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EDITORIAL

Multipotent nestin-expressing hair follicle stem cells

HAIR FOLLICLE CYCLICALLY REGENERATES

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

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 originated 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]

IMAGING HAIR FOLLICLE STEM CELLS *IN VIVO*

A breakthrough occurred with the use of transgenic mice in which the neural stem cell marker, nestin, drives the expression of green fluorescent protein (ND-GFP cells). 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.[4] The hair follicle stem cells could then be tracked by their green fluorescence. These relatively small, oval-shaped, ND-GFP-expressing cells in the hair follicle stem cell 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 hair follicle stem cell area but not in the hair matrix bulb (Figs 1 and 2). 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.* subsequently used GFP to isolate hair follicle stem cells in transgenic mice.[5] Fuchs' group also subsequently used GFP to identify hair follicle stem cells and possibly other skin stem cells in transgenic mice.[6,7] Yu *et al.*[8] showed that nestin was present in human hair follicle stem cells confirming our original observation.[4]

Li *et al.*[9] 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.[4]

The evidence that nestin-expressing cells in the hair follicle stem cell area are hair follicle stem cells

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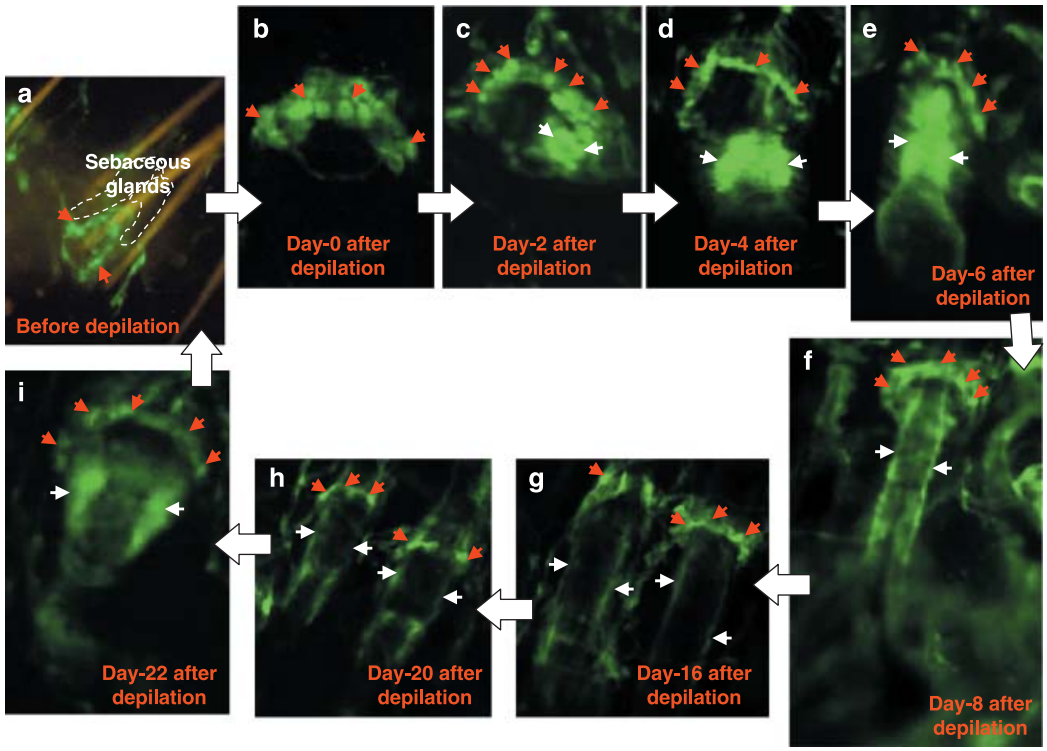

Figure 1. Hair follicle stem cells in the hair-growth cycle.[9] (a,b) ND-GFP hair follicle stem cells (red arrows) are located in the hair follicular stem cell area (the permanent upper hair follicle immediately below the sebaceous glands in the hair follicle stem cell area surrounding the bulge area) in telogen phase. Day 2 (c) and day 4 (d) after anagen induction by depilation. Note the new hair follicle outer-root sheath cells (white arrows) formed directly from the ND-GFP hair follicle stem cells. Day 6 (e) and day 8 (f) after anagen induction by depilation. Note the ND-GFP outer-root sheath cells (white arrows) in the upper two-thirds of the hair follicle. Day 16 (g), day 20 (h) and day 22 (i) after depilation. Note in (g–i) that the hair follicles are in the catagen phase and are undergoing regression and degeneration, including the ND-GFP cells in the outer-root sheath. The hair follicle stem cell area containing ND-GFP stem cells remains.

