

Chapter 8

Hair Follicle Pluripotent Stem (hfPS) Cells

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Abstract Our laboratory has discovered that nestin, a protein marker for neural stem cells is also expressed in hair follicle stem cells and their immediate, differentiated progeny. The fluorescent protein, GFP, whose expression is driven by the nestin regulatory element in transgenic mice (ND-GFP mice), served to mark hair follicle stem cells and enabled us to make this observation. The ND-GFP hair-follicle stem cells are positive for the stem cell marker CD34 but negative for keratinocyte marker keratin 15, suggesting their relatively undifferentiated state. We have shown that these hair follicle stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes in vitro. In vivo studies show the hair follicle stem cells can differentiate into blood vessels and neural tissue after transplantation to the subcutis of nude mice. Hair follicle stem cells implanted into the gap region of severed sciatic or tibial nerves greatly enhance the rate of nerve regeneration and the restoration of nerve function. When transplanted to severed nerves in mice, the follicle cells transdifferentiate largely into Schwann cells, which are known to support neuron regrowth. The transplanted mice regain the ability to walk normally. We have also shown that hair follicle stem cells can affect the functional joining of the severed spinal cord. When the hair follicle stem cells are injected into the severed spinal cord, they differentiate into Schwann cells enabling the cord to rejoin and the mouse to regain function of its rear legs. Thus, hair follicle pluripotent stem (hfPS) cells can provide an effective, accessible, autologous source of stem cells for treatment of peripheral nerve injury and appear to be a paradigm for adult stem cells.

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8.1 Introduction

8.1.1 The Hair Follicle as a “Mini-Organ”

The hair follicle produces a terminally differentiated keratinized end product, the hair shaft, that is eventually shed. The follicle undergoes cyclical regeneration with at least 10 different epithelial and mesenchymal cell lineages [1]. Hair is formed by rapidly proliferating matrix keratinocytes in the bulb located at the base of the growing (anagen) follicle. The duration of anagen varies greatly between hairs of differing lengths. Nevertheless, matrix cells eventually stop proliferating, and hair growth ceases at catagen when the lower follicle regresses (telogen). After telogen, the lower hair-producing portion of the follicle regenerates, starting the new anagen phase [1].

Hair follicle stem cells, located in the hair follicle bulge, possess stem cell characteristics, including multipotency, high proliferative potential, and ability to enter quiescence. Lineage analysis has demonstrated that all epithelial layers within the adult follicle and hair originate from bulge cells [1, 2]. The hair follicle stem cells, therefore appear to be responsible for regenerating the hair follicle in each hair cycle.

After wounding, hair follicles form *de novo* in adult mice. The nascent follicles arise from epithelial cells outside of the hair follicle stem cell niche, suggesting that epidermal cells in the wound assume a hair follicle stem cell phenotype. The newly generated hair follicles establish a stem cell population, express known molecular markers of follicle differentiation, produce a hair shaft, and progress through all stages of the hair follicle cycle [3].

8.1.2 Tracking Hair Follicle Stem Cells In Vivo

The insufficiency of markers to identify and track hair follicle stem cells in the bulge area has hindered the study of hair follicle stem cells. CD34 expression, as first defined by Trempus et al. [4], is a marker for hair follicle stem cells. Antibodies recognizing CD34 were used to collect viable bulge cells by fluorescent activated cell sorting [4, 5]. K15 is expressed at high levels in the bulge, but lower levels of expression can be present in the basal layers of the lower follicle outer-root sheath (ORS) and the epidermis [6, 7]. A K15 promoter used for generation of transgenic mice was active only in the bulge in the adult mouse [8].

A breakthrough occurred with the use of transgenic mice in which the neural stem cell marker, nestin, drove the expression of green fluorescent protein (GFP) (ND-GFP). We observed in these mice that nestin was also a marker for hair follicle stem cells which suggested that hair follicle stem cells could form neurons and were pluripotent [9]. The hair follicle stem cells could then be tracked by their green fluorescence. These relatively small, oval-shaped, ND-GFP-expressing cells in the bulge area surround the hair shaft and are interconnected by short dendrites. In mid- and late anagen, the ND-GFP-expressing cells are located in the upper outer-root

sheath as well as in the bulge area but not in the hair matrix bulb. These observations show that the ND-GFP-expressing cells form the outer-root sheath. Following our report that ND-GFP can serve as a marker for hair follicle stem cells to track them in the live animal, Morris et al. [10] subsequently used GFP to isolate hair follicle stem cells in transgenic mice. Fuchs' group also subsequently used GFP to identify hair follicle stem cells and possibly other skin stem cells in transgenic mice [11, 12]. Mignone et al. [13] have confirmed our results that hair follicle stem cells express nestin. Yu et al. [14] showed that nestin was present in human hair follicle stem cells also confirming our original observation [9].

