Chapter 28

Isolation and Culture of Hair Follicle Pluripotent Stem (hfPS) Cells and Their Use for Nerve and Spinal Cord Regeneration

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Abstract

The hair follicle is dynamic, cycling between growth (anagen), regression (catagen), and resting (telogen) phases throughout life. We have demonstrated that nestin-expressing hair follicle stem cells give rise to follicle structures during early anagen or growth phase of the hair follicle. Nestin-expressing hair follicle stem cells appear in the hair follicular stem cell area, the permanent upper hair follicle immediately below the sebaceous glands and above the bulge area. The nestin-expressing hair follicle stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro. Furthermore, the hair follicle stem cells promote the recovery of peripheral nerve and spinal cord injury. We have termed these cells hair follicle pluripotent stem (hfPS) cells. These results suggest that hfPS cells provide an important accessible, autologous source of adult stem cells with potential for use in regenerative medicine.

Key words: Hair follicle, Nestin, Stem cell, Hair follicular stem cell area, Differentiation, Neuron, Glial cell, Melanocyte.

1. Introduction

The hair follicle bulge area has an abundance of, easily accessible actively-growing, pluripotent adult stem cells. Nestin, a protein marker for neural stem cells, also is expressed in follicle stem cells and their immediate, differentiated progeny (1). The fluorescent protein GFP, whose expression is driven by the nestin regulatory element in transgenic mice, can be used to image the fate of the hair follicle stem cells. The pluripotent nestin-driven GFP stem cells are positive for the stem cell marker CD34 but negative for keratinocyte marker keratin 15, suggesting their relatively undifferentiated state. These cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells,

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and melanocytes in vitro (2). In vivo studies show the nestin-driven GFP hair follicle stem cells can differentiate into blood vessels and neural tissue after transplantation to the subcutis of nude mice (3).

Equivalent hair follicle stem cells, derived from transgenic mice with beta-actin-driven GFP, implanted into the gap region of a severed sciatic nerve greatly enhance the rate of nerve regeneration and the restoration of nerve function. The follicle cells transdifferentiate largely into Schwann cells, which are known to support neuron regrowth. Function of the rejoined sciatic nerve was measured by contraction of the gastrocnemius muscle upon electrical stimulation. After severing the tibial nerve and subsequent transplantation of hair follicle stem cells, walking print length and intermediate toe spread significantly recovered, indicating that the transplanted mice recovered the ability to walk normally (4).

Hair follicle stem cells were also transplanted to the severed spinal cord in mice. Most of the transplanted cells also differentiated into Schwann cells that apparently facilitated repair of the severed spinal cord. The rejoined spinal cord re-established extensive hind-limb locomotor performance. These results suggest that hair follicle stem cells can promote the recovery of spinal cord injury. Thus, hair follicle stem cells provide an effective accessible, autologous source of stem cells for the promising treatment of peripheral nerve and spinal cord injury (5).

In the mouse, nestin-expressing stem cells are located in the upper hair follicle immediately below the sebaceous glands just above the hair follicle bulge area. The nestin-expressing stem cells are K15-negative. Nestin-negative, K15-positive cells, on the other hand, are located in the bulge area of the mouse hair follicle and can differentiate only to keratinocytes (6).

Analogous to the mouse, in the intact human hair follicle dissected from the scalp, the cells immediately below the sebaceous glands just above the bulge area are nestin-positive and K15-negative. In contrast, the hair follicle stem cells in the bulge are nestin-negative, K15-positive (6).

Thus, the hair follicles of mice and men appear to have two populations of stem cells: a pluripotent type and an apparent unipotent type. These stem cells have potential for regenerative medicine and hair growth, respectively. Our results suggest that, the hair follicle stem cells have clinical potential as adult stem cells due to easy access and capability for differentiation (6). We have termed these stem cells hair follicle pluripotent stem (hfPS) cells (12).

2. Materials

2.1. Reagents

1. Transgenic mice with nestin-regulatory-element-driven green fluorescent protein (ND-GFP mice) (7)

2. GFP-expressing transgenic mice (GFP mice) (Jackson Laboratories) (8); C57BL/6 mice and C57BL *nu/nu* mice (e.g., Charles River Labs, Wilmington, MA, Taconic Labs Germantown, NY or Harlan Teklad, Los Angeles, CA)
3. DMEM-F12 (GIBCO-BRL, Grand Island, NY) containing B-27 (GIBCO-BRL) and 1% penicillin–streptomycin (GIBCO-BRL) with 1% methylcellulose (Sigma-Aldrich St. Louis, MO). The culture was supplemented every 2 days with basic FGF at 20 ng/ml (Chemicon, Temecula, CA)
4. RPMI medium 1640 (Cellgro, Herndon, VA) containing 10% FBS
5. 96-well uncoated tissue-culture dishes (BD Biosciences, San Jose, CA)
6. SonicSeal four-well chamber slides (Nunc)
7. Anti-βIII-tubulin mAb (1:500, Tuj1 clone; Covance Research Products, Berkeley, CA)
8. Anti-neurofilament 200 polyclonal Ab (1:80; Sigma-Aldrich)
9. Anti-GABA polyclonal Ab (1:200; Chemicon)
10. Anti-neuronal-specific enolase mAb (1:800; Lab Vision, Fremont, CA)
11. Anti-tyrosine hydroxylase polyclonal Ab (1:100; Chemicon)
12. Anti-glial fibrillary acidic protein (GFAP) mAb (1:100; Molecular Probes, Invitrogen, Carlsbad, CA)
13. Anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) mAb (1:50; Lab Vision)
14. Anti-keratin 5/8 (K5/8) mAb (1:250; Chemicon)
15. Anti-keratin 15 (K15) mAb (1:100; Lab Vision)
16. Anti-smooth muscle actin mAb (1:200; Lab Vision)
17. Anti-BrdUrd mAb (1:10; BD PharMingen, San Jose, CA)
18. Anti-CD31 mAb (1:50; Chemicon)
19. Anti-CD34 mAb (1:10; BD PharMingen)
20. The following are 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)
21. Mouse-on-Mouse (MOM) immunodetection kit (Vector Laboratories, Burlingame, CA)
22. Ig horseradish peroxidase detection kit (BD PharMingen)

2.2. Equipment

1. OV-100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan)

2. IMT-2 inverted microscope equipped with a mercury lamp power supply (Olympus)
3. Hamamatsu C5810 three-chip cooled color CCD camera (Hamamatsu Photonics Systems, Hamamatsu-city, Japan)
4. FluorVivo Imaging System (Indec Systems, Santa Clara, CA) iBox Imaging System (UVP LLC, Upland, CA)
5. Sony VCR model SLV-R1000 (Sony)
6. Image Pro Plus 3.1 software (Media Cybernetics, Bethesda, MA)
7. 1 ml 27G2 latex-free syringe (BD Bioscience)
8. 25-ml Hamilton syringe (Fisher Scientific, Santa Ana, CA)
9. D470/40 excitation filter (Chroma Technology, Rockingham, VT)
10. GG475 emission filter (Chroma Technology)
11. Cloning cylinders (Bel-Art Products, Pequannock, NJ)
12. Hemocytometer (Reichert Scientific Instruments)
13. Blunt-end hook (Fine Science Tools, Foster City, CA)
14. 33-gauge needle (Fine Science Tools)

2.3. Equipment Setup

2.3.1. Whole-Body Imaging Equipment

The Olympus OV-100 small animal imaging system (Olympus), 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 magnifications (9). The optics of the OV100 fluorescence imaging system have been specially developed for macro-imaging as well as micro-imaging with high light gathering capacity. The instrument incorporates a unique combination of high numerical aperture and long working distance. Four individually optimized objective lenses, parcentered and parfocal, provide a 10^5-fold magnification range for seamless imaging of the entire body down to the subcellular level without disturbing the animal. The OV100 has lenses mounted on an automated turret with a high magnification range of $\times 1.6$ to $\times 16$ and a field of view ranging from 6.9 to 0.69 mm. The optics and antireflective coatings ensure optimal imaging of multiplexed fluorescent reporters in small animals. High-resolution images are captured directly on a PC (Fujitsu Siemens). Images are processed for contrast and brightness and analyzed with the use of Paint Shop Pro 8 (Corel) and Cell (Olympus Biosystems).

