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Derivation of sensory neurons and neural crest stem cells from human neural progenitor hNP1

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Abstract

Although sensory neurons constitute a critical component for the proper function of the nervous system, the in vitro differentiation of functional sensory neurons from human stem cells has not yet been reported. This study presents the differentiation of sensory neurons (SNs) from a human neural progenitor cell line, hNP1, and their functional maturation in a defined, in vitro culture system without murine cell feeder layers. The SNs were characterized by immunocytochemistry and their functional maturation was evaluated by electrophysiology. Neural crest (NC) precursors, as one of the cellular derivatives in the differentiation culture, were isolated, propagated, and tested for their ability to generate sensory neurons. The hSC-derived SNs, as well as the NC precursors provide valuable tools for developing in vitro functional systems that model sensory neuron-related neural circuits and for designing therapeutic models for related diseases.

Keywords

Sensory neuron; stem cell; cell differentiation; human; neural crest stem cell; neural progenitor; Schwann cell

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1. Introduction

Sensory neurons are responsible for conveying internal, external, and environmental stimuli to the central nervous system (CNS). They act as signal initiators in all reflex responses, and constitute an indispensable component for the correct function of the nervous system. Multiple insults can cause damage or disease in sensory neurons, such as traumatic injury [1], infection, toxin exposure, metabolic disease, immune system disorders, cancer and chemotherapy [2] and heredity [3, 4]. The subsequent cellular dysfunction caused by such insults is associated with many disorders ranging from abnormal sensation, numbness and pain to loss of coordination in voluntary movement [2–4]. The development of stem cells and their differentiated products are critical in biomaterials for use in regenerative medicine, in addition, understanding the environment needed to control these cells is a key area of research in biotechnology and bioengineering. An in vitro source of human sensory neurons would therefore generate invaluable material for fabricating functional human disease models for pathological studies and drug screening as well as providing a renewable cell source for applications in regenerative medicine.

Compared with primary human tissue, which is limited in quantity and strictly regulated, stem cells provide a potentially unlimited source for generating specialized neuron subtypes for disease modeling and cell therapy [5–8]. During embryonic development, the neural crest is an important population of stem cells that gives rise to diverse derivatives, including the PNS and the craniofacial skeleton [9]. It has been used as an excellent system for studying fundamental developmental processes, such as tissue induction [10]. Recently, with the progress of stem cell technology, the potential of utilizing neural crest as a source for regenerative medicine has also been explored [11–16]. Although progress in the specification of neuron subtypes within the CNS, i.e. spinal motoneurons (MNs) [5, 7] and midbrain dopamine neurons [6, 17, 18], has evolved rapidly, the study in the specification of peripheral neurons, especially sensory neurons, from stem cells is somewhat limited.

During development, the generation of sensory neurons from embryonic stem cells (ESCs) proceeds through a series of stages: 1) the induction of neural ectoderm, which distinguishes the developing nervous system from other systems, 2) the induction of neural crest cell fate which segregates the peripheral nervous system (PNS) from the CNS, and 3) the specification of sensory neurons which distinguishes them from other neural crest derivatives. To date, almost all the studies utilizing ESCs focused on the generation of neural crest stem cells (NCSCs) instead of sensory neurons [13, 19, 20].

Initial studies describing the differentiation of NCSCs or sensory neurons utilized a technique labeled “stromal cell-derived inducing activity” (SDIA), which relies on the co-culture of stem cells with a mouse stromal cell line [14, 15, 17]. However, this method raises the concern that contamination of the desired cell population with undefined components from a murine feeder layer may cause poor consistency and reproducibility. More recent studies have reported the development of a feeder-free protocol for the differentiation of NCSCs from pluripotent stem cells [20, 21]. However, only one study was able to differentiate peripheral sensory neurons and neural crest cells directly from non-ESCs; in this case, ESC-derived neurospheres were used as the cell source in conjunction with a murine stromal AP6 cell feeder layer [22].

In general, ESC culture is a lengthy process, requiring the handling of aggregated embryoid bodies and neurospheres, which to date has only produced small yields for the differentiated cellular phenotypes. In addition, neural crest cells in such cultures are typically found interspersed with neural rosettes and cell-sorting is required to obtain a highly enriched cell population [13, 21, 22]. These issues highlight the limitations of current approaches and,

consequently, the limitations with regards to the applications of stem cell derived sensory neurons in regenerative medicine, drug screening and tissue engineering. Therefore, by developing systems with defined components to allow stem cell differentiation in a controlled environment it makes these stem cells and their differentiated products more easily integratable with current biomaterials for in vivo and in vitro applications.

