

The bulge area is the major hair follicle source of nestin-expressing pluripotent stem cells which can repair the spinal cord compared to the dermal papilla

Fang Liu,^{1,2,3} Aisada Uchugonova,^{1,2,4} Hiroaki Kimura,^{1,2,5} Chuansen Zhang,³ Ming Zhao,¹ Lei Zhang,^{1,2} Karsten Koenig,⁴ Jennifer Duong,¹ Ryoichi Aki,^{1,6} Norimitsu Saito,^{1,6} Sumiyuki Mii,^{1,6} Yasuyuki Amoh,⁶ Kensei Katsuoka⁶ and Robert M. Hoffman^{1,2,*}

¹AntiCancer Inc.; San Diego, CA; ²Department of Surgery; University of California at San Diego; San Diego, CA USA; ³Department of Anatomy; Second Military Medical University; Shanghai, China; ⁴Department of Biophotonics and Laser Technology; Saarland University; Saarbruecken, Germany; ⁵Department of Orthopaedic Surgery; Kanazawa University; Kanazawa, Japan; ⁶Department of Dermatology; School of Medicine, Kitasato University; Kanagawa, Japan

Key words: hair follicle, vibrissa, mouse, nestin, GFP, bulge area, dermal papilla, spinal cord injury, repair, confocal fluorescence imaging

Nestin has been shown to be expressed in the hair follicle, both in the bulge area (BA) as well as the dermal papilla (DP). Nestin-expressing stem cells of both the BA and DP have been previously shown to be pluripotent and be able to form neurons and other non-follicle cell types. The nestin-expressing pluripotent stem cells from the DP have been termed skin precursor or SKP cells. The objective of the present study was to determine the major source of nestin-expressing pluripotent stem cells in the hair follicle and to compare the ability of the nestin-expressing pluripotent stem cells from the BA and DP to repair spinal cord injury. Transgenic mice in which the nestin promoter drives GFP (ND-GFP) were used in order to observe nestin expression in the BA and DP. Nestin-expressing DP cells were found in early and middle anagen. The BA had nestin expression throughout the hair cycle and to a greater extent than the DP. The cells from both regions had very long processes extending from them as shown by two-photon confocal microscopy. Nestin-expressing stem cells from both areas differentiated into neuronal cells at high frequency *in vitro*. Both nestin-expressing DP and BA cells differentiated into neuronal and glial cells after transplantation to the injured spinal cord and enhanced injury repair and locomotor recovery within four weeks. Nestin-expressing pluripotent stem cells from both the BA and DP have potential for spinal cord regeneration, with the BA being the greater and more constant source.

Introduction

The hair follicle has recently been shown to be a source of nestin-expressing pluripotent stem cells that can form neurons and other non-follicular cell types.¹⁻⁷ These relatively small, oval-shaped cells express the stem cell marker nestin and are located in the bulge area (BA) surrounding the hair shaft and are interconnected by short dendrites. Nestin-expressing pluripotent stem cells were also found in the dermal papilla (DP).⁵ The expression of nestin in both neural stem cells and the hair follicle BA cells suggested the ability of nestin-expressing stem cells in the hair follicle to be able to differentiate to neurons and related cell types.¹

Subsequently, bulge explants from adult mouse whisker follicles were shown to give rise to cells which formed neurons, smooth muscle cells, Schwann cells, and melanocytes,²⁻⁴ thereby confirming the observation of Li et al.

Transgenic mice, with GFP expression driven by the nestin regulatory element [nestin-driven GFP (ND-GFP)], were

used to identify nestin-expressing cells in the hair follicle bulge area (BA).¹ The nestin-expressing BA cells were found to be positive for the stem cell marker CD34, as well as keratin 15-negative, suggesting their relatively undifferentiated state. Furthermore, BA nestin-expressing cells were shown to differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes *in vitro*.⁴

When nestin-expressing cells from the mouse vibrissa BA were implanted into the gap region of the severed sciatic nerve, they greatly enhanced the rate of nerve regeneration and the restoration of nerve function. The transplanted follicle BA cells transdifferentiated largely into Schwann cells, which are known to support neuron regrowth. The transplanted mice recovered the ability to walk normally.⁸

Nestin-expressing mouse vibrissa BA cells were transplanted to the injury site of C57B16 mice in which the thoracic spinal cord was severed. Most of the transplanted cells also differentiated into Schwann cells that apparently facilitated repair of the

*Correspondence to: Robert M. Hoffman, Ph.D., E-mail: all@anticancer.com

Submitted: 12/28/10; Accepted: 01/28/11

DOI: 10.4161/cc.10.5.14969

severed spinal cord. The rejoined spinal cord recovered reestablished extensive hind-limb locomotor performance.⁹

Yu et al.^{10,11} isolated a population of human nestin-expressing hair follicle BA cells. These cells also expressed neural crest and neuron stem cell markers as well as the embryonic stem cell transcription factors Nanog and Oct4. The human BA cells proliferated as spheres, were capable of self-renewal, and differentiated into multiple lineages including myogenic, melanocytic, and neuronal cell lineages after in vitro clonal single cell culture. In addition, the human nestin-expressing BA cells differentiated into adipocyte, chondrocyte, and osteocyte lineages. The human BA cells shared a similar gene expression pattern with murine skin immature neural crest cells.^{10,11}

Human nestin-expressing hair follicle cells from the mouse vibrissa BA were transplanted in the severed sciatic nerve of the mouse where they differentiated into glial fibrillary-acidic-protein (GFAP)-positive Schwann cells and promoted the recovery of pre-existing axons, leading to nerve regeneration. The regenerated nerve recovered function and, upon electrical stimulation, contracted the gastrocnemius muscle.¹²

Biernaskie et al.¹³ showed what they termed skin-derived precursors (SKPs), are present in the hair follicle DP. SKPs could be cultured from adult whisker follicle DPs.¹⁴⁻¹⁶ SKPs were shown to differentiate into mesodermal and peripheral neural progeny including adipocytes, skeletogenic cell types, and Schwann cells.¹⁷ The DP of hair follicles appeared to comprise the niche for SKPs. SKPs were also shown to effect spinal cord repair.¹³

The question is what is the major source of hair follicle nestin-expressing pluripotent stem cells which can effect spinal cord repair—the BA or DP? In the present study, we show, in the mouse vibrissa follicle, that nestin-expressing pluripotent stem cells, residing in the BA and DP, can effect spinal cord injury repair, with the BA being the major source.

Results

Localization of nestin-expressing cells in the vibrissa BA and DP. In order to visualize the localization of nestin-expressing cells in the mouse hair follicle, the anagen vibrissa follicle from ND-GFP mice (8 weeks) was dissected. The follicle was covered by a rigid capsule. It had two blood-filling areas (Fig. 1A1). One was at the BA, the other was at the bulb area, which contained the DP. The BA and DP could be clearly visualized after the capsule was removed (Fig. 1A2-A7). Very bright GFP fluorescence was found at the BA and DP using the OV100 Small Animal Imaging System (Olympus Corp., Tokyo, Japan) (Fig. 1A3, A5, A7), indicating that nestin-expressing cells existed in these two regions.

Nestin expression in the BA and DP varies with follicle stage. To understand whether the expression of nestin in BA and DP was related to animal age or follicle stage, vibrissa follicles were dissected from 2-week and 9-month old ND-GFP mice. The BA in all the follicle stages expressed nestin very strongly in animals of both ages. In middle anagen follicles (Fig. 2B1-B4, C1-C4, Fig. 3B1, B2), the area between the BA and DP was extended and nestin-expressing blood vessels were present. Some

blood vessels were associated with the DP directly (Fig. 2C4). The DP had the most extensive nestin expression in middle anagen (Fig. 2, B1-B4, C1-C4). In late anagen follicles, the DP lost nestin expression (Fig. 2, D1-D4). Similar patterns were seen with 9-month old mice (data not shown).