Many other fluorescence imaging systems can also be used. For example, a Leica fluorescence stereo microscope (model MZ16) equipped with a mercury 50 W lamp power supply can be used. Selective excitation of GFP is produced through a D425/60 band pass filter and 470 DCXR dichroic mirror. Emitted fluorescence is collected through a longpass filter (GG475; Chroma Technology). Under anesthesia, the experimental animals can be examined with the microscope and the images can be

acquired with a Hamamatsu C5810 three-chip cooled color charge-coupled-device camera (Hamamatsu Photonics Systems). Images can also be processed for contrast and brightness and analyzed with the use of Image-Pro Plus software (Media Cybernetics). High-resolution images of 1,024 × 724 pixels can be captured directly on a PC or continuously through video output on a high-resolution VCR (e.g., Sony model SLV-R1000).

Alternatively, the FluorVivo Imaging System (Indec Systems) can be used with any type of CCD camera. The FluorVivo has its own measurement software. FluorVivo uses extremely bright, solid state, LED illuminators and a full color CCD camera to provide high-speed, multi-color imaging of up to three animals with single exposures. The instrument's high speed acquisition permits *in vivo* monitoring of both static and dynamic processes, as well as real-time recordings of fluorescence-guided surgeries. FluorVivo's fully integrated software provides complete control of the instrument, ease of use, and powerful analytical tools for extracting quantitative data from acquired images.

The UVP iBox Small Animal Imaging System (UVP LLC) is capable of fluorescent protein imaging with a range of cameras that use front and back illuminated CCDs with sizes up to a 43 mm diagonal, greatly expanding the applications for high resolution, large-field-of-view and increased-throughput imaging. The iBox imaging system can be configured with both monochrome and color CCDs, with CCD resolution currently up to 8.3 megapixels and sensitive to a wide range of spectrum (CFP to near infrared). The range of fast lenses includes several interchangeable, fully automated optics: a 50 mm f1.2, 28 mm f 1.8, and a 24 -70 mm f2.8 zoom lens. These lenses give maximum imaging flexibility, with the field of view ranging from one to several animals. At f1.2, the typical exposures are less than 50 msec, minimizing the effect of animal movement. The camera, optics, sample platform position, and excitation and emission filters are under full software control, permitting reproducible and rapid imaging with software presets and macros.

Please refer to **Note 1** when using any of the imaging systems.

3. Methods

3.1. Isolation of Mouse Nestin-Expressing Pluripotent Hair Follicle Stem Cells from the Hair Follicle Stem Cell Area

1. Isolate ND-GFP cells from the hair follicle stem cell area. To isolate the vibrissa follicles, cut the upper lip containing the vibrissa pad of ND-GFP mice and expose its inner surface. 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 (GIBCO-BRL) containing B-27 (GIBCO-BRL) and 1%

penicillin–streptomycin (GIBCO-BRL). Do all surgical procedures under a sterile environment. Please refer to **Notes 2–4** when performing the surgical procedures. The hair follicle stem cell area contains ND-GFP-expressing cells. Isolate the cells under fluorescence microscopy. Suspend the isolated cells in 1 ml DMEM-F12 containing B-27 with 1% methylcellulose (Sigma-Aldrich), and 20 ng ml1 basic FGF (bFGF) (Chemicon) (3, 10). Culture the cells in 24-well tissue-culture dishes (Corning) 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 colonies. Please refer to **Notes 5–8**.

2. For differentiation, centrifuge the colonies to remove growth factor-containing DMEM-F12 medium. Resuspend the cells into fresh RPMI medium 1640 (Cellgro, Herndon, VA) containing 10% FBS. Culture colonies in SonicSeal four-well chamber slides (Nunc). Please refer to **Notes 9 and 10**.
3. Clone cells from ND-GFP cell colonies that had been cultured for 2 months by trypsinizating and serially diluting cells into DMEM-F12 containing B-27 in 96-well uncoated tissue-culture dishes (BD Biosciences). Assess each chamber microscopically for the presence of a single cell. 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 colonies to RPMI medium 1640 containing 10% FBS in SonicSeal four-well chamber slides for differentiation. Label ND-GFP cells with BrdUrd for 7 days. Immunostain the cells for anti-βIII-tubulin and anti-BrdUrd mAbs.
4. Isolate ND-GFP-expressing hair follicles from telogen dorsal skin pelage hair follicles under fluorescence microscopy. Wash the follicles in DMEM-F12 containing B-27 and 1% penicillin–streptomycin. Suspend the ND-GFP-expressing hair follicle stem cells in 1 ml DMEM-F12 containing B-27 and 20 ng/ml bFGF. Culture the cells in 24-well tissue-culture dishes at 37°C in 5% CO_2 95% air. The cells form ND-GFP colonies by 4 weeks. For differentiation, transfer the ND-GFP colonies to SonicSeal four-well chamber slides, where the colonies are resuspended in fresh RPMI medium 1640 containing 10% FBS. Transfer ND-GFP colonies to SonicSeal four-well chamber slides in the presence of DMEM-F12 containing B-27 and 20 ng ml bFGF. Please refer to **Notes 9 and 10**.
5. For immunohistochemistry use the following primary Abs: anti-βIII-tubulin mAb (1:500, Tuj1 clone; Covance Research Products, Berkeley, CA); anti-neurofilament 200 polyclonal Ab (1:80; Sigma-Aldrich); anti-GABA polyclonal Ab (1:200; Chemicon); anti-neuronal-specific enolase mAb (1:800; Lab Vision, Fremont, CA); anti-tyrosine hydroxylase polyclonal Ab (1:100; Chemicon); anti-GFAP mAb (1:100; Molecular

Probes); anti-CNPase mAb (1:50; Lab Vision); anti-keratin 5/8 (K5/8) mAb (1:250; Chemicon); anti-keratin 15 (K15) mAb (1:100; Lab Vision); anti-smooth muscle actin mAb (1:200; Lab Vision); anti-BrdUrd mAb (1:10; BD PharMingen); anti-CD31 mAb (1:50; Chemicon); and anti-CD34 mAb (1:10; BD PharMingen). The following secondary Abs were used: 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). For BrdUrd immunocytochemistry, treat cells as described above. For quantification of the percentage of cells producing a given marker protein, photograph at least three fields in any given experiment and determine the number of positive cells relative to the total number of cells. Immunocytochemical staining of βIII-tubulin and K15 in the ND-GFP cells is detected with the Mouse-on-Mouse (MOM) immunodetection kit (Vector Laboratories). CD31 and CD34 are detected with the Ig horseradish peroxidase detection kit (BD PharMingen).

3.2. Sciatic Nerve Regeneration Using Hair Follicle Stem Cells

1. Prepare GFP-expressing hair follicle stem cells as described above, but in this case, using C57BL/6 GFP mice.
2. Differentiate hair follicle stem cells as described above.
3. Transplant differentiated GFP-expressing hair follicle stem cell colonies between severed sciatic or tibial nerve fragments in immunocompetent C57BL6 mice under tribromoethanol anesthesia. The skin incision is closed with nylon sutures (6–0). After 2 months, directly observe the sciatic nerve of the transplanted mouse by fluorescence microscopy under anesthesia.
4. Embed the sciatic nerve samples in tissue-freezing embedding medium and keep at –80°C overnight. Cut frozen sections 5 μm thick with a Leica CM1850 cryostat and air-dry. Observe the sections under fluorescence microscopy.
5. Use the frozen sections for the immunofluorescence staining of βIII-tubulin, glial fibrillary acidic protein, K15, and smooth muscle actin as described above. For quantification of the percentage of cells producing a given marker protein in any given experiment, photograph at least three fields and determine the number of positive cells relative to the total number of cells.
6. Observe the sciatic nerve in the live mouse and the excised sciatic nerve directly under an Olympus IMT-2 inverted microscope or equivalent equipped with a mercury lamp power supply. The microscope needs a GFP filter set (Chroma Technology, Brattleboro, VT).

