Protocols for Cryopreservation of Intact Hair Follicle That Maintain Pluripotency of Nestin-Expressing Hair-Follicle-Associated Pluripotent (HAP) Stem Cells

Satoshi Kajiura, Sumiyuki Mii, Ryoichi Aki, Yuko Hamada, Nobuko Arakawa, Katsumasa Kawahara, Lingna Li, Kensei Katsuoka, Robert M. Hoffman, and Yasuyuki Amoh

Abstract

Hair follicles contain nestin-expressing pluripotent stem cells, the origin of which is above the bulge area, below the sebaceous gland. We have termed these cells hair-follicle-associated pluripotent (HAP) stem cells. Cryopreservation methods of the hair follicle that maintain the pluripotency of HAP stem cells are described in this chapter. Intact hair follicles from green fluorescent protein (GFP) transgenic mice were cryopreserved by slow-rate cooling in TC-Protector medium and storage in liquid nitrogen. After thawing, the upper part of the hair follicle was isolated and cultured in DMEM with fetal bovine serum (FBS). After 4 weeks culture, cells from the upper part of the hair follicles grew out. The growing cells were transferred to DMEM/F12 without FBS. After 1 week culture, the growing cells formed hair spheres, each containing approximately 1×10^2 HAP stem cells. The hair spheres contained cells which could differentiate to neurons, glial cells, and other cell types. The formation of hair spheres by the thawed and cultured upper part of the hair follicle produced almost as many pluripotent hair spheres as fresh follicles. The hair spheres derived from cryopreserved hair follicles were as pluripotent as hair spheres from fresh hair follicles. These results suggest that the cryopreservation of the whole hair follicle is an effective way to store HAP stem cells for personalized regenerative medicine, enabling any individual to maintain a bank of pluripotent stem cells for future clinical use.

Key words Hair follicle, HAP stem cell, Nestin, Pluripotent, Hair spheres, Cryopreservation

1 Introduction

Nestin-expressing stem cells of the hair follicle were discovered by our laboratory [1]. We subsequently demonstrated that the nestin-expressing cells of the hair follicle are able to form neurons and other non-follicle cell types [2]. We have termed these cells as hair-follicle-associated pluripotent (HAP) stem cells. The nestin-expressing stem cells from the hair follicle can effect the repair of peripheral nerves and the spinal cord [3–5] when transplanted at

the site of injury. The hair follicle stem cells differentiated into neuronal and glial cells after transplantation to the injured peripheral nerve and spinal cord, and enhanced injury repair and locomotor recovery.

When the excised hair follicle, with its sensory nerve stump, was placed in Gelfoam® 3D histoculture, HAP stem cells grew and extended the hair follicle nerve which consisted of βIII-tubulin-positive fibers with F-actin expression at the tip [6] indicating the fibers were growing axons. The growing hair follicle nerve could interact with the sciatic nerve, the trigeminal nerve, and the trigeminal nerve ganglion in Gelfoam® histoculture [6, 7]. These results suggest that a major function of the HAP stem cells in the hair follicle is for growth of the follicle sensory nerve.

HAP stem cells have critical advantages over embryonic stem cells and induced pluripotent stem (iPS) cells because they are highly accessible, require no genetic manipulation, are non-tumorigenic, and do not present ethical issues for regenerative medicine [8].

The present chapter describes the cryopreservation methods of the whole hair follicle by slow-rate cooling and storage in liquid nitrogen that preserve the differentiation potential of HAP stem cells [9].

2 Materials

2.1 Animals

1. Transgenic C57/B6-GFP mice (AntiCancer Inc., San Diego, CA) [10].

2.2 Instruments

1. Binocular microscope (SZX16, Olympus, Tokyo, Japan).
2. Fluorescence microscope (BX51, Olympus).
3. CFX96 real-time PCR detection (Bio-Rad, Hercules, CA).
4. Scissors and forceps (Fisher Scientific, Waltham, MA).
5. Needle holder (Fisher Scientific).
6. Micro-scissors and micro-forceps (Fisher Scientific).
7. Exel International Disposable Scalpels (Fisher Scientific).

2.3 Reagents

1. DMEM/F12 medium (GIBCO Life Technologies, Carlsbad, CA).
2. B-27 (GIBCO Life Technologies).
3. Gentamicin (GIBCO Life Technologies).
4. DMEM (Sigma Aldrich, St. Louis, MO).
5. l-Glutamine (GIBCO Life Technologies).
6. HEPES (MP Biomedicals, Santa Ana, CA).
7. Fetal bovine serum (Omega Scientific, Tarzana, CA).

8. Accumax (Innovative Cell Technologies, Inc., San Diego, CA).

9. TC-Protector (DS Pharma Biomedical Co., Osaka, Japan).

10. StemCell Keep (Bio Verde, Inc.).

11. Anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; Covance, San Diego, CA).

12. Anti-glial fibrillary acidic protein (GFAP) chicken polyclonal (1:300; Abcam, Cambridge, MA).

13. Anti-keratin 15 (K15) monoclonal (1:100; Lab Vision, Fremont, CA).

14. Anti-smooth muscle actin (SMA) monoclonal (1:200; Lab Vision).

15. Alexa Fluor® 568-conjugated goat anti-mouse (1:400; Molecular Probes, Eugene, OR).

16. Alexa Fluor® 568-conjugated goat anti-chicken (1:1000; Molecular Probes).

17. 4′,6-Diamidino-2-phenylindole dihydrochloride (Molecular Probes).

