



Original article

Neural crest stem cells can be induced *in vitro* from human-induced pluripotent stem cells using a novel protocol free of feeder cells

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Abstract

Objective: Our knowledge of human neural crest stem cells (NCSCs) is expanding, owing to recent advances in technologies utilizing human-induced pluripotent stem cells (hiPSCs) that generate NCSCs. However, the clinical application of these technologies requires the reduction of xeno-materials. To overcome this significant impediment, this study aimed to devise a novel method to induce NCSCs from hiPSCs without using a feeder cell layer.

Materials and Methods: hiPSCs were cultured in feeder-free maintenance media containing the Rho-associated coiled-coil forming kinase inhibitor Y-27632. When the cells reached 50–70% confluence, differentiation was initiated by replacing the medium with knockout serum replacement (KSR) medium containing Noggin and SB431542. The KSR medium was then gradually replaced with increasing concentrations of Neurobasal medium from day 5 to 11.

Results: Immunocytochemistry and flow cytometry were performed 12 days after induction of differentiation and revealed that the cells generated from hiPSCs expressed the NCSC markers p75 and HNK-1, but not the hiPSC marker SOX2.

Conclusion: These findings demonstrate that hiPSCs were induced to differentiate into NCSCs in the absence of feeder cells.

Key words: induced pluripotent stem cell, neural crest stem cell, feeder-free, p75, HNK-1

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Introduction

Neural crest stem cells (NCSCs) represent a transient, multipotent, and migratory population of cells unique to vertebrates that emerge at the interface of the neural and non-neural ectoderm layers and migrate extensively to form various neural crest derivatives such as peripheral neurons, glia, melanocytes, endocrine cells, craniofacial tissues, bone, smooth muscle, and enteric neurons¹. Because NCSCs play major roles in development and are associated with

various human diseases, they are highly relevant for clinical applications, such as in regenerative medicine².

Human NCSCs are difficult to obtain because they are mainly harvested from fetal cells, which accounts for a few relevant published studies. Therefore, our knowledge of the role of NCSCs in development comes from studies on organisms such as chickens and zebrafish³.

Lee *et al.* developed protocols designed for differentiating human-induced pluripotent stem cells (hiPSCs) into NCSCs, which employ pharmacological inhibition of the bone morphogenetic protein (BMP) and transforming growth factor- β (TGF- β) signaling pathways. The availability of these protocols has led to numerous studies in the past decade, dedicated to establishing robust and efficient methods to induce the differentiation of hiPSCs into NCSCs. However, most methods are cumbersome because they require numerous compounds and procedures and, more importantly, rely on nonhuman materials such as stromal feeder cells and murine embryonic fibroblast-conditioned medium (MEF-CM)^{1, 4–7}. Feeder- and xeno-free methods are required for the effective clinical application of NCSCs.

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To solve these problems, we devised a novel method for generating NCSCs from hiPSCs. In this study, we evaluated the efficacy of this feeder-free method in establishing a novel and useful protocol.

Materials and Methods

Cell culture

hiPSCs were cultured in feeder-free maintenance medium mTeSRTM1 (STEMCELL Technologies, Vancouver, Canada) supplemented with 10 μ M of the Rho-associated kinase inhibitor Y-27632 (Cayman Chemical Company, Ann Arbor, MI, USA) on dishes coated with Matrigel (BD Biosciences, San Jose, CA, USA)^{8,9}.

Generation of NCSC from hiPSCs

The hiPSCs were plated on a Matrigel-coated dish (10,000–25,000 cells/cm²) in mTeSR1 in the presence of Y-27632. When the cells reached 50–70% confluence, they were treated with different ratios of KSR:Neurobasal medium to induce differentiation. The KSR medium contained knockout DMEM, 15% KSR, 1% L-glutamine, 1% MEM-nonessential amino acids (each from Life Technologies, Carlsbad, CA, USA), and 55 μ M β -mercaptoethanol (Wako, Osaka, Japan). The Neurobasal media were made of NeurobasalTM supplemented with 2% B27 supplement, 1% N2 supplement, and 1% L-glutamine (each from Life Technologies, Carlsbad, CA, USA). Cells were incubated daily with fresh KSR:Neurobasal medium supplemented with 10 μ M SB431542 (Tocris, Bristol, UK) and 500 ng/mL Noggin (R&D systems, Minneapolis, MN, USA). SB431542 is a drug candidate that inhibits activin receptor-like kinase receptors, ALK5, ALK4, and ALK7 and inhibits TGF- β . Noggin is a protein that inhibits BMP signaling. The changes made to the ratio of KSR:Neurobasal medium were as follows: days 0–3 (100:0), day 5 (75:25), day 7 (50:50), day 9 (25:75), and day 11 (0:100)^{4, 10, 11} (Figure 1).

