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Robert M. Hoffman *Editor*

Multipotent Stem Cells of the Hair Follicle

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Edited by

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Preface

This volume describes methods for the study of multipotent and pluripotent stem cells of the hair follicle. The stem cells described are involved in both the growth of the hair follicle and its production of the hair shaft, as well as in growth of the hair follicle sensory nerve. Also described in this volume are very unexpected results accumulated in the first 15 years of the present century demonstrating a set of hair follicle-associated pluripotent (HAP) stem cells which not only have the capability for regenerating the hair follicle sensory nerve, but also can differentiate ex vivo and in vivo to multiple cell types not associated with the hair follicle, including glial cells, motor neurons, and beating cardiac muscle cells. The potential for HAP stem cells for regenerative medicine is also described in detail.

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Chapter 1

Introduction to Hair-Follicle-Associated Pluripotent Stem Cells

Robert M. Hoffman

Abstract

Nestin-expressing stem cells of the hair follicle, discovered by our laboratory, have been shown to be able to form outer-root sheaths of the follicle as well as neurons and many other non-follicle cell types. We have termed the nestin-expressing stem cells of the hair follicle as hair-follicle-associated pluripotent (HAP) stem cells. We have shown that the HAP stem cells from the hair follicle can effect the repair of peripheral nerve and spinal cord injury. The hair follicle stem cells differentiate into neuronal and glial cells after transplantation to the injured peripheral nerve and spinal cord, and enhance injury repair and locomotor recovery. When the excised hair follicle with its 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. These findings indicate that βIII-tubulin-positive fibers elongating from the whisker follicle sensory nerve stump were growing axons. The growing whisker sensory nerve was highly enriched in HAP stem cells, which appeared to play a major role in its elongation and interaction with other nerves in 3D Gelfoam® histoculture, including the sciatic nerve, the trigeminal nerve, and the trigeminal nerve ganglion. These results suggest that a major function of the HAP stem cells in the hair follicle is for growth of the follicle sensory nerve. Recently, we have shown that HAP stem cells can differentiate into beating cardiac muscle cells. HAP stem cells have critical advantages for regenerative medicine over embryonic stem (ES) cells and induced pluripotent stem (iPS) cells in that they are highly accessible from each patient, thereby eliminating immunological issues since they are autologous, require no genetic manipulation, are non-tumorigenic, and do not present ethical issues.

Key words Hair follicle, Bulge, Nestin, Pluripotent, Stem cells, GFP, Neurons, Schwann cells, Sciatic nerve, Spinal cord repair, Cardiac muscle cells

1 Introduction

1.1 Hair-Follicle-Associated Pluripotent (HAP) Stem Cells

Our laboratory discovered nestin-expressing hair-follicle-associated pluripotent (HAP) stem cells. This discovery resulted in a new era of adult stem cells [1].

The totally unexpected serendipitous discovery of nestin-expressing HAP stem cells was with the use of transgenic mice in which the regulatory elements of the neural stem cell marker, nestin, drive the expression of green fluorescent protein (GFP)

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(ND-GFP). We visualized the HAP stem cells by their bright GFP expression in the hair follicle. The HAP stem cells are relatively small, oval-shaped, surrounding the hair shaft and interconnected by short dendrites. In mid- and late-anagen, the HAP stem cells were located in the upper outer-root sheath as well as in the bulge area but not in the hair matrix bulb. Mignone et al. [2] have confirmed these results in ND-GFP mice. Yu et al. [3] showed that nestin was present in human hair follicle stem cells confirming our original observation in mice [1]. Yu et al. suggested that the nestin-expressing stem cells isolated from the human hair follicle are different from keratinocyte stem cells and melanocyte stem cells which were also present in the hair follicle. These observations indicate that the hair follicle probably contains several distinct populations of stem cells [4–6].

1.2 HAP Stem Cells Differentiate to Many Types of Cells

After our discovery of HAP stem cells, we originally predicted HAP stem cells could differentiate to neurons and possibly other cell types [1]. Subsequently, it was demonstrated that nestin-expressing HAP stem cells could differentiate to neurons, glia, keratinocytes, smooth muscle cells, melanocytes, and heart muscle cells [7–13]. The existence of HAP stem cells has been confirmed by at least four laboratories [2, 3, 7, 8, 12, 14–19].

1.3 HAP Stem Cells Can Repair Severed Nerves

Mouse HAP stem cells were injected in the region of a severed sciatic nerve of nude mice which subsequently rejoined. The regenerated nerve recovered function and contracted the gastrocnemius muscle upon electrical stimulation [20]. HAP stem cells can be readily isolated from the human scalp and can be used to regenerate the injured mouse sciatic nerve [16].

1.4 Human HAP Stem Cells

Yu et al. [3, 19] characterized human nestin-expressing HAP stem cells. These cells expressed Nanog and Oct4 which are neural-crest and neuron stem-cell markers as well as embryonic stem-cell transcription factors. The human HAP cells formed spheres in vitro and differentiated into myogenic, melanocytic, and neuronal cell lineages in single-cell culture. The human nestin-expressing HAP stem cells also differentiated into adipocyte, chondrocyte, and osteocyte lineages [8].

When transplanted to a severed sciatic nerve in mice, human HAP stem cells differentiated into glial fibrillary-acidic-protein (GFAP)-positive Schwann cells and promoted the recovery of pre-existing axons, leading to nerve generation upon transplantation to the severed sciatic nerve [16].

1.5 HAP Stem Cells Can Effect Spinal Cord Repair

Mouse HAP stem cells were injected into the injured spinal cord of nude mice. The HAP stem cells promoted the recovery of the

injured spinal cord, by differentiating into glial-like cells with subsequent locomotor improvement [15, 21].

1.6 HAP Stem Cells Originate in the Bulge and Can Traffic to the Dermal Papilla

HAP stem cells are present in the BA throughout the hair cycle, but in the DP only in early anagen where they have apparently migrated from the bulge. HAP stem cells from both regions have very long processes extending from them [21–24].

HAP stem cells from the BA trafficked to the DP as well as into the epidermis, including during wound healing, indicating that the bulge is the source of HAP stem cells [22].

HAP stem cells from both the BA and the DP could differentiate into neurons and other cell types in vitro. HAP stem cells from both the BA and the DP had equal capabilities for functional spinal cord repair into Schwann and neural type cells [21].

1.7 HAP Stem Cells Can Differentiate to Motor Neurons and Reduce Muscle Due to Atrophy Nerve Injury

HAP stem cells expressing RFP were induced by retinoic acid and fetal bovine serum to differentiate, and when transplanted together with Matrigel into the transected distal sciatic or tibial nerve stump of nude mice, differentiated into neurons with large round nuclei and long extensions expressing the neuron marker Tuji1 as well as motor neuron markers Isl 1/2 and EN1. Muscle fiber areas in the HAP stem cell-transplanted animals were much larger than those in control animals. HAP stem cells can thus differentiate into motor neurons and reduce muscle atrophy after peripheral nerve transection [25]. These results suggest HAP stem cells may have regenerative potential for ALS as well.

1.8 HAP Stem Cells Produce Neurons in 3D Gelfoam Histoculture

It was observed over a 2-week period of Gelfoam® histoculture of whiskers that HAP stem cells trafficked from the BA toward the DP area and extensively grew out onto the Gelfoam® forming nerve-like structures [24].

In a subsequent study, mouse vibrissa hair follicles, including their sensory nerve stump, were excised from ND-GFP mice and were placed in Gelfoam® histoculture. HAP stem cells in the nerve stump produced β-III tubulin-positive fibers, extending up to 500 mm from the whisker nerve stump, with their tips expressing F-actin indicating they were growing axons [26].

1.9 HAP Stem Cells Can Differentiate to Beating Heart Muscle Cells

Mouse vibrissa hair follicle were separated into three parts (upper, middle, and lower), and each part was suspended separately in DMEM containing 10% FBS. All three parts of hair follicle differentiated to beating cardiac muscle cells as well as neurons, glial cells, keratinocytes, and smooth muscle cells. The differentiation potential to cardiac muscle is greatest in the upper part of the hair follicle. The beat rate of the cardiac muscle cells was stimulated by isoproterenol and inhibited by propanolol [10].

2 Conclusions

HAP stem cells originate in the BA [1] and migrate to the DP [22, 24] and hair-follicle-associated nerve [26].

Mouse HAP stem cells can differentiate into many cell types including neurons, glial cells, and heart muscle cells [7, 11, 12]. HAP stem cells can effect peripheral nerve [7, 16] and spinal cord repair [15, 21] by differentiating into neural and glial type cells. Human HAP stem cells have also been shown to be multipotent and can effect repair of peripheral nerves [7, 16].

Compared to embryonic stem cells or iPS cells [27–29], HAP stem cells are superior in that they can be used autologously, they are non-oncogenic, and do not have ethical issues.

Recently, Sakaue and Sieber-Blum [30] have shown that highly pure populations of human Schwann cells can be formed from HAP stem cells from the BA of hair follicles. Ex vivo expansion of the HAP stem cells isolated from hair bulge explants and manipulation of WNT, sonic hedgehog and TGFβ expression as well as incubation with growth factors resulted in differentiation to Schwann cells expressing SOX10, KROX20 (EGR2), p75NTR (NGFR), MBP, and S100B by day 4 in virtually all cells. The Schwann cells matured by 2 weeks. In co-culture of the HAP stem cells-derived human Schwann cells with rodent dorsal root ganglia, the Schwann cells interacted with axons. These results confirm our earlier conclusions and results [1, 7, 20, 21, 25, 26, 31].

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Chapter 2

Nestin-Based Reporter Transgenic Mouse Lines

John Mignone, Natalia Peunova, and Grigori Enikolopov

Abstract

Nestin expression marks stem and progenitor cells of the neural lineage. Transgenic mouse lines, originally generated to identify neural stem cells, can also help to identify, track, and isolate stem and progenitor cells in a range of tissues of the ectodermal, endodermal, and mesodermal origin. Here, we describe the generation of transgenic mouse lines expressing fluorescent proteins (FP) under the control of critical regulatory elements of the nestin gene and their use for identifying and analyzing adult stem and progenitor cells in various tissues.

Key words Nestin, Hair-follicle-associated pluripotent (HAP), Stem cells, Transgenic animals, Reporter lines, Immunocytochemistry, Nestin-GFP

1 Introduction

Nestin is an intermediate filament protein, which became a valuable reporter of stem cells. First identified over 20 years ago [1], it gradually became a standard marker of stem and progenitor cells of the neural lineage and, more recently, of several other lineages as well. The first forays that characterize the gene were made when monoclonal antibodies against embryonic neural tissue were raised [2] and one of the clones (Rat-401) was shown to recognize an epitope of a novel type IV intermediate filament protein. This was followed by the cloning of the gene, designated as nestin, for its expression in neuroepithelial stem cells [1]. Nestin is expressed in proliferative zones of the embryonic and adult mammalian brain and is not detected in differentiated cells of neural tissue.

The gene for nestin has been thoroughly studied and several crucial regulatory elements that are responsible for expression in embryonic (and, later, in adult) tissues have been identified [3–5]. The most important element controlling nestin expression in cells of neuroepithelial and neural stem lineage is a strong transcriptional enhancer located in the second intron of the mouse, rat, and human nestin gene. This enhancer contains POU/Pbx binding sites that

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are crucial for nestin expression in neural stem cells. The neural enhancer is so strong and specific that it can reliably direct expression of heterologous genes, even when combined with unrelated promoters. These properties of the nestin neural enhancer have been used to generate lines of transgenic animals expressing fluorescent proteins (GFP or other colors) in the developing and adult nervous system [6–8]. Several lines of evidence demonstrate that FP-positive cells in these nestin-based reporter transgenic animals accurately represent neural stem and early progenitor cells and cover the vast majority of such cells [7, 9–12]. These reporter animals can be used to identify neural stem and early progenitor cells, track them in vivo, isolate them for profiling or culturing, determine quantitative changes, and detect their proliferation, differentiation, or death.

Remarkably, after the nestin-based transgenic mouse lines have been shown to reliably highlight stem cells of the neural lineage, it gradually became apparent that their utility is broader and that nestin-driven transgenes can report the location of stem and progenitor cells in other tissues as well. The panel of tissues in which expression of nestin-FPs reveals cells with stem-like properties is continuously growing, but even an incomplete list includes liver (oval cells; [13]), anterior pituitary (multipotent adult stem cells; [14]), bone marrow (mesenchymal stem cells; [15]), testes (precursors of testosterone-producing Leydig cells; [16]), muscle (satellite cells; [17]), skin [18], and hair-follicle-associated pluripotent (HAP) stem cells [19, 20, 26–30]. This provides the ability to identify and analyze stem and progenitor cells of these tissues in the same animal, thus facilitating experimental design and helping to reveal potential coordinated changes in the stem cell compartments of various tissues of the same reporter animal. Here, we describe the generation of nestin-FP transgenic animals and their use for detecting and counting tissue-specific stem and progenitor cells.

2 Materials

2.1 Cloning, Isolation of Tail DNA, PCR

Standard reagents and procedures are described in [21, 22] and in paragraphs below. Restriction enzymes, DNA ligase, and DNA polymerase are purchased from New England Biolabs (Beverly, MA). Plasmid isolation columns and QIAquick gel extraction kit are purchased from Qiagen (Santa Clarita, CA). AmpliTaq© enzyme and 1× AmpliTaq© PCR buffer are purchased from Boehringer-Mannheim (Indianapolis, IN) and pBluescript vector (pBSM13+, Stratagene, La Jolla, CA).

2.2 Immunocytochemistry

1. Polyclonal antibody to GFP (host—chicken; Aves Laboratories, Tigard, OR) and is used at a working dilution 1:400.
2. Monoclonal antibody to GFP (host—mouse; Developmental Studies, Hybridoma Bank, Iowa City, IA) and is used at working dilution of 1:1000.

3. Polyclonal antibodies to 5-bromo-2-deoxyuridine (BrdU) (host—rat; Accurate Chemicals, Westbury, NY).
4. AlexaFluor 488 goat anti-chicken antibody (Molecular Probes, Eugene, OR). Secondary antibodies are used at a working dilution 1:500.
5. AlexaFluor 594 goat anti-mouse antibody (Molecular Probes, Eugene, OR). Secondary antibodies are used at a working dilution 1:500.
6. AlexaFluor 568 goat anti-rat antibody (Molecular Probes, Eugene, OR). Secondary antibodies are used at a working dilution 1:500.
7. BrdU, paraformaldehyde, Triton X-100, sodium azide, PBS, and goat serum (Sigma, St. Louis, MO).

3 Methods

3.1 Generation of Nestin-FP Transgenic Animals

Here, we describe the production of reporter transgenes and transgenic animals. Procedures are described using the original nestin-GFP line [7] as an example.

3.1.1 Nestin-ZGF and Nestin-GFP Expression Constructs

To generate the expression vector, we used plasmids containing the nestin promoter and the second intron from the rat nestin gene that were generously provided by Drs. R. McKay and L. Zimmerman. We also used constructs containing an enhanced version of GFP, a polyadenylation sequence from the genome of simian virus 40 (SV40), and a pBluescript vector. The major steps for generating the nestin-ZGF expression vector and nestin-GFP constructs are as follows:

1. A cloned SV40 fragment containing a polyadenylation sequence (polyA) was digested with XbaI and BamHI restrictases and the resulting 0.25 kb long fragment was subcloned into pBSM13+ vector. The Xbal site of the resulting polyA-pBSM13+ plasmid was then blunt-ended by treatment with Klenow DNA polymerase. A 5′-AGGCGCGCCT-3′ linker containing a recognition sequence for AscI was cloned into this site, reestablishing the Xbal sites on either side of the now-present AscI restriction site.
2. A 1.8 kb fragment containing the second intron of the rat nestin gene was isolated using BamHI and SmaI restrictases and then inserted into the polyA-pBSM13+ plasmid near the 3′-end of the polyA sequence (polyA-intron2-pBSM13+ plasmid). Then the HindIII site of the plasmid was cleaved, blunt-ended, and re-ligated, thus creating an NheI site.
3. A 5.8 kb fragment containing the promoter of the rat nestin gene was isolated using SpeI and SalI restrictases and inserted

into the polyA-intron2-pBSM13+ plasmid opened by NheI and SalI enzymes (the SpeI restriction site is compatible with the NheI site), placing the nestin promoter near the 5′-end of the polyA sequence. The resulting nestin promoter-polyA-intron2-pBSM13+ plasmid (nestin-ZGF) was used as a basic vector for cloning genes of interest (e.g., GFP).

4. An 0.82 kb fragment containing EGFP was isolated from the pEGFP-N1 plasmid (Clontech) using the NotI enzyme, blunt-ended by Klenow DNA polymerase, and then ligated to an AscI linker (as above). This created an AscI restriction site in place of the NotI site. The XmaI restriction site of the EGFP-N1 5′ polylinker was blunt-ended and religated in order to destroy the SmaI site.
5. The resulting eGFP fragment was then digested with SalI and AscI enzymes, creating a 780 bp DNA fragment which was ligated into the nestin-ZGF plasmid digested with SalI and AscI, positioning it between the nestin promoter and polyA site (nestin-GFP plasmid). The same strategy was used for inserting other genes of interest (e.g., CFPnuc, dTimer, mCherry).

3.1.2 Generation of Transgenic Mice

1. The nestin-GFP plasmid was purified using cesium chloride centrifugation or a Qiagen plasmid isolation column (Qiagen, Santa Clarita, CA).
2. 10 μg of the purified plasmid was digested with the restriction enzyme SmaI.
3. The DNA fragment containing the nestin promoter-EGFP-polyA-intron2 sequences was separated from the pBSM13+ on an 0.8% agarose gel.
4. The band was cut from the agar and purified using the Qiagen QIAquick gel extraction kit.
5. 3 μg of the isolated and purified fragment was used for generating transgenic mice.
6. DNA was injected into the pronuclei of ~500 oocytes of the C5BL/6xBalb/cBy hybrid mouse strain (or other appropriate mouse strain) using established procedures [21].
7. The injected oocytes were then transferred to 12 pseudo-pregnant female mice. 80–100 pups were born in a typical experiment and transgenic animals were determined using PCR.

3.1.3 Genotyping of Nestin-GFP Transgenic Mice

1. DNA was isolated from tails using established procedures [21].
2. The sequences of the primers used for PCR were 5′-GATCACTCTCGGCATGGACGAGC-3′ (corresponding to the last 40 bases of the EGFP sequence) and 5′-GGAGCTGCA CACAACCCATTGCC-3′ (corresponding to 225 bases into the nestin second intron).

3. PCR was performed in 30 μl containing 1.5 mM $MgCl_2$, 1× AmpliTaq© PCR buffer (Boehringer-Mannheim, Indianapolis, IN), 0.2 nM each of dNTP, 0.4 μM of each primer, and 1 U AmpliTaq© (Boehringer Mannheim).
4. Thirty-five cycles of PCR with an annealing temperature of 65° (30 s) and an extension temperature of 72° (1 min) were used.
5. The expected fragment of 510 bp was detected in eight out of the 86 F-0 mice (in the experiment described in [7]. Of these eight transgenic mice, three were male and five were female.
6. Seven transgenic animals produced progeny, which was analyzed using flow cytometry and immunohistochemistry. All seven substrains demonstrated the expected pattern of EGFP expression. Choice of strain was eventually limited to two after demonstrating high sensitivity and specificity for EGFP cell labeling.

3.1.4 Genotyping of Nestin-GFP Transgenic Mice (Alternative Protocol)

1. Primers: 5′-ATCACATGGTCCTGCTGGAGTTC-3′ (GFP, GFP, or YFP) and 5′-GGAGCTGCACACAACCCATTGCC-3′ (nestin second intron).
2. PCR performed in 25 μl containing 2.5 mM $MgCl_2$, 1× AmpliTaq© PCR buffer, 0.2 nM of each dNTP, 0.5 μg of each primer, and 1 U AmpliTaq©.
3. Cycling conditions: step 1—94° for 3 min; step 2—94° for 30 s, 62° for 45 s, 72° for 45 s; repeat step 2 for a total of 32 cycles; step 3—72° for 2 min; step 4—hold at 4°. Product size—700 bp.

3.2 Immunocytochemical Detection of Nestin-FP-Expressing Stem and Progenitor Cells

In many cases, the fluorescence signal of nestin-FP cells can be detected directly using an epifluorescence or confocal microscope. However, in some tissues this signal is too weak to detect directly and requires amplification by immunocytochemistry. Furthermore, immunocytochemical detection of FPs is required when fluorescence is abolished by a particular procedure, for example, by treatment of samples with acid when fluorescent proteins are analyzed in conjunction with detection of BrdU-labeled proliferating cells. Here, we describe immunocytochemical detection of dividing (BrdU-labeled) cells expressing nestin-GFP, nestin-CFP, or nestin-YFP cells in sections of adult mouse brains. The same protocol can be used for most of the other (neural or non-neural) tissues. Further details for immunocytochemistry, microscopy, and cell quantitation can be found in [9, 12, 23–25].

1. Nestin-FP mice are injected with BrdU (150 mg/kg) 2 or 24 h before perfusion.
2. Mouse tissues are fixed by transcardial perfusion with 30 ml phosphate buffered saline (PBS) and 30 ml of 4% paraformaldehyde in PBS (pH 7.4). Brains are removed and further

post-fixed in 4% paraformaldehyde in PBS overnight at 4 °C; if necessary, samples are stored in PBS with 0.1% sodium azide at 4 °C until sectioning.

3. Brains are sagittally sectioned at a thickness of 50 μm with a vibratome. Sections are sequentially collected and subsets of sections at 300 μm intervals are taken for immunohistochemistry and analysis.
4. Sections are rinsed with PBS and denatured in 2 M HCl at 37 °C for 1 h for detection of BrdU-incorporating dividing stem and progenitor cells. The denatured sections are neutralized with 0.1 M borate (pH 8.0) twice for 20 min each.
5. Sections are rinsed with washing solution (PBS with 0.2% Triton X-100), and incubated for blocking and permeabilization in PBS with 2% Triton X-100 and 5% goat serum at room temperature for 2 h.
6. After rinses with washing solution, the sections are incubated at 4 °C overnight in antibody solution (PBS with 0.2% Triton X-100 and 3% goat serum) containing primary antibodies: chicken anti-GFP (1:400 dilution) and rat anti-BrdU (1:400 dilution). If necessary, mouse monoclonal anti-GFP antibody can be used.
7. The sections are rinsed with washing solution and incubated for 2 h at room temperature with Alexa Fluor-conjugated goat secondary antibodies (AlexaFluor 488 anti-chicken antibody and AlexaFluor 568 goat anti-rat antibody).
8. After rinses with washing solution, the sections are mounted on gelatin-coated slide glasses with DakoCytomation Fluorescent Mounting Medium and cover-slipped for microscopy.

4 Notes

1. In addition to PCR-based genotyping, nestin-GFP transgenic mice can be also identified by a characteristic green fluorescence in the brains of newborn mice, in the retina of adult live mice, or in the tails of adult live mice when analyzed at a minimum of 100× magnification.
2. Transgenic mice that are generated on a particular genetic background should be crossed to wild-type mice of a desired genotype (e.g., C57BL/6) for at least five generations.
3. Nestin-FP heterozygous transgenic mice can be bred to generate homozygous animals, which can then be crossed to other lines or to wild type mice without the need for further genotyping of the heterozygous progeny.

4. If additional antigens need to be analyzed by confocal microscopy (e.g., glial fibrillary acidic protein, GFAP), a rabbit polyclonal primary antibody (Sigma, cat. no. G9269) and AlexaFluor 594 goat anti-rabbit secondary antibody (cat. no. A11012) can be used. If the protocol necessitates the use of a mouse monoclonal antibody to GFP, Rat-401 antibody and AlexaFluor 594 goat anti-mouse antibody (cat. no. A11005) can be used.
5. The pattern of reporter expression in nestin-based transgenic lines is usually highly reliable in marking neuroepithelial cells in the developing embryo and neural stem and progenitor cells in the adult neurogenic zones (provided the construct contains the second intron enhancer element). However, the expression pattern can be different between different lines for non-neural tissues. This may reflect both the overall intensity of the signal (e.g., fluorescence failing to reach the threshold for detection in particular tissues) and subtle differences in the genomic integration site.

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Chapter 3

Discovery of HAP Stem Cells

Lingna Li and Robert M. Hoffman

Abstract

Cells expressing the stem cell marker, nestin, were selectively labeled in transgenic mice by placing green fluorescent protein (GFP) under the control of the nestin promoter in transgenic mice. In these transgenic mice, neural and other stem cells brightly expressed GFP. The mice were termed nestin-driven GFP (ND-GFP) mice. During early anagen or growth phase of the hair follicle, ND-GFP appeared in the permanent upper hair follicle immediately below the sebaceous glands in the follicle bulge. The relatively small, oval-shaped, nestin-expressing cells in the bulge area surrounded the hair shaft and were interconnected by short dendrites. The location of the nestin-expressing cells in the hair follicle varied with the hair cycle. During telogen or resting phase and in early anagen, the GFP-positive cells are mainly in the bulge area. However, in mid- and late-anagen, the GFP-expressing cells were located in the upper outer-root sheath as well as in the bulge area. The expression of the unique protein, nestin, in both neural stem cells and hair follicle stem cells, which suggested their relationship. The ND-GFP hair follicle stem cells were later termed hair-follicle-associated pluripotent (HAP) stem cells.

Key words Hair follicle, Bulge, Nestin, GFP

1 Introduction

Hair growth is a unique cyclic regeneration phenomenon. The hair follicle undergoes repeated cycles of periods of growth (anagen), regression (catagen), and rest (telogen) throughout the life of mammals. Stem cells for the outer-root sheath of the hair follicle were found in the bulge area (BA) [1, 2].

Taylor et al. [3] reported that hair follicle bulge stem cells were bipotent because they can give rise to the hair follicle as well as epidermal cells. Other experiments [1] also have shown that the upper outer-root sheath of whisker follicles of adult mice contains multipotent stem cells, which can differentiate into hair follicle matrix cells, sebaceous gland basal cells, and epidermis.

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We discovered the expression of nestin, a marker for neural progenitor cells, in the cells of the follicle bulge. The nestin promoter was linked to GFP, allowing us to observe that the nestin-expressing cells formed the major part of the hair follicle in each cycle [7].

2 Nestin-GFP Transgenic Mice

Nestin is an intermediate filament (IF) gene that is a marker for central nervous system (CNS) progenitor cells and other stem cells [4]. Enhanced green fluorescent protein (EGFP) transgenic mice carrying green fluorescent protein (EGFP) under the control of the nestin promoter were originally developed for studying and visualizing the self-renewal and multipotency of CNS stem cells [4–6]. We have termed these mice as nestin-driven GFP (ND-GFP) mice. We discovered ND-GFP expression in the hair follicle while trying to image these cells externally in the brain. The skin expressing GFP was so bright that we could not image the brain. Upon opening of the skin, we discovered ND-GFP expressing cells in the BA and outer-root sheath [7].

3 Induction of Anagen

In order to induce anagen, ND-GFP transgenic mice, 6–8 weeks old, in the telogen phase of hair growth were depilated by a hot mixture of rosin and beeswax [7].

4 Fluorescence and Confocal Microscopy

The ND-GFP-expressing skin samples, after dissection, were directly observed with dermis up and epidermis down under a fluorescence microscope (Nikon, Tokyo, Japan). An MRC-600 confocal imaging system (Bio-Rad, Hercules, CA) mounted on a Nikon Optiphot with a 10× PlanApo objective was also used [7].

5 Immunohistochemical Staining

Colocalization of nestin and GFP in the paraffin-embedded ND-GFP mouse skin sections was detected with a DAKO ARK animal research kit (nestin) and DAKO EnVision doublestain system following the manufacturer's instructions [7].

The cells with ND-GFP expression were located in the permanent upper region of telogen hair follicles immediately below the sebaceous glands and in the bulge area (Figs. 1–3). These cells are relatively small, oval- or round-shaped, and interconnected by dendrite-like structures (Fig. 2) [7].

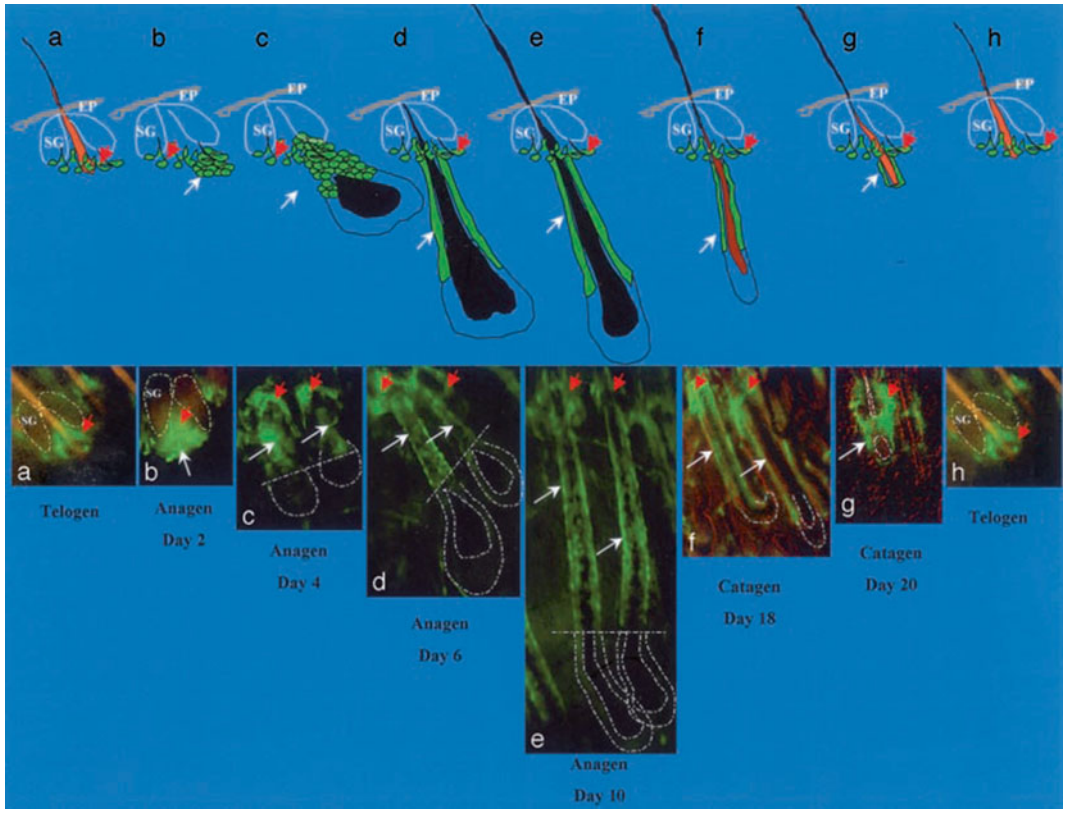

Fig. 1 Hair follicle stem cells in the hair growth cycle. (*Upper*) Cartoon showing position of nestin-driven (ND-GFP) expressing GFP stem cells at each stage of the hair follicle cycle. *SG* sebaceous gland, *EP* epidermis. (*Lower* **a**) ND-GFP-expressing hair follicle stem cells (*red arrow*) located in the hair follicle bulge area in telogen phase. (**b**) Day 2 after anagen induction by depilation. Note the new hair follicle cells (*white arrow*) formed directly from the bulge nestin-GFP-expressing stem cells. (**c**–**e**) Day 4 (**c**), day 6 (**d**), and day 10 (**e**) after anagen induction by depilation. Note the ND-GFP-expressing outer-root sheath cells (*white arrows*) in the upper two-thirds of the hair follicle. (**f**, **g**) Day 19 (**f**) and day 20 (**g**) after depilation. Note in (**f**) and (**g**) that the hair follicles are in the catagen phase and are undergoing regression and degeneration, including the ND-GFP-expressing cells in the outer-root sheath. The bulge area ND-GFP-expressing stem cells remain. (**h**) Hair follicle cycling in telogen phase [7]

6 Results

The location and number of the nestin-expressing cells was hair-cycle-dependent (Fig. 1). The progression and proliferation of the ND-GFP cells in the developing hair follicle was followed in detail in mice (6–8 weeks old) after inducing anagen in telogen follicles by depilation. At telogen, the ND-GFP cells in the hair follicles were located only at the upper permanent bulge region (Figs. 1a, 2, and 3). Two to 3 days after depilation, ND-GFP cells proliferated, migrating down from the bulge (Figs. 1b, c and 3). During the middle and late anagen phases, the ND-GFP-expressing hair follicle cells occupied the upper two-thirds of the outer-root sheath and were absent from the lower one-third of the follicle and the hair matrix bulb (Fig.

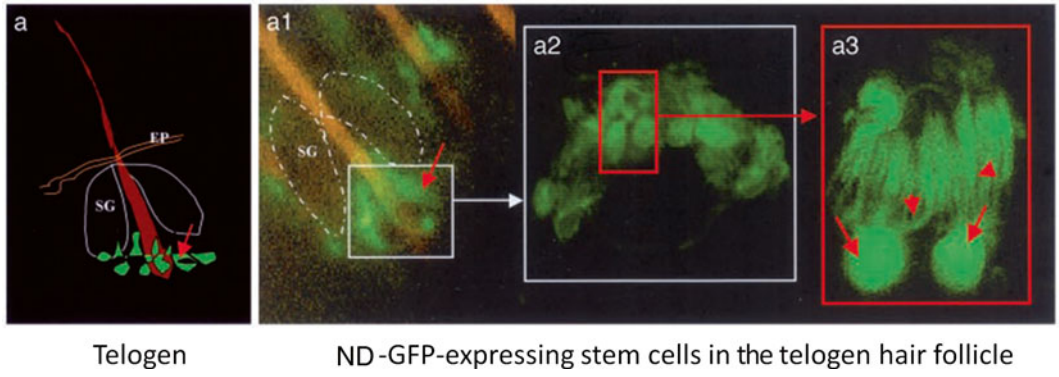

Fig. 2 Hair follicle ND-GFP-expressing cells in the telogen phase of ND-GFP transgenic mouse skin. The skin sample was prepared freshly right after excision from the back skin of a ND-GFP transgenic mouse. The skin sample then was directly observed by fluorescence or confocal microscopy with the dermis side up after subcutaneous tissue was dissected out. (**a**) Cartoon of telogen hair follicle showing position of ND-GFP-expressing hair follicle stem cells. (**a1**) Low-magnification fluorescence-microscopy image showing the ring of bulge ND-GFP-expressing stem cells (*small white box*, see **a**). (**a2**) High-magnification confocal-microscopy image reflecting the small white box in (**a1**). Note the small round- or oval-shaped ND-GFP-expressing cells in the bulge area of the hair follicle (*small red box*). (**a3**) High-magnification fluorescence-microscopy image showing two individual ND-GFP-expressing stem cells reflecting the red box in (**a2**). Note the unique morphology of the hair follicle stem cells and multiple dendrite-like structures of each cell. *Red arrows* indicate the cell body, and *red arrowheads* show the multiple dendritic structure of each cell. (Original magnifications: **a1**, 100×; **a2**, 400×; **a3**, 1600×). SG: sebaceous gland, EP: epidermis [7]

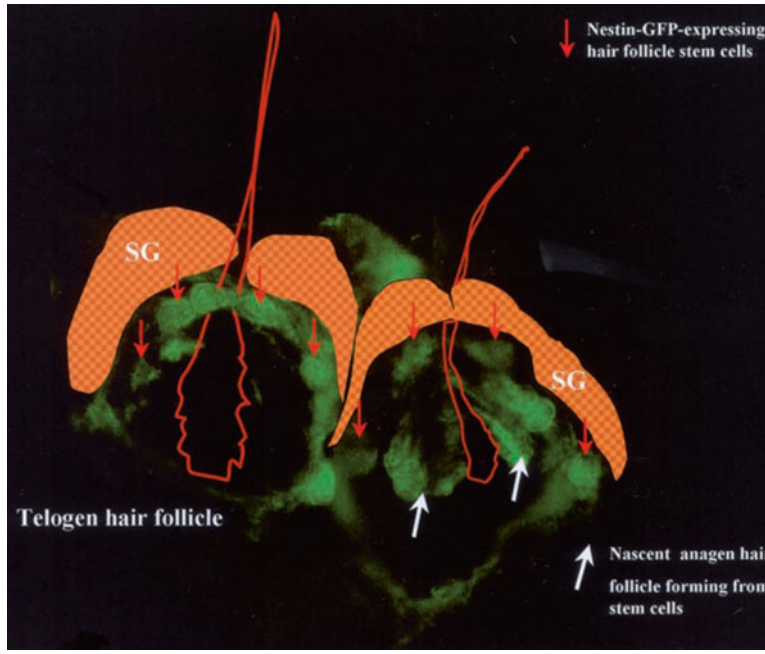

Fig. 3 Telogen–anagen transition showing ND-GFP-expressing hair follicle stem cells forming nascent hair follicles. The image was taken by fluorescence microscopy 18 h after depilation. Note the bulge ND-GFP-expressing hair follicle stem cells in the telogen-phase (left hair follicle, *red arrows*) and the nascent anagen hair follicle directly formed from the bulge ND-GFP-expressing stem cells (right hair follicle, *white arrows*). (Original magnification, 400×) [7]

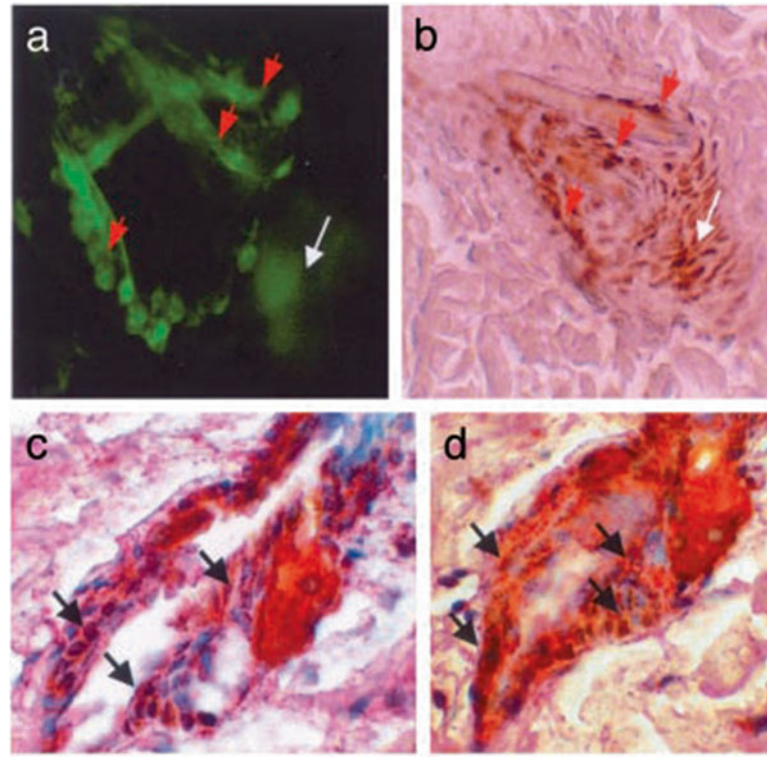

Fig. 4 Colocalization of GFP and nestin in hair follicle bulge stem cells and outer-root sheath cells determined by immunohistochemical staining. (**a**) Confocal image of live tissue showing nestin-GFP-expressing hair follicle bulge stem cells (*red arrows*) forming the nascent anagen hair follicle (*white arrow*). (**b**) Paraffin-embedded tissue section immunohistochemically stained with nestin antibody showing the localization of nestin expression in the nascent hair follicle and bulge stem cells. *Red arrows* indicate nestin-positive hair follicle bulge stem cells. The *white arrow* indicates nestin-positive nascent hair follicle. (**c**, **d**) GFP is detected by chromogen fast red. (Magnification, 400×) [7]

1c–e). Figure 1c–g shows ND-GFP-expressing outer-root sheath cells during the entire anagen and catagen phases. In catagen, when hair bulb matrix cells underwent regression and degeneration, the number of outer-root sheath ND-GFP-expressing cells decreased along with shrinkage of the hair follicle. Eventually, by the next telogen these cells localized only in the bulge (Fig. 1h) [7].

Nestin and GFP colocalized in the hair follicle bulge cells, outer-root sheath cells, and basal cells of the sebaceous glands as shown by immunohistochemistry staining (Fig. 4) [7].

These original findings suggested a possible relation between the ND-GFP hair follicle cells and neural stem cells. Subsequent studies in ours and other laboratories confirmed our hypothesis as reviewed in other chapters in the present volume, that for ND-GFP cells in the hair follicle are indeed hair-follicle-associated pluripotent (HAP) stem cells [8] that can differentiate to neurons, glial cells, beating cardiac muscle cells, and many other cell types [9–13]

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Chapter 4

Peripheral-Nerve and Spinal-Cord Regeneration in Mice Using Hair-Follicle-Associated Pluripotent (HAP) Stem Cells

Yasuyuki Amoh, Kensei Katsuoka, and Robert M. Hoffman

Abstract

Nestin, a neural stem cell marker protein, is expressed in hair follicle cells above the bulge area. These nestin-positive hair follicle-associated-pluripotent (HAP) stem cells are negative for the keratinocyte marker K15 and can differentiate into neurons, glia, keratinocytes, smooth muscle cells, cardiac muscle cells, and melanocytes in vitro. HAP stem cells are positive for the stem cell marker CD34, as well as K15-negative, suggesting their relatively undifferentiated state. HAP stem cells promoted the functional recovery of injured peripheral nerves and the spinal cord. HAP stem cells differentiated into glial fibrillary acidic protein (GFAP)-positive Schwann cells when implanted in severed sciatic nerves and spinal cords in mice. These results suggest that HAP stem cells provide an important accessible, autologous source of adult stem cells for regenerative medicine, that have critical advantages over ES and iPS stem cells.

Key words Hair follicle, Bulge area, Nestin, Stem cell, Pluripotent, Differentiation, Nerve, Spinal cord, Repair

1 Introduction

The stem cell marker, nestin, is expressed in hair follicles in cells located above the bulge area (BA), below the sebaceous gland. The nestin-expressing hair follicle cells were discovered in transgenic mice with ND-GTP-driven green fluorescent protein (ND-GFP) [1–4]. The nestin-expressing cells of the hair follicle can differentiate into neurons, glial cells, smooth muscle cells, keratinocytes, and other cell types [3]. Nestin-expressing hair follicle stem cells can also differentiate to beating cardiac muscle cells [5]. We have termed these cells as hair-follicle-associated pluripotent (HAP) stem cells [6]. HAP stem cells were shown to effect functional-nerve and spinal-cord repair [7–11].

HAP stem cells originate in the BA and migrate to the dermal papilla (DP) (Fig. 1). HAP stem cells from the DP and BA differentiated into neuronal and glial cells after transplantation to the

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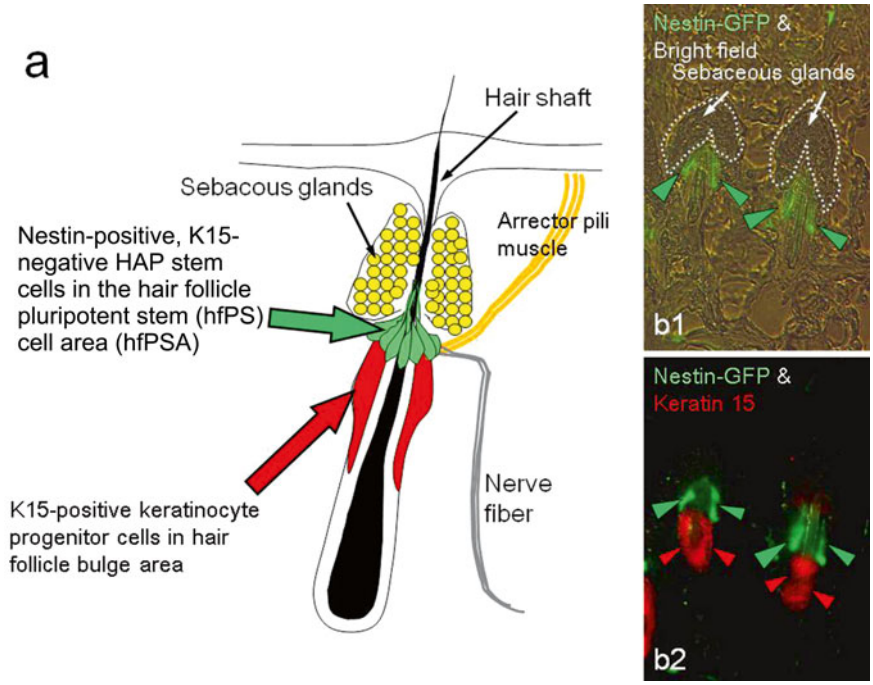

Fig. 1 Nestin-positive, K15-negative HAP stem cells and nestin-negative, K-15 positive keratinocyte progenitor cells are in seprate locations in the hair follicles [9]

injured nerve or spinal cord and enhanced injury repair and locomotor recovery within 4 weeks [2–4, 11].

