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Original Article

A robust method to derive functional neural crest cells from human pluripotent stem cells

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Abstract: Neural crest (NC) cells contribute to the development of many complex tissues of all three germ layers during embryogenesis, and its abnormal development accounts for several congenital birth defects. Generating NC cells—including specific subpopulations such as cranial, cardiac, and trunk NC cells—from human pluripotent stem cells will provide a valuable model system to study human development and disease. Here, we describe a rapid and robust NC differentiation method called "LSB-short" that is based on dual SMAD pathway inhibition. This protocol yields high percentages of NC cell populations from multiple human induced pluripotent stem and human embryonic stem cell lines in 8 days. The resulting cells can be propagated easily, retain NC marker expression over multiple passages, and can spontaneously differentiate into several NC-derived cell lineages, including smooth muscle cells, peripheral neurons, and Schwann cells. NC cells generated by this method represent cranial, cardiac and trunk NC subpopulations based on global gene expression analyses, are similar to *in vivo* analogues, and express a common set of NC alternative isoforms. Functionally, they are also able to migrate appropriately in response to chemoattractants such as SDF-1, FGF8b, and Wnt3a. By yielding NC cells that likely represent all NC subpopulations in a shorter time frame than other published methods, our LSB-short method provides an ideal model system for further studies of human NC development and disease.

Keywords: Neural crest, induced pluripotent stem cells, human, SMAD inhibition, differentiation, migration

Introduction

Neural crest (NC) cells are multipotent cells that are specified at the prospective neural plate border, undergo an epithelial-to-mesenchymal transition to form NC cells, and migrate from the dorsal neural tube to their target tissues where they differentiate into various cell types [1]. The abnormal development of NC cells can result in severe congenital birth defects that affect craniofacial, cardiac, and peripheral nervous system structure and function. These defects include some of the most frequently observed birth defects such as cleft palate, conotruncal heart malformations, and Hirschprung disease [2]. In addition, NC-derived tumors such as neuroblastomas represent 8% of all childhood tumors and account for 15% of cancer-related deaths in children. While many studies have begun to elucidate the mechanisms governing NC development, many questions remain unanswered, particularly with respect to how alterations in NC development can cause disease.

It is known that the regulation of specific developmental pathways, including the bone morphogenetic protein (BMP)/Activin and Wnt signaling axes, are required for proper NC development during embryogenesis [3]. To better understand the precise role of these signaling pathways, much effort has been devoted to developing methods to induce NC cell formation *in vitro*. However, the first methods to do

Table 1. Human pluripotent cell lines used in this study

	Cell Line Information	NC Cell Formation
iPS-1	WTC-10 integration-free iPS	Yes
iPS-2	WTB-6 integration-free iPS	Yes
iPS-3	WTC-11 integration-free iPS	Yes
iPS-4	WTC-11 integration-free-H2B-GFP	Yes
H9	Human ES stem cell line	Yes

The LSB-short NC differentiation method was tested on multiple human pluripotent stem cell lines. All starting pluripotent lines could be differentiated into NC cells as verified by ICC or flow cytometry analysis. Cell lines are referenced as iPS-X for ease of reference throughout the manuscript.

this were complex, multi-step procedures that relied on the formation of embryoid bodies and the use of stromal feeder co-cultures [4, 5]. The first embryoid body–independent methods to generate NC cells were focused on producing neural stem cells [6], whereby NC cells were observed as a by-product of neural differentiation. However, it was soon realized that these neural precursor methods could be used to obtain larger numbers of NC cells. These newer methods included the use of Noggin, an endogenous secreted peptide that antagonizes BMP signaling by blocking BMP interaction with its receptors, and SB435142, a small molecule that inhibits the Activin type–I receptor ALK4 and the nodal type–I receptor ALK7, in human induced pluripotent stem (iPS) cells and human embryonic stem (ES) cells [6]. More recently, it was demonstrated that LDN-193189 [7], a potent, synthetic small-molecule inhibitor of BMP type–I receptors ALK2 and ALK3, could substitute for Noggin during peripheral nerve differentiation of iPS cells [8]. Blocking BMP and Activin A/Nodal signaling resulted in the inhibition of downstream SMAD signaling, thereby facilitating differentiation into neural and NC lineages [6]. Concurrent activation of Wnt signaling can also induce NC cell formation from pluripotent stem cells [9, 10].

In spite of this progress, most existing methods for *in vitro* NC cell formation remain relatively inefficient. They yield highly heterogeneous populations and require cell-sorting techniques to enrich for NC cells, making it difficult to study NC-specific phenotypes in culture. Also, the functionality of these derived NC cells, such as their migratory potential in response to known chemoattractants, has not been extensively evaluated *in vitro*. Understanding this intrinsic migratory potential will be important for improving our understanding of NC cell migratory defects in various NC disorders. Furthermore, NC cells are comprised of diverse subpopulations, including cranial, cardiac, and trunk NC cells, and it is unclear whether the existing methods can generate all of these subpopulations. This consideration is critical when studying specific NC disorders that result in craniofacial, cardiac, or enteric developmental abnormalities. Therefore, more robust and efficient methods for NC derivation will be required for applications in regenerative medicine and drug discovery.

The advent of human iPS cell technology provides an invaluable tool for studying both normal NC development as well as NC disease mechanisms [11]. In this report, we applied this technology to develop a rapid, robust, and reproducible method, known as "LSB-short", to derive NC cells from human pluripotent stem cells. The LSB-short method is similar to the previously published LSB method, which uses a combination of LDN-193189 and SB435142 to induce NC formation in approximately two weeks [8]. Our method generates high yields of NC cells in a shorter time frame compared to other published methods. The NC cells could be cultured over multiple passages while maintaining NC marker expression, and could spontaneously differentiate into different NC lineages. Global gene-expression analyses revealed the multipotency potential of NC cells, while migration assays confirmed their migratory potential. The LSB-short method is therefore well suited for regenerative medicine and drug-screening applications, and could be used to advance our understanding of NC biology in development and disease.

