiting a peak migration rate of $14.1 \mu\text{m per 10 min}$ and an average migration rate of $4.97 \pm 0.64 \mu\text{m per 10 min}$ (Figure 2f and Supplementary Movie 1).

When suspension-cultured on ultra-low attachment plates in NCCM, FACS-purified HNK1+/p75+ cells formed spheres (Supplementary Figure 5A). These spheres could be passaged every 3–4 days and maintained their sphere-formation ability for at least 10 passages *in vitro*. We also found that neither plating density nor passage number influenced the sphere-forming efficiency of NCSCs cultured in NCCM (Supplementary Figures 5B and C). Immunostaining indicated that most cells within spheres of passage 10 expressed the NCSC markers HNK1, p75 and Sox10 and the proliferation marker Ki67 (Supplementary Figure 5D).

Multilineage differentiation of hiPSC-derived NCSCs

NCSCs migrate along defined pathways to various tissues and differentiate into a wide array of derivatives, including neurons and glia of the peripheral nervous system, bone, cartilage and

smooth muscle.³⁸ We thus explored the potential of hiPSC-derived NCSCs to give rise to various cell types. For neuronal differentiation, NCSCs were cultured in medium containing brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, nerve growth factor and 10 ng ml^{-1} neurotrophin-3, ascorbic acid and dibutyryl-cAMP for 3–4 weeks. Of the resulting cells in these cultures, $81.46 \pm 4.22\%$ ($n=3$) co-expressed peripherin and TUBB3 (tubulin- β 3), thus confirming their identity as peripheral neurons. We also found that NCSCs could differentiate into Brn3a+ and peripherin-positive (Brn3a+/peripherin+) sensory neurons, tyrosine hydroxylase- and TUBB3-positive (TH+/TUBB3+) peripheral sympathetic neurons and Mash1- and peripherin-positive (Mash1+/peripherin+) autonomic neurons with variable efficiency under neural-differentiation conditions (Supplementary Figure 6A). qPCR analysis further confirmed that peripherin, TH, Brn3a and Mash1 mRNA were significantly upregulated during the differentiation process, reaching levels $\sim 2.5\text{--}5$ -fold higher in differentiated cells than in undifferentiated NCSCs (Supplementary Figure 6B).

When cultured initially in medium containing ciliary neurotrophic factor, neuregulin and dibutyryl-cAMP for 2–3 weeks, NCSCs subsequently cultured *in vitro* for more than 2 months could be induced to efficiently differentiate into Schwann cells. Immunostaining showed that $53.75 \pm 9.57\%$ ($n=3$) of the resulting cells co-expressed the Schwann cell markers S100B and glial fibrillary acidic protein (GFAP) (Supplementary Figure 6C), a result that was further validated by qPCR detection of the corresponding mRNAs (Supplementary Figure 6D).

To assess their mesodermal differentiation potential, we cultured NCSCs in fetal bovine serum-containing medium. Cells with spindle-shaped morphology that exhibited a parallel or spiral arrangement emerged and proliferated actively. FACS analysis showed that NCSC-derived mesenchymal stem cells (MSCs) expressed surface markers typical of human MSCs including CD44, CD73 and CD90, and nearly all were negative for CD34 and CD45. However, CD105 and CD166 were rarely expressed by the NCSC-MSC population (Supplementary Figure 7A), a result consistent with a previous report.²⁸ We further cultured NCSC-MSCs under conditions that specifically promote differentiation into either osteoblasts, adipocytes, chondrocytes or smooth muscle. As confirmed by Alizarin Red S staining, Oil Red O staining, Toluidine Blue staining and anti- α -smooth muscle cell immunostaining, the NCSC-MSCs showed (respectively) osteogenic, adipogenic, chondrogenic (Supplementary Figure 7B) and smooth muscle cell differentiation capacity (Supplementary Figure 7C).³⁹ The qPCR results further confirmed the multilineage differentiation ability of NCSCs-MSCs (Supplementary Figure 7D).

Derivation of enteric-like neurons from hiPSC-NCSCs *in vitro*

To investigate whether hiPSC-NCSCs can differentiate to enteric-like neurons, we co-cultured them with gut explants for 10 days using a Transwell system⁴⁰ (Supplementary Figure 8A). After culturing for 6–8 more weeks, NCSCs seeded onto the lower chamber in peripheral neuron differentiation medium were assessed by immunostaining. This analysis showed that the differentiated NCSCs expressed the functional enteric neural markers VIP ($16.2 \pm 2.29\%$), choline acetyltransferase (ChAT; $20.08 \pm 2.18\%$), calretinin ($24.54 \pm 2.40\%$), tyrosine hydroxylase (TH) ($9.56 \pm 1.04\%$) and neuronal nitric oxide synthase (nNOS; $18.4 \pm 2.12\%$) ($n=5$; Figures 3a and b), indicating that these NCSCs could be induced to differentiate into enteric-like neurons efficiently in a co-culture system. In addition, $< 5\%$ of the differentiated cells expressed the sympathoadrenal markers chromogranin A, dopamine and phenylethanolamine *N*-methyltransferase (Supplementary Figure 8B). However, without co-culturing with gut explants, few NCSCs ($< 1\%$) differentiated into enteric-like neurons in peripheral neural differentiation conditions. Electrophysiological analysis of enteric-like neurons exhibiting