The evidence that nestin-expressing cells in the hair follicle bulge are hair follicle stem cells rather than a population of stem cells that reside in the hair follicle whose purpose is to regenerate the neuronal and endothelial components associated with the pilosebaceous unit is that the nestin-expressing (and GFP-expressing) cells have been imaged over time to regenerate a large portion of the hair follicle as described above [9]. The ND-GFP marker may have enabled the identification and isolation of the most pluripotent cells in the hair follicle bulge area.

8.1.3 The Ability of Hair Follicle Stem Cells to Differentiate to Follicular and Non-Follicular Cell Types

Hair follicle stem cells from adult mice, when combined with neonatal dermal cells, formed hair follicles after injection into immunodeficient mice [5, 10]. Cultured, individually cloned bulge cells from adult mice also were shown to form hair follicles in skin reconstitution assays [5].

Taylor et al. [15] reported that hair follicle bulge stem cells are potentially bipotent because they can give rise to not only cells of the hair follicle but also to epidermal cells. However, hair follicle stem cells may form epidermal stem cells only when the epidermis is wounded [16]. Other experiments [17] also have provided new evidence that the upper outer-root sheath of vibrissal (whisker) follicles of adult mice contains multipotent stem cells, which can differentiate into hair follicle matrix cells, sebaceous gland basal cells, and epidermis. Toma et al. [18] reported that multipotent adult stem cells isolated from mammalian skin dermis, termed skin-derived precursors (SKP), can proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells, and adipocytes. However, the exact location of these stem cells in skin is unknown, and their functions are still unclear. They may have arisen in hair follicles.

8.1.4 Blood Vessels Derived from Hair-Follicle Stem Cells

We observed that in ND-GFP mice, skin blood vessels express ND-GFP and appear to originate from hair follicles and form a follicle-linking network. This was seen most clearly by transplanting ND-GFP-labeled vibrissa (whisker) hair follicles to

unlabeled nude mice. New vessels grew from the transplanted follicle, and the number of vessels increased when the local recipient skin was wounded. The ND-GFP-expressing blood vessels display the characteristic endothelial-cell-specific markers CD31 and von Willebrand factor [19].

8.1.5 Differentiation of Hair Follicle Stem Cells to Neural and Other Cell Types

ND-GFP hair follicle stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro. These pluripotent ND-GFP stem cells are positive for the stem cell marker CD34, and negative for keratin 15, suggesting their relatively undifferentiated state as mentioned above. The apparent primitive state of the ND-GFP stem cells is compatible with their pluripotency. The ND-GFP hair follicle stem cells may be more primitive than those hair follicle stem cells previously isolated [2]. Furthermore, we showed that the hair follicle stem cells differentiated into neurons after transplantation to the subcutis of nude mice [20].

Mignone et al. [13] confirmed our data that hair follicle stem cells are pluripotent when isolated from the ND-GFP mice. In addition, the hair follicle stem cells formed neuronal cells when implanted in chick embryos. Transcriptional profiling showed that the nestin-expressing hair follicle stem cells are similar to neural stem cells.

Li et al. [21] have reported that nuclei from hair follicle stem cells can be successfully used as nuclear transfer (NT) donors, resulting in live cloned mice. Thus, the nuclei of hair follicle stem cells can be reprogrammed to the pluripotent state by exposure to the cytoplasm of unfertilized oocytes. These results confirm our earlier results demonstrating the pluripotency of hair follicle stem cells [9].

8.1.6 Hair Follicle Stem Cells Can Effect Nerve Repair

When the GFP hair follicle stem cells were implanted into the gap region of a severed sciatic nerve they greatly enhanced the rate of nerve regeneration and the restoration of nerve function. After transplantation to severed nerves, the hair follicle stem cells differentiated 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. The transplanted mice recovered the ability to walk normally [22].

