Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves

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The hair follicle bulge area is an abundant, easily accessible source of 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. The fluorescent protein GFP, whose expression is driven by the nestin regulatory element in transgenic mice, served to mark the follicle cell fate. 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, 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. Equivalent hair follicle stem cells derived from transgenic mice with β-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. These results suggest that hair follicle stem cells provide an important, accessible, autologous source of adult stem cells for regenerative medicine.

green fluorescent protein | nerve regeneration | nestin | glial cell | bulge area

Considerable recent interest has focused on adult stem cells for both research and clinical applications. Such cells can circumvent some of the problems associated with embryonic stem cells, such as immunologic incompatibility. However, most adult stem cells are relatively sparse and in indeterminate locations and growth states. A highly promising source of relatively abundant and accessible, active, pluripotent adult stem cells is afforded by hair follicles. Hair growth is a unique, cyclic regeneration phenomenon and the hair follicle undergoes repeated cycles of periods of growth (anagen), regression (catagen), and rest (telogen) throughout the life of mammals (1). The follicle bulge region contains cells that are, by several criteria, true stem cells. During the anagen phase of the hair growth cycle, the bulge stem cells periodically differentiate into all of the follicle cell types including the outer-root sheath, hair matrix cells, and inner-root sheath as well as sebaceous-gland basal cells, and epidermis (2, 3). In response to wounding, some cells exit the follicle, migrate and proliferate to repopulate the infundibulum and epidermis (4). Morris *et al.* (5) used a keratinocyte promoter to drive GFP expression in the hair-follicle bulge cells. They showed that bulge cells in adult mice generate all epithelial cell types within the intact follicle and hair during normal hair-follicle cycling. Toma *et al.* (6) reported that multipotent adult stem cells isolated from mammalian skin dermis, termed skin-derived precursors, can proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells, and adipocytes. However, the exact location of the skin-derived precursors was not identified.

We have previously reported that nestin, a marker for neural progenitor cells, also is expressed in stem cells of the hair follicle bulge, suggesting a potential relationship between the cell types (7). We initially used nestin-driven-GFP (ND-GFP) transgenic mice to trace the fate of follicle stem cells *in vivo* during the hair cycle (7). Subsequently, we observed that blood vessels in the skin express ND-GFP and originate from hair-follicle cells during the anagen phase in the ND-GFP mice (8). The ND-GFP blood vessels interconnect the hair follicles and contribute to vascularizing the dermis. The follicular origin of the blood vessels was most evident when transplanting ND-GFP-labeled follicles to unlabeled nude mice. In the transplanted mice, fluorescent new blood vessels originate only from the ND-GFP-labeled follicles and can respond to presumptive angiogenic signals from healing wounds (8). These studies suggested the potential of hair follicle stem cells to form diverse cell types.

A very extensive characterization of cells grown from hair follicle bulge explants was presented by Sieber-Blum *et al.* (9). These authors showed that neural crest cells grew out when the bulge was explanted, resulting in differentiation to a variety of cell types including neurons, smooth muscle cells, rare Schwann cells, and melanocytes. However, the differentiated cell types obtained by *in vitro* culture could be heavily skewed by morphogenetic signals. Thus, exposure to bone morphogenetic protein-2 largely produced chondrocytes, whereas neuregulin-1 directed differentiation to Schwann cells, an observation relevant to the nerve regrowth studies reported in the present study (9). In a recent study, we started with isolated bulge hair follicle stem cells expressing the ND-GFP marker (10). These cells appear primitive in that they express the stem cell marker CD34 but do not express the keratinocyte marker keratin-15. We showed that these ND-GFP stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes *in vitro* (10). Furthermore, we showed that the ND-GFP-expressing stem cells can extensively differentiate into neurons after transplantation to the subcutis of nude mice (10). In the present study, we show that transplanted hair follicle stem cells can enhance the regrowth of severed sciatic and tibial nerves in immunocompetent mice and are therefore candidate adult stem cells for widespread individualized application in regenerative medicine.

Conflict of interest statement: No conflicts declared.

Abbreviation: ND-GFP, nestin-driven-GFP.