Immunocytochemistry

The hiPSCs were seeded onto plastic chamber slides coated with Matrigel, cultured, and differentiated into NCSCs as described above. The hiPSCs or differentiated cells were fixed with 4% paraformaldehyde in a phosphate buffer (0.1 M, pH 7.4) for 15 min and rinsed with phosphate buffered saline (PBS) (Wako, Osaka, Japan). The cells were permeabilized by incubation for 10 min at room temperature in a blocking solution containing 5% BSA and PBS with 0.3% Triton X-100. Next, the cells were treated with a blocking solution for 30 min and incubated overnight at 4 °C with the following mouse antibodies (diluted with blocking solution): SOX2 (R&D Systems, Minneapolis, MN, USA), p75 (Advanced Targeting Systems, San Diego, CA, USA), and HNK-1 (BD Biosciences, San Jose, CA, USA). After incu-

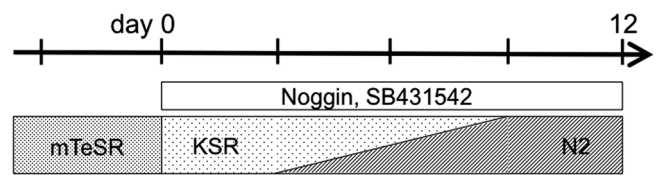


Figure 1 Schematic of the NCSC induction methods. hiPSCs were maintained in mTeSR. Cell differentiation was initiated by replacing the medium with KSR medium containing Noggin and SB431542. KSR medium was then exchanged for Neurobasal medium, which was added in gradually increasing concentrations from day 3 to 12.

bation with the primary antibody, the cells were incubated with Alexa Fluor 488-conjugated goat anti-mouseantimouse immunoglobulin G (Abcam, Cambridge, UK). After each step, the cells were rinsed three times in PBS and observed under a fluorescence microscope (Olympus, Tokyo, Japan). DAPI (Thermo Fisher Scientific, Waltham, MA, USA) was used to counterstain the nuclei.

Flow cytometry

Cells were harvested using Accumax (Innovative Cell Technologies, San Diego, CA, USA) for 5 min at 37 °C until they detached from the dishes. Cells were pelleted by centrifugation at 160 \times g for 5 min, resuspended in PBS, washed with a chilled FACS buffer containing 0.5% BSA and 2-mM EDTA in Dulbecco's PBS (D-PBS), and kept on ice until further use. Cells were resuspended in an appropriate cell density in FACS buffer containing an antibody against p75 or HNK-1 (BD Biosciences, San Jose, CA, USA). Cells were filtered through a 40- μ m filter, transferred to a FACS tube, and immediately analyzed using a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

Results

Immunocytochemistry

Immunocytochemistry was performed to evaluate the differentiation of hiPSCs into NCSCs. We used SOX2 as a marker for hiPSCs, and p75 and HNK-1 were used as markers for NCSCs. SOX2, but not p75 or HNK-1, was expressed by hiPSCs before they were induced to differentiate. In contrast, after differentiation, the cells expressed p75 and HNK-1, but not SOX2 (Figure 2).

Flow cytometry

Flow cytometry revealed that 63.4% \pm 7.4% of the cells expressed p75 and HNK-1 after they were induced to differentiate. In contrast, these markers were undetectable in hiPSCs (Figure 3).