18. RNeasy Plus Mini kit (QIAGEN, Valencia, CA).

19. High-capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA).

20. TaqMan Gene Expression Assays (Applied Biosystems).

21. TaqMan Probes r18s:Hs99999901_s1 (Applied Biosystems).

22. TaqMan Probes Nestin: Mm00450205_m1 (Applied Biosystems).

23. TaqMan Probes Sox2: Mm03053810_s1 (Applied Biosystems).

24. TaqMan Probes SSEA1: Mm00487448_s1 (Applied Biosystems).

25. 4–20% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel.

26. Immobilon-P membranes (Millipore Corp., Tokyo, Japan).

27. Anti SSEA1 antibody (1:250, BioLegend, San Diego, CA).

28. Peroxidase-conjugated anti-mouse IgA,IgG,IgM (CHEMICON, Temecula, CA).

29. Enhanced chemiluminescence plus a Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

3 Methods

3.1 Isolation of Vibrissa Hair Follicles

1. Isolate the vibrissa follicles from GFP-transgenic mice at the upper lip containing the vibrissa pad under anesthesia and expose the inner surface.

2. Dissect the whole vibrissa hair follicles under a binocular microscope and pluck the whisker from the pad by pulling them gently by the neck with fine forceps.

3. Wash the isolated vibrissa in DMEM/F12 with 2% B-27 and 50 μg/ml gentamicin. Perform all surgical procedures under a sterile environment [10].

3.2 Hair Follicle and Hair Sphere Culture

1. Isolate the upper part of the vibrissa hair follicle and culture in DMEM with 10% FBS (*see* **Note 1**).

2. After 4 weeks culture, treat cells growing out from the upper follicle enzymatically with Accumax, in order to detach them.

3. Transfer the detached cells to non-adhesive culture dishes with DMEM/F12 containing 2% B-27 in order to produce hair spheres (*see* **Notes 2–4**).

3.3 Cryopreservation of the Whole Hair Follicle

3.3.1 Slow-Rate Cooling Method

1. Transfer five whole vibrissa follicles to cryovials and add TC-Protector medium (500 μl).

2. Store 18 cryovials with the vibrissa follicles overnight in a −80 °C freezer.

3. Transfer to a liquid nitrogen tank the next day. Use three mice for this method for three independent experiments involving one mouse each.

4. Thaw the cryopreserved vibrissa follicles at 37 °C in a water bath for 60–90 s (slow recovery) with gentle shaking and separate the follicles into three parts (upper, middle, and lower).

5. Isolate the upper part of hair follicle and culture in DMEM with 10% FBS (*see* **Note 5**).

3.4 Immuno-histochemistry of Differentiated Hair Follicles

1. After 1 week culture of hair spheres in DMEM with 10% FBS, stain the hair spheres in each individual well (*see* **Note 6**).

2. Use the following primary antibodies: anti-III-β-tubulin mAb; anti-glial fibrillary acidic protein (GFAP) chicken polyclonal Ab (1:200); anti-smooth muscle actin (SMA) mAb (1:400); and anti-keratin 15 (K15) mAb (1:100).

3. Use the following secondary antibodies: Alexa Fluor 568 labeled goat anti-mouse IgG (1:400) for anti-III-β-tubulin, anti-SMA, and anti-K15. Use Alexa Fluor 568-labeled goat anti-chicken IgG for anti-GFAP (1:1000).

4. In order to quantify the percentage of cells producing a given marker protein, photograph at least four fields in any given experiment. Determine the number of positive cells relative to the total number of cells strained with 4′,6-diamidino-2-phenylindole dihydrochloride in the nucleus.

3.5 Expression of Stem Cell Marker Genes

3.5.1 RT-PCR

1. Examine the mRNA levels of stem cell marker genes (nestin, Sox2, SSEA-1) using the real-time polymerase chain reaction (RT-PCR) analysis.

2. Extract total RNA from 100 hair spheres using an RNeasy Plus Mini kit.

3. Synthesize c-DNA with a high-capacity RNA-to-cDNA kit.

4. Use real-time PCR on a CFX96 TaqMan Gene Expression Assays and TaqMan Probes as follows; r18s:Hs99999901_s1; Nestin: Mm00450205_m1; Sox2: Mm03053810_s1; SSEA1: Mm00487448_s1. Normalize the mRNA levels by comparison with r18s.

3.5.2 Western Blotting

1. Use Western blot analysis to detect the expression of SSEA-1.

2. Subject total proteins (30 μg/well) from hair spheres to 4–20% SDS-PAGE.

3. Transfer to Immobilon-P membranes.

4. Detect SSEA1 with an anti-SSEA1 primary antibody (1:250), followed by a mixture of peroxidase-conjugated anti-mouse IgA,IgG,IgM with enhanced chemiluminescence plus a Western Blotting Detection System. Express results as mean ± standard deviation (SD) for each of the three samples.

4   Notes

1. Only the upper part of vibrissa hair follicle was isolated and cultured in DMEM with 10% FBS. The middle and lower parts of hair follicle were not used.

2. Pluck the intact vibrissa hair follicles from the pad by pulling them gently by the neck with fine forceps.

3. After 1 week of culture, the growing cells formed hair spheres containing nestin-expressing HAP stem cells.

4. After change of medium to DMEM containing 10% FBS, and 2 days additional culture, the GFP-expressing HAP stem cells differentiated to βIII-tubulin-positive neurons, GFAP-positive glial cells, K15-positive keratinocytes, and smooth muscle actin-positive smooth muscle cells [10].

5. When the upper part of the vibrissa hair follicle is cultured, change the medium (DMEM with 10% FBS) every 3 or 4 days.

6. To make hair spheres, use non-adhesive culture dishes.

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