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Materials and Methods

GFP-Expressing Transgenic Mice (Green Mice). Transgenic C57/B6-GFP mice were obtained from 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.

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 with 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) stainings.

GFP-Expressing, Hair Follicle Stem Cells Cultured from Isolated Vibrissa Follicle Bulge Area. 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 vibrissa follicle bulge area was isolated from the vibrissa follicles. The GFP-expressing vibrissa follicular bulge-area cells 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 very 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% CO^2/95% air tissue-culture incubator. After 4 weeks, GFP-expressing vibrissa follicle bulge-area 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.

Immunofluorescence Staining and Quantification. Immunofluorescence staining of cells that differentiated from GFP-expressing hair follicle stem cell colonies and of the frozen mouse vibrissa follicle sections was performed. The primary antibodies that were used were: monoclonal anti-β-III-tubulin (1:500, Tuj1 clone; Covance Research Products, Berkeley, CA), polyclonal anti-GABA (1:200; Chemicon), monoclonal anti-glial fibrillary acidic protein (1:200; Lab Vision, Fremont, CA), monoclonal anti-2′-3′-cyclic nucleotide 3′-phosphodiesterase (1:50; Lab Vision), monoclonal anti-K15 (1:100; Lab Vision), and monoclonal anti-smooth muscle actin (1:200; Lab Vision). Secondary antibodies were Alexa Fluor 568 goat anti-mouse (1:200; Molecular Probes) and 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 was determined relative to the total number of cells.

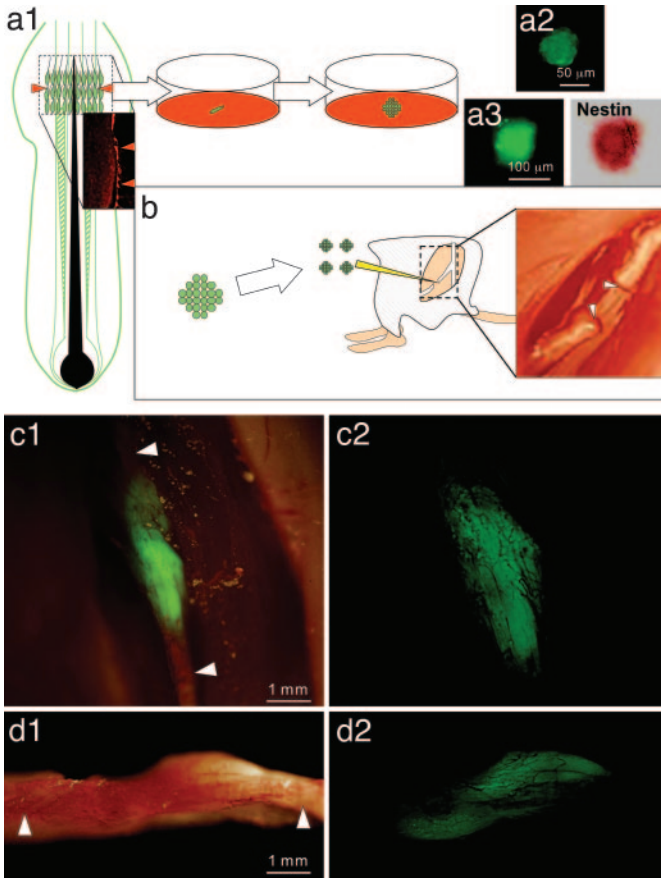

Fig. 1. Rejoining severed sciatic nerve with hair follicle stem cells. (*a1*) Schematic of vibrissa follicle of GFP transgenic mice showing the position of GFP- and nestin-expressing vibrissa follicle bulge area (red arrowheads). (*a2*) Colony formed from GFP-expressing hair follicle stem cells from 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 two months in DMEM-F12 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 joined 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 preexisting sciatic nerve is denoted by white arrowheads.

