Chapter 6

Nestin-Expressing Hair-Follicle-Associated Pluripotent (HAP) Stem Cells Promote Whisker Sensory-Nerve Growth in Long-Term 3D-Gelfoam® Histoculture

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Abstract

Mouse whiskers containing hair-follicle-associated pluripotent (HAP) stem cells, from nestin-driven green fluorescent protein (ND-GFP) transgenic mice, were placed in 3D histoculture supported by Gelfoam®. β-III tubulin-positive fibers, consisting of ND-GFP-expressing HAP stem cells, extended up to 500 mm from the whisker nerve stump in histoculture. The growing fibers had growth cones on their tips expressing F-actin indicating they were growing axons. The growing whisker sensory nerve was highly enriched in ND-GFP HAP stem cells which appeared to play a major role in its elongation and interaction with other nerves placed in 3D culture, including the sciatic nerve, the trigeminal nerve, and the trigeminal nerve ganglion. The results suggested that a major function of HAP stem cells in the hair follicle is for growth of the hair follicle sensory nerve.

Key words Hair follicle, Vibrissa, Peripheral nerve, Nerve ganglion, Stem cells, Nestin, Pluripotent, GFP, Confocal fluorescence imaging, Histoculture, Gelfoam®

1 Introduction

Transgenic mice, under the control of the nestin promoter, termed nestin-driven green fluorescent protein (GFP) (ND-GFP), enabled our laboratory to discover small oval-shaped, nestin-expressing cells in the bulge area of the hair follicle surrounding the hair shaft [1, 2]. In vitro, the hair follicle nestin-expressing cells, termed hair-follicle-associated pluripotent (HAP) stem cells, differentiated into neurons, glia, keratinocytes, smooth muscle cells, cardiac muscle cells, and melanocytes [3]. Using confocal imaging of whisker follicles from ND-GFP mice, it was found that the bulge area is the source of the nestin GFP-expressing HAP stem cells of the hair follicle. The nestin GFP-expressing HAP stem cells migrate

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from the bulge area to the dermal papilla as well as into the surrounding skin tissues including the epidermis [4].

Li et al. [5, 6] reported that mouse skin and human scalp histocultured on Gelfoam® showed continuous hair growth for up to 40 days in vitro, thereby demonstrating that Gelfoam®-supported histoculture can be used to evaluate the dynamic state of hair follicles. Duong et al. [7] showed that isolated mouse whiskers could be cultured for long periods on Gelfoam® and that trafficking of ND-GFP-expressing cells could be imaged longitudinally within the histocultured whisker by confocal microscopy.

Gelfoam®-supported histoculture was shown to be useful not only for hair follicles but also for peripheral nerves and nerve ganglions as well [8]. We could observe peripheral nerve growth and interaction with hair follicle nerves and other nerves in Gelfoam® histoculture [9]. These reports suggested that Gelfoam® 3D histoculture is a physiologic system to support nerve growth and interaction which should have broad application.

2 Materials

2.1 Animals

1. Transgenic mice expressing nestin-driven green fluorescent protein (ND-GFP) (AntiCancer Inc., San Diego, CA).
2. β-Actin-driven red fluorescent protein (RFP) transgenic mice (AntiCancer Inc.) at different ages (4 weeks up to 5 months).

2.2 Gelfoam® Whisker Histoculture and Growth Medium

1. Ketamine solution (25 mg/ml) for animal anesthesia (Henry Schein, San Diego, CA).
2. 70% isopropyl alcohol and PBS for washing whisker pads.
3. Binocular microscope (MZ6, Leica, Buffalo Grove, IL).
4. Gelfoam® (Pharmacia and Upjohn Co., Kalamazoo, MI).
5. 35 mm culture dishes.
6. CO_2 incubator.
7. Culture medium: DMEM-F12 medium (GIBCO/BRL Life Technologies, Inc., Gaithersburg, MD) containing B-27 (2.5%) (GIBCO/BRL), N2 (1%) (GIBCO/BRL), and 1% penicillin and streptomycin (GIBCO/BRL).

2.3 Confocal Laser Scanning Microscopy

1. Confocal laser scanning microscope (Fluoview FV1000, Olympus Corp., Tokyo, Japan).

2.4 Histology and Immunofluorescence Staining

1. 4% paraformaldehyde for tissue fixation.
2. Tissue freezing medium (Triangle Biomedical Science, Durham, NC).
3. Liquid nitrogen.

