Chapter 15

Protocols for Gelfoam® Histoculture of Hair-Shaft-Producing Mouse Whisker Follicles Containing Nestin-GFP-Expressing Hair-Follicle-Associated Pluripotent (HAP) Stem Cells for Long Time Periods

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Abstract

Gelfoam®-histocultured whisker follicles from nestin-driven-green fluorescent protein (ND-GFP) mice produced growing pigmented and unpigmented hair shafts. Hair-shaft length increased rapidly by day 4 and continued growing until at least day 12 after which the hair-shaft length was constant. By day 63 in histoculture, the number of ND-GFP hair follicle-associated pluripotent (HAP) stem cells increased significantly and the follicles were intact. Three-dimensional Gelfoam® histoculture of hair follicles can provide a very long-term period for evaluating novel agents to promote hair growth.

Key words Gelfoam®, Whisker culture, GFP, Nestin, Transgenic mice, HAP stem cells, Hair-shaft growth

1 Introduction

Gelfoam® is a physiological substrate that is highly effective for three-dimensional culture of tumor and normal tissue [1, 2].

We previously reported the long-term growth, shaft elongation, and spontaneous regression of human hair follicles in Gelfoam® histoculture of intact scalp skin. Human scalp skin, with abundant hair follicles in various stages of the hair growth cycle, was grown for up to 40 days on Gelfoam®. Isolated human scalp follicles placed on Gelfoam® also supported hair-shaft elongation [3]. When mouse skin was histocultured on Gelfoam®, hair-shaft elongation was observed for up to 14 days [4].

Hair-follicle-associated pluripotent (HAP) stem cells can differentiate to multiple cell types, including neurons [5], heart muscle cells [6], and can effect nerve repair [7, 8] and spinal cord repair [8, 9].

Vibrissa hair follicles, including their sensory nerve stump, were excised from ND-GFP mice in which the nestin promoter drives green fluorescent protein (ND-GFP mice) [10], and were

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placed in Gelfoam® histoculture. β-III tubulin-positive fibers, consisting of ND-GFP-expressing cells, extended up to 500 μm from the whisker nerve stump in histoculture. The growing fibers had growth cones on their tips expressing F-actin indicating they were growing axons [11, 12].

We describe in this chapter the use of Gelfoam® histoculture of isolated whiskers from ND-GFP mice to determine hair-shaft growth, as well as the extent of proliferation of ND-GFP-expressing HAP stem cells, over very long periods. Our results demonstrate that extensive hair-shaft elongation occurred in Gelfoam® histoculture of mouse whisker follicles from ND-GFP transgenic mice along with extensive proliferation of the HAP stem cells within the whisker follicles [13]. In the present chapter, protocols for long-term Gelfoam® histoculture of hair-shaft producing hair follicles are described.

2 Materials

2.1 Animals

1. Nestin-GFP transgenic mice and non-transgenic nude mice (AntiCancer, Inc., San Diego, CA).

2.2 Instruments

1. Stereomicroscope (MZ6, Leica, Buffalo Grove, IL).
2. Scissors and forceps (Fisher Scientific, Waltham, MA).
3. Micro-scissors and micro-forceps (Fisher Scientific).
4. Exel International disposable scalpels (Fisher Scientific).
5. Illumatool™ fluorescence imaging system (Lightools Research, Encinitas, CA).
6. OV100 small animal fluorescence imaging system (Olympus Corp., Tokyo, Japan).

2.3 Reagents and Consumable Items

1. Gelfoam® (Pharmacia and Upjohn, Kalamazoo, MI).
2. DMEM/F12 medium (GIBCO Life Technologies, Carlsbad, CA).
3. B-27 (GIBCO Life Technologies).
4. N2 (GIBCO Life Technologies).
5. Penicillin and streptomycin (GIBCO Life Technologies).
6. Ketamine mixture: ketamine (100 mg/ml) 10 ml, xylazine (20 mg/ml) 10 ml, acepromazine (10 mg/ml) 4 ml, PBS 26 ml, total 50 ml.
7. Six-well dishes (Nest Biotechnology Co., Wuxi, Jiangsu, China).