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 μm thick were cut with a Leica CM1850 cryostat and air dried. The sections were first directly observed

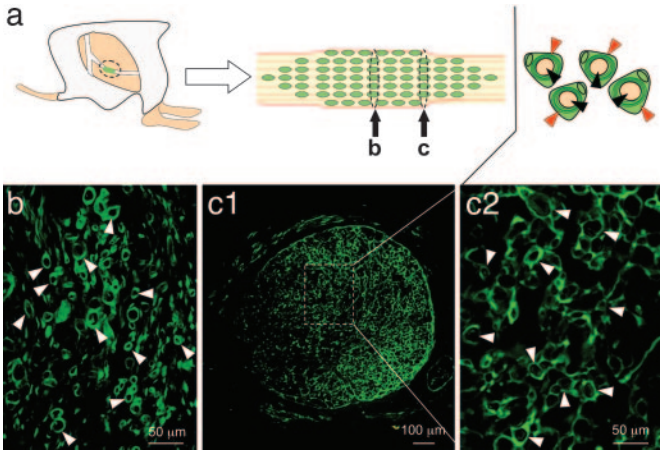

Fig. 2. Cell types growing in area of sciatic nerve joined by hair follicle stem cells. (*a*) GFP-expressing vibrissa hair follicle stem cells were growing in the joined sciatic nerve. Most of the GFP-expressing vibrissa hair follicle stem cells differentiated to Schwann cells and formed myelin sheaths surrounding axons (red arrowheads). The axons are denoted by black arrowheads. (*b*) Transverse section of joined nerve. In the central area of the joined nerve, GFP-expressing cells formed many small myelin sheaths (white arrowheads). (*c1*) In the marginal area of the joined nerve, GFP-expressing cells formed many myelin sheaths (white arrowheads). (*c2*) Higher magnification of area of *c1* indicated by the white dashed box.

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.

Fluorescence Microscopy. The sciatic nerve in the live mouse and the excised sciatic nerve were directly observed under an Olympus IMT-2 inverted microscope equipped with a mercury lamp power supply. The microscope had a GFP filter set (Chroma Technology, Brattleboro, VT).

Sciatic Nerve Stimulation with an Electric Stimulator. 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 m/sec was used to stimulate control mice, mice with severed sciatic nerves, and mice that had GFP hair follicle stem cells injected to join the severed nerve. The gastrocnemius muscle lengths were measured, and the difference of the gastrocnemius muscle lengths (from lateral epicondyle of femur to heel) before and after contraction by the electric stimulator was calculated. Each experimental group consisted of 10 mice.

Walking Track Analysis. Walking tracks were obtained by using a 6- × 44-cm corridor open at one end to a darkened compartment (11). The animal's feet were soaked in Higgins7 black waterproof ink (Sanford, Bellwood, IL), and the animal was walked multiple times to obtain measurable prints. The tracks were evaluated for print length and intermediate toe spread. Multiple linear regression analysis was performed with factors derived from each of the parameters. Factors were 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 (11). Each experimental group consisted of seven mice. Groups included control mice, mice with a severed tibial nerve only, and mice with the tibial nerve enjoined by injected hair follicle stem cells.

Table 1. Cell differentiation of GFP-expressing hair follicle stem cells

Cell type	From vibrissa follicular bulge area *in vitro*, %	In severed sciatic fragments, %
Neurons	51 ± 6*	8 ± 4
Glial cells	34 ± 8*	84 ± 5
Keratinocytes	10 ± 10†	4 ± 5
Smooth muscle cells	4 ± 2‡	0
Melanocytes	1 ± 1§	0

Values indicate the percentage of GFP-expressing hair follicle stem cells that differentiated into the indicated cell types under the following conditions: *, 7 days after being switched into RPMI medium 1640 containing 10% FBS; †, 2 weeks after being switched into RPMI medium 1640 containing 10% FBS; ‡, 1 month after being switched into RPMI medium 1640 containing 10% FBS; §, 2 months after culture in DMEM-F12 plus basic FGF. Also shown are the percentages of cell types resulting 2 months after transplantation of GFP-expressing hair follicle stem cells between severed sciatic nerve fragments of C57BL/6 mice.

$$PL\ \text{factor} = \frac{EPL - NPL}{NPL} \qquad IT\ \text{factor} = \frac{NIT - EIT}{NIT},$$

where *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.

Statistical Analysis. The experimental data are expressed as the mean ± SD. Statistical analysis was performed by using a two-tailed Student *t* test.

