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Report

Multipotent hair follicle stem cells promote repair of spinal cord injury and recovery of walking function

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The mouse hair follicle is an easily accessible source of actively growing, pluripotent adult stem cells. C57BL transgenic mice, labeled with the fluorescent protein GFP, afforded follicle stem cells whose fate could be followed when transferred to recipient animals. These cells appear to be relatively undifferentiated since they are positive for the stem cell markers nestin and CD34 but negative for the keratinocyte marker keratin 15. These hair follicle stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes in vitro. Implanting hair follicle stem cells into the gap region of severed sciatic or tibial nerves greatly enhanced the rate of nerve regeneration and restoration of nerve function. The transplanted follicle cells transdifferentiated mostly into Schwann cells, which are known to support neuron regrowth. The treated mice regained the ability to walk essentially normally. In the present study, we severed the thoracic spinal chord of C57BL/6 immunocompetent mice and transplanted 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 promising treatment of peripheral nerve and spinal cord injury.

Introduction

The hair follicle is a remarkably active miniature organ. It undergoes repeated cycles of periods of growth (anagen), regression (catagen) and rest (telogen) throughout the life of mammals.[1] The follicle bulge region contains progenitor (stem) cells that repopulate the hair follicle during the anagen phase of the hair growth cycle. At this time, the bulge stem cells 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 follicle infundibulum and surrounding epidermis.[4] Morris et al.,[5] used a keratinocyte promoter to drive green fluorescent protein (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, while the exact source of the skin-derived precursors was not identified, it is possible they originated in the hair follicles.

We have previously reported that nestin, a marker for neural progenitor cells, is also expressed in stem cells of the hair follicle. This suggested the existence of hair follicle stem cells pluripotent for neurons and as well as other cells types.[7] We first 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] Transplanting ND-GFP-labeled follicles to unlabeled nude mice clearly showed the follicular origin of new blood vessels.

We isolated and characterized hair follicle stem cells expressing the ND-GFP marker.[9] These cells express the stem cell marker CD34 but not the keratinocyte marker keratin-15 suggesting they are primitive. We showed that these ND-GFP stem cells could differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes when cultured in vitro.[9,10] These results have recently been independently confirmed.[11] In a subsequent study, we showed that transplanted hair follicle stem cells could enhance the regrowth and functional rejoining of severed sciatic and tibial nerves in immunocompetent mice.[12]

Yu et al.,[13] isolated a population of stem cells from human hair follicles that express nestin, proliferate as spherical aggregates and can differentiate into multiple lineages. However, the location of these stem cells was not clear in this study.

Sieber-Blum et al.,[14] 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.,[15] characterized

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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, the Schwann-cell marker glial fibrillary acidic protein (GFAP) was not detected by immunofluorence.

We report here that injected multipotent mouse hair follicle stem cells can enhance the rejoining of the severed spinal cord. This is effected, in large part, by their differentiation into Schwann cells, which apparently promote axon regrowth and, thus, partial recovery of hind-leg function.

Results and Discussion

Differentiated state of hair follicle stem cells after two months in culture. GFP-expressing hair follicle stem cells from the vibrissa follicle, cultured in vitro for 2 months, formed colonies (spheres). The cells are nestin- and CD34-positive[9,12] and K15- and βIII-tubulin-negative[9,12] indicating they are undifferentiated stem cells.

Spinal cord repair after follicle stem cell implantation. The GFP-expressing hair follicle stem cells were implanted between the severed thoracic region of spinal cord in C57BL/6 immunocompetent mice (Fig. 1). Two months after transplantation, GFP-expressing cells had migrated into the joined region of the previously severed thoracic region of the spinal cord (Fig. 1C). Longitudinal frozen sections offer another view of the GFP-expressing cells growing in the joined thoracic region of spinal cord (Fig. 2A). Most of the GFP-expressing stem cells differentiated to GFAP-, and CNPase-positive Schwann cells (Fig. 2B and C, Table 1). The GFP-expressing Schwann cells formed myelin sheaths, which surrounded the host βIII-tubulin-positive axons (Fig. 3).