This study reports a simplified, feeder free, and efficient protocol for the generation of functional sensory neurons, as well as NCSCs, from a human neural progenitor cell line, hNP1. The SNs were characterized by immunocytochemistry and their functional maturation was evaluated by electrophysiology. Neural crest (NC) precursors, one of the cellular derivatives in the differentiation culture, were isolated, propagated, and evaluated for their ability to generate sensory neurons.

2. Materials and Methods

2.1. DETA Surface Modification

Previous studies have proven that DETA (a self-assembled monolayer (SAM) of N-1(3-(trimethoxysilyl) propyl) diethylenetriamine) supports neuronal growth as well as biological surfaces, if not better [23, 24], and it has been shown to support the growth of both embryonic and adult MNs [25, 26]. DETA has also been shown to be an analog to spermine, a growth factor known to promote cellular survival [27, 28]. The differentiation pattern of the human spinal stem cells on DETA coverslips was similar to that on PDL/fibronectin coated chamber slides regarding the numbers and morphology of MNs induced [5]. For DETA modification, glass coverslips (6661F52, 22×22 mm No. 1; Thomas Scientific, Swedesboro, NJ, USA) were cleaned using HCl/methanol (1:1) for at least 2 hours, rinsed with deionized water, soaked in concentrated H₂SO₄ for at least 2 hours and rinsed again with deionized water. Coverslips were boiled in nanopure water and then oven dried. The DETA (T2910KG; United Chemical Technologies Inc., Bristol, PA, USA) film was formed by the reaction of the cleaned surfaces with a 0.1% (v/v) mixture of the organosilane in freshly distilled toluene (T2904; Fisher, Suwanee, GA, USA). The DETA coated coverslips were heated to ~80°C, cooled to room temperature (RT), rinsed with toluene, reheated to approximately the same temperature, and then cured for at least 2 hours at 110°C. Surfaces were characterized by contact angle and X-ray photoelectron spectroscopy as described previously [23, 25, 26].

2.2 Culture of the Human Neural Progenitor hNP1

Human neural progenitor cells, STEMEZ™hNP1, were obtained from Neuromics (Edina, Minnesota). The cells were derived from the human embryonic stem cell line WA09 (H9), which have been differentiated past the point of requiring feeder cells or expensive feeder free systems, and could be cultured as adherent monolayers. The cells were expanded and maintained as described in the STEMEZ™hNP1 expansion kit. Briefly, the cells were proliferated on a Matrigel-coated surface in the proliferation medium (supplemented AB2™ basal medium from Neuromics containing 20 ng/ml bFGF (R&D system, cat. 234-FSE-025/CF)). The Matrigel-coated surface was prepared by incubating BD Matrigel™ (BD Biosciences, cat. 356234, 1:200 diluted in DMEM) with the cell culture surface for 1 hour at room temperature, rinsed briefly with DMEM and used immediately for cell plating. The cells were plated at ~75% confluence and harvested for passaging by manual dissociation when ~100% confluence was reached. At various passages, the cells were frozen in growth medium, plus 10% dimethyl sulfoxide (DMSO), at 1×10⁶ cells/ml using a programmable freezer. The frozen cells were then stored in liquid nitrogen.