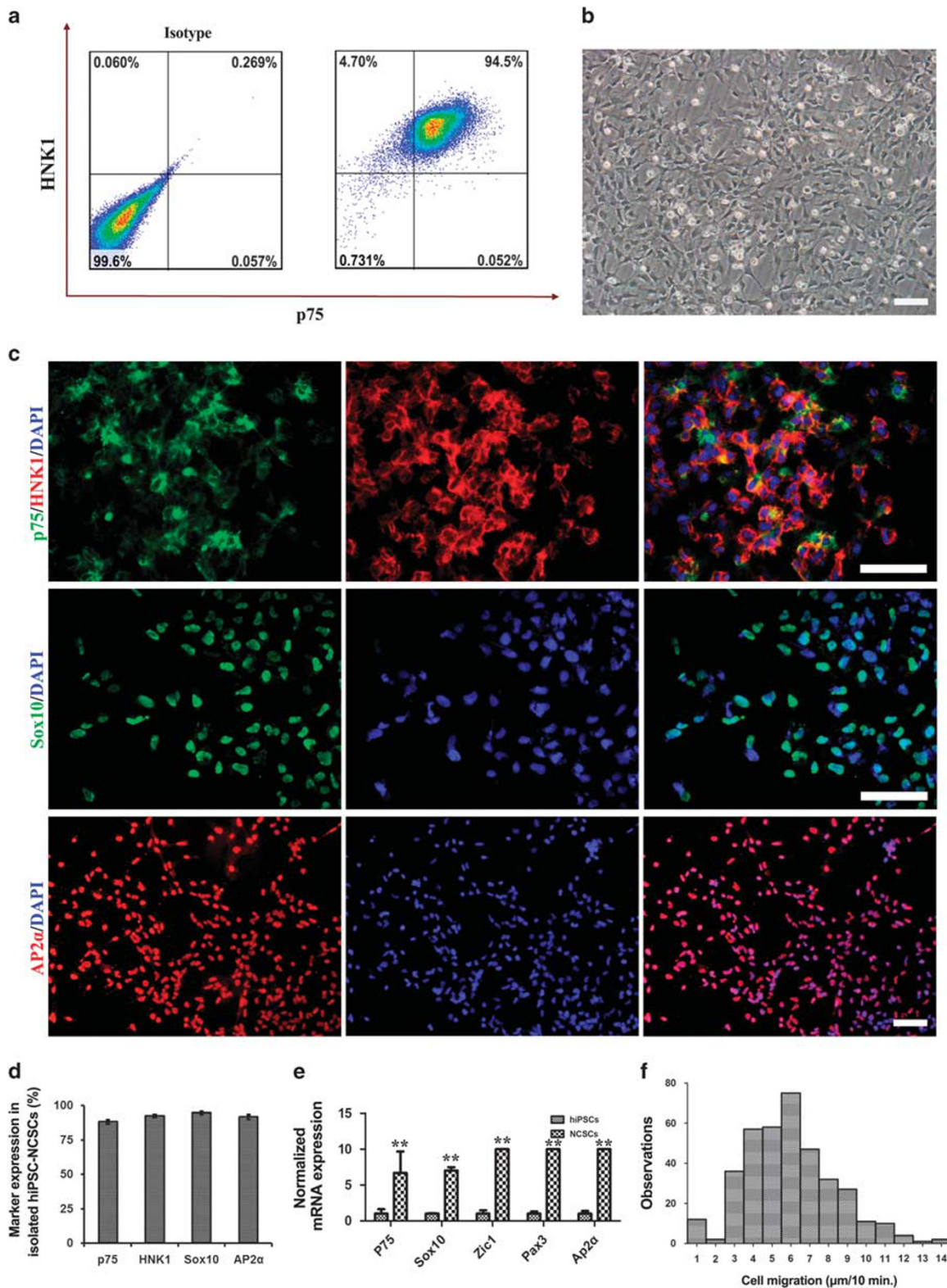


Figure 2. Enrichment and characterization of human induced pluripotent stem cell-derived neural crest stem cells (hiPSC-NCSCs). **(a)** HNK1⁺/p75⁺ cells were isolated by fluorescence-activated cell sorting (FACS) after differentiation for 10–12 days. **(b)** NCSCs cultured on PO/LN-coated dishes maintained their typical cellular morphology. **(c)** hiPSC-NCSCs expressed the neural crest stem cell markers p75, HNK1, Sox10 and AP2α. **(d)** The percentages of p75⁺/HNK1⁺, Sox10⁺, and AP2α⁺ cells in isolated hiPSC-NCSCs were analysed, respectively. **(e)** Quantitative PCR (qPCR) analysis showed that mRNA expression of the NCSC markers p75, Sox10, Zic1, Pax3 and AP2α was 5–10-fold higher in enriched NCSCs than in undifferentiated hiPSCs (***P* < 0.01). **(f)** Time-lapse microscopy revealed that these NCSCs possessed considerable migration ability, reaching a peak migration rate of 84.6 μm h⁻¹ (14.1 μm per 10 min). Scale bar, 100 μm.