Nestin-expressing cells from BA and DP have very similar morphology. Confocal two-photon microscopy was used to visualize individual nestin-expressing cells in the DP and BA. The nestin-expressing oval-shaped cells with long dendrite-like structures were observed in both the BA and DP within isolated vibrissa follicles (Fig. 3A and B). The BA and DP oval-shaped cells with long dendrite-like structures were also observed from each region when the cells from each region were cultured on Gelfoam® (Fig. 3C and D). These cells from both the BA and DP were essentially identical in morphology.

Nestin-expressing cells from both BA and DP can form spheres. Nestin-expressing cells from the BA and DP were cultured in serum-free medium supplemented with bFGF and EGF (Fig. 1B, C). After primary culture for 4 to 7 days, nestin-expressing cells grew out from the BA and DP. BA cells derived from whiskers from red fluorescent protein (RFP)-expressing mice were also cultured using the same medium as that for ND-GFP BA cells and DP cells (Fig. 1D). Immunocytochemistry demonstrated that the RFP-BA cells also expressed nestin (Fig. 1E).

Cells from BA and DP floated in culture. At day 7, spheres were formed from cells from both regions (Fig. 4A1, A2). Cells from both regions expressed nestin, identified by immunocytochemistry (Fig. 4B1, C1). Nestin-expressing cells from the BA had higher sphere-forming efficiency than those from the DP (Fig. 4A3). The spheres started to differentiate after bFGF and EGF were removed from the culture medium. Most cells were Tuj1- or GFAP- positive suggesting that nestin-expressing cells from both the BA and DP can differentiate into neuronal or Schwann cells (Fig. 4B2, B3, C2, C3). Nestin-expressing DP cells were versican-negative (Fig. 4C4), which is a marker for DP cells. This result suggests that nestin-expressing DP cells may not be native to the DP.

Three-dimensional culture of nestin-expressing BA and DP cells on Gelfoam®. Nestin-expressing cells from both the BA and DP grew very well on Gelfoam®. The cells attached to the Gelfoam® within one hour. They grew along the grids of the Gelfoam® during the first 2 or 3 days. Later they spread into the Gelfoam® (Fig. 3, 4D, 4E).

Fate of nestin-expressing BA and DP cells transplanted into the injured spinal cord. After transplantation of Gelfoam® cultures of nestin-expressing BA or DP cells into the injured spinal cord (including the Gelfoam®) nestin-expressing BA and DP cells were observed to be viable over 100 days post surgery. The transplanted cells attached to the surrounding tissue very well. The surface of the transplanted area appeared smooth (Fig. 5A2, B1).

When RFP-BA cells plus Gelfoam® were transplanted into the spinal cord of GFP transgenic nude mice (Fig. 5A1-A8), many RFP-BA cells were found in the transplanted area (Fig. 5A3-A7). RFP BA cells were observed migrating toward adjacent spinal cord segments (Fig. 5A7). GFP host cells and

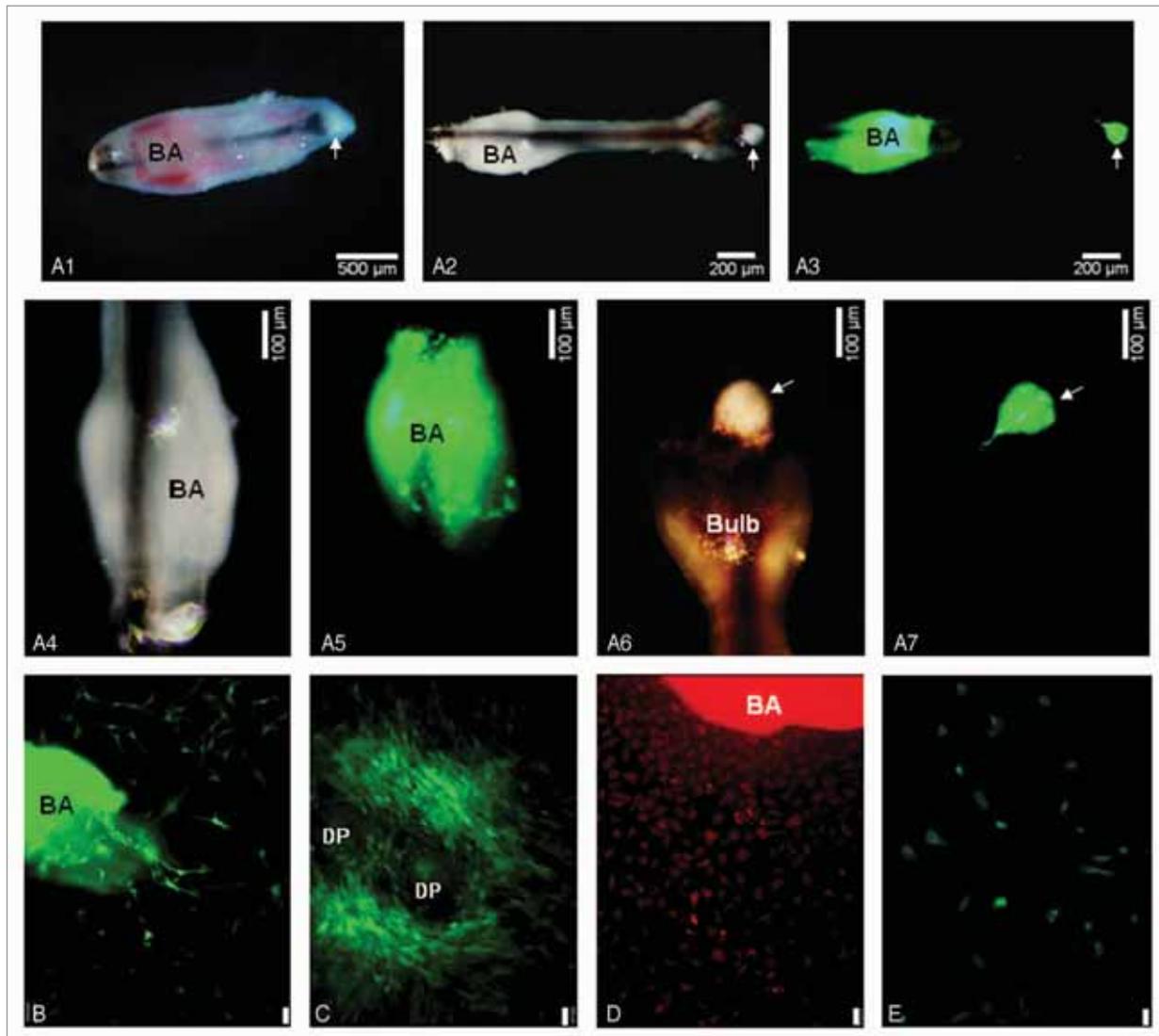


Figure 1. Nestin-expressing cells in the bulge area (BA) and dermal papilla (DP) of mouse vibrissal follicle. A1~A3: Whole view of a ND-GFP follicle. The follicle capsule was intact in A1. In A2 and A3, the capsule was removed. The BA and DP had strong nestin-GFP fluorescence. A4~A7 are the higher magnification of A2 and A3. The DP is shown by arrows. B: Primary culture of ND-GFP BA cells. C: Primary culture of ND-GFP DP cells. Two ND-GFP dermal papilla are shown. D: Primary culture of RFP BA cells. E: Nestin-immunofluorescence staining of RFP BA cells. The BA cells were nestin-positive. F: Bulge area. G: Dermal papilla. Panel A1~A7 were observed with the Olympus OV100 Small-Animal Imaging System. Panel B~E were observed under fluorescence microscopy. Scale bar in A1: 500 μm ; Scale bars in A2 and A3: 200 μm ; Scale bars in A4~A7: 100 μm ; Scale bars in B, D, E: 25 μm ; Scale bar in C: 50 μm .

fibers also grew into the injured area (Fig. 5A5–A7). H&E staining showed connections between the transplanted cells and the host tissue (Fig. 5A8).