8.1.7 Hair Follicle Stem Cells Can Effect Spinal Cord Repair

We severed the thoracic spinal chord of C57BL/6 immunocompetent mice and transplanted mouse GFP-expressing hair follicle stem cells to the injury site. Most of the transplanted cells also differentiated into Schwann cells that apparently facilitated

repair of the severed spinal cord. The rejoined spinal cord reestablished 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 treatment of peripheral nerve and spinal cord injury [23].

8.2 Discussion

Sieber-Blum et al. [24] showed that neural crest cells grew out when the hair follicle was explanted, resulting in differentiation to a variety of cell types including neurons, smooth muscle cells, rare Schwann cells, and melanocytes. The location of these cells within the follicle was not determined. Sieber-Blum et al. [25] characterized the behavior of implanted neural crest stem cells from the hair follicle in the contusion-lesioned murine spinal cord. The grafted neural crest cells survived, integrated, and intermingled with host neurites in the lesioned spinal cord. They did not proliferate and did not form tumors. Subsets expressed neuron-specific beta-III tubulin, the GABAergic marker glutamate decarboxylase 67 (GAD67), the oligodendrocyte marker, RIP, or myelin basic protein (MBP). However, glial fibrillary acidic protein (GFAP) was not detected by immunofluorescence.

This apparent puzzle is probably due to different cell types transplanted by Sieber-Blum et al. [25] compared to the cell types we transplanted to the lesioned spine. Sieber-Blum et al. explanted the bulge area of a whisker (vibrissa) *in vitro*. Within 3–4 days, cells migrated from the explanted bulge area and grew on the surface of the culture dish. Glial markers were not expressed or expressed only at low levels in the migrating cells. Four days after onset of migration, these cells were harvested and further expanded in culture for another four days. After four days of expansion, the cells were implanted in the lesioned spinal cord. Although neurons and oligodendrocytes formed after transplantation, glial cells did not appear.

Our approach was to actually isolate the vibrissa stem cells, culture them for two months and then implant the cells in the lesioned spinal cord. In contrast to Sieber-Blum et al., in our study, the vast majority of the implanted cells (82%) formed glial cells in the lesioned spinal cord. Our hypothesis is that the glial cells promoted axon growth and recovery of spinal cord function. Perhaps the outgrowth method of Sieber-Blum et al. [25] did not allow for recovery of sufficient numbers of cells capable of glial differentiation, which in turn did not allow for sufficient axon growth for spinal cord recovery.

Toma et al. [18] reported that multipotent adult stem cells isolated from mammalian skin dermis, the SKP mentioned above, can proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells, and adipocytes. However, while the exact source of the skin-derived precursors was not identified, it is possible they originated in the hair follicles. This same laboratory then observed that the SKPs could form myelinating Schwann cells when injected into the injured sciatic nerve [26] which is similar to our earlier results with the nestin-expressing hair follicle stem cells [22]. The same laboratory then showed that SKPs could promote

spinal cord repair. The SKPs were released from skin by collagenase treatment of the skin which produced a mixture of cells [27]. The origin of SKPs within the skin is thus unclear. In contrast, our results show that the hair follicle stem cells, a defined population, can functionally repair the severed spinal cord. It should also be noted that our studies as well as the studies with SKPs used fluorescent proteins to track the transplanted cells, a technology pioneered in our laboratory [28–32].

Soluble factors secreted from host cells as well as hair follicle stem cells may play a role in the regeneration of spinal cord injury. For example, brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF-1) were shown to be involved in nerve regeneration [33]. Future experiments will examine this issue in the case of hair follicle stem cells.

Cell-replacement therapies show particular promise in the nervous system, where transplanted embryonic or bone marrow stem cells have been shown to promote recovery of function in animal models of spinal cord or peripheral nerve injury [34, 35]. Although the therapeutic potential of such transplants is clear, a number of problems remain. In particular, fetal tissue is the current tissue source for human neuron-specific and embryonic stem cells, raising significant ethical issues. Moreover, the use of human tissue involves heterologous transplantation with attendant immune response. The requisite accompanying immuno-suppression is particularly problematic in individuals with long-term neuron-specific problems. Recently, induced pluripotent stem (iPS) cells have been derived from skin and other organs by gene transfer [36–38]. However, the vectors used for gene transfer have made these stem cells potentially malignant. In this regard, nestin-expressing hair follicle pluripotent stem (hfPS) cells are available from autologous, accessible adult tissue source, normal skin and they do not form tumors. hfPS cells can readily generate neuron and glial cells and provide a potential solution to these problems.