3 Methods

3.1 Isolation of Mouse Vibrissa Follicles

1. Prepare the culture medium before Gelfoam® whisker histoculture. Use DMEM/F12 medium containing B-27 (2.5%), N2 (1%) and 1% penicillin and streptomycin.
2. Cut Gelfoam® in approximately 1 cm × 1 cm pieces and put into six-well dishes. Add 1 ml cell-culture medium onto the Gelfoam® (*see* **Note 1**). Place Gelfoam® in a 37 °C incubator for hydration overnight (*see* **Note 2**).
3. Anesthetize nestin-driven-GFP (ND-GFP) transgenic mice (6–8 weeks) with the ketamine mixture and cut the whisker hair shafts. Remove both whisker pads from the mice with sterilized scissors.
4. Lay the whisker pads on a black pad, expose the inner side of whisker pad and pin the whisker pads on the black pad.
5. Under a stereoscopic microscope or equivalent, carefully remove the tissue attached to the whisker pad with a micro-scissors and micro-forceps. Gently pull off each hair follicle by grasping the hair shaft near the skin surface and pull firmly and smoothly. Place the freshly-isolated hair follicle into DMEM immediately (*see* **Note 3**).
6. After collecting all hair follicles, choose only those which are intact and transfer them to the Gelfoam® presoaked in cell culture medium. Support each hair follicle with one piece of Gelfoam®.
7. Place the hair follicle with Gelfoam® in six-well dishes and culture in a 37 °C CO_2 incubator. Change the medium every 2–3 days. Obtain images of hair follicles on Gelfoam® at day 1, 4, 7, 9, 12, 19, and 63 using the OV100 fluorescence imaging system [14] (*see* **Note 4** and **5**).

4 Notes

1. Gelfoam®, which is derived from gelatinized pig skin, provides a three-dimensional physiological scaffold for the hair follicle to attach and grow [10–13]. Gelfoam® appears to preserve the integral hair follicle.
2. The purpose of this step is to allow the Gelfoam® soak up the culture medium.
3. When isolating the hair follicles, try not to grasp and press the bulge area. Maintain the hair follicle intact by not squeezing it. When whisker hair follicles are freshly isolated, the follicle is covered by a rigid and intact capsule filled with red blood (*see* Fig. 1).
4. The culture medium should be changed every 2–3 days. The interval time cannot exceed 1 week, otherwise the Gelfoam® will become thin and digested by 2–3 weeks. The isolated