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.[4] The ND-GFP marker may have enabled the identification and isolation of the most pluripotent cells in the hair follicle stem cell area.

PLURIPOTENCY OF HAIR FOLLICLE STEM CELLS TO FORM NEURONS, GLIA AND OTHER 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.[10]

Recently, Taylor *et al.*[11] 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.[12] Other experiments 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.[13] Toma *et al.* reported that multipotent adult stem cells isolated from mammalian skin dermis, termed skin-derived precursors (SKP), can proliferate and differentiate in

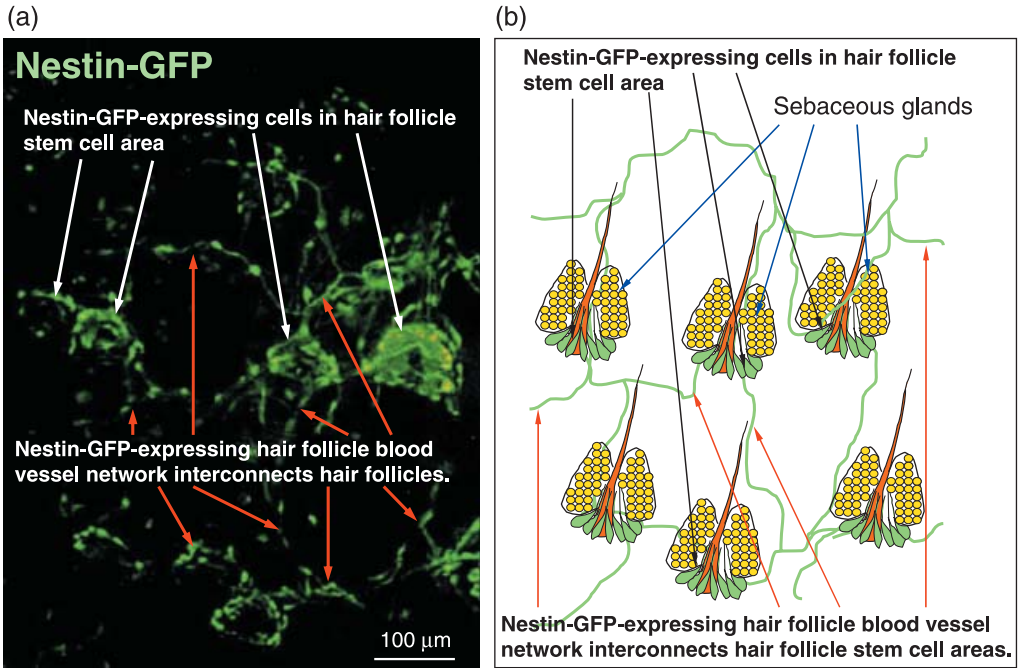

Figure 2. View from the dermis side of the dorsal skin in ND-GFP transgenic mice.[15] (a) ND-GFP cells are visualized in the follicle stem cell area (white arrows) along with blood vessels (red arrows). The ND-GFP blood vessels (red arrows) are connected to ND-GFP hair follicle cells (white arrows). (b) Schematic of telogen hair follicle showing position of ND-GFP hair follicle stem cell areas (black arrows) and blood vessel network (red arrows). The ND-GFP blood vessels (red arrows) are connected to ND-GFP hair follicle cells (white arrows). The hair follicle stem cell area (black arrows) is located beneath the sebaceous gland (blue arrows). (Scale bars, 100 μm.)

culture to produce neurons, glia, smooth muscle cells and adipocytes.[14] However, the exact location of these stem cells in skin is unknown, and their functions are still unclear.