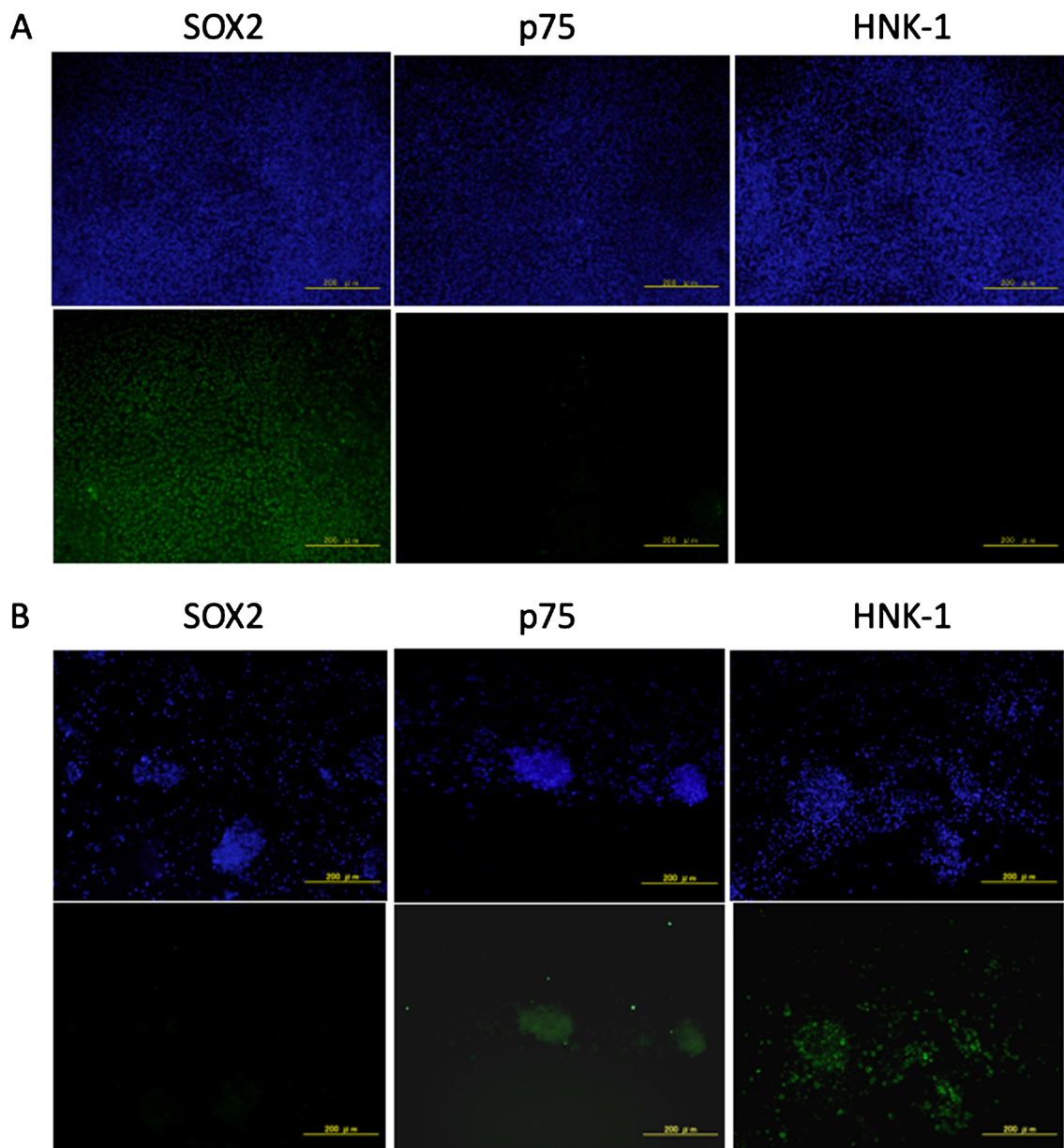


Figure 2 Immunocytochemical analysis of cells on day 12.

The upper images show DAPI-stained cells. Scale bar=200 μm. A) hiPSCs expressed SOX2 but not p75 and HNK-1.

B) After induction of differentiation, the cells expressed p75 and HNK-1 but not SOX2.

Discussion

In this study, we established a protocol for generating NCSCs from hiPSCs that did not require feeder cells or xeno-materials. The hiPSCs that were induced to differentiate expressed the NCSC markers p75 and HNK-1, in contrast with untreated hiPSCs that expressed SOX2, which was assessed by immunocytochemistry and flow cytometry.

NCSCs represent embryonic migratory cells with the potential to differentiate into various cell types that populate the peripheral nervous system, craniofacial cartilage,

bone, and teeth. Furthermore, they differentiate into endocrine cells of the adrenal and thyroid glands, and melanocytes of the skin. There are only a few published studies of human NCSCs because of the difficulty in obtaining 3–5-week-old human embryos and the transient nature of this stem cell population³. However, recent developments in improving technologies for using hiPSCs have expanded research efforts focused on human NCSCs, NC-derived tissues, or both. The most important advantages of these advanced technologies include the high numbers (e.g., $>10^6$) of NCSCs that can be generated from hiPSCs, and NCSCs or