8.3 Conclusions

We have shown that the hair follicle bulge area is an abundant, easily accessible source of actively growing pluripotent adult stem cells that could serve a clinical source in humans. The availability of the ND-GFP mice has enabled the identification, isolation, and characterization of the hfPS cells. These hair follicle stem cells express the stem cell marker CD34 and nestin but are negative for the keratinocyte marker keratin 15, indicating their relatively undifferentiated state. The hair follicle stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes *in vitro*. *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. Hair follicle stem cells implanted into the gap region of a severed sciatic or tibial nerve greatly enhance the rate of nerve regeneration and the restoration of nerve function. After transplantation to the severed nerve, the follicle cells transdifferentiate largely into Schwann cells, which are known to support neuron regrowth. The transplanted mice regain the ability to walk normally.

Thus, hfPS cells provide an effective, accessible, autologous source of stem cells for treatment of peripheral nerve injury.

The hfPS cells thus have the potential as an alternative to the use of embryonal stem cells or fetal cells for regenerative medicine. The hfPS cells do not have the ethical problems that embryonal or fetal stem cells have. Even more important, the hfPS cells are much more easily accessible than other stem-cell types and offer the potential for autologous treatment as they can be readily expanded in culture after isolation from the patient. The fact that Yu et al. [14] have shown nestin expression and pluripotency of human hair follicle stem cells further suggests the clinical potential of hair follicle stem cells for regenerative medicine. Hair follicle stem cells also have great potential for hair restoration [1].

Li et al. [21] have shown that nuclei from hair follicle stem cells can be used to clone mice, further demonstrating the pluripotency of these stem cells.

It is also important to note that the dermal papilla is a potential source of multipotent stem cells that may have use in regenerative medicine. For example, Jahoda's group has demonstrated that hair follicle dermal cells repopulate the mouse haematopoietic system [39], can differentiate into adipogenic and osteogenic lineages [40] and participate in wound healing and induction [41].

8.4 Materials and Methods

8.4.1 *GFP-Expressing Transgenic Mice (Green Mice)*

Transgenic C57/B6-GFP mice were originally obtained from Professor M. Okabe at the Research Institute for Microbial Diseases (Osaka University, Osaka). The C57/B6-GFP mice expressed the *Aequorea victoria* GFP under the control of the chicken β -actin promoter and cytomegalovirus enhancer (β -actin-driven GFP). All of the tissues from this transgenic line, with the exception of erythrocytes and hair, express GFP [22].

8.4.2 *ND-GFP Transgenic Mice*

Transgenic mice carrying GFP under the control of the nestin second-intron enhancer (9, 19) were obtained from G. Enikolopov (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) [20].

8.4.3 *GFP-Expressing, Hair Follicle Stem Cells Cultured from Isolated Vibrissa Follicles*

To isolate the vibrissa follicles, the upper lip containing the vibrissa pad was cut, and its inner surface was exposed. The vibrissa follicles were dissected under a binocular microscope and plucked from the pad by pulling them gently by the neck with fine forceps. The follicles were then washed in DMEM-F12 (GIBCO/BRL,

Grand Island, NY) containing B-27 (GIBCO/BRL) and 1% penicillin-streptomycin (GIBCO/BRL). All surgical procedures were made in a sterile environment. The GFP-expressing vibrissa follicular stem cells located under the sebaceous gland (9) were isolated under a binocular microscope and suspended in 1 ml of DMEM-F12-containing B-27 with 1% methylcellulose (Sigma-Aldrich). The culture was supplemented every 2 days with basic FGF at 20 ng·ml⁻¹ (Chemicon). Cells were cultured in 24-well tissue culture dishes (Corning) in a 37°C, 5% CO₂/95% air tissue-culture incubator. After 4 weeks, GFP-expressing vibrissa follicle stem cells formed GFP-expressing colonies. For differentiation, GFP-expressing cell colonies were centrifuged, the growth factor-containing supernatant was removed, and the colonies were resuspended in fresh RPMI medium 1640 (Cellgro, Herndon, VA) containing 10% FBS in SonicSeal four-well chamber slides (Nunc). After 8 weeks of expansion, the GFP-expressing cell colonies were switched to RPMI medium 1640 containing 10% FBS in the SonicSeal four-well chamber slides and then differentiated [22].