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 (Fig. 2).[15]

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.[16] The ND-GFP hair follicle stem cells may be more primitive than those hair follicle stem cells previously isolated (Figs 3 and 4).[2] These results have subsequently been independently confirmed.[17] Furthermore, we showed that the hair follicle stem cells differentiated into neurons after transplantation to the subcutis of nude mice.[18]

HAIR FOLLICLE STEM CELLS AND 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

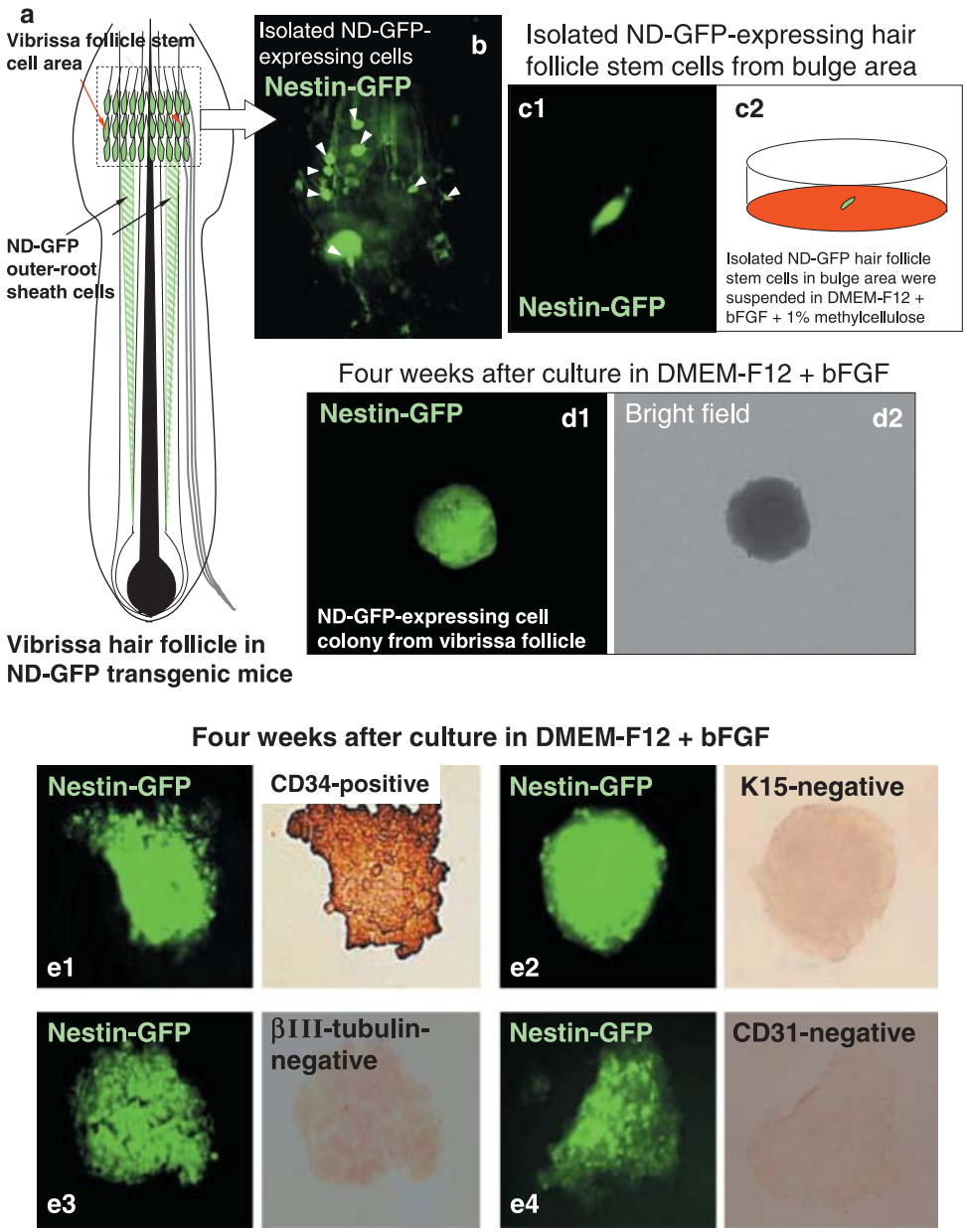

Figure 3. Isolation, culture, and characterization of ND-GFP hair follicle stem cells.[16] (a) Schematic representation of a vibrissa hair follicle in ND-GFP transgenic mice showing the position of the ND-GFP-expressing vibrissa follicle stem cell area (red arrows) and ND-GFP-expressing outer-root sheath cells (black arrows). (b) Isolated ND-GFP-expressing vibrissa follicle stem cell area contains ND-GFP-expressing hair-follicle stem cells (white arrow heads). (c1,c2) The ND-GFP-expressing hair-follicle stem cells in the vibrissa follicle stem cell area were isolated and suspended in Dulbecco's minimum essential medium (DMEM)-F12 containing B-27 and 1% methylcellulose supplemented with basic fibroblast growth factor (bFGF) every 2 days. (d1,d2) After 4 weeks, ND-GFP-expressing hair-follicle stem cells from the vibrissa follicle stem cell area formed the ND-GFP-expressing cell colony. (e) ND-GFP-expressing cells within the colony from the vibrissa follicle stem cell area were CD34-positive (e1), and the ND-GFP-expressing cells within the colony were negative for K15 (e2), III β-tubulin (e3) and CD31 (e4). GFP, green fluorescent protein.