Materials and methods

Human iPS cell culture, NC differentiation, and maintenance

Human iPS cells were generated in our lab (Hayashi et al, unpublished) using the episomal reprogramming method described in [12]. The cells are referenced as iPS-1, iPS-2, iPS-3, and iPS-4 in this study (**Table 1**). The human iPS cells and H9 ES cells were maintained on growth factor–reduced matrigel (BD) in mTesR1

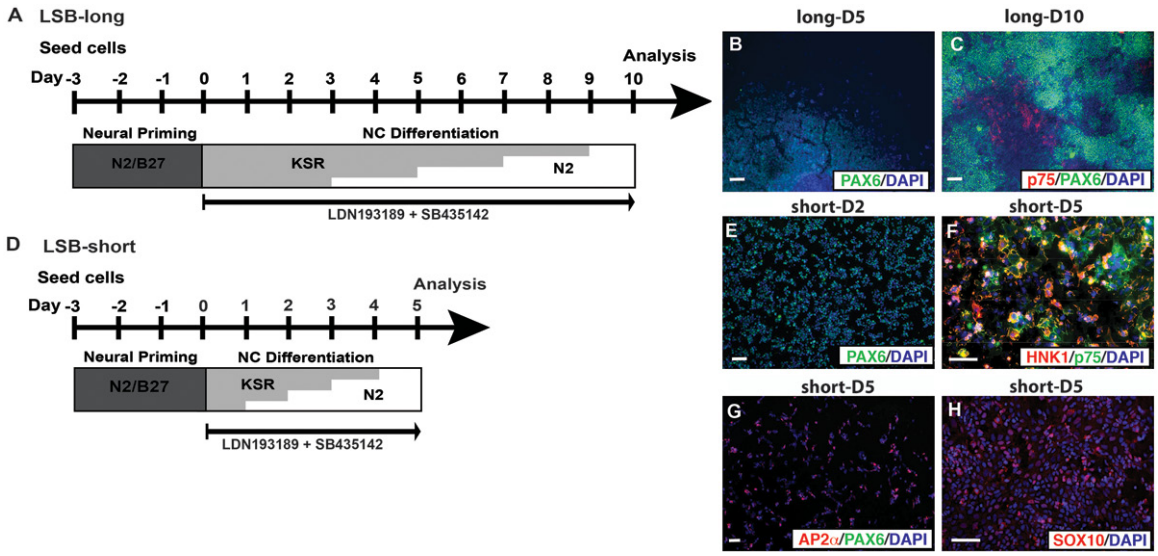

Figure 1. Modified NC differentiation methods, LSB-long and LSB-short, generate NC cells from human pluripotent stem cells via a neural precursor stage. A: Schematic of LSB-long method, which takes 13-14 days to complete. B: PAX6+ neural precursor cells appear as early as day 5 using the LSB-long method (iPS-1, representative image). C: By day 10-11 of the long method, a small number of p75+ NC cells were observed next to large regions of neural precursor (PAX6+) cells (H9, representative image). D: Schematic of LSB-short method showing a shorter NC differentiation phase. E: With the short method, PAX6+ neural precursor cells were observed at day 2 of NC differentiation (iPS-1, representative image). F: NC cells co-expressed HNK1/p75 (iPS-5, representative image). G: At day 5 of the short method, AP2a-expressing NC cells predominated in the culture wells, which contained almost no PAX6+ neural precursor cells (iPS-1, representative image). H: Day 5 NC cells from the short method also expressed SOX10, a marker of migrating NC cells (H9, representative image). Scale bar = 100 μm.

medium (STEMcell Technologies). Cells were passaged every 3-4 days with Accutase (Millipore) and replated with mTesR1 supplemented with 10 μM Y-27632 (Millipore), with daily media changes.

To initiate differentiation, cells were plated at a density of 10^4 cells/cm^2 on growth factor–reduced matrigel in N2B27-CDM consisting of DMEM/F12 medium containing 1% N2 supplement, 2% B27 supplement, 0.05% BSA fraction V, 1% Glutamax, 1% MEM-non-essential amino acids, and 110 μM 2-mercaptoethanol (Life Technologies), supplemented with 10 μM Y-27632 and 20 ng/mL bFGF (Peprotech). Twenty-four hours later, N2B27-CDM was replaced with medium lacking Y-27632.

Once cells reached approximately 60% confluency, they were treated with different ratios of KSR:N2 media (see **Figure 1**). KSR media consisted of KO DMEM medium containing 15% knockout serum replacement, 1% glutamax, 1% MEM-NEAA, and 55 μM 2-mercaptoethanol. N2 media consisted of DMEM/F12 medium containing 0.15% glucose, 1% N2 supplement, 20 μg/mL insulin, 5 mM HEPES (Life Technologies).

For the LSB-long method, cells were incubated daily with fresh KSR:N2 media supplemented with 50 nM LDN-13189 (Stemgent) and 5 μM SB431542 (Tocris) with varying ratio changes of KSR:N2 on day 0 (100:0), day 3 (75:25), day 5 (50:50), day 7 (25:75), and day 9 (0:100). Similarly, the LSB-short method has a 3-day neural priming step, following by incubation in varying ratio changes of KSR:N2. However, cells were treated with KSR:N2 media containing 50 nM LDN-13189 (Stemgent) and 5 μM SB431542 (Tocris) on day 0 (100:0), day 1 (75:25), day 2 (50:50), day 3 (25:75), and day 4 (0:100) (**Figure 1D**). Twenty-four hours later, NC cells were harvested with Accutase or TrypLE (Invitrogen) and prepared for analyses or maintenance on growth factor–reduced matrigel in SFM media (KO DMEM/F12 medium containing 2% StemPro neural supplement and 1% glutamax) (Life Technologies) supplemented with 20 ng/mL bFGF and 20 ng/mL EGF (Sigma).