8.4.4 Nestin-, CD34-, and K15-Expression in Vibrissa Follicles of Green Mice

Skin samples were dissected from 6- to 8-week-old β-actin-driven GFP mice. These mice were anesthetized with tribromoethanol (i.p. injection of 0.2 ml per 10 g of body weight of a 1.2% solution), and samples were excised from the skin containing vibrissa follicles. Immediately after excision, the vibrissa follicle samples were frozen in liquid nitrogen, embedded in tissue-freezing embedding medium (Triangle Biomedical Sciences, Durham, NC) and stored at -80°C until further processing. Frozen vibrissa follicle sections (5 μm thick) were cut with a Leica CM1850 cryostat and were air-dried. The sections were directly observed by fluorescence microscopy and used for immunofluorescence (nestin and K15) and immunohistochemical (CD34) staining [22].

8.4.5 Transplantation of GFP-Expressing Hair Follicle Stem Cells to the Thoracic Region of the Severed Spinal Cord in C57BL/6 Immunocompetent Mice

The GFP-expressing stem cells, cultured for two months in DMEM-F12 containing B-27 and 1% methylcellulose, were used for transplantation. Six- to eight-week old C57BL/6 immunocompetent mice (Harlan, San Diego, CA) were anesthetized with tribromoethanol. Using a binocular microscope, a laminectomy was made at the 10th thoracic spinal vertebra, followed by transversal cut. The GFP-expressing hair follicle stem cells were transplanted between the severed thoracic region (spinal level T10) of the spinal cord in C57BL/6 immunocompetent mice. After 2 months, the spinal cords of the transplanted mice were directly observed by fluorescence

microscopy under anesthesia. A total of 12 mice were transplanted with hair follicle stem cells [22].

8.4.6 Transplantation of GFP-Expressing Hair Follicle Stem Cells Between Severed Sciatic or Tibial Nerve Fragments In Immunocompetent C57BL/6 Mice

The GFP-expressing hair follicle stem cell colonies from the vibrissa follicle bulge area were transplanted between the severed sciatic or tibial nerve fragments in immunocompetent C57BL/6 mice under tribromoethanol anesthesia. The skin incision was closed with nylon sutures (6–0). After 2 months, the sciatic nerve of the transplanted mouse was directly observed by fluorescence microscopy under anesthesia. The sciatic nerve samples were embedded in tissue freezing embedding medium and frozen at -80°C overnight. Frozen sections $5\ \mu\text{m}$ thick were cut with a Leica CM1850 cryostat and air dried. The sections were first directly observed under fluorescence microscopy. The frozen sections were then used for the immunofluorescence staining of β -III-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, at least three fields were photographed, and the number of positive cells determined relative to the total number of cells [22].

8.4.7 Histology and Immunohistochemistry

Spinal cord or nerve biopsies of the transplanted mice were excised under anesthesia. Tissues were embedded in tissue-freezing embedding medium (Triangle Biomedical Sciences, Durham, NC) and frozen at -80°C overnight. Frozen sections, $5\ \mu\text{m}$ thick, were cut with a Leica CM1850 cryostat, and were air-dried. The sections were directly observed by fluorescence microscopy. The sections were then used for immunofluorescence (IF) staining of β III-tubulin, GFAP, CNPase, K15, and SMA. The primary antibodies used were: anti- β III-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). Secondary antibodies were 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, in any given experiment at least three fields were photographed, and the number of positive cells determined relative to the total number of GFP-expressing cells. For each mouse, a minimum of three fields of 400x were photographed and analyzed.

8.4.8 Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale

Behavioral analyses of mice with nerves or spinal cords repaired by hfPS cells were conducted for 12 weeks using the BBB locomotor rating scale [42, 43]. Each experimental group consisted of seven mice.

8.4.9 Fluorescence Microscopy

The spinal cord or peripheral nerve in the live mouse, transplanted with GFP-expressing hfPS cells, was directly observed under an Olympus IMT-2 inverted fluorescence microscope equipped with a mercury lamp power supply. The microscope had a GFP filter set (Chroma Technology, Brattleboro, VT).

8.4.10 Statistical Analysis

The experimental data are expressed as the mean \pm SD. Statistical analysis was performed using the two-tailed Student's t test.

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