Chapter 7

Isolation and Culture of Neural Crest Stem Cells from Human Hair Follicles

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Abstract

The hair follicle undergoes lifelong cycling and growth. Previous studies have been focused on epithelial stem cells in the hair follicles. Neural crest stem cells (NCSCs) are pluripotent cells that can persist in adult tissues. We have previously demonstrated that human NCSCs can be isolated from hair follicles. Here, we present a protocol to isolate NCSCs from human hair follicles based on their specific surface-marker expression of CD271/HNK1 or CD271/CD49D (alpha4 integrin). NCSCs can be expanded in the culture as neural spheres or attached cells.

Key words Protocol, Neural crest stem cells, Hair follicle, Bulge

1 Introduction

Hair follicles undergo lifelong growth. The hair cycle is a well-controlled process involving stem cell proliferation and quiescence. The bulge area, where arrector pili muscles insert into hair follicles, is a well-characterized niche for adult stem cells [1]. This segment of the outer-root sheath contains a number of different types of stem cells, including epithelial stem cells [2], melanocyte stem cells [3], and neural crest stem cells [4–7]. Hair follicles represent an accessible and rich source of different types of human stem cells.

Nestin-expressing pluripotent stem cells were discovered in the hair follicle by Li et al. [8]. We and others have isolated these neural crest stem cells (NCSCs) from human fetal and adult hair follicles [4, 5]. These human stem cells are label-retaining and are capable of self-renewal through asymmetric cell division in vitro. They express immature neural crest cell markers but not differentiation markers. Our expression-profiling study showed that they share a similar gene expression pattern with murine skin immature neural crest cells. They exhibit clonal multipotency that can give rise to myogenic, melanocytic, and neuronal cell lineages after in vitro clonal single cell culture. Differentiated cells not only acquire

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lineage-specific markers but also demonstrate appropriate functions in ex vivo conditions. In addition, the hair-follicle NCSCs show differentiation potential toward mesenchymal lineages, and they can be differentiated into adipocyte, chondrocyte, and osteocyte lineages. Differentiated neuronal cells can persist in mouse brain and retain neuronal differentiation markers. It has been shown that hair-follicle-derived NCSCs can promote nerve regrowth, and improve motor function in mice transplanted with these stem cells following transecting spinal cord injury [9]. Furthermore, peripheral nerves have been repaired with hair follicle stem cells [10]. Implantation of skin-derived precursor cells adjacent to crushed sciatic nerves has resulted in remyelination [11]. Therefore, the hair-follicle/skin-derived NCSCs have already shown promising results for regenerative therapy in preclinical models [9, 12–14].

Somatic cell reprogramming to induced pluripotent stem (iPS) cells has shown enormous potential for regenerative medicine. However, there are still many issues with iPS cells, particularly the long-term effect of oncogene/virus integration and potential tumorigenicity of pluripotent stem cells which have not been adequately addressed. There are still many hurdles to be overcome before iPS cells can be used for regenerative medicine. In contrast, the adult stem cells are known to be safe and they have been used clinically for many years, such as for bone marrow transplants. Many patients have already benefited from this treatment. Autologous adult stem cells are still preferred cells for transplantation. Therefore, the readily accessible and expandable adult stem cells in human hair follicles are a valuable source for regenerative medicine.

2 Materials

1. Dispase solution (0.48%): dispase (grade II, 0.5 U/mg; Boehringer Mannheim, Indianapolis, IN, #165859) 0.48 g is dissolved in 100 ml phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Cellgro, Herndon, VA, #MT21-031-CM). Sterilize the enzyme solution through a 0.2 mm filter, aliquot into 5 ml/tubes, and store at −20 °C for up to 3 months.
2. Collagenase solution (1 mg/ml): collagenase type IV (Invitrogen, Carlsbad, CA, #17104-019). 100 mg is dissolved in 100 ml DMEM to yield a final concentration of 1 mg/ml. Sterilize the enzyme solution through a 0.2 mm filter, aliquot into 5 ml/tubes, and store at −20 °C for up to 3 months.
3. Poly D lysine: Biomedical Technologies, Stoughton, MA. Dissolve 5 mg in 33 ml of sterile distilled water (BioWhittaker, Walkersville, MD). Store at −20 °C.
4. Fibronectin (human): Biomedical Technologies, Stoughton, MA. Dissolve 1 mg in 0.5 ml of water. Incubate at 37 °C for at least 1 h up to overnight. Add 5.5 ml of D-PBS before use.