Cells were passaged when they reached 80-90% confluency by rinsing once with Ca2+/

Table 2. Primary antibodies used to characterize NC cells by ICC

	Company	Catalog No.	Dilution	Application
HNK1	Sigma	C6680	1:300	ICC
HNK1 (CD57) FITC	BD Biosciences	555619	1:5	Flow Cytometry
P75	Advanced Targeting Systems	AB-N07	1:200	ICC
P75 (CD271)-AF647	BD Biosciences	560877	1:5	Flow Cytometry
AP2α	DSHB	3B5	1:50	ICC
S0X10	Abcam	ab108408	1:100	ICC
Oct3/4	Santa Cruz	Sc-5279	1:100	ICC
GFAP	Dako	Z0334	1:1000	ICC
SMAα	Sigma	A2547	1:800	ICC
Peripherin (C19)	Santa Cruz	Sc-7604	1:100	ICC
Pax6	Proteintech	12323-1-AP	1:100	ICC
S100	Abcam	Ab4066	1:200	ICC
βIII-tubulin	Santa Cruz	Sc-9104	1:100	ICC

Mg2+-free-PBS and treating with Accutase for 3 minutes. Cells were collected, pelleted, and re-plated at a density of 3 x 10^4 cells/cm^2 on growth factor–reduced matrigel (BD) and grown in SFM.

Immunocytochemistry (ICC)

Cells were seeded onto plastic chamber slides (Ibidi) treated with 50 μg/mL fibronectin (Roche) and allowed to attach for at least 2 days before ICC. For the spontaneous NC differentiation experiments, NC cells were cultured in the chamber slides for at least 10 days after seeding. Cells were fixed with 4% PFA (Santa Cruz Biotechnology) for 20 min and rinsed with PBS. To permeabilize cells, cells were incubated in blocking solution--2% BSA (Sigma) in PBS (Gibco)--containing 0.1% Triton-X 100 for 5 min. Cells were then incubated in blocking solution for 30 min at room temperature with mild agitation and then incubated in blocking solution containing primary antibodies for 1 h, rinsed 3 x 5 min in PBS, and incubated with secondary antibodies for 1 h at room temperature. Cells were then rinsed 3 x 5 min in PBS and treated with DAPI Vectashield Mounting media (Vector Labs) or Hoescht (Molecular Probes) at room temperature to stain cell nuclei. The slides were imaged immediately using an inverted epifluorescence microscope (Zeiss; Keyence). All primary antibodies used in this study are listed in **Table 2**. Secondary antibodies used in this study were from Invitrogen. Images were captured using Axiovision or Keyence BZ 9000 software, and images were processed using ImageJ. Figures were assembled using Adobe Illustrator and Photoshop.

Flow cytometry analysis

Cells were harvested by enzymatic dissociation using TrypLE for 8 min at 37°C until cells were visibly detached. TrypLE activity was quenched and collected with a 5x volume of 10% FBS in KO DMEM/F12 per 1x volume of TrypLE used. Cells were pelleted by centrifugation at 800 rpm for 3 minutes, resuspended in KO DMEM/F12, filtered through a 40 μm cell strainer (BD Biosciences) to obtain a single-cell suspension and then counted using the Countess® Automated Cell Counter (Invitrogen). Cells were pelleted and washed with chilled FACS buffer consisting of 0.5% BSA (w/v) and 2 mM EDTA in D-PBS and kept on ice for all further procedures. Cells were resuspended at the appropriate cell density in FACS buffer containing the appropriate concentration of antibody according to the manufacturer's specifications (see **Table 2** for the list of BD Biosciences Antibodies used in this study). Cell Trace™ Calcein Violet, AM was added to samples as a live-cell stain for viability, according to the manufacturer's specifications. Live, stained cell samples were transferred to a FACS tube and analyzed immediately on the BD™ LSR II flow cytometer.

Microarray profiling

Total RNA from biological triplicates of reprogrammed iPS cells (iPS-1), obtained from the fibroblasts of healthy donors, and day 5 iPS-NC

cells (iPS-1), generated using the LSB-short method, were extracted with Trizol and prepared for hybridization to Human Gene 1.0 ST Affymetrix microarrays as described [13]. As starting material, ~1 μg of total RNA was purified with the RiboMinus human transcriptome isolation kit (Invitrogen), cDNA was fragmented and labeled with the GeneChip WT cDNA synthesis and WT terminal labeling kits (Affymetrix), hybridized to individual GeneChip arrays and scanned according to the manufacturer's instructions. The data were deposited in NCBI's Gene Expression Omnibus database (GSE44727).

Transcriptome data analysis

NC and pluripotent stem cell microarray data from this study, as well as several additional studies (GSE21511, GSE14340, GSM540717), were reanalyzed from Affymetrix CEL files using the software AltAnalyze version 2.0.8 with the EnsMart65 database [14]. All NC comparisons were made to the appropriate ES cell reference samples. Default analysis options were used, which include analysis of gene expression via Empirical Bayes–moderated t-test with a Benjamini-Hochberg-adjusted *p*-value. For Affymetrix Exon and Gene array analysis, alternative exon analysis was performed using the FIRMA analysis option. GO-Elite [15] analysis and pathway/network visualization was employed for all available gene-sets using the default options (Fisher Exact t-test) with a false-discovery rate *p*-value cutoff of 0.05. RNA-Seq analyses for comparing ES-derived NC (GSM714814, GSM602289) were performed following genome and junction alignment with TopHat [16] to produce junction.bed and exon.bed files for gene-expression and exon/junction cooperative-alternative-exon analyses in AltAnalyze. Since only one sample was analyzed for this RNA-Seq data, differentially expressed genes were based on a threshold of greater than two-fold. Expression clustering and heatmap visualization were performed in AltAnalyze using the HOPACH clustering algorithm option [17]. oPPOSUM single-site transcription-factor binding site analysis was performed using default options [18].