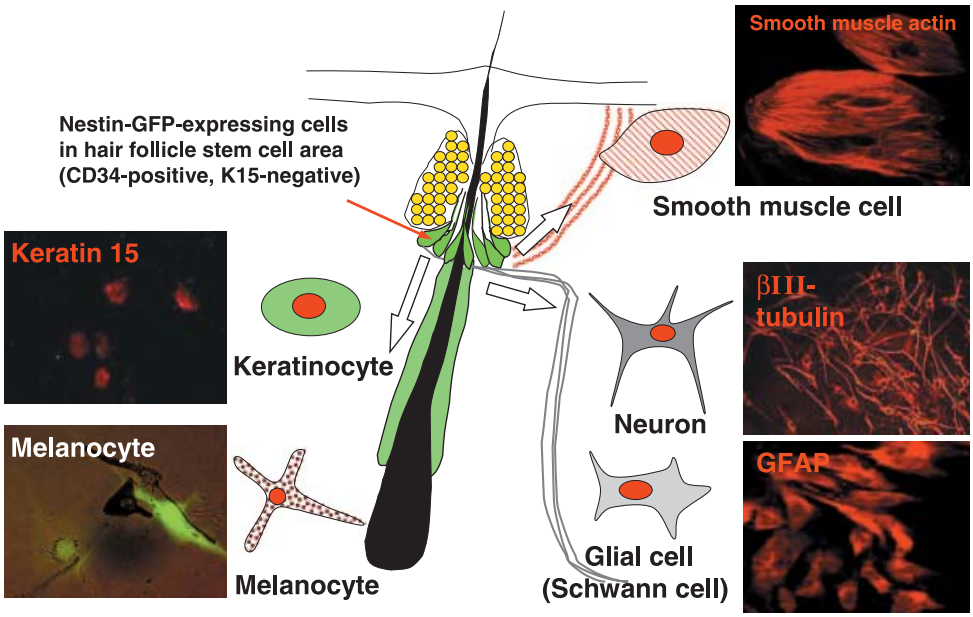

Figure 4. Differentiation of ND-GFP hair follicle stem cells *in vitro*.[16] The ND-GFP-expressing cell colony was switched to RPMI-1640 containing 10% fetal bovine serum (FBS) from Dulbecco's minimum essential medium (DMEM)-F12 containing B-27 and 1% methylcellulose supplemented with basic fibroblast growth factor (bFGF) every 2 days. Two days after switching into RPMI-1640 containing 10% FBS, differentiating cells migrated out of the ND-GFP-expressing cell colony. Seven days after switching to RPMI-1640, many differentiating cells migrated out of the ND-GFP-expressing cell colony. ND-GFP-expressing cells differentiated to III β-tubulin-positive neurons which maintain ND-GFP-expression. Five days after switching to RPMI-1640, ND-GFP-expressing cells differentiated to K15-positive cells. The K15-positive cells still expressed ND-GFP. ND-GFP-expressing cells differentiated to K5/8-positive cells 2 weeks after switching to RPMI-1640. Seven days after switching to RPMI-1640, ND-GFP-expressing cells differentiated to glial fibrillary acidic protein (GFAP)-positive astrocytes. Seven days after switching to RPMI-1640, ND-GFP-expressing cells differentiated to 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)-positive oligodendrocytes. One month after culture in RPMI-1640 containing 10% FBS, ND-GFP-expressing cells differentiated to smooth muscle actin-positive smooth muscle cells. Two months after culture in DMEM-F12 containing B-27 and 1% methylcellulose supplemented with bFGF every 2 days, ND-GFP-expressing cells differentiated to melanocytes containing melanin. Some melanocytes still expressed ND-GFP.