2.3. Induction of Sensory Neurons from hNP1 cells

Neuromics product information specifies that their cells can be expanded through 10 passages before any genotypic monitoring is necessary (<http://www.neuromics.com/>). In the present study, passage 9 or 10 cells were used and the induction procedure consisted of three steps. For proliferation, 1×10^6 cells were seeded into a 35 mm cell culture dish, pre-coated with BD Matrigel, and maintained in the proliferation medium supplemented with bFGF (20 ng/ml); the medium was changed every other day. The cells were proliferated for 3 to 5 days until 100% confluence was reached. Next, they were manually dissociated, re-plated onto glass coverslips pre-coated with DETA, followed by Poly-ornathine/Laminin/Fibronectin [21], at a density of 400 cells/mm². The cells were expanded in proliferation medium for 2 to 3 days to enable ~90% confluence before induction. To initiate sensory neuron differentiation, the medium was replaced with KSR medium that contained 10 μ M SB43152 and 500 ng/ml Noggin. KSR medium was prepared by supplementing 800 ml Knockout DMEM (Invitrogen, Cat. 11330-032) with 150 ml KSR (knockout serum replacement, (Invitrogen, Cat. 10828-028), 10 ml L-Glutamine (Invitrogen, Cat. 21051-016), 10 ml Penicillin/ Streptomycin (100 $\times$, Invitrogen, Cat. 15070-063), 10 ml 10 mM MEM (100 $\times$, nonessential amino acids, Invitrogen, Cat. 11140-050) and 1 ml β -mercaptoethanol (1,000 $\times$, Invitrogen, Cat. 21985-023). To feed the cells during differentiation, the medium was replaced and gradually switched from KSR medium to N2B medium (NeuralStem Inc) according to the following schedule: day 2 (75% KSR, 25% N2B), day 4 (50% KSR, 50% N2B), day 6 (25% KSR, 75% N2B), days 8 & 10 (0% KSR, 100% N2B). However, the content of SB43152 and Noggin (10 μ M and 500 ng/ml, respectively) remained constant throughout the procedure. Starting with day 12, the cells were fed with a differentiation medium by changing 1/3 of the medium every 2 days. The differentiation medium consisted of N2B medium supplemented with BDNF (Cell Sci. cat. CRB600B, 10 ng/ml), L-ascorbic Acid (Sigma, cat. 396-HB, 200 μ M), GDNF (Cell Sci. Cat. CRG400B, 10 ng/ml), NGF (R&D systems, cat. 256-GF, 10 ng/ml), NT-3 (Cell Sci. cat. CRN500B, 10 ng/ml), cAMP (Sigma, Cat. A9501, 20 μ M), and Wnt-1 (Sigma, cat. SRP4754, 10 ng/ml). The cells were analyzed by immunocytochemistry and electrophysiology starting at day 14.

2.4. Isolation of Neural Crest Stem Cells (NCSCs)

Following the initiation of sensory neuron differentiation, cells were harvested on day 10 by manual dissociation, and re-plated onto a surface pre-coated with Poly-ornathine/Laminin/Fibronectin as above at a density of 200 cells/mm². The cells were maintained in N2B medium containing bFGF (20 ng/ml) and hEGF (10 ng/ml) and named as P1. The cells were fed every 2 days with the same medium until they reached confluence. They were then harvested by manual dissociation and passaged onto coverslips with an identical surface treatment in the same medium. At various passages, the cells were frozen in the growth medium plus 10% Dimethyl Sulfoxide (DMSO) at 1×10^6 cells/ml using a programmable freezer. The frozen cells were then stored in liquid nitrogen.

2.5. Differentiation of Sensory Neurons and Schwann cells from NCSCs

To initiate the differentiation of sensory neurons and Schwann cells from NCSCs, the cells were allowed to proliferate until confluence was almost achieved as described above. To induce differentiation to sensory neurons, the cells were fed with differentiation medium as in 2.3 by changing 1/3 of the medium every 2 days. To induce differentiation into Schwann cells, the cells were similarly fed, but with Schwann cell medium as described in [21] with slight modifications. Briefly, the medium consisted of N2 medium supplemented with CNTF (10 ng/ml), Neuregulin (20 ng/ml), bFGF (10 ng/ml) and cAMP (5 μ M). The cells were fixed for immunocytochemistry after 15 days of differentiation.

2.6. Immunocytochemistry and Microscopy

Cells were fixed in freshly prepared 4% paraformaldehyde for 15 min. Cells were washed twice in Phosphate Buffered Saline (PBS) (pH 7.2, w/o Mg^{2+} , Ca^{2+}) for 10 min each at room temperature, and permeabilized with 0.1% triton X-100/ PBS for 15 min. Non-specific binding sites were blocked with 5% Donkey serum plus 0.5% BSA in PBS (blocking buffer) for 45 min at room temperature. Cells were incubated with primary antibodies overnight at 4°C. After being washed with PBS 3×10 min, the cells were incubated with secondary antibodies for 2.5 hours at room temperature. The cells were then washed with PBS 3×10 min and mounted with Vectashield with 4'-6-Diamidino-2-Phenylindole (dapi) (Vector laboratories, Inc.). Primary antibodies used in this study were as follows: Rabbit-anti-Nestin (Chemicon, 1:200), Rabbit-anti- β III Tubulin (Sigma, 1:1000), Rabbit-anti-SOX1 (Millipore, 1:100), Goat-anti-Peripherin (Santa Cruz Biotech, 1:25), Mouse-anti-HNK1 (Sigma, 1:400), Rabbit-anti-Brn3a (millipore, 1:1000), Rabbit-anti-S100 (sigma, 1:400), Rabbit-anti-MASH1 (abcam, 1:500), Guinea pig-anti-Parvalbumin (millipore, 1:100), vGluT1 (Millipore, 1:400), Rat-anti-Substance P (Millipore, 1:50), Rabbit-anti-P75 (Millipore, 1:200), and Mouse-anti-GFAP (Chemicon, 1:150). Secondary antibodies were as follows: Donkey-anti-Goat-594, Donkey-anti-Goat-488, Donkey-anti-Mouse-488, Donkey-anti-Mouse-594, Donkey-anti-Rabbit-594, Donkey-anti-Rabbit-488, and Donkey-anti-Guinea Pig-488. All secondary antibodies were from Invitrogen and used at 1:250 dilution. All antibodies were diluted in Blocking Buffer.