Generation of stable iPS cell line

The pSicoR-PGK-H2B-GFP-Puro lentiviral construct was created by replacing the Ef1α-mCherry cassette of pSicoR-Ef1α-mCh-Puro [19] (Addgene plasmid 31845) with the PGK-H2B-eGFP cassette from PGK-H2BeGFP [20] (Addgene plasmid 21210). Lentivirus was packaged in HEK293 cells (6 x 10^6 cells in a 10 cm dish), co-transfected with pSicoR-PGK-H2B-GFP-Puro (3 μg) and 1 μg of each packaging plasmid: pVSV-G (Clontech), pRSV-Rev (Addgene plasmid 12253), and pMDLg/pRRE (Addgene plasmid 12251) [21], using FuGENE (Promega). HEK293 cells were grown on 0.1% gelatin-coated plates in high-glucose DMEM supplemented with 10% fetalplex (Gemini), 1% glutamax, and 1% sodium pyruvate (Life Technologies). Viral supernatant was collected 48 h post-transfection, filtered through a 0.45 μm filter and frozen down until use. To titer the virus, 100 μL of filtered supernatant was incubated with 1.5 x 10^5 HEK293 cells for 48 h and GFP expression was measured using the VYB flow cytometer (Miltenyi). iPS-3 cells were transduced with virus in mTesR1 media supplemented with 10 μM Y-27632. Twenty-four hours post-transduction, cells were given fresh mTesR1. Once cells were grown for 3-4 days, transduced cells were selected for using 0.5 μg/mL puromycin (Sigma). Stable cell colonies were expanded and cryopreserved. This line is referred to as iPS-4 in this study (**Table 1**).

Migration assays

NC cells were harvested with TrypLE and pelleted in DMEM/F12 + 10% FBS. Cells were rinsed once in DMEM/F12 and incubated with 1 μM Draq5 (Biostatus Limited) for 10 min at 37°C. Cells were rinsed in PBS and pelleted in DMEM/F12 media without growth factors. Draq5-labeled NC cells were seeded at 4 x 10^4 cells/well into Boyden-chamber (Neuroprobe) assays. For Boyden-chamber assays, the chemoattractants SDF1a at 3 ng/mL (Peprotech), FGF8b at 3 ng/mL (R&D Systems), BMP2 at 0.3 ng/mL (R&D Systems), and Wnt3a at 1 ng/mL (R&D Systems) were diluted in SFM and loaded into the bottom chamber, while cells were diluted in DMEM/F12 and loaded onto the 50 μg/mL fibronectin-treated 5 or 8 μm filter (Neuroprobe) of the upper chamber. Once assembled, the apparatus was incubated at room temperature for 10 min and subsequently moved into a 37°C, 5% CO_2 incubator for 4 h. The filter was removed and scanned (Fc Odyssey LiCor) and fluorescence intensity was quantified (LiCor Image Studio Software).

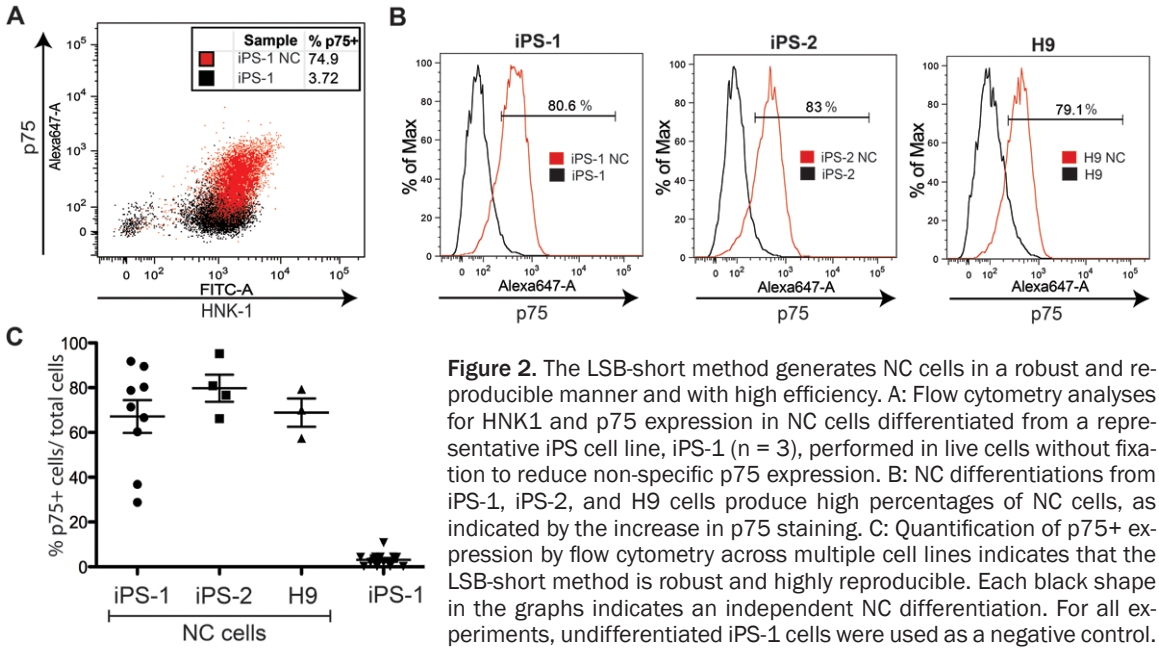

Figure 2. The LSB-short method generates NC cells in a robust and reproducible manner and with high efficiency. A: Flow cytometry analyses for HNK1 and p75 expression in NC cells differentiated from a representative iPS cell line, iPS-1 (n = 3), performed in live cells without fixation to reduce non-specific p75 expression. B: NC differentiations from iPS-1, iPS-2, and H9 cells produce high percentages of NC cells, as indicated by the increase in p75 staining. C: Quantification of p75+ expression by flow cytometry across multiple cell lines indicates that the LSB-short method is robust and highly reproducible. Each black shape in the graphs indicates an independent NC differentiation. For all experiments, undifferentiated iPS-1 cells were used as a negative control.

Graphs and statistical analysis

Graphs and statistics (i.e., Student's t-test for comparisons between two groups or one-way ANOVA followed by Dunnett's post-hoc test for multiple comparisons) were generated using Prism 5 software (GraphPad).