7. Stimulate the sciatic nerve with an electric stimulator. Use an electric stimulator (FGK-1S, Medical Access, Tokyo) that can deliver repetitious electric pulses of 0.05 mA at 10 Hz with pulse widths of 0.5 msec to stimulate control mice, mice with severed sciatic nerves and mice that had GFP hair follicle stem cells injected to join the severed nerve.
8. Measure the gastrocnemius muscle lengths and calculate the difference of the gastrocnemius muscle lengths (from lateral epicondyle of femur to heel) before and after contraction by the electric stimulator. Each experimental group consists of 10 mice.
9. Obtain walking tracks by using a corridor open at one end to a darkened compartment. The animal's feet are soaked in Higgins7 black waterproof ink (Sanford, Bellwood, IL), and the animal is allowed to walk multiple times to obtain measurable prints. Evaluate the tracks for print length and intermediate toe spread.
10. Perform multiple linear regression analysis with values derived from each of the parameters. Values are derived for each parameter by subtracting the normal value (normal left side) from the hair follicle stem cell-transplanted value (experimental right side) and dividing by the normal value. Each experimental group consists of seven mice. Groups include control mice, mice with a severed tibial nerve only, and mice with the tibial nerve enjoined by injected hair follicle stem cells. PL is the print length, NPL is the normal left print length, EPL is the experimental right print length, IT is the intermediate toe spread, NIT is the normal left intermediate toe spread, and EIT is the experimental right intermediate toe spread.
11. Express the experimental data as the mean ± SD. Perform statistical analysis by using a two-tailed Student's *t*-test.

Regarding repair of peripheral nerve injury by hair follicle stem cells, refer to **Note 11**.

3.3. Spinal Cord Regeneration Using Hair Follicle Stem Cells

1. Transplant differentiated GFP-expressing stem cell colonies to the thoracic region of the spinal cord in C57BL/6 immunocompetent mice. Perform a laminectomy at the 10th thoracic spinal vertebrae, followed by transversal cut using a binocular microscope. Transplant the GFP-expressing stem cells between the severed thoracic region (spinal level T10) of the spinal cord in C57BL/6 immunocompetent mice.
   a. After 2 months, directly observe the spinal cord of the transplanted mice by fluorescence microscopy under anesthesia. Excise spinal cord samples of the transplanted mice under anesthesia. Embed the spinal cord samples in tissue freezing embedding medium and freeze at –80°C overnight.
   b. Cut frozen sections 5 μm thick with a Leica CM1850 cryostat and air-dry. Directly observe the sections by fluorescence microscopy that are then used for immunofluoresence

stain with βIII-tubulin, GFAP, CNPase, K15, and SMA. Use the following primary antibodies: anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; Covance Research Products, Inc., Berkeley, CA), anti-glial fibrillary acidic protein (GFAP) monoclonal (1:200; Lab Vision, Fremont, CA), anti-2′-3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) monoclonal (1:50; Lab Vision), anti-K15 monoclonal (1:100; Lab Vision), and anti-smooth muscle actin (SMA) monoclonal (1:200; Lab Vision). Use the following secondary antibodies: Alexa Fluor® 568 goat anti-mouse (1:200; Molecular Probes, Eugene, OR) or Alexa Fluor® 568-conjugated goat anti-rabbit (1:200; Molecular Probes). For quantification of the percentage of cells producing a given marker protein, photograph at least three fields and determine the number of positive cells relative to the total number of GFP-expressing cells.

2. Transplant differentiated GFP-expressing stem cell colonies to the lumbar region of the spinal cord in C57BL/6 immunocompetent mice. Using a binocular microscope, perform a laminectomy at the second lumbar spinal vertebrae, followed by a transversal cut. Transplant the GFP-expressing stem cell colonies between the severed lumbar region (spinal level L2) of the spinal cord in C57BL/6 immunocompetent mice.
3. Evaluate the regeneration efficacy of GFP-expressing stem cells transplanted in the severed thoracic and lumbar regions of the spinal cord. Conduct performance analyses for 12 weeks using the Basso–Beattie–Bresnahan (BBB) locomotor rating scale (11). Each group consists of five to seven mice.
4. Observe the spinal cord in the live mouse with transplanted GFP-expressing hair follicle stem cells using an Olympus IMT-2 inverted microscope equipped with a mercury lamp power supply. The microscope has a GFP filter set.
5. Express the experimental data as the mean ± SD. Perform statistical analysis using the two-tailed Student's *t*-test (5).

Regarding repair of spinal cord injury by hair follicle stem cells, refer to **Note 12**.

3.4. Isolation of Human Nestin-Expressing Pluripotent Hair Follicle Stem Cells from the Hair Follicle Stem Cell Area

3.4.1. Culture of Whole and Plucked Hair Follicles from Human Scalp

1. Obtain samples from surgical specimens of normal human scalp skin. To isolate whole hair follicles, cut the scalp skin containing the hair follicle pad and expose its inner surface. Dissect the scalp hair follicles under a binocular microscope and pluck from the pad by pulling them gently by the neck with fine forceps and wash in DMEM-F12 (GIBCO-BRL, Grand Island, NY) containing B-27 (GIBCO-BRL) and 1% penicillin–streptomycin (GIBCO-BRL). Perform all surgical procedures in a sterile environment. Isolate the hair follicle stem cell area from the hair follicles. Immediately locate the

hair follicle stem cell area below the sebaceous glands and above the hair follicle bulge area. The hair follicle stem cell area contains nestin-positive and K15-negative cells. Isolate the hair follicle stem cell area under a binocular microscope and suspend in 1 ml DMEM-F12 containing B-27 with 1% methylcellulose (Sigma-Aldrich) and add 20 ng ml^{-1} basic FGF (bFGF) (Chemicon, Temecula, CA) every 2 days. Culture cells in 24-well tissue-culture dishes (Corning, Aliso Viejo, CA) in a 37°C, 5% CO^2/95% air tissue-culture incubator. After 4 weeks, the nestin-positive human hair follicle cells should form a colony. For differentiation, centrifuge the hair follicle cells, remove the growth factor-containing supernatant, and resuspend the colony in fresh RPMI 1640 medium (Cellgro, Herndon, VA) containing 10% fetal bovine serum (FBS) in the SonicSeal 4-well chamber slides (Nunc Inc., Rochester, NY).

2. Obtain plucked hair follicles by removing the scalp hairs with a depilation forceps and wash in DMEM-F12 containing B-27 and 1% penicillin–streptomycin. Perform all surgical procedures under a sterile environment. Divide the hair follicle into three parts (upper, middle, and lower parts) and suspend in DMEM-F12 containing B-27 supplemented with 20 ng ml^{-1} bFGF added every 2 days. Culture cells in 24-well tissue-culture dishes in a 37 °C, 5% CO^2/95% air tissue-culture incubator. After 4 weeks, the middle parts of the hair follicle should form colonies. For differentiation, centrifuge the cell colonies, remove the growth factor-containing supernatant, and resuspend the colonies into fresh RPMI 1640 medium containing 10% FBS in SonicSeal 4-well chamber slides.