In Gelfoam® histoculture, HAP stem cells from mouse whisker follicles formed nerve-like structures containing β-III tubulin-positive fibers [12]. The growing fibers had growth cones on their tips expressing F-actin, indicating they were growing axons. These results suggest a major function of HAP stem cells is for growth of the follicle sensory nerve [12]. HAP stem cells can be cryopreserved with full function [13].

2 Materials

2.1 Reagents

1. Transgenic mice with nestin-regulatory-element-driven green fluorescent protein (ND-GFP mice) (AntiCancer Inc., San Diego, CA).
2. GFP-expressing transgenic mice (GFP mice) (AntiCancer Inc.).
3. Non-transgenic nude mice (AntiCancer Inc.).
4. Human scalp skin samples. (The human scalp skin samples were obtained from surgical specimens of normal human scalp skin. All experiments were performed according to Helsinki guidelines, in compliance with national regulations for the experimental use of human material.)

5. Immuno-competent and immuno-deficient mice (AntiCancer Inc.).
6. DMEM-F12 medium (GIBCO-BRL); (Life Technologies, Inc., Gaithersburg, MD).
7. B-27 (GIBCO-BRL) 1% penicillin-streptomycin (GIBCO-BRL).
8. 1% methylcellulose (Sigma–Aldrich).
9. Basic FGF at 20 ng/ml (Chemicon).
10. RPMI medium 1640 (Cellgro) containing 10% FBS.
11. 96-well uncoated tissue-culture dishes (BD Biosciences).
12. SonicSeal four-well chamber slides (Nunc Inc.).
13. Anti-β3-tubulin mAb (1:500, Tuj1 clone; Covance Research Products).
14. Anti-neurofilament 200 polyclonal Ab (1:80; Sigma–Aldrich).
15. Anti-GABA polyclonal Ab (1:200; Chemicon).
16. Anti-neuronal-specific enolase mAb (1:800; Lab Vision).
17. Anti-tyrosine hydroxylase polyclonal Ab (1:100; Chemicon).
18. Anti-glial fibrillary acidic protein (GFAP) mAb (1:100; Molecular Probes).
19. Anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) mAb (1:50; Lab Vision).
20. Anti-keratin 5/8 (K5/8) mAb (1:250; Chemicon).
21. Anti-keratin 15 (K15) mAb (1:100; Lab Vision).
22. Anti-smooth muscle actin mAb (1:200; Lab Vision).
23. Anti-BrdUrd mAb (1:10; BD PharMingen).
24. Anti-CD31 mAb (1:50; Chemicon).
25. Anti-CD34 mAb (1:10; BD PharMingen).
26. Secondary Abs: Alexa Fluor 568-conjugated goat anti-mouse (1:200; Molecular Probes); Alexa Fluor 568-conjugated goat anti-rabbit (1:200; Molecular Probes); and Alexa Fluor 647-conjugated chicken anti-rat (1:200; Molecular Probes).
27. Mouse-on-mouse (MOM) immunodetection kit (Vector Laboratories).
28. Ig horseradish peroxidase detection kit (BD PharMingen).

2.2 Equipment

1. OV100 Small Animal Imaging System (Olympus).
2. IMT-2 inverted microscope equipped with a mercury lamp power supply (Olympus).
3. Hamamatsu C5810 3-chip cooled color CCD camera (Hamamatsu Photonics Systems).
4. Lightools Fluorescence Imaging System (Lightools Research).
5. Sony VCR model SLV-R1000 (Sony).

6. Image Pro Plus 3.1 software (Media Cybernetics).
7. 1 ml 27G2 latex-free syringe (Becton Dickinson).
8. 25-ml Hamilton syringe (Fisher Scientific).
9. D470/40 excitation filter (Chroma Technology).
10. GG475 emission filter (Chroma Technology).
11. Cloning cylinders (Bel-Art Products).
12. Hemocytometer (Reichert Scientific Instruments).
13. Blunt-end hook (Fine Science Tools).
14. 33-G needle (Fine Science Tools).
15. Electric stimulator (FGK-1S, Medical Access).
16. Higgins7 black waterproof ink (Sanford).
17. Leica CM1850 cryostat (Leica Biosystems).

2.3 Equipment Setup

2.3.1 Whole-Body Imaging Equipment

The Olympus OV100 Small Animal Imaging System, containing an MT-20 light source and a DP70 CCD camera, can be used for whole-body and skin-flap imaging in live mice at variable magnification. The optics of the OV100 fluorescence imaging system have been specially developed for macroimaging as well as microimaging with high light-gathering capacity [14]. Many other fluorescence imaging systems can also be used to acquire subcellular images of HAP stem cells.

3 Methods

3.1 Isolation and Culture of Nestin-Positive HAP Stem Cells and Spheres

1. Isolate vibrissa follicles by exposing the upper lip containing the vibrissa pad of ND-GFP mice. Dissect the vibrissa follicles under a binocular microscope. Pluck the vibrissa from the pad by pulling them gently by the neck with fine forceps. Wash the isolated vibrissae in DMEM-F12, containing B-27 and 1% penicillin/streptomycin. Perform all surgical procedures under a sterile environment. Isolate ND-GFP HAP stem cells under fluorescence microscopy. Suspend the isolated cells in 1 ml DMEM-F12 containing B-27 with 1% methylcellulose, and 20 ng/ml basic-FGF (bFGF) [1, 15]. Culture cells in 24-well tissue-culture dishes at 37 °C in a 5% CO_2 95% air tissue-culture incubator. After 4 weeks, the ND-GFP-expressing hair follicle stem cells form spheres.
2. For differentiation, centrifuge the spheres and remove the growth factor-containing DMEM-F12 medium. Resuspend the spheres in fresh RPMI 1640 medium containing 10% FBS. Culture the spheres in SonicSeal four-well chamber slides.
3. For cloning experiments, trypsinize ND-GFP spheres that had been cultured for 2 months and serially dilute them into DMEM-F12 containing B-27 in 96-well uncoated tissue-culture

dishes. Supplement the medium with 1% methylcellulose and 20 ng/ml bFGF. Change the medium every 2 days. After 4 weeks of clonal expansion, switch the ND-GFP spheres to RPMI 1640 medium containing 10% FBS in SonicSeal four-well chamber slides. Label ND-GFP cells with BrdUrd for 7 days. Immuno-stain the cells for anti-βIII-tubulin and BrdU.

4. Detect the immuno-cytochemical staining of βIII-tubulin and K15 in the ND-GFP cells with the Mouse-on-mouse (MOM) immuno-detection kit. Detect CD31 and CD34 with the Ig horseradish peroxidase detection kit, using the antibodies listed in Materials. For quantification of the percentage of cells producing a given marker protein, photograph at least three microscopic fields in any given experiment and determine the number of positive cells relative to the total number of cells.

3.2 Sciatic Nerve Regeneration with HAP Stem Cells

1. Culture HAP stem cells and spheres from vibrissa follicles of GFP transgenic mice using the techniques described above (*see* **Note 1**).
2. For differentiation, centrifuge GFP-expressing spheres and remove the growth factor-containing supernatant and resuspend the spheres in fresh RPMI 1640 medium containing 10% FBS in Sonic-Seal four-well chamber slides. After 8 weeks of expansion, switch the GFP-expressing spheres to RPMI 1640 medium containing 10% FBS in the SonicSeal four-well chamber slides (*see* **Note 2**).
3. Transplant HAP stem cell spheres, isolated as described above, between the severed sciatic or tibial nerve fragments in immunocompetent C57BL6 mice under tribromoethanol anesthesia. Close the skin incision with nylon sutures (6–0). After 2 months, directly observe the sciatic nerve of the transplanted mouse by fluorescence microscopy under anesthesia (*see* **Note 3**).
4. Embed sciatic nerve samples in tissue freezing-embedding medium and freeze at −80 °C overnight. Cut frozen sections 5 μm thick with a Leica CM1850 cryostat and air dry. Directly observe the sections under fluorescence microscopy.
5. Use the frozen sections for immuno-fluorescence staining of β-III-tubulin, glial fibrillary acidic protein, K15, and smooth muscle actin as described above.
6. Directly observe GFP fluorescence in the sciatic nerve in the live mouse with the OV100 and the excised sciatic nerve under an Olympus IMT-2 inverted microscope equipped with a mercury lamp power supply and a GFP filter set (*see* **Note 4**).
7. Use an electric stimulator to deliver repetitious electric pulses of 0.05 mA at 10 Hz with pulse widths of 0.5 ms to stimulate control mice, mice with severed sciatic nerves, and mice that had HAP stem cells injected to join the severed nerve.

8. Measure the difference of the gastrocnemius muscle length (from lateral epicondyle of femur to heel) before and after contraction by the electric stimulator in each case in 7 above.
9. Obtain walking tracks by using a corridor open at one end to a darkened compartment. Soak the animal's feet in Higgins 7 black waterproof ink and walk the animal multiple times to obtain measurable prints. Evaluate the tracks for print length and intermediate toe spread.
10. For each experimental group, use at least seven mice, including control mice, mice with a severed tibial or sciatic nerve only, and mice with the tibial or sciatic nerve enjoined by injected HAP stem cells.
11. Express the experimental data as the mean ± SD. Perform statistical analysis by using a two-tailed Student's *t*-test.

3.3 Spinal-Cord Regeneration with HAP Stem Cells (Fig. 2)

1. Using a binocular microscope, perform a laminectomy at the tenth thoracic spinal vertebra, followed by a transversal cut.
2. Transplant GFP-expressing HAP stem cells between the severed thoracic region (spinal level T10) of the spinal cord in C57BL/6 immunocompetent mice.
3. After 2 months, directly observe the spinal cord of the transplanted mice by fluorescence microscopy.
4. Under anesthesia, excise spinal-cord samples of the transplanted mice.
5. Freeze the spinal-cord sample sections and embed them in tissue freezing-embedding medium and store overnight at −80 °C.
6. Cut frozen sections 5 μm thick with a Leica CM1850 cryostat, and air-dry.
7. Directly observe the sections by fluorescence microscopy.
8. Perform immuno-fluorescence staining of βIII-tubulin, GFAP, CNPase, K15, and SMA as described above.
9. Conduct walking analyses for 12 weeks, as described above, using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale.
10. Express the experimental data as the mean ± SD. Perform statistical analysis using the two-tailed Student's *t*-test (*see* **Note 5**).

3.4 Isolation of Human HAP Stem Cells (Figs. 3 and 4)

1. Obtain surgical specimens of normal human scalp skin. Perform all experiments according to Helsinki guidelines, in compliance with national regulations for the experimental use of human material.
2. Isolate whole hair follicles in the scalp skin by cutting the hair follicle pad and expose its inner surface.
3. Dissect the scalp hair follicles under a binocular microscope. Pluck the follicles from the pad by pulling them gently by the

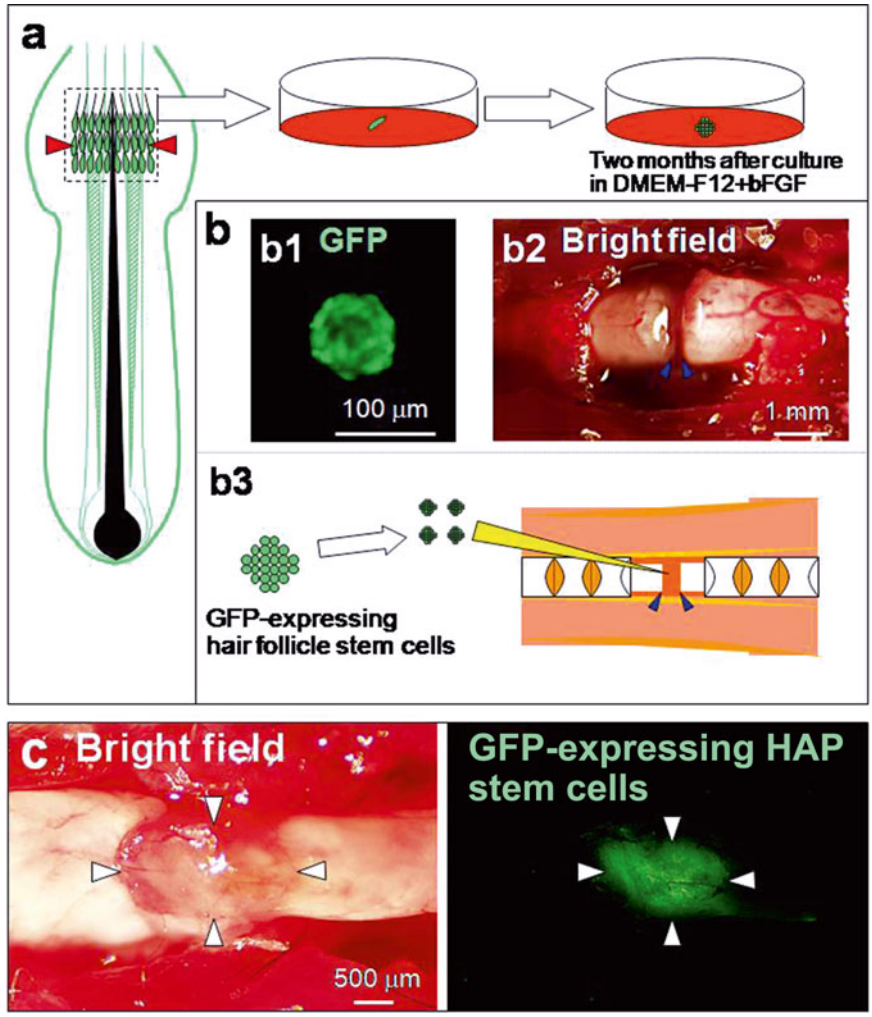

Fig. 2 Rejoining the severed thoracic region of the spinal cord with HAP stem cells. (**a**) Schematic of vibrissa follicle of GFP-transgenic mice shows the position of GFP-expressing HAP stem cells (*red arrowheads*). The HAP stem cells were cultured into DMEM-F12 containing B-27 supplemented with bFGF every 2 days, for 2 months. After 2 months, GFP-expressing HAP stem cells formed spheres. (**b**) (**b1**) Shows a sphere formed from GFP-expressing HAP stem cells. (**b2**, **b3**) GFP-expressing HAP stem cell spheres were transplanted to the severed thoracic region of the spinal cord in C57BL/6 immunocompetent mice (*blue arrowheads*). (c) Two months after transplantation of GFP-expressing HAP stem cell spheres between the severed thoracic region of the spinal cord, the GFP-expressing HAP stem cells joined the spinal cord (*white arrowheads*) [8]

neck with fine forceps. Wash in DMEM-F12 containing B-27 and 1% penicillin/streptomycin.

4. Perform all surgical procedures under a sterile environment.
5. Isolate HAP stem cells under a binocular microscope, suspend in 1 ml DMEM-F12 containing B-27 with 1% methylcellulose, 20 ng/ml basic FGF (bFGF).

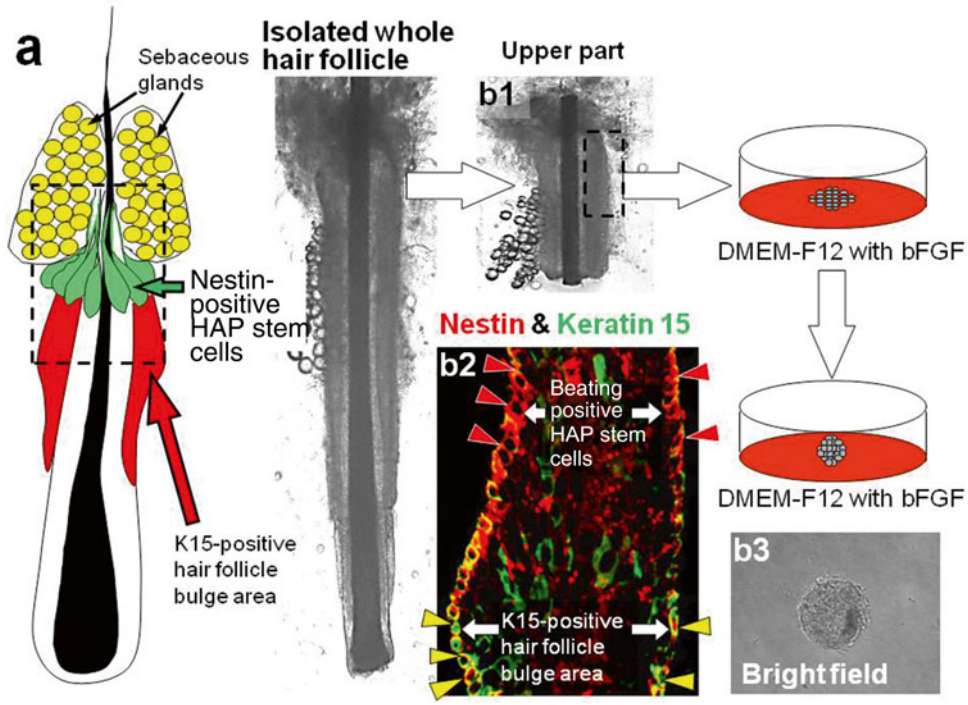

Fig. 3 (**a**) Schema of a human hair follicle in the scalp. The human hair follicle was divided into three parts (*upper*, *middle*, and *lower parts*). (**b1**) HAP stem cells are located immediately below the sebaceous glands and above the hair-follicle bulge area in the *upper part*. The HAP stem cells were suspended in DMEM-F12 containing B-27 supplemented with bFGF every 2 days. (**b2**) The bulge area contained ND-GFP-expressing HAP stem cells (*red arrowheads*). Below the HAP stem cells in the hair follicle bulge area, nestin-negative, K15-positive cells (*yellow arrowheads*) are located (*yellow arrowheads*). (**b3**) Four weeks after culture in DMEM-F12, containing B-27 supplemented with bFGF every 2 days, HAP stem cells formed spheres

6. Culture cells in 24-well tissue culture dishes in a 37 °C, 5% CO_2/95% air tissue-culture incubator for two months (*see* **Note 6**).
7. Centrifuge HAP stem-cell spheres.
8. For differentiation, resuspend the spheres in fresh RPMI 1640 medium containing 10% fetal bovine serum in SonicSeal 4-well chamber slides (*see* **Note 7**).

3.5 Direct Transplantation of Upper Part of Hair Follicle-Containing HAP Stem Cells Promotes the Recovery of Peripheral-Nerve Injury (Fig. 5)

1. Isolate vibrissa follicles from transgenic C57/B6-GFP mice, as described above.
2. Transplant the upper part of hair follicle containing HAP stem cells between the severed sciatic nerve in C57BL/6 immunocompetent mice as described above (*see* **Note 8**).

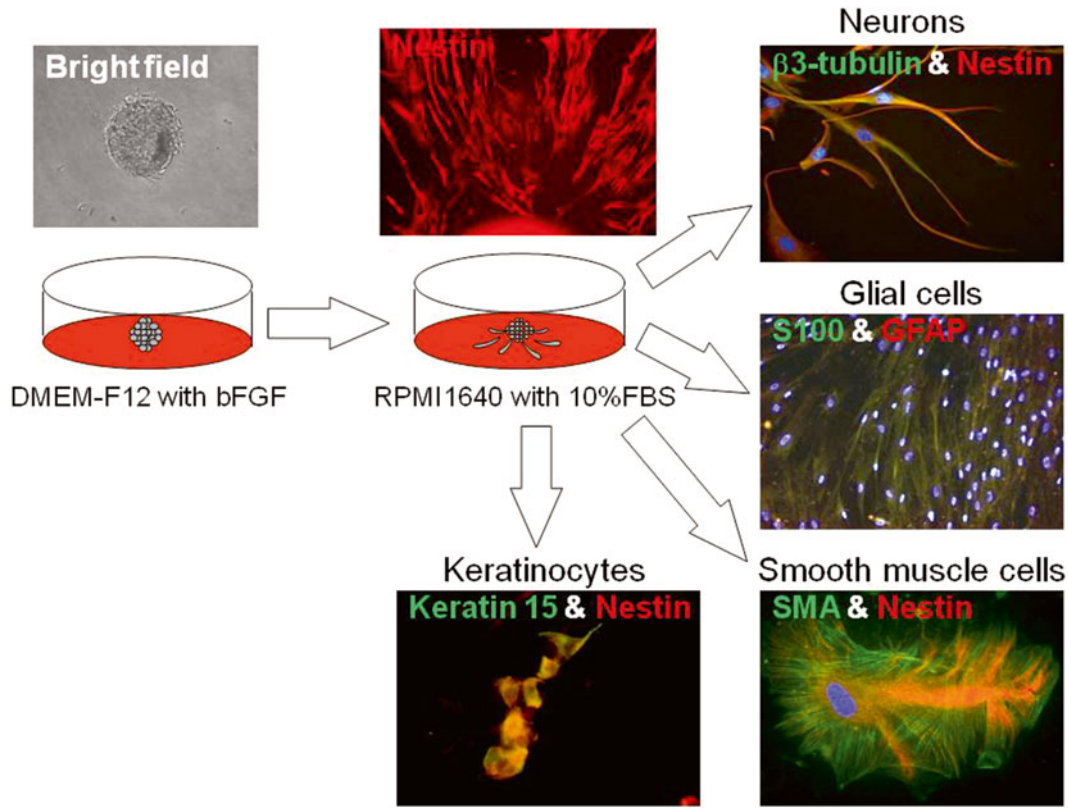

Fig. 4 (**a**) HAP stem cells were switched into RPMI 1640 containing 10 % fetal bovine serum (FBS) from DMEM-F12 containing B-27 supplemented with bFGF every 2 days. Ten days after switching into RPMI 1640 medium containing 10 % FBS, the differentiating cells migrated away from the colonies. The nestin-positive, K15-negative HAP stem cells differentiated into β3-tubulin-positive neurons, S100- and GFAP-positive glial cells, K15-positive keratinocytes, and SMA-positive smooth muscle cells [16]

4 Notes

1. After 4 weeks, GFP-expressing HAP stem cells formed GFP-expressing colonies (spheres).
2. HAP stem cells isolated from the hair-follicle bulge area are negative for the keratinocyte marker keratin 15, and can differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro. The HAP stem cells are positive for the stem cell marker CD34, as well as keratin 15-negative, suggesting their relatively undifferentiated state [3].
3. After 2 months, the sciatic nerve has rejoined. At the healed juncture, of the nerve, the strong GFP fluorescence of the HAP stem cells can be observed. Implanting HAP stem cells into the gap region of the severed sciatic or tibial nerves greatly

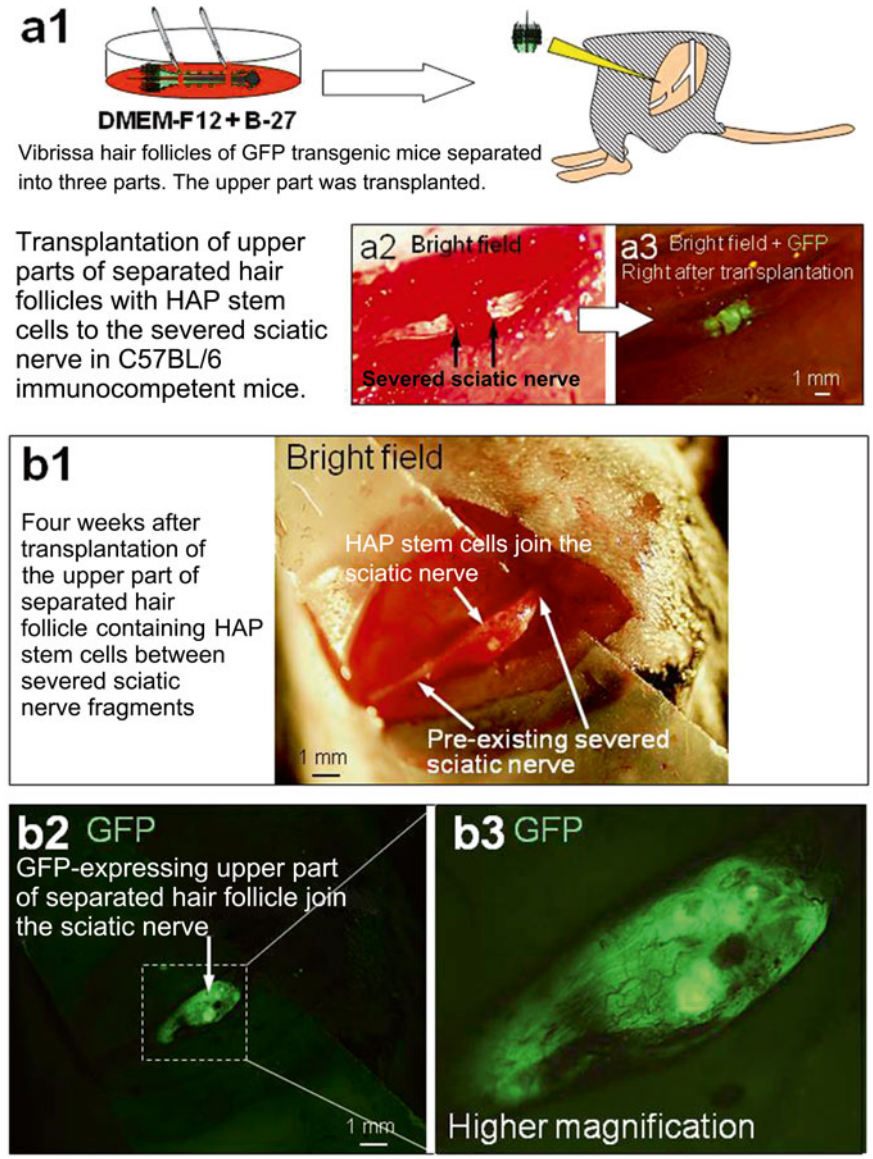

Fig. 5 Rejoined severed sciatic nerve effected by transplantation of the upper part of separated hair follicle containing HAP stem cells to the severed sciatic nerve in C57BL/6 immunocompetent mice. (**a1**) Vibrissa hair follicle from GFP-transgenic mice were separated into three parts. (**a2**) Before transplantation. (**a3**) Right after transplantation of GFP-expressing upper parts of separated hair follicles. (**b**) Four weeks after transplantation, the GFP-expressing HAP stem cells joined the severed sciatic nerve [10]

enhanced the rate of nerve regeneration and restoration of nerve function. The transplanted HAP stem cells transdifferentiated mostly into Schwann cells, which are known to support neuron regrowth. The treated mice regained the ability to walk essentially normally.

4. Auto-fluorescence: It is important to minimize auto-fluorescence interference from the tissue and body fluids by using proper filters. Excitation filters should have a narrow band as close to 490 nm as possible to specifically excite GFP whose excitation peak is distinct from that of the skin, tissues, and fluid of the animal. In addition, proper band-pass emission filters should be used with a cutoff of approximately 515 nm.
5. We severed the thoracic spinal cord of C57BL/6 immunocompetent mice and transplanted GFP-expressing HAP stem cells to the injury site. Most of the transplanted cells differentiated into Schwann cells that apparently facilitated repair of the severed spinal cord. The rejoined spinal cord reestablished extensive hind-limb locomotor performance [8].
6. After 4 weeks, nestin-expressing HAP stem cells formed colonies.
7. Human HAP stem cells can also be used to regenerate severed peripheral nerves in mice as described above.
8. Previously, HAP stem cells were cultured for 1–2 months before transplantation to the injured nerve or spinal cord which would not be optimal for clinical application of these cells for nerve or spinal-cord repair, since the patient should be treated soon after injury. We subsequently addressed this issue by directly using the upper part of the hair follicle, which is highly enriched in HAP stem cells in the bulge area, without culture, for injection into the severed sciatic nerve in mice. After injection of upper part of the whisker, the implanted HAP stem cells grew and promoted joining of the severed nerve. The transplanted HAP stem cells differentiated mostly to glial cells forming myelin sheaths, which promoted axonal growth and functional recovery of the severed nerve. These results suggest that direct transplantation of the uncultured upper part of the hair follicle containing HAP stem cells is an important method to promote the recovery of peripheral-nerve injuries and has significant clinical potential [10].

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Chapter 5

Construction of Tissue-Engineered Nerve Conduits Seeded with Neurons Derived from Hair-Follicle Neural Crest Stem Cells

Fang Liu, Haiyan Lin, and Chuansen Zhang

Abstract

Tissue-engineered nerve conduits are widely used for the study of peripheral nerve injury repair. With regard to repairing long nerve defects, stem-cell-derived neurons are recommended as seed cells. As hair-follicle neural crest stem cells (hfNCSCs) are easily to be harvested from patients and have the potential to differentiate into neuronal cells, hfNCSCs-derived neurons are an ideal candidate choice. Acellular nerve grafts, a type of biological material scaffold, with intact collagen structure, with biocompatibility and less toxicity are obtained through removing live cells with 1 % lysolecithin, are also an ideal choice. In the present report, we describe a tissue-engineered nerve conduit seeded with rat hfNCSCs-derived neurons into the beagle acellular sciatic nerve scaffold. Our goal is to provide a novel engineered therapeutic for repairing peripheral nerve injury with long distance defects.

Key words Hair follicle, Neural crest stem cells, Neuron, Acellular nerve scaffold, Tissue-engineered nerve conduit, Nerve injury repair

1 Introduction

Many kinds of stem cells have been used for the study of peripheral nerve injury repair [1–5], such as neural stem cells, induced pluripotent stem cells, adipose tissue-derived stem cells, bone marrow mesenchymal cells, and hair-follicle pluripotent stem cells. Most can be induced to differentiate into Schwann cells in vitro or in vivo and be used for the repair of short-nerve defects. With regard to long-nerve defects, it would be better to transplant neurons than Schwann cells, as neurons may extend axons to form neuromuscular junctions and reduce muscle atrophy [6–10]. Hair-follicle-associated pluripotent (HAP) stem cells or hair-follicle neural crest stem cells (hfNCSC) are easily harvested and have the potential to differentiate into neuronal cells [11–13]. Therefore, hfNCSCs are an ideal source of neurons. We have used Sonic hedghog (Shh) and retinoic acid (RA) to differentiate hfNCSCs into neurons [13].

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As for long nerve defects, a scaffold is often used to connect the proximal and distal parts of the injured nerve. For scaffolds, biological materials provide better biocompatibility and biodegradability than synthetic materials, which have desirable features for axonal regeneration. Among biological material scaffolds, acellular nerve grafts were widely used with better biocompatibility and less toxicity [14–16]. Acellular nerve grafts with intact collagen structure, impermeable and semipermeable, obtained through removing live cells by 1% lysolecithin, are an ideal choice [17]. In this regard, we seeded hfNCSC-derived neurons into acellular canine nerves to construct a tissue-engineered nerve conduit. Our goal is to provide a novel engineered therapeutic for repairing peripheral nerve injury with long distance defects.

2 Animals and Materials

2.1 hfNCSCs Culture and Neuronal Differentiation Components

1. Animals: Sprague–Dawley rats.
2. Hair follicle sources: whisker follicles.
3. Dissection or surgery equipment: stereomicroscope, microforceps, microscissors, syringe needles (10 ml, 5 ml, 1 ml syringe).
4. Tissue and cell culture plate or flask: tissue culture dishes, collagen I-coated 12-well plates, collagen I-coated 25 cm^2 flasks.
5. Primary culture medium: Dulbecco's modified Eagle's medium/F12 (Gibco, Grand Island, NY); 10% fetal bovine serum (FBS) (Gibco); 1% N2 (Gibco); 2% B27 (Gibco); 200 mM L-glutamine (Gibco); 0.025% ITS + 3 (Sigma Aldrich, St. Louis, MD); 20 ng/ml epithelial growth factor, 20 ng/ml basic fibroblast growth factor (Invitrogen, Carlsbad, CA).
6. hfNCSCs neuronal differentiation medium: Dulbecco's modified Eagle's medium/F12 (Gibco), 1% N2 (Gibco); 2% B27 (Gibco); 200 mM L-glutamine (Gibco); 0.025% ITS + 3 (Sigma); 20 ng/ml epithelial growth factor; 20 ng/ml basic fibroblast growth factor (Invitrogen); Sonic hedgehog (Shh 500 ng/ml, R&D Systems, Minneapolis, MN) retinoic acid (RA, 2 mM, Sigma-Aldrich).

2.2 Scaffold Preparation Components

1. Scaffold sources: beagle sciatic nerves.
2. Scaffold preparation medium: calcium-and-magnesium-free phosphate-saline (CMF-PBS) (Gibco); lysolecithin (1%) (Sigma-Aldrich); $CaCl_2$ (10 mM) (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China); DNase I (600 U/ml) (Sigma-Aldrich); RNase A (10 U/ml) (Sigma-Aldrich).

3 Methods

3.1 Primary Culture of Rat hfNCSCs

1. Anesthetize (40 mg/kg phentobarbital sodium, Sigma-Aldrich) SD rats deeply and cut the whisker pad carefully using dissecting scissors (*see* **Note 1**). Suture the wounds and keep the animals in an animal warming chamber until they recover from anesthesia.
2. Put whisker pads in 75 % ethanol for further sterilization for 3 min (*see* **Note 2**). Wash the whisker pad in PBS three times, 5 min each.
3. Cut the whisker pad into several strips along the hair follicle lines and separate them into individual hair follicles with scissors under the stereomicroscope. An ice bag is recommended to be put under the petri dish during the dissection of the hair follicle.
4. Discard the fat tissue and connective tissue around each hair follicle with syringe (5 ml) needles under a stereomicroscope (*see* **Note 3**), then wash the hair follicles with PBS three times.
5. Make a transverse section between the two blood-filled areas of each hair follicle under the stereomicroscope. The upper one is around the bulge area and the lower one is in the dermal papilla area. Keep the upper part and discard the lower part.
6. Use the syringe (1 ml) needle to tear off the connective capsule of the hair follicle and expose the bulge area with hair shaft on (*see* **Note 4**). Wash it in PBS for three times.
7. Place a collagen I-coated 12-well plate with primary culture medium (200 μl each well) in an incubator before the dissection of the hair follicle. Remove the medium in each well with a pipettor. Put the bulge area, prepared as above, at the base of the plate in radial orientation, 5–8 in each well. Remove the extra medium carefully around each bulge area with a pipettor (*see* **Note 5**).
8. Place the plate with hair follicle bulges in an incubator (37 °C, 95 % CO_2, 5 % O_2). Add the primary culture medium slowly and gently to each well of the plate 40 min later; avoid floating of the bulge (*see* **Note 6**). Culture for 6 days, add fresh medium into each well at day 3 if necessary.

3.2 Subculture of Rat hfNCSCs

1. Remove the hair follicle bulges carefully with a syringe (1 ml) needle under an inverted phase-contrast microscope. Make sure not to disturb the cells growing from the bulge area.
2. Rinse the cells with PBS three times. Add 200 μl 0.25 % trypsin to each well and incubate for 5–10 min. Observe cell morphology under an inverted phase-contrast microscope. Terminate the digestion of trypsin with trypsin inhibitor when over 80 % of the cells become rounded (*see* **Note 7**).

3. Add 800 μl serum-free culture medium in each well. Pipette the medium up and down about 20 times gently and place the medium in a centrifuge tube (10 ml). Centrifuge at 1000 rpm for 5 min. Discard the supernatant and add 5 ml of fresh culture medium to the centrifuge tube. Pipette gently to make a cell suspension.
4. Add the cell suspensions to a collagen I-coated 25 cm^2 flask, then place it in an incubator for culture. Renew half of the liquid volume every 2–3 days.

3.3 Neuronal Differentiation of hfNCSCs

1. Remove the culture medium and the neuronal differentiation medium after the subculture of hfNCSCs becomes 80% confluence. Renew half of the liquid volume every 2–3 days.
2. Incubate the hfNCSCs in neuronal differentiation medium for 7–10 days (*see* **Note 8**). Then change the medium to serum-free culture medium. Culture for another 7 days.

3.4 Preparation of Scaffold

1. Harvest beagle sciatic nerves from another experiment that has no effect on nerve tissues. Cut the nerves into 4 cm segments.
2. Immerse the nerve segments in calcium-and-magnesium-free phosphate-saline (CMF-PBS), pH 7.3, for 5 days at 4 °C. Incubate with 1% lysolecithin in 0.01 M PBS at room temperature for 4 days. Change the incubation solution every 24 h. For the last 2 days, supplement the solution with 10 mM $CaCl_2$.
3. Wash the extracted nerve segments in CMF-PBS for 30 min and incubate them in CMF-PBS supplemented with 600 U/ml DNase I and 10 U/ml RNase A at 37 °C for 24 h.
4. Wash the nerve scaffolds with CMF-PBS, three times for 20 min each (*see* **Note 9**). Lyophilize the nerve scaffolds. Irradiate them with ^{60}Co gamma rays and store at −80 °C.

3.5 Construction of Nerve Conduit

1. Trypsinize the hfNCSCs-induced neurons cultured in the Collagen I coated 25 cm^2 flask, suspend with PBS, and count with a hemocytometer under an inverted phase-contrast microscope. Adjust the cell density to 1.5×10^6 cells/ml. Put the cell suspensions in a 500 μl eppendorf tube and keep it on ice until use (*see* **Note 10**).
2. Rehydrate the scaffold in PBS. Inject a 160 μl suspension of induced neurons into the acellular nerve scaffold at 5-mm intervals with a 31-G insulin syringe under a stereomicroscope, 20 μl each time (*see* **Note 11**).
3. Incubate the nerve conduits in Dulbecco's-modified Eagle's medium/F12 medium containing 10% FBS, penicillin (1:100), and streptomycin (1:100) at 37 °C with 95% CO_2 and 5% O_2.
4. Turn over the conduits every 2 days with half of the medium replenished every 4 days (*see* **Note 12**).

4 Notes

1. Before dissecting the whisker pad, it is necessary to sterilize all the surgical instruments including dissecting scissors and forceps. Spray 75 % ethanol on the whisker pad and be careful not to stimulate the animal's eyes. It is recommended to make the whisker shorter with scissors for convenience of dissecting the hair follicle later.
2. Bubbles are formed at the surface of the skin among whiskers after the whisker pad is placed in ethanol. Vibrate the whisker pad gently with forceps, clear the bubbles and soak it entirely in ethanol in order to avoid contamination.
3. A syringe needle works very well during the dissection of hair follicles. The tip of the syringe needle is just like a small knife. It is very convenient to handle the follicle under a microscope. You can choose the appropriate size of the syringe needle to dissect different tissues.
4. The bulge areas are too tiny to be held tightly. Sometimes you may lose some of them during dissection. Be careful to maintain the hair shaft together with the bulge area. It is easier to hold the hair shaft when transferring the bulge areas.
5. If the bulge area floats, the culture fails. Removing the extra medium around each bulge area with a 10 μl pipette tip is an effective way to avoid floatation of the bulge area.
6. Adding the primary culture medium as slow as possible along the wall of the well is another good way to avoid floating of the bulge area.
7. It is necessary to observe the cells under the microscope during trypsin digestion. Too long a time of trypsin digestion may influence cell viability.
8. Observe carefully under the microscope during neuronal differentiation. If the projections of the cells interweave and appear neuron-like, neuronal differentiation is successful.
9. A shaker is recommended to be used during washing the nerve scaffolds in order to remove DNase I and RNase A as much as possible.
10. Keeping the cells on ice is good for maintaining cell viability as long as possible.
11. The scaffold becomes soft after rehydration. Be careful not to destroy the structure of the scaffold during cell injection.
12. Turning over the conduits helps the cells grow evenly in the scaffold. This is important for the construction of an effective tissue-engineered nerve conduit.

Acknowledgement

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Chapter 6

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

Sumiyuki Mii, Jennifer Duong, Yasunori Tome, Aisada Uchugonova, Fang Liu, Yasuyuki Amoh, Norimitsu Saito, Kensei Katsuoka, and Robert M. Hoffman

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.