Results

Inhibition of BMP and activin a/nodal signaling pathways with LDN-193189 and SB435142 is sufficient to induce NC formation in 8 days

To develop a robust method for generating NC cells to study human NC development and disease, we modified published methods based on dual SMAD inhibition with Noggin or LDN-193189 and SB435142 [6, 8]. We cultured human iPS cells on matrigel in feeder-free conditions at low density and "primed" them with N2/B27 medium to promote neural induction. The cells were then switched to gradients of KSR:N2 media containing 50 μM LDN-193189 and 5 μM SB435142 to promote neural precursor formation followed by NC differentiation (**Figure 1**). We first carried out a NC differentiation method similar to published dual SMAD inhibition protocols, called "LSB-long" [6, 8]. Using this method, we found that PAX6+ cells were present by day 5, albeit at low levels (**Figure 1B**). By day 10, the majority of cells were strongly PAX6+, similar to described methods [6], with small clusters of p75+ NC cells (**Figure 1C**). Even when starting with lower iPS seeding densities, due to the high proliferation rate of neural precursors, cell confluency was maximal by day 10, resulting in significant cell death (data not shown).

Since lower iPS-seeding densities are thought to favor the differentiation of both neural precursor and NC cell types [6], we hypothesized that NC cells could be generated from fewer neural precursor cells in a shorter time frame than published methods, and at high yields. We therefore modified our LSB-long method to generate "LSB-short", in which we kept the neural priming step the same, but shortened the NC differentiation time by half. In this method, neural-primed cells were incubated in a KSR:N2 media gradient over a period of 4 days, so that the NC-differentiation phase spanned 5 instead of 10+ days (**Figure 1D**). Compared to the equivalent stage of the LSB-long method (day 5), PAX6+ neural precursor cells were clearly visible by day 2 of the LSB-short method (**Figure 1E**). At day 5, we were able to identify NC cells by the presence of the NC markers HNK1, p75 (**Figure 1F**) and AP2a (**Figure 1G**), concomitant with the absence of PAX6 protein (**Figure 1G**). In addition, we detected SOX10 protein in almost all day 5 cells (**Figure 1H**), indicative of migratory NC cells [22, 23]. These data suggest

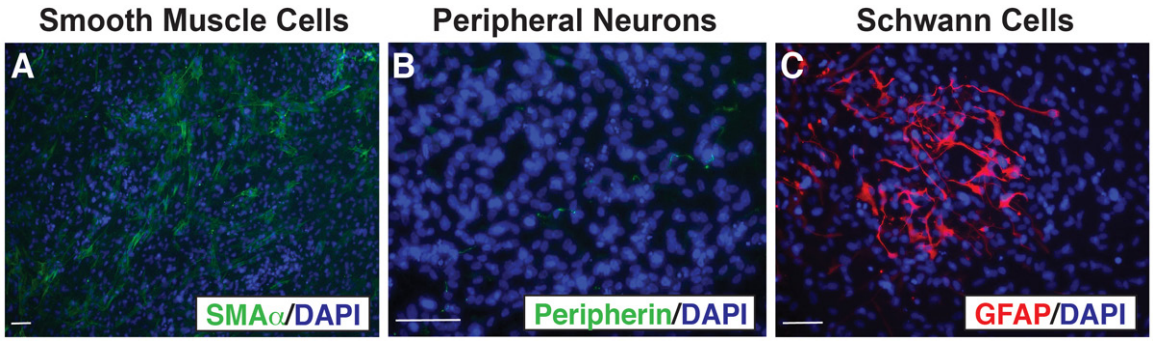

Figure 3. NC cells from the LSB-short method can differentiate into multiple NC lineages. NC cells spontaneously differentiate into A: smooth muscle cells (SMAa+), B: peripheral neurons (peripherin+), C: Schwann cells (GFAP+) (iPS-3 cells, representative images). Scale bar = 100 μm.

that our LSB-short method, which combines low-density iPS-cell seeding, neural priming, and the SMAD pathway inhibitors LDN193189 and SB435142, could induce *bona fide* NC cell production in a significantly shorter time frame than published methods.

The LSB-short method generates NC cells in a rapid, robust and reproducible manner in multiple human pluripotent cell lines with high efficiency

To assess the robustness and efficiency of the LSB-short method, we tested it on two biologically distinct and at least two clonally distinct iPS cell lines [24] (**Table 1**). We also tested the method with the H9 human ES cell line [25]. The parental (undifferentiated) iPS cell line, iPS-1, was used as a negative control. Initially, we carried out double staining for NC markers, HNK1 and p75, as described [10, 26] (**Figure 2A**). However, we found that our iPS cell lines were also HNK1+, albeit at moderate levels (**Figure 2A**). Therefore, we carried out the rest of our NC differentiation analyses using mainly p75 expression as a readout. All pluripotent cell lines tested produced NC cells, as determined by flow cytometry for p75+ cells on day 5 of NC differentiation (**Figure 2B** and **Table 1**). To quantify differentiation efficiency, we carried out at least three independent NC differentiations on two human iPS cell lines, iPS-1 and iPS-2, and one ES line, H9. At day 5, almost all NC differentiation experiments produced populations of at least 60% p75+ cells (**Figure 2C**), with ~80% on average. At best, we observed ~90% NC differentiation efficiency from both human iPS cell lines.

LSB-short method generates multipotent NC cells capable of differentiating into all NC sub-populations with a migratory NC gene-expression program

To verify the multipotency potential of NC cells generated with the LSB-short method, NC cells were cultured to confluency in SFM maintenance medium to allow for spontaneous differentiation. We observed that these pools contained NC cells of multiple lineages, which could give rise to smooth muscle cells, peripheral neurons, and Schwann cells (**Figure 3A-C**).