4. PBS.
5. CM1850 cryostat (Leica).
6. 5 % normal goat serum.
7. Primary antibodies: anti-β III tubulin mAb (mouse, 1:100, Santa Cruz Biotechnology, Dallas, TX); anti-glial fibrillary acidic protein (GFAP) mAb (mouse, 1:250, BD Pharmingen, San Diego, CA); anti-S100 mAb (mouse, 1:200, Millipore, Billerica, MA); anti-$p75^{NTR}$ mAb (rabbit, 1:3200, Cell Signaling Technology, Danvers, MA); anti-TrkA mAb (rabbit, 1:50, Santa Cruz); and anti-TrkB mAb (rabbit, 1:50, Santa Cruz).
8. Secondary antibodies; goat anti-mouse IgG Alexa Fluor® 555 (1:1000, Cell Signaling); goat anti-rabbit IgG (H + L) Alexa Fluor® 555 (1:1000, Cell Signaling Technology, Danvers, MA).
9. Alexa Fluor® 647 phalloidin (1:40, Invitrogen, Calsbad, CA) for F-actin detection.
10. DAPI (1:48000, Invitrogen).
11. Fluoremount (Sigma, St. Louis, MO).

3 Methods

3.1 Isolation of Vibrissa Hair Follicles, Trigeminal and Sciatic Nerves and Trigeminal Nerve Ganglions

1. Anesthetize the mice with 30–50 μl ketamine solution (25 mg/ml).
2. Make an incision line in a circle around the whisker pad. Remove the whisker pad containing vibrissa hair follicles with fine tweezers and scissors.
3. Sterilize whisker pads from ND-GFP transgenic mice with 70 % isopropyl alcohol and wash in PBS three times.
4. Dissect the whisker pad with forceps and fine needles with a binocular microscope to obtain a single vibrissa hair follicle.
5. Remove trigeminal nerves from whisker pads from mice and sterilize with 70 % isopropyl alcohol. Expose the trigeminal nerve from its infraorbital foramen in each vibrissa on the inside of the whisker pad. Using the binocular microscope, excise the trigeminal nerve with fine forceps. The length of the excised trigeminal nerve is 1.5–2 mm.
6. Isolate the sciatic nerve by making a skin incision in the medial side of the thigh of mice. Expose the nerve between the short adductor muscle and long adductor muscle. Using the binocular microscope, excise the sciatic nerve with fine forceps. The length of the excised sciatic nerve is 5–8 mm.
7. Isolate trigeminal nerve ganglions by making a skin incision on top of the head and open the skull with a drill. Expose the trigeminal nerve ganglions after the skull base is removed. Using

the binocular microscope, excise the trigeminal nerve ganglions with fine forceps.

8. Wash the isolated vibrissae hair follicles, nerves, and ganglions in PBS three times before placing them in Gelfoam® histoculture (please see below).

3.2 Gelfoam® Histoculture

1. Cut Gelfoam® 2 cm square by 0.5 cm thick with a sterile razor.
2. Soak Gelfoam® in a 35 mm culture dish filled with 3 ml culture medium.
3. Place the culture dish in the incubator for 2 h.
4. Culture the vibrissae hair follicles on Gelfoam®, with or without capsules, maintaining their sensory nerve stumps, along with trigeminal and sciatic nerves and trigeminal nerve ganglions described above (Fig. 1).
5. Arrange the vibrissa follicle nerve stump and the trigeminal nerve or sciatic nerve with severed ends opposed to each other. Arrange the vibrissae follicle containing the sensory nerve stump and the trigeminal nerve ganglion with the nerve stump opposed to the ganglion (*see* **Notes 1–5**).
6. Incubate the cultures at 37 °C, 5% CO_2 100% humidity. Change the medium every other day.