2.7. Electrophysiological Recording

For electrophysiological recordings, the hNP1-derived sensory neurons were plated and differentiated on glass coverslips coated with DETA. The cells were characterized in cultures differentiated for 2 to 4 weeks using whole-cell patch-clamp recording techniques [23, 29]. The recordings were performed in a recording chamber located on the stage of a Zeiss Axioscope 2FS Plus upright microscope [30]. Sensory neurons were visually identified under an infrared DIC-video microscope. The large round bipolar or pseudo-unipolar cells (15–25 μ m diameter) with bright illuminance in the culture were tentatively identified as sensory neurons. Patch pipettes with a resistance of 5–10 M Ω were made from borosilicate glass (BF 150-86-10; Sutter, Novato, CA) with a Sutter P97 pipette puller (Sutter Instrument Company). Current-clamp and voltage-clamp recordings were performed utilizing a Multiclamp 700A amplifier (Axon, Union City, CA). The pipette (intracellular) solution contained (in mM) K-gluconate 140, $MgCl_2$ 2, Na_2ATP 2, Phosphocreatine 5, Phosphocreatine kinase 2.4 mg, Hepes 10; pH 7.2.

After the formation of a G Ω seal and membrane puncture, the cell capacitance was compensated. The series resistance was typically < 23 M Ω , and it was compensated > 60% using the amplifier circuitry. Signals were filtered at 3 kHz and sampled at 20 kHz using a Digidata 1322A interface (Axon instrument). Data recording and analysis were performed with pClamp8 software (Axon instrument). Membrane potentials were corrected by the subtraction of a 15 mV tip potential, which was calculated using Axon's pClamp8 program. Membrane resistance and capacitance were calculated using 50 ms voltage steps from –85 to –95 mV without any whole-cell or series resistance compensation. The resting membrane potential and depolarization-evoked action potentials (APs) were recorded in current-clamp mode. Depolarization-evoked inward and outward currents were examined in voltage-clamp mode.

2.8. Quantification

The gradual clustering of the sensory neurons in culture presented a challenge for accurately quantifying the differentiated cells. Cell clusters started to appear from about day 10 after differentiation and become more apparent as the culture matured. Generally cells in clusters

cannot be quantified unless they are dissociated, however the dissociation process causes cell death and then inaccurate cell counts, especially for neurons. Moreover, this dissociation-caused cell death increases as the cells mature and clusters become more solid. On the other hand, the expression of most immunological markers is usually not evident until the culture reaches a more advanced differentiation level. As a compromise, quantification of the differentiated cultures were conducted between day 17 to day 38, in which the cells in clusters accounted for less than half of the total number of cells and the marker for immunostaining were clearly identifiable. Specifically, quantification of the differentiated cultures utilized a minimum of 6 pictures taken from randomly chosen, un-clustered areas on each coverslip. The ratio of marker-positive to DAPI-positive cells was quantified from a minimum of three coverslips in each group. In all cases, the presented data were expressed as an average $\pm$ the standard deviation.

3. Results

3.1. Characterization of hNP1

The hNP1 cells were derived from the human embryonic stem cell line WA09 and were cultured as adherent monolayers. Before differentiation induction, the undifferentiated hNP1 cells were examined with markers for neural progenitors, neural crest and sensory neurons to establish a baseline composition. They were plated on DETA-coated glass coverslips at 400 cells/mm² in growth medium, and fixed for immunostaining 2 days later. As indicated in Figure 1, all the cells were positive for the neural progenitor markers Nestin and SOX1 (Figure 1A). Conversely, they were negative for both the neural crest cell marker HNK1 (Figure 1B) and the general neuronal marker β III Tubulin (Figure 1C), as well as the sensory neuron marker Peripherin (Figure 1D). The results from the immunostaining confirmed that this cell line provided a pure population of neural progenitors.