We next wished to assess the transcriptome of these differentiated cells. To do this, gene expression and alternative-exon-usage profiles were evaluated using whole genome exon–sensitive expression arrays (Affymetrix Gene 1.0 ST) in the program AltAnalyze. In total, 694 genes were induced and 661 genes were repressed (fold > 2, FDR moderated t-test $p < 0.05$) in differentiated NC cells compared to iPS cells. Examination of known markers for distinct NC lineages, NC migration, and NC specification further suggested that our method produced migratory cranial, cardiac, and trunk NC cells (**Figure 4A**). As a means for unbiased classification, we examined cellular biomarkers, pathways, Gene Ontology (GO) terms, and transcriptional targets using the GO-Elite module of AltAnalyze. Genes induced upon differentiation were highly enriched in predicted NC stem–cell markers (18 out of 55), while repressed genes were most associated with pluripotent stem cells (25 out of 83). In addition, markers for neural progenitors were also enriched among the induced genes (FDR p <

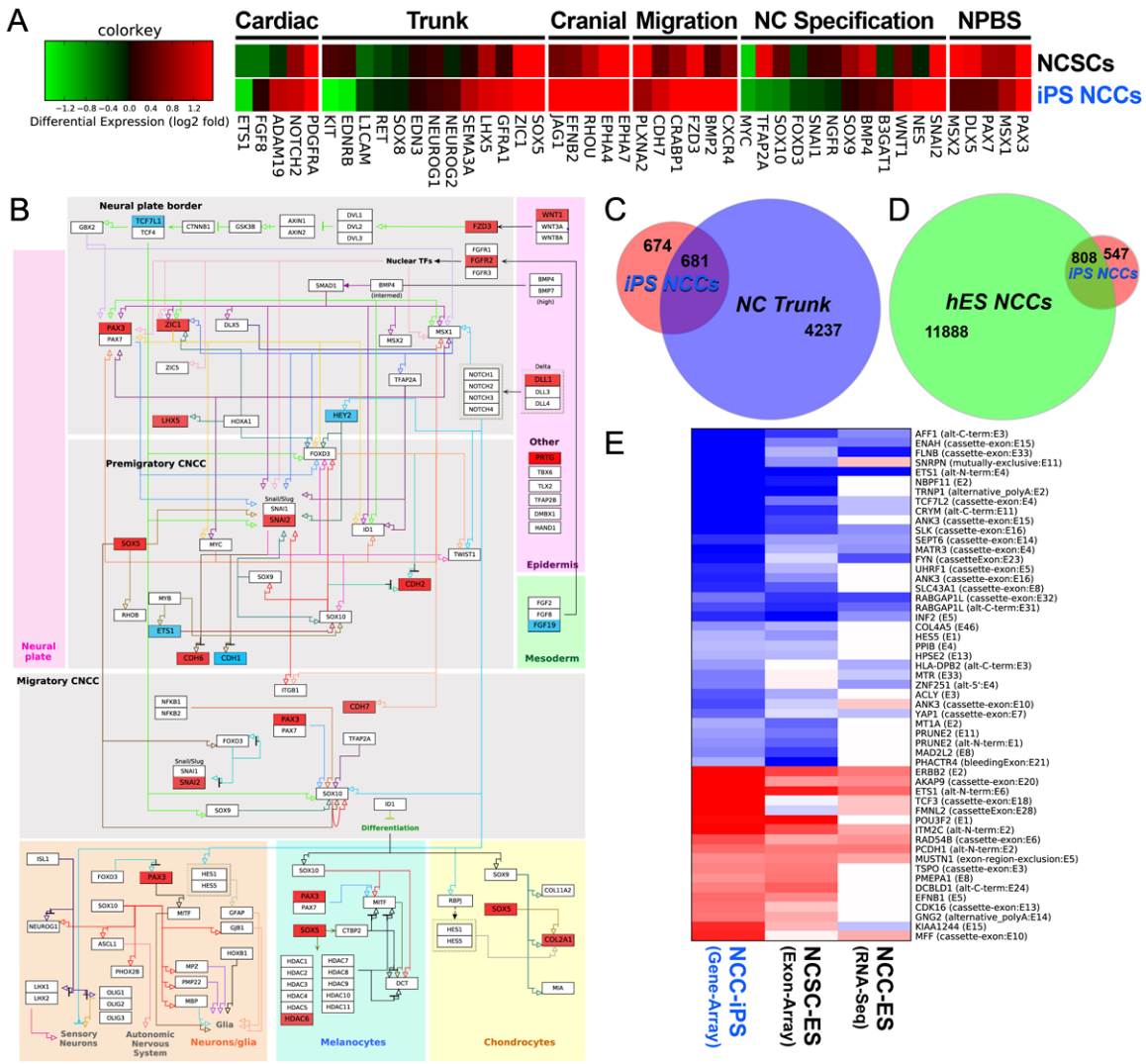

Figure 4. Activation of migratory NC expression program. iPS-derived NC cells (iPS NCCs) analyzed in the context of well-described NC markers and previously reported *in vitro* and *in vivo* NC transcriptome datasets. A: Various markers for distinct NC sub-populations are compared between the iPS NCC method and a previous method to derive NC stem cells (NCSC). These include markers for neural plate border specifiers (NPBS), NC specification, and NC migration, and distinct markers for cranial, trunk and cardiac NC cells. Red = upregulation; green = downregulation. B: Differential gene-expression changes in iPS NCCs relative to iPS cells are visualized upon a previously developed model of NC cell differentiation (WikiPathway WP2064 revision 47071). Differentially expressed genes are colored red or blue (up or downregulation, respectively) from AltAnalyze. C, D: Comparison of differentially expressed genes from RNA transcriptome profiles of human embryo–derived NC trunk and ES-derived NC cells to iPS NCCs. For ES-derived NC cells, RNA-Seq of single samples was performed resulting in lower stringency results (see *Materials and Methods*). E: Hierarchically clustered heatmap of alternative exons differing between NCCs and ES or iPS cells from three distinct differentiation methods using the software AltAnalyze. Blue indicates exon-exclusion and red exon-inclusion relative to pluripotent stem cell profiles.