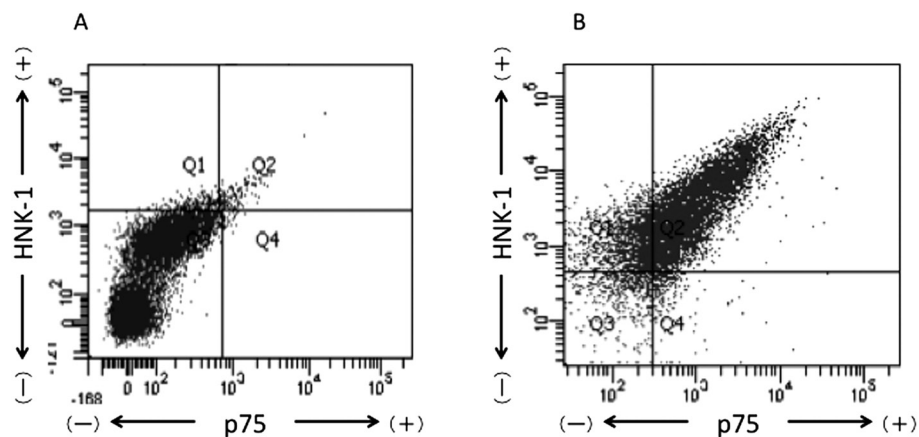


Figure 3 Flow cytometric analysis of cells on day 12. A) hiPSCs were not detected using antibodies against p75 or HNK-1. B) After hiPSC cultures were induced to differentiate, 63% cells expressed p75 and HNK-1.

NC-derived tissues that can be isolated from patients with certain diseases¹⁾.

The regulation of specific developmental pathways, such as the BMP/activin and Wnt signaling axes, is required for proper development of NCs during embryogenesis¹²⁾. Efficient methods for generating NCSCs from hiPSCs employ specific inhibitors such as Noggin and SB 431542 that inhibit the BMP and activin A/nodal signaling pathways, respectively^{4, 10)}. Furthermore, NCSCs are generated by supplementing cultures of hiPSCs with an activator of Wnt signaling and an inhibitor of activin/nodal/TGF- β signaling, although modulation of BMP signaling is not required²⁾. However, these methods require xeno-materials such as feeder cells and sera, which are problematic for research aimed at clinical applications such as regenerative medicine.

For clinical purposes, the cell induction protocol should not include the xeno-materials mentioned above and should use a chemically defined medium. Recently, Fukuta *et al.* reported a modification of the method reported by Menendez¹³⁾ that employs a feeder- and xeno-free protocol using an activator of Wnt signaling and inhibitor of activin/nodal/TGF- β signaling without BMP inhibitors. In contrast, in this study, we developed a feeder-free protocol using a BMP inhibitor based on the report by Lee *et al.* We used a feeder-free medium instead of MEF-CM, which was used in Lee's protocol⁴⁾. Although MEF-CM does not contain feeder cells, it contains fetal bovine serum and requires feeder cells for its production, indicating that it is not technically feeder- or xeno-free.

Another advantage of this protocol is its high efficiency and use of a concise methodology. For example, 63% of hiPSCs were induced to differentiate into NCSCs, which is consistent with previous studies using xeno-materials. Previous protocols induced 30–80% of hiPSCs to differentiate into NCSCs^{1, 3, 11, 13)}. Moreover, although certain protocols employ more than 15 compounds in the medium^{2, 13)}, our method

requires only eight compounds and fewer xeno-materials. Furthermore, this simple protocol achieved efficient generation of NCSCs within 12 days, which is shorter than that required using the protocol published by Menendez *et al.*¹³⁾.

This study has several limitations. First, the ability of the induced NCSCs to differentiate was not evaluated. Other studies have reported that all cells induced from hiPSCs that express both p75 and HNK-1 express NCSC markers such as SOX10 and ERBB3¹⁾. Therefore, it is likely that the induced NCSCs produced here that expressed p75 and HNK-1 were authentic. Second, this protocol is not completely xeno-free because mTeSR and KSR medium contains some xeno-materials. However, in using this method, we were able to reduce the xeno-materials. Third, we evaluated only one hiPSC cell line, despite the availability of several other cell lines with varying phenotypes¹⁴⁾. However, all media and compounds employed in this study, including mTeSRTM1 and KSR, are widely used to maintain hiPSCs and embryonic stem cell lines, suggesting that this method is generally applicable, although further studies are required to confirm this assumption.

Conclusion

We developed a feeder-free protocol to induce hiPSCs to differentiate into NCSCs. Therefore, this novel protocol shows promise as a resource for generating NCSCs and NC-derived tissues that are suitable for preclinical research.

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