3.2. Induction of sensory neurons from hNP1

The hNP1 cell line was induced to generate sensory neurons by treating the cells with trophic factors and/ or chemicals that had previously been shown to be important for the generation of sensory neurons; Noggin, SB431542 and Wnt1 [10, 20, 21, 31]. From day 8 after differentiation, most of the cells had gradually adopted a neural morphology with smaller somas and long processes (Figure 2B). From day 12, the medium was switched to a hSN differentiation medium (Table 1) and as differentiation continued, the cells with neuronal morphology became more prominent. These neurons tended to form clusters with their axons aligning uniformly and were located between the clustered cell bodies (Figure 2C). This feature has also been observed in cell cultures derived from rat embryonic DRGs in which sensory neurons formed clusters with their axons developing “highway” bundles traveling between individual clusters (Figure 2D) [32].

To confirm the induction from progenitor cells to sensory neurons, the cultures were fixed and immunostained with a marker for sensory neurons (Brn3a) [33], one for peripheral neurons (Peripherin) and with a general neuronal marker (β III Tubulin). The immunocytochemical analysis indicated that there were a significant number of sensory neurons induced in the cultures (Figure 3A, B). Closer observation of the stained cells also revealed sensory neurons at different developmental stages: bipolar neurons which were in an early stage of differentiation, cells with two axons moving closer together, indicating an intermediate stage, and pseudo-unipolar neurons, indicating a more mature stage (Figure 3C). DETA pre-coating on glass coverslips was necessary for differentiation as clean glass alone, after similar Poly-ornathine/Laminin/Fibronectin coating, did not allow for differentiation (data not shown). An alternative to the DETA-coated glass coverslip is the surface of polystyrene petri-dishes, which produces comparable sensory neuron

differentiation after Poly-ornathine/Laminin/Fibronectin coating, however this result would not have the ability to be extended as a general technique.

3. 3. Analysis of the cellular derivatives from the differentiation culture

The cellular compositions derived from this differentiated population were analyzed by immunostaining the cultures with additional cellular markers. First, the generation of Schwann cells was investigated, since these cells are also derived from neural crest stem cells and are closely related to sensory neurons in physical distribution and function. Immunocytochemical analysis utilizing the Schwann cell marker S100 demonstrated the presence of abundant Schwann cells in the differentiated culture. These Schwann cells were found mingled with dispersed sensory neurons, and as cultures matured, also in the neuronal clusters (Figure 4A) and the axonal bundles (Figure 4B). No obvious proliferation was observed for these Schwann cells in the differentiated culture. Quantification of the immunostaining indicated that neurons (positive for β III Tubulin and Peripherin) and Schwann cells (positive for S100) accounted for 51.3 $\pm$ 3.7 % and 48.0 $\pm$ 2.5 %, respectively, of the cells in culture following this differentiation protocol.

Since Peripherin-positive neurons can be sensory or autonomic neurons, it was important to determine how many autonomic neurons were in the derived neuronal population. The cultures were immunostained for MASH1, a transcription factor specific for autonomic neurons [34], and co-immunostained for Peripherin. Surprisingly, none of the Peripherin-positive cells were MASH1-positive. However, all the examined Peripherin-positive cells were Brn3a-positive (Figure 3B). Both results suggest that all of the Peripherin-positive neurons were sensory neurons (Figure 5A). To further determine the subtype of the sensory neurons, the differentiated cultures were immunostained for Parvalbumin, a marker for large type I proprioceptive sensory neurons [29, 35, 36] (Figure 5A). The result indicated that most of the neurons were positive for Parvalbumin, and therefore belong in the type I category (Figure 5B).

Glutamate is used as the neurotransmitter in the afferents of all DRG neurons, including both glutamatergic and peptidergic neurons [37]. Glutamate transporters (vGluT) are proteins located on synaptic vesicles that are responsible for packaging the neurotransmitter glutamate into vesicles. vGluT1 is mostly present in medium to large-sized, CGRP (calcitonin gene related peptide)-negative DRG neurons, which are type I sensory neurons [38]. The cultures were analyzed by immunocytochemistry for the expression of vGluT1. The vGluT1 positive result (Figure 5C) also indicated that they were primarily type I sensory neurons.