0.05). The WikiPathway of NC Differentiation (**Figure 4B**) was the most highly enriched pathway when all regulated genes or only induced genes were considered (16 and 11 out of 72 genes, respectively). Among the most induced biological processes were cell migration and axonal guidance, neuron, osteoblast, hepatocyte and muscle differentiation, epithelial proliferation, regulation of cell-cell adhesions, neuron fate commitment and the ephrin receptor, Wnt receptor, SMAD and TGF-beta signaling pathways (FDR $p < 0.05$). Along with these groups, six out of 38 genes associated with NC cell migration were also regulated (FGF19,

ZEB2, EDNRB, LEF1, EFNB1, PAX3), further suggesting the migratory potential of these cells.

To determine which transcriptional pathways were active in these cells, we used both experimentally derived (PAZAR, Amadeus, GO Elite) and sequence-based (oPOSSUM) transcription factor (TF) target predictions. Enriched TF targets (upregulated genes) included SRY, LHX3, SOX5, FOXD3 (oPPOSUM), and TWIST1, TCF3/TCF7L1, HIF1A-ARNT GO-Elite), all of which have previously been implicated in NC specification. In addition, several novel TFs targets were identified (oPOSSUM: NKX2-5, PDX1; GO-Elite: SP1, SP3, SOX2, NANOG, SRF and PDX1). In our microarray analysis, the TFs SOX5 and LHX3 were upregulated while NANOG and TCF7L1 were downregulated. Among the most highly regulated TFs in our dataset was NR2F2, a predicted NC master regulator [27], along with a number of other zinc ion–binding TFs (NR2F1, ZEB1, ZEB2 and ZIC1).

To better understand how these iPS-derived NC cells compare to known NC populations, we analyzed and compared microarray and transcriptome profiles from previously profiled NC cells relative to a pluripotent baseline. From these analyses, we found that over half of all transcriptionally regulated iPS-derived NC genes were common to NC trunk RNA obtained from human embryos [28] (**Figure 4C**). The same trend was also observed when comparing iPS-derived NCs to RNA-Seq data for hES-derived NC cells [27] with 59.6% of regulated genes in common (**Figure 4D**). As a further demonstration of these similarities, we compared changes in exon expression among NC derivation methods. Similar to the transcriptional regulation analyses, a common set of alternatively expressed exons was identified, including several important transcriptional regulators (ETS1, HES5, POU3F2, TCF3, TCF7L2) (**Figure 4E**).

NC cells are functional and can migrate to appropriate cues

To contribute to tissue development during embryogenesis, NC cells migrate over great distances in response to chemoattractant and chemorepellent cues [29]. Studies in animal models have demonstrated that for proper migration, cranial NC cells require Wnt3a and BMP2 [30-32], while cardiac NC cells require FGF8b [31] and trunk NC cells require BMP2 [33]. In addition, SDF-1 is required as a chemoattractant for all NC cells. To determine whether the NC cells generated here could respond to these cues, we analyzed NC cell chemotaxis using migration assays. Each NC guidance molecule was loaded into the lower well of a Boyden chamber and, over time, formed a concentration gradient (**Figure 5A**). Draq5-labeled NC cells were loaded into the top half of the Boyden chamber (**Figure 5A**) and NC cells that responded to the gradient of chemoattractant migrated through the 5–8 μm pores of the fibronectin-coated filter membrane. These migrating cells were quantified by fluorescence imaging (**Figure 5C** and **5D**). We found that NC cells migrated towards the chemoattractants SDF-1, FGF8b, BMP2, and Wnt3a (**Figure 5E**). To verify that NC cells were chemotaxing and not exhibiting chemokinesis (i.e., random, non-directional motility), checkerboard experiments were performed in which the chemoattractant was loaded in both the top and bottom of the well (**Figure 5B**) to avoid the establishment of a gradient. We found that BMP2 induced chemokinesis (**Figure 5F**), suggesting that the chemotaxis observed with BMP2 exposure was probably negligible. Overall, these data suggest that the NC cells are functional and can migrate in response to appropriate cues.

Discussion

In this report we present a robust, efficient and rapid method—LSB-short—for NC cell differentiation from human pluripotent stem cells (iPS and ES cells). Our method is a modification of a previously published method based on dual SMAD inhibition using LDN13189 and SB431542 [8]. Our data suggests that we can generate *bona fide* NC cells expressing the appropriate NC markers, including HNK1 and p75, in a total of 8 days, which is significantly faster than previously described [6, 8-10, 26, 34]. Furthermore, cells expressed PAX6 protein by day 2, an observation suggesting that the neural precursor stage is a necessary pre-requisite for NC differentiation. Interestingly, we did not see neural rosettes, which have been observed using other NC differentiation methods. This may result from differences in cell density, as our method seeds cells at lower