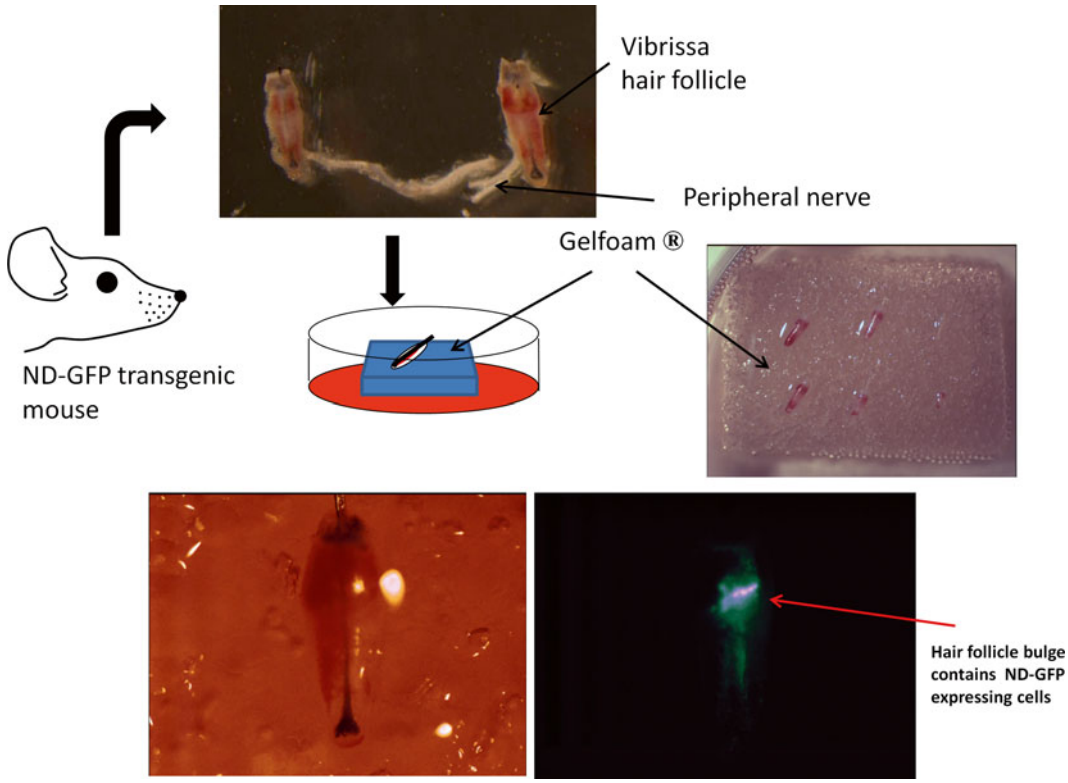

Fig. 1 Isolated vibrissa hair follicles from an ND-GFP mouse were placed on Gelfoam® and suspended in culture medium

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

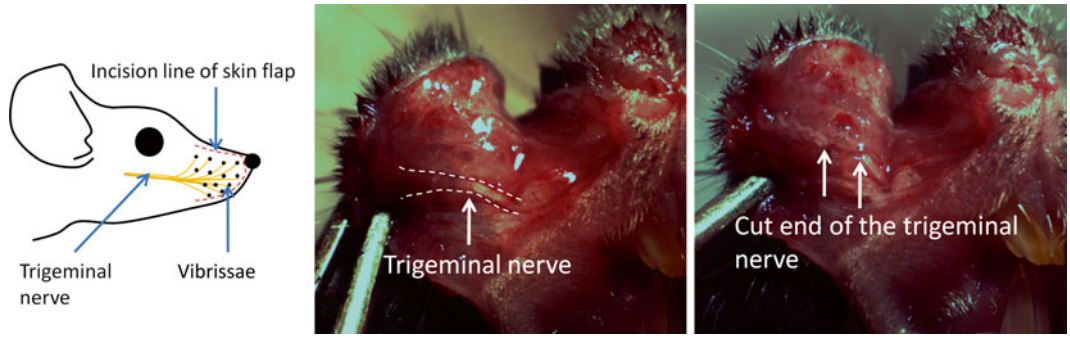

Fig. 2 A skin-flap is made to visualize the whisker pad and trigeminal nerve

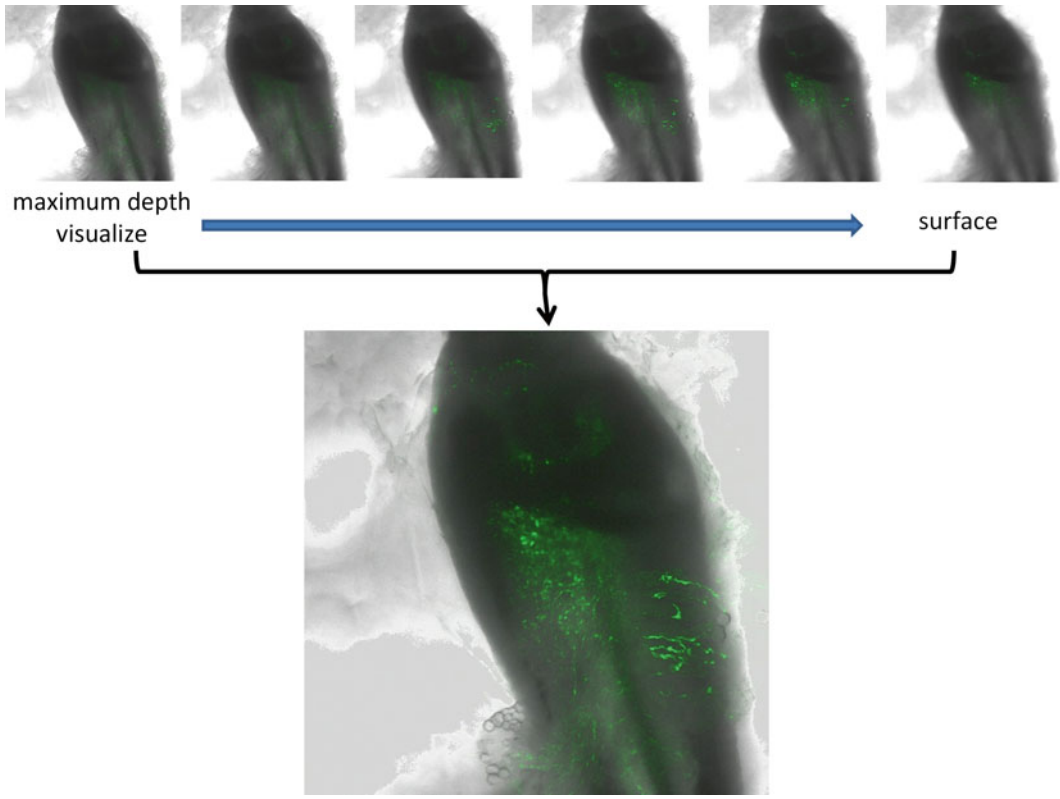

Fig. 3 The hair follicle bulge area is shown by integrating images of different depths from the surface of the hair follicle

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**).

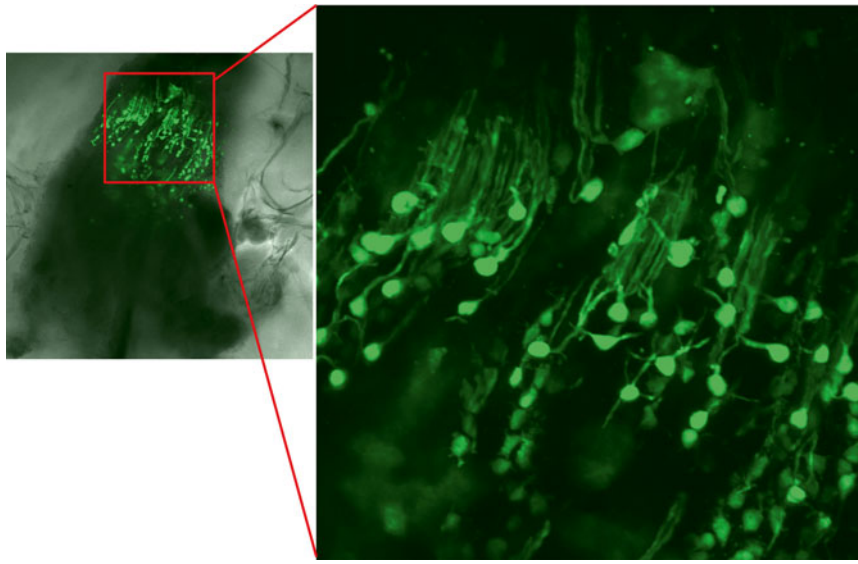

Fig. 4 A high magnification image of nestin-expressing HAP stem cells of the hair follicle bulge

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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Chapter 7

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

Ruifeng Yang and Xiaowei Xu

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.

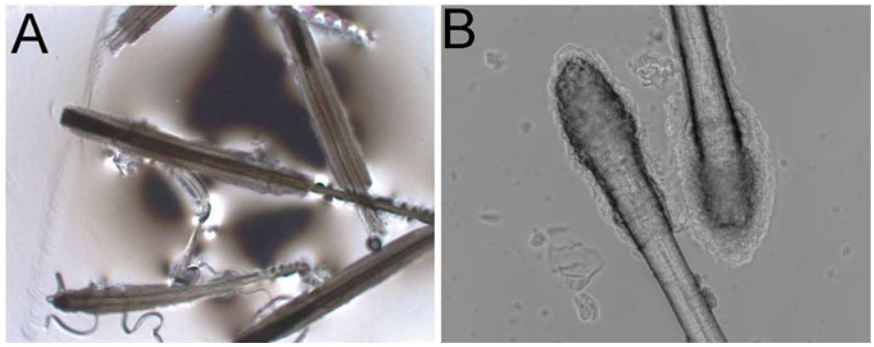

Fig. 1 Plucked anagen and telogen hair follicles. (**a**) Anagen hair follicles. (**b**) Telogen hair follicles

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

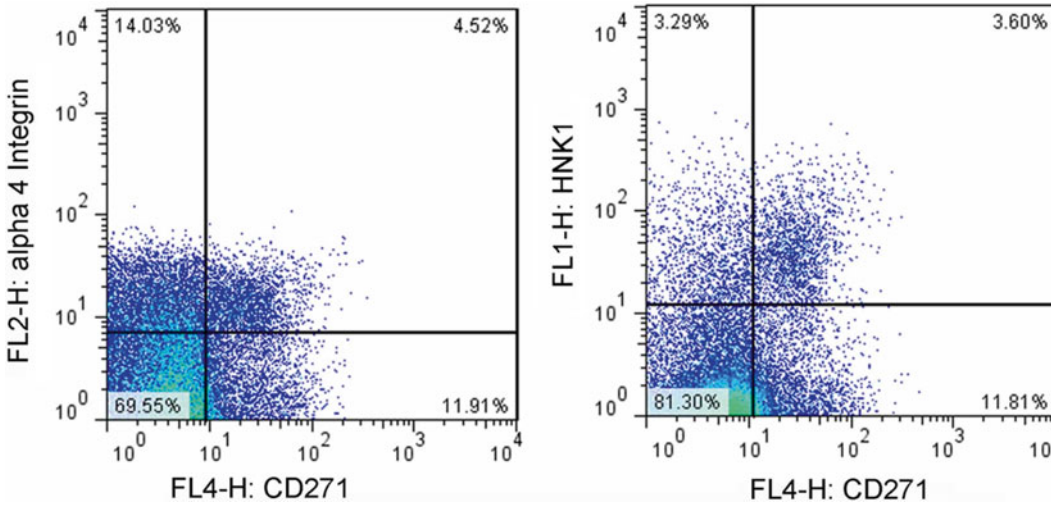

Fig. 2 Representative images of FACS analysis of hair follicle NCSCs. *Left panel*: Cells are gated using anti-CD271 and anti-alpha4 integrin antibodies. *Right panel*: Cells are gated using anti-CD271 and anti-HNK1 antibodies

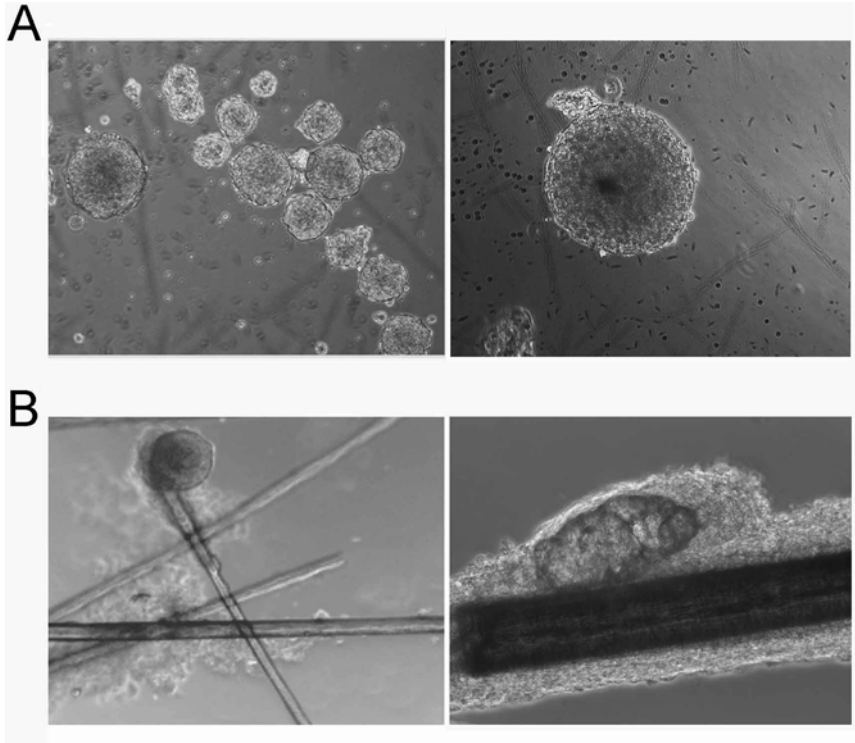

Fig. 3 Morphology of hair spheres. (**a**) Morphology of floating hair spheres, *left panel*: low power, *right panel*: high power. (**b**) Morphology of hair sphere in situ at the bulge region, *left panel*: a hair sphere formed at a telogen bulge, *right panel*: a hair sphere formed at the bulge region 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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Chapter 8

Isolation of Mouse Hair Follicle Bulge Stem Cells and Their Functional Analysis in a Reconstitution Assay

Ying Zheng, Jen-Chih Hsieh, Julia Escandon, and George Cotsarelis

Abstract

The hair follicle (HF) is a dynamic structure readily accessible within the skin, and contains various pools of stem cells that have a broad regenerative potential during normal homeostasis and in response to injury. Recent discoveries demonstrating the multipotent capabilities of hair follicle stem cells and the easy access to skin tissue make the HF an attractive source for isolating stem cells and their subsequent application in tissue engineering and regenerative medicine. Here, we describe the isolation and purification of hair follicle bulge stem cells from mouse skin, and hair reconstitution assays that allows the functional analysis of multipotent stem cells.

Key words Hair follicle stem cell, Bulge, Hair reconstitution assay, Regeneration

1 Introduction

Stem cells (SC) have the unique capacity to self-renew and to differentiate into more mature cell types; these properties allow stem cells to play a crucial role in the homeostasis of adult tissues and organs as well as during healing and regenerative responses following injury. Characteristics of epithelial stem cells are quiescence and slow cycling, persistence during the lifetime of the organism, having a distinct biochemical makeup, and localized in a protected reservoir [1]. Studies have revealed that adult stem cells reside in specific niches within various tissues.

Cell lineage tracing studies have demonstrated that the hair follicle bulge, a specialized structure of the skin located in the mid to upper portion of the follicle at the arrector pili muscle attachment site [2], harbors stem cells necessary for hair follicle regeneration and maintenance. These cells express specific markers, such as keratin 15 [3] and CD34 [4] in the mouse. The stemness of HFSC has been demonstrated by reconstitution assays where these cells regenerate de novo hair follicles in vivo, by repopulating all lineages of cells in the nascent hair follicles with HFSC progeny.

Robert M. Hoffman (ed.), *Multipotent Stem Cells of the Hair Follicle: Methods and Protocols*, Methods in Molecular Biology, vol. 1453, DOI 10.1007/978-1-4939-3786-8_8,

Moreover, similar to native hair follicles, these de novo hair follicles go through cyclic regression and regeneration that rely on the function of the isolated hair follicle bulge stem cells.

Morris et al. first established a method for skin cell isolation in 1990 [5]. Subsequently, several methods to test the hair-forming ability of the isolated cells were developed, including the patch assay [1, 5, 6] and the silicone chamber assay [7, 8] described in details here. Liang et al. [9] compared these methods and concluded that the patch assay offered the following advantages over others: easy to perform, requires a manageable number of cells, shorter assay times, and cost-effective. However, the patch assay also suffers some shortcomings: uncontrolled hair shaft orientation and inability of the de novo hairs to cycle properly. The chamber assay, on the other hand, is labor-intensive, requires longer assay times and cell numbers that are at least an order of magnitude higher than the Patch assay. However, the chamber assay results incorrectly oriented hairs capable of normal cycling for an extended period of time. In this chapter, we describe these two assays that are most commonly used for reconstitution of the hair follicle, so that researchers are able to choose an assay according to their needs.

2 Materials

2.1 Hair-Follicle Cell-Isolation Reagents

1. C57/BL mice, 6–8 weeks (Jackson Laboratory, Sacramento, CA).
2. Scissors.
3. Tweezers.
4. Oster Golden A5 hair clipper with #40 blade (Fisher, NC 9378235).
5. Povidone iodine prep solution 16 oz (More Medical, Farmington, CT, 11602).
6. 70% ethanol.
7. 100 mm × 20 mm CELLSTAR TC Petri dish (BioExpress, Kaysville, UT, T-2881-2).
8. #22 blade, sterile (Fisher, 08-918-5C).
9. MooreBrand® scalpel handle #4 (More Medical, 30976).
10. 1× DPBS, no calcium, no magnesium (Invitrogen, 14190-144).
11. Gentamicin (Gibco, Grand Island, NY, 15750-060).
12. 0.25% trypsin/saline without EDTA (Invitrogen, 25200-056).
13. SMEM (Bio-Whittaker, Walkersville, MD, 12-1260).
14. 70 μm cell strainer (BD Falcon 35-2350).
15. 50 mL conical tube.

2.2 FACS Reagents

1. Antibody-staining buffer: 1× PBS + 2% FBS.
2. CD49f-PE (BD Pharmingen, San Diego, CA, 555736).
3. CD34-FITC (BD Pharmingen, 553733).
4. DAPI, FluoroPure™ grade (Invitrogen, D21490).

2.3 Mouse Neonatal Dermal Cell Isolation Reagents

1. Postnatal C57BL/6 Mice day 0–2.
2. Scissors (Geoge Tiemann & Co., Hauppauge, NY, 110-1250SS or equivalent).
3. Tweezers (Geoge Tiemann & Co. 160-55PP or equivalent).
4. Collagenase type I (Invitrogen, 17100-017).
5. DNase (Roche, 104159).
6. 1× DPBS, no calcium, no magnesium (Invitrogen, 14190-144).
7. 100× AB/AMY (Invitrogen, 14190-144).
8. 40 μm cell strainer (BD Falcon 35-2340).
9. 100 μm cell strainer (BD Falcon 35-2360).
10. 0.25% trypsin/saline without EDTA (Invitrogen, 25200-056).
11. Dispase (Invitrogen, 17105-041).
12. $CaCl_2$-free Eagles minimal essential medium (Sigma, St. Louis, MD, M8167).
13. Fetal calf serum (Hyclone, SH30037.03).
14. Chelex (Sigma, C7901-500G).
15. 1 M solution of $CaCl_2$ (Sigma, C-7902).
16. 200 mmol/L L-Glutamine (Invitrogen, 25030-081).
17. 1 M Tris–Cl (Sigma, T3038).
18. 5 M NaCl (Sigma, S-5150).
19. Dithiothreitol (DTT) (Sigma, 43815-1G).
20. Glycerol (Sigma, G5516).
21. Distilled water (Invitrogen, 15230-147).

2.4 Reconstitution Assay Reagents

1. Nude/nude mice (6 weeks, Jackson Laboratories).
2. 1 mL TB syringe with 25 G needle (BD, 309626).
3. DMEM/F12(1:1) medium (Gibco, 11039-021).
4. Alcohol swabs (Kendall Webcol, Brooksville, FL, alcohol prep 70% isopropyl alcohol).
5. Tattoo ink (Permanent Black Animal Identification and Marking Systems, Inc., Hornell, NY).
6. Silicone chamber dome sets (*see* **Note 18**).
7. Eye ointment (Puralube ointment, E. Fougera & Co., Melville, NY 11747).

8. Curved scissors.
9. Tweezers.
10. Heating pad.

3 Methods

3.1 Hair-Follicle Cell Isolation

1. Sacrifice 6- to 8-week mice by cervical dislocation or CO_2 euthanasia (*see* **Note 1**).
2. Clip dorsal fur on the entire back. Place mice in a jar with enough Betadine to cover them. Roll the jar in your hands until the mice are completely wet. Pour out the Betadine and rinse with distilled water until the water is clear. Repeat the process.
3. Add 70% ethanol to cover the mice and let them soak for 5–10 min. Pour the ethanol out. Repeat the process.
4. Remove the entire dorsal skin using scissors and rinse in a 100 mL cup with 30–40 mL 1× DPBS and 2× gentamycin (100 mg/L).
5. Lay skin flat on a square Petri dish with the dermal side facing up; scrape off subcutaneous tissue with a scalpel until the remaining skin is nearly translucent. Keep scraped skin in the PBS until all pieces have been scraped (*see* **Note 2**).
6. Flatten skins on a fresh Petri dish, and cut the skin samples into ~1 × 1.5 cm strips. Transfer the skin pieces, epidermal side up, to a Petri dish containing 25 mL of 0.25% trypsin/saline and 2× gentamycin. Incubate at 37 °C for 70 min (*see* **Note 3**).
7. Transfer skin pieces from trypsin to a Petri dish with 10 mL SMEM containing 2× gentamycin and 5% FBS. Scrape off the epidermis with a sterile scalpel. Discard the dermis (*see* **Note 4**).
8. Pour the epidermis samples into a sterile 50 mL conical tube. Rinse the Petri dish with extra medium, and add the rinse medium into the conical tube containing scraped epidermis. Then add medium to 40 mL, and gently pipette the suspension up and down with a 10 mL pipette for 20 times (*see* **Note 5**).
9. Filter the suspension through a sterile 70 μm cell strainer into a 50 mL conical tube, and spin at 180 × *g* for 7 min at room temperature.
10. Resuspend the pellet in 10 mL SMEM medium and count the cell number.

3.2 FACS of Hair Follicle Stem Cells

1. Set up incubation of cells with antibodies
   (a) Compensation tubes (*see* **Note 6**):
   - Negative control: Aliquot 1×10^6 cells in 250 μL staining buffer (no stain).
   - FITC control: Aliquot 1×10^6 cells in 100 μL staining buffer. Add 20 μL CD49f-FITC antibody.

- PE control: Aliquot 1×10^6 cells in 100 μL staining buffer. Add 20 μL CD34-PE antibody.
- DAPI control: Aliquot 1×10^6 cells in 250 μL staining buffer. DAPI should be added immediately before FACS without additional incubation.

(b) FACS tubes:

Add CD49f-FITC and CD34-PE antibodies, 20 μL each in 100 μL staining buffer for every 1×10^6 cells (*see* **Note 7**).

2. Incubate for 30 min at room temperature.
3. Add 1× PBS to 1 mL and spin at $180 \times g$ for 5 min to remove supernatant.
4. Add 1–2 mL 1× PBS and suspend cells. Spin again to collect pellets.
5. Resuspend DAPI control samples in 250–500 μL staining buffer, and FACS samples in 1 mL staining buffer containing up to 10×10^6 cells.
6. Dilute DAPI 1:1000 in antibody-staining buffer. When all samples are ready for FACS, add 20 μL of the diluted DAPI into DAPI control tube (250–500 μL).
7. FACS is performed on a BD FACS Vantage with Diva Option, using a 100 μM nozzle, 15 PSI pressure and 30,000 kHz drop drive frequency (*see* **Note 8**).

3.3 Mouse Neonatal Dermal Cell Isolation for Reconstitution Assay

3.3.1 Medium Preparation

1. Chelex-treated fetal-calf serum
   (a) 500 mL fetal calf serum.
   (b) 25 g Chelex (Sigma, C7901-500G).

   Stir gently for 1 h on a magnetic platform at room temperature. Filter through 90 mm filter unit (Nalge Nunc, Rochester, NY, 162-0045), and then aliquot into 50 mL conical tubes. Store the treated serum at –20 °C. Thaw at 37 °C before using.
2. Complete EMEM Media:
   (a) $CaCl_2$-free Eagles minimal essential medium 500 mL (Sigma, M8167).
   (b) 50 mL Chelex-treated fetal calf serum.
   (c) 0.05 mmol/L $CaCl_2$ (25 μL of 1 M solution per 500 mL, Sigma, C-7902).
   (d) 2 mmol/L L-glutamine (Invitrogen, 25030-081, 5 mL per 500 mL).
   (e) 1× AB/AMY (Invitrogen 14190-144, 5 mL per 500 mL).
3. DNase I (40 mg/mL, 80 U/μL, 2.5 mL) stock solution
   (a) DNase I 100 mg (200,000 U) (Roche, Indianapolis, IN, 104159)
   (b) 40 mM Tris–Cl.

(c) 100 mM NaCl.

(d) 2 mM DTT (to make 10 mL 100 mM DTT stock solution, use 154 mg DTT (Sigma, 43815-1G), add distilled water to 10 mL).

(e) 1.15 mL distilled water (Invitrogen, 15230-147).

(f) 1.25 mL glycerol (Sigma, G5516) (Fig. 1).

3.3.2 Procedure

1. Sacrifice neonatal pups (day 0–2) according to the IACCU-approved protocol.
2. Remove heads, limbs, and tails from pups with sharp scissors.
3. Remove the skin from the muscle, rinse in DPBS with 1× AB/AMY three times.
4. Float isolated skins with epidermal side up in 2 mg/mL dispase/DPBS solution overnight at 4 °C in a 100 × 20 mm Petri dish. One dish can hold 20–30 mL dispase solution with ~20 pup skins (*see* **Note 9**).
5. Use sterilized forceps; peel off the epidermis from the dermis. Transfer dermis to a Petri dish containing complete EMEM to rinse off the dispase.
6. Transfer the dermis to a 50 mL conical tube, add 10 mL complete EMEM with 0.35% collagenase per ten pieces of dermis (*see* **Note 10**).
7. Use scissors to cut the dermis to very fine pieces (2–3 mm^2) until they can be easily pipetted up and down with a 10 mL pipette.
8. Incubate with moderate agitation in a 37 °C water bath for 25 min (*see* **Note 11**).
9. Add 20 μL of 20,000 U/mL DNase I/PBS solutions to each 10 mL collagenase digestion mixture. Incubate for another 5 min at 37 °C with agitation.
10. Dilute each 10 mL digestion mixture to 50 mL with complete EMEM, and pipet the mixture up and down for 20 times using a 10 mL pipet. Filter solution through 100 μm cell strainer.
11. Spin at 180 × g for 5 min at 4 °C. This is supernatant A and pellet A (Fig. 2).
12. Transfer supernatant A to a 50 mL tube; resuspend pellet A in 25 mL media and keep on ice.
13. Spin supernatant A at 520 × *g* at 4 °C for 5 min. This is supernatant B and pellet B (Fig. 2). Discard supernatant B, and resuspend pellet B in 25 mL media. Keep on ice.
14. Spin the resuspended pellet A mixture at 29 × *g* for 3 min at 4 °C. This is supernatant C and pellet C (Fig. 2). Transfer supernatant C to a new tube, and discard the pellet.

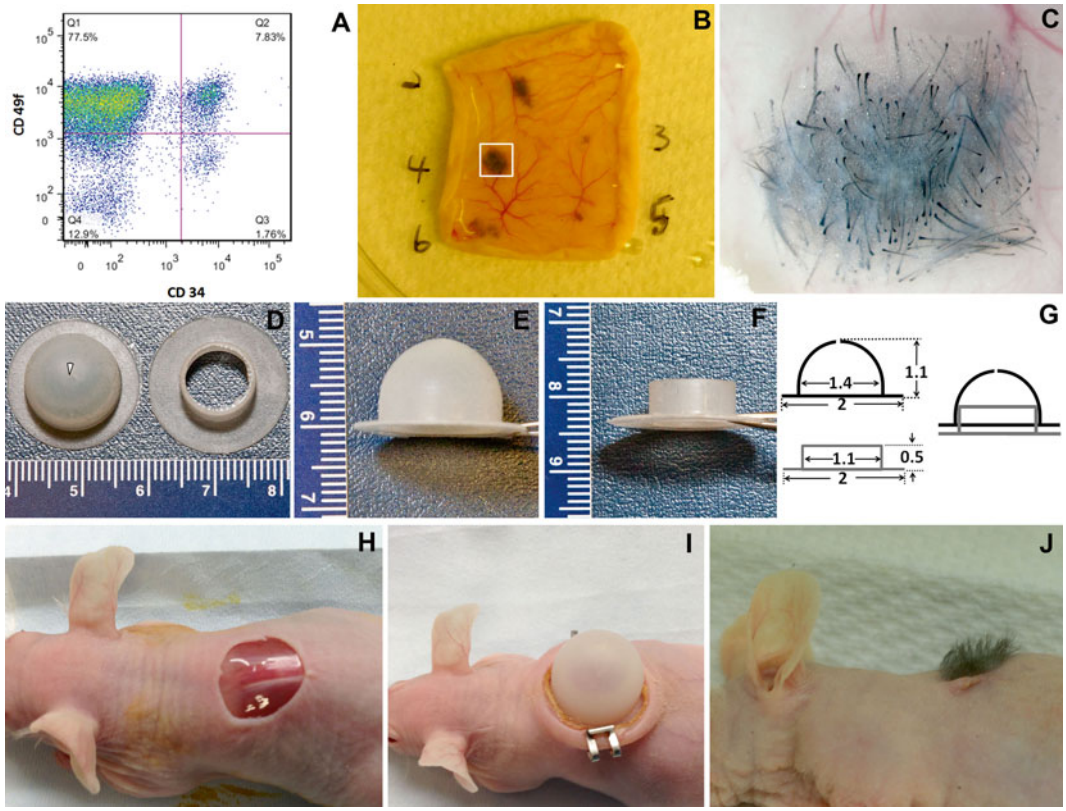

Fig. 1 FACS analysis and reconstitution assays for hair-follicle stem cells. (**a**) FACS analysis revealing the presence of bulge stem cells ($CD34^{+}CD49f^{+}$, approximately 8 % of the total input cells) among the keratinocytes isolated from the back skin of a 2-month-old mouse. (**b**) Patch-assay skin sample showing dermal view of skin harvested from a nude mouse which received multiple injections of a mixed suspension of dermal and epidermal cells. The *boxed area* shows pigmented de novo hair follicles reconstituted from injected cells. (**c**) An enlarged view of a representative injected site similar to the one in the *boxed area* in (**b**). As many as several hundred hair follicles with hair shafts can be seen at one injection site. (**d**–**g**) The dimensions of the silicone chamber used in the chamber assay. The chamber assembly consists of two parts, a dome and a base onto which the dome snaps. The rulers have metric units, with the smallest division representing 1 mm. (**d**) Top view of the dome (*left*) and the base (*right*). The *arrowhead* indicates the small hole on top of the dome, through which cell slurry is pipetted into the chamber. (**e**) Side view of the dome. (**f**) Side view of the base. (**g**) Schematic representations of the chamber, with the dimensions indicated in cm. The *right* illustrates the assembled chamber in use. The thickness of the chamber is uniformly 0.8 mm. (**h**–**j**) Chamber assays in nude mice. (**h**) Excision of the back skin on a nude mouse to prepare for the insertion of the chamber. (**i**) The inserted chamber is securely anchored to the skin with clips. (**j**) Hairs growing out of the site where the chamber was implanted

15. Spin resuspended pellet B and supernatant C at 180×*g* for 5 min at 4 °C. Aspirate supernatant. This is pellet D and E, respectively (Fig. 2). Resuspend both pellets in 10 mL complete EMEM each and combine in one tube.
16. Spin the combined suspension at 180×*g* for 5 min at 4 °C, and aspirate the supernatant. Resuspend the pellet in 20 mL complete EMEM. Spin the combined suspension at 180×*g* for 5 min at 4 °C and collect the pellet.

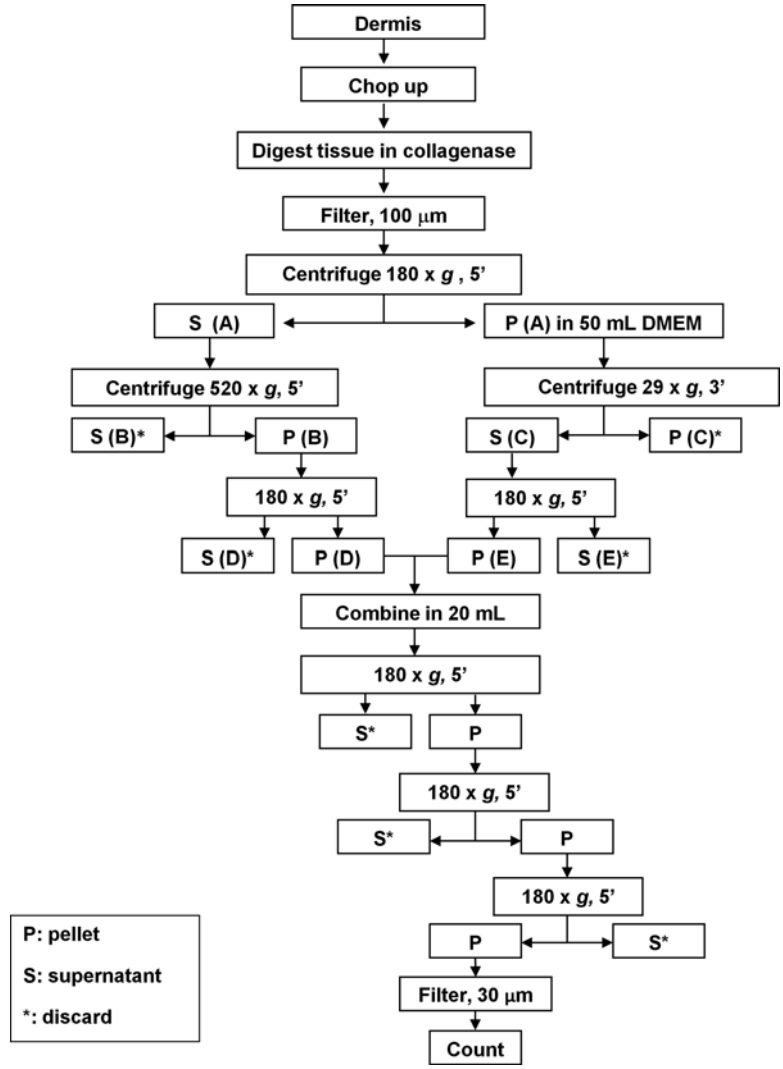

Fig. 2 Flow chart of mouse neonatal dermal-cell isolation. Details are described in the procedures for neonatal dermal cell isolation. S (A): supernatant A; P (A): pellet A; S (B): supernatant B; P (B): pellet B; S (C): supernatant C; P (C): pellet C; S (D): supernatant D; P (D): pellet D; S (E): supernatant E; P (E): pellet E. Asterisk denotes fractions to be discarded

17. Resuspend final pellet in 20 mL medium. Filter through a 40 μm cell strainer.
18. Count the cells. Store cells on ice until grafting (*see* **Note 12**).

3.4 Reconstitution Assays (See Note 13)

3.4.1 Patch Assay Procedure

1. For a single injection, mix 1×10^6 mouse neonatal dermal cells with 0.5×10^6 epidermal cells in 10 mL DMEM/F12 medium. Spin down the mixed cells at $350 \times g$ for 5 min at 4 °C (*see* **Note 14**).
2. Aspirate most of the supernatant, but leave approximately 70–80 μL of residual medium in the tube.

3. Transport the cell pellet on ice immediately to the animal facility.
4. Place nude mice in an induction container with 5% isoflurane and 1 L/min oxygen. Use the pedal (paw pinch) reflex to determine the depth of anesthesia.
5. Once mice are properly anesthetized, transfer them to a nose cone with 1% isoflurane and 1 L/min oxygen. Use the pedal (paw pinch) reflex again to determine the depth of anesthesia.
6. Disinfect the dorsal skin with iodine followed by alcohol swab. Tattoo the site of cell injection by piercing the mouse skin adjacent to the injection site once with a 25 G needle dipped in tattoo ink to mark the location of injection. Up to six injections can be performed per mouse.
7. Resuspend the cell pellet by pipetting. Draw the cells slowly into a 1 mL TB syringe with a 25 G needle to avoid shearing of the cells (*see* **Note 15**).
8. Insert the needle parallel to the skin, making sure the needle tip does not penetrate the whole dermis so that the cells, once injected, can remain in a confined space in the dermis. Inject the cell suspension into the dermis slowly, followed by gradual withdrawal of needle to avoid back flow of the fluid (*see* **Note 16**).
9. Place injected mice on their belly into pre-warmed cages lined with a paper towel. Use a heating pad on low setting to warm the cage. Moniter mice until they can get up by themselves when placed on their backs.
10. Sacrifice the mouse 2 weeks after cell injection. Harvest the skin and lay flat on a Petri dish. Visualize the hair follicles formed at each injection site from the dermal side of the skin under a dissecting scope (Fig. 1b and c).
11. Quantify the number of hair follicles formed in each injection before or after fixation (*see* **Note 17**).

3.4.2 Chamber Assay

1. For each single assay, mix 10×10^6 mouse neonatal dermal cells with 5×10^6 epidermal cells in DMEM/F12 medium. Spin down the mixed cells at $350 \times g$ for 5 min.
2. Aspirate most of the supernatant, but leave approximately 200 μL of residual medium in the tube.
3. Transport the cell pellet on ice immediately to animal facility.
4. Administer bubrenorphine (0.5 mg/kg) by subcutaneous injection 30 min before the procedure and 6–8 h post surgery.
5. Place mice in an induction container with 5% isoflurane and 1 L/min oxygen. Use the pedal (paw pinch) reflex to determine the depth of anesthesia.

6. Once mice are anesthetized, transfer them to a nose cone with 1% isoflurane and 1 L/min oxygen. Use the pedal (paw pinch) reflex again to determine the depth of anesthesia. If the mouse responds to pedal pressure, increase isoflurane (e.g. by 1–2%) until the mouse does not respond to a pedal pinch. Monitor respiration and heartbeat continuously during surgery.
7. Place eye ointment on each eye of the mouse to prevent drying out. Disinfcet the dorsal skin with iodine followed by alcohol swab.
8. Using curved scissors, cut a piece of full thickness skin (about 1 cm diameter) from the back of the mouse by lifting it with forceps (Fig. 2h).
9. Moisturize the area where the chamber dome will be grafted with PBS. Pull the skin by forceps to create a slit-like opening and insert the dome flange under the skin. Securely anchor the chamber to the skin using wound clips (Fig. 2i, *see* also **Note 18**).
10. Apply cell slurry through the hole on top of the grafting dome (Fig. 2d).
11. After cell implantation, place mice on their belly into prewarmed cages lined with a paper towel. Use a heating pad on low setting to warm the cage. Apply additional eye ointment as necessary. Monitor mice until they are awake.
12. Remove domes after 7 days. The grafted area heals within 3 weeks. Euthanize animals and collect skins 30 days post-grafting or later as needed to fit study purpose (*see* **Note 19**).

4 Notes

1. We routinely use 6- to 8-week C57BL female mice for isolation of bulge stem cells, since hair follicles are in the telogen phase at this stage and can be easily scrapped off the skin surface after trypsin treatment. Each female mouse will yield ~25×10^6 total epidermal cells prior to sorting, while the yield from a male mouse is ~usually 20–30% lower.
2. Use a new scalpel for each mouse skin, and keep scalpel holder and forceps in 70% ethanol between samples.
3. We found that when younger mice (such as 42–45 days) are used, the trypsin digestion time can be reduced by 5–10 min. On the other hand, when older mice are used, the trypsin digestion time may need to be increased by 5–10 min. However, we do not normally recommend more than 70 min of trypsin treatment as prolonged trypsin digestion would increase cell death. The trypsin treatment can also be replaced

by overnight (less than 16 h) dispase (2 mg/mL/1× DPBS) treatment at 4 °C. However, in our experience the trypsin treatment usually gives a higher yield than the overnight dispase treatment.

4. To maximize the yield of epidermal cells, hold one corner of the skin with forceps, scrape in the direction away from the forceps. Then hold the skin at another corner, and repeat the scraping. Repeat this procedure for the remaining two corners.
5. The pipetting time can be optimized based on the force and speed of the pipettor. The goal is to fully dissociate the tissues without causing damage to the cells.
6. Cell number for compensation samples can be reduced to as few as 0.25×10^6 if not enough cells were obtained.
7. Antibody titer optimization: We found the BD Pharmingen antibodies listed here work well for the FACS of bulge stem cells. The antibody concentrations listed are based on the manufacturer's recommendation. We found that using 1/5 to 1/10 of the recommended concentration also worked well. However, the concentrations of antibodies used need to be optimized by each user when they first perform this experiment.
8. FACS parameter optimization: Cells are collected using the following filters: CD49f FITC 530/30-A, CD34 PE 585/42-A, and DAPI 450/20-A with UV excitation. Sorting parameters are set at: 15 psi, a 100 μm nozzle, and 30,000 kHz drop drive frequency. This configuration permits a trigger rate of approximately 6000 cell/s with abort rates of less than 20%. Purity check on the $CD34^+CD49f^+$ population ranges, typically around 95–98%. Data are analyzed using DIVA software. When 6- to 8-week telogen skin is used for cell isolation, the percentage of the $CD34^+CD49f^+$ population is usually between 5% and 10% of the total cells (*see* Fig. 1a).
9. Dispase treatment overnight at 4 °C can be shortened to 2 h by incubation at 37 °C, if it is necessary to speed up the isolation process.
10. Collagenase I solution: Weigh 35 mg collagenase I, dissolve in 10 mL complete EMEM medium, filter by pushing through a syringe attached to a 0.22 μm filter unit. Use 10 mL solution for every ten pup dermis.
11. Invert the tube and resuspend the tissue pieces in the collagenase solution thoroughly 4–5 times during incubation can help increase the yield of isolated cells.
12. Dermal cells are best used fresh after isolation for reconstitution assay. Dermal cells can be frozen in complete EMEM containing 5% DMSO for future use. However, we found that

previously-frozen dermal cells yield approximately 50% less hair follicles than freshly-isolated dermal cells in reconstitution assays.

13. Both reconstitution assays, the patch assay, and the silicone chamber dome grafting surgery should be carried out with strict adherence to institutional guidelines for minimizing distress in experimental animals. Surgery instruments should be sterilized before use.
14. As a positive control for the constitution experiment, 1×10^6 freshly-isolated mouse neonatal dermal cells, combined with 1×10^6 freshly-isolated mouse neonatal epidermal cells per injection, will yield roughly 200–300 hair follicles. We found that the ratio of dermal to epidermal cells, ranging 5:1 to 1:5, while maintaining the total number of injected cells, had little effect on hair-forming efficiency in the reconstitution assay. When sorted cells are used, the number of hair follicles formed may vary due to the purity of the cells and the level of cell damage resulting from sorting. The number of cells used per injection will need to be adjusted and optimized by individual researchers, based on the available number of sorted cells and hair-forming potential of the cells used.
15. Withdraw the plunger prior to loading the cell suspension in the syringe, so that an air space forms between the plunger and the cell suspension. Then carefully inject the cell suspension only, while avoiding injecting the air into the skin. This is to prevent losing cells to the dead space of the needle hub during the injection.
16. Given the indispensible roles of the reciprocal interactions between dermal and epidermal cells during hair morphogenesis, it is important to maintain the target cells in close proximity following injection. To achieve this, the injected cells should be placed above the panniculus carnosus layer of the skin. Therefore, it is critical to maintain the needle-entry angle in parallel to the skin surface and make sure that the needle does not penetrate the full thickness of the skin during the injection. This is to ensure that the cell mixture stays in a confined space in the skin, without being diluted through subcutaneous diffusion.
17. One advantage of the patch assay is that it is a semi-quantitative assay. The number of hair follicles formed by various cell populations in different injections can be counted under a dissecting scope once the skin is harvested. The number of hair follicles formed per injection can be manipulated by injecting less or more cells per test in order to make the quantification manageable.
18. Currently, the silicone chamber is not available from any commercial source as far as we know. However, the chambers can

be custom-made by individual researchers. To assist interested researchers, the chamber and its dimensions are shown in Fig. 1d–j.

19. If the number of hair follicles formed in a chamber assay needs to be quantified, the harvested fresh skin can be submerged in 2 mg/mL dispase solution at 4 °C overnight. The epidermis can be peeled off from the dermis, and the dermis can be fixed with 4% paraformaldehyde/PBS solution at room temperature for 1 h, followed by chromogenic staining for alkaline phosphatase activity in the dermal papilla (DP). The number of stained DPs can be counted under a dissection microscope or in digital images of the stained skin using ImageJ software, which can be downloaded from NIH website.

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Chapter 9

Hair Follicle Regeneration by Transplantation of a Bioengineered Hair Follicle Germ

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Abstract

Hair follicle morphogenesis is first induced by epithelial–mesenchymal interactions in the developing embryo. In the hair follicle, various stem-cell populations are maintained in specialized niches to promote repetitive hair follicle-morphogenesis, which is observed in the variable lower region of the hair follicle as a postnatal hair cycle. In contrast, the genesis of most organs is induced only once during embryogenesis. We developed a novel bioengineering technique, the Organ Germ Method, that employs three-dimensional stem cell culture for regenerating various organs and reproducing embryonic organogenesis. In this chapter, we describe a protocol for hair follicle germ reconstitution using adult follicle-derived epithelial stem cells and dermal papilla cells with intracutaneous transplantation of the bioengineered hair-follicle organ germ. This protocol can be useful not only for the clinical study of hair regeneration but also for studies of stem cell biology and organogenesis.