Results and Discussion

Transplanted Hair Follicle Stem Cells Foster Regrowth of the Severed Sciatic Nerve. The GFP-expressing vibrissa follicular bulge area stem cells were isolated and suspended in DMEM-F-12 containing B-27 and 1% methylcellulose. The cultures were supplemented with basic FGF every 2 days. After 2 months, GFP-expressing, hair follicle stem cells from the vibrissa follicular bulge area formed colonies (Fig. 1). GFP-expressing cells within the colony were nestin- and CD34-positive and K15- and β-III-tubulin-negative. These data suggest that these cells were primitive stem cells.

The GFP-expressing, hair follicle stem cell colonies were then

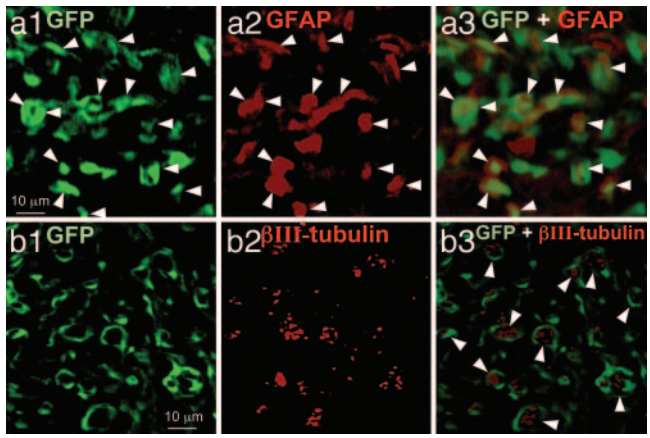

Fig. 3. Cell types growing in joined part of sciatic nerve. (*a1–a3*) GFP-expressing hair follicle stem cells differentiated to glial fibrillary acidic protein-positive Schwann cells after injection between the fragments of the severed sciatic nerve (white arrowheads). (*b1–b3*) GFP-expressing Schwann cells formed myelin sheaths and surrounded β-III-tubulin-positive axons (white arrowheads).