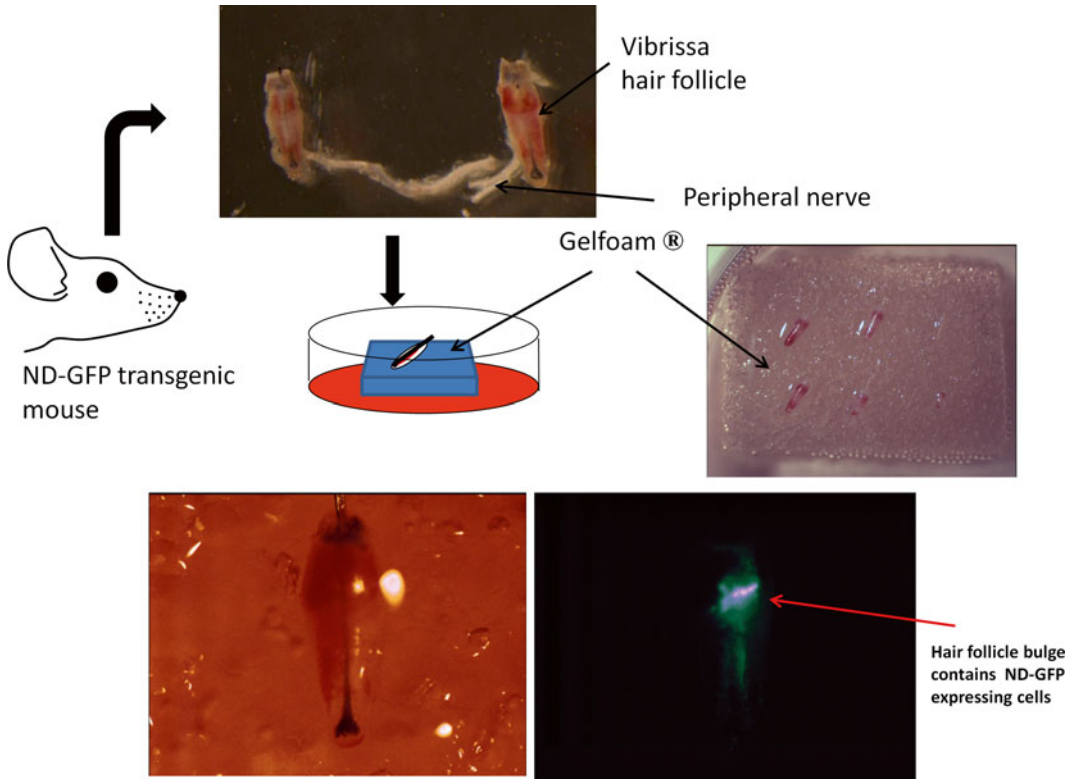

Fig. 1 Isolated vibrissa hair follicles from an ND-GFP mouse were placed on Gelfoam® and suspended in culture medium

3.3 Immunofluorescence Staining of Sections

1. Fix tissues in pre-cooled 4% paraformaldehyde at room temperature (RT) for 2 h.
2. Embed in tissue freezing medium.
3. Freeze in liquid nitrogen for 10 min and store at −80 ° C.
4. Prepare frozen sections of 7–10 μm thickness with a cryostat.
5. Wash the frozen sections with PBS three times.
6. Apply 5% normal goat serum at RT for 1 h, then wash with PBS three times.
7. Apply primary antibodies at RT for 2 h, then wash with PBS three times.
8. Apply secondary antibodies at RT, dark, 1 h, then wash with PBS three times.
9. Apply DAPI at RT, dark, 3 min, then wash with PBS three times.
10. Mount slides with Fluoremount and observe under confocal laser scanning microscopy. Compare all findings of immunofluorescence staining with positive and negative controls.

3.4 Immunofluorescence Staining of Tissues

1. Fix histocultured tissues on Gelfoam® in pre-cooled 4% paraformaldehyde at room temperature (RT) for 2 h.
2. Wash the histocultured tissues on Gelfoam® with PBS three times.
3. Apply 5% normal goat serum at RT for 1 h, then wash with PBS three times (*see* **Note 6**).
4. Apply primary antibodies at RT for 2 h, then wash with PBS three times.
5. Apply secondary antibodies at RT, dark, 1 h, then wash with PBS three times.
6. Apply DAPI at RT, dark, 3 min, then wash with PBS three times.

3.5 Injuring the Trigeminal Nerve of ND-GFP Transgenic Mice

1. Anesthetize the ND-GFP transgenic mouse with the ketamine solution.
2. Make a skin flap including the whisker pad and trigeminal nerve (Fig. 2).
3. Sever the trigeminal nerve.
4. Close the skin flap with a 6-0 suture (Ethibond extra polyester suture, Ethicon). Perform all surgical procedures under sterile conditions.
5. Open the wound 1 week later and remove the whisker pad, including the injured trigeminal nerve, and observe microscopically with an MVX-10 microscope.