When ND-GFP DP Gelfoam® cultures were transplanted into the injured spinal cord (Fig. 5B1–B8), the transplanted cells grew very well and also migrated toward adjacent spinal cord segments (Fig. 5B2, B3, B7). Some cells had long extensions (Fig. 5B4, B5).

Immunohistochemistry showed many Tuj1-, Isl 1/2, and EN1- positive cells and nerve fibers in the transplanted area of the spinal cord after BA Gelfoam® or DP Gelfoam® cultures were transplanted (Fig. 6A1–A3, B1–B3). Fewer Tuj1-, Isl 1/2-, and EN 1- positive cells or nerve fibers were observed in the

Gelfoam®-only transplant group (Fig. 6C1–C3). Some cells in the transplanted area were also GFAP - positive (Fig. 6A4, B4, C4) in the above three groups. The average grey value of the stained fluorescence showed that the BA Gelfoam® transplant group had significantly higher Tuj1-, and EN-1-positive fluorescence grey value than the DP Gelfoam® group or Gelfoam®-only group. The Gelfoam®-only group had significantly lower Tuj1-, Isl 1/2, and EN-1-positive fluorescence grey value than the BA Gelfoam® and DP Gelfoam® group (Fig. 6D).

In the non-transplanted group (Fig. 5C1), Tuj1-immunofluorescence staining was negative at the injured area (Fig. 5C2). Many GFAP-positive cells aggregated together and formed a “wall” at the injured site (Fig. 5C2). In the untransplanted

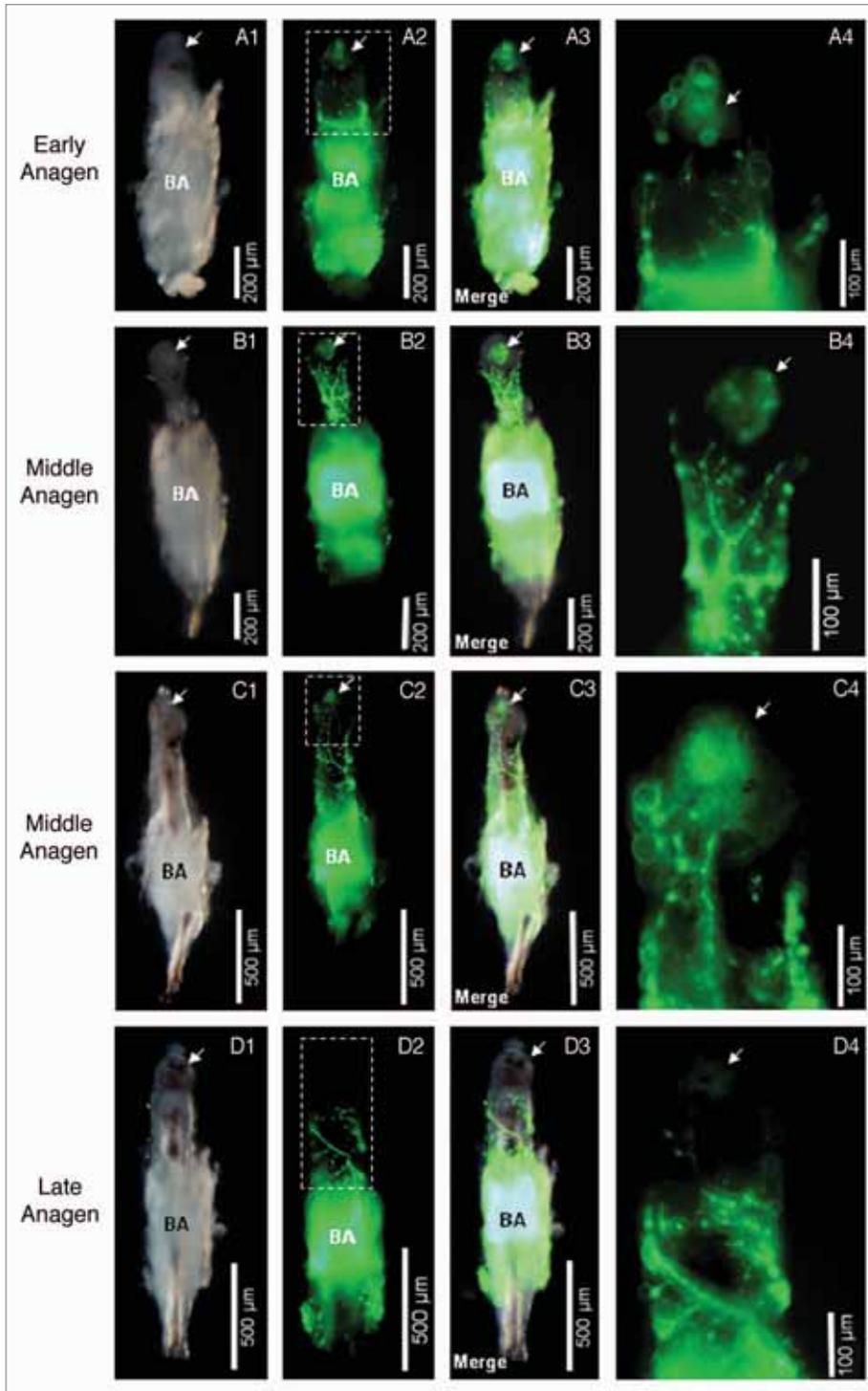


Figure 2. Vibrissa follicles from 2-week-old nestin-GFP mouse. The capsule of the follicles was removed. The BA in all the follicles expressed nestin very strongly. Panel (A1~A4) show follicles in early anagen. The area between BA and DP was small and the blood vessels in this area were not yet formed. Panel (B1~B4) and (C1~C4) show follicles in middle anagen. The area between the BA and DP was extended and blood vessels were formed. Some blood vessels connected to the DP directly (C4). The DP had strong nestin expression. Panel (D1~D4) shows a follicle in late anagen. The DP lost nestin-GFP fluorescence. All imaging was with the Olympus OV100. Arrows point to the DP. Panel (A4, B4, C4, D4) are the higher magnification of dotted squares in (A2, B2, C2, and D2), respectively. Scale bars in (A1~A3 and B1~B3): 200; Scale bars in (C1~C3 and D1~D3): 500 μm ; Scale bars in (A4, B4, C4, and D4): 100 μm .

group, glia cells formed a barrier at the spinal cord injured site. It prevented the regeneration of nerve fibers. The Gelfoam®-only group induced less neuronal marker-positive cells in the injured site than that of the BA Gelfoam® or DP Gelfoam® transplant groups.