5. Expansion medium (100 ml): 96 ml DMEM:F12, 1 ml penicillin/streptomycin (10,000 U/ml) (Gibco), 1 ml N-2 supplement (Gibco 17502-048), 2 ml B-27 supplement (Gibco, Grand Island, NY, 17504-044), 100 μl 50 mM 2-mercaptoethanol stock (to make a 50 μM final concentration) (Sigma, St. Louis, MD), 80 μl FGF (25 μg/ml stock solution) (to make a final concentration of 20 ng/ml), 80 μl EGF (25 μg/ml stock) (20 ng/ml final concentration; add after filter sterilization). Filter sterilize.
6. FACS buffer/staining medium: L15 medium without phenol red (132 ml), 1% penicillin/streptomycin (1.5 ml, 10,000 U/ml stock), 1 mg/ml BSA (150 mg, tissue culture grade), 10 mM HEPES pH 7.4 (1.5 ml of a 1 M stock), 10% Biowhittaker water (15 ml).

3 Methods

3.1 Preparation of Tissue Culture Plates

1. Coat each well with sufficient Poly-D-Lysine (PDL) to cover the bottom of the well. Allow the plates to dry in the hood.
2. After the wells are dry, rinse with sterile water, and aspirate. Allow the plates to dry in the hood.
3. When dry, coat with fibronectin (that was dissolved overnight at 37 °C at a concentration of 1 mg in 6 ml).
4. Add medium before fibronectin dries in the plates.

3.2 Isolate Hair Follicles from Human Scalp

1. Before starting the procedure of isolating NCSCs, prepare the respective media and reagents.
2. Collect fresh adult human scalp skin from facelift procedures or fetal scalp tissue. Wash with PBS containing penicillin/streptomycin.
3. Transfer the skin into 50 ml tubes and incubate in DMEM with dispase (10 mg/ml) overnight at 4 °C. Incubate for 2–4 h at 37 °C is also effective. Section skin pieces should yield a maximum width of 1 cm to allow for optimal enzyme penetration.
4. Transfer the skin into a sterilized Petri dish; pull off each hair follicle from the skin by grasping the hair shaft near the skin surface and pulling firmly and smoothly. The hair follicles show morphology of either anagen (Fig. 1a) or telogen (Fig. 1b).
5. Incubate the isolated follicle fragments in 0.05% trypsin-EDTA for 15–20 min at room temperature with periodic shaking and add 4 ml DMEM with 10% FBS to stop the reaction. The follicular epithelium is trypsinized and filtered through a 40 μm filter to obtain a single-cell suspension containing cells of varying size and shape.

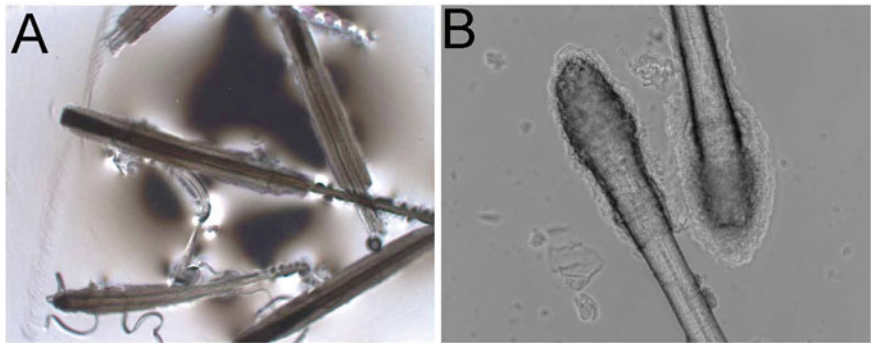

Fig. 1 Plucked anagen and telogen hair follicles. (**a**) Anagen hair follicles. (**b**) Telogen hair follicles

6. Spin at 200 × *g* for 5 min, discard supernatant carefully, resuspend in 1 ml PBS containing 2% serum (FBS).
7. Alternatively, plucked hair follicles can be placed in culture without trypsin digestion to grow hair spheres in situ.