3.6 Confocal Laser Scanning Microscopy

1. Use a FV1000 confocal laser scanning microscope for two- (X,Y) and three-dimensional (3D, X,Y,Z) high-resolution imaging of vibrissa follicles and nerves in histoculture. Obtain

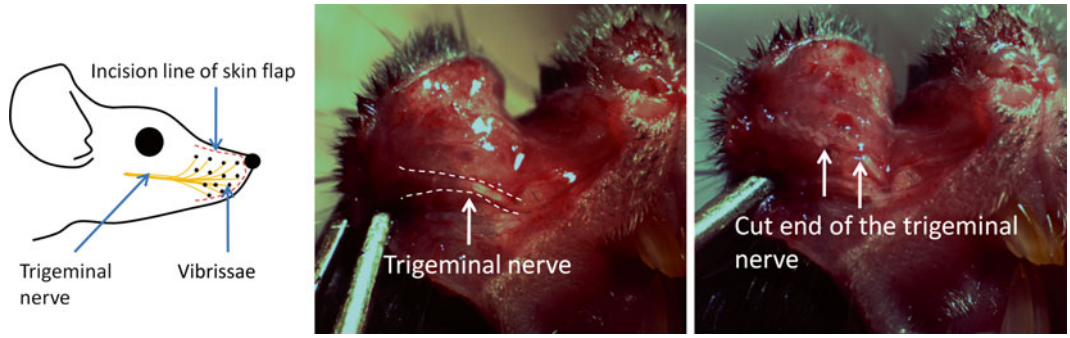

Fig. 2 A skin-flap is made to visualize the whisker pad and trigeminal nerve

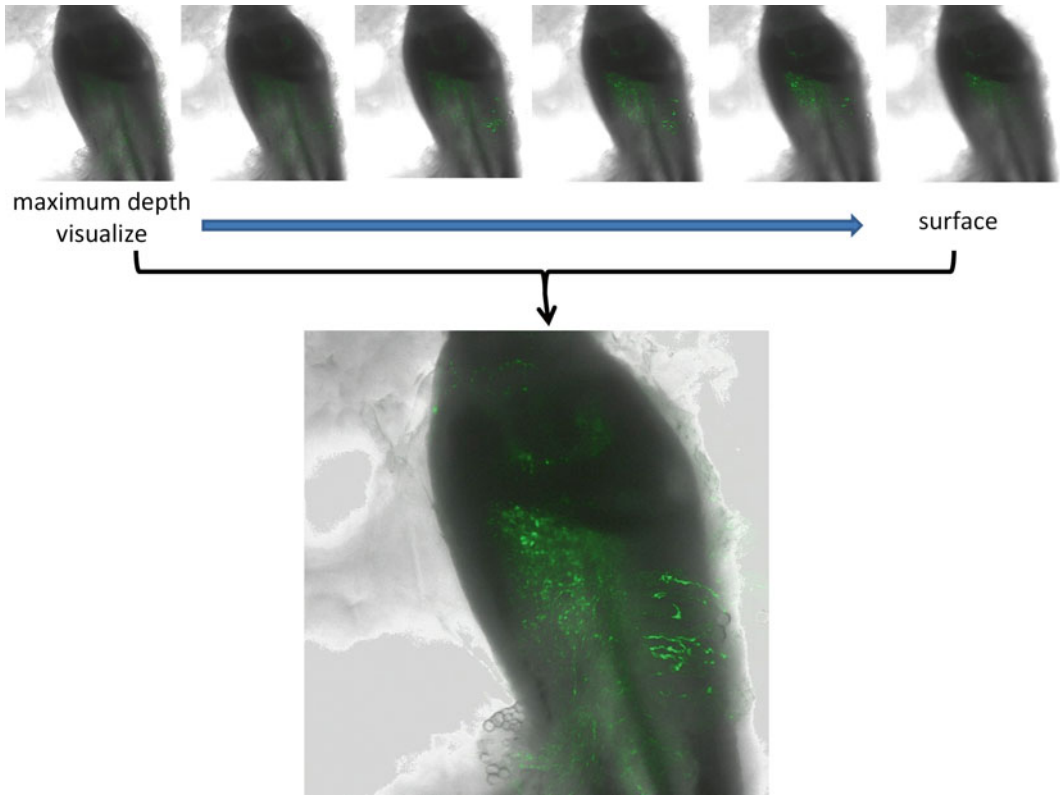

Fig. 3 The hair follicle bulge area is shown by integrating images of different depths from the surface of the hair follicle

fluorescence images using the 4×/0.10 Plan N, 10×/0.30 Plan-NEOFLUAR, 20×/0.50 UPlan FL N, or 20×/1.00w XLUMplan FL objectives.

2. Obtain images of 30 optical sections of 10 μm each starting from the surface of the hair follicle. Reconstruct three-dimensional images using the FV1000 software (Fig. 3) (*see* **Notes 4**, **7**–**9**).