Spinal cord recovery after transplantation of nestin-expressing BA or DP cells. All the animals had paralysis of the hindlimb at the same side where the spinal cord was injured. Most animals in the BA Gelfoam® and DP Gelfoam® transplant groups recovered plantar placing of the paw and frequent dorsal stepping within 3 days after transplantation. It took the Gelfoam®-only group 7 days to recover this behavior. It took the untransplanted group 14 days for locomotion recovery. Twenty-eight days after transplantation, both the BA Gelfoam® and DP Gelfoam® groups had normal locomotion. Animals walked with consistent plantar stepping, mostly coordinated, and paws in parallel at initial contact and liftoff. Though animals in the Gelfoam®-only group also had consistent plantar stepping at day 28, they only had some coordination or the paws rotated at initial contact and liftoff. The untransplanted group had the least locomotion recovery. At day 28, only a few Gelfoam®-only animals walked with occasional plantar stepping. After 21 days, the BBB locomotor recovery scores¹⁸ of the BA Gelfoam® and the DP Gelfoam® transplant animals were similar (Fig. 7). Those two groups achieved significantly greater locomotor recovery than groups transplanted with Gelfoam®-only and untransplanted animals from 21 days to 63 days post surgery. The data suggest that transplant nestin-expressing pluripotent stem cells from both the BA and DP have similar effects on locomotor recovery, which are better than that of Gelfoam®-only transplantation.

Discussion

Nestin-expressing pluripotent stem cells were found in the BA and DP of the mouse whisker follicle. Nestin-expressing BA cells and DP cells co-existed in the mouse vibrissal follicle only during part

of the hair follicle cycle, including middle anagen. Nestin-expressing cells were present in the DP only in the early and middle anagen stage, independent of age.

From the early to the late anagen stages, the BA became smaller and the area between the DP and BA became larger. In late anagen, nestin expression in the DP became weaker and disappeared. There were many nestin-expressing blood vessels between the BA and DP that connected those two structures. They started to be formed in the early anagen and formed a blood vessel network in the middle anagen. Regression of blood vessels occurred in late anagen. It seemed that nestin expression in the DP coincided with the existence of nestin-expressing blood vessels, which may originate from the BA.

Skin-derived precursor cells (SKPs) were shown to be multipotent neural crest-related stem cells that grow as self-renewing spheres and are capable of generating neurons and myelinating glial cells. Hunt et al.¹⁵ demonstrated that the DP of the rodent vibrissa follicle is 1,000-fold enriched for sphere-forming neural crest-derived cells compared with whole facial skin. Biernaskie et al.¹³ used SKPs to repair the contused rat spinal cord. SKPs reduced the size of the contusion cavity, myelinated endogenous host axons and recruited endogenous Schwann cells into the injured cord.

Cells from the BA of the mouse and human express nestin and can differentiate into many cell types, including neurons and glial cells^{1,2,4,8,10} and affect nerve^{8,9} and spinal cord repair in mouse models. Yu et al.^{10,11} demonstrated that stem cells with neural crest characteristics from the BA of cultured human hair follicle can form spheroid structures. SKP cells can also differentiate to neurons and glial cells.^{14, 15} In the present study, nestin-expressing cells from the BA had higher sphere-forming efficiency than those from the DP.

The present study showed that transplanted nestin-expressing BA and DP cells in the injured spinal cord both expressed Tuj1, and the motor neuron markers Isl 1/2 and EN1 as well. In the transplanted area of the spine, some GFAP-positive cells were observed in both groups. Nestin-expressing BA cells and DP cells differentiated into neuronal cells which appeared to accelerate spinal cord injury repair. The BA Gelfoam® and DP Gelfoam® transplant groups had similar BMS scores suggesting that nestin-expressing pluripotent stem cells from both regions have similar effects on locomotor recovery and both cell types are candidates for use for spinal cord repair. The greater and more constant expression of nestin in the BA and than the DP suggest the BA may be the major source of pluripotent stem cells.

Materials and Methods

Animals. Nestin-driven GFP (ND-GFP) transgenic mice,^{1, 19} at different ages, were used to observe the expression of nestin in BA and DP, and also for the culture of the nestin-expressing

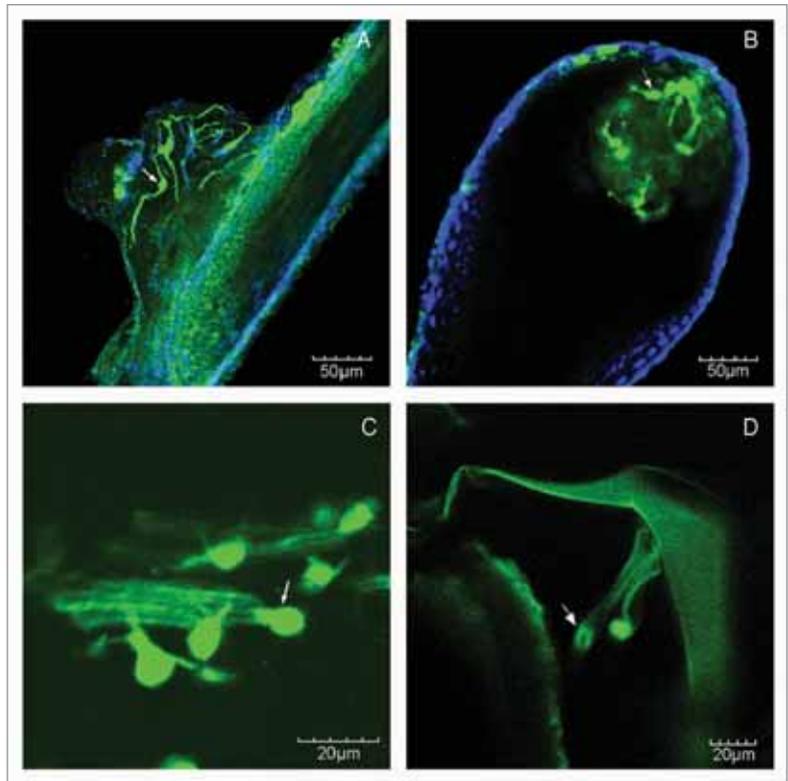


Figure 3. (A, B): Isolated vibrissa of an ND-GFP mouse was imaged by optical sectioning in 3D using a confocal two-photon microscope (Fluoview FV1000, Olympus; Tokyo Japan) with a high NA objective (40x/NA1.3 Oil). The figures demonstrate one optical plane image out of 3D stacks of images. BA (A) and DP (B) are in middle anagen. ND-GFP expressing cells in the BA and DP have similar morphology (oval/round shaped body with a diameter of approximately 5-7 μ m) with long dendrite-like structures. Arrows depict typical cells in each region. The blue fluorescence is from Hoechst staining and green fluorescence is from GFP. (C, D): ND-GFP expressing hair follicle stem cells grew out from the BA (C) and DP (D) when cultured on Gelfoam® after several days of incubation. In both images, nestin-expressing cells from both areas have similar oval/round morphology (diameter of 5-7 μ m) with long dendrite-like structures. Arrows depict typical cells in each region.

cells. RFP transgenic mice²⁰ were also used as a source of RFP BA cells. GFP transgenic nude mice²¹ were used as hosts for transplantation of RFP BA cells into the injured spinal cord. Non-transgenic nude mice were also used for spinal cord transplantation experiments.