Key words Hair follicle, Organ Germ Method, Stem cells, Epithelial–mesenchymal interaction

1 Introduction

Hair plays important roles in physical insulation, sensitivity to noxious stimuli, and social communication in humans [1]. Hair follicle morphogenesis is first induced by reciprocal epithelial and mesenchymal interactions in the developing embryo [1, 2]. The hair follicle is composed of a permanent region, which consists of the infundibulum and isthmus, and a lower variable region, including differentiated epithelial cells, hair matrix, and dermal papilla (DP) cells. After morphogenesis, various stem cell types are maintained in specialized regions: follicle epithelial stem cells in the bulge region [3, 4], follicle mesenchymal precursors in the DP [5, 6], neural crest-derived melanocyte progenitors in the sub-bulge region [7, 8], and follicle epithelial stem cells in the bulge region that is connected to the arrector pili muscle [1, 9]. These follicle stem cells contribute to repeated hair follicle morphogenesis, which

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is observed in the variable lower region of hair follicle as a postnatal hair cycle. In contrast, the genesis of most organs is induced only once during embryogenesis [1].

As a next-generation regenerative therapy, organ regeneration is expected to provide a novel medical therapeutic system to replace lost organs or organs damaged by disease, injury, or aging with a bioengineered organ [10]. A concept has been proposed to reproduce the organogenesis that occurs in the developing embryo through epithelial–epithelial interactions for generating a bioengineered organ [11]. To achieve hair-follicle organ regeneration, many studies have described technologies to reconstitute the variable lower region of the hair follicle [12], to reproduce de novo folliculogenesis via replacement with hair follicle-inductive dermal cells [13], and to direct the self-assembly of skin-derived epithelial and mesenchymal cells [14–16]. These technologies provide the basic potential to reconstruct a regenerated hair follicle for hair regeneration in vivo [17]. However, several technical issues, including precise cell processing methods in three-dimensional (3D) stem cell culture, eruption of a bioengineered hair by intracutaneous transplantation in vivo, restoration of the correct connection to surrounding tissues such as arrector pili muscle and nerve fibers, and the enduring hair cycle in a lifetime, must be resolved [17].

Recently, a novel bioengineering method, designated the Organ Germ Method, was developed to generate a bioengineered organ germ by multicellular organization of epithelial and mesenchyme cells in 3D stem cell culture [18]. The bioengineered organ germs reconstituted by the Organ Germ Method could regenerate various fully functional bioengineered ectodermal organs, such as teeth [18, 19], salivary glands [20], lacrimal glands [21], and hair follicles [22, 23] in vivo. Hair regeneration has successfully been demonstrated by intracutaneous transplantation of a bioengineered pelage and vibrissae hair follicle germ, which were regenerated with embryonic skin-derived cells and adult vibrissae follicle-derived stem cells, respectively [22, 23]. These bioengineered hair follicle germs could develop the correct hair follicle structures and repeat hair cycles through the rearrangement of stem cells and their niches. The bioengineered hair follicle could further form the correct connections with surrounding host tissues, such as the epidermis, arrector pili muscle, and nerve fibers [22, 23]. These results indicate that the technique for creating bioengineered hair follicles can be applicable to clinical regeneration as well as basic studies of stem cell biology during organogenesis.

In this chapter, we describe a protocol to generate bioengineered hair follicle germs with adult hair follicle-derived epithelial and mesenchymal stem cells using our Organ Germ Method. We also provide an intracutaneous transplantation protocol using bioengineered hair follicle germs for hair regeneration.

2 Materials

All solutions should be prepared using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents, sterilized by autoclave treatment or 0.22 μm filtration. All surgical instruments should be washed and sterilized in an autoclave prior to each use to prevent contamination. Enzymes, cytokines and serum reagents should be evaluated for activity and primary cell culture efficiency, respectively, prior to experimental use.

1. Donor animals: An inbred mouse strain (e.g., C57BL/6) should be used in these experiments. To discriminate between newly-reproduced hair follicles and host hair follicles, use a mouse with a pigmented coat and/or a fluorescent protein-tagged transgenic mouse (i.e., C57BL/6-TgN (act-EGFP) OsbC14-Y01-FM131) for experiments.
2. Recipient animals: Immunodeficient mice lacking a normal hair coat (e.g., Balb/c nu/nu or hairless SCID) are ideally suited for intracutaneous transplantation of the bioengineered hair follicle germ and for reproducing hair follicles.
3. Disinfectants: 10% povidone-iodine and 70% rubbing alcohol.
4. Ca^{2+}/Mg^{2+}-free, phosphate-buffered saline (PBS): 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 8.0 mM anhydrous disodium hydrogen orthophosphate (Na_2HPO_4), and 1.5 mM potassium phosphate monobasic (KH_2PO_4). Store at 4 °C (*see* **Note 1**).
5. Cell culture medium: Dulbecco's Modified Eagle's Medium (high glucose) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (DMEM10).
6. Buffering medium: Isolate hair follicles and dissociate single cells in cell culture medium supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (DMEM10/HEPES). For organ culture, use this medium with the above-mentioned supplements, excluding HEPES (*see* **Note 2**).
7. Dermal papilla cell culture medium: Cultivate dermal papilla cells in DMEM10 supplemented with 10 ng/mL fibroblast growth factor (FGF)-2.
8. 0.05% trypsin, 1 mM EDTA solution.
9. Dispase solution: Dispase (50 U/mL, Cat. #354235; BD, Franklin Lakes, NJ, USA). Dilute tenfold in Hanks' balanced salt solutions (HBSS) and store at −20 °C until use (*see* **Note 3**).

10. Collagenase solution: Adjust the concentration of collagenase I (Cat. #4196; Worthington, Lakewood, NJ, USA) to 1 mg/mL using distilled water and store at −20 °C until use (*see* **Note 4**).
11. 4.8 U/mL dispase and 100 U/mL collagenase solution in PBS.
12. 70 U/mL Deoxyribonuclease I (DNase) from bovine pancreas (*see* **Note 5**).
13. Collagen gel: Combine 100 μL tenfold concentrated α-Minimum Essential Medium (αMEM) (Sigma, St. Louis, MD) and 100 μL mixed buffer (0.08 N sodium hydroxide and 200 mM HEPES) in 800 μL acidic collagen solution (3 mg/ml, pH 3.0). Gently mix and keep on ice until usage (*see* **Note 6**).
14. Nylon thread (8–0 nylon surgical suture; Natsume, Tokyo, Japan) (*see* **Note 7**).
15. Surgical tools for the separation and dissection of adult vibrissa hair follicles from the whisker pad: Use a large surgical scissors and forceps to cut and collect whole layers of the mystacial pad. Use a small surgical scissors and forceps are used to isolate the vibrissa hair follicle (*see* **Note 8**).
16. Microsurgical instruments for intracutaneous transplantation of the bioengineered hair follicle germ: Use a disposable microsurgical knife (e.g., 20-G Ophthalmic V-Lance, Alcon Japan, Tokyo, Japan) to prepare a micrograft site in the skin and use microforceps for implantation of the bioengineered hair follicle germ (*see* **Note 9**).
17. Dressings: Sterile surgical tape to cover the engrafted site (*see* **Note 10**).
18. Dissecting microscope (*see* **Note 11**).
19. Sterile disposable 1-mL syringes and 25-G needles (5/8; 0.50 × 16 mm) for hair germ extraction.
20. Sterile disposable Petri dishes (35 and 100 mm).
21. Sterile disposable polypropylene conical tube (15 mL) to collect single cells isolated from hair germ.
22. Sterile disposable 1.5-mL microtubes (*see* **Note 12**).
23. Sterile disposable gel loader tips and pipette tips for reconstitution of the bioengineered hair germ (*see* **Note 13**).
24. Culture at the medium-gas interface using a cell culture insert (0.4-μm pore size membrane) (*see* **Note 14**).
25. Silicone grease.
26. Sterile micropipette.

3 Methods

The hair regeneration protocol comprises three steps: (1) the preparation of epithelial stem cells in the bulge region and primary cultured DP cells from adult vibrissa hair follicles (3.1. in Fig. 1), (2) reconstitution of the bioengineered hair follicle using the Organ Germ Method (3.2 in Fig. 1; Ref. 18), and (3) intracutaneous transplantation into the back of recipient mice (3.3 in Fig. 1; Ref. 22).

3.1 Preparation of Hair Folicle Inducible Stem Cells

3.1.1 Preparation of Adult Murine Vibrissa Follicles

1. Dissect the mystacial pad region from 7- to 9-week-old mice and briefly rinse with povidone-iodine disinfectant, 70% ethanol, PBS and DMEM10/HEPES for 10–30 s in each step.
2. Keep the donor tissues in a 6-cm petri dish filled with cold DMEM10/HEPES for further preparation (Fig. 2A-a, b, *see* **Note 15**).
3. Remove the connective tissues from the reverse side of each mystacial pad (Fig. 2A-c).
4. Isolate whole vibrissa follicles from donor tissues using a surgical knife and fine forceps (Fig. 2B-a).
5. Remove the connective tissues, nerve fibers and adipose tissues attached to the hair follicles (Fig. 2B-b).
6. Separate vibrissa hair follicles into early-mid anagen (I–V), late anagen (VI), and telogen–catagen phases [24].
7. Keep the hair follicles at 4 °C in cold DMEM10/HEPES until the next step.

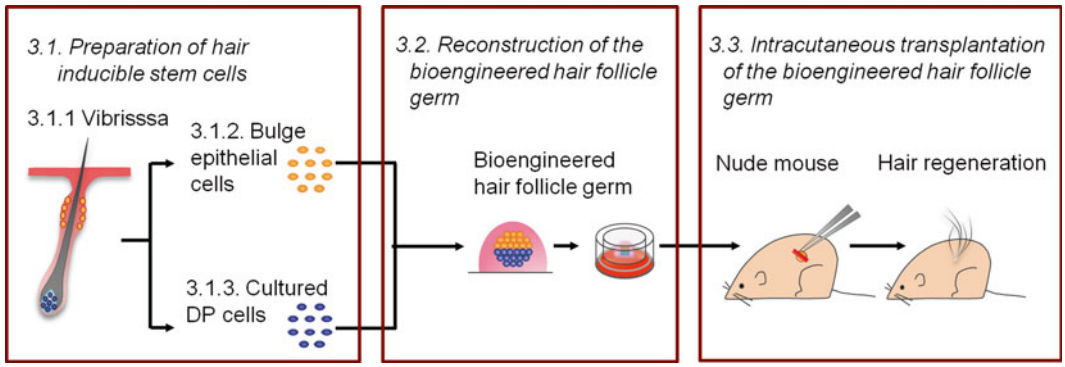

Fig. 1 Overview of hair regeneration. Schematic representation of the methods used for the preparation of inducible hair cells from adult hair follicles, their reconstruction, and the transplantation of a bioengineered hair-follicle germ

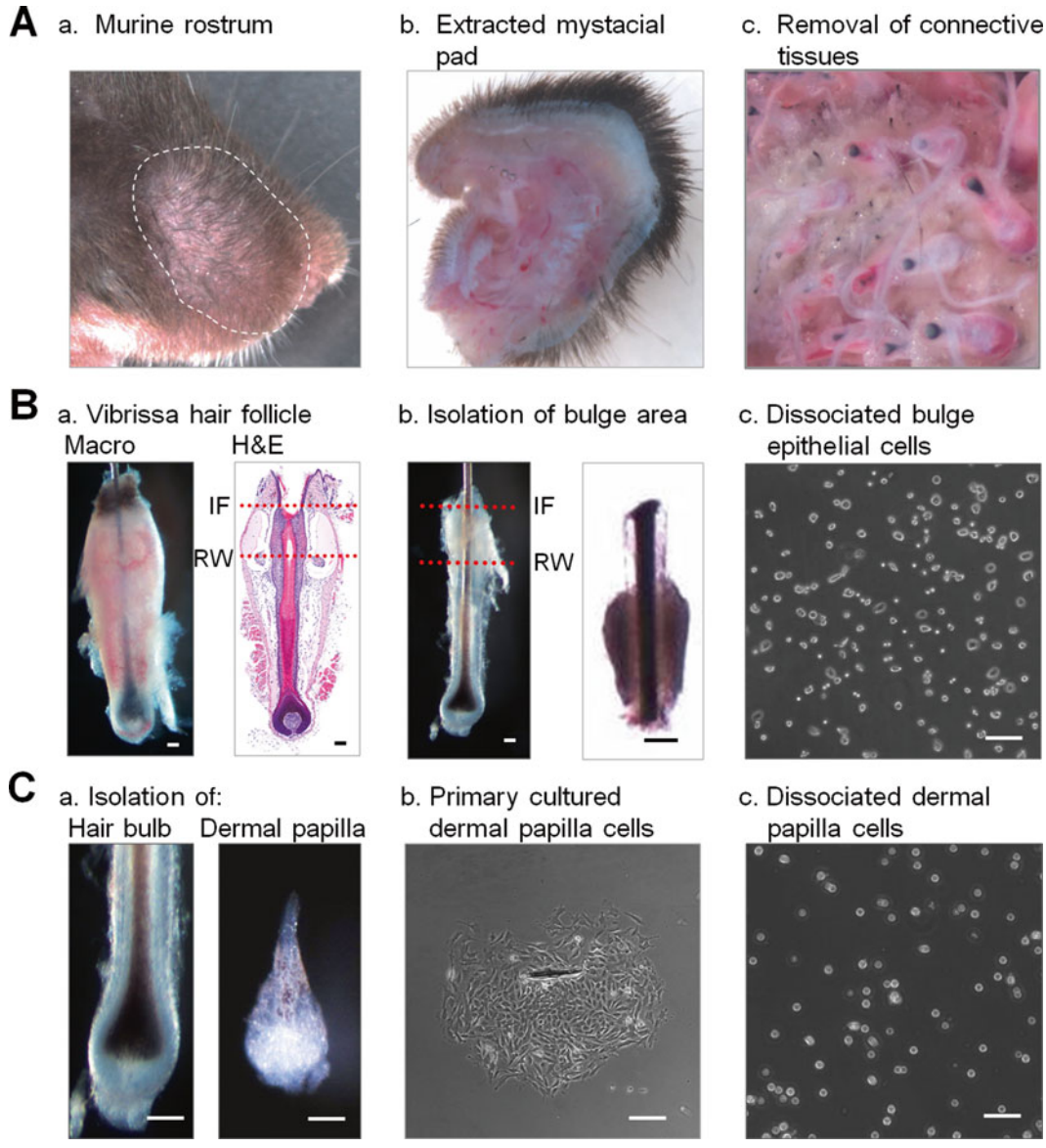

Fig. 2 Preparation of single bulge epithelial and dermal papilla cells. (**A**) Macroscopic images of the murine vibrissa hair follicle. Mystacial pad area in a murine rostrum area (*dotted lined area* in *a*). Image from dermis side of the extracted mystacial pad area (*b*). The mystacial pad after removal of connective tissues (*c*). (**B**) Bulge epithelial tissue and cells of vibrissa isolated from an adult mouse. Macroscopic and histological definition of a bulge epithelial tissue and cells of vibrissa isolated from an adult mouse. Macroscopic and H&E staining of whole vibrissa (*a*). *Dashed lines* (*red*) via macro-morphological observations (*left*) and H&E staining (*right*) indicate the location of the dissection. *IF* infundibulum, *RW* ringwulst. Scale bars, 100 μm. Macroscopy of a whole vibrissa hair follicle after removal of the collagen sheath and the dissected bulge region (*b*). Phase-contrast image of single cells from the bulge epithelium (*c*). Scale bars, 100 μm. (**C**) Dermal papilla tissue and primary cultured dermal papilla cells of the vibrissa from an adult mouse. Stereomicroscopic images of a hair bulb region (*left*) and the dermal papilla (*right*) of an adult vibrissa at anagen phase (*a*). The isolated dermal papilla tissue is explanted and cultured in vitro for cell propagation. Phase-contrast image of outgrowth colony (*b*) and dissociated primary-cultured dermal papilla cells at 9 days after explantation (*c*). Scale bars, 100 μm

3.1.2 Enzymatic Separation of Single Epithelial Cells from the Bulge Region of Adult Murine Vibrissa Follicles

1. Remove the collagen sheath from early-mid and late-anagen vibrissa hair follicles.
2. Separate the bulge region, which is defined as an area from below the sebaceous gland to above the ringwulst, under a microscope using 25-G needles (Fig. 2B-b, *see* **Note 15**).
3. Keep the bulge region at 4 °C in cold DMEM10/HEPES until enzymatic digestion.
4. Wash the bulge region twice with PBS (*see* **Note 16**).
5. Incubate the bulge region with 4.8 U/mL dispase and 100 U/mL collagenase solution at 37 °C for 5 min (*see* **Note 16**).
6. Stop the enzymatic reaction by washing the bulge region twice in cold DMEM10/HEPES (*see* **Notes 17** and **18**).
7. Remove the connective tissue sheath from the bulge region under a microscope using 25-G needles (*see* **Note 15**).
8. Collect the epithelial tissues.
9. Wash the bulge epithelial tissues twice with PBS (*see* **Note 17**).
10. Incubate the bulge epithelial tissues in 0.05% trypsin solution for 1 h at 37 °C.
11. Stop the enzymatic reaction by adding DMEM10/HEPES at three times the volume of the trypsin solution.
12. Dissociate the bulge epithelial cells by pipetting three times with a P-1000 tip.
13. Wash the single bulge epithelial cells twice in cold DMEM10/HEPES (*see* **Note 17**).
14. Immediately add 1 mL of DMEM10/HEPES and 1 μL of DNase solution to the cells (*see* **Note 19**). Create a single-cell suspension by passing the solution through a 70 μm cell strainer (Fig. 2B-c).
15. Resuspend with DMEM10/HEPES, count the single bulge epithelial cells and store on ice until reconstruction of the bioengineered hair follicle germ (*see* Subheading 3.2).

3.1.3 Primary Cultivation and Enzymatic Single-Cell Preparation of Dermal Papilla Cells

1. Isolate dermal papillae from early-mid anagen vibrissa follicles, explant onto a plastic culture dish (BD), and maintain the cells in DMEM10 supplemented with FGF-2 as primary cultures for 9 days, as described previously [5, 12, 25] (Fig. 2C-a, b).
2. Wash the primary cultured dermal papilla cells twice in PBS and aspirate the PBS.
3. Incubate with 0.5 mL 0.05% trypsin and 1 mM EDTA solution for 3 min at 37 °C (*see* **Note 20**).
4. Stop the enzymatic reaction by adding DMEM10/HEPES and resuspend the dermal papilla cells using a P-200 tip (*see* **Note 21**).

5. Wash the cells twice with DMEM10/HEPES (*see* **Note 16**).
6. Aspirate the supernatant to a residual volume of 200 μL and add 1 μL of DNase solution to the sample (*see* **Note 19**). Create a single-cell suspension by passing the solution through a cell strainer (Fig. 2C-c).
7. Resuspend with DMEM10/HEPES, count the single dermal papilla cells and store on ice until reconstruction of the bioengineered hair-follicle germ (*see* Subheading 3.2).

3.2 Reconstruction of the Bioengineered Hair Follicle Germ

1. Prepare siliconized 35-mm petri dishes and 1.5-mL tubes coated with silicon grease (*see* **Note 22**).
2. Transfer the isolated epithelial or mesenchymal single cell suspensions into separate siliconized 1.5-mL tubes.
3. Centrifuge at 600 × *g* for 3 min and remove the supernatant using a micropipette and a P1000 or P200 tip (Fig. 3A-a, *see* **Note 23**).
4. Centrifuge at 600 × *g* for 3 min and remove the residual supernatant from the cell pellets using a micropipette and a gel-loading tip under a microscope.
5. Prepare a droplet of 30 μL collagen gel on a siliconized petri dish (*see* **Note 24**).
6. Aspirate a 0.05-μL volume of the dermal-papilla cell pellet using a micropipette and a 0.1–10-μL pipette tip under a microscope (*see* **Note 25**).
7. Apply the cell pellet slowly into the collagen drop and make a spherical cell aggregate (Fig. 3A-b, c).
8. Similarly, apply a 0.05-μL volume of the epithelial cell pellet into the same collagen drop and make contact with the dermal-papilla cell aggregate (Fig. 3A-d, e, *see* **Notes 26–28**).
9. To form intraepithelial tissue connections between the host skin epithelium and the bioengineered hair follicle, insert a nylon thread guide (8–0 nylon surgical suture) into a bioengineered hair germ through the epithelial and dermal-papilla cell portions (Fig. 3A, f).
10. Incubate the petri dish holding the collagen gel drop for 15 min at 37 °C for gelation. Set the cell culture insert into a six-well plate filled with culture medium (1 mL/well) (Fig. 3B-a).
11. Pick up the collagen gel drop with tweezers and transfer the drop onto the cell-culture insert (Fig. 3B-b, *see* **Note 29**).
12. To prepare the bioengineered hair-follicle germ for transplantation, culture for 2 days.

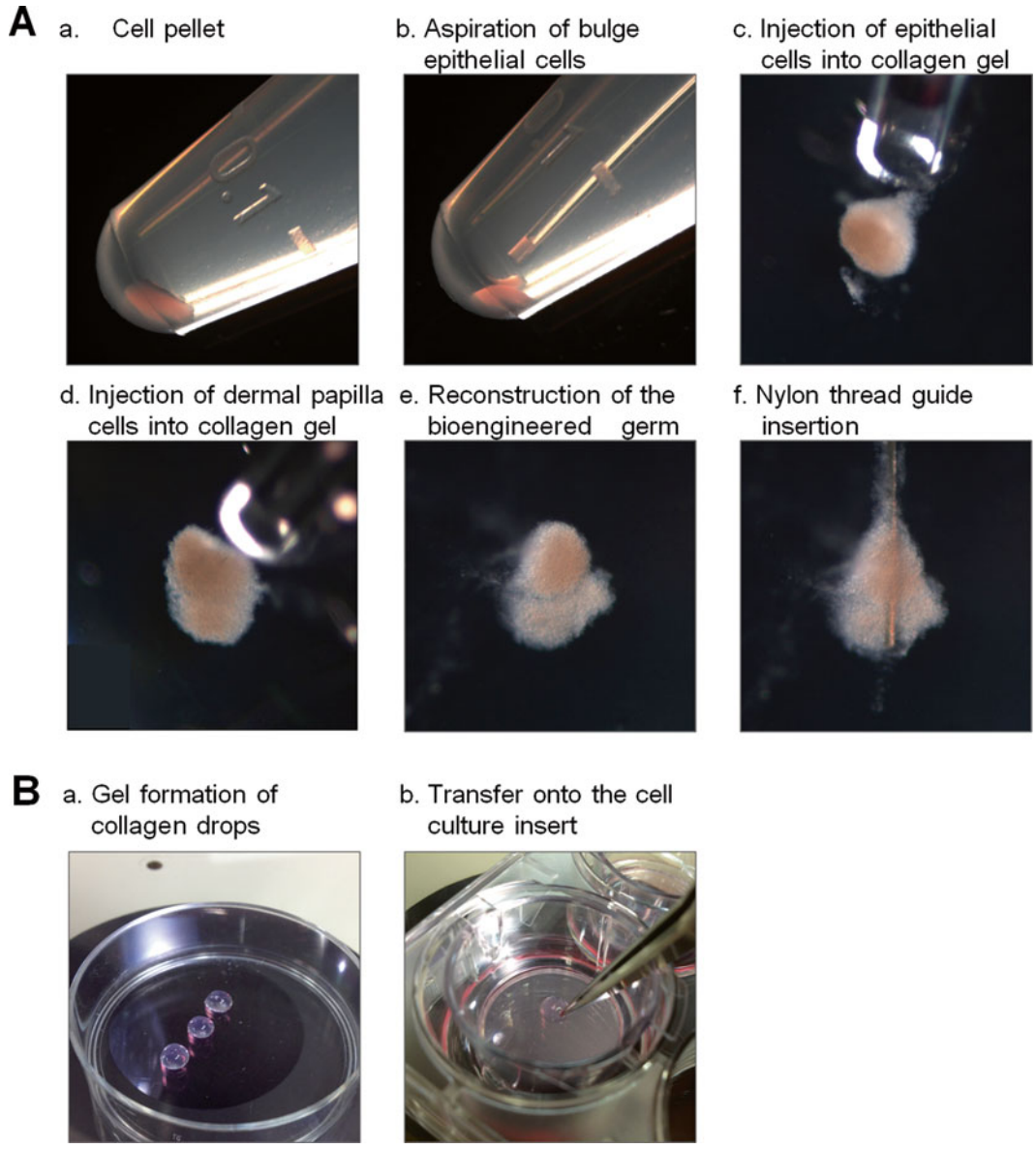

Fig. 3 Reconstruction of steps to generate a bioengineered hair follicle germ. (**A**) Reconstitution of the bioengineered hair follicle germ. Centrifuge and remove the residual supernatant around the cell pellet to obtain highly-concentrated bulge epithelial cells (*a*) and primary cultured dermal-papilla cells. Aspirate and inject the total volume of bulge epithelial cells into the center of the collagen drop (*b*, *c*). Subsequently, aspirate and inject the primary cultured papilla cells into the same drop adjacent to the epithelial-cell aggregate (*d*, *e*). Insert a nylon thread guide into a bioengineered hair germ through the epithelial and dermal-papilla cell portions (*f*). Scale bars, 100 μm. (**B**) Gel formation and short-term organ culture of the bioengineered hair follicle germ. Coagulate the collagen gel drops at 37 °C (*a*). Pick up the collagen gel drop, transfer onto a cell culture insert (*b*) and culture for 2 days until transplantation of the bioengineered hair-follicle germ

3.3 Intracutaneous Transplantation of the Bioengineered Hair Follicle Germ

1. Anesthetize the recipient mouse (*see* **Note 30**).
2. Position the mouse on it's side.
3. Briefly disinfect the skin surface of the recipient mouse with povidone-iodine and 70 % ethanol.
4. Create a shallow stab wound that is nearly parallel to the skin surface on the back of nude mice using a 20-G Ophthalmic V-Lance (Fig. 4A-a).
5. Engraft a bioengineered hair follicle germ, which contains a nylon guide, and arrange the germ so the epithelial portion is on top, into the shallow stab wound (Fig. 4A-b).
6. Hold the nylon guide using surgical tape on the skin surface (Fig. 4A-c).
7. Completely dress the engraftment using surgical tape.
8. Repeat this intracutaneous transplantation process, described in **steps 4**–7, until the density and area of the bioengineered hairs reaches the experimental goal (*see* **Note 31**).
9. Approximately 21 days after transplantation, the bioengineered hair follicle will exhibit correct tissue structures similar to the murine natural vibrissa follicle and erupt from the skin surface (Fig. 4B). The bioengineered hair follicle will reproduce the proper arrangement and connections with the cutaneous tissues of the recipient skin (Fig. 4C).

4 Notes

1. PBS is used to wash the tissues and cells. Therefore, it should be an isotonic solution that does not cause cell injury.
2. Choose the cell culture medium (e.g., DMEM) after performing a pilot experiment. HEPES is added to keep the pH constant, and prevent cytotoxicity during extended culture, HEPES is not added to organ culture medium.
3. Dispase dissolves the basement membrane between the epithelial and the mesenchymal tissues of the hair germ. Even when using the same reagents described in this protocol, we recommend evaluating the temperature and reaction time because enzymatic activity is easily decreased at 4 °C.
4. Collagenase is an enzyme used to digest collagen. Intercellular collagen molecules are dissolved by collagenase, and tissues are separated into single cells. Even when using the same reagents described in this protocol, we recommend evaluating the temperature and reaction time because enzymatic activity is easily decreased at 4 °C.
5. DNase can prevent cell aggregation by digesting the DNA.

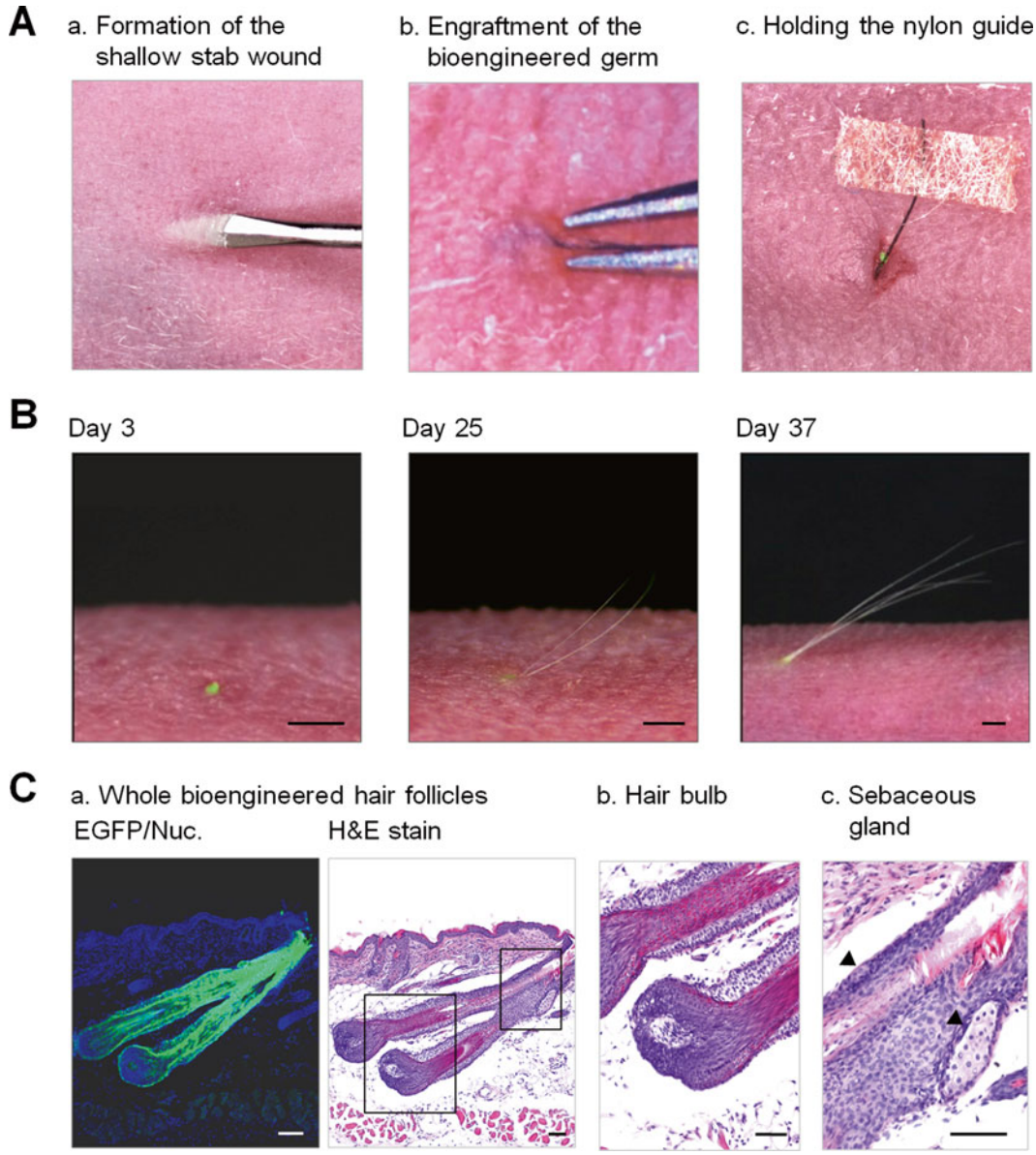

Fig. 4 Intracutaneous transplantation of the bioengineered hair follicle germ. (**A**) Surgical formation of a shallow stab wound for an engraftment site for the bioengineered hair-follicle germ. Create a shallow stab wound in the back skin of nude mice with a microsurgical knife (*a*). Engraft the bioengineered hair follicle germ into the shallow stab (*b*) and hold the nylon thread so it protrudes from the skin surface (*c*). (**B**) Macro-morphological observations of the bioengineered hairs during eruption and growth of the bioengineered vibrissa. (**C**) Histological analyses of the bioengineered vibrissa follicles. Fluorescence microscopy and low-magnification H&E panels present the same bioengineered hair follicles. The *boxed areas* in the low-magnification H&E panels are shown at a higher magnification in the right panels. The *arrowhead* indicates a sebaceous gland. Scale bars, 100 μm in *a*, 50 μm in *b* and *c*

6. Collagen gel can be easily made using Cellmatrix Type I-A (Nitta Gelatin, Inc., Osaka, Japan).
7. Choose smooth and tough materials as an equivalent.
8. Surgical instruments should be washed and sterilized by autoclaving prior to each use to prevent contamination.
9. The microsurgical knife should be disposable.
10. We recommend the use of 1-in. wide surgical tape, which can easily cover the engraftment sites.
11. We recommend the use of a dissecting microscope capable of 6.5–50× magnification with a transmitted beam applied as the light source.
12. Round-bottom microtubes should be used because square-bottom microtubes are unsuitable for forming a cell pellet by centrifugation.
13. A 0.1–10-μL pipette tip is suitable for making a highly-concentrated cell aggregate in the collagen gel.
14. A membrane should be used with a pore size that is sufficient for liquid components to pass through. We use a cell culture insert with a 0.4-μm pore size membrane (BD Biosciences).
15. Needle manipulation should be performed carefully to avoid injury to the epithelial and dermal-papilla tissue.
16. Carefully follow the time and the temperature of enzyme reactions because long enzyme reactions can injure the hair follicle.
17. The washing procedures of this protocol should be repeated by centrifuging at 590 × *g* for 3 min, removing the supernatant carefully, and then resuspending in PBS or DMEM10/HEPES.
18. Quick and precise washing is required for the dispase solution.
19. Tissues and cells that have been enzymatically treated can easily aggregate due to released DNA, making subsequent manipulations difficult. The addition of DNase digests DNA and can prevent cell aggregation.
20. When cell aggregates are not dispersed by tapping, the cell pellet can be manipulated into a single-cell suspension by gently pipetting up and down.
21. Micropipette manipulation should be performed gently at a constant speed.
22. Be careful not to apply too much silicone grease and not to apply it to the inside of the 1.5-mL tube cap.
23. Remove as much of the residual supernatant as possible to create a high-density cell pellet. If residual supernatant remains, the cell aggregate will not form in the collagen gel.

24. The cell manipulation that reconstitutes the bioengineered hair germ should be performed quickly because the collagen gel solidifies with a change in temperature and the passage of time.
25. To reconstitute a bioengineered hair-follicle organ germ, aspirate only the required amount of cells using a pipette tip.
26. Insert the pipette tip into the collagen gel using a P-2 micropipette. The cell aggregate is extruded slowly, and the pipette tip must be precisely operated so that the cell aggregate becomes spherical. Cell insertion should be stopped precisely when all cells have been extruded from the pipette tip in order to prevent air bubbles from getting into the gel.
27. When bioengineering a hair germ using both epithelial and/or mesenchymal hair germ tissues (i.e., reconstitution of tissue with tissue or tissue and cell), ensure that there is sufficient contact between the tissues and/or the cell aggregate.
28. When creating a bioengineered hair germ using this cell-manipulation method, the number of developed bioengineered hair germs can be reduced by creating a small volume for the cell aggregate.
29. Remove the collagen gel from the bottom of the siliconized dish. The side of the collagen gel should be picked up with tweezers and carefully placed onto the cell-culture insert.
30. Anesthetize the mouse using an intraperitoneal injection of 5 mg/mL pentobarbital or inhalation anesthesia.
31. The density and area of the bioengineered hairs also can be regulated by repeatedly transplanting bioengineered hair follicle germs.

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Chapter 10

Hair Induction by Cultured Mesenchymal Cells Using Sphere Formation

Kazuo Kishi and Ruka Hayashi

Abstract

Isolated dermal cells possess the capacity to induce hair growth. The cells cannot be expanded while they retain the capacity for hair induction, and lose their potential immediately after cultivation. Sphere-forming multipotent cells derived from the dermis (skin-derived precursors [SKPs]) possess hair-inducing activity. These observations provide two possibilities for the determination of the capacity for hair induction: capacity is dependent on either identity as a dermal cell or on the process of sphere formation. We developed a method that demonstrates cultivated mesenchymal cells derived from dermis and lung tissue possess in vivo hair-inducing capacity via sphere formation.

Key words Mesenchymal cells, Sphere formation, Skin-derived precursors (SKPs)

1 Introduction

Interactions between epithelial and dermal cells are essential for hair follicle morphogenesis and maintenance [1]. The hair-inducing capacity of dermal components is critical for realization of regenerative medicine of hair follicles. Indeed, dermal papilla cells and fetal dermal mesenchymal cells have been shown to possess the capacity to regenerate hair follicles [2–5].

Miller and colleagues first demonstrated that a distinct population of multipotent dermal cells, skin-derived precursors (SKPs), is found in juvenile and adult rodent skin and the adult human scalp [6]. When skin tissues are dissociated and cultured in uncoated flasks, a small population of floating cells proliferates to generate larger spheres. These sphere-forming cells can differentiate into neurons, adipocytes, and smooth muscle cells. Moreover, clonally expanded SKPs induce hair morphogenesis, and form a hair follicle niche upon transplantation [7]. Hoffman and colleagues isolated multipotent cells, utilizing nestin-EGFP mice, from the hair

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follicle bulge area [8–10]. These findings raise the following question: On what does hair induction capacity depend, a history of sphere formation or identity as a dermal cell?

We have shown that sphere-forming dermal cells, but not two-dimensionally cultured dermal cells, possess the capacity to induce hair morphogenesis in vivo [11]. Moreover, sphere-forming mesenchymal cells derived from adult lungs of mice also possess the capacity for in vivo hair induction, suggesting that sphere formation alone is sufficient to impart capacity to mesenchymal cells other than dermal cells. Here, we present the technique of regenerating hair from dermal and lung mesenchymal cells by making spheres on agarose gels.

2 Materials

Animal care and all experimental procedures should be performed in accordance with organizational guidelines and policies.

2.1 Mice

1. Pregnant, newborn and 8-week-old male C57bl/6 J mice from a breeder.
2. Seven-week-old SCID mice from a breeder.
3. CAG-EGFP transgenic mice from the Jackson Laboratory (Bar Harbor, ME, USA).

2.2 Two-Dimensional (2D) Expansion of Mesenchymal Cells

1. Microscope, scissors, and forceps.
2. Enzymatic dissociation solution: 0.2% collagenase Type I (Wako, Osaka, Japan) diluted in phosphate-buffered saline (PBS).
3. Cell strainer: 40 μM (BD Falcon, Franklin Lakes, NJ, USA).
4. Culture medium for expansion: Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco-BRL, Carlsbad, CA, USA).
5. Passage solution: 0.25% trypsin/EDTA (Sigma Chemical Co., St. Louis, MO, USA).

2.3 Sphere-Forming Induction of Mesenchymal Cells

1. Agarose gel: 1% (Takara, Osaka, Japan) in PBS.
2. Plastic dishes: 10 cm in diameter.
3. Culture medium for sphere formation: three parts DMEM, one part F-12 containing B-27 (Gibco-BRL), 20 ng/ml EGF and 40 ng/ml bFGF (both from Sigma).

2.4 Cell Transplantation into the Skin of SCID Mice

1. Microscope, scissors, micro forceps, and needle with a 4-0 nylon suture.
2. Dispase: 1000 units (Godo Shusei, Tokyo, Japan).

3. Trypsin/EDTA, 0.25% (Sigma).
4. DMEM containing 10% FBS.
5. Cell strainer: 40 μm nylon filter (Cell Strainer R, BD Falcon).
6. Type I collagen solution: Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan).
7. Cap separated from a 1.5-ml polypropylene microfuge tube. Heat an 18 G needle and make four tiny holes, one of which must be in the center of the cap (for transplantation) while the other three are for suturing.
8. Vinyl tape.
9. Pipette and tips: 200 μl.

3 Methods

3.1 Two-Dimensional Expansion of Mesenchymal Cells

1. Anesthetize mice and perform cervical spine dislocation for euthanasia. Dissect E17 fetal skin and adult lungs (*see* **Note 1**). Mince the tissues with a scalpel, add 0.2% collagenase Type I and incubate at 37 °C for 20–30 min, depending on the type of tissue.
2. Pass the tissues through a 40-μm cell strainer and centrifuge at 1500 rpm (380 × *g*) for 5 min. Place the suspended cells in a plastic flask with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37 °C in 5% CO_2.
3. Passage the cultured cells with 0.25% trypsin. Use cells from passages 8–10 for sphere formation.

3.2 Sphere-Forming Induction of Mesenchymal Cells

1. Make a 1% agarose solution by dissolving 5 g of agarose in 495 ml of PBS. Heat the mixture in a microwave oven until completely dissolved.
2. Pour the agarose solution into 10-cm plastic dishes and keep them covered or in a hood until they cool and can be stacked (*see* **Note 2**) (Fig. 1).
3. Prepare sphere-forming culture medium.
4. Suspend 2D-cultured mesenchymal cells with 0.25% trypsin, neutralize with culture medium, centrifuge, resuspend in sphere-forming culture medium, and spread on solidified agarose. Incubate the dishes at 37 °C in 5% CO_2 (*see* **Note 3**) (Fig. 2). Change medium twice a week (*see* **Note 4**).

3.3 Cell Transplantation into the Skin of SCID Mice (see **Note 5***)*

1. Prepare type I collagen gel solution using the Cellmatrix type I-A: mix eight parts collagen, one part concentrated medium, and one part reconstitution buffer. Mix well by pipetting. Keep gel mixture on ice in a cooler box to prevent gelling (*see* **Note 6**).

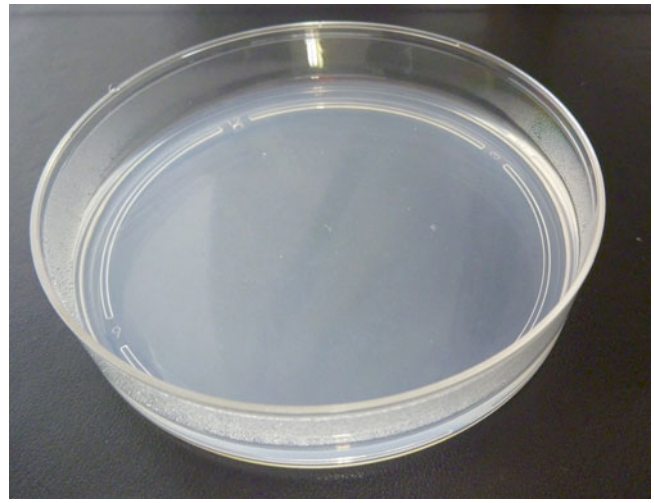

Fig. 1 Agarose gel spread over the bottom of a 10-cm plastic dish. It is important to cover the entire surface of the bottom of the dish

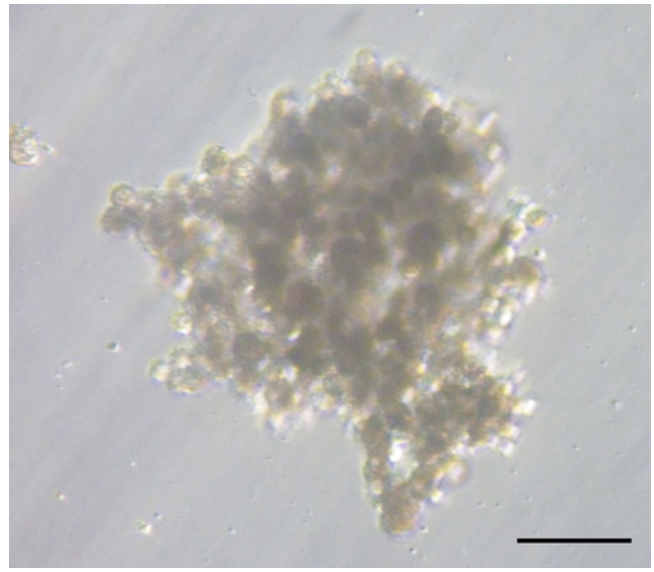

Fig. 2 Spheroid on an agarose gel 1 day after cultivation. Bar = 50 μm

2. Dissect approximately 1 cm^2 of full thickness skin from newborn C57bl/6J mice under a microscope.
3. Immerse and incubate the skin in 1000 units of dispase solution for 30 min at 37 °C.
4. Detach the epidermal sheets from the dermis using two microforceps (*see* **Note** 7).