3.4.2. Immunofluorescence Staining and Quantification

Use immunofluorescence to stain the cells which differentiate in cell colonies formed from the nestin-positive and K15-negative cells in the intact hair follicle. Use the following primary antibodies: anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; Covance Research Products Inc., Berkeley, CA), anti-nestin polyclonal (1:200; Chemicon, Temecula, CA), anti-S100 polyclonal (1:200; Chemicon), anti-glial fibrillary acidic protein (GFAP) monoclonal (1:200; Lab Vision, Fremont, CA), anti-2′-3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) monoclonal (1:50; Lab Vision), anti-K15 monoclonal (1:100; Lab Vision), and anti-smooth muscle actin (SMA) monoclonal (1:200; Lab Vision). Secondary antibodies are fluorescein-conjugated sheep anti-mouse IgG (1:10; Chemicon) and tetramethylrhodamine-conjugated swine anti-rabbit IgG (1:80; Nordic Immunological laboratories, Tilburg, The Netherlands). DAPI (Molecular Probes) is used for nuclear counterstaining.

3.4.3. Nestin RT-PCR

Extract total RNA from human endothelium (HMvEC) using ISOGEN or equivalent (Nippon Gene Co., Toyama, Japan) according to the manufacturer's instructions. Reverse transcribe 1 μg of the total RNA into cDNA in a 20 μl reaction mixture containing 1 × RT buffer (GIBCO-BRL, Rockville, MD, USA), 0.5 mM of dNTPs (Takara Shuzo Co., Otsu, Japan), 0.5 μg of oligo-dT primer, 40 U of RNase inhibitor (Boehringer Mannheim Co., Mannheim, Germany), 200 U M-MLV reverse transcriptase (GIBCO-BRL), and 10 mM DTT (GIBCO-BRL). After incubation at 43°C for 1 hour and then at 95°C for 3 min, amplify the cDNA using 1 μl of the cDNA preparation for nestin in a 25 μl reaction mixture containing 10 mM of Tris-HCl, pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% (W/V) gelatin, 0.2 mM dNTPs, 25 pM 5′ and 3′ oligonucleotide primers, and 2.5 U Taq polymerase (Perkin-Elmer, Branchburg, NJ). Pre-incubate the reactions in a DNA thermocycler 480 (Perkin-Elmer Cetus, Norwalk, CT) for 94°C for 2 min and then 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 45 seconds and extention at 72°C for 1 min), followed by a final 7 min extension at 72°C. Use the following primers for cDNA amplification: nestin (5′ GGCAGCGTTGGAACAGAGGTTGGA 3′, 5′ CTCTAAACT GGAGTGGTCAGGGCT 3′, 718 bp) (10), and β-actin (5′ primer: GTGGGGCGCCCCAGGCACCA; 3′ primer: CTCCTTAATGTCACGCACGATTTC). Electrophoresis the PCR product in 1.5% agarose gel which is visualized by staining with ethidium bromide.

3.4.4. In Situ Hybridization of Nestin mRNA

Use a human nestin cDNA PCR product containing a 718 bp fragment. Subclone the cDNA using a TA cloning kit. Generate sense and antisense nestin RNA probes using SP6 or T7 RNA polymerases. Label the probes with digoxigenin-11-UTP using in vitro transcription. Digoxigenin-label RNA probes at a concentration of 100 μg per ml. Mount serial cryostat sections of biopsy material on aminopropylsilane-coated slides, followed by fixation in freshly prepared 4% paraformaldehyde. Permeate with 1 μg/ml proteinase K for 15 min at 37°C. Acetylate the sections for 10 min with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), and dehydrate in ethanol. Perform hybridization in a sealed, humid box for 16 hours at 50°C in hybridization solution (Boehringer Mannheim). Wash the slides in 50% formamide/2 × sodium citrate/sodium chloride buffer, treated with RNase A (20 μg per ml); further wash in 2 × sodium citrate/sodium chloride buffer for 20 min at 50°C, two changes of 0.2 × sodium citrate/sodium chloride buffer for 20 min at 50°C, and then block with blocking solution (Boehringer Mannheim) at room temperature for 1 hour. After blocking, wash the sections and incubate with anti-digoxigenin and Fab fragments conjugated to alkaline phosphatase (Boehringer Mannheim.) at room temperature for 30 min. Wash the

sections and then incubate with nitroblue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate at 37°C for 20 hours. Mount the slides and view by light microscopy.

3.5. Nestin, K15, and CD34 Expression in Plucked and Intact Hair Follicles of Human Scalp

Stain by immunofluorescence and immunohistochemistry for nestin, K15, and CD34 in plucked and intact human hair follicles. Detect with the secondary fluorescent antibodies described above using an anti-mouse immunoglobulin horseradish peroxidase detection kit (DAKO, Carpinteria, CA) following manufacturer's instructions. Use the following primary antibodies: anti-nestin polyclonal, anti-K15 monoclonal, and anti-CD34 monoclonal (1:10; Nichirei, Tokyo, Japan) (6).

4. Notes

1. Autofluorescence: It is important to minimize autofluorescence of 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 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.
2. Bleeding : Bleeding should be avoided at the surgical site as hemoglobin will absorb the incident excitation light.
3. Dehydration : During biopsy procedures, it is essential to hydrate the animal by spraying normal saline on the open tissue.
4. Infection : When doing open biopsies or other invasive procedures, it is crucial to maintain a proper sterile operation field.
5. The procedures in these protocols describe the isolation and differentiation of hair follicle stem cells that can be used for the regeneration of injured peripheral nerves and the spinal cord. Moreover, we can locate the nestin-positive and K15-negative stem cells in mouse and human hair follicles.
6. **Figure 28.1** demonstrates that ND-GFP is highly expressed in the hair follicle stem cell area and newly formed outer-root sheath cells of the mouse (1).
7. **Figure 28.2** demonstrates that ND-GFP-expressing cells in the hair follicle stem cell area are CD34-positive and K15-negative. ND-GFP-expressing cells (keratinocyte progenitor cells) in the bulge area are K15-positive and CD34-negative (1).