Isolation and culture of nestin-expressing cells from the bulge area (BA) and dermal papilla (DP) of mouse vibrissa follicles. The whisker pad of the nestin-GFP or RFP transgenic mice was cut free and its inner surface was exposed. The vibrissa follicles were dissected under a binocular microscope. Small scissors were used to separate each follicle. Anagen follicles were chosen for cell culture. The isolated vibrissae were washed in PBS three times. Fine needles were used to remove the follicle capsule. The BA and DP areas were separated with fine needles. All surgical procedures were performed under a sterile environment. A 12-well plate coated with Collagen I was pre-incubated with DMEM-F12 (GIBCO/BRL), containing B-27 (GIBCO/BRL), N2 (GIBCO/BRL) and 1% penicillin/streptomycin (GIBCO/

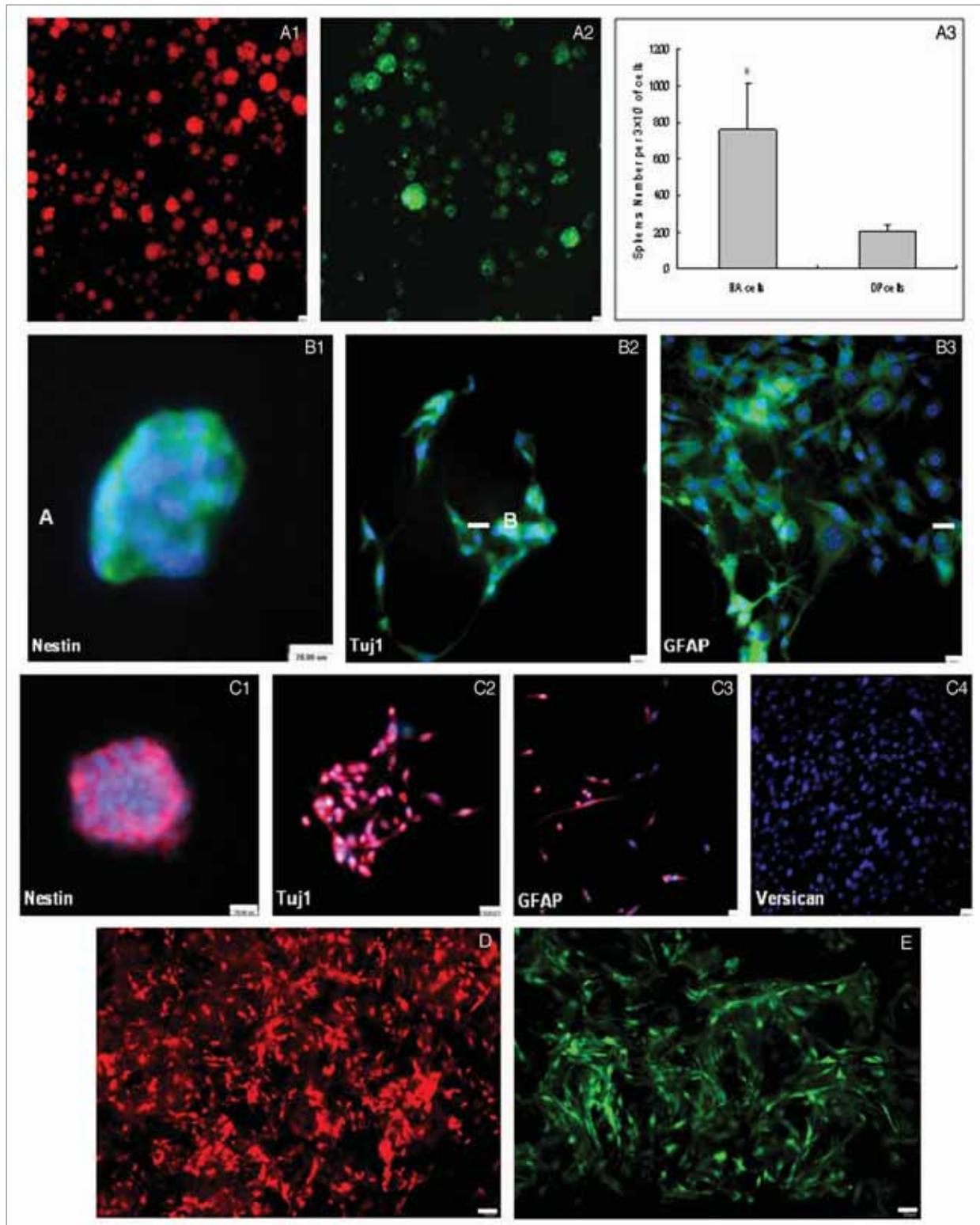


Figure 4. Sphere-forming of cells from the BA and DP. (A1) Spheres formed from RFP BA cells. (A2) Spheres formed from ND-GFP DP cells. (A3) Cells from the BA had higher sphere-forming efficiency than those from the DP. Immunofluorescence-staining showed spheres were nestin-positive (B1: BA sphere; C1: DP sphere). Tuj1- or GFAP-positive cells were detected in the spheres after bFGF and EGF were removed from the culture medium (B2~B3, cells from a BA sphere; C2~C3, cells from a DP sphere). (C4) showed versican-negative staining of ND-GFP DP cells. Green fluorescence: FITC, Red fluorescence: TRITC, Blue fluorescence: DAPI. Scales in (A1) and (A2): 50 μ m; Scales in (B1~B3, C1~C4): 20 μ m. (D) RFP BA cells grown on Gelfoam[®] for 10 days. (E) ND-GFP DP cells grown on Gelfoam[®] for 10 days. Scale bar: 50 μ m.