5. Incubate the epidermal sheets with 0.25% trypsin-EDTA for 15 min at 37 °C. Stop the digestion reaction with a two-fold volume of DMEM containing 10% FBS. Suspend the mixture by pipetting up and down through an 18 G needle. Pass the suspended epidermal cells through a cell strainer and centrifuge at 1500–2000 rpm (380–670 × *g*) for 5 min. Discard the supernatant (*see* **Notes 8** and **9**).
6. Use a pipet to collect the cultured spheroids and centrifuge them at 1500 rpm (380 × *g*) for 5 min. Discard the supernatant. Each transplant contains approximately 1×10^7 epidermal cells and 1×10^7 mesenchymal cells.
7. Mix the newborn epidermal cells with either centrifuged mesenchymal-cell spheroids or 2D cultured mesenchymal cells. Mix the same volume of type I collagen-gel solution with the pellet. Keep on ice until transplantation.
8. Inject pentobarbital into the abdominal cavity. Make full skin thickness wounds, 7 mm in diameter, on both sides of the back skin of 7-week-old male SCID mice.
9. Separate a polypropylene cap from a 1.5-ml microfuge tube and push it into the skin wounds. Suture the surrounding skin with 4-0 nylon (Fig. 3).
10. Inject the mixture through the tiny hole in the center of the cap. Cover with vinyl tape (Fig. 4).
11. One week later, remove the polypropylene caps under anesthesia.
12. Four weeks later, sacrifice the transplanted animals with an inhalational overdose of ether followed by cervical-spine dislocation. Collect the transplants (Fig. 5).

Fig. 3 Polypropylene cap from a microfuge tube containing three holes for a suture, and one central hole for transplantation

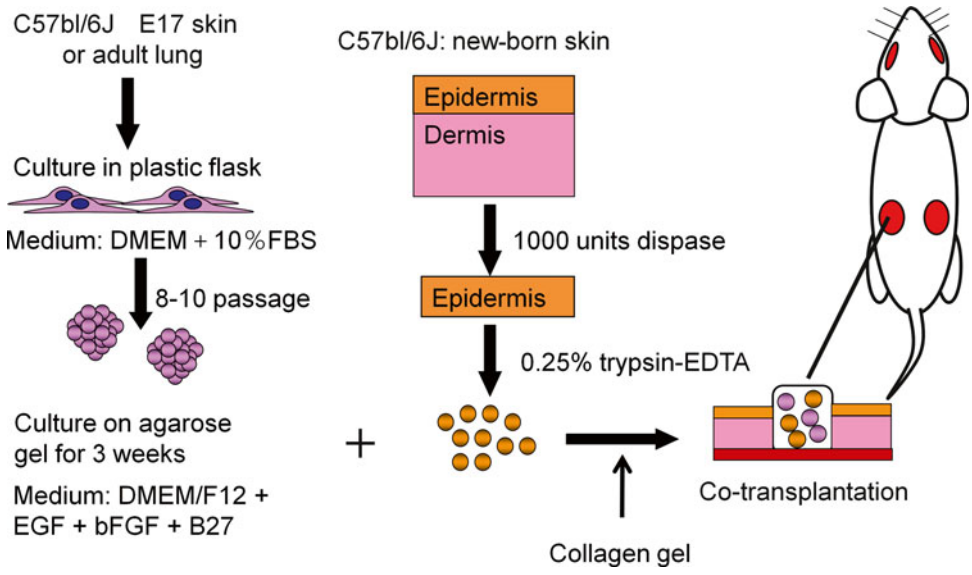

Fig. 4 Schematic of the transplantation process

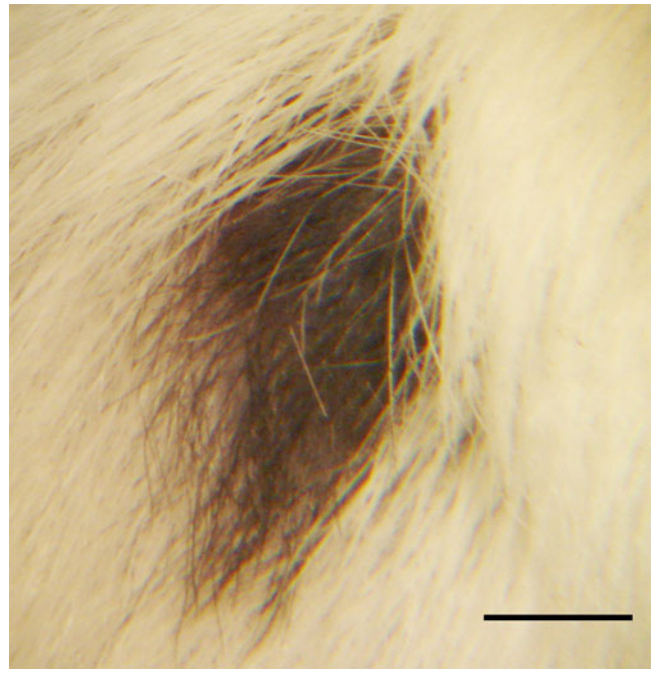

Fig. 5 Regenerated hairs induced by dermal fibroblasts. Bar = 5 mm

4 Notes

1. We tested hair induction using cells from E17 skin and adult lungs, and fibroblasts from passages 8–10. Other fibroblast sources or conditions have not been tested.
2. It is important to cover the entire area of the base of the dish with agarose. Thus, the amount of agarose solution may be increased.
3. Most of the cells start making aggregates within a day after culturing on agarose in dishes.
4. Spheroids can be observed with the naked eye. The spheroids sink to the bottom of the culture medium (approximately 20 ml/dish). One-half to two-thirds of the supernatant is removed when the medium is changed.
5. Cell and animal preparation for the transplantation procedure should be performed simultaneously. Thus, it is recommended that two teams perform the procedures.
6. The collagen solution does not gel when the procedure is performed on ice. However, it easily gels when it becomes warm. To prevent gel formation before transplantation, all equipment and reagents that might attach to the collagen gel solution, including the cell mixture, pipet tips, cylinder, and 18 G needle, should be cooled.
7. If the epidermis does not detach from the dermis, the incubation time may be increased. In order to separate the epidermis effectively, the skin should be spread thoroughly in the dispase solution.
8. To dissolve the epidermal cells in trypsin solution, pipet up and down using a 1-ml syringe with an 18 G needle every 5 min during incubation.
9. Since the cells of fetal or newborn mice are sticky, apply pressure to the filter with a syringe.

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Chapter 11

Stereological Quantification of Cell-Cycle Kinetics and Mobilization of Epithelial Stem Cells during Wound Healing

Eduardo Martínez-Martínez, Eileen Uribe-Querol, Claudio I. Galván-Hernández, and Gabriel Gutiérrez-Ospina

Abstract

We describe a stereology method to obtain reliable estimates of the total number of proliferative and migratory epithelial cells after wounding. Using pulse and chase experiments with halogenated thymidine analogs such as iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU), it is possible to track epithelial populations with heterogeneous proliferative characteristics through skin compartments. The stereological and tissue processing methods described here apply widely to address important questions of skin stem-cell biology.

Key words Stereology, Hair follicles, Halogenated thymidine analogs, Iododeoxyuridine, Chlorodeoxyuridine, Bromodeoxyuridine, Nucleoside, Stem cells, Bulge, Epidermis, Wound, Keratinocytes, Random sampling, Confocal microscopy

1 Introduction

One fundamental role of adult stem cells is to reestablish tissue integrity after injury by supplying new cells to the wounded area. In the skin epithelium, it is known that epidermal wounds activate the proliferation and differentiation of a group of stem cells that reside in an area of the hair follicle called the bulge [1–3]. The initial identification of the regions in the skin epithelium that contained putative stem cells was made possible by using tritiated thymidine labeling and autoradiographic techniques. Using this method, the epithelial cells that retained the tritiated thymidine labeling, even for periods longer than 1 year, were found to be a population of cells (label-retaining cells) mainly located at the bulge area. These also display clonogenic properties in culture [1, 4]. Later identification of bulge-cell markers enabled their isolation and molecular characterization which led to the confirmation of the multipotency of these cells, both in vivo and in vitro [5–7].

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Pulse and chase experiments with nucleoside analogs have also been employed to understand the contribution of the bulge stem cells in skin wound repair. The co-labeling of hair follicle stem cells with tritiated thymidine and bromodeoxiuridine (BrdU) was the first approach used to show that hair follicle cells migrate toward the epidermis in response to an injury [3, 4]. This mobilization process suggested the existence of different populations of epithelial progenitors, which have been confirmed over the past few years with the generation of sophisticated lineage tracing models [8]. Nucleoside analog labeling is an ideal tool in applications such as the determination of cell cycle kinetics of progenitors in different skin compartments, the examination of the fate of stem-cell progeny in dermatologically-relevant transgenic models, and the understanding of stem-cell dynamics in different species where genetically-modified models are not available.

The versatility of the nucleoside-analog labeling technique has greatly increased with the introduction of nonradioactive thymidine analogs and the development of reagents suited for fluorescence microscopy. The original combination of tritiated thymidine and BrdU has some limitations: (1) time-consuming because it requires at least 2 weeks for exposure, (2) expensive due to high cost of the radioactive analog, and (3) restricted within 3D tissue analysis because only thin sections can be used for autoradiography. These disadvantages can be overcome with the sequential pulse of halogenated thymidine analogs such as iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU). These analogs can be detected by different monoclonal antibodies and visualized by confocal microscopy. Moreover, tissue sections could potentially be immunostained for other markers of interest to determine cell identity. Recently, the IdU/CldU double-labeling technique has shown to be a useful approach to address diverse aspects of stem cells (SC) and skin biology. In a chromosome segregation study in vivo, the double-labeling approach was used to show that the vast majority of the bulge SCs segregate their chromosomes in a random manner [9]. Regarding the physiological role of hair follicle stem cells, it has been possible to demonstrate, with the double-labeling technique, that the migration of the SC progeny is fundamental to assure the neonatal growth of the skin and accelerate the re-epithelialization process after wounding [2, 10].

Traditionally, the analysis of morphological parameters in dermatological research has relied on qualitative or 2D sampling methods that do not assure unbiased quantification of biological structures [11]. For example, the changes in cell number in a pathological condition may be counted from a small fraction of tissue that is not representative of the global response. This kind of issue is one of the most common problems in all morphometric analyses, independent of the biological field of study. Thus, miscounting could lead to interpretative errors. In response to these caveats,

stereologists have refined over the last three decades, a variety of geometrical probes to obtain reliable and unbiased estimates of volume, number, area, and length of biological objects. Design-based stereology methods or probes do not make assumptions regarding the size, shape, spatial orientation, and spatial distribution of the objects to be quantified. One of the advantages of stereological quantification is that data of the measured parameters are expressed as total quantities (i.e., millions of cells) and not as ratio quantities (i.e., cell/unit area), which are meaningless if the reference space is unknown. The advent of computer-based systems has optimized data collection and facilitated systematic random sampling within tissue sections. In fact, the sampling procedure is a critical component of design-based stereology studies. The term "systematic random sampling" refers to how animals, sections, and counting sites are determined for quantification in stereological studies. Under this scheme, the first object is randomly sampled and then the rest of the objects are sampled with a constant periodicity.

In addition to following a strict sampling scheme, stereological probes require tissue sections with certain characteristics. For counting cells, the optical fractionator is the most common probe used. This method requires thick tissue sections (>20 μm) and a homogenous staining thorough the entire thickness of the section. The estimation of the total number of cells is performed by counting the cells inside a virtual box or optical dissector. As the fraction of tissue sampled is known, the total number of cells can be estimated in a region of interest. Rather than give a strong theoretical background about the principles of stereology [12, 13], the main intention of this chapter is to provide practical cues to design a stereological study that can be applied to estimate the total number of cells, single- or double-labeled with thymidine analogs, in the skin epithelium of the rat. We will describe step by step how to produce quality tissue sections suitable for any stereological application.

2 Materials

2.1 Skin Wounding

1. Isoflurane vaporizer.
2. Surgical soap.
3. Double-edged razor blades.
4. Disposable dermal biopsy punch (6 mm).
5. Cotton.
6. Surgical scissors and forceps.

2.2 Thymidine Analog Administration

1. Bromodeoxyuridine.
2. Chlorodeoxyuridine (CldU; 150 nmol/g).
3. Iododeoxyuridine (IdU; 150 nmol/g).

4. Nucleoside vehicle 1: 0.007 N NaOH, 0.9% NaCl for BrdU and CldU.
5. Nucleoside vehicle 2: 0.0014 N NaOH, 0.9% NaCl for IdU.

2.3 Tissue Collection

1. Sodium pentobarbital.
2. Perfusion pump.
3. 0.9% NaCl.
4. Zamboni's fixative: 4% paraformaldhyde, 15% (v/v) saturated picric acid in 0.1 M phosphate buffer (PB), pH 7.4.
5. 20% and 30% sucrose in PB.
6. Optimal Cutting Temperature (OCT) compound.
7. 2-Methylbutane.
8. Cryostat.
9. Cryoprotectant solution: 25% ethylene glycol, 25% glycerol in 0.05 M PB.
10. 48-Well plates.

2.4 Immunohistochemistry Using DAB

1. PB containing 0.3% Triton X-100 (PBT).
2. PB containing 1% H_2O_2.
3. Immuno/DNA retriever (Bio SB).
4. 1 N HCl in distilled water.
5. 0.1 M sodium borate buffer.
6. Biotin–avidin blocking kit (Vector Laboratories, Burlingame, CA, USA).
7. Mouse anti-BrdU (Roche Applied Science, Indianapolis, IN, USA).
8. Blocking solution #1: 5% normal horse serum in PBT.
9. Biotinylated donkey anti-mouse antibody.
10. VECTASTAIN ABC kit (avidin–biotin complex, Vector Laboratories).
11. DAB peroxidase substrate kit (Vector Laboratories).
12. 0.05 M sodium bicarbonate buffer pH 9.6.
13. DAB enhancing solution (Vector Laboratories).
14. Methyl Green.
15. Acetone containing 0.05% acetic acid.
16. 95% and 100% Ethanol.
17. Xylene.

2.5 Immunofluorescence

1. Tris-buffered saline: 150 mM NaCl, 100 mM Tris, pH 7.4 (TBS).
2. TBS containing 0.3% Triton X-100 (TTBS).

3. Blocking solution #2: 5% normal goat serum, 5% bovine serum albumin in TTBS.
4. Mouse anti-BrdU (Becton Dickinson [Franklin Lakes, NJ, USA], clone B44) for IdU.
5. Rat anti-BrdU (AbD Serotec [Bio-Rad, Raleigh, NC, USA], clone BU1/75) for CldU.
6. Goat anti-mouse conjugated to Alexa 488.
7. Goat anti-rat conjugated to Alexa 594.
8. DAPI.
9. Dako (Carpinteria, CA, USA) Fluorescence Mounting Medium.
10. Stereo Investigator system (or equivalent) configured for bright-field and fluorescence microscopy with a structured illumination system and a motorized *x*–*y*–*z* stage.

3 Methods

3.1 Excisional Wounding on the Back Skin

All the experiments described here are designed for Wistar rats between 7 and 8 weeks old (*see* **Note 1**).

1. Induce anesthesia in a closed chamber with 4% isoflurane. Do not remove the rat from the chamber until the absence of tail clamp response is detected. Place the rat on a flat surface with its back facing up. Maintain anesthesia with 1.5% isoflurane through a face mask.
2. Trim the hair from the back skin corresponding to the thoracic area with a hair clipper or scissors, as close as possible to the skin surface. Rub the trimmed area with surgical soap until foam is created and carefully shave an area of 3 × 3 cm (*see* **Note 2**). Clean the shaved area with cotton soaked with sterile water.
3. Insert the 6 mm biopsy punch at the center of the shaved area with a circular motion taking care not to move the punch from its original position. In case of bleeding, clean the area with sterile cotton and stop anesthesia until the bleeding stops. Otherwise, transfer the rat immediately to a clean cage to monitor its recovery (*see* **Note 3**).

3.2 BrdU Injection

1. Weigh rats and calculate the amount of BrdU for each rat to achieve a dose of 50 mg/kg. Dissolve the BrdU in the corresponding volume of nucleoside vehicle #1 in order to inject approximately 1 mL solution per rat.
2. Administer the BrdU solution via an intraperitoneal route with a 23-G needle. Euthanize the rat and harvest the skin samples at the desired time points according to Subheading 3.4.

3.3 IdU/CldU Injection

1. Determine the amount of IdU and CldU in order to administer equimolar concentrations of the thymidine analogs (*see* **Note 4**). Weigh the rats and calculate the concentration of each analog to achieve a dose of 150 μmol/kg.
2. Dissolve the IdU in the nucleoside vehicle #2 in an appropriate volume to inject 1 mL solution through an intraperitoneal injection (*see* **Note 5**). The separation time between the first analog and the second analog should be adjusted according to the needs of the researcher. If one would like to determine the duration of the cell cycle, an interval of 2 h between pulses is recommended over a period of 16–20 h or until the cell population of interest is double-labeled.
3. Dissolve CldU in nucleoside vehicle #1 and inject a dose of 150 μmol/kg to each rat, accordingly to the experimental design. We recommend between three and five animals per time point. The final number of animals will depend on the frequency of single- or double-labeled cells. An ultimate goal of stereology is to obtain valid statistical estimates with the minimum use of resources.
4. Collect samples for immunostaining and stereology analysis as detailed in the next section.

3.4 Perfusion Fixation to Harvest Nucleoside-Labeled Tissue

1. Inject an overdose of sodium pentobarbital (>100 mg/kg) to one rat. Once the animal is deeply anesthetized, expose the heart and insert a 25-G needle into the left ventricle connected to a peristaltic pump. Make a small incision in the right atrium to allow for the outflow of blood from the animal. Start perfusion of 100–150 mL of 0.9% NaCl followed by 200 mL of Zamboni's fixative (*see* **Note 6**).
2. With scissors, remove the area of skin containing the wound (~2 × 2 cm). In some cases, hair growth can be observed around the wound. Shave the whole area to facilitate tissue processing before dissection. The orientation of the sample in relation to the position of the head and tail should be recorded. Make a small diagonal cut in one of the corners that corresponds to the head side (*see* **Note 7**). Post-fix the skin sample for 24 h in Zamboni's fixative.
3. Rinse the skin sample with distilled water and then immerse the sample in 20% sucrose for 48 h. Change to 30% sucrose for an additional 48 h.
4. Embed samples in OCT compound in order to obtain longitudinal sections of hair follicles [14]. Cut the borders of the original block in order to generate a square of 1.5 × 1.5 cm. Add OCT compound on the side of the epidermis. Fold the skin so that the tail-side touches the head-side. Immerse the sample for 2 min in a freezing bath of dry ice and 2-methylbutane. Using an L-shaped aluminum profile, place the sample in

the center of one drop of OCT compound. The folded side of the sample should be perpendicular to the drop of OCT. Immerse in the freezing bath for 30 s. Cover the rest of the sample with OCT compound, freeze again in the bath, and store at −80 °C.

3.5 Tissue Sectioning

1. Add 300 μL cryoprotectant solution per well in a 48-well plate (*see* **Note 8**).
2. Obtain 50 μm serial sections using a cryostat (*see* **Note 9**). Start collecting all the sections as soon as the complete profile of the skin sample is obtained. Put one section per well to maintain the serial order. The actual number of sections collected will depend of the reference space defined by the researcher. For example, the starting point could be defined at a certain distance from the first section where the wound is visible. In our studies, we have analyzed up to 4 mm from the border of the wound (*see* **Note 10**).
3. Store sections at −20 °C (*see* **Note 11**).
4. Select sections for immunostaining under a systematic random sampling regime. The sampling interval and number of sections to be counted are defined in a pilot study (*see* **Note 12**). Mount series sections on slides in order (*see* **Note 13**). Once the sections are dried, they can be stored overnight in a sealed plastic bag at 4 °C.

3.6 Immunohistochemistry for BrdU

1. Rinse the slides twice in PB in order for 5 min. For permeabilization, incubate for 15 min in PBT.
2. Incubate for 1 h in 1% H_2O_2 in PB to inactivate endogenous peroxidase (*see* **Note 14**). Wash three times for 5 min in PB.
3. Incubate the slides in immuno/DNA retriever for 30 min at 70 °C. Remove the staining jar from the oven, let cool for 15 min at room temperature. Wash three times in PB (*see* **Note 15**).
4. Denature DNA with 1 N HCl for 30 min at room temperature.
5. Neutralize HCl by incubation in sodium borate buffer for 10 min at room temperature then rinse three times in PB.
6. Using a biotin-avidin blocking kit, incubate slides for 15 min in avidin solution (100 μL avidin per mL PB), rinse once in PB, and incubate for 15 min in biotin solution (100 μL/mL). Incubate for 30 min in blocking solution (*see* **Note 16**).
7. Dilute anti-BrdU antibody (1:500-1:1000) in blocking solution #1. Place slides in a humid chamber. Add ~300 μL of the antibody solution per slide and incubate overnight at room temperature.

8. Wash the sections three times for 5 min in PB. Dilute secondary antibody in blocking solution (1:500). After 2 h incubation with the secondary antibody, rinse sections three times in PBS.
9. Incubate sections in avidin–biotin-complex (ABC) solution for 1.5 h at room temperature. Prepare ABC solution by mixing solution A (3 μL/mL) and solution B (3 μL/mL) in PB at least 30 min prior to use.
10. Rinse sections with PB three times for 5 min.
11. Prepare DAB substrate solution with nickel chloride immediately before use. Cover the sections with the substrate and rinse the section after 8–10 min with distilled water.
12. Incubate slides in sodium bicarbonate for 10 min. Blot off excess buffer and then cover the sections with DAB enhancing solution for 15 s. Immediately rinse sections with water to stop the reaction (*see* **Note 17**).
13. After rinsing in tap water, immerse the slides for 3 min in methyl green, preheated at 60 °C. Wash slides for 30 s in distilled water. Quickly dip slides two times in acetone and sequentially transfer slides to 95 % ethanol, 100% ethanol, and 100 % ethanol (1 dip in each case). Clear the sections in xylene for 5 min. Do not let the sections dry after xylene. Add mounting media, taking care to avoid creating bubbles, and place a no. 1.5 cover glass (thickness 0.17 mm).

3.7 Double Immunofluorescence

1. Rinse the slides twice in TBS and then permeabilize for 15 min with TTBS. Incubate the slides in immuno/DNA retriever for 30 min at 70 °C and let cool for 15 min. Rinse three times in TBS (*see* **Note 18**).
2. Incubate sections in 1 N HCl at 37 °C then neutralize with sodium borate buffer for 10 min. Wash three times in TBS.
3. Block sections for 1 h with blocking solution #2.
4. Dilute in the same tube anti-IdU (BD clone B44, 1:1000) and anti-CldU (Serotec clone BU1/75, 1:300) in blocking solution #2. Place the slides in a humid chamber and cover the sections with antibody solution. Incubate overnight at room temperature.
5. After three rinses with TBS, incubate sections in blocking solution #2 for 1 h at room temperature. Dilute anti-mouse Alexa 488 (1:1000) and anti-rat Alexa 594 (1:1500) in blocking solution #2. Incubate 2 h with this solution (*see* **Note 19**).
6. Rinse three times in TBS.
7. Counterstain for 5 min with DAPI. Never allow sections to dry during the whole procedure. Drying of sections will affect final section thickness. While mounting, place the cover glass while the tissue is still wet. The resulting stain can be checked in a fluorescence microscope configured for FITC and Texas Red (Fig. 1).

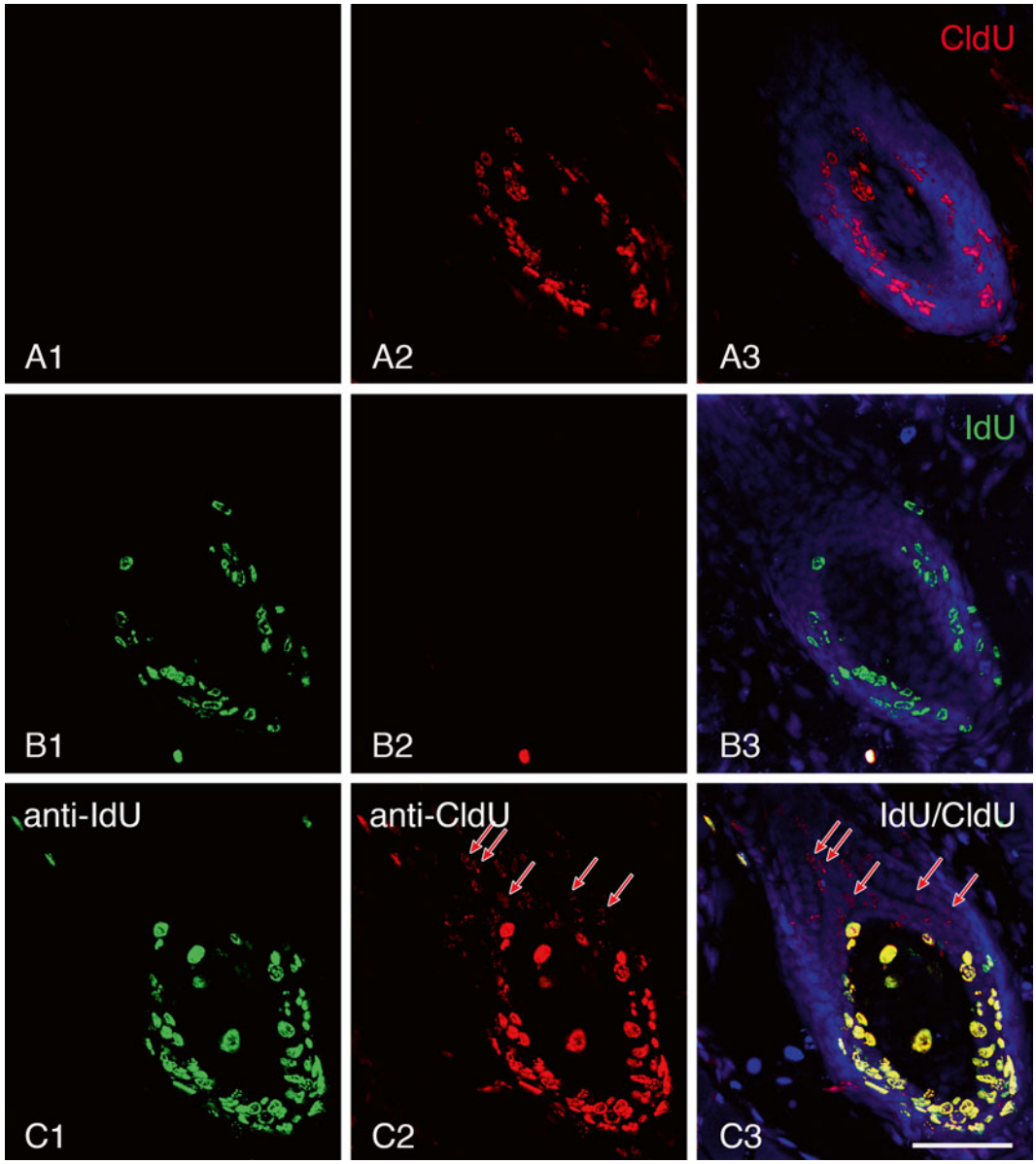

Fig. 1 Differential detection of iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU) by immunofluorescence. Neonatal rats received a single intraperitoneal dose of either CldU (**a**) or IdU (**b**) and were sacrificed 1 h later. CldU was detected specifically by a rat monoclonal antibody from clone BU1/75 (Serotec). Similarly, IdU was only detected by a mouse monoclonal antibody from clone B44 (Becton Dickinson). After a chase period of 48 h, the CldU-labeled cells were detected outside the hair bulb (*arrows*). Simultaneous delivery of an equimolar dose of IdU and CldU in the same animal labels the same population ($IdU^+/CldU^+$) in the hair bulb, suggesting that CldU-labeled cells were originally located in this region (**c**)

3.8 Estimation of the Total Number of Nucleoside-Labeled Cells Using an Optical Fractionator

1. Check thickness, after tissue processing, in 2–3 sections to verify that sections are suitable for optical fractionator use. Locate hair follicles with a low-magnification objective (4× or 10×). Change to a high numerical aperture objective with small depth-of-field (water or oil immersion; >1.0 N.A.). Locate the top of the section, focus in the opposite direction of the tissue until all the field of view is out of focus. Slowly move the microscope knob in order to move the objective close to the section. Once some features of the section appear in focus, register this position as 0 in the *Z*-axis. Repeat the same approach at the bottom of the section. If Stereo Investigator is used, the section thickness can be read from the "Z focus meter" (*see* **Note 20**).
2. Determine the size of the counting box. The basic procedure to set sampling parameters within sections is the same, independent of whether cells are being counted by bright-field or by fluorescence microscopy. It is recommended that the size of the counting frame be adjusted in order to contain less than ten events of interest. Ideally, 3–5 events per counting frame would allow the researcher to keep track of the events and avoid double counting of cells. It is possible to use the same counting frame to simultaneously count two populations of cells. Nonetheless, it is preferable to set independent sampling parameters for different populations in order to avoid under- or oversampling of a particular cell population. For example, a pulse of IdU at 21 h after wounding followed by a pulse of CldU would result in a higher number of IdU-labeled cells than CldU-labeled cells. Moreover, double-labeled cells ($IdU^+/CldU^+$) could represent less than 30% of IdU-labeled cells [2]. In addition to *X* and Υ dimensions, a height for the counting box should be established based on the mean section thickness. The height of the optical dissector should be less than the total thickness to avoid artifacts at the section surface. This volume at the top and bottom of the section is known as the guard zone (*see* **Note 21**).
3. Determine the size of the sampling grid. It has been shown that efficient stereological design involves the counting of 100–200 events per animal [12, 13]. The actual number of sampling sites within sections needs to be empirically determined in order to achieve these figures. There is a battery of statistical tools to estimate the precision of stereological estimates. The most common estimator employed is the Gundersen–Jensen coefficient of error (CE) which is an indicator of how much the estimate would vary if it were repeated many times [15]. A value of $CE < 0.1$ in a sample can be considered as an indication that the estimate is precise (*see* **Note 22**).
4. Count nucleoside-labeled cells. In each sampled site, identify the nucleoside-labeled cells of interest inside the counting

box. Strictly follow the same counting rules throughout sections and animals. For the counting box, do not count cells touching exclusion surfaces (left and bottom lines of the counting frame). The total number of nuclei is calculated using the formula: $N = \Sigma Q^{-} * t/h * 1/\text{asf} * 1/\text{ssf}$, where ΣQ^{-} is the number of particles counted, t the mean section thickness, h the counting-frame height, asf the relation of the area of the counting frame to the area of sampling grid, and ssf the serial section fraction, which refers to the periodicity of sampling.

4 Notes

1. The protocols described in this chapter have been used primarily on adult rats. However, we have experienced that these protocols can also be used on mice and neonatal rats without further modifications. The suggested time of 7–8 weeks is the approximate time when most of the hair follicles are in telogen in the back skin of Wistar rats [16]. Therefore, most of the hair-follicle stem cells would be in a resting period. It is recommended that the researcher check on the specific rodent strain to determine when the follicles are in telogen in order to avoid an activated state of the stem cells.
2. Extreme care must be taken at this step to avoid skin irritation or accidental cuts with a razor blade.
3. With practice, the whole procedure can be completed in 10–15 min. The proliferative response around the wound depends on the diameter of the biopsy punch used. In preliminary studies, we determined that a 6 mm punch evidently increased the BrdU labeling and thickness of the epithelium up to 5–6 mm from the central edge of the wound. The injection of thymidine analogs in our experiments was performed after at least 21 h when it is known that bulge cells are fully activated [3]. Depending on the objective of the study, the researcher can use one pulse of BrdU or sequential pulses of IdU and CldU at times that correspond to the experimental design.
4. Due to differences in molecular weight between thymidine analogs, the dose should be calculated based on the same molar concentration to guarantee a similar proportion of labeled cells with the pulses. It has been shown that different populations of proliferating cells are labeled with the simultaneous administration of non-equimolar pulses of thymidine [17].
5. All nucleoside solutions are prepared fresh. IdU solution is more difficult to prepare than BrdU and CldU solutions. In addition to using a higher concentration of NaOH, we found that warming the solution in a water bath at 30 °C for short periods helps to dissolve IdU. Intercalate cycles of vigorous

vortexing and warming until all IdU grains disappear. Preparation of this solution may take more than 20 min.

6. Saline is perfused until the fluid coming out from the right atrium is clear which is an indication of a good perfusion. It is also advisable to check that the liver has lost its characteristic reddish coloration. In our experience, Zamboni fixative produces less tissue autofluorescence than 4% PFA. Thus, we prefer Zamboni fixative for double immunofluorescence and immunostaining of protein antigens. For BrdU, 4% PFA gives excellent results for bright-field microscopy.
7. It is important to maintain the orientation of the sample because hair follicles are not perpendicular to the vertebral line. Longitudinal sections of hair follicles are only obtained if the tissue block is sectioned parallel to the vertebral line.
8. We found that the tissue is better preserved if we maintain the sections by storing them in cryoprotectant solution rather than mounting them on glass slides. Moreover, cryoprotectant solution allows for long-term storage without affecting the quality of the staining. We have maintained tissue sections for up to 6 months in this solution without noticing any change. This method also allows us to keep sections for additional labeling and quantification on the same animals.
9. Some stereology probes require the use of thick sections (>20 mm). All histologic processing significantly collapses the height of the section. It is not recommended to cut below 40 μm because a 50–60% reduction in the thickness of the section may occur.
10. A clear definition of the reference space should be established at the beginning of the study. The comparison between experimental groups only will be valid if the estimate of the parameter is obtained through an exhaustive and random sampling of equivalent anatomical regions. The challenge for dermatology is that in some cases, there are no anatomical cues that guide the definition of a reference space, as it is the case for other areas such as the brain. However, it is possible to collect skin from equivalent regions from different animals. The dimension of the reference space can be established by the researcher according to the goals of the project. Considering our example of 4 mm from the wound edge, the number of collected sections is 281 of 50 μm each. The dimension of this reference space is a square of 14 mm × 14 mm. According to the magnitude of the response studied, the borders of the reference space can be increased or decreased.
11. We have observed that cryoprotectant solution can be frozen at −80 °C.

12. A pilot study with a few animals should be conducted to determine the sampling parameters, such as the number of sections to be analyzed and the frequency of the sampling within the sections. In the pilot study, several points are addressed: (1) shrinkage of the tissue after histological processing, (2) quality of staining through the entire thickness of the tissue, and (3) frequency and distribution of the cells of interest. Commonly, the number of sections needed to obtain a valid estimate is in the range of 8–15 per animal per region of interest. As a starting point, ten sections could be considered by the researcher.
13. Mounting skin sections with brushes can be challenging, especially if the tissue sticks to the brush. To avoid damage of the sections, we recommended use of glass tools which can be easily prepared from a pasteur pipet. Flame a pipet while the tip is pulled. A gentle movement will create a fine, rounded tip. If the tip is too fine, continue flaming to increase its thickness.
14. In most peroxidase-inactivation protocols, the concentration of H_2O_2 is 3%. However, this concentration is aggressive for skin sections and bubbles formed during the incubation may detach the sections from the slide.
15. This step is optional. The quality of BrdU staining is not affected by the omission of this step. Incubation with a citrate-based retriever is a good option when BrdU staining is combined with labeling of protein antigens. It minimizes stain variability between experiments.
16. We use a biotin–avidin blocking kit in order to eliminate unspecific staining of the sebaceous gland of the hair follicle. This background can interfere with BrdU labeling identification. The blocking step with horse serum can be omitted.
17. The potentiation step was added after noticing that counterstaining fades peroxidase staining. Do not use the enhancing solution for more than 20 s because a general darkening of the section will be produced.
18. For IdU and CldU staining, TBS gives better results than PB. TBS eliminates primary antibody cross-reactivity and decreases general background.
19. The anti-rat antibody binds unspecifically to the dermis, especially in adult rats. This background does not impede identification of labeled cells within the epithelium.
20. The numerical aperture of an objective determines its resolution. Only objectives of high numerical aperture should be used for thickness measurements and optical sectioning. To guarantee accuracy of optical sectioning, the microscope should be equipped with a *Z*-axis encoder. In addition, optical

sectioning requires Köhler illumination and a fully-open condenser to ensure the thinnest optical sections.

21. It is recommended to measure section thickness at every sampling site in order to consider in the calculations the effect of differential thickening throughout the section. If the Stereo Investigator is used, the guard zone can be set as a percentage of the section thickness at each sampling site. In this way, the counting box is centered according to the thickness in a particular counting site.
22. Additional estimates could be calculated to determine the relative weight of different sources of variation. The two sources of group variance (CV2) for a theoretically unbiased estimate are given by the biological variance (BV2) and the sampling variance (CE2). The biological variance, which arises from inter-individual differences, cannot be controlled by the researcher. In case sampling variation is one of the main sources of error, the sampling frequency should be increased [13, 15].

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Chapter 12

Culture of Dermal Papilla Cells from Ovine Wool Follicles: An In Vitro Model for Papilla Size Determination

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Abstract

Common human balding or hair loss is driven by follicle miniaturization. Miniaturization is thought to be caused by a reduction in dermal papilla size. The molecular mechanisms that regulate papilla size are poorly understood, and their elucidation would benefit from a tractable experimental model. We have found that dermal papilla cells from sheep spontaneously aggregate in culture to form papilla-like structures. Here, we describe methods for microdissecting dermal papillae from wool follicles, for initiating and maintaining cultures of ovine papilla cells, and for using these cells in an in vitro assay to measure the effect of bioactive molecules on aggregate size.

Key words Hair follicle, Dermal papilla, Morphogenesis, Aggregation, In vitro, Sheep, Ovine, Wool, Hair loss

1 Introduction

The biology of wool follicles from sheep has a long history of scientific investigation, due its relevance to the wool industry [1]. The anatomy, development, physiology, and molecular biology of wool follicles are generally similar to those from other species. However, some idiosyncrasies of wool follicles and their constituent cells make them a good model for studying particular aspects of mammalian hair biology.

Dermal papilla cells (DPC) exhibit marked morphogenetic activity in addition to their multipotency [2, 3]. When cultured cells are implanted in the skin, they are able to reorganize to form a papilla and induce the growth of a new hair follicle [4–6]. The spontaneous aggregation of DPC in culture is likely to be another expression of this morphogenetic activity [6–8]. We have found that DPC from wool follicles aggregate particularly robustly. We have used these cells to develop an in vitro assay for bioactive molecules that influence aggregate size [9]. The molecular mechanisms that determine dermal papilla size are of interest because the

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size of the papilla influences the size of the whole follicle [10, 11]. Importantly, alterations in papilla size are thought to underlie follicle miniaturization associated with human hair loss [12, 13].

In this chapter, we describe methods for harvesting wool follicles from sheep skin and microdissecting the dermal papillae. The isolated papillae are then used to initiate DPC cultures which maintain robust aggregative behavior over numerous passages. We describe a standardized in vitro assay in which these cells are used to measure the effect of bioactive molecules on aggregate size. We have also adapted these methods to culture DPC from the pelage follicles of cattle [14], deer, and rats.

Microdissection of wool follicles to isolate the dermal papillae is technically difficult due to their small size. A certain amount of practice is usually required to develop the necessary manual skills. For beginners, we recommend starting with larger vibrissa follicles, either from rats or sheep. Vibrissa can be isolated from the upper lip skin of sheep by modifying the methods in Subheadings 3.1 and 3.2, **steps 1–9**. The papilla can then be isolated using methods previously described for rat vibrissae [15].

2 Materials

1. Sheep. We usually obtain sheep skin specimens from a nearby abattoir. Lambs (typically <18 months old) are preferable, as cells from younger animals seem to grow more robustly (*see* **Note 1**).
2. Stereomicroscope and cold light source (*see* **Note 2**).
3. Serrated tip forceps (*see* **Note 3**).
4. No. 4 watchmakers' forceps (at least 2).
5. No. 5 watchmakers' forceps (at least 3).
6. No. 24 scalpel blades with no. 4 handle.
7. Curved-blade microscissors (World Precision Instruments, Sarasota, FL).
8. Angled-blade microscissors (World Precision Instruments).
9. 26-G hypodermic needles, with 10 or 20 ml syringes to use as handles.
10. Plastic universal tubes (30 ml, screw top).
11. 10-cm Petri dishes. Non-tissue-culture-treated dishes are better for follicle dissection, because they are shallower. Tissue-culture-treated dishes are necessary for growing DPC.
12. Tissue-culture-treated 35 mm Petri dishes.
13. Tissue-culture-treated 25 cm^2 flasks (or other culture vessels as desired).
14. Collagen-coated 12-well culture plates (Becton Dickinson, Oxford, UK).

15. Dissection medium: minimum-essential medium (MEM) supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 12.5 ng/ml amphotericin B, and 10% lamb serum. Make up the day before to ensure the antibiotics are fresh.
16. Culture medium: MEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 12.5 ng/ml amphotericin B, and 20% lamb serum.
17. PBS (phosphate buffered saline): 8.0 g/l sodium chloride, 0.2 g/l potassium chloride, 1.15 g/l disodium hydrogen phosphate, 0.2 g/l potassium dihydrogen phosphate, pH 7.3 (tablets from Oxoid, Basingstoke, UK, cat. no. BR0014).
18. PBS/EDTA: PBS with 0.6 mM EDTA.
19. Trypsin solution: 0.25% trypsin in PBS/EDTA.
20. Freezing mix: 90% lamb serum with 10% dimethyl sulfoxide.
21. Van Gieson's solution (Sigma-Aldrich, St. Louis, MO).

3 Methods

3.1 Skin Collection

1. Wear latex or nitrile cloves, and periodically rub them with 70% ethanol as an antimicrobial precaution.
2. Swab the work area with 70% ethanol and paper towels.
3. Swab the skin to be harvested with 70% ethanol and paper towels (*see* **Note 4**).
4. Shave the wool from the harvest site using a no. 24 scalpel blade in a standard holder (*see* **Note 5**).
5. Clean the shaved site by wiping it with 70% ethanol-saturated paper towels. Blot away any excess fluid.
6. Make four scalpel incisions through the skin, to form a 2 × 2 cm square.
7. Grasp one corner of the square with serrated-tip forceps. Use a fresh no. 24 scalpel to cut through the connective tissue beneath the skin, beginning at the same corner. Continue to cut beneath the square, lifting the free skin as you proceed, until the square of skin has been removed from the surrounding tissue (*see* **Note 6**).
8. Place the skin in a Universal bottle approximately half-full with dissection medium. Place on ice for short-term storage and transport.

3.2 Follicle Dissection

1. Work in a laminar flow cabinet; wipe down with 70% ethanol and paper towels before beginning.
2. Preferably, place a stereomicroscope and cold light source inside the cabinet and wipe down with 70% ethanol (*see* **Notes 7** and **8**).