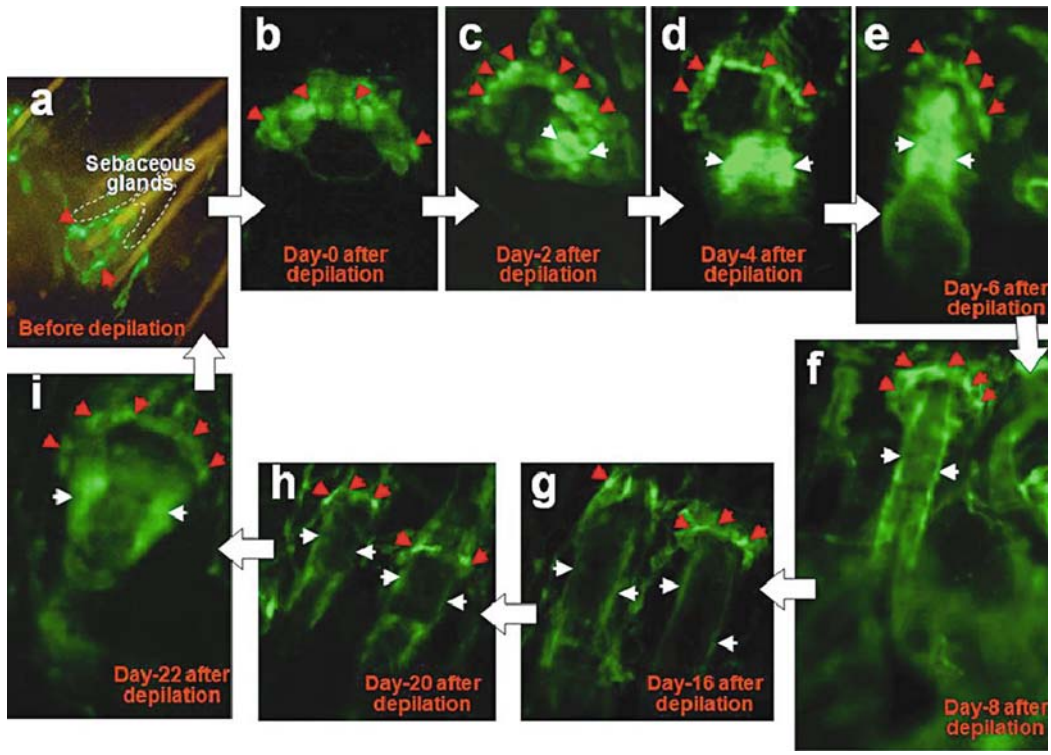

Fig. 28.1. Hair follicle stem cells in the hair growth cycle. (**a, b**) ND-GFP-expressing hair follicle stem cells (*red arrows*) located in the hair follicular stem cell area (the permanent upper hair follicle immediately below the sebaceous glands in the hair follicular stem cell area surrounding the bulge) in telogen phase. (**c, d**) Days 2 (**c**) and 4 (**d**) after anagen induction by depilation. Note the new hair follicle outer-root sheath cells (white arrow) formed directly from the nestin-GFP-expressing hair follicle stem cells. (**e, f**) Day 6 (**e**) and day 8 (**f**) after anagen induction by depilation. Note the nestin-GFP-expressing outer-root sheath cells (white arrows) in the upper two-thirds of the hair follicle. (**g–i**) Day 16 (**g**), day 20 (**h**), and day 22 (**i**) after depilation. Note in (**g**, **h,** and **i)** that the hair follicles are in the catagen phase and are undergoing regression and degeneration, including the ND-expressing cells in the outer-root sheath. The hair follicular stem cell area ND-GFP-expressing stem cells remain.

8. **Figure 28.3** demonstrates that ND-GFP-expressing cells in the hair follicle stem cell area are CD34-positive and K15-negative. ND-GFP-expressing cells in the hair follicle bulge area are K15-positive and CD34-negative (1).
9. **Figure 28.4** demonstrates that hair follicle cells labeled with ND-GFP behave as stem cells, differentiating to form much of the hair follicle in each hair growth cycle. Nestin also occurs in new perifollicular blood vessels, which are formed in response to follicular angiogenic signals during the anagen growth phase. This is seen most clearly by transplanting ND-GFP-labeled vibrissa (whisker) hair follicles to unlabeled nude mice. New ND-GFP-expressing vessels grow from the transplanted follicle, and these vessels increase when the local recipient skin is wounded (3).

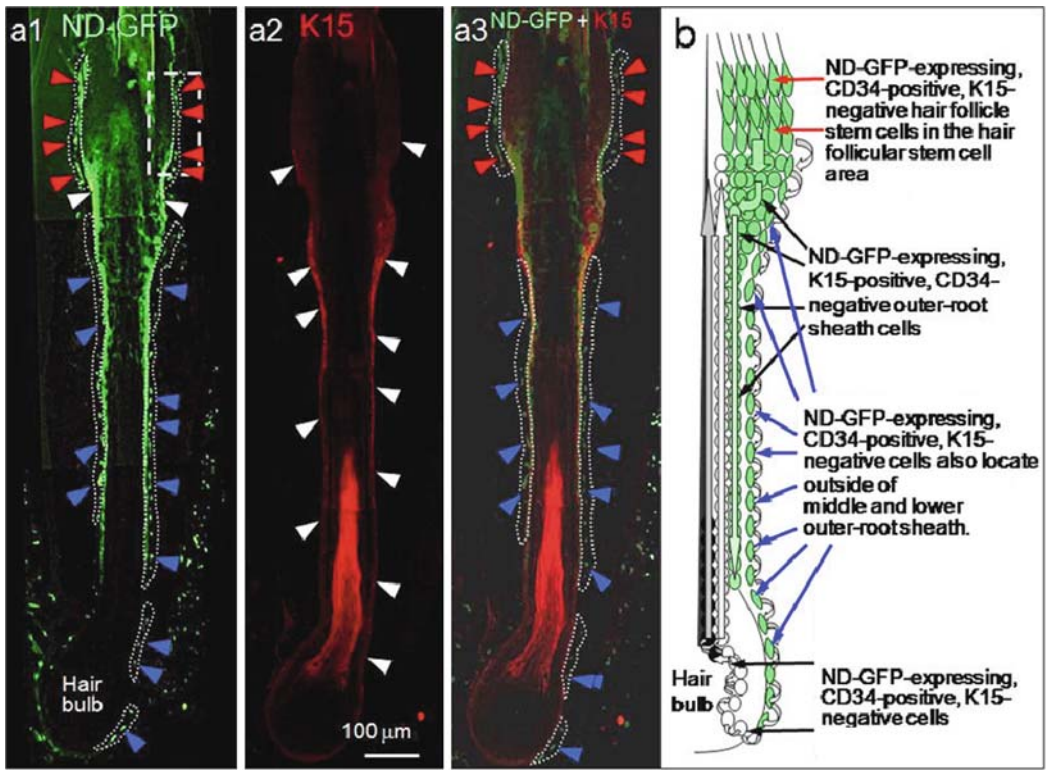

Fig. 28.2. ND-GFP-expressing, K15-negative, and CD34-positive cells locate not only in the hair follicular stem cell area but also outside of the middle and lower outer-root sheath. (**a1**) In frozen section of vibrissa follicle of ND-GFP transgenic mice, ND-GFP-expressing cells locate in hair follicular stem cell area (*red arrowheads*, white dashed areas), outside of the middle and lower outer-root sheath (*blue arrowheads, white dashed areas*), and outer-root sheath (*white arrowheads*). (**a2, a3**) Immunofluoresence staining of K15 showed that ND-GFP-expressing cells in hair follicular stem cell area (*red arrowheads, white dashed areas*) and ND-GFP-expressing cells around the middle and lower outer-root sheath (*blue arrowheads, white dashed areas*) were K15-negative. ND-GFP-expressing outer-root sheath cells in the bulge area were K15-positive and CD34-negative. ND-GFP-expressing cells in the outer-root sheath were K15-positive, (*white arrowheads*). (**b**) Schematic of vibrissa follicle of ND-GFP transgenic mice showing the position of ND-GFP-expressing cells in the section. ND-GFP-expressing cells in hair follicle stem cell area were CD34-positive and K15-negative (*red arrows*). ND-GFP-expressing outer-root sheath cells were K15-positive and CD34-negative (*black arrows*). ND-GFP-expressing cells outside of the outer-root sheath (*blue arrows*) were CD34-positive and K15-negative. ND-GFP-expressing, CD34-positive, K15-negative hair follicle stem cells in the growing outer-root sheath are directly derived from the follicle stem cell area (black arrows).