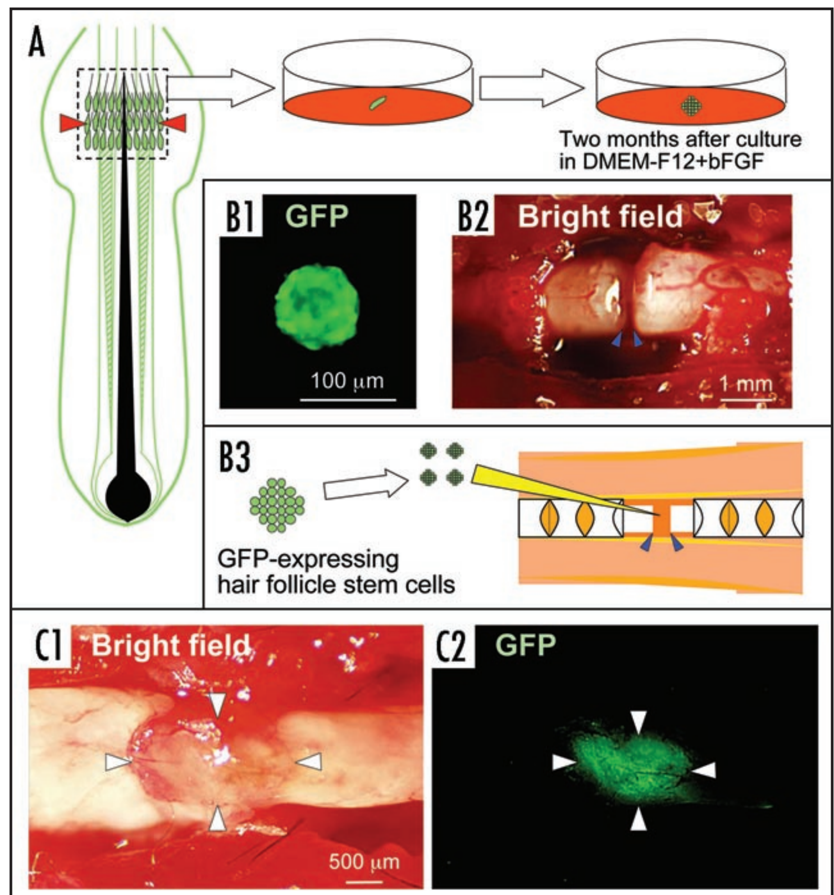

Figure 1. Rejoining the severed thoracic region of the spinal cord with hair-follicle stem cells. (A) Schematic of vibrissa follicle of GFP-transgenic mice shows the position of GFP-expressing vibrissa follicle stem cells (red arrowheads). The GFP-expressing stem cells from the vibrissa follicle were cultured for two months in DMEM-F12 containing B-27 supplemented with bFGF every two days. (B1) Colony formed from GFP-expressing vibrissa follicle stem cells. (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, the spinal cord rejoined (white arrowheads). (C1) Brightfield, (C2) GFP.

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Degree of functional recovery after stem cell implantation. Between 6–12 weeks after the hair follicle stem-cell transplantation, the mice recovered significant hind-limb function as reflected in their BBB locomotor rating scale compared to the untransplanted control mice (Fig. 4, see Suppl. Movies 1 and 2).

The recent upsurge of interest in hair follicle biology has revealed a surprising complexity of functions and cell types in the hair follicle.[2,3,15-17] We showed that hair follicle stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells and melanocytes.[9]

We previously showed that transplanted hair follicle stem cells facilitated the joining of the severed sciatic nerve.[12] After implantation into the severed region of the sciatic nerve, hair follicle stem cells differentiated into GFAP- and CNPase-positive Schwann cells. These formed a myelin sheath surrounding growing axons in the nerve.[12]

In the present study, we implanted the GFP-expressing hair follicle stem cells between the severed thoracic regions of spinal cord. Many of the GFP-expressing hair follicle stem cells differentiated into GFAP- and CNPase-positive Schwann cells, which facilitated joining the severed spinal cord in C57BL/6 immunocompetent mice. Pearse et al.,[18] reported that Schwann cells promote axonal growth and functional recovery after spinal cord injury. These results suggest that the nestin-expressing stem cells from hair follicles differentiated into immature Schwann cells that promote axonal growth and recovery of function after spinal cord injury.

Sieber-Blum et al.[15] have transplanted neural crest cells derived from the spinal cord to spinal-cord-injured mice. However, there was no detected recovery of function. Sieber-Blum et al.,[15] found that the hair follicle-associated neural crest cells did not express the Schwann-cell marker GFAP when implanted in the spinal cord: a result that differs from ours.