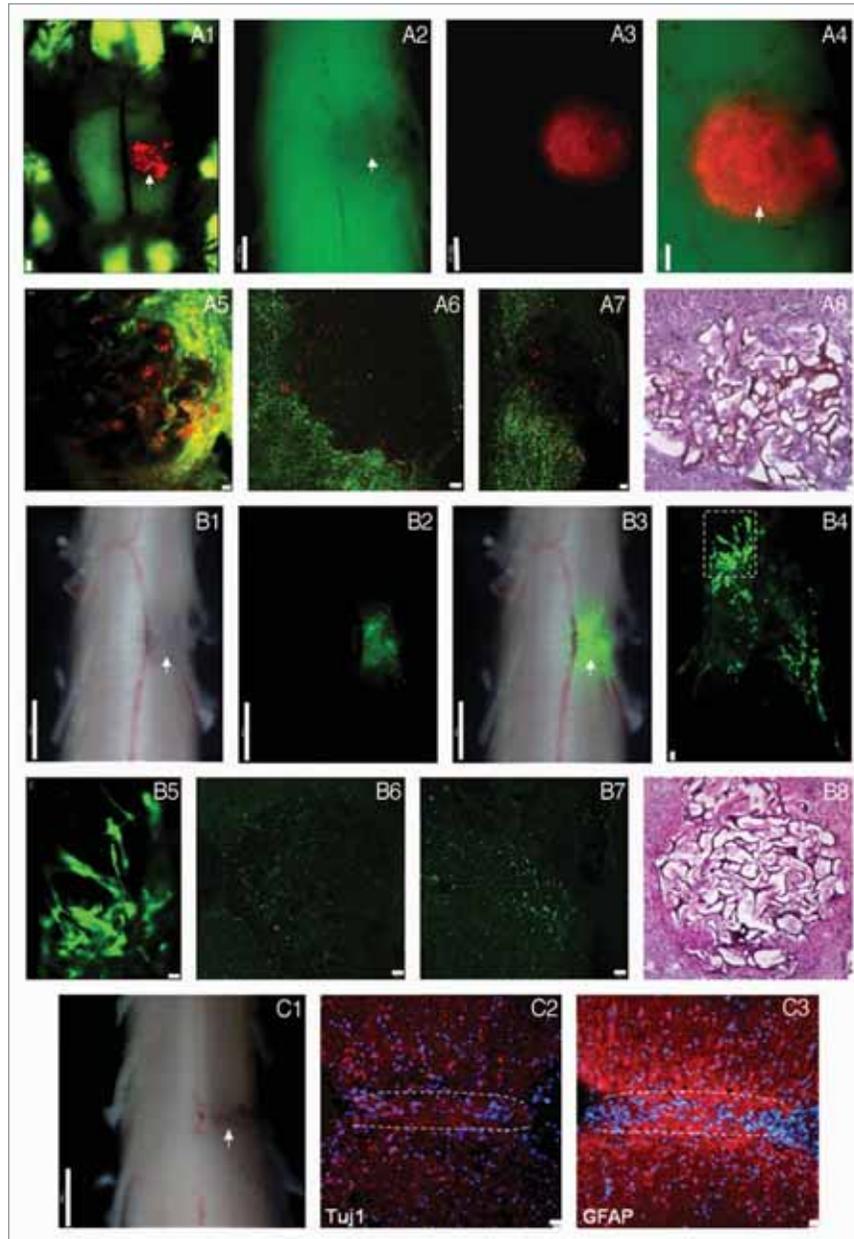


Figure 5. Appearance of RFP BA cells or ND-GFP DP cells plus Gelfoam® in the injured spinal cord. (A1~A8): RFP BA cells grown on Gelfoam® were transplanted into the injured spinal cord of GFP mice. (A1) is day 0 after surgery, (A2~A7) is 5 weeks post surgery. In panel (A3 ~ A7), many RFP BA cells were found in the transplanted area. GFP host cells and fibers grew into the injured area (A5~A7). In panel (A7), RFP BA cells are observed migrating toward spinal cord segments. Panel (A8) is H&E staining of a transplanted area 7 weeks post-surgery. Many cells and fibers were found at the injured site. (B1~B8): ND-GFP DP Gelfoam® was transplanted into the injured spinal cord 8 weeks post-surgery. Transplanted cells grew very well and also migrated toward spinal cord segments (B2, B3, B7). Some cells had long extensions (B4, B5). Panel (B4) is the higher magnification of panel (B3). Panel (B5) is the higher magnification of the dotted square in panel (B4). H&E staining in panel (B8) also showed cells and fibers migrating into the injured site. Arrows in panel (A1, A2, A4, B1, and B3) showed the injured and transplanted area of the spinal cord. Panel (A1~A4, B1~B3) were observed with the Olympus OV100. Panel (A5~A7, B4~B7) were observed with the Olympus IV100 Laser Scanning Microscope. Scale bar in (A1): 100 μ m; Scale bars in (A2), (A3): 500 μ m; Scale bar in (A4): 200 μ m; Scale bars in (A5, A8, B5, B8): 20 μ m; Scale bars in (A6, A7, B4, B6, B7): 50 μ m; Scale bars in (B1~B3): 1 mm. C. Spinal cord injured on the right side without transplantation, 7 weeks post surgery. In the injured area, many GFAP-positive cells aggregated together forming a "wall". The Tuj1-immunofluorescence staining was negative at the injured area. Dotted lines in panels 2 and 3 represent the injured area. Red fluorescence: TRITC, Blue fluorescence: DAPI. Scale bar in (A): 1mm; Scale bar in (B), 20 μ m. Panel (C1) show the injured site of the spinal cord on the right side (arrow). It was observed with the OV100. Panel (C2) and panel (C3) show the immunofluorescent staining of the frozen sections of the injured spinal cord with Tuj1- and GFAP- primary antibodies, observed under fluorescence microscopy. The injured area is shown by the dotted lines. It was negative for the Tuj1-immunofluorescence staining at the injured area. Many GFAP-positive cells (red fluorescence) aggregated toward the injury area to form a "glial wall". Red fluorescence was stained by TRITC-combined secondary antibody; Blue fluorescence was the nuclear counter-staining by DAPI. Scale bar in (C1): 1mm; Scale bars in (C2 and C3): 20 μ m