10. **Figure 28.5** demonstrates the isolation of multipotent nestin-positive, keratin-negative hair follicle stem cells. Hair follicle stem cells are isolated using the ND-GFP marker. These ND-GFP-expressing stem cells are primitive, because they express the stem cell marker CD34 but do not express the keratinocyte marker K15. The ND-GFP-expressing hair follicle stem cells in ND-GFP transgenic mice are isolated and suspended in DMEM-F12 containing B-27 supplemented with basic FGF every 2 days. After 4 weeks, ND-GFP-expressing

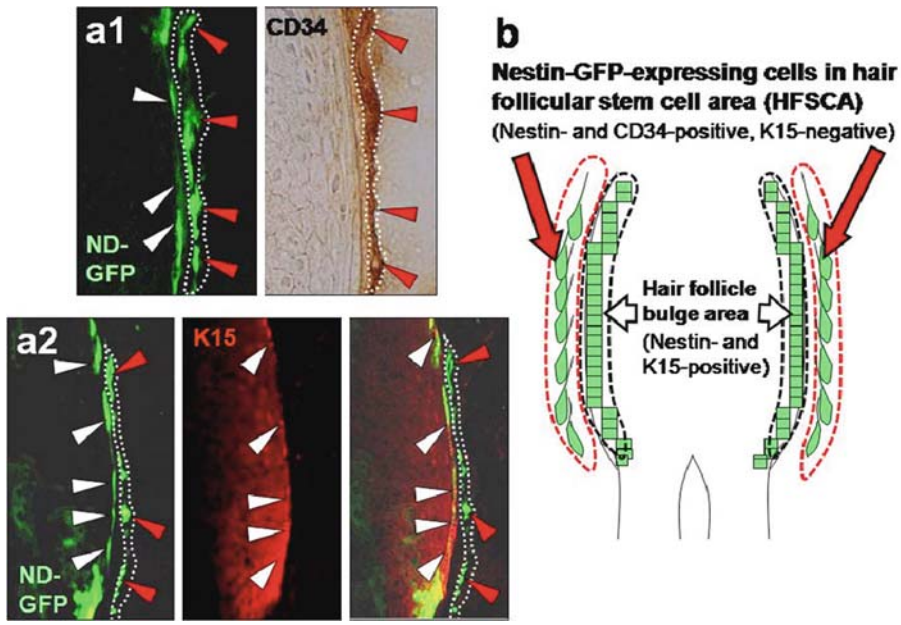

Fig. 28.3. (**a**) Higher magnification of white dashed box in **Fig. 28.2a**. ND-GFP-expressing cells in hair follicle stem cell area (*white dashed area, red arrowheads*) are CD34-positive and K15-negative. ND-GFP-expressing outer-root sheath cells in outer layer of the outer-root sheath (*white arrowheads*) are K15-positive and CD34-negative. (**b**) Schematic of hair follicular stem cell area and bulge area of ND-GFP transgenic mice. ND-GFP-expressing cells in hair follicle stem cell area (*red dashed area, red arrows*) are CD34-positive and K15-negative. ND-GFP-expressing outer-root sheath cells (keratinocyte progenitor cells) in outer layer of the outer-root sheath (hair follicle bulge area) (*black dashed area, white arrows*) are K15-positive and CD34-negative.

hair follicle stem cells formed the ND-GFP-expressing, CD34-positive, and K15-negative cell colonies. Upon transfer to RPMI-1640 medium containing 10% fetal bovine serum (FBS), the ND-GFP-expressing cells differentiated to neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro (2).

11. **Figure 28.6** demonstrates that hair follicle stem cells promote the recovery of peripheral nerve injury. We isolated the nestin- and CD34-positive and K15-negative stem cells from the hair follicle stem cell area in GFP transgenic mice (GFP mice). The nestin-expressing hair follicle stem cell area contains multipotent stem cells, which can differentiate into neurons, glial cells, keratinocytes, smooth muscle cells, and melanocytes. The isolated immature multipotent hair follicle stem cells differentiated to GFAP-positive glial cells and βIII-tubulin-positive neurons and could join the severed sciatic nerve. Most of the GFP-expressing hair follicle stem cells differentiated into GFAP-positive Schwann cells in the sciatic nerve of C57BL/6 immunocompetent mice. The regenerated sciatic nerve contracted the gastrocnemius muscle by

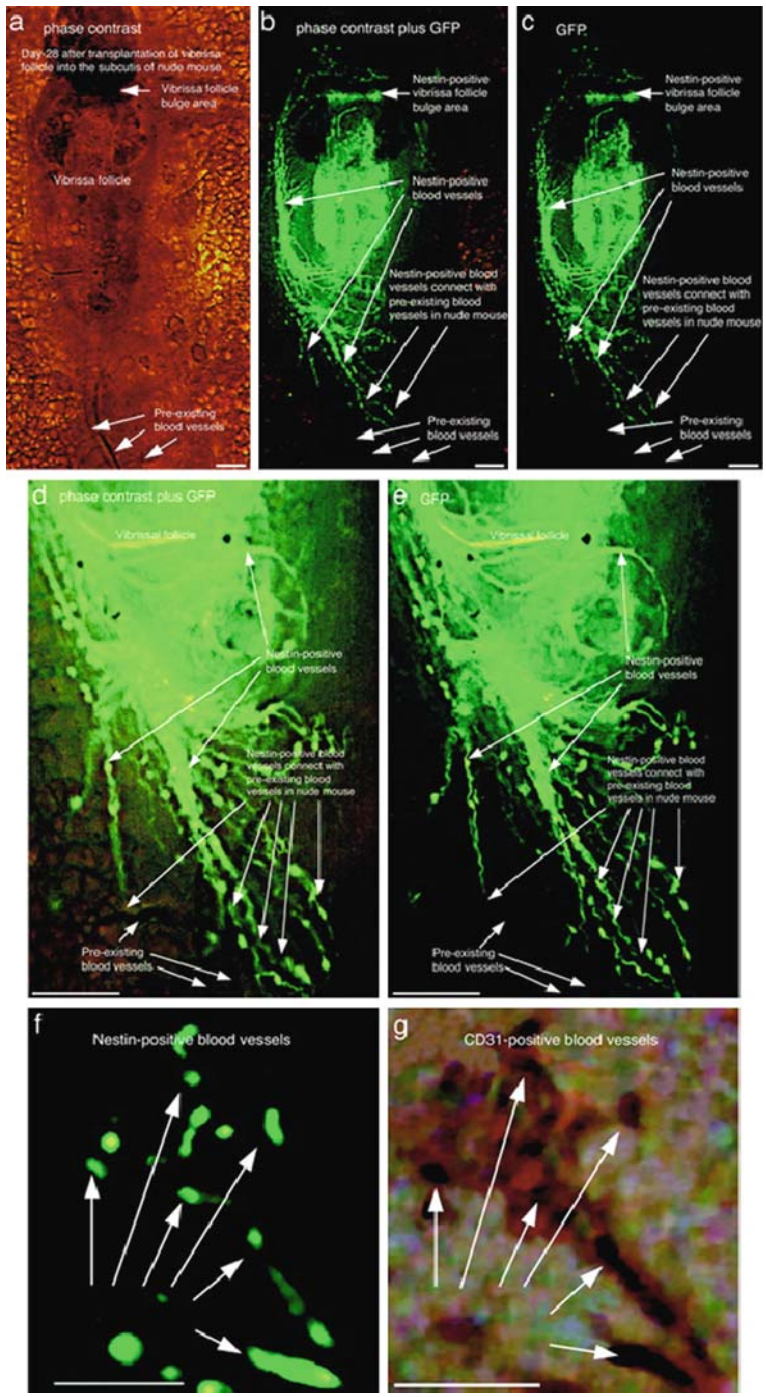

Fig. 28.4. Transplantation of an ND-GFP vibrissa follicle into the subcutis of a nude mouse. (**a**) Phase-contrast micrograph of follicle 28 days after transplantation. (**b**) Phase-contrast micrograph plus GFP fluorescence. (**c**) GFP fluorescence. In **(b and c)**, ND-GFP blood vessels (*arrows*) are seen growing from the transplanted ND-GFP hair follicle and associating with pre-existing blood vessels in the nude mouse skin. **(d and e)** Higher magnification of the ND-GFP vessels of (**b)** and (**c)**, respectively. (**f** and **g**) Colocalization of GFP and the endothelial cell marker CD31 (*arrows*). (**f)**is a fluorescent image, and (**g)** shows the same field air-dried and immunohistochemically stained with CD31. Scale bars, 100 μm (3).