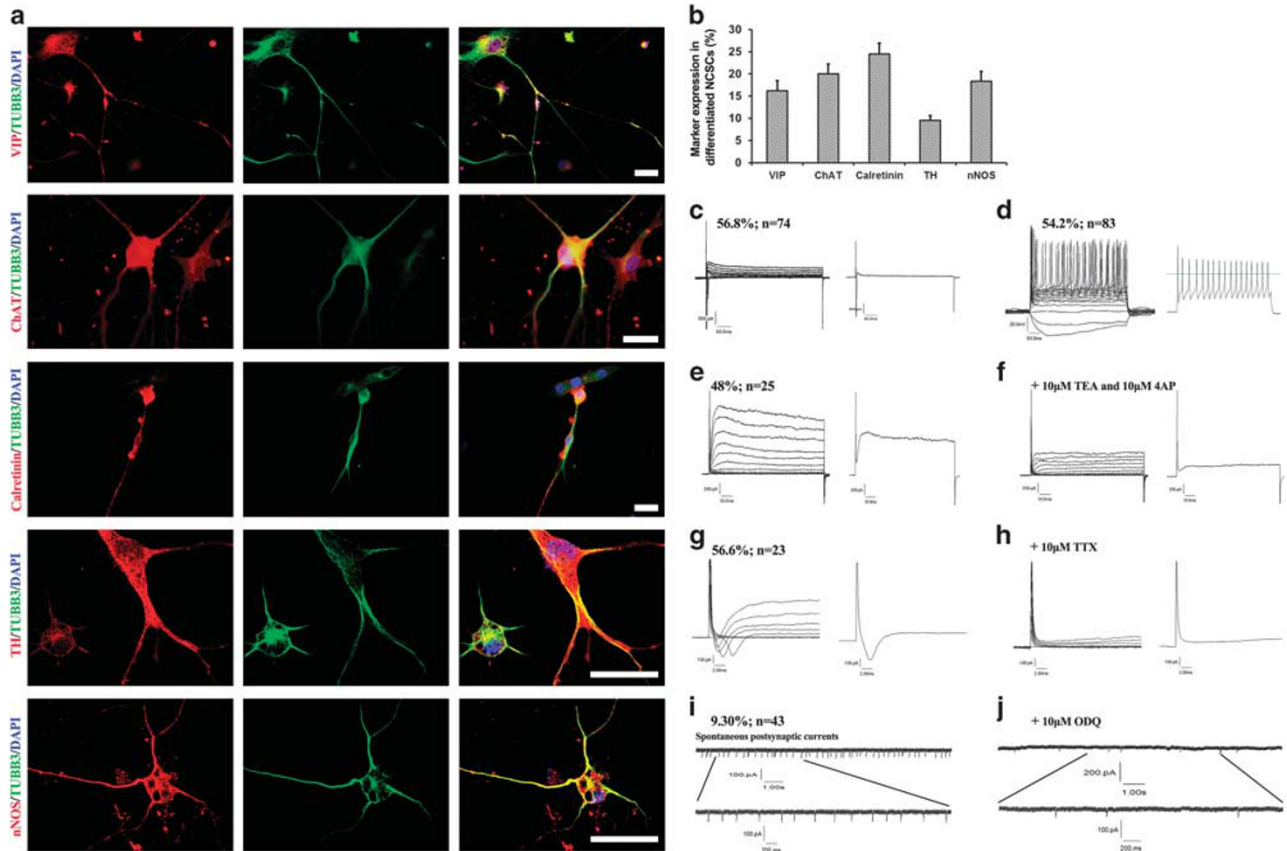


Figure 3. Functional enteric-like neurons derived from human induced pluripotent stem cell-derived neural crest stem cells (hiPSC-NCSCs) *in vitro*. **(a)** Differentiated hiPSC-NCSCs were found to express functional enteric neural markers such as vasoactive intestinal polypeptide (VIP), choline acetyltransferase (ChAT), calretinin, tyrosine hydroxylase (TH) and neuronal nitric oxide synthase (nNOS). **(b)** The percentages of VIP+, ChAT+, calretinin+, TH+ and nNOS+ enteric-like neurons differentiated from hiPSC-NCSCs were analysed, respectively. **(c)** Electrophysiological recordings obtained in voltage-clamp mode from differentiated cells with a typical neuronal morphology suggested that voltage-dependent currents could be invoked in these cells (56.8%; $n = 74$). **(d)** Electrophysiological recordings obtained in current-clamp mode (54.2%; $n = 83$). **(e, f)** Outward current was observed in voltage-clamp mode from differentiated hiPSC-NCSCs and could be blocked by the potassium channel blockers tetraethylammonium (TEA) and 4-aminopyridine (4-AP) (48%; $n = 25$). **(g, h)** Inward current was observed in voltage-clamp mode from differentiated hiPSC-NCSCs and could be blocked by the sodium channel blocker tetrodotoxin (TTX) (56.6%; $n = 23$). **(i, j)** Spontaneous activity was recorded in differentiated hiPSC-NCSCs and could be blocked by the soluble guanylyl cyclase inhibitor oxadiazolo-quinoxalineone (ODQ) (9.30%; $n = 43$). Scale bar, 50 μm .

typical neuronal morphology suggested that voltage-dependent currents could be invoked using a voltage-clamp protocol in 56.8% of the recorded cells ($n = 74$; Figure 3c). Moreover, in cells held in current-clamp mode, a short depolarizing pulse elicited an action potential in 54.2% of the recorded cells ($n = 83$; Figure 3d). We also observed rapidly inactivating persistent outward currents and inward currents in response to depolarizing voltage steps that could be blocked by the potassium channel blockers tetraethylammonium and 4-aminopyridine (48%; $n = 25$; Figures 3e and f) and the sodium channel blocker tetrodotoxin (56.5%; $n = 23$; Figures 3g and h), respectively. Moreover, spontaneous postsynaptic currents were recorded and could be blocked by the soluble guanylyl cyclase inhibitor oxadiazolo-quinoxalineone (the binding of oxadiazolo-quinoxalineone is competitive with NO) in 9.30% of patched cells ($n = 43$; Figures 3i and j). These results confirm that hiPSC-derived NCSCs have the ability to differentiate into electrophysiologically mature neurons.