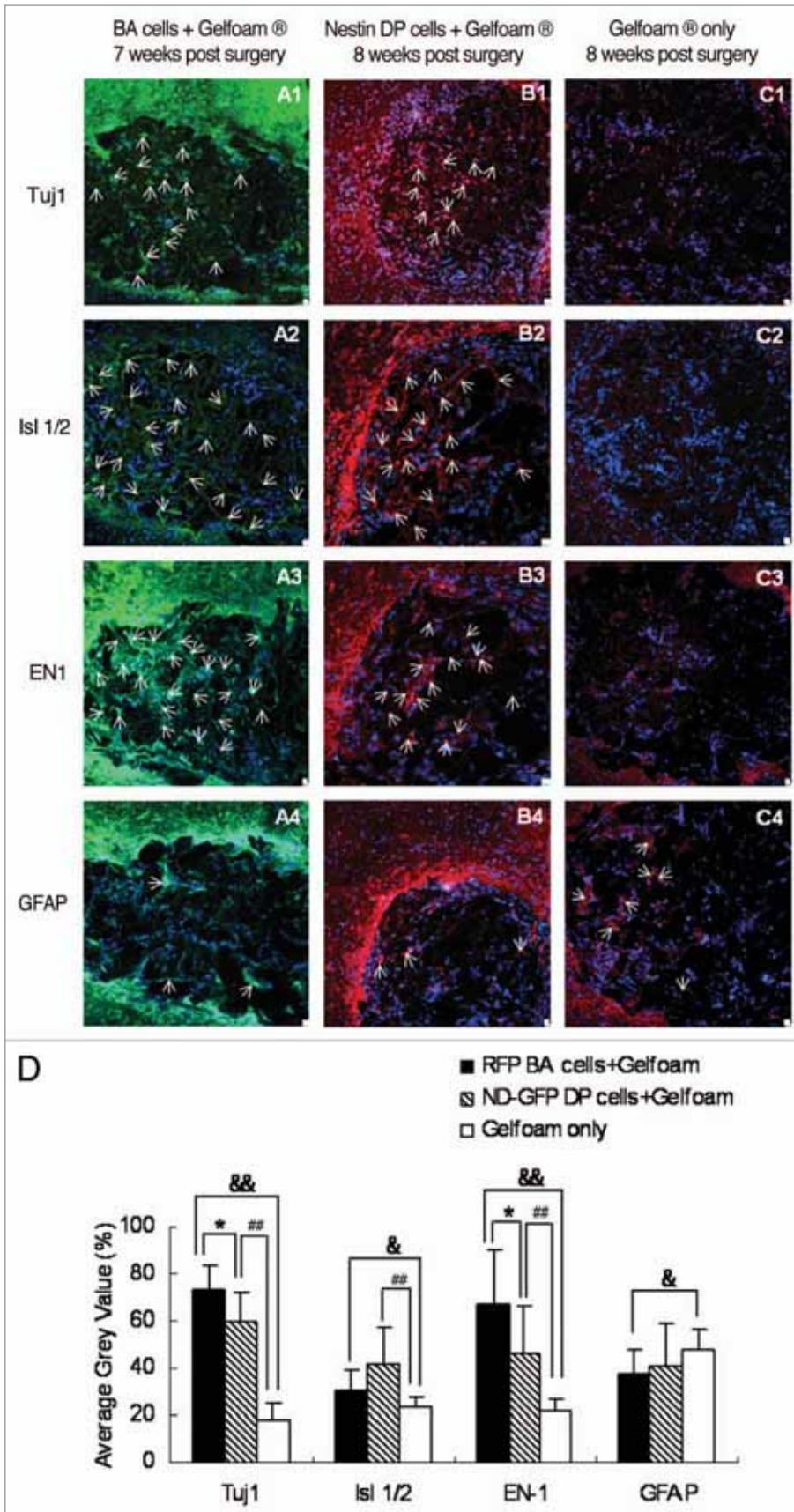


Figure 6 (See previous page). Immunofluorescence staining of the transplanted area of the spinal cord. Primary antibodies used were Tuj1-, Isl 1/2, EN1, and GFAP. Secondary antibodies were conjugated with FITC (green fluorescence) or TRITC (red fluorescence). Nuclei were counterstained by DAPI (blue fluorescence). Positive staining in the sections from the BA Gelfoam® transplanted group appear green. Positive staining in the sections of the other two groups is red. In the BA or DP plus Gelfoam® transplanted groups, many of Tuj1-, Isl 1/2-, and EN 1- positive cells or nerve fibers (arrows) were found in the transplanted area (panel A1~A3, B1~B3). The Gelfoam® transplanted-only group induced less neuronal marker-positive cells in the injured area than that of the BA cells + Gelfoam® or DP cells + Gelfoam® transplanted groups. However, the Gelfoam® only group had more GFAP- positive cells than that in the BA or DP- Gelfoam® groups. Scale bar: 20 µm. Glial cells could form a barrier in the spinal cord injured area. The barrier prevented the regeneration of nerve fibers. In the Gelfoam®-only group, some neuronal-like cells appeared in the transplanted area (C1, C3). Those cells may come from the surrounding spinal cord tissue. More neuronal-like cells or nerve fibers appeared in the BA- or DP-Gelfoam® groups, which stimulated the recovery of spinal cord injury much more than Gelfoam® only. The neuronal-like cells in these two groups could come from the implanted BA cells or DP cells or migrate from the surrounding tissue. (D) Average grey value of the stained fluorescence in the transplanted area of spinal cord. The BA-Gelfoam® group had significantly higher Tuj1-, and EN-1-positive fluorescence grey value than the DP-Gelfoam® group and Gelfoam®-only group. The Gelfoam®-only group had significantly lower Tuj1-, Isl 1/2, and EN-1-positive fluorescence grey value than that in the BA-Gelfoam® and DP-Gelfoam® groups, except for the GFAP-positive fluorescence grey value. *, BA-Gelfoam® group vs DP-Gelfoam® group, $p < 0.05$; &, BA-Gelfoam® group vs Gelfoam®-only group, $p < 0.05$; &&, $p < 0.01$; ##, ND-GFP DP cells + Gelfoam® group vs Gelfoam®-only group, $p < 0.01$.

BRL). The separated BA and DP attached to the plate bottom within 40 minutes. Five to eight BA or DP were placed in each well. Serum-free culture medium was then added very carefully into the well with a 1ml pipette. Half the medium was changed every other day. 20 ng/ml bFGF and EGF were added daily. After 4 to 6 days, the attached BA or DP was removed from the plate bottom. The cells were incubated with 0.25% trypsin for 10 minutes at 37. Fetal bovine serum was added to inhibit trypsin. Cells were washed with serum-free culture medium and were seeded in a collagen I coated T25 flask.

High-resolution imaging of BA and DP nestin-expressing cells with a confocal two-photon microscope. A confocal two-photon microscope (Fluoview FV1000, Olympus Corp., Tokyo Japan) was used for two- (x,y) and three-dimensional (3D, x,y,z) high-resolution imaging of vibrissa follicles from a ND-GFP transgenic mice. Excitation sources included cw semiconductor lasers at 473 nm for GFP excitation and 405 nm for Hoechst excitation. A tunable Mai-Tai HP1040 laser emitting at 700-1020 nm (Newport-Spectra Physics, Irvine, CA) was used for deep multicolor imaging including two-photon induced autofluorescence. Fluorescence images were obtained using the 20x/0.50 UPlan FLN and 40x/1.3 Oil Olympus UPLAN FLN objective. Emission filters used were BA 430-455 nm (at exc. 405 nm, Hoechst) and BA 490-590 (at exc. 473 nm, GFP).

Sphere-forming assay. BA and DP cells, at passage two, were seeded at a density of 30,000 cells per ml in 10 ml of serum-free culture medium in a 25 cm² tissue culture flask with low attachment. Sphere numbers were determined at day seven by counting under a low-power fluorescence microscope.

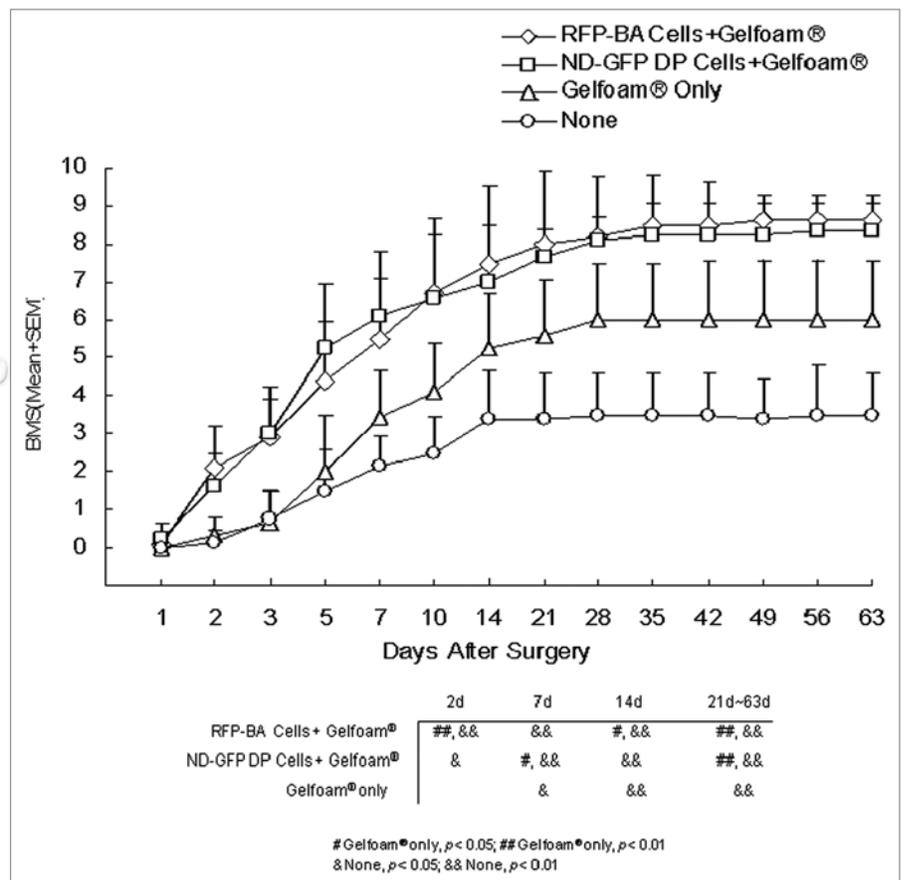


Figure 7. Mean Basso mouse scale (BMS) for locomotion (BMS) scores. The locomotor recovery pattern of the BA - Gelfoam® and the DP - Gelfoam® animals was similar. Those two groups achieved significantly greater locomotor recovery than the Gelfoam®-only and non-transplanted control animals.

Three-dimensional culture of cells on Gelfoam®. BA and DP cells passage 2 or 3 were collected and centrifuged at 4, 1000 rpm for 5 minutes. The cell density was 1×10^7 cells/ml. Sterile Gelfoam® (Pharmacia & Upjohn Co., Kalamazoo, MI) sponges comprised of absorbable gelatin prepared from purified porcine skin, were trimmed into 3 mm³ cubes. Cell suspensions (20 µl) were injected into each sponge cube with a 31G insulin syringe.

The Gelfoam® cubes, together with the injected cells, were put in 48-well plates and placed in an incubator (37°C, 5% CO₂, 95% humidity) for one hour before 1 ml culture medium was added in each well. Half of the medium was changed every other day. After ten days, the cultures, including the Gelfoam® were used for spinal cord transplantation.