3. Wear latex or nitrile cloves, and periodically rub them with 70% ethanol.
4. Throughout the dissection, work at room temperature but store tissue in dissection medium on ice while it is not being worked on.
5. Using forceps to handle the skin, wash it through three changes of dissection medium, in three fresh 10 cm Petri dishes. Make sure the skin is fully immersed in medium. Leave the inner surface of the skin facing upward in the last dish, ensuring there is sufficient medium to just cover the skin.
6. Place the dish and skin under the stereomicroscope, set to low-power magnification.
7. Use a pair of curved microscissors to cut away any subcutaneous muscle, while using a pair of no. 4 watchmakers' forceps in your other hand to hold the skin in place. This will expose a layer of connective tissue which typically has a fibrous texture.
8. Use the microscissors to mince away the connective tissue from the centre of the skin piece, until the lower follicle bulbs begin to emerge (*see* **Note 9**).
9. To harvest follicles, hold the forceps as close to horizontal as possible and pinch a small area of the minced skin. This will make the follicles stand up from the surrounding connective tissue. Use a pair of angled microscissors with the blades parallel to the forceps to cut away a cluster of follicles (*see* **Note 10** and Fig. 1a). Transfer to a fresh 10 cm Petri dish containing dissection medium. Continue until sufficient follicles for the day have been harvested before proceeding to the next step.
10. Set the microscope to medium power magnification to isolate individual follicles. Use a pair of no. 5 watchmakers' forceps to hold a cluster of follicles, pinning it to the bottom of the dish. Use a 26-G hypodermic needle to tease a follicle away from the attached remnants of connective tissue (*see* **Note 11** and Fig. 1b, c). Transfer the isolated follicle to a fresh 35 mm dish of dissecting medium, using no. 5 watchmakers' forceps to grasp it by the cut end. Do not touch the bulb containing the papilla to avoid damaging it. Continue until sufficient follicles for the day have been isolated before proceeding to the next step.
11. Place seven drops of dissection medium in the lid of a 10 cm Petri dish (*see* **Note 12**). Transfer one follicle to each drop (*see* **Note 13**).
12. Place 2.0 ml culture medium in a 35 mm Petri dish, prewarmed to 37 °C. Place the 35 mm Petri dish inside a 10 cm dish to facilitate handling.

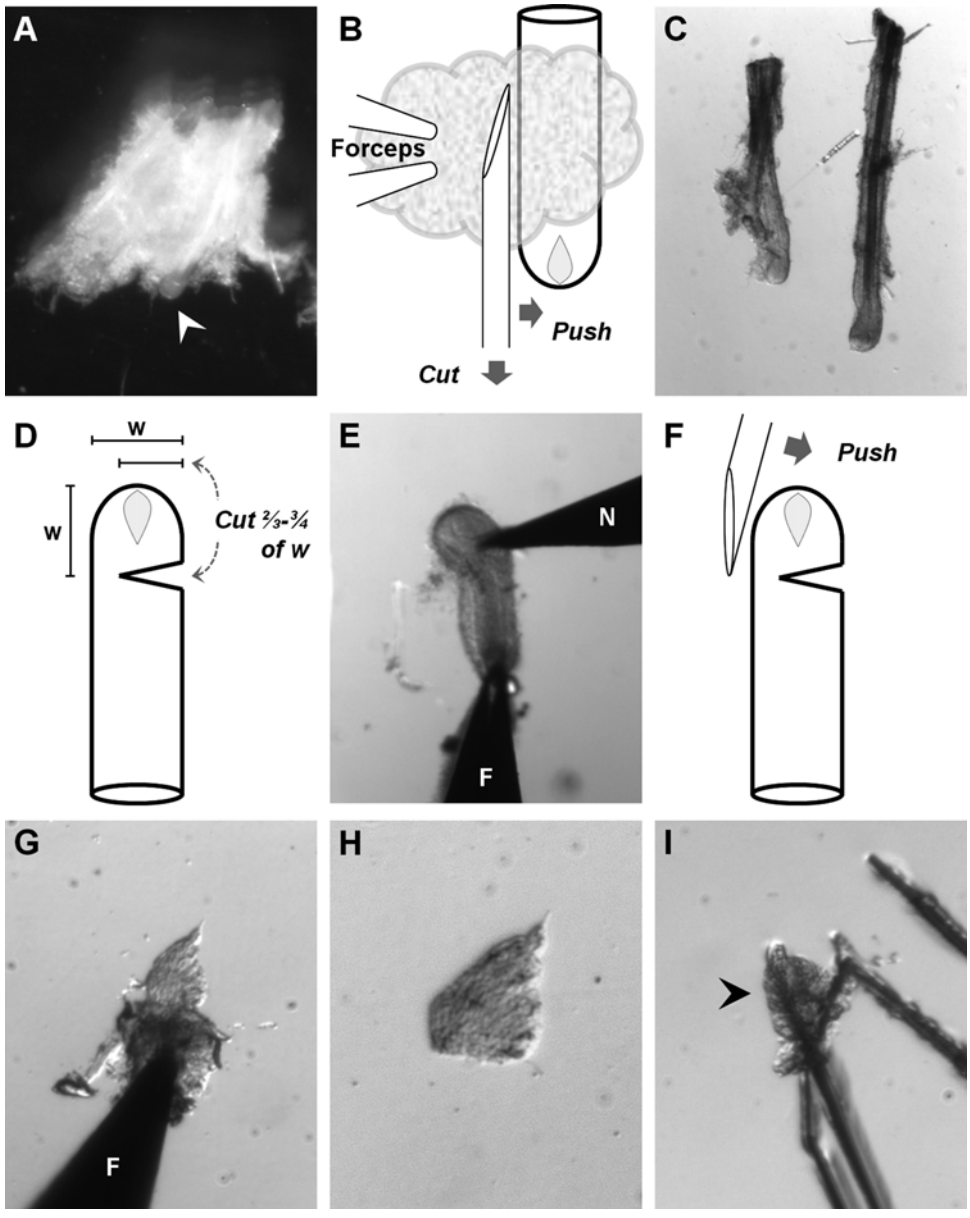

Fig. 1 Dissection and isolation of dermal papillae. (**a**) A cluster of wool follicles embedded in connective tissue. The end-bulb of one follicle can be seen protruding from the connective tissue (*arrowhead*). (**b**) To cut follicles from the connective tissue, use the side of the needle to push against the follicle, stretching the connective tissue, while simultaneously drawing the tip of the needle through the tissue to cut it. (**c**) Two isolated wool follicles. (**d**) Cut $\frac{2}{3} - \frac{3}{4}$ of the way through the follicle, a distance from the end approximately equal to the follicle width (w). (**e**) A follicle being cut. *N* needle, *F* forceps. (**f**) To invert the bulb of the cut follicle, push against it with the side of the needle. (**g**) An inverted follicle bulb, showing the papilla protruding toward the top of the image. *F* forceps. (**h**) An isolated dermal papilla. (**i**) A papilla (*arrowhead*) scratched onto the bottom of the culture dish

13. Set the microscope to high power to isolate dermal papillae. Grasp the cut end of the follicle with a pair of no. 5 watchmakers' forceps, simultaneously pinning it to the bottom of the Petri dish lid. Use a fresh 26-G hypodermic needle to make an incision $^{2}/_{3} - {}^{3}/_{4}$ of the way through the follicle, positioned a distance above the very base of the bulb approximately equal to the follicle width (Fig. 1d and e).
14. Use the non-beveled side of the needle tip to push against the follicle bulb on the non-cut side, to turn the bulb inside out (Fig. 1f and g). Semi-keratinized tissue from the nascent hair shaft (i.e., the keratogenous zone) should fall away easily, leaving a leaf-shaped dermal papilla protruding from the inverted dermal sheath. There may be some germinative epithelial tissue visible at the junction between the papilla and the dermal sheath (*see* **Note 14**).
15. Use the needle tip to tease away excess germinative epithelium. Use the needle tip to cut the clean dermal papilla away from the dermal sheath (*see* **Note 15** and Fig. 1h).
16. Use a pair of no. 5 watchmakers' forceps to transfer the papilla to a 35 mm Petri dish containing 2.0 ml culture medium, prewarmed to 37 °C (*see* **Note 16**). Use a 26-G needle to gently stab through the papilla and make a small scratch on the bottom of the dish. The cut papilla should adhere to the scratch, holding it in place (Fig. 1i).
17. Place 6–10 papillae in one 35 mm dish of culture medium (i.e., the papillae isolated from 1–2 batches of seven follicles, as in **step 11** above, allowing for lost or damaged papillae). Place each dish in a tissue-culture incubator at 37 °C/5 % CO_2 once it contains sufficient papillae (*see* **Note 17**). Do not move or disturb the dish for 1 week, to avoid the risk of papillae moving within the dish as cell outgrowth is initiating. Set up 2–4 dishes in a day.

3.3 DPC Culture

1. For all DPC culture procedures, observe standard aseptic technique for mammalian cell culture, and standard procedures for discarding biological waste material.
2. After 1 week, check the cultures for outgrowth of cells. DPC initially appear as scattered fibroblastic cells around the explants. Approximately ¾ of explants should produce cells. Replenish the culture medium every 3–4 days from this point on (i.e., twice per week).
3. Continue to observe cell growth. Cell density should increase as they proliferate, and three-dimensional aggregates of cells should be seen in denser regions toward the centre of the culture. Passage the cells once they cover approximately half of the culture dish (*see* **Note 18**).

4. To passage DPC, wash with three changes of PBS/EDTA (pre-warmed to 37 °C).
5. Replace PBS/EDTA with 2.0 ml trypsin solution (pre-warmed to 37 °C). Immediately remove 1.5 ml.
6. Incubate at 37 °C until the cells round up and detach, typically after 5–10 min. Tap the dish on a flat surface to aid detachment.
7. Add 4.5 ml culture medium (pre-warmed to 37 °C). Gently aspirate the cell suspension and transfer to a tube for centrifugation.
8. Centrifuge at $400 \times g$ for 5 min to pellet the cells. Decant and discard the supernatant and resuspend the cells in 5.0 ml culture medium (pre-warmed to 37 °C).
9. Seed into a 25 cm^2 culture flask. Maintain at 37 °C/5 % CO_2. Cells should be reattached by the next day. Continue to replenish the culture medium every 3–4 days.
10. Once the cells are confluent, passage into a 10 cm culture dish (*see* **Note 19**). Follow the procedure described above, with volumes scaled up appropriately.
11. For subsequent passages, re-seed the cells into fresh 10 cm dishes, splitting 1:2 or 1:4.
12. To freeze cells, trypsinize as described above in **steps 4–7**.
13. Count cells on a hemocytometer before centrifuging. Obtain $1.0–1.5 \times 10^7$ cells from one confluent 10 cm dish. However this number is quite variable, because cells continue to proliferate after first appearing confluent.
14. Centrifuge at $400 \times g$ for 5 min to pellet the cells.
15. Resuspend at $4.0–5.0 \times 10^6$ cells/ml in freezing mix. Transfer 1 ml aliquots into cryovials.
16. Cool slowly in a cell freezing canister in a −80 °C freezer.
17. Store under liquid nitrogen.
18. To thaw cells, warm a cryovial rapidly in a 37 °C waterbath.
19. Seed cells from one vial into one 10 cm culture dish containing 9 ml culture medium (pre-warmed to 37 °C).
20. Replenish the culture medium the next day. Maintain cultures and passage as described above.

3.4 Aggregation Assays

1. Perform standardized aggregation assays in collagen-coated 12-well plates.
2. Prepare cells by culturing in 10 cm dishes as described in Subheading 3.3. Allow cells to begin aggregating before beginning a standardized assay.

3. Harvest the cells by trypsinization, count, and resuspend in culture medium at 1.33×10^6 cells/ml (as in Subheading 3.3).
4. Prepare a 12-well plate by adding 750 µl/well culture medium containing a bioactive at twice its final concentration, pre-warmed to 37 °C (*see* **Note 20**).
5. Add 750 µl/well of the cell suspension (1.0×10^6 cells/well). Maintain at 37 °C/5 % CO_2.
6. Begin half-volume medium changes the next day. Remove 750 µl of spent medium and add 750 µl of fresh medium. Ensure that the correct concentration of bioactive is maintained in each well.
7. Perform a half-volume medium change every second day (*see* **Note 21**).
8. Continue until numerous spheroid aggregates are seen throughout, typically after 1–2 weeks.
9. Fix and stain the cells via the following steps:
   - PBS, two brief washes.
   - 90 % ethanol with 5 % acetic acid and 5 % water, 20 min fixation.
   - Water, three brief washes.
   - Van Gieson's solution, staining for 3 min.
   - Water, four 2 min washes.
   - 50 % ethanol, 5 min wash.
   - 70 % ethanol, brief wash.
   - 95 % ethanol, brief wash.
   - 100 % ethanol, two 3-min washes.
10. Photograph the cells straight away, while still in the last wash. Use an Olympus SZ40 stereomicroscope and Leica DFC290 camera or equivalent. Ideally, photograph the entire well in a single field of view.
11. Stained aggregates appear red-orange, with minimal staining of cell monolayers between the aggregates.
12. Measure the diameter of aggregates from a digital photomicrograph, either manually or by image analysis.

4 Notes

1. The lamb head is a convenient body site for collecting skin, because it has no commercial value and is removed from the carcass at an early stage of processing. It can therefore be obtained shortly after death with minimal disruption to abattoir operations. Wool-type follicles are found around the neck

and the back of the head, whereas the muzzle has hair-type follicles (producing shorter but coarser fibers) and vibrissae. We routinely use wool-type follicles to initiate DPC cultures, and further standardize the body site by collecting the skin specimen from the neck, at a position that forms an equilateral triangle with the eye and the ear. However, we have successfully initiated cultures from other body sites with no obvious differences in cell growth or behavior. We aim to begin the skin collection within 15 min of death, but we have successfully initiated DPC cultures from lambs' heads that were stored on ice for 2–3 h while they were transported to the lab. We have also initiated cultures from skin which was collected, washed as in Subheading 3.2, **step 5**, stored in dissection medium overnight at 4 °C, and then dissected the next day.

2. We use 10× eyepieces with a microscope body that zooms to at least 40× magnification (preferably more). We illuminate the specimen from above using a cold light source with two fiber optic arms, positioned at around 45° each side of the back of the microscope. We prefer a base as flat as possible, and large enough to accommodate at least two 10 cm dishes. We then slide these alternatively in and out of the field of view without removing them from the base (*see* Subheading 3.2, **step 16** and **Note 16**).
3. Not all no. 5 watchmakers forceps have tips of equal fineness, but it is useful to have the finest available. It is also helpful to dedicate a pair of forceps for transferring isolated papillae from the dissection dish to the culture dish. Bend the tips slightly inward, so that they close at a larger than standard angle, accommodating an isolated papilla in the space between the tines just back from the tips (*see* Subheading 3.2, **step 16** and **Note 16**). More generally, we use a different pair of forceps for different tasks, in order to minimize the transfer of tissue fragments and so keep both the instruments and dissection medium clean for the delicate final steps of the procedure.
4. As the wool is typically quite dense, the ethanol needs to be worked through it by rubbing with saturated paper towels.
5. Wet the wool thoroughly with more 70% ethanol. Hold the edge of the scalpel blade at 45° to the skin surface while scraping the wool away. Place your free hand on the surrounding skin to stabilize it, taking care to shave away from your fingers. Keep a 70% ethanol-saturated paper towel nearby, and remove shaved wool from the scalpel by wiping it onto the towel.
6. As an additional antimicrobial precaution, the scalpel and forceps can be dipped into a Universal bottle half-full with 70% ethanol, followed by one with dissection medium. This is useful if the instruments accidently come into contact with the

work bench or with sheep skin that has not been swabbed with ethanol.

7. If it is not possible to work with the stereomicroscope inside the cabinet, it can be set up on a nearby bench. Wipe down the bench and surrounding area liberally with 70 % ethanol. Try to set up the microscope away from any air currents that might be caused by opening doors or passing lab workers, etc.
8. It is convenient to place an empty 10 cm Petri dish within easy reach on each side of the microscope. These can be used as temporary "tool racks" for dissection instruments (forceps, microscissors, and needles). Rest the instruments on the rim of the dish so that the working ends are over the centre of the dish, not touching any surface that might compromise sterility.
9. The lower follicles are only loosely attached to the surrounding skin, so they can appear to move in and out of the surrounding connective tissue as the skin is manipulated during the mincing procedure. The follicles can be recognized by their smoother, less fibrous texture compared with the connective tissue. Adjust the magnification of the microscope as necessary to identify the follicles. Excessive mincing will begin to cut through follicles, which can then be seen as small cylinders within the connective tissue. If this happens, move to an adjacent area of skin and mince just sufficiently to expose intact follicles.
10. The cluster of follicles will be held together by fragments of loosely attached connective tissue. Aim to adjust the size of the pinched area of skin so that clusters contain 5–10 follicles. Aim to pinch and cut the follicles so that the length of each follicle removed is at least four times its diameter. If the follicles do not stand up sufficiently on pinching, try mincing some more connective tissue away (*see* Subheading 3.2, **step 7**).
11. The tip of the needle can be used like a scalpel to cut through connective tissue. Place the tip gently against the bottom of the dish and draw it through the connective tissue. Use the side of the needle to slightly stretch the connective tissue, to facilitate cutting close to the follicle (Fig. 1b).
12. It is convenient to work in the lid instead of the dish because the rim is lower, facilitating access with instruments over a wide range of angles.
13. Working with only one follicle per drop minimizes the presence of tissue fragments that are an inevitable by-product of dissection, thereby helping to keep the isolated papilla clean and readily identifiable.
14. The germinative epithelium has a softer, less translucent appearance than the dermal papilla and dermal sheath tissue. The germinative epithelium is more sticky and more elastic when touched with the needle tip.

15. It is important that the isolated dermal papilla is as free as possible of germinative epithelial tissue, as this can give rise to keratinocyte cells contaminating the subsequent culture. Keratinocytes persist after sub-culturing through several passages, and so cannot readily be eliminated once present.
16. The isolated papilla is typically too small to be grasped by the forceps without crushing it. Instead, close the tips of the forceps first, and place them adjacent to the papilla, gently touching the underlying plastic. Then use the needle tip to push the papilla into the gap between the forceps' tines, just back from the tips. Carefully withdraw the forceps containing the papilla from the drop of dissection medium. The papilla should stay in a small volume of medium trapped between the forceps' tines. Using your free hand, remove the 10-cm Petri dish lid containing follicles from the microscope's field of view, and replace it with the dish of culture medium. Lower the forceps holding the papilla into this dish, placing the papilla on the bottom. It is helpful to keep both the Petri dish lid containing follicles and the 35 mm culture dish on the microscope platform, so they can be readily slid in and out of the field-of-view with one hand. When transferring the papilla, there is a risk that it will fall out of the forceps as it is drawn through the meniscus of the medium. It can be helpful to adjust the focus of the microscope to observe the papilla throughout the transfer, allowing it to be seen in and recovered from the meniscus if necessary.
17. Although wool follicle papillae are small, cell outgrowth is generally quite robust, therefore a small number of papillae are sufficient to initiate a culture. Larger numbers of papillae in one dish increase the risk of contaminating keratinocytes, or of microbiological contamination.
18. Cell death can occasionally be visualized in the primary culture, particularly as patches of cells become larger and denser. We aim to passage the cells before this occurs, or while it is only minor. Continued growth of cells after passaging does not appear to be affected.
19. From this point on, the cells can be maintained at high density without compromising their viability. Aggregative behavior is typically seen once the cells become confluent. It is not necessary to passage the cells as soon as they reach confluence; they can be allowed to aggregate without losing viability. However twice-weekly medium changes should be continued regardless of cell density.
20. When conducting a dose–response experiment, it is convenient to prepare a stock solution of the bioactive which allows the correct final concentration to be achieved by adding different volumes of the stock to each well. An example to test the effect of lithium chloride is shown in Table 1 (*see* also [9]).

Table 1
Component volumes for a lithium chloride dose-response experiment

Per well	A	B	C	D	E	F	G	H
µl of culture medium	750	675	600	525	450	300	150	0
µl of 50 mM LiCl in culture medium	0	75	150	225	300	450	600	750
µl of cell suspension (1.33×10^6 cells/ml)	750	750	750	750	750	750	750	750
Final concentration of LiCl (mM)	0	2.5	5.0	7.5	10.0	15	20	25

21. Alternatively, the culture volume can be increased to 3.0 ml per well and half-volume medium changes undertaken on the third day. If necessary, it is possible to fit all medium changes into a Monday–Friday schedule, as follows:
    - On Friday, add 1500 µl fresh medium to a 1500 µl culture without removing any spent medium. This constitutes a half-volume change while increasing the culture volume.
    - On Monday, remove 2250 µl spent medium and add 750 µl fresh medium. This constitutes a half-volume change while decreasing the culture volume.
    - On Wednesday, remove 750 µl spent medium and add 750 µl fresh medium.

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Chapter 13

Isolation and Fluorescence-Activated Cell Sorting of Mouse Keratinocytes Expressing β-Galactosidase

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Abstract

During the past decade, the rapid development of new transgenic and knock-in mouse models has propelled epidermal stem-cell research into "fast-forward mode". It has become possible to identify and visualize defined cell populations during normal tissue maintenance, and to follow their progeny during the processes of homeostasis, wound repair, and tumorigenesis. Moreover, these cells can be isolated using specific labels, and characterized in detail using an array of molecular and cell biology approaches. The bacterial enzyme, β-galactosidase (β-gal), the product of the *LacZ* gene, is one of the most commonly used in vivo cell labels in genetically-engineered mice. The protocol described in this chapter provides a guideline for the isolation of viable murine epidermal cells expressing β-gal, which can then be subjected to further characterization in vivo or in vitro.

Key words Beta-galactosidase (β-galactosidase), Genetically engineered reporter mice, Keratinocytes, Skin, Epidermis, Stem cells, FACS sorting

1 Introduction

Advances in mouse transgenic and knock-in technologies have enabled labeling and fate mapping of defined cell groups in vivo. These cells can then be isolated and used for studies in vitro or can be transplanted into immunodeficient hosts to study their tissue regenerative properties. The repertoire of techniques used for genetic cell labeling in vivo is fairly broad and can be categorized into three major classes: (a) techniques taking advantage of the expression of enzymes that are normally not found in mouse (e.g. β-galactosidase (β-gal) [1] or luciferase [2]); (b) techniques utilizing the expression of fluorescent proteins, either one at a time [3] or in various combinations [4]; and (c) techniques detecting the expression of specific cell-surface antigens originating from an organism other than mouse (e.g. human CD4 [3]).

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In order to characterize a newly identified cell population, the cells need to be extracted from the tissue and isolated from the bulk of other cell types. The most commonly used method, next to magnetic bead-mediated cell isolation, is fluorescence-activated cell sorting (FACS), which is used for cells marked with fluorescent proteins or fluorescent tags. The isolation of cells expressing fluorescent proteins or specific cell-surface markers that can be visualized via fluorescently-labeled antibodies is relatively simple, in contrast to the detection of intracellular enzymatic markers. Intracellular detection is more complex, since in order to enable the specific enzyme–substrate reaction to occur, the substrate needs to be loaded intracellularly. Thus, a temporary disruption of the cell membrane, without substantially harming the cell's integrity and viability, is required.

Prior to the availability of modern genetically-engineered mice, keratinocytes with enhanced clonogenic properties were isolated, initially based on their ability to attach rapidly to the growth substrate [5]. A few years later, a variety of cell surface markers including CD34 [6], CD71 [7], Lrig1 [8], and Plet1 [9] were used to isolate mouse epidermal keratinocytes with stem-cell properties. The construction of an elegant transgenic mouse model expressing a nuclear histone 2B-enhanced green fluorescent protein (H2B-EGFP) fusion allowed the direct identification of slow cycling hair follicle keratinocytes as long-term stem cells, demonstrating the great benefit of engineered mice to stem cell research [10]. Recently, knock-in mouse models that utilized EGFP expression under the control of endogenous Hopx or Lgr6 promoters were used to characterize keratinocyte progenitor populations in mouse hair follicles [11, 12].

The bacterial enzyme, β-gal, encoded by the *LacZ* gene, has been widely used to localize the expression of genes of interest, or to label cells originating from a defined cell population (fate mapping). The advantage of this system is that β-gal activity can be detected readily on tissue sections or in whole mount tissue samples using a specific and sensitive biochemical reaction, followed by visualization with a normal light microscope. Due to the availability of reagents, which enable the detection of intracellular β-gal activity using FACS, cell populations from mice expressing bacterial β-gal can also be isolated by cell sorting. The fluorescent signal is generated by the enzymatic degradation of the non-fluorescent fluorescein di-β-D-galactopyranoside (FDG) resulting in free fluorescein, which can be detected by FACS. We have used this technique for the isolation of β-gal-expressing keratinocytes from the back skin of Lgr5-*LacZ* mice [13]. These mice express β-gal under the control of the endogenous Lgr5 promoter, which allowed us to show that Lgr5-expressing hair follicle stem cells display enhanced stem-cell properties in vitro and in vivo [14].

In this chapter, we describe in detail the protocol used to isolate these cells. The keratinocyte-isolation procedure is an adapted version of a method described previously [15]. Please note that this protocol was developed to isolate keratinocytes from dorsal mouse skin in the resting phase of the hair cycle (telogen), and is not applicable to the isolation of keratinocytes during the growth phase (anagen), since the digestion of the skin is insufficient, and the use of other protocols is recommended [16–18]. Once the keratinocytes are separated from the skin, FDG-labeled cells can be stained with antibodies that recognize cell-surface antigens followed by multicolor FACS analysis and sorting. The isolated and FACS-sorted cells are viable and can be grown in vitro or transplanted into immunodeficient hosts.

2 Materials

2.1 Reagents

1. Hanks' balanced salt solution (HBSS), Ca^{2+} and Mg^{2+}-free.
2. 0.25% solution of trypsin from porcine pancreas in HBSS (*see* **Note 1**).
3. Minimum essential medium (MEM), Ca^{2+} and glutamine-free (S-MEM) (*see* **Note 2**).
4. Bovine serum albumin (BSA).
5. Soybean trypsin inhibitor.
6. Defined keratinocyte serum-free medium (DK-SFM) (*see* **Note 3**).
7. Fluorescein di-β-D-galactopyranoside (FDG) (*see* **Note 4**).
8. Dimethyl sulfoxide (DMSO), cell-culture tested.
9. Phosphate buffered saline (PBS) solution (PBS tablets pH 7.4 [Medicago AB]): sterile, filtered and used as a FACS Flow substitute.
10. 0.4% trypan blue in PBS.
11. 96% ethanol.
12. 70% ethanol.
13. Sterile double-distilled water.
14. Sterile autoclaved water.

2.2 Antibodies

1. Anti-CD49f phycoerythrin (PE-Cy5) (551129, BD Biosciences, San Jose, CA).
2. Anti-Mouse CD34 Alexa Fluor® 647 (51-0341-82 eBioscience, Inc., San Diego, CA) (*see* **Note 5**).

2.3 Instruments

1. Animal hair clipper.
2. Scalpels (e.g. BA 210, B. Braun, Melsungen, Germany).

3. Forceps (e.g. "bent pointed" 105 mm forceps; 232-0130, VWR, Sweden).
4. Scissors (e.g. 03-320-090, Allgaier Instruments, Frittlingen/Tuttlingen, Germany).
5. Plastic bottles with flat bottom (*see* **Note 6**).
6. Magnetic stirrer bars.
7. A set of lab pipettes.
8. Spray bottle for 70% ethanol.
9. Refrigerated centrifuge.
10. Rocking platform.
11. Laminar flow hood.
12. Tissue culture incubator (37 °C).
13. Thermal incubator (32 °C).
14. Flow cytometer/cell sorter (*see* **Note 7**).

2.4 Preparation of Working Solutions

1. *Trypsin inhibitor solution:* dissolve 0.5 g BSA and 0.03 g trypsin inhibitor in 500 ml S-MEM, filter sterilize using the 0.22 mm filter unit, aliquot into 50 ml tubes and store at −20 °C. Avoid freeze–thaw cycles.
2. *1% BSA solution in S-MEM (1% BSA/S-MEM):* dissolve 5 g BSA in 500 ml S-MEM, aliquot into 50 ml tubes and store at 4 °C. The solution is stable for 1 month.
3. *20 mM FDG stock solution:* Prepare a 1:1 mixture of 96% ethanol and DMSO, pipette 38 ml of this mixture into a vial containing 5 mg FDG and dissolve the FDG by swirling the vial vigorously. Add 342 ml sterile double-distilled water to the FDG solution and mix thoroughly. Prepare 20–100 ml aliquots in sterile tubes and store at −20 °C. Avoid freeze–thaw cycles.

3 Methods

General considerations: when manipulating mouse keratinocytes one should bear in mind that these cells are extremely sensitive to mechanical and other forms of stress; therefore, please follow the instructions below:

(a) It is essential to avoid the use of pipette tips (e.g. 200 µl or 1 ml) for pipetting keratinocytes unless it is unavoidable, such as in the distribution of a small volume or an exact number of keratinocytes. As a rule the use of serological pipettes is strongly recommended.

(b) When resuspending keratinocyte pellets, do not use a vortex; instead gently tap with fingers on the tube.

(c) Cells should be kept on ice as much as possible.

(d) If the cells are to be used for grafting or further cultivation, aseptic conditions must be maintained at all stages.

3.1 Preparation

1. Prepare 20–30 ml HBSS in 50 ml tubes under sterile conditions for each mouse to be sacrificed.
2. Prepare autoclaved forceps and scissors, a glass beaker, a spray bottle filled with 70% ethanol and sterile autoclaved water. For each mouse, approximately 300 ml sterile water will be required.
3. Place a 0.25% trypsin solution in a 37 °C water bath (20–25 ml per mouse) for thawing.
4. Check that one incubator is set at 32 °C.
5. Drop a magnetic stirrer bar into each flat-bottom plastic-bottle. To sterilize the bottles, add 15–20 ml of 70% ethanol to each and shake vigorously. Spread a few disposable towels on the working surface of the tissue-culture hood and pour the ethanol and the magnetic stirrer bars onto the towels. Place the bottles upside down on the towels and let the bottles and the stirrers dry until needed.
6. Pre-chill 5, 10, and 25 ml serological pipettes (five each) in the refrigerator.

3.2 Harvesting Dorsal Skin

1. Sacrifice mice (*see* **Note 8**).
2. Clip dorsal hair (*see* **Note 9**). Use a fresh disposable towel to cover the dissection table and sterilize the dissection table and towel by spraying with ethanol. For each dissected mouse, the use of a separate fresh disposable towel is recommended in order to maintain aseptic conditions.
3. Sterilize each mouse by spraying with 70% ethanol, then rinse each mouse thoroughly with sterile water.
4. Remove the dorsal skin using forceps and scissors. Avoid contamination with mammary glands by excluding the side and ventral skin.
5. Transfer each dorsal skin specimen into a 50 ml tube containing HBSS (*see* **Note 10**).

3.3 Digesting Dorsal Skin

1. Take a 10 cm tissue culture dish containing 15 ml of HBSS buffer and spread the skin sample, hair side down, in the dish.
2. Remove all subcutaneous tissue by scraping with a scalpel. The skin sample can be held in place with forceps (*see* **Note 11**).
3. Cut the skin longitudinally into two equal pieces and then cut each piece into approximately 0.5 cm wide strips.
4. Fill a new tissue-culture dish with 20–25 ml pre-warmed 0.25% trypsin solution ensuring that the floating strips are

completely covered with trypsin solution. Seal the dishes with parafilm to maintain pH. Incubate for exactly 2 h at 32 °C (*see* **Note 12**).

5. Pipette 10 ml trypsin-inhibitor solution into a 10 cm tissue culture dish. Raise one edge of the dish with the help of a small support (e.g. cover of a scalpel blade). Take the digested skin strips one-by-one from the trypsin solution and scrape the hairy side (epidermis) into the trypsin-inhibitor solution. The material from up to two mice can be pooled into one dish (*see* **Note 13**).
6. Add 10 ml 1% BSA/S-MEM to the trypsin-inhibitor solution containing the pooled epidermal material, pipette the suspension gently five times up and down using a serological pipette and transfer the suspension into one of the sterile flat-bottom plastic bottles that were prepared earlier (Subheading 3.1, **step 5**). Insert the magnetic stirrer bar into the bottle using sterile forceps.
7. Rinse each tissue culture dish with 1% BSA/S-MEM and add the rinse fluid to the bottle. For one mouse, the total volume after rinsing should be 15 ml, for pooled samples from 2 to 5 mice, 30 ml.
8. Fasten the bottle lid and stir slowly on the magnetic stirrer (100–150 rpm) for 20 min at room temperature.
9. Mount a cell strainer (70 μm mesh size) into a 50 ml tube. To facilitate the flow of the liquid, the strainer should be mounted at a slight angle.
10. Titurate the cell suspension gently 5 times using a serological pipette and filter the cell suspension through the 70 μm cell strainer to eliminate fur and larger tissue parts.
11. Rinse the flat-bottom bottle with 10 ml 1% BSA/S-MEM and add the rinse fluid to the rest of the suspension by filtering it through the same cell strainer.
12. Centrifuge the cell suspension for 7 min at 200 rcf and 4 °C (*see* **Note 14**). Carefully decant (pour off, do not aspirate) the supernatant and resuspend the cells in the small amount of remaining liquid by gently tapping the tube with fingers. Put the cell suspension on ice.
13. Dilute the cell suspension with cold DK-SFM and count the cells (*see* **Note 15**).

3.4 FDG Loading (See Note 16)

1. Resuspend the cells in cold DK-SFM at 1×10^7 cells/ml (*see* **Note 17**).
2. Prepare an icebox and chill the DK-SFM on ice.
3. Prepare the 2 mM FDG working solution (equal in volume to the cell suspension): thaw an aliquot of the 20 mM FDG stock

solution in your hand, vortex until the last ice is just melted and dilute it 1:10 in sterile deionized water.

4. Pre-warm the cell suspension and the FDG working solution for 10 min in a 37 °C water bath. NB: Do not switch on the mixing/rocking feature of the water bath since this increases clumping of the keratinocytes! To reduce clumping it is advisable to mix the cell suspension occasionally during the incubation, and prior to adding the FDG loading solution, by tapping the tube with fingers.
5. Add an equal volume of the 2 mM FDG working solution to the cell suspension, mix briefly using a vortex and put the cell suspension immediately back in the 37 °C water bath. Incubate for exactly 1 min (*see* **Note 18**). Tip: 30 s before the end of the incubation time, fix a chilled serological pipette of suitable volume to the pipette aid.
6. Add nine times the volume of chilled DK-SFM added in **step 5** to the cells using a pre-cooled pipette and place the cells immediately on ice.
7. Incubate the cells in the dark on ice for 1 h. If antibody staining is to follow, this incubation step is only 15 min.

3.5 Antibody Staining (See Note 19)

1. Prepare the antibody dilutions in 1% BSA/S-MEM. For CD49f and CD34 (single or co-staining) use 20 µl anti-CD49f-PE-Cy5 (1:5) and/or 5 µl anti-CD34 Alexa Fluor® 647 (1:20) conjugates per 100 µl total volume (prepare a volume of 100 µl per 2×10^6 cells). Place the antibody cocktail on ice.
2. Centrifuge the cell suspension for 7–10 min at 200 rcf and 4 °C (*see* **Note 20**).
3. Decant the supernatant and resuspend the cells by gently tapping the tube where the residual amount of media is running back from the walls of the tube. This step is important to avoid cell clumping.
4. Place the cells in a 50 ml tube and add the antibody solution by simultaneously tapping the tube (*see* **Note 21**).
5. Incubate the cells on ice for 45 min, gently tapping the tube twice every 15 min.
6. Place the cell strainers (one for each sample) on ice to cool.
7. To avoid the attachment of isolated keratinocytes to the tube walls, fill the 5 ml FACS tubes with 2.5 ml of DK-SFM, seal it with the cap, invert the tube several times to cover all plastic at least once with liquid and chill on ice. Alternatively, fill 15 ml tissue culture tubes with 5 ml media, invert several times and chill on ice.
8. Wash the cell suspension twice with 2–10 ml cold 1% BSA/S-MEM, and centrifuge for 5–7 min at 200 rcf at 4 °C. Decant

the supernatant and resuspend the cells in the residual liquid by gentle tapping.

9. Resuspend the cells in cold DK-SFM at a density of 1×10^6 cells/ml for FACS analysis, and up to 3×10^6 cells/ml for FACS sorting (*see* **Note 22**).

3.6 FACS Analysis/ Sorting

1. Filter the cell suspension through a pre-cooled 40 μm cell strainer and place the cells on ice, protected from light (*see* **Note 23**).
2. Analyze/sort the cells using a flow cytometer. During the analysis/sorting, the sample must be kept cool (*see* **Notes 24** and **25**). For comments on the sorting procedure, *see* **Note 26**.
3. Centrifuge cells for 7 min at 200 rcf at 4 °C.
4. Decant the supernatant, resuspend the cells in the residual medium running back from the tube walls by gently snapping the vial. Add 1 ml cold DK-SFM.
5. Centrifuge cells for 7 min at 200 rcf at 4 °C.
6. If the cells are to be lysed or fixed, remove all medium and proceed with the lysis/fixation protocol. If live cells are required, proceed to the next step.
7. Resuspend the cells in the residual medium running back from the tube walls by gently tapping the vial. The cell suspension can now be used for further applications.

4 Notes

1. Trypsin can be obtained ready-to-use, for example in 100 ml bottles (T4424-100 ml) or in 500 ml bottles (T4424-500 ml, Sigma-Aldrich). The empty 100 ml bottles can be used for the skin digestion step. It is recommended to store trypsin in 45 ml aliquots in the freezer and to avoid repeated thaw–freeze cycles.
2. This item is no longer available and has been replaced by an MEM suspension with no calcium, no glutamine (11380-037, Life Technologies, Carlsbad, CA). All our previously published data are based on experiments conducted using the product 11380-052 which is no longer available.
3. DK-FSM has to be reconstituted by adding the frozen growth supplement (1 ml vial) to 500 ml medium at the time of use. In general, the contents of the vial should not be thawed repeatedly. However, if less than 500 ml DK-SFM are needed, it is preferable to prepare an aliquot of 250 ml. In our hands preparing half the DK-SFM and refreezing the rest of the supplement once did not notably decrease the activity of the DK-SFM. The reconstituted medium was aliquoted into

50 ml tubes. Each 50 ml aliquot of the growth medium was warmed up only once and was never used a second time.

4. Life Technologies also provides a Floreporter lacZ Flow Cytometry Kit (F-1930, Life Technologies, Carlsbad, CA), which contains chloroquine to inhibit endogenous β-gal activity. Keratinocytes have low levels of endogenous β-gal activity, and since our goal was to discriminate qualitatively between β-gal-positive and negative cells, we did not find it necessary to use this kit. If required, the manufacturer's website can be consulted for detailed instructions.
5. This antibody is no longer available and has been replaced with anti-mouse CD34 eFluor® 660 (50-0341-82, eBioscience, San Diego, CA). Please note, all our previously-published data are based on the 51-0341-82 eBioscience antibody, which is no longer available.
6. We used 100 ml plastic bottles, which originally contained trypsin (T4424-100 ml; Sigma-Aldrich). Any bottle with a flat bottom made of tissue-friendly plastic can be used (e.g. polyethylene terephthalate [PETE] or PETG, a PET co-polymer, from Nalgene). Avoid using glassware as mouse primary keratinocytes will lose their ability to proliferate if they have been in contact with glass.
7. We used the FACSCalibur flow cytometer (BD Biosciences) for analysis and the FACSAria II and III (BD Biosciences) for cell sorting.
8. This protocol has been optimized for the digestion of skin in the resting phase, when the hair follicles are in telogen. The number of mice to be sacrificed depends on the downstream application, for example an analytical FACS experiment usually requires just one dorsal skin specimen per genotype. For sorting more rare cell populations (0.5% and smaller), 2–7 mice should be sacrificed depending on the relative number of the LacZ-labeled cells. In our hands one 7- to 8-week-old (second telogen) female mouse yields a maximum of 16×10^6 cells (max. 6% dead cells). If available, female mice are preferred to males as their skin is easier to digest, resulting in higher cell yields. If younger mice (first telogen) are required for analysis, postnatal days 19, 20, or 21 may be suitable time points depending on the mouse's genetic background. Additionally, it is advisable to harvest negative-control keratinocytes from a mouse that does not express β-gal. These cells should be processed in parallel with the experimental samples and can be used for setting the baseline for the FDG signal for FACS analysis.
9. The fur of the animals needs to be clipped. Skipping this step will result in the attachment of keratinocytes to the hairs and decrease the cell yield. Wet shaving, however, is not necessary.

10. Alternatively, one can float the skin fur-side up in HBSS buffer in a 10 cm tissue culture dish until the next step.
11. Optimal removal of the subcutaneous tissue results in the skin appearing translucent and is crucial for successful trypsinization of the skin. However, to avoid damaging the epidermis itself, scraping should not be so rough that the skin is torn apart.
12. It is advisable to label the dishes with the exact incubation start time. This allows sequential processing of a large number of mouse skin samples and avoids prolonged trypsinization, which is harmful to the cells. The use of a CO_2 atmosphere instead of sealing with parafilm is not recommended since it reduces the pH of the trypsin solution too much.
13. The epidermis and hair follicles should detach easily. However, in some areas the hair follicles might be retained in the dermis (e.g. patches of insufficient trypsin digestion due to incomplete removal of subcutaneous tissue, or anagen areas if the mice were not sacrificed during the synchronized first two telogen phases). These patches should be left as they are, since trying to scrape them off could result in fibroblast contamination. Pooling the cells of more than two mice will reduce the cell yield.
14. We used an Eppendorf 5702R centrifuge at 200 rcf ($200 \times g$ or 1100 rpm).
15. In our hands the number of keratinocytes isolated from one dorsal skin specimen varies within broad limits: $7–12 \times 10^6$ at P21 and $9–16 \times 10^6$ at 8 weeks of age. The number of dead cells is normally below 6%.
16. Since free fluorescein easily leaks out of cells at higher temperatures, the sample should be kept on ice or cooled to 4 °C at all times, beginning from the FDG loading step up to cell analysis and/or sorting. Failure to do so will result in a notable reduction in signal intensity or even loss of the FDG signal. To improve the preservation of the fluorescein signal at higher temperatures, a modified version of FDG, 5-chloromethylfluorescein di-β-D-galactopyranoside (CMFDG, D-2920, Life Technologies, Carlsbad, CA) is available. We have not found it necessary to use this β-gal substrate in our experiments since maintaining low temperatures during the manipulations is essential to preserve the viability of mouse keratinocytes. However, the use of CMFDG might be feasible if the resulting fluorescence signal is very low. Nevertheless, the viability of the cells treated with CMFDG should be evaluated carefully before proceeding with functional tests.
17. The choice of the tube size for this step depends on the initial number of cells. If the starting volume exceeds 0.5 ml, the use of a 50 ml tube is recommended. In extreme cases (sorting of rare cell populations), several 50 ml tubes may be required.

18. Since FDG does not penetrate the cell membrane under normal conditions, it needs to be loaded into the cells "artificially". The manufacturer of FDG provides a specific reagent in the FDG staining kit that facilitates FDG loading via a pinocytosis-mediated mechanism. An alternative to this method is the use of a hypotonic shock treatment also described in the kit protocol. During the optimization of the protocol for mouse keratinocytes we found that, in our hands, hypotonic shock treatment resulted in a considerably stronger fluorescein signal.
19. In order to preserve the fluorescein signal, all procedures described in this section must be conducted on ice and under reduced light conditions. The use of chilled solutions, chilled serological pipettes, and pipette tips is *mandatory*. If the cells are to be subjected to sorting and subsequent culturing, or transplantation and aseptic conditions need to be maintained, use a small ice bucket under the hood for cooling the samples.
20. The centrifugation time depends on the height of the cell suspension in the test tube. Larger volumes in larger tubes require prolonged centrifugation times. Normally, we use 50 ml tubes and have found that 7 min is optimal for up to 20 ml and 10 min for up to 50 ml. Centrifugation for longer than 10 min is not recommended since it may negatively affect cell viability.
21. The residual volume in which the cells remain at this point is approximately 100 μl. Thus the antibody solution will be diluted approximately 1:1. However, the protocol takes this "uncontrolled" dilution factor into account.
22. Higher cell concentrations are required for FACS sorting in order to reduce the sample pressure and to maintain cell viability. Although DK-SFM contains phenol red, we have not noted any negative effects on flow cytometry analysis. However, we have observed a positive effect on cell viability when compared with cells suspended in PBS prior to FACS analysis/cell sorting.
23. We have routinely used the large 70 μm and 40 μm cell strainers from BD Biosciences, and some patience is needed to direct the flow exactly into the opening of a 5 ml FACS or a 15 ml tissue-culture tube. As an alternative to the 40 μm filter, tubes with 35 μm filter mesh caps (5 ml polystyrene round-bottom tube; 352235, BD Falcon) can be used. However, we have not tested their effect with respect to long-term cell viability when used for colony assays or transplantation. In order to maximize the cell yield using the large strainers, it is recommended to pre-wet the mesh with approximately 500 μl medium and tap gently on the strainer until a drop of medium has passed through.
24. For analysis using a flow cytometer without a cooling module (e.g. FACSCalibur, BD Biosciences), a cooled sample holder

can be constructed as follows: cut off approximately 4 cm of the conical end of a 50 ml tissue culture tube to make an "ice container". Fill this half-way with ice and insert the 5 ml FACS tube containing the sample into the bottom of the container. Set the pad on the sample support arm of the FACSCalibur to its lowest possible position and install the sample tube, together with the cooling device (Fig. 1).