We further performed RNA sequencing to examine changes in global expression profile during the enteric neuron differentiation of hiPSCs. The samples included undifferentiated hiPSCs, freshly isolated p75+/HNK1+ hiPSC-NCSCs, enteric neural crest precursors (hiPSC-ENCPs) from hiPSC-NCSCs (co-cultured with gut explants for 10 days) and enteric-like neurons (hiPSC-ENs) from hiPSC-

NCSCs (co-cultured with gut explants for 42 days). The results (GSE79928) demonstrated that the expression profile of undifferentiated hiPSCs was enriched for genes related to pluripotency (Oct4, Sox2, Nanog, Rex1 and others). However, the freshly isolated hiPSC-NCSCs strongly expressed neural crest-specific transcription factors (for example, Sox10, Sox9, FoxD3, Msx1, Msx2, Pax3, Pax7). Significantly, the hiPSC-ENCPs were highly enriched in transcripts associated with vagal neural crest and/or enteric neural progenitors, including Hoxb2, Hoxb3, Hand2 and Ednr β . The hiPSC-ENs differentiated from hiPSC-NCSCs expressed genes involved in neuronal morphogenesis and maintenance, ion channels and functional synaptic components (Mapt, Gap43, Stmn3, Stmn4, Syn1, Syp and Syt1) (Supplementary Figure 9A).

To further confirm these results, we also isolated primary enteric neurons (pENs) (Supplementary Figures 9B and C) from human gut tissues as described previously⁴¹ and performed qPCR analysis to compare the expression level of specific genes among hiPSC-ENCPs, hiPSC-ENs and pENs. The results demonstrated that pENs expressed relatively low levels of NCSC markers (Sox10 and p75) and vagal markers (Hoxb2 and Hoxb3); however, they strongly expressed neuronal markers, including peripherin, calretinin, TH and ChAT, that constitute a gene expression profile that is more characteristic of hiPSC-ENs than hiPSC-ENCPs (Supplementary

