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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.

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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

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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 cells 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





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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 spermamine, 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 h, rinsed with deionized water. soaked in concentrated H₂SO₄ for at least 2 h 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, Suwanne, 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 h 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 can be cultured as adherent monolayers. The cells were expanded and maintained as described in the STEMEZ^{IM} hNP1 expansion kit. Briefly, the cells were proliferated on a Matrigel-coated surface in the proliferation medium (supplemented AB2^{IM} 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^{IM} (BD Biosciences, cat. 356234, 1:200 diluted in DMEM) with the cell culture surface for 1 h 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 3-5 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 as in Refs. [21], at a density of 400 cells/mm². The cells were expanded in proliferation medium for 2-3 days to enable ~90% confluence before induction. To initiate sensory neuron differentiation, the medium was replaced with KSR medium that contained 10 µM 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 (100X, Invitrogen, Cat. 15070-063), 10 ml 10 mM MEM (100X, nonessential amino acids, Invitrogen, Cat. 11140-050) and 1 ml β-mercaptoethanol (1000X, 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 µM 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 µM), 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 µm), 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 Polyornathine/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 × 10⁶ 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 Ref. [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 (5μ 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 Mg²⁺, Ca²⁺) 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

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