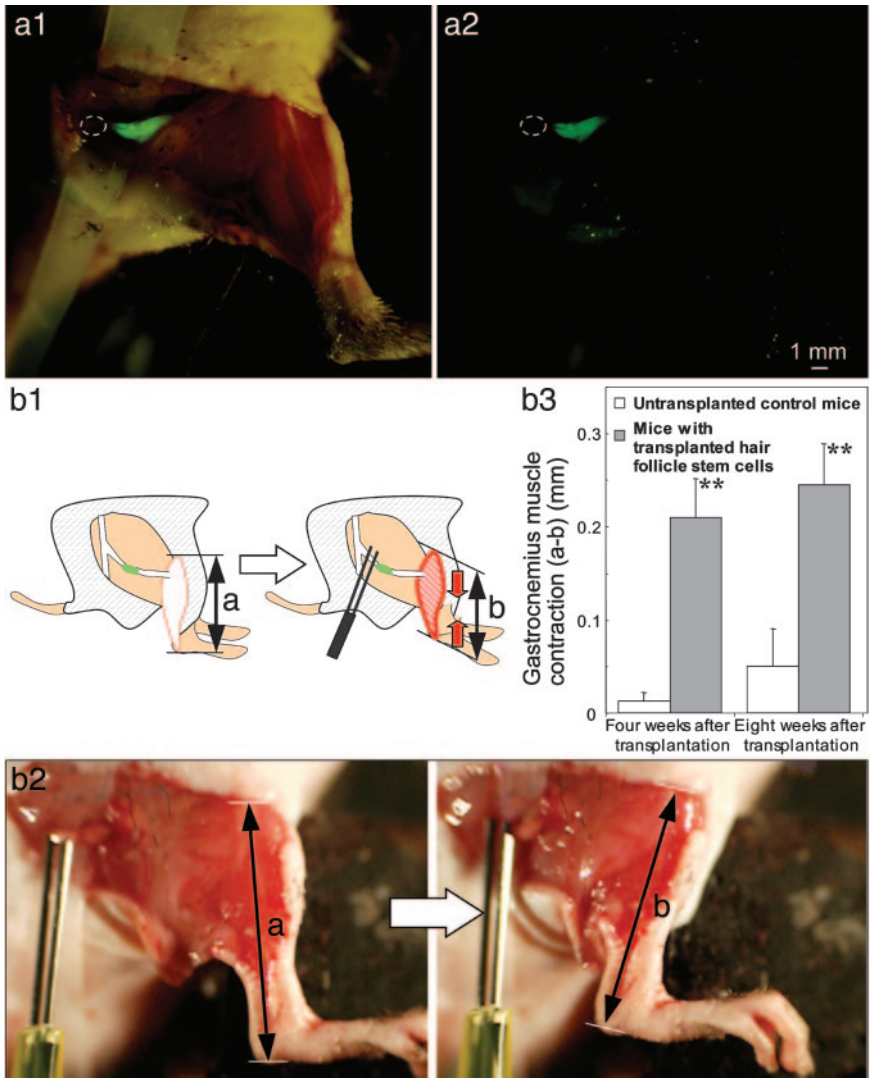

Fig. 4. Electrical stimulation of rejoined sciatic nerve. (*a*) Four weeks after transplantation of GFP-expressing hair follicle stem cells between the severed sciatic nerve fragments, the rejoined sciatic nerve contracted the gastrocnemius muscle upon electrical stimulation. The sciatic nerve was stimulated above where the nerve was severed (white dashed area). (*a1*) Brightfield and fluorescence. (*a2*) Fluorescence. (*b1*and *b2*) Electrical stimulation of the sciatic nerve above where the nerve was severed after the nerve was rejoined by hair follicle stem cells. (*Left*) Before electrical stimulation. (*Right*) After electrical stimulation. (*b3*) Comparison of the extent of gastrocnemius muscle contraction in transplanted and untransplanted control mice. **, $P < 0.01$ vs. control.

transplanted between the severed sciatic nerve fragments in immunocompetent C57BL/6 mice. Two months after transplantation, the GFP-expressing cells joined the severed sciatic nerve. GFP-expressing cells were visualized growing in the joined sciatic nerve (Fig. 1). Blood vessels formed a network around the joined sciatic nerve.

Many of the GFP-expressing vibrissa, hair follicle stem cells differentiated to glial fibrillary acidic protein-positive Schwann cells and formed a myelin sheath surrounding axons while the severed sciatic nerve regrew (Fig. 2). In the central and marginal area of the joined nerve, GFP-expressing Schwann cells formed many small myelin sheaths. The myelin sheaths surrounded unlabeled, host β-III-tubulin-positive axons (Table 1 and Fig. 3).

Rejoined Sciatic Nerve Contracts the Gastrocnemius Muscle upon Electrical Stimulation. Upon recovery of function by the severed sciatic nerve in the presence of hair follicle stem cells, the gastrocnemius muscle contracted with electric stimulation (Fig. 4). The degree of contraction was >0.2 mm (Fig. 4). In contrast, in all mice with a severed sciatic nerve without the hair follicle stem cell transplantation, the gastrocnemius muscles did not contract at all upon electrical stimulation. Thus, the hair follicle stem cell transplantation stimulated recovery of the sciatic nerve, enabling gastrocnemius muscle contraction.

Rejoined Tibial Nerve Enabled Mice to Walk Normally. Walking tracks prepared by using a 6- × 44-cm corridor open at one end to a darkened compartment were evaluated for print length and intermediate toe spread. Multiple linear regression analysis was performed with factors derived from each of the parameters. Factors were derived for each parameter by subtracting the normal value (left side measurements) from the experimental value (right side measurements) and dividing by the normal value. After 6–12 weeks, hair follicle stem cell transplantation enabled in the mice an increasing ability to walk normally as determined by walking print length (Fig. 5). Intermediate toe spread also continually recovered 6–12 weeks after hair follicle stem cell transplantation (Fig. 5).