Surgical procedure, spinal cord injury and transplantation of Gelfoam®-cultured BA and DP cells. Six to eight-week-old non-transgenic nude mice and transgenic GFP nude mice were used. Animals were anesthetized with tribromoethanol. The skin overlaying the vertebral column was cut and a pair of fine scissors was used to separate the muscles along the spine. A partial laminectomy was performed at T8. A 31G insulin syringe needle was used to cut the right side of the spinal cord. The lesion surface size was 500 µm × 300 µm. The depth of the lesion was from the dorsal to the abdominal surface of the spinal cord. Gelfoam® cultures (including the Gelfoam®) were introduced into the lesion site with fine forceps within 30 minutes of injury. The animals were divided into four groups, 10 nude mice in each group: (1) mice transplanted in the injured spine with BA cells on Gelfoam®; (2) mice transplanted in the injured spine with DP cells on Gelfoam®; (3) mice transplanted in the injured spine with Gelfoam® only; (4) negative control mice with no transplantation. Spinal cord samples from the injured and transplanted area were obtained at different time points, embedded in OCT, dipped in liquid nitrogen, and kept in a -80°C freezer.

Immunocytochemistry and immunohistochemistry. Cultured BA and DP cells or frozen sections from the spinal cord were fixed in pre-cooled 4% paraformaldehyde at RT for 10

minutes and washed with PBS three times. Some frozen sections were processed for H&E staining. Cells and some frozen sections were used for immunofluorescence staining. The main procedures were as follows: (1) Slides treated with 0.3% Triton X-100 were incubated at RT for 30 minutes; (2) 5% normal goat serum was applied at RT for 30 minutes; (3) Primary antibodies were applied at 4°C for 48 hours. The primary antibodies used were nestin (rabbit, 1:100, Sigma), versican (mouse, 1:100, Millipore), Tuj1 (rabbit, 1:30, Sigma), GFAP (rabbit, 1:100, Sigma), Isl 1/2 (rabbit, 1:100, Santa Cruz), EN-1 (rabbit, 1:100, Santa Cruz). (4) Secondary antibodies used were: goat anti-rabbit IgG FITC (Santa Cruz), donkey anti-rabbit IgG TRITC (Santa Cruz), donkey anti-mouse IgG TRITC (Santa Cruz), 1:100, RT, dark, 2.5 hours. (5) DAPI, 1:1000, diluted in PBS, RT, 5 minutes. (6) Slides were mounted with Fluoromount (Sigma) and observed under fluorescence microscopy.

BMS measurement of locomotor recovery. According to the Basso mouse scale, all mice with spinal cord injury (SCI) were tested in an open field for 4 min pre-operatively and weekly for 63 days post-operatively. At the first week post surgery, the mice were observed every day. Ankle movements, plantar placing, dorsal stepping, frequency of plantar stepping, stepping coordination and the position of the paws were carefully observed by two people.

Statistical analyses. Images of the immunostaining were processed for average grey value of fluorescence with the use of Paint Shop Pro 8 and Cell (Olympus) software. All data are presented as mean ± SEM. Group differences were obtained using unpaired t-tests. The significance level for all tests was set at p < 0.05.

References

- Li L, Mignone J, Yang M, Matic M, Penman S, Enikolopov G, Hoffman RM. Nestin expression in hair follicle sheath progenitor cells. *Proc Natl Acad Sci USA* 2003; 100:9658-61.
- Sieber-Blum M, Grim M, Hu YF, Szeder V. Pluripotent neural crest stem cells in the adult hair follicle. *Dev Dyn* 2004; 231:258-69.
- Sieber-Blum M, Schnell L, Grim M, Hu YF, Schneider R, Schwab ME. Characterization of epidermal neural crest stem cell (EPL-NCSC) grafts in the lesioned spinal cord. *Mol Cell Neurosci* 2006; 32:67-81.
- 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; 102:5530-4.
- Biernaskie J, Paris M, Morozova O, Fagan BM, Marra M, Pevny L, Miller, F.D. SKPs derive from hair follicle precursors and exhibit properties of adult dermal stem cells. *Cell Stem Cell* 2009; 5:610-23.
- Hoffman RM. The pluripotency of hair follicle stem cells. *Cell Cycle* 2006; 5:232-3.
- Amoh Y, Kanoh M, Niiyama S, Kawahara K, Satoh Y, Katsuoka K, Hoffman RM. Human and mouse hair follicles contain both multipotent and monopotent stem cells. *Cell Cycle* 2009; 8:176-7.
- Amoh Y, Li L, Campillo R, Kawahara K, Katsuoka K, Penman S, Hoffman RM. Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves. *Proc Natl Acad Sci USA* 2005; 102:17734-8.
- 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; 7:1865-9.
- Yu H, Fang D, Kumar SM, Li L, Nguyen TK, Acs G, et al. Isolation of a novel population of multipotent adult stem cells from human hair follicles. *Am J Pathol* 2006; 168:1879-88.
- Yu H, Kumar SM, Kossenkov AV, Showe L, Xu X. Stem cells with neural crest characteristics derived from the bulge region of cultured human hair follicles. *J Invest Derm* 2010; 130:1227-36.
- Amoh Y, Kanoh M, Niiyama S, Hamada Y, Kawahara K, Sato Y, et al. Human hair follicle pluripotent stem (hfPS) cells promote regeneration of peripheral-nerve injury: An advantageous alternative to ES and iPS cells. *J Cell Biochem* 2009; 107:1016-20.
- Biernaskie J, Sparling JS, Liu J, Shannon CP, Plemel JR, Xie Y, et al. Skin-derived precursors generate myelinating Schwann cells that promote remyelination and functional recovery after contusion spinal cord injury. *J Neurosci* 2007; 27:9545-59.
- Fernandes KJL, McKenzie IA, Mill P, Smith KM, Akhavan M, Barnabe-Heider F, et al. A dermal niche for multipotent adult skin-derived precursor cells. *Nat Cell Biol.* 2004; 6:1082-93.
- Hunt DP, Morris PN, Sterling J, Anderson J, Joannides A, Jahoda C, et al. A highly enriched niche of precursor cells with neuronal and glial potential within the hair follicle dermal papilla of adult skin. *Stem Cells* 2008; 26:163-72.
- Joannides A, Gaughwin P, Schwiening C, Majed H, Sterling J, Compston A, Chandran S. Efficient generation of neural precursors from adult human skin: Astrocytes promote neurogenesis from skin-derived stem cells. *Lancet* 2004; 364:172-8.
- 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; 26:6651-60.
- Basso DM, Fisher LC, Anderson AJ, Jakeman LB, McTigue DM, Popovich PG. Basso Mouse Scale for locomotion detects differences in recovery after spinal cord injury in five common mouse strains. *J Neurotrauma* 2006; 23:635-59.
- Mignone JL, Kukekov V, Chiang AS, Steindler D, Enikolopov G. Neural stem and progenitor cells in nestin-GFP transgenic mice. *J Comp Neurol* 2004; 469:311-24.
- Yang M, Reynolds J, Bouvet M, Hoffman RM. A transgenic red fluorescent protein-expressing nude mouse for color-coded imaging of the tumor microenvironment. *J Cell Biochem* 2009; 106:279-84.
- Yang M, Reynolds J, Jiang P, Li L, Moossa AR, Hoffman RM. Transgenic nude mouse with ubiquitous green fluorescent protein expression as a host for human tumors. *Cancer Res* 2004; 64:8651-6.