of the gastrocnemius muscle upon electrical stimulation. The transplanted mice recovered the ability to walk normally (Figs 5 and 6).[19]

CONCLUSIONS

We have shown that the hair follicle stem cell area is an abundant easily accessible source of actively growing pluripotent adult stem cells that could serve as a clinical resource in humans. The availability of the ND-GFP mice has enabled the identification, isolation and characterization of these highly pluripotent hair follicle stem 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 regained the ability to walk normally. Recently we have repaired the severed spinal cord by transplantation of hair follicle stem cells which differentiated mostly to Schwann

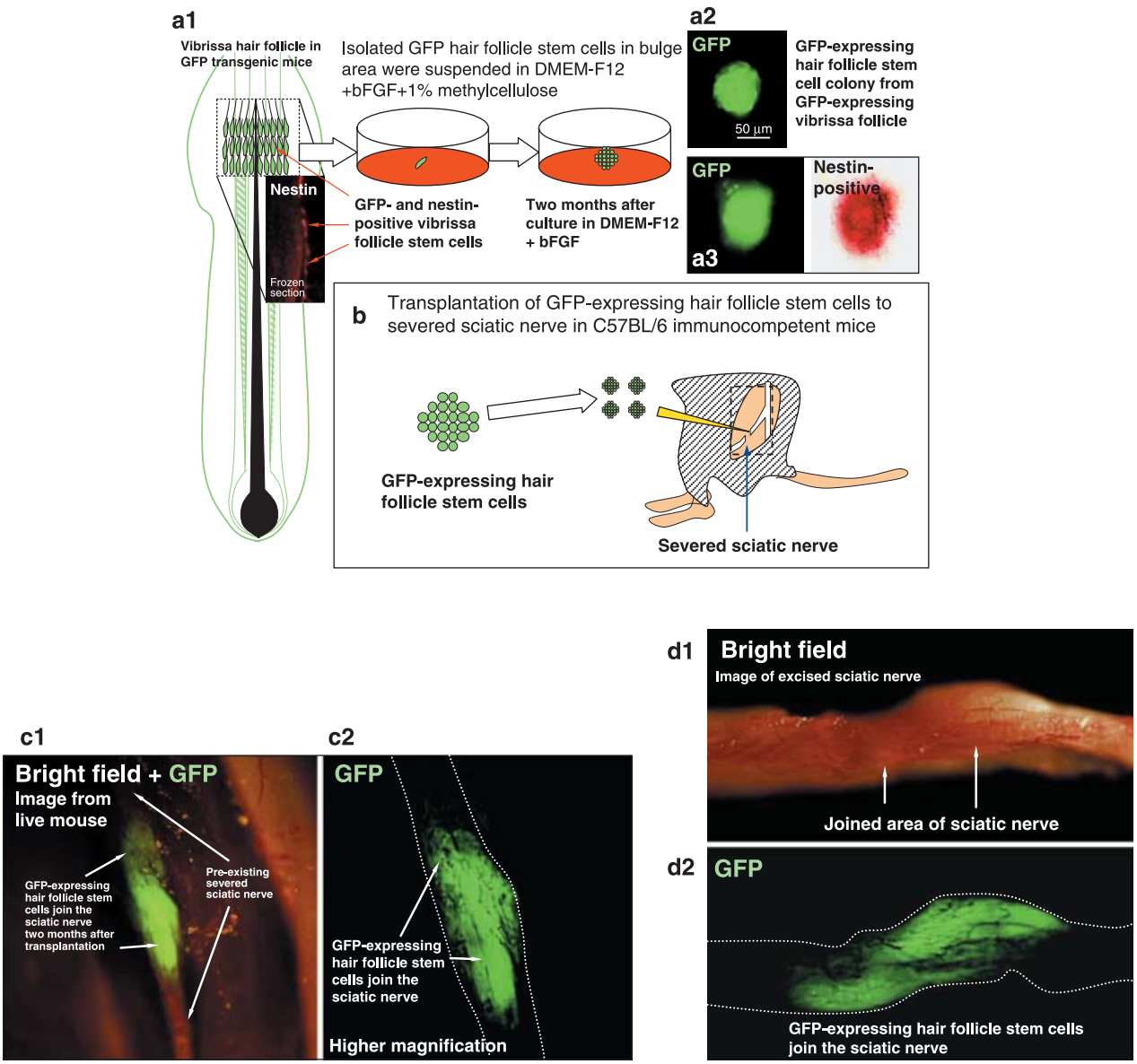

Figure 5. Rejoining severed sciatic nerve with hair follicle stem cells.[19] (a1) Schematic of vibrissa follicle of GFP transgenic mice showing the position of GFP- and nestin-expressing vibrissa follicle stem cell area (red arrows). (a2) Colony formed from GFP-expressing hair follicle stem cells from the vibrissa 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 Dulbecco's minimum essential medium (DMEM)-F12 containing B-27, 1% methylcellulose, and basic fibroblast growth factor (bFGF) were transplanted between the severed sciatic nerve fragments in C57BL/6 immunocompetent mice (blue arrow). (c1,c2) Fluorescence images from a live mouse. Two months after transplantation between the severed sciatic nerve, the GFP-expressing cells joined the severed sciatic nerve. (c2) Higher magnification of (c1). Bright field (d1) and fluorescence (d2) images of an excised sciatic nerve.

cells. The mice recovered most of their hind leg function.[29] Thus, hair follicle stem cells provide an effective, accessible, autologous source of stem cells for treatment of peripheral nerve and spinal cord injury.

The hair follicle stem cells thus have the potential as an alternative to the use of embryonal stem cells or fetal cells for regenerative medicine. The hair follicle stem cells do not have the ethical problems