To determine if any nociceptive sensory neurons were generated, the differentiated cultures were immunostained for Substance P, the peptidergic neurotransmitter for nociceptive sensory neurons. In general, very few Substance P-positive fibers or neurons were found in the differentiated cultures (<1%) (Figure 5D), which was consistent with the above findings that the differentiated neurons were mostly proprioceptive sensory neurons.

3.4. Electrophysiological analysis of the differentiated sensory neurons

The functional maturation of the differentiated sensory neurons was determined by electrophysiological analysis (Figure 6). The voltage clamp analysis revealed that voltage-dependent currents could be invoked (Figure 6A), and the delay and dynamics of the inward and outward currents were consistent with sodium and delayed rectifier potassium currents. The maximum inward and outward currents reached values of -1150 pA and 728 pA, respectively. Prolonged stepped, current clamp recordings ($0-100$ pA $\times$ 1 second) indicated that 23 of the 24 recorded cells were capable of firing APs. Of the firing cells, 19 fired

single APs and 4 displayed repetitive firing behavior; the maximum firing frequency among these cells was 9 Hz (Figure 6B). Single APs elicited by a brief saturated depolarization current had an amplitude of 95.4 ± 22.4 mV (Figure 6C).

Previously, the only study that demonstrated electrophysiological recordings from human stem cell-derived sensory neurons reported results without any quantification utilizing only 6 neurons during a two month culture [39]. Due to the unavailability of primary human sensory neurons and the sparse literature for the electrophysiological characterization for sensory neurons derived from human stem cells, it was difficult to compare these electrophysiological parameters to other studies on human-derived sensory neurons. However, the reliable elicitation of APs and the presence of repetitive APs strongly suggested that these sensory neurons were functional. The positive staining for vGluT1 suggested that the sensory neurons synthesized the correct neurotransmitter, another indicator of functional maturation.

3.5. Derivation of neural crest stem cells from hNP1 stem cells

To determine whether NCSCs were generated during the sensory neuron induction protocol, the differentiated cells (day 10) were re-plated and subjected to proliferation by including bFGF and EGF in the N2B medium [21]. Treatment with bFGF was found to enhance the proliferation of NCSCs [40], and the combination of bFGF with EGF promoted the neuronal fate induction from NCSCs [41]. After 2 passages, almost all of the cells in the culture presented a stem cell morphology and were active in proliferation. Immunostaining with NCSC markers indicated that most were positive for HNK1 (90%) and P75 (99%) (Figure 7A).

To test whether these NCSCs retained the potential to generate sensory neurons, an aliquot of passage 4 cells were induced with sensory neuron differentiation medium and Schwann cell differentiation medium, respectively. After approximately 2 weeks, the cells under each differentiation condition demonstrated a different morphology, and both were distinguishable from undifferentiated NCSCs (Figure 7B). Immunocytochemical analysis of the NCSCs subjected to the sensory neuron differentiation protocol utilizing the peripheral neuronal marker Peripherin, demonstrated a significant number of sensory neurons (Figure 7C). Similarly, immunostaining for the Schwann cell markers S100/ GFAP on cells cultured in the Schwann cell differentiation medium revealed a large number of Schwann cells (Figure 7D). Therefore, these NCSCs preserved the potential for generating sensory neurons and Schwann cells, which are the essential components of dorsal root ganglion structures.

4. Discussion

This study reports on the ability to differentiate functional sensory neurons, and to isolate NCSCs, from a human neural progenitor cell line, hNP1, which was originally derived from the embryonic stem cell line WA09 (H9). The identity of the sensory neurons was confirmed by immunocytochemistry and their functional maturation was analyzed utilizing electrophysiology. The isolated NCSCs preserved the ability to generate sensory neurons and Schwann cells as indicated by immunocytochemical staining for relevant markers. The generation of functional human sensory neurons as well as NCSCs will provide valuable tools for developing in vitro functional systems that model sensory neuron-related neural circuits and for designing therapeutics for these diseases.