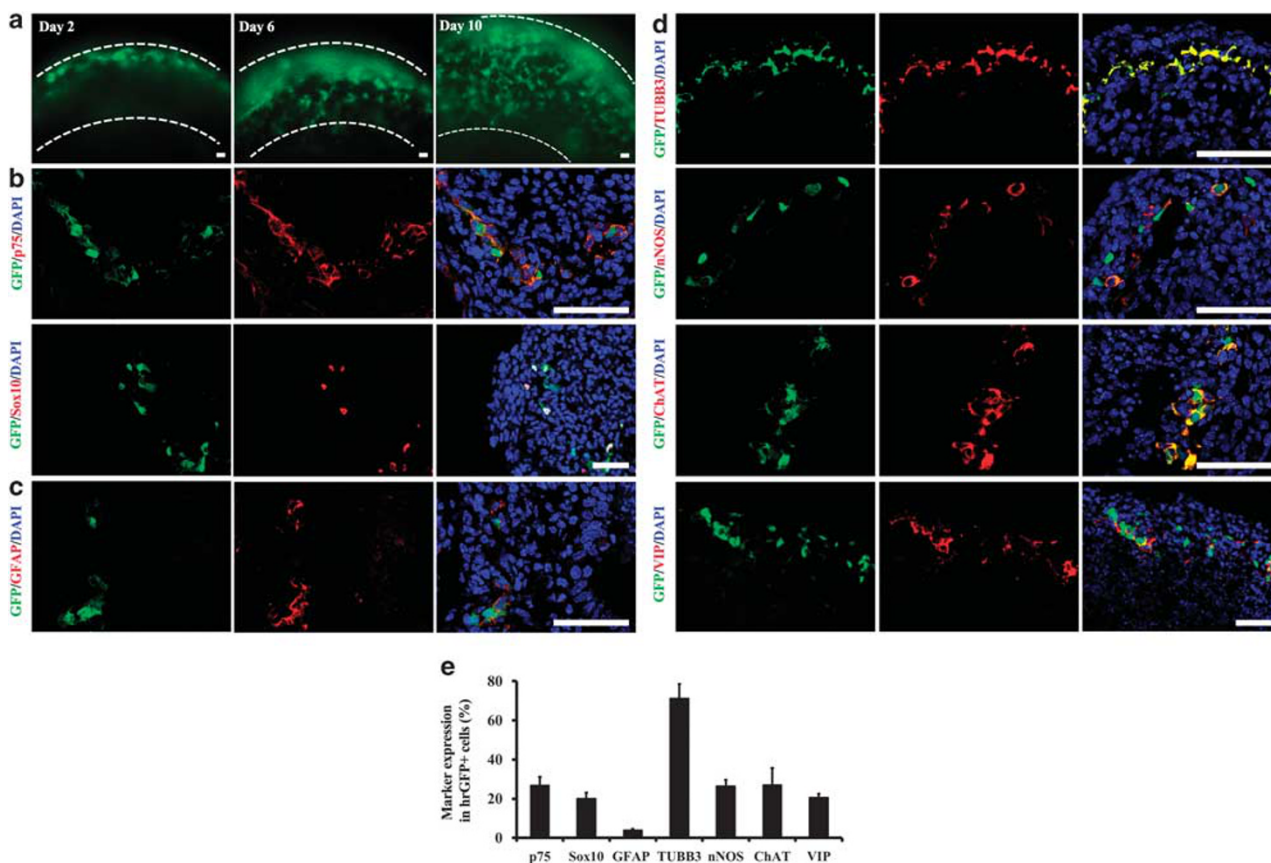


Figure 4. Human induced pluripotent stem cell-derived neural crest stem cells (hiPSC-NCSCs) are capable of migration and differentiation in aneural mouse hindgut *in vitro*. (a) hiPSC-NCSCs were co-cultured with E11.5 aneural mouse hindgut for 7–12 days. hrGFP+ cells progressively migrated into the same distal hindgut during the co-culture process as observed by serial observation under a fluorescence microscope. The areas between dotted lines indicate the hindgut area. (b) Some of the engrafted cells retained expression of the NCSC markers p75 and Sox10. (c) GFAP-positive cells were detected in the mouse hindgut after NCSC transplantation. (d) Immunostaining showed that most NCSCs differentiated into TUBB3+ neurons, whereas some cells differentiated into a more mature neuronal nitric oxide synthase (nNOS)-, choline acetyltransferase (ChAT)- or vasoactive intestinal polypeptide (VIP)-expressing neuronal subtype. Scale bar, 50 μ m. (e) The percentages of p75+, Sox10+, GFAP+, TUBB3+, nNOS+, ChAT+ and VIP+ neurons differentiated from transplanted hrGFP+ hiPSC-NCSCs were analysed, respectively.

Figure 9D). Taken together, these results indicate that the enteric-like neurons can be efficiently differentiated from hiPSC-NCSCs.

Migration and differentiation of hiPSC-NCSCs in aneural mouse gut *in vitro* and chick embryos *in ovo*

It has been reported that the distal one-third of the mouse hindgut at E11.5 is aneural because it is not yet colonized by vagal NCSCs.⁴² Here, to assess the enteric neuron differentiation potential of hiPSC-NCSCs in aneural mouse gut, we investigated the migration and differentiation capacity of hiPSC-NCSCs *in vitro* using a hanging drop method, with aneural embryonic hindgut as the recipient tissue. Spheres were formed by culturing hrGFP-NCSCs in suspension for 2 days and were then co-cultured with aneural embryonic hindguts (Supplementary Figure 10). Continuous observation under fluorescence microscopy revealed that cells inside single hrGFP-NCSC spheres progressively migrated into the same hindgut during the co-culture process (Figure 4a). Immunostaining showed that ~20–30% of the migrated cells retained NCSC characteristics as evidenced by the expression of p75 (26.94 ± 4.31%) and Sox10 (20.20 ± 2.97%) (*n* = 5; Figure 4b). GFAP-positive cells (3.96 ± 0.58%) were detected in the mouse hindgut after NCSC transplantation (*n* = 5; Figure 4c). Most of the NCSCs gave rise to TUBB3+ neurons (71.28 ± 7.28%), whereas some of the hrGFP+ cells differentiated into a more mature nNOS-

(26.42 ± 3.17%), ChAT- (26.98 ± 8.76%) or VIP-expressing (20.76 ± 1.84%) neuronal subtype (*n* = 5; Figures 4d and e).

We also evaluated the migration and differentiation ability of hiPSC-NCSCs using aneural gut from chick embryos (*n* = 4) and ganglionic hindgut of severe combined immunodeficiency (SCID) mice (*n* = 5) as recipient tissues. hrGFP-NCSCs transplanted into embryonic (E5) chick hindgut appeared to organize into ganglia within the submucosal and myenteric regions, and they were capable of longitudinal migration (Supplementary Figure 11A). A small fraction of engrafted NCSCs retained expression of the neural crest marker p75 (7.54 ± 1.89%) or Sox10 (7.83 ± 1.35%), but some differentiated into neurons expressing TUBB3 (43.47 ± 3.31%) (Supplementary Figure 11B) and a more mature neuron marker VIP (19.80 ± 2.91%) or nNOS (22.59 ± 3.30%) (Supplementary Figure 11C). When transplanted into the hindgut of SCID mice, hrGFP-NCSCs were also capable of migration toward the myenteric and submucosal regions (*n* = 4; Supplementary Figure 12A) in recipient mice. Few of the transplanted cells retained their NCSC identity (as shown by p75 immunostaining; 4.70 ± 1.24%; Supplementary Figure 12B), and some hrGFP+ cells differentiated into GFAP+ glia cells (9.16 ± 1.40%; Supplementary Figure 12C) and mature neurons (VIP+, 17.88 ± 1.55%; calretinin+, 21.28 ± 1.13%; nNOS+, 8.98 ± 0.75%; Supplementary Figure 12D). The migration distance of transplanted hrGFP+ cells was ~4–8 mm from the transplanted site in recipient SCID mice that