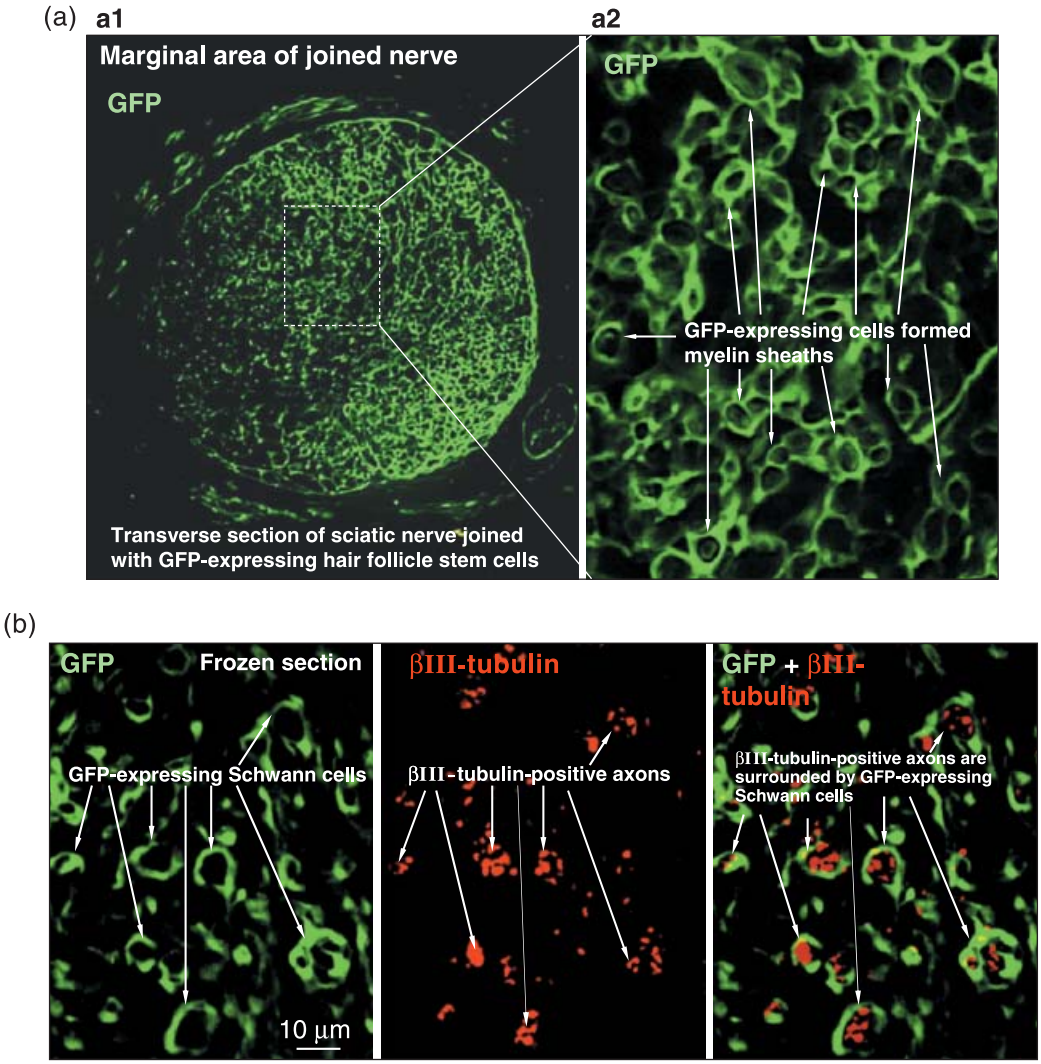

Figure 6. Cell types growing in area of sciatic nerve joined by hair follicle stem cells.[19] GFP-expressing vibrissa hair follicle stem cells were growing in the joined sciatic nerve. (a) Transverse section of joined nerve. In the marginal area of the joined nerve, GFP-expressing cells formed many myelin sheaths (white arrows). (a2) Higher magnification of area of (a1) indicated by the white dashed box. (b) Most of the GFP-expressing vibrissa hair follicle stem cells differentiated to Schwann cells and formed myelin sheaths surrounding axons.

that embryonal or fetal stem cells have. Even more important, the hair follicle stem cells are much more easily accessible than the other stem cell types and offer the potential for autologous treatment, as they can be readily expended in culture after isolation from the patient. The fact that Yu *et al.* 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.[8]

Sieber-Blum *et al.* 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.[20] The location of these cells within the follicle was not determined. Sieber-Blum *et al.* characterized the behavior of implanted neural crest stem cells from the hair follicle in the contusion-lesioned murine spinal cord.[18] 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 β-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 immunofluorence.

Toma *et al.* 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.[21] This laboratory then observed that the SKP could form myelinating Schwann cells when injected into the injured sciatic nerve,[22] which is similar to our earlier results with hair follicle stem cells.[19] The same laboratory then showed that SKP could promote spinal cord repair. The SKP were released from skin by collagenase treatment of the skin which produced a mixture of cells.[23] The origin of the SKP within the skin is thus unclear. In contrast, our results presented here show that the hair follicle stem cells, a defined population, can functionally repair nerves and the spinal cord. It should also be noted that our studies, as well as the studies with SKP, used fluorescent proteins to track the transplanted cells, a technology pioneered in our laboratory.[24]

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 hematopoietic system,[25] can differentiate into adipogenic and osteogenic lineages,[26] and participate in wound healing and induction.[27]

Hair follicle stem cells also have great potential for hair regeneration.[1,28]