Compared to previous studies, which primarily utilized ESCs, this work provides a simplified and more efficient method for the generation of sensory neurons, Schwann cells and NCSCs. Their differentiation was accomplished in an in vitro, adherent culture system in which neither multicellular aggregates nor stromal cells were required. The culture and

differentiation procedures are simpler, and the undefined contaminating element, provided by feeder cells, was also eliminated. The induction of sensory neurons was also faster, probably because the source was a neural progenitor instead of ESCs. This protocol directed ~50% of the cells in culture to a sensory neuron fate and the other 50% to a Schwann cell fate. Compared with previous studies, which produced <1% and ~10% sensory neurons when utilizing hESCs [15] or neurospheres [22], respectively, this procedure is much more efficient. The NCSCs derived from this protocol demonstrated ~90% purity without any subsequent sorting procedures. This outcome is also very high compared with previous reports, which all relied on cell sorting for enrichment [19, 42]. Thus, this study describes a major advancement for the generation of sensory neurons and NC cells from undifferentiated cell sources.

Several crucial signaling pathways were revealed in the developmental stages of sensory neuron generation during this study. The combination of the TGF β super-family and Wnt signaling pathways are of particular significance as several members in the TGF β super-family have been identified as important factors for sensory neuron differentiation. Those that are involved in the induction of neural crest and sensory neurons include: BMP, TGF β , Activin and Nodal. The BMP antagonist, Noggin, binds to BMP molecules and prevents their attachment to the corresponding receptors. Noggin is a critical neural-inducing factor in *Xenopus* [43] and Noggin-mediated BMP signaling is required for the growth and patterning of the neural tube in vertebrates [44]. Treatment of recombinant Noggin has been used in the protocol of neural induction from hESCs [45]. BMP2 and 4 promote autonomic neurogenesis, while the TGF β factor family promotes the development of smooth muscle-like cells [46]. Noggin blocks the effects of BMPs, which may explain why sensory neurons, but not autonomic neurons, constituted the major population of the generated peripheral neurons. The drug SB431542 can inhibit the Lefty/ Activin/TGF β pathways by blocking the phosphorylation of their receptors. This drug was also shown to enhance neural induction in an embryoid body-based hES-cell neural induction protocol [47]. Dual inhibition of SMAD signaling with Noggin and SB431542 could effectively induce neural differentiation [48] and neural crest generation [21] from human ES and iPS cells. In this study, the application of SB431542 may contribute to the inhibition of smooth muscle cell induction by blocking TGF β pathways since very few SMA4+ cells were observed (data not shown). In addition to the TGF β super-family factors, Wnt signaling has been found to promote sensory neurogenesis in a β -catenin-dependent manner [9, 10, 31]. Activation of the canonical Wnt signaling and concurrent suppression of the Activin A/Nodal/BMP pathway with Noggin and SB431542 can generate a highly enriched neural crest stem cell pool from human pluripotent stem cells [20]. Consistent with the literature [9, 10, 21, 31, 48], the combined application of Wnt1, Noggin and SB431542 in this study presented an optimized condition for the generation of neural crest cells as indicated by the almost pure population of NC cells obtained. This regime also favors the induction of sensory neurons vs. autonomic neurons, since no autonomic neurons were identified in the culture.

Two major populations of terminally differentiated cells were derived from this protocol: sensory neurons and Schwann cells. Since neither is protected by the Blood Brain Barrier, they are more vulnerable than CNS cells. Sensory neurons are the major players for sensory input to the CNS. Damage to the sensory neurons can cause a range of symptoms from numbness and pain to a lack of coordination. Schwann cells are the myelinating cells of the PNS, and one of the major cell groups that provide trophic support to peripheral neurons as well. Schwann cell damage or disease [49], such as Charcot-Marie-Tooth disease, can cause symptoms such as sensory changes, paralysis and changes to involuntary functions, depending on which neurons they are associated with. Stem cell-derived sensory neurons and Schwann cells provide valuable tools for developing in vitro cellular models for the study of these diseases. Especially, by combining with state of the art bio-engineering

technologies such as Bio-MEMs (MicroElectroMechanical systems) devices, these cells can be utilized in the development of platforms for high content drug screening. They can also be utilized for regenerative medicine by either direct grafting or after combining with tissue engineering constructs or scaffolds. Moreover, the growth factor combinations and differentiation regime presented in this study would provide important guidelines for utilizing stem cells as the source to generate functional sensory neurons for both in vitro and in vivo applications in general.

5. Conclusion

This study reports a simplified and efficient avenue for the generation of sensory neurons and neural crest cells by utilizing human neural progenitor cells as the initial source, a feeder-free defined system, and a combination of factors that facilitate neural crest and sensory neuron differentiation. The derived functional sensory neurons will also provide valuable tools for the modeling and treatment of related diseases.