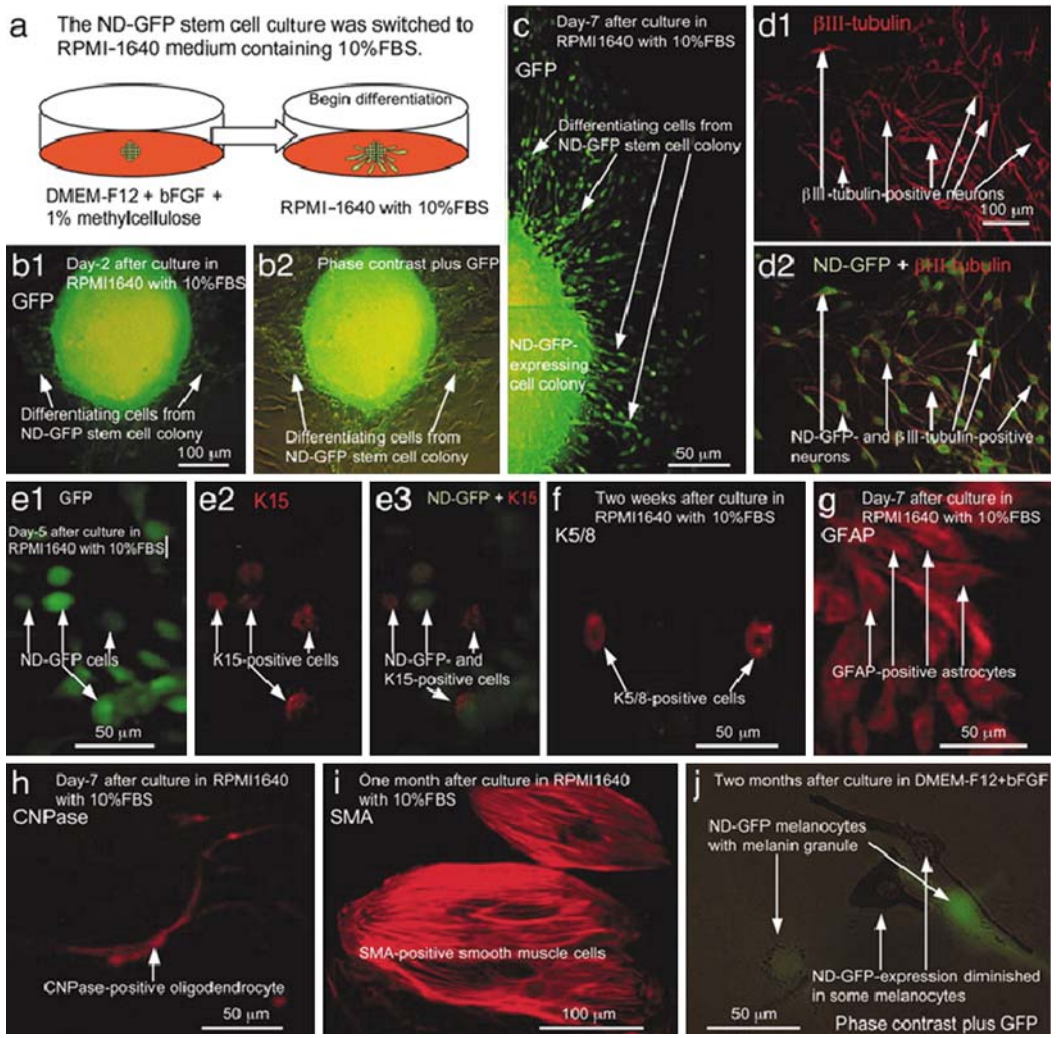

Fig. 28.5. Differentiation of ND-GFP hair follicle stem cells in vitro. (*a*) The ND-GFP-expressing cell culture was switched to RPMI-1640 medium containing 10% FBS from DMEM-F12 containing B-27 and 1% methylcellulose supplemented with bFGF added every 2 days. **(b1 and b2)** Two days after switching into RPMI-1640 medium containing 10% FBS, differentiating cells migrated out of the ND-GFP-expressing cell colony. **(c)** Seven days after switching to RPMI-1640 medium, many differentiating cells migrated out of the ND-GFP-expressing cell colony. **(d1 and d2)** ND-GFP-expressing cells differentiated to βIII -tubulin-positive neurons which maintain ND-GFP expression. **(e1–e3)** Five days after switching to RPMI-1640 medium, ND-GFP-expressing cells differentiated to K15-positive cells (red fluorescence, *arrows*) **(e2)**. The K15-positive cells still expressed ND-GFP. **(f)** ND-GFP-expressing cells differentiated to K5/8-positive cells 2 weeks after switching to RPMI-1640 medium. **(g)** Seven days after switching to RPMI-1640 medium, ND-GFP-expressing cells differentiated to GFAP-positive astrocytes. **(h)** Seven days after switching to RPMI-1640 medium, ND-GFP-expressing cells differentiated to 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)-positive oligodendrocytes. **(i)** At 1 month after culture in RPMI-1640 medium containing 10% FBS, ND-GFP-expressing cells differentiated to SMA-positive smooth muscle cells. **(j)** Two months after culture in DMEM-F12 containing B-27 and 1% methylcellulose supplemented with bFGF added every 2 days, ND-GFP-expressing cells differentiated to melanocytes containing melanin. Some melanocytes still expressed ND-GFP (2).