This apparent puzzle is probably due to different cell types transplanted by Sieber-Blum et al.,[15] compared to the cell types we transplanted to the lesioned spine in the present study. 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

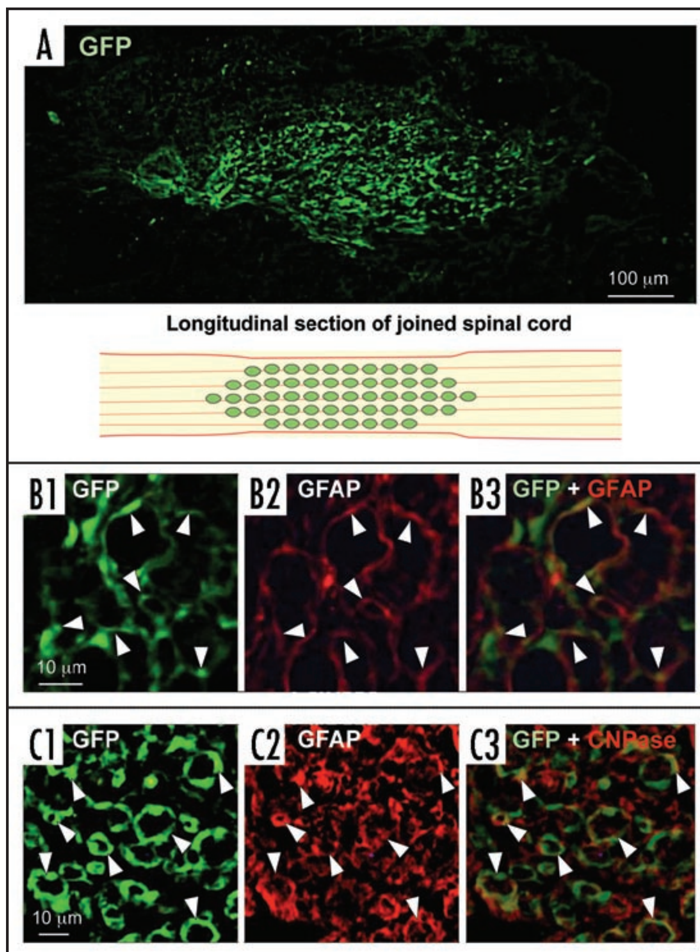

Figure 2. GFP-expressing cell types growing in the joined area of the thoracic region of the spinal cord implanted with hair-follicle stem cells. (A) GFP-expressing hair follicle stem cells were growing in the thoracic region of the spinal cord of C57BL/6 immunocompetent mice (frozen longitudinal section and diagram of transplanted cell growing in the spinal cord). (B and C) Most of the GFP-expressing stem cells differentiated to GFAP- and CNPase-positive Schwann cells after transplantation (white arrowheads).

Table 1 **GFP-expressing cell types growing in the thoracic region of the spinal cord joined by injected GFP hair-follicle stem cells**

Cell type	Percentage of differentiated GFP-expressing cells
Glial cells	82 ± 5%
Neurons	9 ± 4%
Keratinocytes	7 ± 2%
Smooth muscle cells	0%
Melanocytes	0%

the surface of the culture dish. Glial (Schwann cell) 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.,[15] 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.[6] reported that multipotent adult stem cells isolated from mammalian skin dermis, termed skin-derived precursors (SKPs), 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 laboratory then observed that the SKPs could form myelinating Schwann cells when injected into the injured sciatic nerve[19] which is similar to our earlier results with hair follicle stem cells.[12] 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.[20] The origin of SKPs 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 the severed spinal cord. It should also be noted that out studies as well as the studies with SKPs used fluorescent proteins to track the transplanted cells, a technology pioneered in our laboratory.[21-25]

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.[26] 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.[27,28] 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, pluripotent stem cells have been derived from skin and other organs by gene transfer.[29-31] However, the vectors used for gene transfer have made these stem cells potentially malignant. In this regard, nestin-expressing hair follicle stem cells are available from autologous, accessible adult tissue source, normal skin and they do not form tumors. These can readily generate neurons, glial cells and other cell types provide a potential solution to these problems.

Materials and Methods

GFP-expressing transgenic mice (green mice).[12] 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

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cytomegalovirus enhancer (β-actin-driven GFP). All of the tissues from this transgenic line, with the exception of erythrocytes and hair, express GFP.

GFP-expressing, hair follicle stem cells cultured from isolated vibrissa follicles.[12] 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,[7] 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% CO_2/95% 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.

Nestin-, CD34- and K15-expression in vibrissa follicles of green mice.[12] 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 immunofluorescencer (nestin and K15) and immunohistochemical (CD34) staining.