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Figure 1.

Immunocytochemical characterization of the human neural progenitor cell line hNP1. A) hNP1 cells were immunostained with the neural progenitor markers SOX1 and Nestin. All cells were positive for both markers. B,C,D) hNP1 cells were immunostained with the neural crest cell marker HNK1 (B), neuronal marker β III Tubulin (C) and peripheral neuronal marker Peripherin (D). A small number of cells were positive for HNK1, and all of the cells were negative for β III Tubulin and Peripherin.

Figure 2.

Phase contrast images of the cultures before and after the sensory neuron induction. A) hNP1 culture before sensory induction. B) hNP1 culture 10 days after sensory induction. C) hNP1 culture 30 days after sensory induction. Neuronal clusters and axonal bundles, which resemble rat DRG cell cultures, were typically observed. D) For comparison, an image of a rat embryonic DRG cell culture at 7 DIV is provided.

Figure 3.

Abundant sensory neurons were induced after differentiation. A,B) Day 14 differentiated cultures were co-immunostained with sensory neuronal marker Brn3a and neuronal marker β III Tubulin (A), and Brn3a and peripheral neuronal marker Peripherin (B). C) Sensory neurons at different developmental stages were demonstrated with immunostaining for Peripherin in a day 14 culture. (i): bipolar. (ii) mid-stage. (iii) pseudo-unipolar.

Figure 4. Generation of Schwann cells from the differentiated culture. Immunostaining of a day 38 culture with the Schwann cell marker S100 demonstrating a significant number of Schwann cells in the culture. Schwann cells were located either within the neuronal clusters (A) or along the axonal bundles (B). The neuronal clusters and axonal bundles were marked by Peripherin immunostaining.

Figure 5.

The identities of the derived peripheral neurons were analyzed with immunocytochemistry.

A) No cell was positive for MASH1, the autonomic neuronal marker, in a 38 DIV culture.

B) Most of the peripheral neurons were positive for Parvalbumin, a marker for type I

sensory neurons, in a 50 DIV culture. C) vGluT1 signal was observed abundantly in derived

neurons in a 25 DIV culture. D) Very few Substance P signals were observed in a 78 DIV culture.

Figure 6. Electrophysiological recording from differentiated sensory neurons. Representative voltage clamp (A), current clamp (B) and action potential (C) traces recorded from sensory neurons in the differentiated culture. An image of the recorded cell is also included (D).

Figure 7.

Analysis of neural crest (NC) cells isolated from this culture. A) Immunostaining of NC cells (passage 4) with NC markers HNK1 and P75 demonstrated an enriched NC population. B) Phase contrast images of NC cells before induction (a), after sensory neuronal induction (b) and after Schwann cell induction (c). C) Peripheral neurons derived from NC cells (P4) after 15 days of sensory neuron induction were demonstrated using immunostaining for Peripherin. D) Schwann cells derived from NC cells (P4) after 15 days. Schwann cell induction was demonstrated using immunostaining for S100 and GFAP.

Table 1

Composition of the Enriched Co-culture Medium.

Component	Full Name	Concentration	Company	Catalog No.
<i>Neural Crest Stem Cell Differentiation Media</i>				
Noggin		500 ng/ml	R&D	6057-NG/CF
SB431542		10 μ M	Tocris Bioscience	1614
Wnt1		10 ng/ml	Sigma	SRP4754
<i>Neural Crest Stem Cell Growth Media</i>				
bFGF	Basic Fibroblast Growth Factor	50 ng/ml	R&D	234-FSE/CF
hEGF	Human Epidermal Growth Factor	10 ng/ml	Invitrogen	PHG0314
<i>hSN Differentiation Media</i>				
N2B		NeuralStem Inc		
BDNF	Brain-derived Neurotrophic Factor	10 ng/ml	Cell Sciences	CRB600B
NT-3	Neurotrophin-3	10 ng/ml	Cell Sciences	CRN500B
GDNF	Glial cell-derived Neurotrophic Factor	10 ng/ml	Cell Sciences	CRG400B
NGF	Nerve Growth Factor	10 ng/ml	R&D	256-GF
cAMP	Adenosine 3',5'-cyclic Monophosphate	0.5 μ M	Sigma	A-9501
Ascorbic Acid		200 μ M	Sigma	396-HB