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# Direct Transplantation of Uncultured Hair-Follicle Pluripotent Stem (hfPS) Cells Promotes the Recovery of Peripheral Nerve Injury

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## ABSTRACT

We previously showed that the stem cell marker nestin is expressed in hair follicle stem cells which suggested their pluripotency. We subsequently showed that the nestin-expressing hair-follicle pluripotent stem (hfPS) cells can differentiate in culture to neurons, glial cells, keratinocytes, and other cell types and can promote regeneration of peripheral nerve and spinal cord injuries upon injection to the injured nerve or spinal cord. The location of the hfPS cells has been termed the hfPS cell area (hfPSCA). Previously, hfPS cells were cultured for 1–2 months before transplantation to the injured nerve or spinal cord which would not be optimal for clinical application of these cells for nerve or spinal cord repair, since the patient should be treated soon after injury. In the present study, we addressed this issue by directly using the upper part of the hair follicle containing the hfPSCA, without culture, for injection into the severed sciatic nerve in mice. After injection of hfPSCA, the implanted hfPS cells grew and promoted joining of the severed nerve. The transplanted hfPS cells differentiated mostly to glial cells forming myelin sheaths, which promoted axonal growth and functional recovery of the severed nerve. These results suggest that the direct transplantation of the uncultured upper part of the hair follicle containing the hfPSA is an important method to promote the recovery of peripheral nerve injuries and has significant clinical potential. J. Cell. Biochem. 110: 272–277, 2010. 

**KEY WORDS:** HAIR FOLLICLE; STEM CELL; GREEN FLUORESCENT PROTEIN; DIFFERENTIATION; NEURON; GLIAL CELL; PERIPHERAL NERVE; REGENERATION

We have shown that the stem cell marker nestin is expressed in hair-follicle pluripotent stem (hfPS) cells using nestin-driven green fluorescent protein (ND-GFP) transgenic mice. We have demonstrated that during early anagen or growth phase of the hair follicle, nestin-expressing cells, marked by GFP fluorescence in ND-GFP transgenic mice, appear in the permanent upper hair follicle immediately below the sebaceous glands and above the hair follicle bulge area [Li et al., 2003].

The ND-GFP-expressing hair follicle stem cells are keratin 15 (K15)-negative. These cells formed colonies in culture and differentiated into neurons, glial cells, keratinocytes, melanocytes, and smooth muscle cells [Amoh et al., 2005a]. The nestin-expressing cells can also induce angiogenesis, and maintain the network of nestin-expressing microvasculature in the skin [Amoh et al., 2004]. We have therefore termed these as hfPS cells [Amoh et al., 2009]. The location of the hfPS cells has been termed the hfPS cell area (hfPSCA).

We also have previously shown that cultured hfPS cells can promote regeneration of peripheral nerve and spinal cord injuries upon injection to the injured nerve [Amoh et al., 2005b, 2009] or spinal cord [Amoh et al., 2008]. In our previous method, we cultured the hfPS cells for 1–2 months before transplantation to the injured nerve or spinal cord. Long culture time of autologous hfPS cells would not be optimal for clinical application of these cells for nerve or spinal cord repair, since the patient should be treated soon after injury.

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In the present study, we directly transplanted the hfPSCA from whiskers without culture into the severed sciatic nerve of C57B16 mice. The hfPSCA promoted rejoining of the sciatic nerve, which regained the ability to effect contraction of the gastrocnemius muscle upon electric stimulation.

## MATERIALS AND METHODS

### GFP-TRANSGENIC MICE (GREEN MICE)

Transgenic C57/B6-GFP mice [Okabe et al., 1997] were obtained from the Research Institute for Microbial Diseases (Osaka University, Osaka).