25. Due to the relatively broad emission spectrum of fluorescein, the LacZ-expressing cells are detected in both FL1 and FL2 channels (Fig. 2). Therefore, the simultaneous use of phycoerythrin (PE) or other fluorochromes with similar characteristics is not recommended. The bleed-through of the fluorescence signals between the detectors should be tested using singly-stained cells and corresponding cross-channel compensation should be applied. Instead of the fluorochrome-conjugated primary antibodies, one may consider a combination of an unconjugated primary antibody and a fluorochrome-conjugated secondary antibody. In this case wash the cells twice with 1 % BSA-SMEM and then incubate with the secondary antibody for 30 min followed by two washes with 1 % BSA-SMEM. A good option for analyzing the cell cycle, in conjunction with FDG staining, is the use of the cell-cycle dye, Vybrant DyeCycle Violet stain (V35003, Life

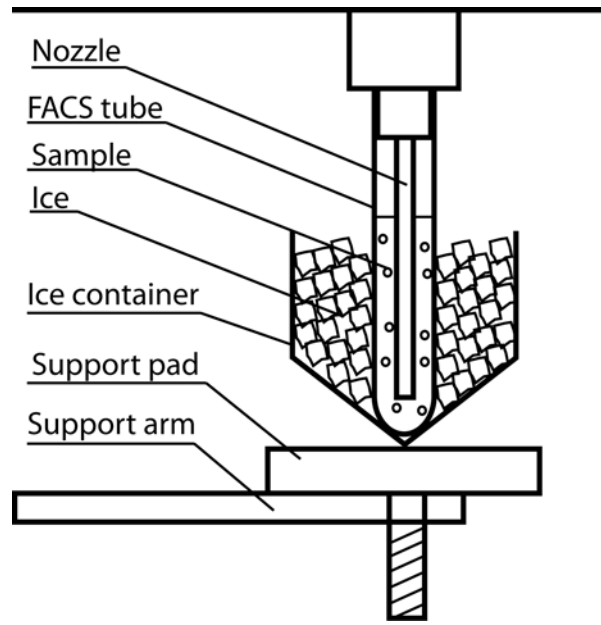

Fig. 1 Schematic representation of the home-made cooling device fitted on a FACSCalibur flow cytometer (*see* **Note 24** for further comments)

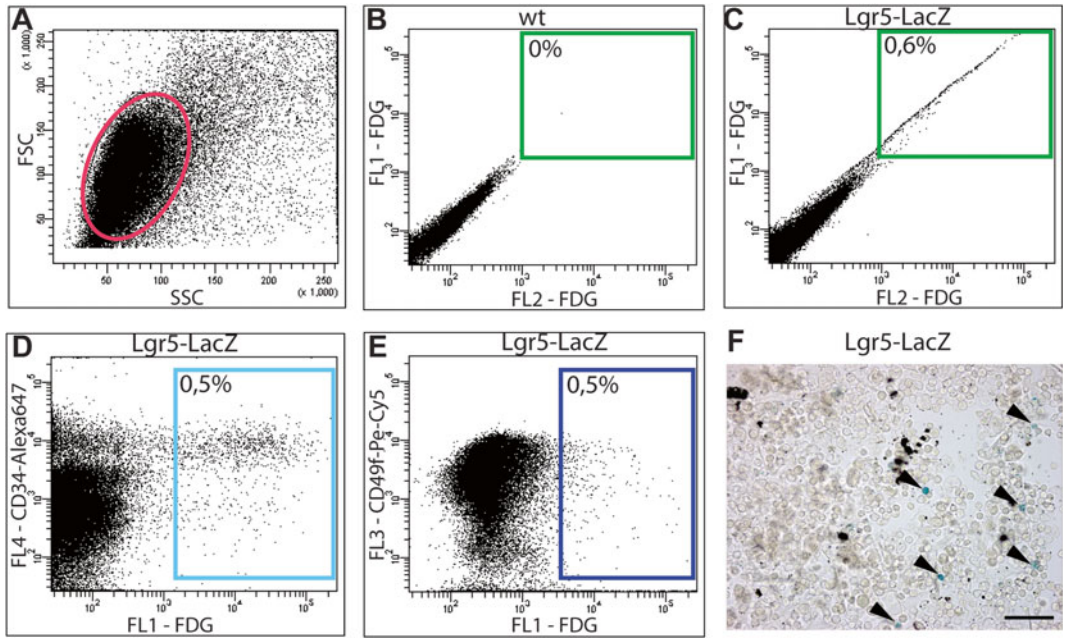

Fig. 2 Identification of β-gal-expressing keratinocytes. (**a**) Forward-scatter (FSC) and side scatter (SSC) plots of keratinocytes isolated from mouse epidermis. The gate used for further analyses is shown as the *red circle*. (**b**, **c**) Flow-cytometric analysis of FDG-stained keratinocytes isolated from a wild-type mouse (**b**) and as Lgr5-*LacZ* mouse (**c**). The percentage of β-gal-expressing cells is shown in the gate. (**d**, **e**) Flow cytometric analysis of FDG-stained keratinocytes co-stained with CD34 Alexa Fluor® 647 (**d**) or CD49f-PE-Cy5 (**e**). The percentage of β-gal-expressing cells is shown in the corresponding gates. (**f**) X-gal staining of cytospun and fixed keratinocytes isolated from the epidermis of an Lgr5-*LacZ* mouse. Arrows indicate β-gal-expressing keratinocytes. Scale bar: 50 μm

technologies, Carlsbad, CA) on live cells. However, this is only possible on systems equipped with a 405 nm laser.

26. For the cell-sorting procedure itself, it is advisable to seek the help of an expert in this technique. If the sorted cells are to be used for cultivation or transplantation, the cell sorter should be decontaminated according to the manufacturer's instructions prior to sorting, and aseptic conditions should be followed throughout the sorting procedure. We use a 100 μm nozzle and the lowest system pressure (20 PSI) to sort primary keratinocytes. Moreover, we use sterile-filtered 1× PBS (pH 7.0–7.4) as the sheath fluid. Cells should be sorted into 5 ml tubes containing DK-SFM prepared earlier. Both the sample tubes and the collection tubes should be cooled. When sorting is complete, the tubes should be sealed with their caps and placed on ice.

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Chapter 14

Protocols for Ectopic Hair Growth from Transplanted Whisker Follicles on the Spinal Cord of Mice

Wenluo Cao, Fang Liu, Yasuyuki Amoh, and Robert M. Hoffman

Abstract

Isolated whisker follicles from nestin-driven green fluorescent protein (ND-GFP) mice, containing hair-associated pluripotent (HAP) stem cells, were histocultured in three dimensions on Gelfoam® for 3 weeks for subsequent transplantation to the spinal cord in order to heal an induced injury with the HAP stem cells. The hair shafts were removed from Gelfoam®-histocultured whisker follicles, and the remaining parts of the whisker follicles, containing GFP-nestin-expressing (HAP) stem cells, were transplanted into the injured spinal cord of nude mice, along with the Gelfoam®. After 90 days, the mice were sacrificed and the spinal cord injuries were observed to have healed. ND-GFP expression was intense at the healed area of the spinal cord, as observed by fluorescence microscopy, demonstrating that the HAP stem cells were involved in healing the spinal cord. The transplanted whisker follicles produced remarkably long hair shafts in the spinal cord over 90 days and curved and enclosed the spinal cord. This result changes our concept of hair growth, demonstrating it is not limited to the skin and that hair growth appears related to HAP stem cells as both increased in tandem on the spinal cord.

Key words Mice, Ectopic hair growth, Gelfoam whisker culture, Transplantation, Spinal cord, Nestin, HAP stem cells, GFP, Imaging

1 Introduction

We previously discovered nestin-expressing hair-follicle-associated pluripotent (HAP) stem cells, in the permanent upper hair follicle immediately below the sebaceous glands in the hair follicle bulge area (BA), in nestin-driven green fluorescent protein (GFP) (ND-GFP) transgenic mice. The ND-GFP-expressing HAP stem cells in the bulge area surrounded the hair shaft and were interconnected by short dendrites [1].

We subsequently demonstrated that ND-GFP HAP stem cells isolated from the BA could differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro [2].

HAP stem cells from ND-GFP mice were transplanted into the gap region of severed sciatic nerves of nude mice. The transplanted HAP stem cells enhanced the rate of nerve regeneration and the

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restoration of nerve function. The HAP stem cells differentiated mostly into Schwann (glial) cells, which supported neuron regrowth [3].

HAP stem cells were subsequently transplanted to the injured spinal cord of nude mice. Most of the transplanted cells also differentiated into Schwann cells which facilitated repair of the severed spinal cord. The rejoined spinal cord resulted in extensive hind-limb locomotor performance recovery [4].

BA and dermal papilla (DP) HAP stem cells were histocultured on Gelfoam® and were separately transplanted to the injured spinal cord of nude mice. Both DP and BA ND-GFP cells differentiated into neuronal and glial cells after transplantation to the injured spinal cord. ND-GFP cells from both areas enhanced injury repair and locomotor recovery [5, 6].

Whiskers from ND-GFP mice were placed in 3D Gelfoam® histoculture. The whiskers produced β-III tubulin-positive fibers, consisting of ND-GFP-expressing HAP stem cells which extended up to 500 mm from the whisker nerve stump in Gelfoam® histoculture. These fibers had growth cones on their tips expressing F-actin indicating the fibers were growing axons which were highly enriched in ND-GFP HAP stem cells [7].

We previously demonstrated the surprising result that Gelfoam®-histocultured whisker follicles, transplanted to the injured spine, along with the Gelfoam® on which they were histocultured, sprouted long hair shafts from the spinal cord during the period the HAP stem cells rejoined the injured spinal cord (10). The present chapter describes a protocol for transplanting Gelfoam® whisker follicles to the injured spine.

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, Hanover Park, IL).
3. Needle holder (Fisher Scientific).
4. Microscissors and microforceps (Fisher Scientific).
5. Exel International Disposable Scalpels (Fisher Scientific).
6. 10 ml syringe (BD).
7. Illumatool™ in vivo fluorescence imaging system (Lightools Research, Encinitas, CA).
8. OV100 small animal fluorescence imaging system (Olympus Corp.).
9. FV1000 confocal laser scanning microscope (Olympus Corp.).

2.3 Reagents and Consumable Items

1. Gelfoam® (Pfizer Inc., New York, NY).
2. DMEM/F12 medium (GIBCO Life Technologies, Grand Island, NY).
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 dish (Nest Biotechnology Co., Rahway, NJ).
8. PBS (phosphate buffered saline) (GIBCO Life Technologies).
9. 0.9% saline.
10. Fixation solution: 4% paraformaldehyde or 10% buffered formalin acetate.

3 Methods

3.1 Isolation of Mouse Whisker Follicles

1. Prepare the Gelfoam® culture medium before hair follicle Gelfoam® histoculture: DMEM/F12 medium containing B-27 (2.5%), N2 (1%) and 1% penicillin and streptomycin.
2. Use a disposable scalpel to cut Gelfoam® into approximately 1 cm × 1 cm and place into six-well dishes, and add 1 ml cell-culture medium to the Gelfoam® (*see* **Note 1**). Place the Gelfoam® with the cell culture medium in a 37 °C CO_2 incubator overnight (*see* **Note 2**).
3. Anesthetize nestin-GFP transgenic mice (6–8 weeks) with the ketamine mixture and cut the whisker hair shafts.
4. Remove both of the whisker pads from the mice with sterilized scissors.
5. Lay whisker pads on a black pad, expose the inner side of whisker pad and pin the whisker pad to the black pad.
6. Under a stereo-microscope, carefully remove the attached tissue with microscissors and microforceps. Gently remove each whisker follicle by grasping the hair shaft near the skin surface and pulling firmly and smoothly. Put the freshly-isolated hair follicle in DMEM immediately (*see* **Note 3**).
7. Collect all hair follicles and choose the hair follicles which are intact and transfer onto the prepared soaked Gelfoam® in the tissue-culture well. Each whisker follicle has one piece of Gelfoam® for support.
8. Place the six-well dishes with the Gelfoam® histocultures in a 37 °C CO_2 incubator for 3 weeks. Change the medium every 2–3 days (*see* **Note 4**).

9. Use a confocal laser scanning microscope for two- (X,Y) and three-dimensional (3D, X,Y,Z) high-resolution imaging of the whiskera follicles in Gelfoam® histoculture. Obtain fluorescence images using the 4×/0.10 Plan N, 10×/0.30 Plan-NEOFLUAR, 20×/0.50 UPlan FL N, and 20×/1.00w XLUMplan FL objectives.

3.2 Surgical Procedures for Spinal Cord Injury and Transplantation of Gelfoam Whisker Histocultures

1. Anesthetize non-transgenic nude mice with the ketamine mixture.
2. Place the mouse in a flat prone horizontal position. Identify the last rib by palpation. This allows one to estimate the location of the XIIIth thoracic vertebra (T13).
3. Cut the skin overlaying the vertebral column.
4. Observe and localize the spine process at T13 by palpating the XIIIth ribs, and identify T8 by counting spine processes cranially from T13 carefully, under an MZ6 stereo-microscope.
5. Use fine scissors and forceps to separate the muscles along the spine under the stereo-microscope.
6. Remove the spinous processes and lamina from T8 in order to achieve a partial laminectomy.
7. Remove the right side of the spinal cord by using a 31 G insulin syringe needle. The lesion surface size should be 500 μm × 500 μm, and the depth of the lesion should be from the dorsal to the abdominal surface of the spinal cord.
8. Cut Gelfoam® whisker cultures (3 weeks) into 500 μm × 500 μm pieces and introduce them into the spinal cord lesion site with fine forceps (*see* **Note 5**).
9. Place the surgically-repaired mice back in their cages and maintain them for 90 days. Use fluorescence and bright light microscopy of the spinal cord to observe spinal cord repair and hair-shaft elongation. Euthanize animals at day 90.

3.3 Whole-Animal Perfusion Fixation

1. Anesthetize transplanted mice at day 90 with a ketamine mixture.
2. Plate the mouse on a shallow tray with its back down. With sharp scissors, cut the skin and the muscles through the abdominal wall beneath the rib cage. Carefully separate the liver and other connective issue from the diaphragm.
3. Hold and lift up the xiphoid process of the sternum with a needle holder, and cut both sides of the ribs up to the collarbone, and open up the thoracic cavity (*see* **Note 6**).
4. Insert a needle into the left ventricle while holding the heart steady with your hand.
5. Pump a small volume of saline into the heart with a 10 ml syringe (*see* **Note 7**) whereby the auricle of right atrium will

become obviously larger and make a quick incision to allow the blood to flow freely.

6. Keep pumping the 0.9% saline into the left ventricle for 2–3 min until the solution coming out from the right atrium is not red and clear. The color of the liver will turn pale.
7. Once blood has been cleared from the body, perfuse 4% paraformaldehyde or 10% buffered formalin acetate solution (10–20 min) (*see* **Note 8**).
8. Stop the perfusion and excise the spinal cord. Under a stereomicroscope, cut the skin and the muscle, and remove the spine, and carefully excise the spinal cord. Place the specimens in vials containing the fixation solution and store at 4 °C for future histological procedures.
9. Obtain fluorescence images of 90-day fixed spinal cords using an OV100 in vivo fluorescence imaging system (*see* **Note 9**).

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, both in vitro and in the injured spinal cord, which may provide nutritional factors for long-term hair shaft growth [5, 6, 8, 9]. Gelfoam® appears to preserve the integral hair follicle both in vitro and in vivo.
2. The purpose of this step is to hydrate the Gelfoam® with culture medium.
3. When isolating the hair follicles, try not to grasp and press the bulge area. Maintain the hair follicle intact and not pressed.
4. The culture medium should be changed every 2–3 days. The interval time cannot exceed 1 week, otherwise the Gelfoam® will became thin and digested by 2–3 weeks.
5. After Gelfoam® histoculture of whiskers for 3 weeks, elongated hair shafts were cut off and the whisker follicles, containing ND-GFP HAP stem cells, which had increased during histoculture, were transplanted along with the Gelfoam® into the injured spinal cord of nude mice (*see* Fig. 1).
6. Lift up the xiphoid process of stemum with a needle holder to avoid or minimize damage to the tissues in the thoracic cavity when cutting the ribs. Once the thoracic cavity is opened, the next 4–7 steps should be completed as soon as possible, since the mice will die soon.
7. Alternatively, a perfusion pump or a flask placed upside down above the animal can be used.
8. First perfuse the fixation solution quickly. When spontaneous movement occurs and the liver became hard, the perfusion is

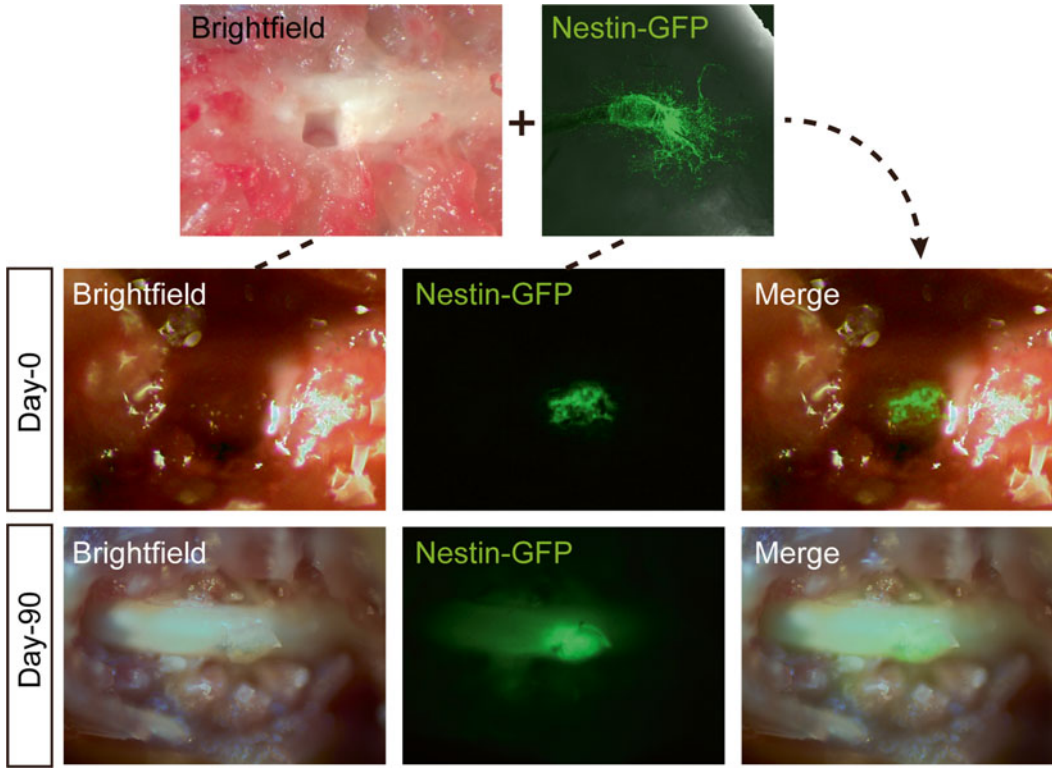

Fig. 1 Transplantation of nestin-driven green fluorescent protein (ND-GFP)-expressing whisker follicle Gelfoam® histocultures to the injured spinal cord of nude mice. After Gelfoam® histoculture of isolated whisker hair follicles from ND-GFP mice for 3 weeks, the long hair shafts of the whisker follicle were cut off, and the follicle, along with the Gelfoam®, was transplanted into the injured nude-mouse spinal cord. The transplanted mice were sacrificed after 90 days. ND-GFP expression intensified by 90 days and expanded in the injured area of the spinal cord, which was apparently healed by ND-GFP expressing HAP stem cells within the whiskers. A total of seven mice were studied. The figure shows typical data

almost complete and then perform perfusion slowly to allow the fixation solution to flow through capillaries in order to fix distal tissue and nerve structures better.

9. After 90 days, the mice are sacrificed. At this time, the spinal-cord lesion will be healed. ND-GFP expression will be visible and intense along the healed area of the spinal cord, indicating the HAP stem cells are viable and healed the injury. We previously reported that implantation of Gelfoam®-supported whisker histocultures to the injured spinal cord resulted in functional healing [5]. In the present experiment, the whiskers were histocultured for a longer period of time and the mice had a longer time after implantation before examination of their spinal cord. It was assumed that the spinal cord was functionally healed, as in our previous experiments [4, 5]. Unexpectedly, stout pigmented hair fibers were observed growing from the implanted whisker follicle Gelfoam® complex in the spinal cord (*see* Fig. 2). The hair shafts grew remark-

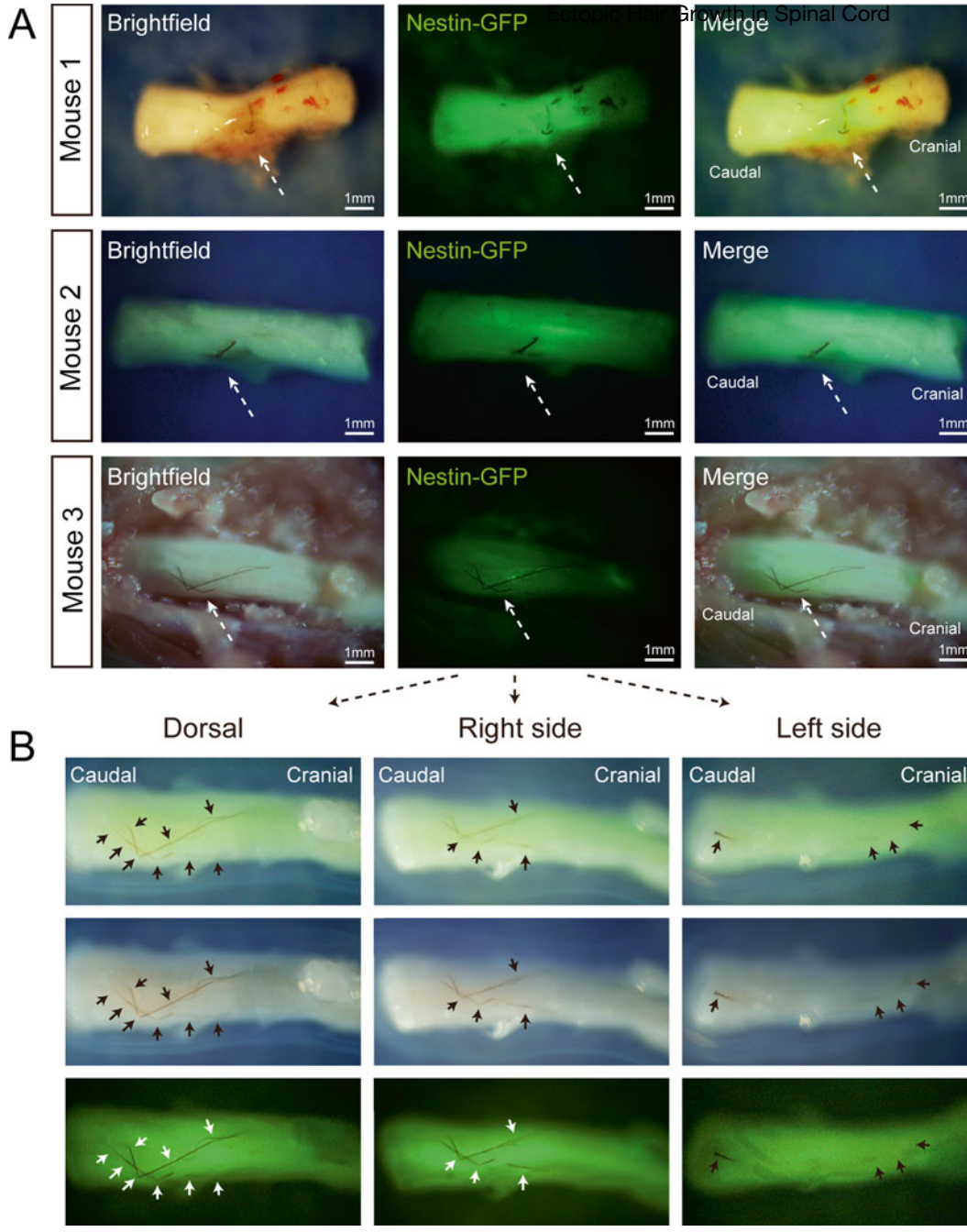

Fig. 2 Ectopic hair growth in the spinal cord. Ninety days after transplantation of the 3-week Gelfoam® ND-GFP-expressing whisker histocultures in the injured spinal cord, long hair shafts (*arrows*), were observed along and around the healed spinal cord. (**a**) Shows the elongated hair shafts that grew from whisker follicles, previously histocultured on Gelfoam®, transplanted into the injured spinal cord in three different mice at day 90 after surgery. Mouse 3 had the most remarkable hair shaft growth, which curved and enclosed the spinal cord. *Arrows* show the hair growth in the spinal cord. (**b**) Panels show the hair-shaft growth from the transplanted Gelfoam®-histoculture whisker follicles in the spine from mouse 3 at higher magnification from different views of the spinal cord (dorsal, *right*, and *left side*). The growing hair shaft reached a length of almost 14 mm and curved around the spinal cord. *Arrows* depict the hair shaft growing from the whisker hair follicles transplanted in the spine. Six out of seven mice implanted with Gelfoam® whisker histocultures showed extensive ectopic hair growth on the spine

ably long in the spinal cord, as much as approximately 14 mm, and curved and enclosed the spinal cord (*see* Fig. 2, Mouse-3). A total of seven mice were implanted with Gelfoam® whisker histocultures and after examination, six mice showed ectopic hair growth ($p = 0.001$ compared to mice implanted with Gelfoam® only). The unanticipated results demonstrate the great potential of hair shaft growth after Gelfoam® histoculture and transplantation of the hair follicle in vivo, even at an ectopic site. The length of the hair shaft observed in mouse 3 of almost 14 mm suggests that the ectopic site of the spine can strongly stimulate hair growth.

Acknowledgements

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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

Wenluo Cao, Fang Liu, Yasuyuki Amoh, and Robert M. Hoffman

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

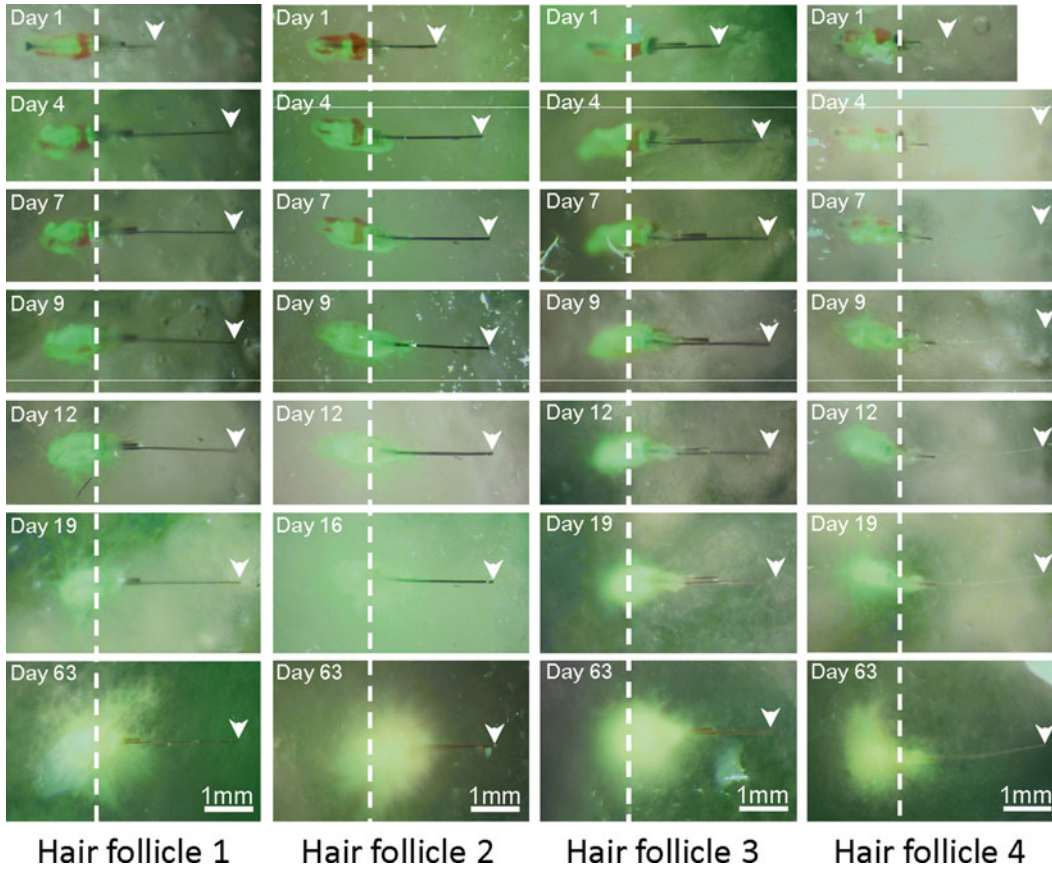

Fig. 1 Hair-shaft elongation of mouse whiskers in Gelfoam® histoculture. Time-course images of hair-shaft growth from individual mouse whisker follicles, isolated from nestin-driven-green fluorescent protein (ND-GFP) mice, histocultured on Gelfoam®. Green fluorescence was from the ND-GFP-expressing hair 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).

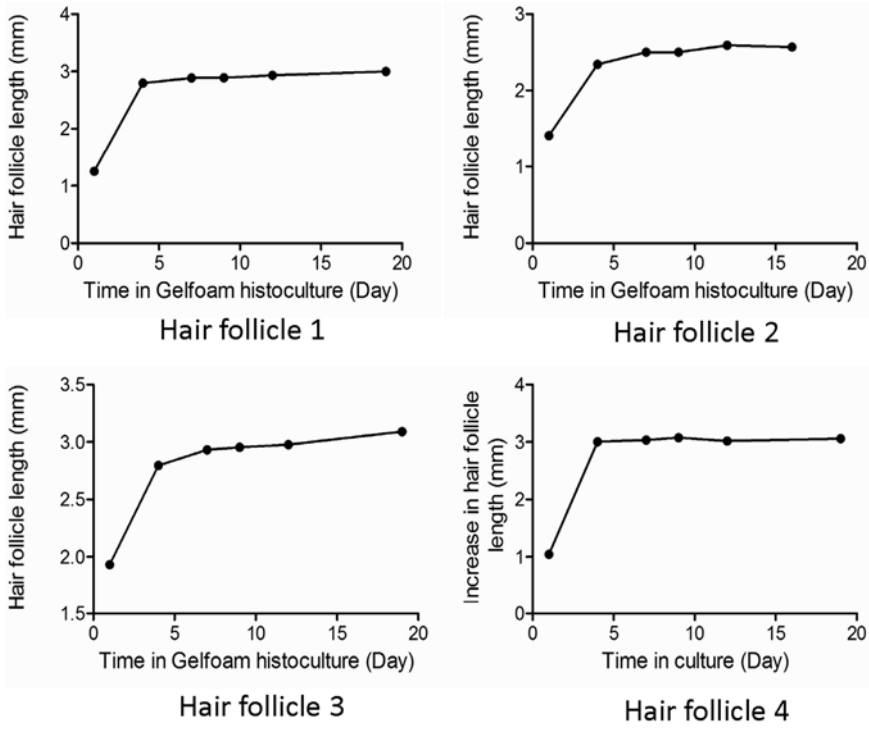

Fig. 2 Graphs quantifying the increase of shaft length over time in individual follicles during Gelfoam® histoculture [13]

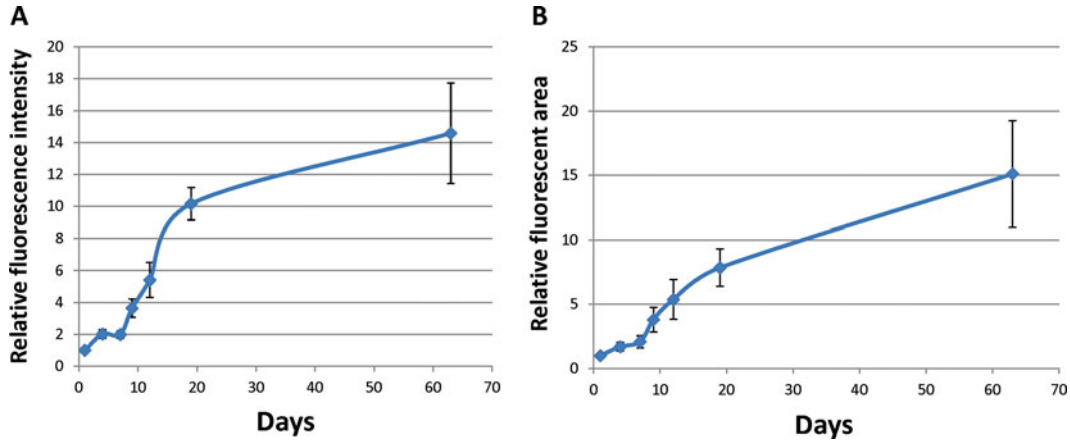

Fig. 3 Graphs quantifying the time-course increase of HAP stem cell GFP fluorescence intensity (**a**) and fluorescent area (**b**). $p<0.01$ in increase of fluorescent area and fluorescence intensity at day 63 compared to day 1 [13]

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).

Acknowledgement

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Chapter 16

Protocols for Efficient Differentiation of Hair Follicle-Associated Pluripotent (HAP) Stem Cells to Beating Cardiac Muscle Cells

Masateru Yashiro, Sumiyuki Mii, Ryoichi Aki, Yuko Hamada, Nobuko Arakawa, Katsumasa Kawahara, Robert M. Hoffman, and Yasuyuki Amoh

Abstract

We have previously demonstrated that the nestin-expressing cells from the upper part of the hair follicle can differentiate to neurons and other cell types. We have termed these cells as hair-associated-pluripotent (HAP) stem cells. In the present chapter, we describe methods for HAP stem cells to differentiate to beating cardiac muscle cells. The mouse vibrissa hair follicle was divided into three parts (upper, middle, and lower), and each part was suspended separately in DMEM containing 10% fetal bovine serum (FBS). All three parts of hair follicle differentiate to neurons, glial cells, keratinocytes, smooth muscle cells, and cardiac muscle cells. The differentiation potential to cardiac muscle is greatest in the upper part of the follicle. Hair spheres comprised of HAP stem cells formed from the upper part of vibrissa hair follicle can differentiate to cardiac muscle cells.

Key words Vibrissa, Hair follicle, HAP stem cell, Cardiac muscle cells, Hair spheres, Differentiation

1 Introduction

Our laboratory discovered that the neuron stem cell marker nestin is expressed in the bulge area [1] of transgenic mice with nestin-regulatory-element-driven GFP (ND-GFP) [1]. The ND-GFP-expressing stem cells can differentiate to neurons, glia, keratinocytes, smooth muscle cells, and melanocytes [2]. We have termed these cells hair-follicle-associated-pluripotent (HAP) stem cells [3, 4]. We demonstrated that the differentiating potential of HAP stem cells is strongest in upper part of the hair follicle compare to the middle and lower parts. These results suggest that the origin of the HAP stem cells is in this part of the follicle [5–9]. The present chapter describes protocols for differentiating beating heart muscle cells from HAP stem cells.

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In the present chapter, we describe materials and methods to produce hair spheres formed from the upper part of vibrissa hair follicle containing HAP stem cells that can differentiate to cardiac muscle cells [10, 11].

2 Materials

2.1 Animals

1. C57BL/6 mice (CLEA, Tokyo, Japan).

2.2 Instruments

1. Binocular microscope (SZX16, Olympus Corp., Tokyo, Japan).
2. Fluorescence microscope (BX51, Olympus).
3. Video microscope camera (ScopPad-500, GelleX, Tokyo, Japan).
4. FACS (FACSVers, BD Biosciences, San Diego, CA).

2.3 Reagents and Consumable Items

1. DMEM/F12 medium (GIBCO Life Technologies, Carlsbad, CA).
2. B-27 (GIBCO Life Technologies).
3. Gentamicin (GIBCO Life Technologies).
4. DMEM (Sigma Aldrich).
5. L-Glutamine (GIBCO Life Technologies).
6. HEPES buffer (MP Biomedicals, Santa Ana, CA).
7. Fetal bovine serum.
8. Anti-βIII-tubulin monoclonal antibody (1:500, Tuj1 clone; Covance, San Diego, CA).
9. Anti-glial fibrillary acidic protein (GFAP) chicken polyclonal antibody (1:300; Abcam, Cambridge, MA).
10. Anti-keratin 15 (K15) monoclonal antibody (1:100; Lab Vision, Fremont, CA).
11. Anti-smooth muscle actin (SMA) monoclonal antibody (1:200; Lab Vision).
12. Anti-cardiac troponin T monoclonal antibody (1:500; GeneTex, Hsinchu City, Taiwan).
13. Anti-desmin rabbit polyclonal antibody (1:25; Cell Signaling, Tokyo, Japan).
14. Anti-nestin monoclonal antibody (1:200, rat401 clone; Millipore, Tokyo, Japan).
15. Alexa Fluor® 568-conjugated goat anti-mouse antibody (1:400; Molecular Probes, Eugene, OR).
16. Alexa Fluor® 488-conjugated goat anti-rabbit antibody (1:400; Molecular Probes).
17. Alexa Fluor® 568-conjugated goat anti-chicken antibody (1:1000; Molecular Probes).
18. Goat anti-mouse IgG H&L phycoerythrin (1:500; Abcam).

19. Goat anti-chicken IgY biotinylated antibody (1:1000; R&D Systems, Minneapolis, MN).
20. Brilliant Violet 421™ streptavidin (1:1000; BioLegend, San Diego, CA).
21. Isoproterenol (Sigma, St. Louis, MO).
22. Propranolol (Sigma).

3 Methods

3.1 Isolation of Vibrissa Hair Follicles and Culture

1. Isolate the vibrissa hair follicles from C57BL/6 mice from the upper lip containing the vibrissa pad.
2. Dissect intact vibrissa hair follicles from the inner surface of the whisker pad under a binocular microscope by pulling them gently by the neck with fine forceps.
3. Wash the isolated vibrissa in DMEM/F12 with 50 μg/ml gentamicin. Perform all surgical procedures under a sterile environment.
4. Separate the vibrissa hair follicles into three parts (upper, middle, and lower) under the binocular microscope.
5. Suspend the separated parts vibrissa hair follicle in fresh DMEM containing 10% fetal bovine serum (FBS), 50 μg/ml gentamicin, 2 mM L-glutamine, 10 mM HEPES to promote differentiation (*see* **Note 1–3**).

3.2 Efficient Generation of HAP Stem Cells from the Upper Part of the Hair Follicle [9]

1. Culture the upper part of hair follicle in DMEM with FBS.
2. Transfer the upper part of hair follicle to DMEM/F12 without FBS after 4 weeks culture of the growing whisker, in DMEM with FBS (*see* **Notes 3–6**).

3.3 Immunofluorescence Staining of the Cells Differentiated from the Hair Follicle

1. Use the following primary antibodies: anti-βIII-tubulin monoclonal (1:500, Tuj1 clone), anti-glial fibrillary acidic protein (GFAP) chicken polyclonal (1:300), anti-keratin 15 (K15) monoclonal (1:100), anti-smooth muscle actin (SMA) monoclonal (1:200), anti-cardiac troponin T monoclonal (1:500), anti-desmin rabbit polyclonal (1:25), and anti-nestin monoclonal (1:200, rat401 clone) antibodies.
2. Use the following secondary antibodies for immunofluorescence: Alexa Fluor® 568-conjugated goat anti-mouse (1:400), Alexa Fluor® 488-conjugated goat anti-rabbit (1:400), and Alexa Fluor® 568-conjugated goat anti-chicken (1:1000) antibodies.
3. Use the following secondary antibodies for FACS: goat anti-mouse IgG H&L phycoerythrin (1:500), goat anti-chicken IgY biotinylated (1:1000). Use Brilliant Violet 421™ streptavidin (1:1000). Repeat immunofluorescence staining and FACS in triplicate.

3.4 Stimulation and Inhibition of the Beating Rate of Cardiac Muscle Cells Differentiated from HAP Stem Cells

1. Stimulate the cardiac muscle cells with 200 nM isoproterenol and observe the cells for changes in beating rate.
2. Treat the cardiac muscle cells with 200 nM of the β1-non-selective blocker propranolol (*see* **Note 6**).
3. Record cells with a video microscope camera.