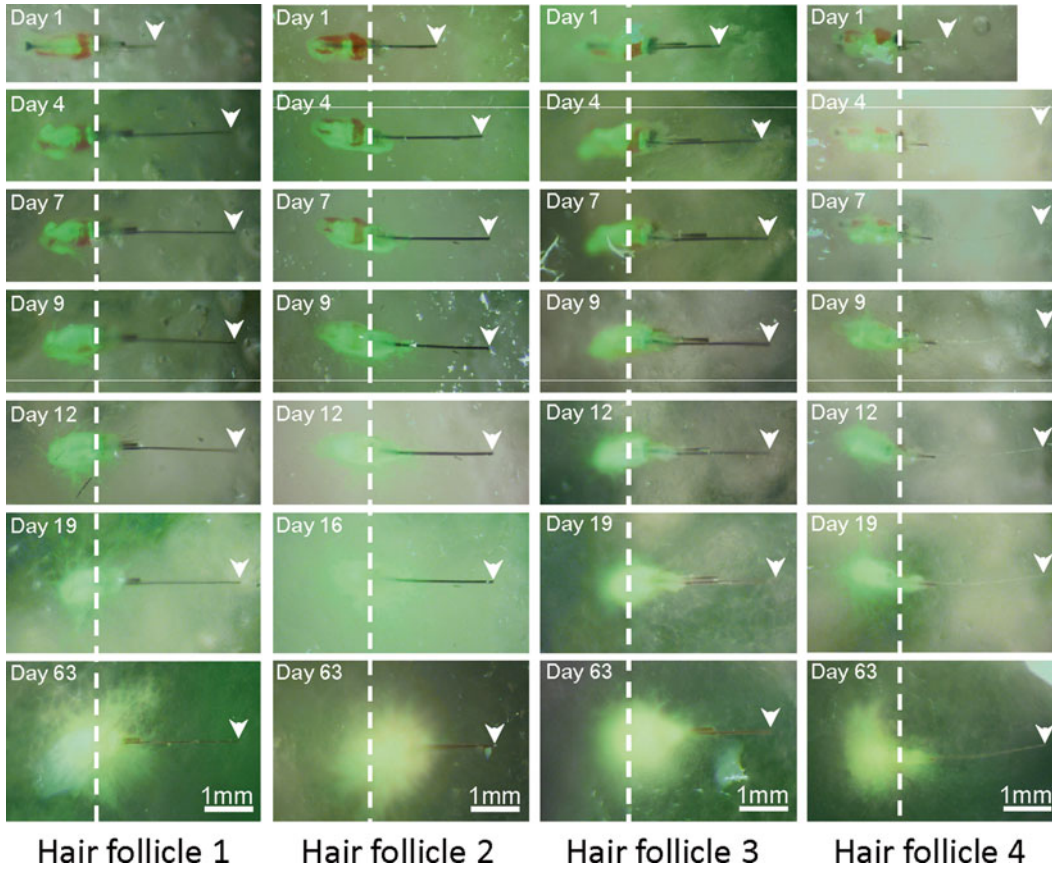

Fig. 1 Hair-shaft elongation of mouse whiskers in Gelfoam® histoculture. Time-course images of hair-shaft growth from individual mouse whisker follicles, isolated from nestin-driven-green fluorescent protein (ND-GFP) mice, histocultured on Gelfoam®. Green fluorescence was from the ND-GFP-expressing hair follicle associated pluripotent (HAP) stem cells in the whisker hair follicles which were enriched during 63 days of histoculture in vitro. Hair shafts lengthened rapidly in the first 4 days, extended over 9–12 days, and remained the same length until day 63 [13]

follicles, with their capsule, are then placed on Gelfoam® histoculture. Hair-shaft length in the follicles increased by 1.32 ± 0.27 mm by day 4 compared to day 1; still growing at day 7 (1.42 ± 0.24 mm) and at day 9 (1.46 ± 0.24 mm). By day 12, the hair-shaft length was 1.50 ± 0.22 mm ($p < 0.001$ compared to day 1) and remained constant until day 63 (*see* Figs. 1 and 2). At day 63 of hair-follicle histoculture, the ND-GFP-expressing HAP stem cells had a large increase in relative fluorescence intensity and fluorescent area ($p < 0.001$ for both). The large increase in ND-GFP expression of the HAP stem cells indicates their extensive proliferation and activity, as well as the very long-term viability of the follicles in Gelfoam® histoculture. Thus ND-GFP-expressing HAP stem cells increased over the 63-day histoculture period even though hair-shaft elongation appeared to cease by day 12 (*see* Figs. 2 and 3).