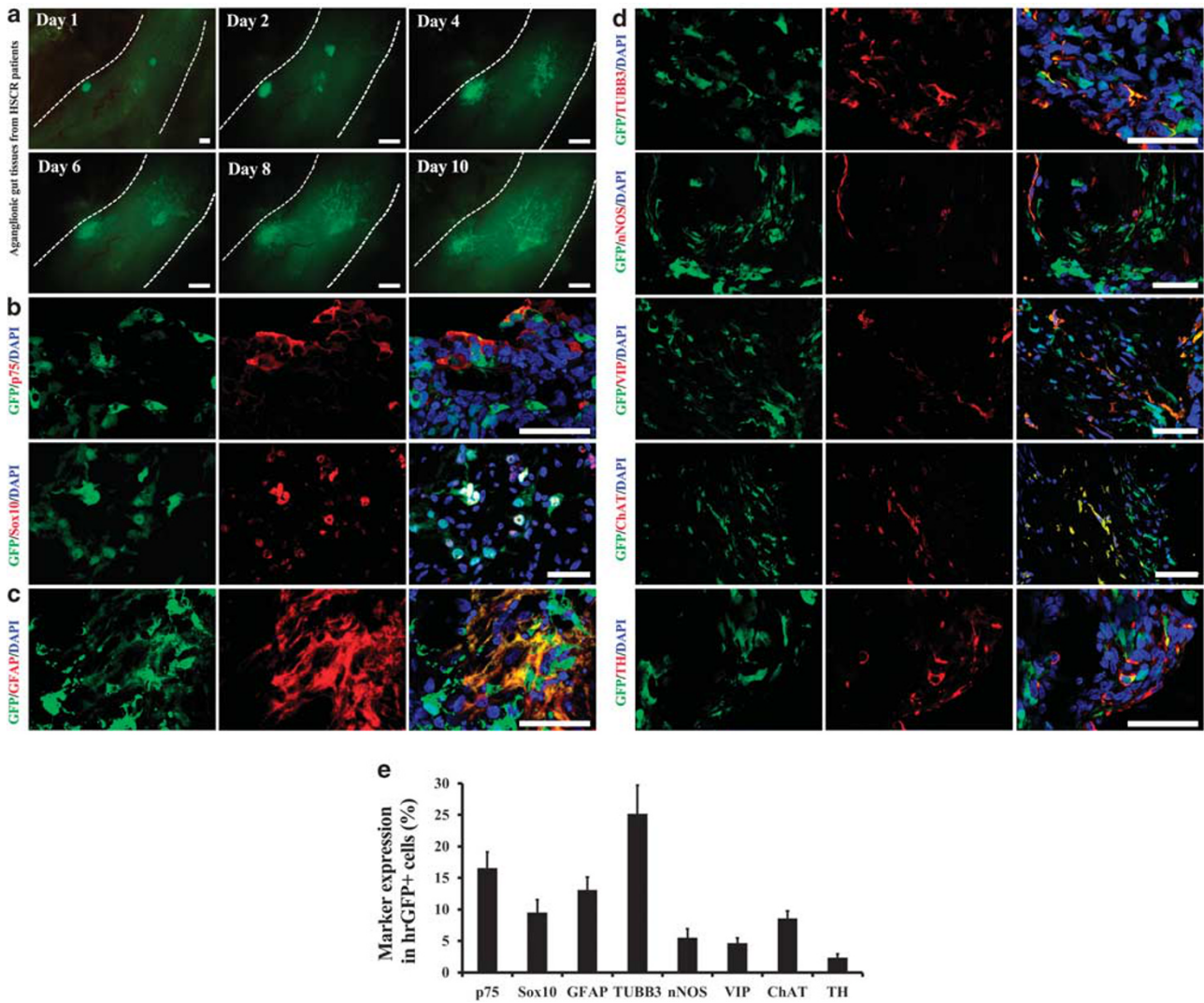


Figure 5. Human induced pluripotent stem cell-derived neural crest stem cells (hiPSC-NCSCs) are capable of migration and differentiation in aganglionic gut tissue from Hirschsprung's disease (HSCR) patients *in vitro*. (a) hrGFP-NCSCs exhibited extensive ability to migrate into the same human aganglionic gut tissue as observed by serial observation under fluorescence microscopy. (b) Some of engrafted cells retained expression of the NCSC marker genes p75 and Sox10. (c) hrGFP+ cells had the ability to differentiate into GFAP+ glial cells. (d) Transplanted cells differentiated into TUBB3+ neurons; different subtypes of mature neurons expressing neuronal nitric oxide synthase (nNOS), choline acetyltransferase (ChAT), vasoactive intestinal polypeptide (VIP) or tyrosine hydroxylase (TH) were also found in recipient gut tissue. Scale bar, 50 μ m. (e) The percentages of p75+, Sox10+, GFAP+, TUBB3+, nNOS+, VIP+, ChAT+ and TH+ neurons differentiated from transplanted hrGFP+ hiPSC-NCSCs were analysed, respectively.

is comparable with a previous report studying the transplantation of mouse gut-derived neural crest cells in postnatal mice colon.⁴³

hiPSC-NCSCs can colonize aganglionic human gut tissue *in vitro*
To determine whether hiPSC-NCSCs have the ability to survive, migrate and differentiate into functional enteric neurons in aganglionic human gut tissue, we further used smooth muscle layers of human aganglionic gut as recipient tissue, including aganglionic gut tissue from HSCR patients and benzalkonium chloride (BAC)-treated ganglionic gut tissue. When co-cultured with the smooth muscle layer of aganglionic gut tissue from HSCR patients, hrGFP-NCSCs exhibited extensive ability to migrate into this recipient tissue by serial observation under fluorescence microscopy (Figure 5a and Supplementary Figure 13A). Most of the gut tissues remained intact after being cultured for 2 weeks (Supplementary Figure 13B). By days 10–13, engrafted cells were

radially distributed throughout the smooth muscle regions. Some of the engrafted cells retained expression of the NCSC marker genes p75 ($16.58 \pm 2.53\%$) and Sox10 ($9.49 \pm 2.08\%$) ($n = 5$; Figure 5b) and of the glial cell marker GFAP ($13.12 \pm 1.99\%$) ($n = 5$; Figure 5c). Moreover, some of the transplanted cells differentiated into TUBB3+ neurons ($25.21 \pm 4.49\%$); different subtypes of mature neurons expressing nNOS ($5.54 \pm 1.40\%$), ChAT ($8.62 \pm 1.11\%$), VIP ($4.63 \pm 0.84\%$) or TH ($2.34 \pm 0.59\%$) were also found in recipient gut tissue ($n = 5$; Figures 5d and e).