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REFERENCES

1 Cotsarelis G. Epithelial stem cells: a folliculocentric view. *J Invest Dermatol* 2006; **126**: 1459–1468.
2 Cotsarelis G, Sun Tt, Lavker Rm. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 1990; **61**: 1329–1337.
3 Ito M, Yang Z, Andl T *et al*. Wnt-dependent de novo hair follicle regeneration in adult mouse skin after wounding. *Nature* 2007; **447**: 316–320.
4 Li L, Mignone J, Yang M *et al*. Nestin expression in hair follicle sheath progenitor cells. *Proc Natl Acad Sci USA* 2003; **100**: 9958–9961.
5 Morris RJ, Liu Y, Marles L *et al*. Capturing and profiling adult hair follicle stem cells. *Nat Biotechnol* 2004; **22**: 411–417.
6 Tumbar T, Guasch G, Greco V *et al*. Defining the epithelial stem cell niche in skin. *Science* 2004; **303**: 359–363.
7 Rhee H, Polak L, Fuchs E. Lhx2 maintains stem cell character in hair follicles. *Science* 2006; **312**: 1946–1949.
8 Yu H, Fang D, Kumar SM, *et al*. Isolation of a novel population of multipotent adult stem cells from human hair follicles. *Am J Pathol* 2006; **168**: 1879–1888.
9 Li J, Greco V, Guasch G, Fuchs E, Mombaerts P. Mice cloned from skin cells. *Proc Natl Acad Sci USA* 2007; **104**: 2738–2743.
10 Blanpain C, Lowry We, Geoghegan A, Polak L, Fuchs E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 2004; **118**: 635–648.
11 Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell* 2000; **102**: 451–461.
12 Ito M, Liu Y, Yang Z *et al*. Stem cells in the hair follicle bulge contribute to wound repair but not to homeostasis of the epidermis. *Nat Med* 2005; **11**: 1351–1354.
13 Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell* 2001; **104**: 233–245.
14 Toma JG, Akhavan M, Fernandes JL *et al*. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001; **3**: 778–784.
15 Amoh Y, Li L, Yang M *et al*. Nascent blood vessels in the skin arise from nestin-expressing hair follicle cells. *Proc Natl Acad Sci USA* 2004; **101**: 13291–13295.
16 Amoh Y, Li L, Katsuoka K, Penman S, Hoffman RM. Multipotent nestin-positive, keratin-negative hair-follicle-bulge stem cells can form neurons. *Proc Natl Acad Sci USA* 2005; **102**: 5530–5534.
17 Mignone JL, Roig-Lopez JL, Fedtsova N *et al*. Neural potential of a stem cell population in the hair follicle. *Cell Cycle* 2007; **6**, 2161–2170.
18 Sieber-Blum M, Schnell L, Grim M, Hu YF, Schneider R, Schwab ME. Characterization of epidermal neural crest stem cell (EPI-NCSC) grafts in the lesioned spinal cord. *Mol Cell Neurosci* 2006; **32**, 67–81.

19 Amoh Y, Li L, Campillo R *et al*. Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves. *Proc Natl Acad Sci USA* 2005; **102**: 17734–17738.
20 Sieber-Blum M, Grim M, Hu YF, Szeder V. Pluripotent neural crest stem cells in the adult hair follicle. *Dev Dyn* 2004; **231**, 258–269.
21 Toma JG, Akhavan M, Fernandes KJ *et al*. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001; **3**, 778–784.
22 McKenzie IA, Biernaskie J, Toma JG, Midha R, Miller FD. Skin-derived precursors generate myelinating Schwann cells for the injured and dysmyelinated nervous system. *J Neurosci* 2006; **26**, 6651–6660.
23 Biernaskie J, Sparling JS, Liu J *et al*. Skin-derived precursors generate myelinating Schwann cells that promote remyelination and functional recovery after contusion spinal cord injury. *J Neurosci* 2007; **27**, 9545–9559.
24 Hoffman RM. The multiple uses of fluorescent proteins to visualize cancer *in vivo*. *Nature Reviews Cancer* 2005; **5**, 796–806.
25 Lako M, Armstrong L, Cairns PM, Harris S, Hole N, Jahoda CA. Hair follicle dermal cells repopulate the mouse haematopoietic system. *J Cell Sci* 2002; **115** (Pt 20): 3967–3974.
26 Jahoda CA, Whitehouse J, Reynolds AJ, Hole N. Hair follicle dermal cells differentiate into adipogenic and osteogenic lineages. *Exp Dermatol* 2003; **12**: 849–859.
27 Gharzi A, Reynolds AJ, Jahoda CA. Plasticity of hair follicle dermal cells in wound healing and induction. *Exp Dermatol* 2003; **12**: 126–136.
28 Hoffman RM. The hair follicle as a gene therapy target. *Nat Biotechnol* 2000; **18**, 20–21.
29 Amoh Y, Li L, Katsuoka K, Hoffman RM. Multipotent hair follicle stem cells promote repair of spinal cord injury and recovery of walking function. *Cell Cycle* 2008; **7**, 1865–1869.