3.3 Isolation of Hair-Follicle NCSCs Using Flow Cytometric Cell Sorting

1. Label the hair follicle cells with antibodies against CD271 (APC-conjugated) and HNK1 (FITC-conjugated) or CD271 and alpha4 integrin (PE-conjugated) for 40 min on ice in the dark. Centrifuge for 5 min at 200 × *g* at room temperature and aspirate the supernatant.
2. Resuspend cells in PBS containing 2% serum (FBS), and before sorting, propidium iodide (PI) is added to gate out the dead cells.
3. Perform cell sorting by flow cytometry (FACS). Collect CD271+/HNK1+ double-positive cells or CD271+/alpha4 integrin+ double-positive cells (Fig. 2) for further culture.

3.4 Culture of Primary NCSCs

1. Culture the dissociated follicular cells or FACS-sorted cells in ultra-low-attachment plates in NCSC medium [95 ml DMEM/F12, 1 ml penicillin/streptomycin (P/S), 1 ml N2, 2 ml B27, 100 μl mercaptoethanol (2 ME; 50 mM stock), βFGF (20 g/ml medium), IGF-1 (20 ng/ml medium), and EGF (20 ng/ml medium)]. Change medium every day.
2. Check cell culture every day under the microscope. NCSCs will start to form floating small aggregates after several days and well-formed spheres in 2–5 weeks in culture, depending on the age of the donors (Fig. 3a).
3. Alternatively, when plucked hair follicles are directly cultured in the NCSC medium, outgrowth will appear at the bulge region in a few days as well as well-formed spheres in situ in several weeks (Fig. 3b).

3.5 Expansion of NCSCs

1. Wash cells with PBS once.
2. Add pre-warmed (to 37 °C, critical) trypsin-EDTA (2×) and incubate at 37 °C for 7 min or longer if necessary for the hair

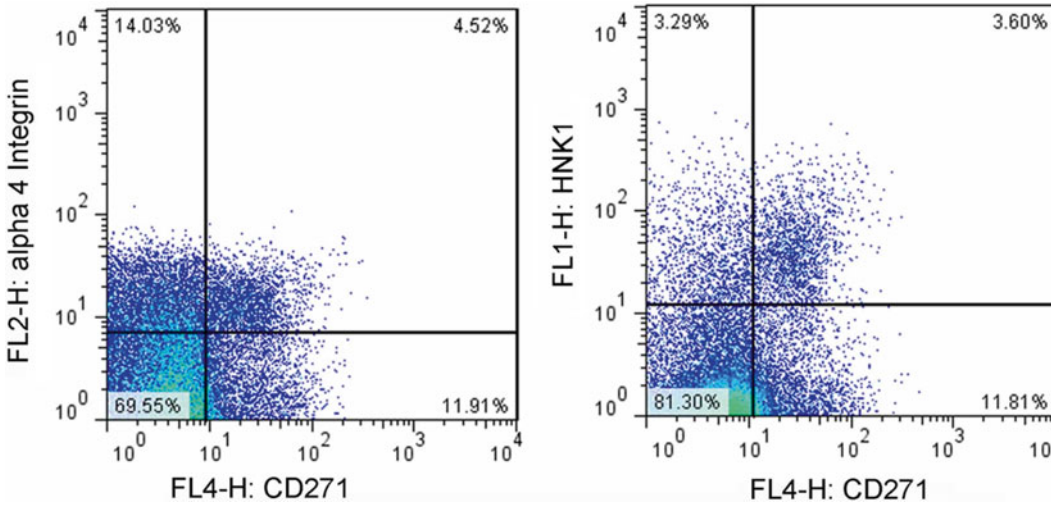

Fig. 2 Representative images of FACS analysis of hair follicle NCSCs. *Left panel*: Cells are gated using anti-CD271 and anti-alpha4 integrin antibodies. *Right panel*: Cells are gated using anti-CD271 and anti-HNK1 antibodies