A previous study showed that muscle contraction of the bowel can be recorded by an optical motion capture technique, and they found that the aneural E11.5 mouse bowel displayed uncoordinated contractions *in vitro*, whereas transplantation of cultured enteric neural progenitors can restore contractility of normal frequency to E11.5 mouse gut explants.⁴² We then evaluated whether hiPSC-NCSCs could restore the muscle contraction frequency of aganglionic gut tissues from HSCR patients, and

we found that the contractions of HSCR aganglionic gut explants were rapid, irregular and significantly more frequent (33.1 ± 3.348 times per s; Supplementary Movie 2) than those of normal ganglionic gut tissues (12.6 ± 1.265 times per s; Supplementary Movie 3; $n=10$; $P < 0.01$). More importantly, aganglionic gut explants transplanted with neurospheres from hiPSC-NCSCs displayed significantly decreased contraction frequency (13.2 ± 1.398 times per s; Supplementary Movies 4 and 5) that was similar to normal controls ($n=10$; $P > 0.05$) (Supplementary Figure 13C). These results indicated that hiPSC-NCSCs can restore the intestinal contractile activity of human aganglionic explants.

BAC treatment was found to efficiently induce chemical ablation of myenteric neurons in ganglionic gut tissue, as demonstrated by immunostaining with anti-TUBB3 antibody (Supplementary Figure 14A). When hiPSC-NCSCs were co-cultured with BAC-treated human gut tissue, similar results were observed, with the extensive migration and enteric neuronal differentiation of grafted hrGFP-positive NCSCs, as demonstrated by whole-mount immunostaining of recipient gut tissues with anti-TUBB3 antibody (Supplementary Figure 14B) or immunostaining of tissue sections (Supplementary Figures 15A–D).

DISCUSSION

We described here the generation of enteric-like neurons from hiPSCs via neural crest specification through co-culturing with human gut explants. NCSCs from hiPSCs exhibited the ability to migrate and differentiate into mature neurons expressing phenotypic markers characteristic of enteric neurons, including nNOS, VIP, ChAT and calretinin, upon transplantation into aneural or aganglionic chick, mouse or human gut tissues.

NCSCs have been suggested as powerful tools for the modelling of GI motility disorders and brain/gut dysfunction.^{5,44} However, although endoscopic full-thickness biopsy of the gastric wall for the derivation of NCSCs from postnatal human gut is a minimally invasive procedure, intestinal biopsies can still cause side effects or complications, including bowel perforation or excessive bleeding at the biopsy site.⁴⁵ Moreover, a previous study has shown that NCSCs inside the rat postnatal gut wall are less likely to be truly multipotent, have reduced self-renewal potential and have lost the ability to differentiate into certain subtypes of neurons compared with their fetal-stage counterparts.⁴⁶ Metzger *et al.*⁴⁷ also found that the efficiency of sphere formation from human gut appeared to decrease with increasing postnatal age. However, the small numbers and reduced potential to proliferate of such cells limit their application for disease modelling.

The hiPSCs that have been reprogrammed from somatic cells have the characteristics of indefinite self-renewal capacity and pluripotency, and as such, they offer an ideal *in vitro* disease model for GI motility disorders. Although it is known that enteric neurons arise from NCSCs, few studies have reported the derivation of enteric neurons from hiPSCs. Therefore, establishing a stable and efficient protocol for the derivation of NCSCs from pluripotent stem cells will provide an ideal cell resource for the generation of enteric neurons. Previous studies showed that human ESCs co-cultured with M55 or PA6 stromal cells (stromal cell-derived inducing activity) differentiate towards a neural crest fate,⁴⁸ but the culture conditions for stromal-derived inducing activity have not been fully defined, and it usually requires a longer differentiation process. Furthermore, Kawaguchi *et al.*⁴⁹ reported that the forced expression of Sox10 enhances the generation of neural crest progenitors from mouse embryonic stem cells. However, the yield of Sox10-GFP-positive cells was only ~10%.⁴⁹ Recent studies demonstrated that inhibition of the bone morphogenetic protein and transforming growth factor- β signalling pathways and/or glycogen synthase kinase 3 greatly improved the yield of NCSCs (>90%) from human pluripotent stem cells in a monolayer differentiation protocol.^{34,50,51} To

compare the efficiency, repeatability and scalability of the differentiation method from hiPSCs to NCSCs between our method (referred to as 'bFGF+EGF') and the published protocols, including 'LDN+SB',³⁰ 'BIO+SB',²⁹ and 'Chir+SB',⁵¹ we used six hiPSC lines, including two HDF-hiPSC subclones (1 and 2) from the same individual, two iPSC lines from human urine-derived cells (hUC-hiPSCs 1 and hUC-hiPSCs 2)⁵² and two iPSC lines from human amniotic fluid-derived cells (hAFDC-hiPSCs 1 and hAFDC-hiPSCs 2).⁵³ FACS analysis of the percentage of p75+/HNK1+ in differentiated cell population demonstrated that the 'LDN+SB' protocol and our method have better differentiation efficiency and consistency among these hiPSC lines (Supplementary Figures 16 and 17A). Nonetheless, the qPCR analysis for neural crest lineage-specific gene expression among these differentiation protocols demonstrated the NCSCs from our differentiation system expressed relatively higher level neural crest-specific genes, such as p75, HNK1, Sox10 and Ap2 α (Supplementary Figure 17B). Therefore, we speculate that the 'bFGF+EGF' method through EB formation may recapitulate the *in vivo* neural crest development and provide a new approach for NCSC differentiation from pluripotent stem cells.