Some insight into the function of the implanted Schwann cell progenitors is afforded by a previous report (12). After injury to a peripheral nerve, axonal elongation may occur (13). Thus, periph-

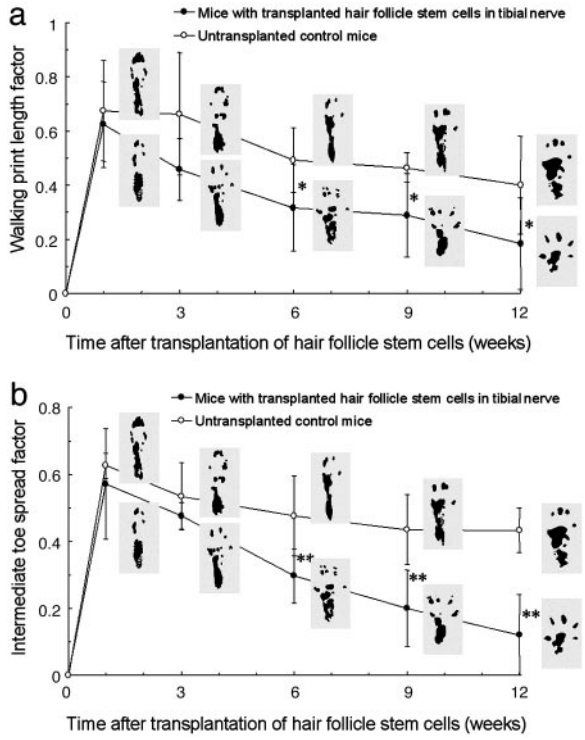

Fig. 5. Walking track analysis of GFP-expressing hair follicle stem cell transplantation in the tibial transection. Walking tracks were prepared as previously described using a 6- × 44-cm corridor open at one end to a darkened compartment. The tracks were evaluated for print length and intermediate toe spread. (*a*) After 6, 9, and 12 weeks, stem cell transplantation enabled the walking print length factor to recover as compared with untransplanted controls. (*b*) After 6, 9, and 12 weeks, stem cell transplantation enabled the intermediate toe spread factors to recover as compared with untransplanted controls. *, $P < 0.05$; **, $P < 0.01$ vs. control (without transplantation).

eral nerves appear to have some spontaneous regenerative capacity. A real-time imaging study of axonal regeneration was carried out in transgenic animals in which yellow fluorescent proteins was driven by the human Thy-1 promoter, where motor and sensory nerves fluoresce brightly yellow (13). In grafting experiments, Schwann cells introduced along the graft promote and guide regeneration of peripheral axons (14). In a rat model of transection of the sciatic nerve, GFP-expressing Schwann cells were seeded in a resorbable nerve conduit for grafting. The Schwann cells stimulated signifi cantly better axonal regenerative distance in comparison with empty conduits (14). In the present experiment, it appears that the primary cell type produced by the hair follicle stem cells are glial (Schwann) cells after injection to the area of the sciatic nerve transection. These Schwann cells appear to produce myelin sheaths that surround nonlabeled host axons. Therefore, by differentiating into Schwann cells, the hair follicle stem cells may stimulate the host axons to extend and, thus, to fill the transection gap.

Cell-replacement therapies show particular promise in the nervous system, where transplanted embryonic or bone-marrow stem cells have been shown to promote functional recovery in animal models of, for example, spinal cord or peripheral nerve injury (12, 15). However, although the therapeutic potential of such transplants is clear, a number of problems remain. In particular, use of fetal tissue raise ethical issues. Moreover, the use of heterologous human tissue requires immunosuppression, which is particularly problematic in individuals with long-term, neuron-specific problems. In this regard, the fact that hair follicle stem cells are generated from an autologous and accessible adult tissue source, skin, and that they can readily generate neural-specific cell types provides a potential solution to these problems.

Conclusion

In the present study, we showed that injection of hair follicle stem cells resulted in the recovery of severed sciatic nerves, which could then contract the gastrocnemius muscle upon electric stimulation. Six weeks after hair follicle stem cell transplantation to the severed tibial nerve, walking print length and intermediate toe spread significantly recovered. These results suggest that hair follicle stem cells promote axonal growth and functional recovery after peripheral nerve injury. We propose that hair follicle stem cells offer an important opportunity for the clinical treatment of peripheral nerve diseases.

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