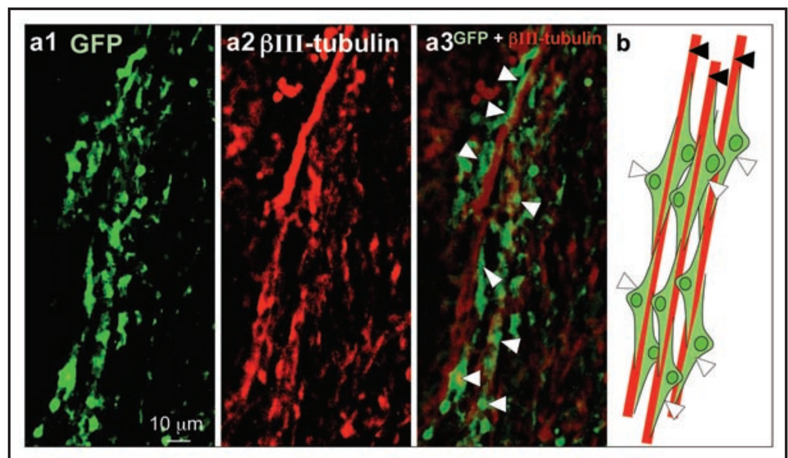

Figure 3. Hair-follicle stem cells differentiated to Schwann cells, and formed myelin sheaths around pre-existing axons after transplantation to the severed spinal cord. (A1–A3) The majority of the GFP-expressing hair follicle stem cells differentiated to GFAP- and CNPase-positive Schwann cells after transplantation in the severed thoracic region of the spinal cord. GFP-expressing Schwann cells formed myelin sheaths, which surrounded βIII-tubulin-positive axons (white arrowheads). (B) Schematic of joined thoracic region of spinal cord. The axons are denoted by black arrowheads and the Schwann cells by white arrowheads.

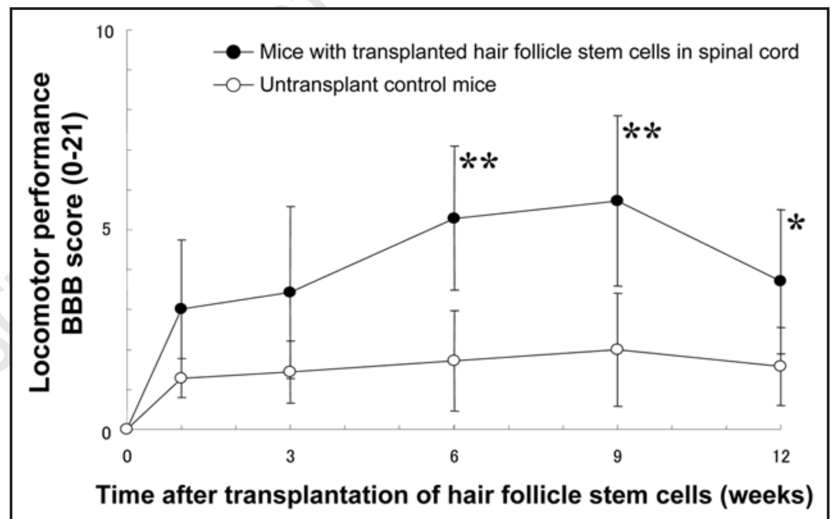

Figure 4. The spinal cord, rejoined after transplantation of hair follicle stem cells, improved hind-limb locomotor performance. GFP-expressing hair follicle stem cells were transplanted to the severed thoracic region of the spinal cord. After 6, 9 and 12 weeks, the hind-limb BBB locomotor rating scale was significantly higher compared to the untransplantated control mice. $p < 0.05$*, $p < 0.01$** vs. control (without transplantation).

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 vertebrae, followed by transversal cut. The GFP-expressing hair follicle stem cells were transplanted in the severed thoracic region (spinal level T10) of the spinal cord in C57BL/6 immunocompetent mice. After 2 months, the spinal cord of the transplanted mice was directly observed by fluorescence microscopy under anesthesia. A total of 12 mice were transplanted with hair follicle stem cells.

Histology and immunohistochemistry. Spinal cord 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 μm, 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 immunofluoresence (IF) staining of βIII-tubulin, GFAP, CNPase, K15 and SMA. The primary antibodies used were: anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; Covance Research Products, Inc.,

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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.

Basso-Beattie-Bresnahan (BBB) locomotor rating scale. Behavioral analyses were conducted for 12 weeks using the BBB locomotor rating scale.[32,33] Each experimental group consisted of seven mice.

Fluorescence microscopy. The spinal cord in the live mouse, transplanted with GFP-expressing hair follicle stem cells, was 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).

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

Acknowledgements

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Note

Supplementary materials can be found at: www.landesbioscience.com/supplement/AmohCC7-12-Sup.pdf

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