4 Notes

1. Two weeks after culture, all three parts of hair follicle differentiate to troponin- and desmin-positive cardiac muscle cells (*see* Fig. 1). The number of cardiac muscle cells was significantly higher in cultures of the upper part of the hair follicle (*see* Fig. 2).
2. Fluorescence-activated cell sorting (FACS) analysis showed that all three parts of the hair follicle could produce troponin-positive cardiac muscle cells as well as βIII-tubulin-positive neurons, K15-positive keratinocytes, smooth muscle actin-

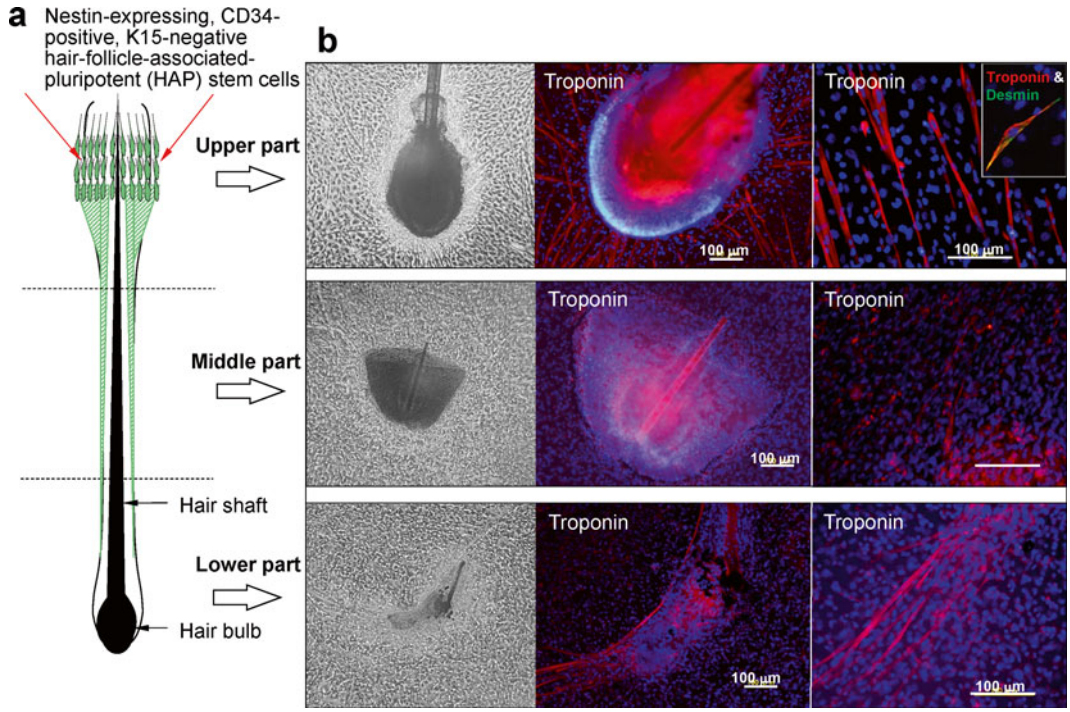

Fig. 1 (**a**) Schematic of the separated mouse vibrissa hair follicle. The *upper part* of the vibrissa hair follicle contains nestin-expressing, CD34-positive, K15-negative hair-follicle-associated-pluripotent (HAP) stem cells. (**b**) Two weeks after culture all three parts (*upper*, *middle*, and *lower*) of the hair follicle, incubated in DMEM with 10 % FBS cells differentiate to troponin- and desmin-positive cardiac muscle cells. The number of cardiac muscle cells was significantly higher in the upper part compared to the middle, and *lower part* of the hair follicle. Bars 100 μm [10]

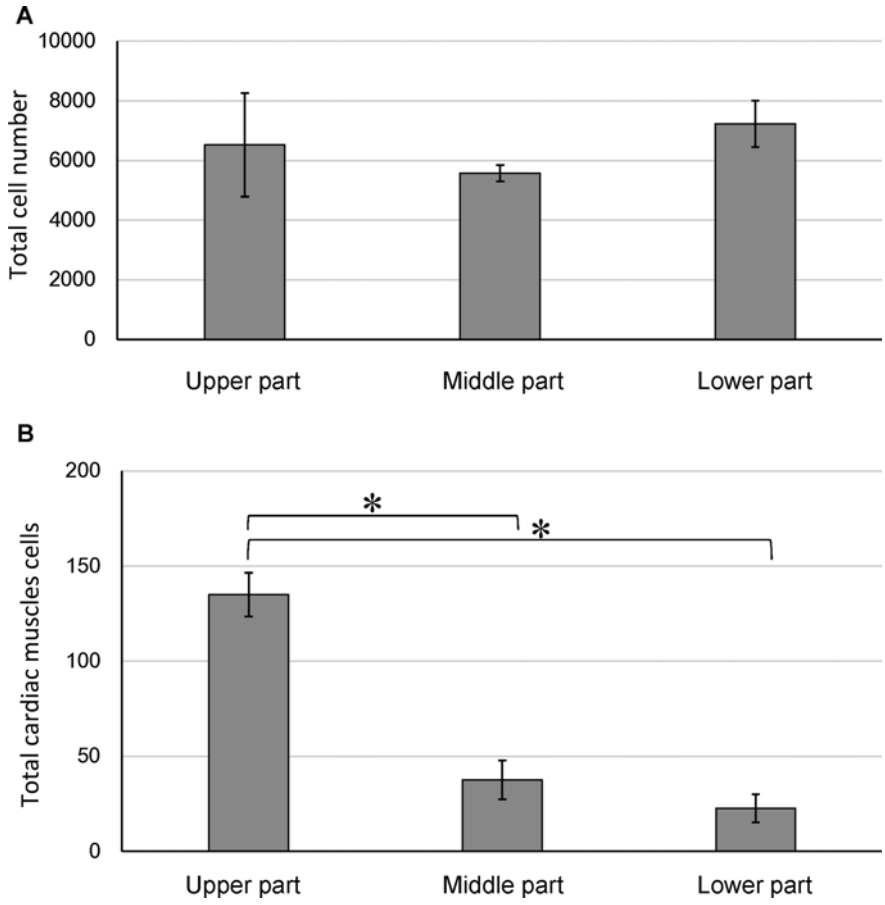

Fig. 2 (**a**) Total cell number differentiated from the *upper*, *middle*, and *lower part* of the hair follicle. (**b**) The number of cardiac muscle cells differentiated from the *upper*, *middle*, and *lower parts* of the hair follicle. $^{*}P < 0.01$ vs upper part [10]

positive cells, and GFAP-positive glial cells (*see* Fig. 3). The differentiation potential to form all these cell types was greatest in the upper part of the hair follicle compared to the middle and lower parts. The upper part of the hair follicle differentiate to neuron cells (15.4 ± 0.9%), glial cells (35.3 ± 11.3%), keratinocytes (7.9 ± 1.3%), smooth muscle cells (15.6 ± 4.6%), and cardiac muscle cells (6.9 ± 2.6%) (*see* Table 1).

3. After 1 week of culture in DMEM/F12 without FBS, the growing cells formed many HAP stem cells.
4. One week after culture in DMEM/F12 without FBS, the growing cells formed many nestin-expressing HAP stem cells containing hair spheres. Two days after transfer to DMEM with FBS, the hair spheres started to differentiate (*see* Fig. 4a–c).
5. One week after switching into DMEM containing 10% FBS, the hair spheres differentiate to nestin- and βIII-tubulin-

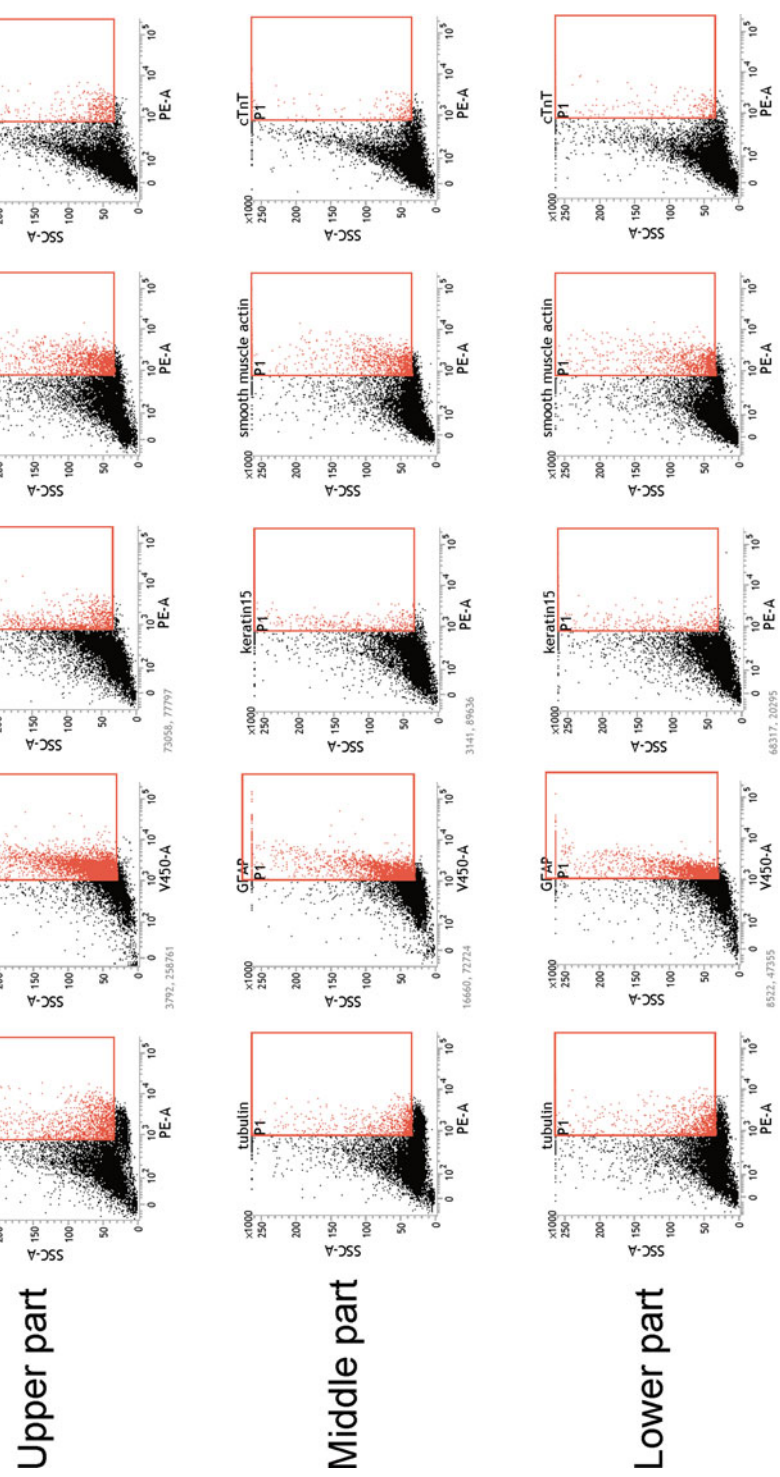

Fig. 3 Fluorescence-activated cell sorting (FACS) analysis showed that all three parts of the hair follicle differentiated to troponin-positive cardiac muscle cells, βIII-tubulin-positive neurons, K15-positive keratinocytes, smooth muscle actin-positive cells, and GFAP-positive glial cells [10]

Table 1
Percentage of cardiac muscle cells and other cell types differentiated from the separated upper, middle, and lower parts of the mouse whisker follicle [10]

	Upper part	Middle part	Lower part
Cardiac muscle cells	6.9 ± 2.6 %	4.9 ± 2.8 %	3.8 ± 1.8 %
Neurons	15.4 ± 0.9 %	10.3 ± 3.4 %	11.5 ± 2.3 %
Glial cells	35.3 ± 11.3 %	26.6 ± 3.0 %	28.4 ± 5.3 %
Keratinocytes	7.9 ± 1.3 %	5.7 ± 2.2 %	5.0 ± 1.3 %
Smooth muscle cells	15.6 ± 4.6 %	12.9 ± 0.8 %	13.7 ± 2.0 %

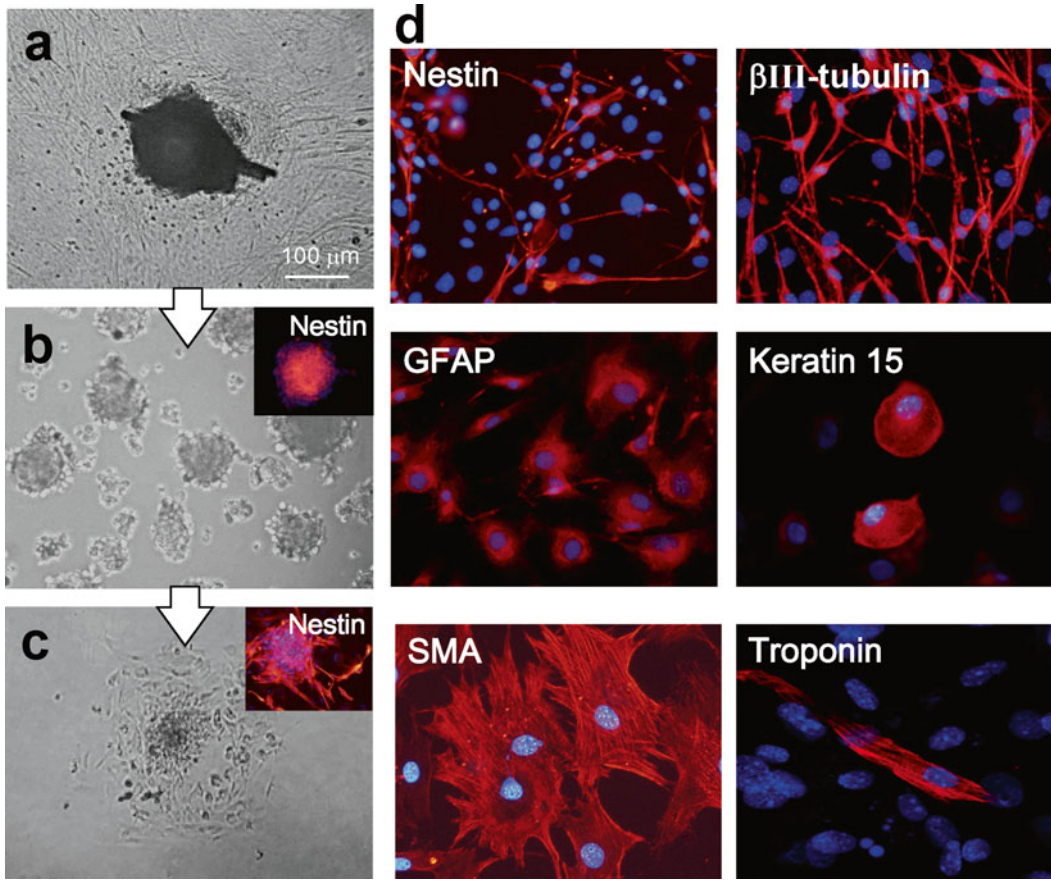

Fig. 4 (**a**) The *upper part* of hair follicle was cultured for 4 weeks in DMEM with 10 % FBS. (**b**) Cells growing out from the *upper part* of the hair follicle were transferred to DMEM/F12 without FBS. Two weeks later, the growing cells formed many nestin-expressing hair spheres. (**c**) Two days after transfer to DMEM with 10 % FBS, the hair spheres started to differentiate. (**d**) One week after switching to DMEM containing 10 % FBS, the hair spheres differentiated to troponin-positive cardiac muscle cells, nestin- and βIII-tubulin-positive neurons, GFAP-positive glial cells, K15-positive keratinocytes, and actin-positive cells [10]

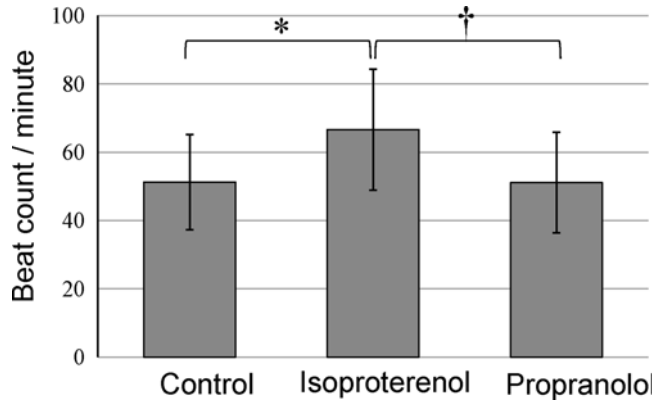

Fig. 5 Effect of isoproterenol and propranolol on the beat rate of cardiac muscle cells differentiated from the upper part of the hair follicle. $^{*}P<0.01$ vs control, $^{\dagger}P<0.01$ vs isoproterenol [10]

positive neurons, GFAP-positive glial cells, K15-positive keratinocytes, smooth muscle actin-positive smooth muscle cells, and troponin-positive cardiac muscle cells (*see* Fig. 4d).

6. The spontaneous, unstimulated beating rate of cardiac muscle cells differentiated from the whisker hair follicle had an average 51.3 ± 14.0 beats/minute ($n=10$). The spontaneous beating rate increased significantly with isoproterenol treatment ($n=10$; average 66.6 ± 17.7). Propranolol reduced the isoproterenol-induced increase in beating rate ($n=10$; average 51.1 ± 14.7) (*see* Fig. 5).

Acknowledgements

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Chapter 17

Highly Efficient Neural Differentiation of CD34-Positive Hair-Follicle-Associated Pluripotent Stem Cells Induced by Retinoic Acid and Serum-Free Medium

Mohsen Sagha and Nowruz Najafzadeh

Abstract

Neural differentiation of hair-follicle-associated pluripotent (HAP) stem cells residing in the bulge area is a promising autologous source for stem cell therapy. In the present chapter, we describe the identification and enrichment of $CD34^+$ HAP stem cells by magnetic-activated cell sorting (MACS), and induce them to differentiate into neuronal and glial cells using defined neural-induction media. The different neural cell populations arising during in vitro differentiation from HAP stem cells are characterized by reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemistry assay.

Key words Hair follicle, Stem cells, Neural differentiation, Magnetic-activated cell sorting, $CD34^+$ hair-follicle-associated-pluripotent (HAP) stem cell

1 Introduction

Hair follicle stem cells were first identified by Cotsarelis et al. [1] in the bulge area of the hair follicles. These cells are slow cycling and retain label for a relatively long time [2]. They are an easily accessible source of stem cells and play a critical role in the replenishment of hair follicles [3], epidermis [4], and sebaceous glands [5].

In 2003, Li et al. [6] discovered stem cells in the bulge are of hair follicles that express the neural stem cells marker, nestin, and suggested the relationship of the nestin-expressing stem cells of the hair follicles and the brain. The nestin-expressing stem cells of the hair follicle were termed hair-follicle-associated pluripotent (HAP) stem cells [7].

Subsequent studies have shown that HAP stem cells can differentiate into keratinocytes, neural cells [8, 9], melanocytes, Schwann cells [10], and endothelial cells [11]. The different markers expressed in HAP stem cells include nestin [9], Lgr6 [12], CD34 [13], CD200, K19 [14], as well as CD34 [2]. The neural potential of HAP stem cells was first described by Li et al. [6] and

Robert M. Hoffman (ed.), *Multipotent Stem Cells of the Hair Follicle: Methods and Protocols*, Methods in Molecular Biology, vol. 1453, DOI 10.1007/978-1-4939-3786-8_17,

subsequently by Sieber-Blum [15] and Amoh [16]. Various neural inducers such as neuregulin 1 [10], NT-3 [17, 18], glial cell-derived neurotrophic factor, brain-derived neurotrophic factor [19], and RPMI-1640 containing 10% fetal bovine serum (FBS) [20] have been used. In the present chapter, we demonstrate that CD34-expressing HAP stem cells can be efficiently isolated from mouse vibrissae follicles by high-gradient magnetic-activated cell sorting (MACS).

Recent studies have shown that using of all trans-retinoic acid (RA) [21], serum-free medium [22], and chemical treatment such as with β-mercaptoethanol (BME), butylated hydroxyanisole (BHA), and dimethyl sulfoxide (DMSO) [23] can induce neural differentiation of embryonic stem cells and bone marrow stem cells. Recently, RA was found to enhance HAP stem cells to differentiate to motor neurons [24].

Thus, we have purified $CD34^+$ HAP stem cells from the mouse hair follicle, and then the neural differentiation potential of these cells was evaluated in the presence of RA, serum-free medium, and neural differentiation medium (NDM).

We found that the serum-free medium and 1 μmol/L RA accelerate neurogenesis in the HAP stem cells and NDM induces rapid changes in cell morphology that leads to cell death.

2 Materials

2.1 Hair Follicle Stem Cells Isolation and Cultivation

1. Female mice (3–5 weeks) (Razi Institute, Karaj, Iran).
2. Trypsin-EDTA (0.05%, Invitrogen, Carlsbad, CA)
3. Dulbecco's modified Eagle's medium: Nutrient mixture F-12 1: 1 (Sigma, St. Louis, MO).
4. Fetal bovine serum (FBS, Invitrogen).
5. Growth media: 81.863 ml DMEM/F12, 10 ml FBS, 1 ml penicillin/streptomycin, 1 ml MEM non-essential amino acids, 2 ml bFGF (1000 ng), 2 ml EGF (2000 ng), 1 ml hydrocortisone (100×), 137 μl insulin, 1 ml (100×) cholera toxin.
6. Ethanol (70%).
7. Betadine.
8. Dispase (Sigma): Dissolve 10 mg dispase II in 10 PBS to prepare 1 mg/ml dispase and use freshly.
9. Stereo microscope (SZX12, Olympus, Tokyo, Japan).
10. Collagen coating: Dilute sterilely collagen type 1 solution (Sigma) to 50 μg/ml in distilled water. Add 4 ml diluted collagen to a T25 flask or 2 ml to six-well plates. Allow the collagen to bind for 2 h at room temperature or overnight at 2–8 °C. Remove excess fluid from the coated surface and allow it to dry for 2 h (*see* **Note 1**).

2.2 Magnetic-Activated Cell Sorting Components

1. Wash buffer: 1000 ml PBS containing 2 mM EDTA, pH 7.2. Add 800 ml PBS to 1-L in graduated cylinder. Weigh 292.24 g EDTA, and transfer to the cylinder. Add 200 ml of wash buffer to bring the volume up to 1 L. Store it at 4 °C (*see* **Note 2**).
2. Separation buffer: Prepare a solution containing phosphate buffer saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.2. Add approximately 1000 ml wash buffer to 1-L graduated cylinder. Weigh 0.5 g BSA and transfer to a cylinder or dilute MACS BSA stock solution (130-091-376) 1:20 with wash buffer. Filter the solution with a 0.2 pore size filter. Keep buffer cold (2–8 °C).
3. PE-conjugated rat anti-mouse CD34 monoclonal antibody (Invitrogen).
4. Anti-PE Micro Bead (Miltenyi Biotec, San Diego, CA).
5. FcR Blocking reagent to avoid Fc receptor-mediated antibody labeling.
6. MiniMACS™ Separator (Miltenyi Biotec).
7. MultiStand (Miltenyi Biotec).
8. MS columns plus tubes (Miltenyi Biotec).
9. Hemocytometer.

2.3 Neural Induction Components

1. Non-essential amino acids (100×) (Invitrogen).
2. Penicillin/streptomycin (10,000 U/ml) (Invitrogen).
3. Fibroblast growth factor-b (bFGF) (Bio Vision, Milpitas, CA, 4037-50 μg): Dissolve 50 μg of bFGF in 10 ml sterilized distilled water, store at −80 °C in 500 ng/ml aliquots.
4. Epidermal growth factor (EGF) (Bio Vision, 4022-100 μg): Dissolve 100 μg of the growth factor in 10 ml sterilized distilled water, store at −80 °C in 1000 ng/ml aliquots.
5. β-Mercaptoethanol (BME, Sigma) (14.3 M). To prepare 100 mM BME: combine 61 μl BME with 9.939 ml of sterile distilled water and store at −30 °C in 1 ml aliquots. To prepare 1 mM BME add 1 mM of 100 mM BME to 99 ml DMEM/F12 and store at 4 °C up to 2 weeks.
6. Butylated hydroxyanisole (BHA, B1253): dissolve 180.24 mg BHA in 100 ml distilled water to make a 100× stock and store at 4 °C.
7. Dimethyl sulfoxide (DMSO) (Merck).
8. KCl (Merck): dissolve 1.864 g KCl in 10 ml distilled water to prepare 2500 mM/ml.
9. Hydrocortisone (Sigma): dissolve 1 mg hydrocortisone in 1 ml ethanol, then increase its volume to 10 ml with distilled water to prepare a 100× stock solution.

10. Heparin (1 mg/ml): Dissolve 27.778 mg heparin in 10 ml PBS medium to prepare 500 U/ml, aliquot and store at −80 °C.
11. N2 supplement (Invitrogen).
12. Valproic acid (Sigma): dissolve 332.38 mg in 10 ml distilled water to prepare a 200 mM stock solution.
13. Insulin (Invitrogen): add 137 µl stock solution to 100 ml DMEM/F12 to prepare 5 µg/ml.
14. Cholera toxin (Sigma): Dissolve 10 ng/ml, then add 10 µl in 10 ml sterile distilled water to dilute the solution to 100× and store at 4 °C (*see* **Note 3**).
15. Pre-induction medium: Sterilely combine: 72 ml DMEM/F12, 20 ml FBS, 1 ml penicillin/streptomycin, 1 ml cholera toxin (100×), 1 ml MEM non-essential amino acids, 1 mM 100 mM BME, 2 ml bFGF, and 2 ml EGF. Medium can be stored at 4 °C for up to 2 weeks.
16. Neural differentiation media (NDM): sterilely combine: 82 ml DMEM/F12, 10 ml FBS, 1 ml penicillin/streptomycin, 2 ml DMSO, 1 ml BHA (100× stock solution), 1 ml heparin (500 U), 1 ml KCl (2500 µg), 1 ml valproic acid, and 1 ml N2 supplement. Medium can be stored at 4 °C for up to 2 weeks.
17. Retinoic acid (RA) (Sigma): Dissolve 3 mg RA in 1 ml DMSO (0.01 M). Aliquot 50 µl into light protected vials and store at −20 °C. Dilute each 50 µl with 4.95 ml ethanol and make subsequent dilutions in growth medium as a working solution. The working solution may be stored up to 2 weeks (*see* **Note 4**).
18. RA neural induction medium: Sterilely combine 100 µl RA working solution (100 µg), 89.9 ml DMEM/F12, and 10 ml FBS. Medium can be stored at 4 °C for up to 2 weeks.
19. Serum-free neural induction medium: Sterilely combine 84 ml DMEM/F12, 1 ml penicillin/streptomycin, 1 ml MEM non-essential amino acids, 2 ml bFGF, and 2 ml EGF. Medium can be stored at 4 °C for up to 2 weeks.

2.4 RT-PCR

1. Trizol reagent (Invitrogen).
2. RNA isolation: For extraction of RNA, use molecular-biology grade chloroform (Merck), isopropanol (Merck), DNase and RNase-free ethanol (Fluka) and diethyl pyrocarbonate (DEPC) (Sigma)-treated water.
3. DEPC-treated water (0.1%): add 1 ml DEPC to liter of the autoclaved water in a fume-hood, and shake vigorously for at least 1 h at 100 rpm to ensure that it is completely dissolved. Finally, autoclave DEPC-treated water and store at 4 °C for further use.

4. cDNA synthesis: To produce complementary DNA (cDNA) from RNA template, use the RevertAid™ Minus First-Strand cDNA Synthesis Kit (Fermentase, Burlington, ON, Canada) containing the Moloney Murine Leukemia Virus (MMlV) Reverse Transcriptase and its buffer, ribonuclease inhibitor (40 U/IU) and random hexanucleotide primers.
5. PCR: 10 mM deoxynucleotide 5′ triphosphates (dNTPs) stocks, SmarTaq DNA polymerase, and its buffer as well as $MgCl_2$ (50 mM) (CinnaGen, Tehran, Iran).
6. Electrophoresis: electrophoresis system and UV transilluminator (UVIdoc, Uvitec), molecular-biology-grade agarose (Cinnagen); ethidium bromide solution (Merck), 6× loading Dye solution (Cinnagen), DNA size marker (Genedirex, SinaClon, Tehran, Iran), $Na_2EDTA{\cdot}2H_2O$ (Sigma), NaOH (Merck), boric acid (Cinnagen) and Tris-base (Sigma).
7. EDTA solution (0.5 M) : Add 1.58 g $Na_2EDTA{\cdot}2H_2O$ and 200 mg NaOH to 8 ml ddH_2O, mix well and increase the solution volume up to 10 ml with ddH_2O and adjust to pH 8.
8. Tris-base EDTA (TBE) solution (0.5×): Add 1 ml 0.5 M EDTA, 1.375 g boric acid and 2.7 g Tris-base to 40 ml ddH_2O. Mix well and adjust the solution volume up to 50 ml.
9. Agarose gel (1.2%): add 480 mg agarose powder to 45 ml 0.5× TBE and warm in microwave to ensure that it is dissolved. Add 5 μl ethydium bromide (10 μg/ml) to the agarose solution and cool at RT.
10. DNase I (Fermentas).
11. Use the Primer3 plus program design-specific primers with 17–21 bp nucleotides long and 50–60% GC content and synthesized with Gene Ziest (Gene Ziest Yakhteh Co., Tehran, Iran). All primer sequences used are shown in Table 1.

Table 1
Primer sequences, annealing temperature (AT), length of products (bp), and number of cycles for different genes

Gene	Primer sequences (5′ → 3′)	Annealing temperature	Bp length	Cycle
β-Tubulin	F: TCACTGTGCCTGAACTTACC R:GGAACATAGCCGTAAACTGC	47	318	30
Nestin	F:TCGAGCAGGAAGTGGTAGG R: TTGGGACCAGGGACTGTTA	49	352	30
MAP2	F:CCGGAAAACCACAGCAGCAAG R:TTGGAGGAGTGCGGATGATGG	57	434	30
GFAP	F: TCCGAGACGGTGGTCA R: GTCCCTCTCCACCTCCA	49.5	375	30

2.5 Immunocytochemistry Components

1. Paraformaldehyde (4%).
2. Blocking buffer: sterilely combine: 8.100 ml PBS, 0.5 ml goat serum (Invitrogen), or 0.5 ml FcR blocking reagent, 300 μl Triton X-100 (Sigma), 100 μl BSA, and 1 ml FBS.
3. Primary antibodies: rabbit anti-MAP2 polyclonal antibody (Sigma, 1:500) and rabbit anti-GFAP polyclonal antibody (Sigma, 1:500) (*see* **Note 5**).
4. Secondary antibody: Alexa Fluor® donkey anti-rabbit IgG (H + L) (Invitrogen, 0.5 ml).
5. 4,6-Diamidino-2-phenylindole (DAPI, GERBU, Heidelberg, Germany): add 1 ml of deionized water (diH_2O) to 1 mg DAPI to make a stock solution (*see* **Note 6**). Add 100 μl of the stock solution to 100 ml PBS to make a 1 μg DAPI stain solution.

3 Methods

3.1 Cell Culture

1. Sterilize the whisker pads with 70% alcohol and betadine.
2. Cut the upper lip and divide into small pieces, then place in 10 ml DMEM/F12 containing 10% penicillin/streptomycin and 0.5 μg/ml amphotericin B.
3. Incubate the tissue pieces in 1 mg/ml dispase II at room temperature for 30 min.
4. After two rinses in DMEM/F12, remove connective tissues around the follicles.
5. Lift out the vibrissa follicles under a stereo microscope.
6. Isolate the bulge region from the hair follicle by making two transverse sections above and below the region. Cut bulge regions into small pieces and add 2 ml trypsin/EDTA, incubate for 10 min.
7. After two washes with PBS, cultivate the bulge pieces in growth medium on collagen type 1-coated plates.

3.2 Separation of CD34-Positive Cells by Magnetic-Activated Cell Sorting (MACS)

Work rapidly, keep cells cold, and use pre-cooled solutions. Wash cells in the culture flask with PBS (*see* **Note 7**).

1. Isolate monolayer cells with 1.5 ml pre-warmed trypsin/EDTA until cells are detached.
2. Pass cells through 30 μm sterile CellTrics® filters in order to remove bulge pieces.
3. Centrifuge the cell suspension at $300 \times g$ for 10 min. Aspirate supernatant and determine the cell number, then wash cells with 10 ml wash buffer.
4. Resuspend 1×10^6 cell pellet in a final volume of 100 μl separation buffer containing 10 μl anti-CD34 primary antibody and

incubate for 20 min at 4–8 °C; then wash cells by adding 10 ml wash buffer and centrifuge at 300×*g* for 10 min (*see* **Note 8**).

5. Resuspend the cell pellet in 80 µl separation buffer and 20 µl anti-PE Micro Beads per 10^6 total cells. Mix well and incubate for 20 min at 4–8 °C.
6. Wash cells by adding 10 ml wash buffer, resuspend in 500 µl separation buffer and proceed to magnetic separation.
7. Place the column in the magnetic field of a mini MACS Separator. Prepare the column by rinsing with 500 µl wash buffer.
8. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells. Wash column with 1.5 ml wash buffer (*see* **Note 9**).
9. Remove MS column from the mini MACS separator and place it on a 15 ml conical tube.
10. Add 1 ml wash buffer onto the MS column and count the cell number. Immediately flush out the $CD34^+$ cells by pushing the plunger into the column (*see* **Note 10**).

3.3 Neural Induction Protocols

A different method was used for differentiating neural cells from HAP stem cells. Mouse HAP stem cells were maintained in growth medium.

3.3.1 NDM

1. Check that the cells have reached 70% confluence; wash the cells once with PBS.
2. Replace the medium with pre-induction medium consisting of DMEM/20% FBS/1 mM β-mercaptoethanol (BME), 10 ng/ml bFGF, 20 ng/ml EGF, and 10^{-9} M cholera toxin for 24 h.
3. Remove the pre-induction medium and wash the cells with PBS.
4. Use neuronal differentiation medium containing DMEM/F12, 10% FBS, 2% DMSO, 100 µM BHA, 5 U/ml heparin, 25 mM KCl, 2 mM valproic acid, and 1× N2 supplement, for 14 days.
5. Change neural induction medium every 3 days and allow the cells to differentiate for 14 days and process for immunocytochemistry.

3.3.2 RA Neural Induction

1. Wash the cells with PBS.
2. Replace the growth medium with neural induction medium containing DMEM/F12, 10% FBS, and 1 µM RA. Medium should be changed every 48 h.

3.3.3 Serum-Free Neural Induction

1. Wash the cells with PBS.
2. Replace the growth medium with serum-free medium containing 20 ng EGF, 10 ng bFGF, 1% N2 supplement, and 1% penicillin streptomycin. Medium should be changed every 48 h.

3.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

3.4.1 RNA Isolation

Since RNA is very sensitive to degradation by RNases, it is necessary that all the following steps for RNA extraction be performed with sterile filter tips.

1. Remove medium and wash the cultured cells with sterile PBS twice.
2. Add 500 µl Trizol to the cells and pass the resulting cell lysate several times through a pipette and collect the lysate into a 1.5 ml Eppendorf tube; store at −80 °C for further use.
3. Vortex tubes containing samples gently for 30 s and put the samples on ice for 5 min.
4. Add 100 µl chloroform to each tube and shake tubes gently by hand for 20 s. Then, incubate the tubes at room temperature for 2–3 min.
5. Centrifuge the samples at 12,000 × *g* for 15 min at 4 °C.
6. Take colorless upper aqueous supernatant and transfer it to a fresh Eppendorf tube.
7. Add one volume chloroform, vortex for 30 s and centrifuge at 4 °C for 5 min at 12,000 × *g*.
8. Transfer the supernatant into a new Eppendorf tube and add one volume isopropanol and mix well by hand.
9. Precipitate RNA at −20 °C for 30 min to 33 h and centrifuge at 12,000 × *g* for 30 min at 4 °C.
10. Discard supernatant and save the pellet (*see* **Note 11**).
11. Add at least 400 µl 75% Ethanol (dissolved in RNase-free water) to the RNA pellet and mix well, then centrifuge at 7500 × *g* for 5 min at 4 °C.
12. Remove the ethanol, and dry the pellet at 37 °C (*see* **Note 12**).
13. Resuspend the pellet in 20–30 µl DEPC-treated water and warm to 55–60 °C for 10 min.
14. Calculate the RNA concentration with Nanodrop kit, dissolve the RNA to obtain a ratio of $A_{260/280}$ ratio > 1.6. Use it for DNase treatment or store at −80 °C for further use (Table 1).

3.4.2 DNase Treatment

To ensure that the extracted RNA is free from any DNA contamination, the following steps are suggested:

1. Mix 1 µg mRNA, 1 µl DNase I enzyme (1 U/µl), 1 µl $MgCl_2$ in DEPC-treated water (to 10 µl) in 0.5 ml PCR tubes (*see* **Note 13**).
2. Incubate the tubes at 37 °C for 30 min.
3. Add 1 µl 50 mM EDTA and heat-inactivate at 65 °C for 10 min.

3.4.3 cDNA Synthesis

To generate complementary DNA, the following steps should be performed for the reverse transcription of DNase-treated RNA in a final 40 µl reaction solution.

1. Prepare a 20 µl RNA-primer mixture in a 0.2 ml microcentrifuge tube. Mix 1 µg RNA, 2 µl random hexamer primer, and 2 µl dNTP mix (10 mM) in sterile water. Spin tube by hand and incubate at 65 °C for 5 min and then chill on ice for 2 min.
2. Prepare a cDNA-synthesis mixture containing 4 µl RT-buffer (5×), 1 µl reverse transcriptase (RT) in 15 µl sterile water.
3. Add a cDNA-synthesis mix into the RNA-primer mixture and incubate at 25 °C for 10 min and then at 42 °C for 60 min to perform the reverse-transcription reaction.
4. Incubate the tube at 85 °C for 5 min to neutralize the RT enzyme reaction. Use cDNA in the PCR reaction mixture or store at −20 °C for further use (*see* **Note 14**).

3.4.4 PCR Reaction

For PCR of different genes, dilute all forward and reverse primers up to 10 pmol/L.

1. Add 30–50 ng template cDNA to the PCR solution containing ddH_2O, 10× PCR buffer, $MgCl_2$ (50 mM), dNTP mix (10 mM), primers (F + R), and SmarTaq DNA polymerase (5 U/µl) in a final volume of 25 µl. Perform PCR according to the following program: initial denaturation (94 °C for 5 min); denaturation in each cycle (94 °C for 45 s); annealing temperature (*see* Table 1), extension (72 °C for 45 s), and final extension (72 °C for 10 min). In the negative control, add ddH_2O instead of cDNA.
2. Add 1 µl loading dye to 10 µl sample and place in an electrophoresis system. Visualize and photograph the resultant bands with a UVIdoc system (*see* Fig. 1a).

3.5 Immunocytochemistry

1. Grow $CD34^+$ cells on collagen-coated six-well plates with growth medium. Replace the medium with fresh neural-induction medium.
2. Remove the culture medium and carefully wash cultures 3× with PBS.
3. Fix cells in 4% paraformaldehyde (pH 7.4) for 15 min at room temperature, wash three times with PBS, and incubate with blocking buffer for 30 min at room temperature.
4. Incubate cells overnight at 4 °C with primary antibodies (*see* **Note 15**).
5. Rinse the cells for 3 × 5 min in PBS to remove unbound primary antibodies. Dilute the secondary antibody in blocking buffer and apply for 2 h at room temperature.

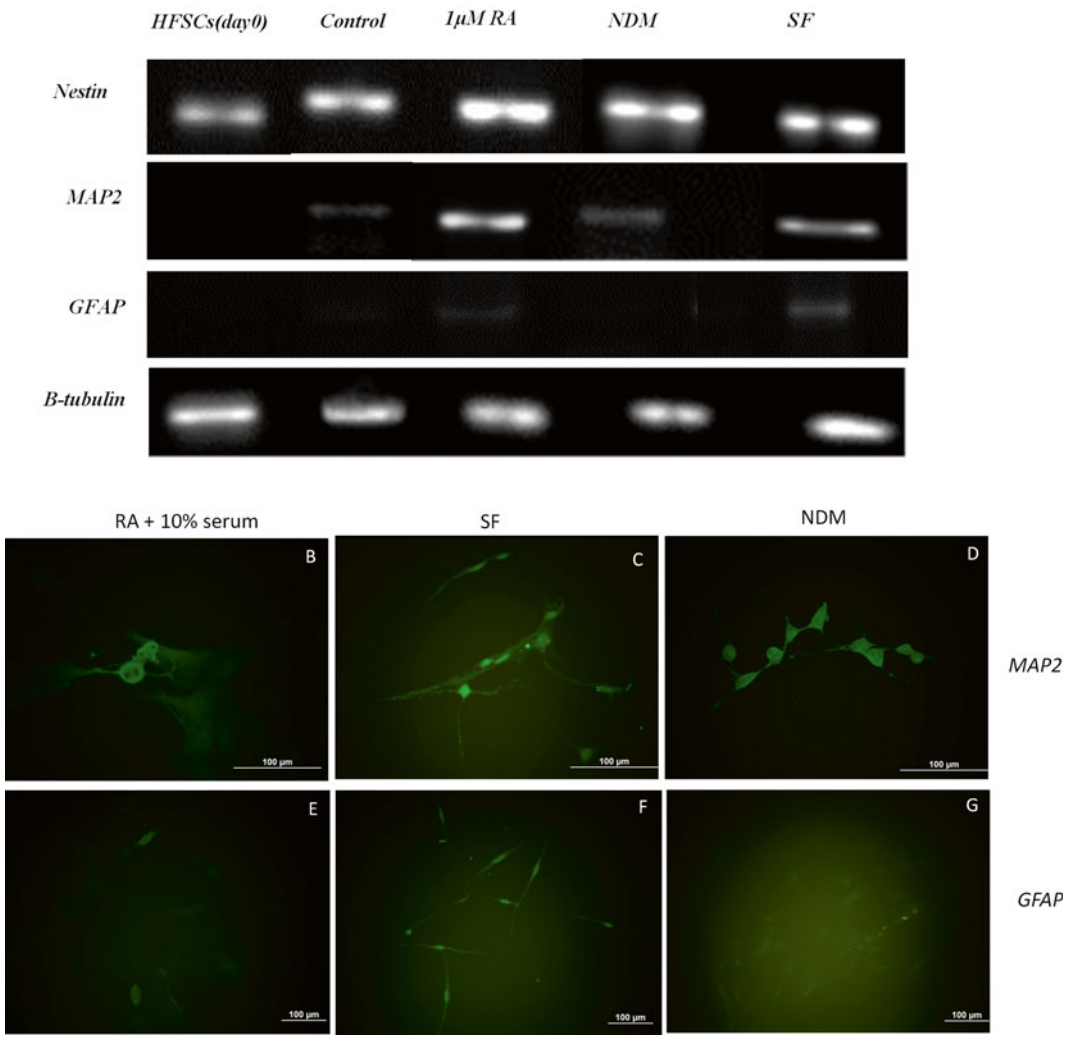

Fig. 1 Expression of neural (nestin and MAP2) genes increased in cells treated with 1 μM RA + 10 % serum, serum-free protocols, and NDM. Moreover, astrocyte differentiation was increased in serum-free medium (**a**). Images (**b**)–(**d**) showing increased MAP2 immunostaining in a week after neural induction in all the treated groups. Images (**e**)–(**g**) showing GFAP expression in the differentiated cells. Intriguingly, more GFAP positive glial cells were seen in the serum free-treated group (**f**). SF = serum free

6. After two washes, stain the cells with DAPI and incubate for 5 min in dark.
7. Wash two times with PBS and observe the cells with a fluorescence microscope (*see* Fig. 1b–g).

4 Notes

1. The coated plates may be stored at 4 °C for up to a month. Rinse the coated plates with sterile PBS or DMEM before cell culture, since the pH of coated surfaces is acidic, which is not suitable for the cell culture.

2. EDTA does not readily dissolve unless the pH is approximately 8. Diluted NaOH (1 N) can be used to adjust the pH to approximately 8 and then turn on a stir plate to mix the EDTA into solution. Adjust pH with HCl and sterilize by filtering through a 0.2 μm pore-size filtration unit.
3. In our experience, cholera toxin is useful in stimulating colony growth, which increases cyclic AMP in cultured keratinocytes.
4. RA solution is sensitive to light, air, and oxidizing agents. It is recommended to use all the stock solution immediately after opening the vials.
5. Primary antibodies are diluted in blocking buffer and secondary antibody is prepared in PBS containing 1 % BSA.
6. To dissolve DAPI sonicate for 1 min. The 1 mg/ml DAPI stock solution is divided in 100 μl aliquots and can be stored at −20 °C for up to 6 months.
7. All buffers and media used contain 1 % antibiotic-antimycotic solution (100 U/ml penicillin and 100 μg/ml streptomycin; 0.5 μg/ml amphotericin B) to avoid possible contamination from dissection. To prevent capping of antibodies on the cell surface and nonspecific cell labeling, carry out all procedures rapidly and keep cells cold.
8. For less than 1×10^6 total cells, use 90 μl separation buffer and 10 μl primary antibody. For 1×10^6–1×10^8 cells use 300 μl separation buffer.
9. Perform washing steps by adding 3×500 μl wash buffer only when the column reservoir is empty.
10. To increase the purity of CD 34^+ cells, repeat the magnetic separation procedure as described in Subheading 3.2, **steps 7–10** with a new MS column.
11. RNA precipitate is sometimes observed in the side or bottom of the tube. If so, it is better to remove the supernatant from the opposite wall.
12. It should be noted that the pellet should not be dried completely, since the extracted RNA may be degraded or its solubility will greatly decrease.
13. DNase I is sensitive to physical denaturing. Therefore, do not vortex the DNase.
14. To ensure that reverse transcription has been carried out, one group should be considered as a negative control (no RT enzyme).
15. Longer incubations at 4 °C may be better to increase specific binding and may be useful for very dilute primary antibodies.

Acknowledgments

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Chapter 18

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

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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

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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 Immunohistochemistry 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

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

3.5.1 RT-PCR

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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