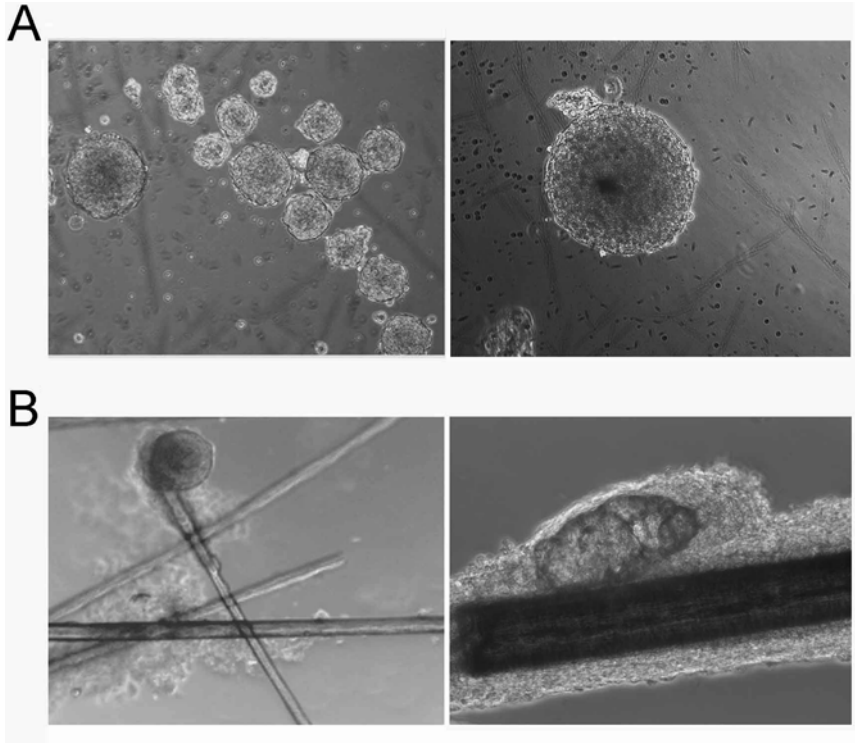

Fig. 3 Morphology of hair spheres. (**a**) Morphology of floating hair spheres, *left panel*: low power, *right panel*: high power. (**b**) Morphology of hair sphere in situ at the bulge region, *left panel*: a hair sphere formed at a telogen bulge, *right panel*: a hair sphere formed at the bulge region of an anagen hair follicle

follicle stem cell to disassociate. No more than 15 min should be allowed for the digestion in order to optimize cell survival.

3. Add DMEM with 10% FBS to stop the trypsin; gently pipette up and down to disperse cells; spin down at 200 × *g*, 5 min.
4. Resuspend cells with NCSC culture medium in ultra-low attachment plates.
5. Alternatively, NCSCs can also be expanded as attached cells. Then resuspended cells are cultured with NCSC medium in the pretreated tissue-culture plates.

4 Notes

1. Although it is best to process the tissue right after harvest, we found that scalp tissues can be safely stored in media on ice for overnight transportation with minimal impact on cell viability.
2. It is important to treat the scalp tissue with antibiotics and use aseptic technique during hair follicle isolation to avoid potential microorganism contamination.
3. Punch biopsy of scalp tissue usually generates a limited number of cells and requires additional tissue culture to produce sufficient cells for further experiments.
4. Discarded facelift skin or fetal scalp tissue yields hundreds of viable follicles and usually generates sufficient tissue for FACS sorting or further experiments in only a few days.
5. NCSC culture medium is sufficient to maintain hNCSCs in an undifferentiated state without the need for feeder cells. Keratinocytes will not proliferate and well gradually die in the medium.
6. Certain small round cells will proliferate and form small aggregates in suspension after 3–5 days.
7. These floating aggregates slowly increased in size, generating three-dimensional sphere-like structures, which we termed neural spheres.
8. If cultured in coated plates, NCSCs will attach to the surface and not form any spheres. The attached NCSCs grow faster than in suspension.
9. The sphere-forming or attached stem cells express NCSC markers.
10. When entire hair follicles are cultured, spheres are formed at the area corresponding to the bulge region.

Acknowledgments

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