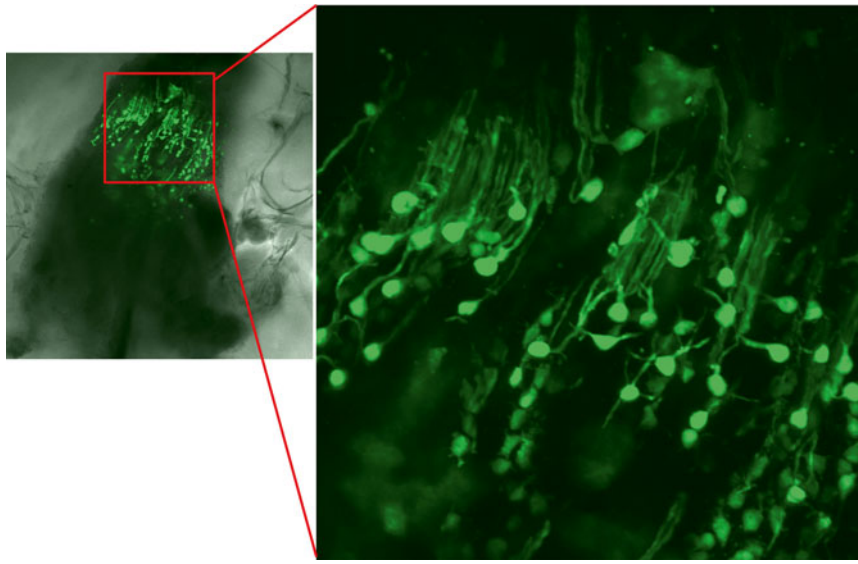

Fig. 4 A high magnification image of nestin-expressing HAP stem cells of the hair follicle bulge

4 Notes

1. The GFP-expressing cord-like structures of the whisker sensory nerve intermingled with the ends of the co-cultured RFP-expressing trigeminal nerve by day 12 of 3D-Gelfoam® histoculture. The ND-GFP-expressing HAP stem cells proliferated at the whisker sensory nerve stump, forming radial cords which extended the nerve. By day 10, the thickest cord extended and intermingled with the RFP-expressing trigeminal nerve. The histocultured whisker nerve intermingled with the trigeminal nerve. The whisker nerve contained spindle-shaped cells highly expressing ND-GFP throughout the nerve, as well as round-shaped cells with less ND-GFP expression.
2. By day 9 of 3D Gelfoam® histoculture, the ND-GFP-expressing HAP stem cells migrated from the whisker sensory nerve stump of the vibrissa hair follicle and invaded deeply into the RFP-expressing sciatic nerve.
3. The ND-GFP-expressing HAP stem cells in the whisker sensory nerve co-expressed $p75^{NTR}$, TrkB, and β-III, tubulin, intermingled with the trigeminal nerve.
4. By 28 days of 3D Gelfoam® histoculture, the β-III tubulin-positive fibers extended widely and radially around the hair follicle sensory nerve. The tips of the β-III tubulin-positive fibers expressed phalloidin-positive F-actin suggesting that the β-III tubulin-positive fibers were axons growing from the whisker sensory-nerve stump.

5. The extending GFP-expressing cord-like structures of the whisker sensory nerve intermingled extensively with the co-cultured trigeminal nerve ganglion by day 41 of 3D Gelfoam® histoculture. Immunofluorescence staining demonstrated that many β-III tubulin-positive fibers extended from both the trigeminal nerve ganglion and the hair-follicle sensory nerve. The fibers consisted of ND-GFP-expressing HAP stem cells. The β-III tubulin-positive fibers extending from the nerve stump of the whisker spread widely like a fan and extended toward the trigeminal nerve ganglion. In long-term 3D Gelfoam® histoculture, there was a thick bundle of fibers linking the trigeminal nerve ganglion and the whisker sensory nerve stump.
6. 300 μl liquid solution of antibodies are required to soak the whole tissue.
7. The nestin-expressing HAP stem cells within the whiskers isolated from ND-GFP mice had round/oval-shaped bodies with a typical diameter of 7 μm with 2–3 elongated processes containing club-like bodies (Fig. 1).
8. The vibrissa sensory nerve penetrates the capsule at the lower part of the vibrissa. It is joined to the hair follicle bulge area via a cavernous sinus. The processes from the ND-GFP-expressing HAP stem cells in the vibrissa hair follicle bulge area began to extend toward the nerve stump by Day 4 in 3-D Gelfoam® histoculture. By day 9, ND-GFP-expressing HAP stem cells reached the whisker sensory nerve stump. The processes extending from the ND-GFP-expressing HAP stem cells co-expressed β-III tubulin, $p75^{NTR}$, and TrkB but no longer expressed S100.
9. The whisker sensory nerve stump became enriched with ND-GFP-expressing HAP stem cells in 3D Gelfoam® histoculture.

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