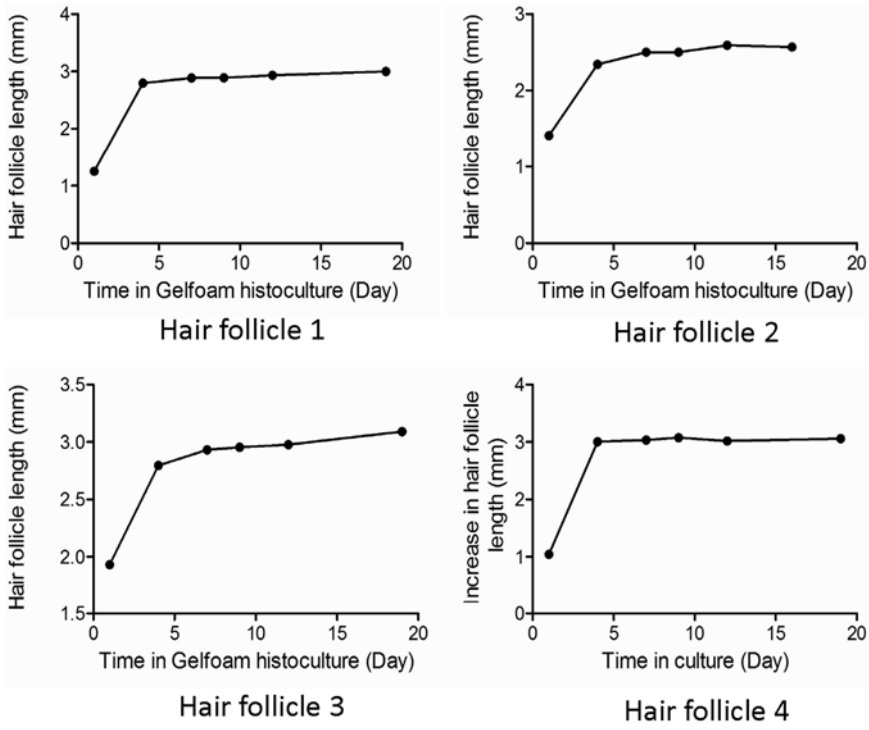

Fig. 2 Graphs quantifying the increase of shaft length over time in individual follicles during Gelfoam® histoculture [13]

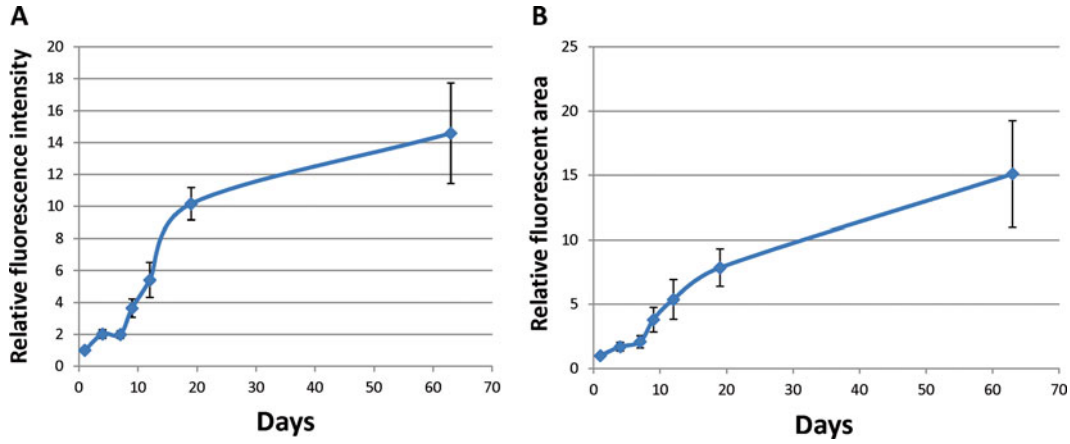

Fig. 3 Graphs quantifying the time-course increase of HAP stem cell GFP fluorescence intensity (**a**) and fluorescent area (**b**). $p<0.01$ in increase of fluorescent area and fluorescence intensity at day 63 compared to day 1 [13]

5. The present chapter describes protocols for improved hair-shaft growth from isolated hair follicles in Gelfoam® histoculture. Isolated free-floating follicles were previously observed to produce elongating hair shafts but apparently were not viable for very long periods of time [15, 16]. Gelfoam® histoculture maintained viability of the follicles and their HAP stem cells for at least 63 days, much longer than free-floating follicles. Gelfoam® also supports ectopic hair growth for the spine after transplantation of

Gelfoam® whisker histocultures [13]. The protocols described in the present chapter can enable long-term experimentation. It is notable that the greatest increase in stem cell fluorescence is during the period of rapid hair-shaft elongation (*see* Figs. 2 and 3).

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