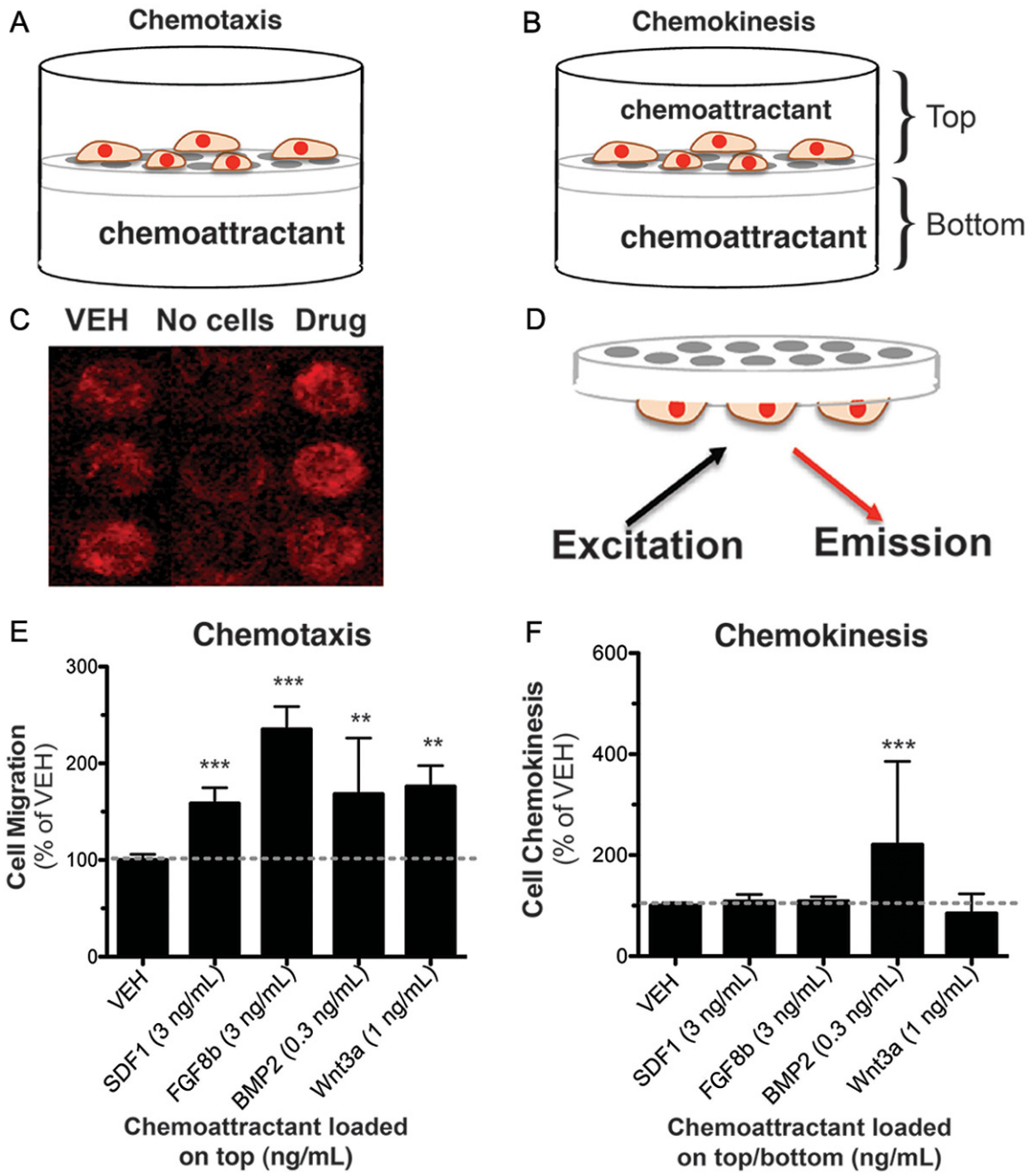

Figure 5. NC cells migrate to appropriate chemoattractants. A, B: Schematics of Boyden chamber experiments. A: NC cell chemotaxis potential was quantified in Boyden chambers by loading cells in the top well and chemoattractant (SDF1, Fgf8b, BMP2, or Wnt3a) in the bottom well. B: NC cell chemokinesis potential was quantified by loading cells on the top and chemoattractant on both the top and bottom as shown. C: Representative image of the bottom filter of the migration chamber showing that Draq5-labeled cells (red) migrated through the filter from the upper chamber in response to drug loaded into the bottom of the lower chamber. D: Schematic of how migrating NC cells are visualized by excitation of Draq5 nuclear dye and emission detection is quantified. E: NC cells migrated to all chemoattractants tested. F: NC cells do not display chemokinetic behavior in response to chemoattractants, except for BMP2. iPS-1, n = 3 experiments performed in triplicate. One-way ANOVA, $^{***}p < 0.001$, $^{**}p < 0.01$.

densities than optimal for promoting neural rosettes, but nonetheless suggests that this step is unnecessary for NC formation.

We were able to differentiate all of the human pluripotent cell lines used in this study into NC cells. By initiating NC differentiation soon after

neural precursor formation, we were able to generate high percentages of NC cells (80% on average, and as high as ~90% in some NC differentiations). These NC cells could proliferate, self-renew, migrate to appropriate cues, and spontaneously differentiate into various NC lineages, including smooth muscle, peripheral nerves and glia. The multipotent potential of these NC cells was supported by the global gene–expression analyses carried out on iPS-1 NC cells, which indicated that the LSB-short method generated migratory NC cells with cardiac, cranial, and trunk transcriptional signatures. Using exon-sensitive microarray profiling, we demonstrated that our iPS-derived NCs possessed a transcriptional and splicing signature highly similar to both *in vitro*– and *in vivo*–profiled NC cells. From this analysis, we also identified novel transcriptional regulatory pathways and common downstream post-transcriptional regulatory changes that may critically underlie NC cell fate. Given the limited availability of human genomic profiles for distinct NC lineages (with only trunk profiles currently available), we restricted our analysis to known NC lineage markers. However, future analyses will benefit from a detailed analysis of cardiac, cranial, trunk, migratory and non-migratory NC cells derived from human embryos.

We hope that our LSB-short protocol will serve as a resource for researchers who are seeking to produce NC-derived tissues. Importantly, this protocol is capable of generating millions of cells from different human pluripotent stem cell lines, opening new opportunities to study NC cells or NC-derived tissues. The NC cells can be passaged for studies of NC biology, such as migration and proliferation. In addition, NC cells derived using the LSB-short method can be differentiated into a wide variety of cell types (e.g. peripheral nerves and smooth muscle), which is of great medical interest. As methods improve, we anticipate that these cells will be useful for a wide variety of studies, ranging from basic cell biology to regenerative medicine, in which NC-derived tissues could be generated using the patient's own cells to replace damaged or lost tissue (such as bone and nerves). The ability to study the mechanisms of NC biology and disease in a patient-specific context should also accelerate the development of novel therapies to treat, or even prevent, NC disorders.

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Disclosure of conflict of interest

None.

Abbreviations

ALK, activin type-1 receptor; BMP, bone morphogenetic protein; BSA, bovine serum albumin; FACS, fluorescence activated cell sorting; ES, embryonic stem; iPS, induced pluripotent stem; ICC, immunocytochemistry; NC, neural crest; NCBI, National Center of Biotechnology Information; LSB, LD-193189 + SB35142; PFA, paraformaldehyde; SFM, serum-free media; WT, wildtype.

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