A previous study demonstrated that human embryonic stem cell-derived NCSCs with self-renewal and multipotency properties could be maintained in SBio-containing media.²⁹ However, when cultured in SBio-containing medium, freshly isolated hiPSC-derived NCSCs underwent massive apoptosis within 2 days in our study (data not shown). These contradictory results may be attributable to differences in the cell lines and culture conditions used. In this latter context, it has been reported that plating density is critical for the survival of sorted NCSCs, with a minimum of $5\text{--}10 \times 10^4$ cells per cm^2 required when NCSCs are cultured in N2B27 medium containing epidermal growth factor and basic fibroblast growth factor.²⁸ However, we found here that the addition of StemPro neural supplement to NCCM greatly enhanced the survival rate and proliferation potential of sorted NCSCs plated at a lower density ($5\text{--}10 \times 10^3$ cells per cm^2).

The iPSC technology has opened new opportunities for modelling human diseases and offers a unique way to investigate molecular mechanisms of disease pathogenesis *in vitro*, such as those of PD⁵⁴ and Alzheimer's disease.⁵⁵ We evaluated the enteric neuron differentiation potential of hiPSC-derived NCSCs, showing that when they were co-cultured with longitudinal muscle layers from gut explants, NCSCs could be induced to differentiate into functional mature enteric-like neurons of different subtypes expressing VIP, ChAT, calretinin or nNOS. Moreover, electrophysiological analysis showed that these cells exhibited functional properties of mature neurons. It is known that a balance between excitatory and inhibitory neurotransmitters controls the movement of gut smooth muscle and that alterations of this balance can contribute to enteric motor dysfunction.³ VIP and NO are primary inhibitory non-adrenergic non-cholinergic transmitters in the gut and are responsible for neurally induced gastric and intestinal relaxation,⁵⁶ whereas ChAT+ neurons are excitatory muscle motor neurons that can induce cholinergic contractions.⁵⁷ It was demonstrated that up to 90% of ASD patients suffer from GI problems, and there is a strong correlation between GI disturbances and autism severity.⁴ Hsiao *et al.*⁵⁸ found that oral treatment of ASD model mice with the human commensal *Bacteroides fragilis* improves gut barrier integrity, to which enteric neurons and glial cells contribute partially,^{59,60} and ameliorates defects in communicative, stereotypic, anxiety-like and sensorimotor behaviours highlighting the importance of gut/brain interaction in health and disease. Braak *et al.*⁶¹ even proposed that a putative environmental pathogen capable of passing the gastric epithelial lining might induce α -synuclein misfolding and aggregation in ENS at a very early stage of PD and reach the brain via the vagal preganglionic innervation of the gut. These results indicate that dysfunction in the gut/brain axis may play an

important role in human brain disorders such as ASD and PD because of unknown causes. Therefore, the enteric neuron differentiation model might be used for the establishment of *in vitro* disease models and provide mechanistic insight into GI disorders or brain/gut dysfunction.

Most recently, Fattahi *et al.*³¹ reported the efficient derivation and isolation of ENS progenitors from human PS cells using CD49D as a surface marker and found that *in vivo* engraftment and migration of these ENS precursors can repopulate the colon and rescue disease-related mortality in the Ednr^b^{S-1/S-1} HSCR mouse model. Compared with the data, our study for we believe the first time demonstrates that hiPSC-NCSCs can differentiate into VIP- or calretinin-positive enteric-like neurons. VIP and calretinin have been found to be involved in the pathogenesis of HSCR. Guo *et al.*⁶² reported a marked reduction of VIP+ nerve cells and fibres in both the myenteric and submucosal plexuses in affected gut of HSCR patients, an alteration that could lead to uncontrolled contractions that are not followed by relaxation, or to spasms of smooth muscles. The calcium binding protein calretinin plays an important role in calcium homeostasis in the nervous system. Barshack *et al.*⁶³ found that calretinin is not expressed in aganglionic segments or associated nerve fibres in HSCR that could lead to an accumulation of excess calcium ions inside the cytoplasm and cause neurodegeneration. We also verified that hiPSC-derived enteric neurons are electrophysiologically functional enteric neurons. More importantly, hiPSC-NCSCs can migrate, generate functional enteric neurons and rescue the spasms of smooth muscles in *in vitro* cultured aganglionic human gut tissues from HSCR patients.

In conclusion, our results indicate that hiPSC-derived enteric NCSCs may supply a powerful tool for studying the pathogenesis of GI disorders, brain/gut dysfunction and ENS development, and they represent a potentially ideal cell resource for autologous cell replacement therapy in GI motility disorders such as HSCR.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

Substantial contributions to conception and design: AP Xiang, H Xia and W Li; acquisition of data or analysis and interpretation of data: W Li, L Huang, J Zeng, W Lin, K Li, J Sun, W Huang, J Chen, G Wang, Q Ke, J Duan, X Lai, R Chen, M Liu, Y Liu, T Wang, X Yang, Y Chen, H Xia and AP Xiang; drafting the article or revising it critically for important intellectual content: W Li, AP Xiang and H Xia; approval of the final version of the manuscript: AP Xiang, H Xia and W Li.

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