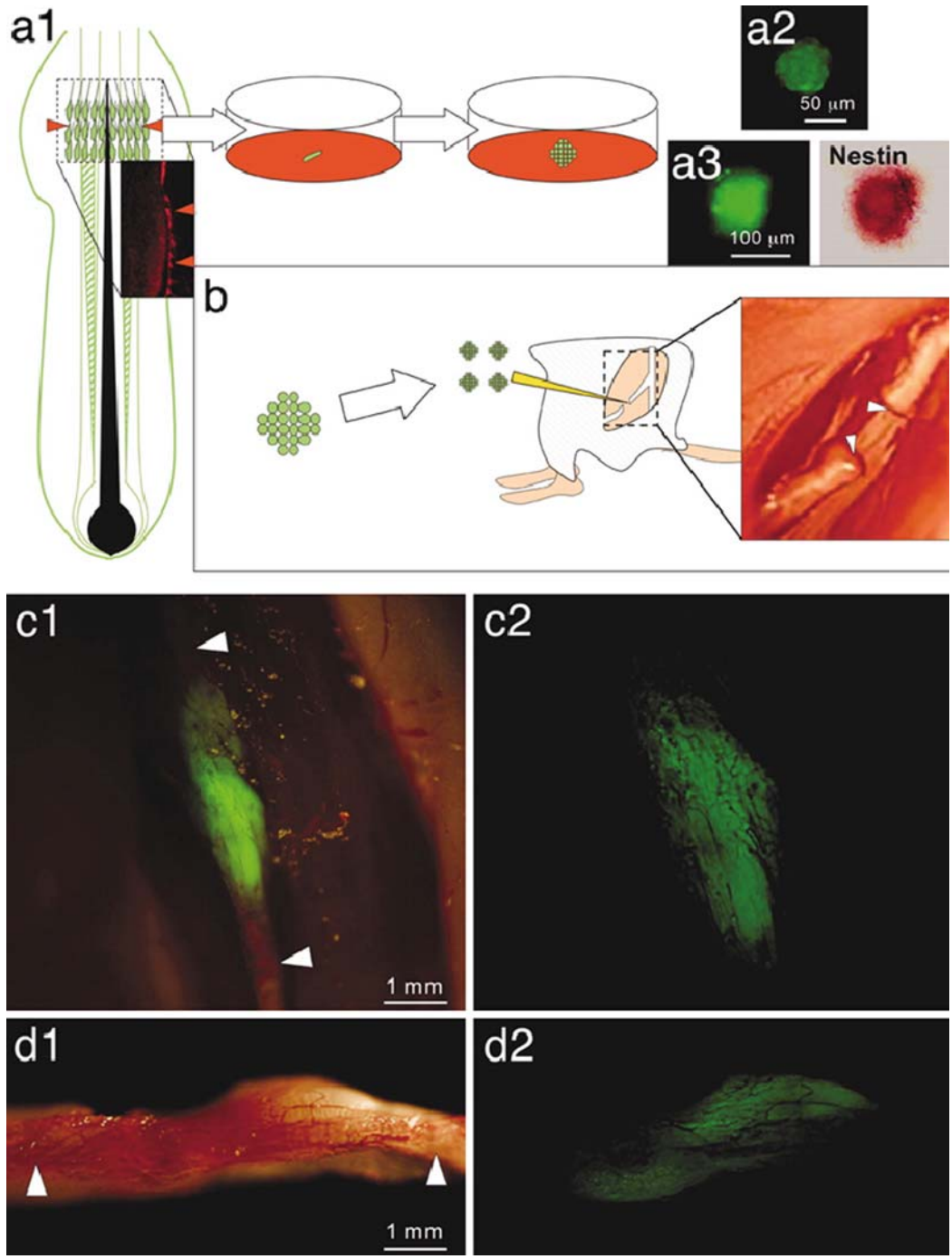

Fig. 28.6. Rejoining severed sciatic nerves with hair follicle stem cells. **(a1)** Schematic of vibrissa follicle of GFP transgenic mice showing the position of GFP- and nestin-expressing hair follicular stem cell area (*red arrowheads*). **(a2)** Colony formed from GFP-expressing hair follicle stem cells after 2 months in culture. **(a3)** GFP-expressing cells within the colony were nestin-positive. **(b)** GFP-expressing hair follicle stem cells grown for 2 months in DMEM-F12 medium containing B-27, 1% methylcellulose, and basic FGF were transplanted between the severed sciatic nerve fragments in C57BL/6 immunocompetent mice (*white arrowheads*). **(c1 and c2)** Fluorescence images from a live mouse. Two months after transplantation between the severed sciatic nerve, the GFP-expressing cells were visualized in the joined region of the severed sciatic nerve. *c2* shows higher magnification of *c1*. **(d1 and d2)** Brightfield (*d1*) and fluorescence (*d2*) images of an excised sciatic nerve. The pre-existing sciatic nerve is denoted by white arrowheads (4).

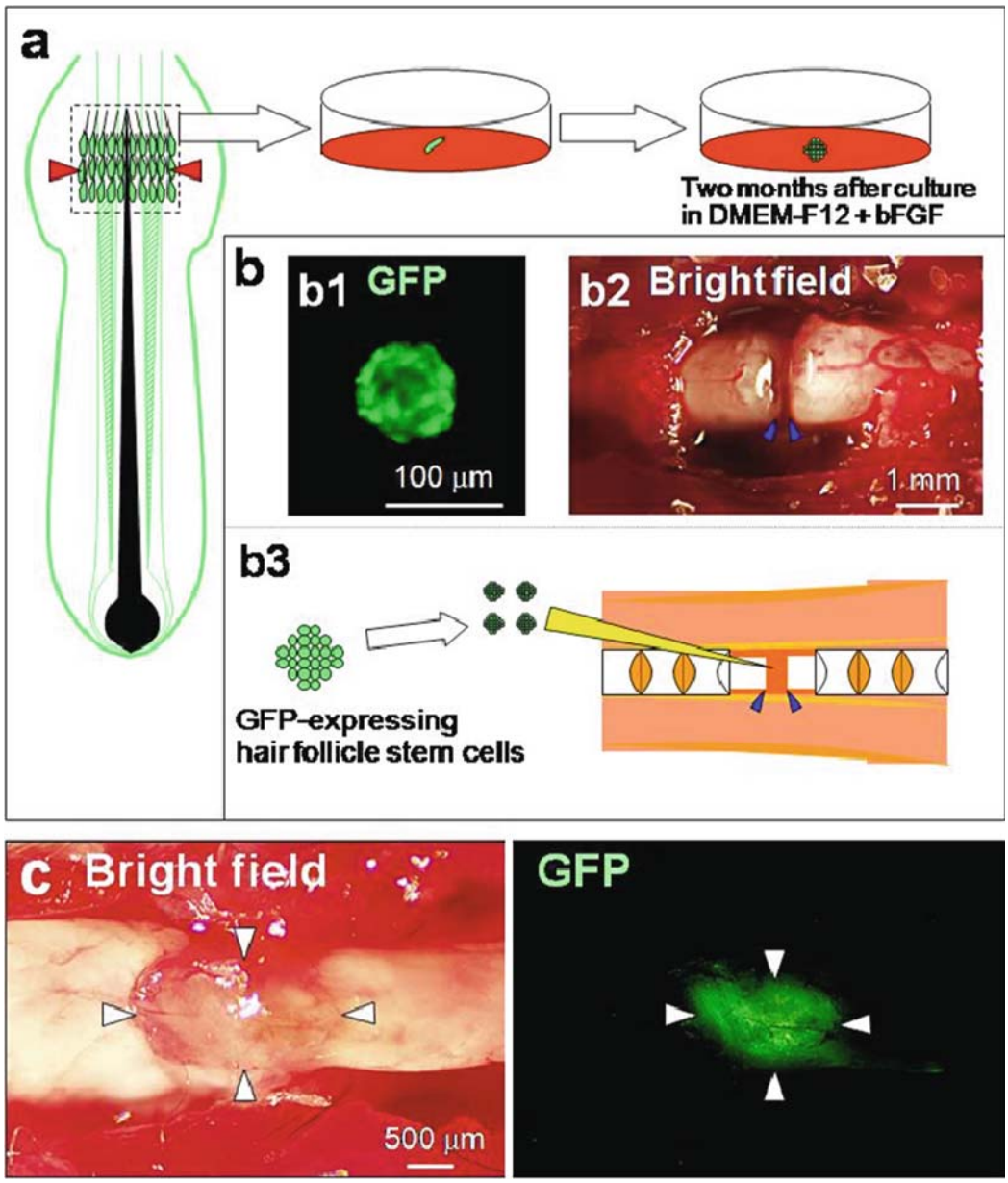

Fig. 28.7. Severed thoracic region of spinal cord joined by transplantation of hair follicle stem cells. **(a)** Schematic of vibrissa follicle of GFP transgenic mice shows the position of the GFP-expressing hair follicular stem cell area (*red arrowheads*). The GFP-expressing stem cells from hair follicular stem cell area were cultured for two months in DMEM-F12 medium containing B-27 supplemented with bFGF added every 2 days. After 2 months, GFP-expressing hair follicle stem cells from the hair follicle stem cell area formed the GFP-expressing hair follicle stem cell colonies. **(b, b1)** denotes a GFP-expressing hair follicle stem cell colony from the GFP-expressing hair follicular stem cell area. **(b2, b3)** The GFP-expressing stem cells 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 hair follicle stem cells between the severed thoracic region of the spinal cord, the GFP-expressing cells effected joining the severed thoracic region of the spinal cord in C57BL/6 immunocompetent mice (*white arrowheads*) (5).

electric stimulation. Walking print length and intermediate toe spread significantly recovered by transplantation of GFP-expressing hair follicle stem cells between the severed tibial nerve. These results suggest that hair follicle stem cells promote the recovery of peripheral nerve injury (4).

12. **Figure 28.7** demonstrates hair follicle stem cells promote the recovery of spinal cord injury. We isolated the nestin- and CD34-positive and K15-negative stem cells from the hair follicular stem cell area in GFP mice. We transplanted the GFP-expressing hair follicle stem cells between the severed thoracic regions of spinal cord. Most of the GFP-expressing hair follicle stem cells differentiated into GFAP- and CNPase-positive Schwann cells and joined the severed spinal cord of C57BL/6 immunocompetent mice. These results suggest that the hair follicle nestin-expressing stem cells differentiated into immature Schwann cells which promote axonal growth and functional recovery after spinal cord injury (5).

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