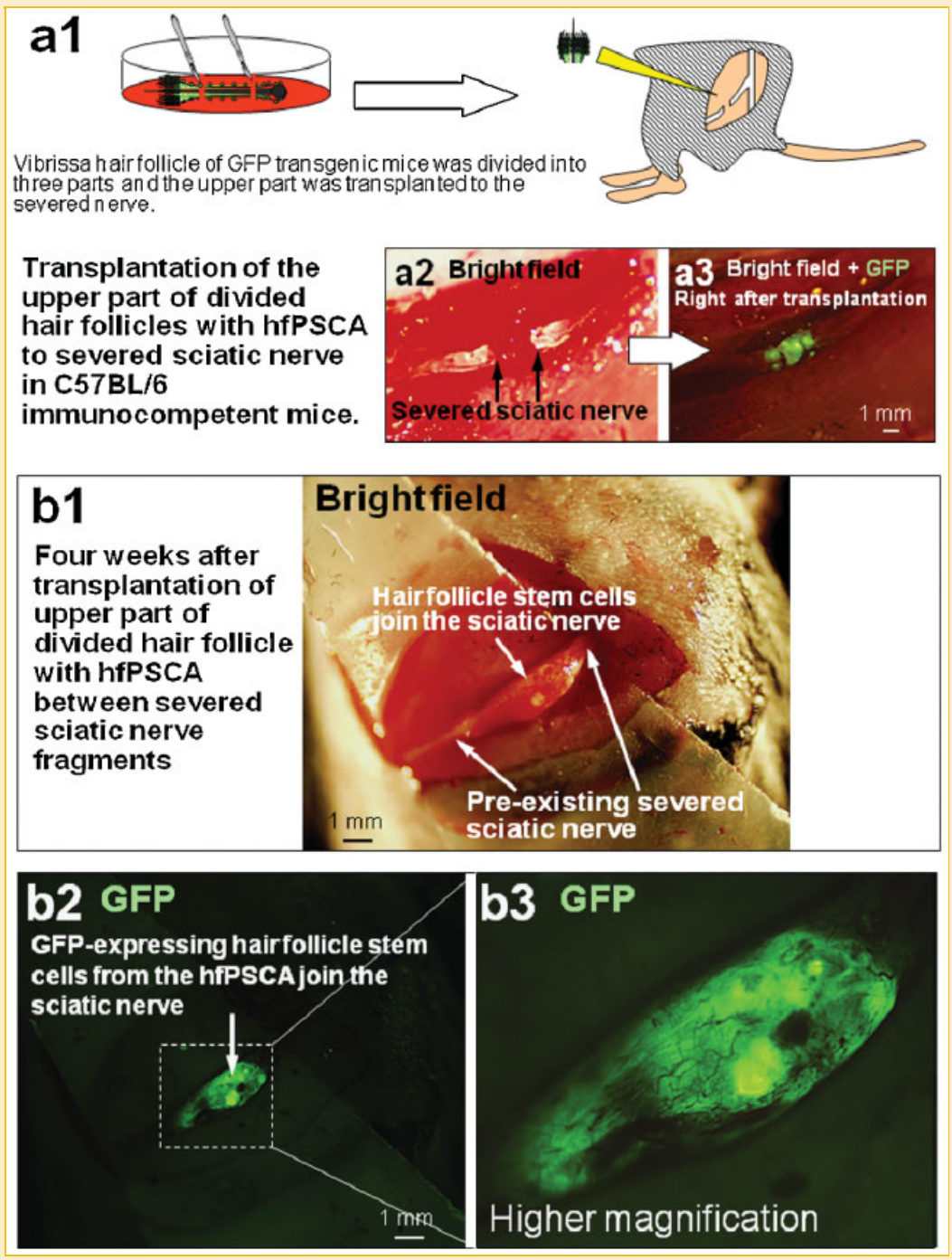


Fig. 1. The severed sciatic nerve of C57BL/6 immunocompetent mice was rejoined by transplantation of the upper part of the whisker containing the hair follicle pluripotent stem cell area (hfPSCA). (a1) A vibrissa hair follicle from a GFP-transgenic mouse was separated into three parts. The upper part containing the hfPSCA was used for transplantation to the severed sciatic nerve. (a2) Brightfield right after transplantation. (a3) Right after transplantation of the GFP-expressing hfPSCA. (b1–b3) Four weeks after transplantation of the GFP-expressing hfPSCA. GFP-expressing cells are observed in the rejoined sciatic nerve. Blood vessels formed a network around the joined sciatic nerve.

The C57/B6-GFP mice expressed the *Aequorea victoria* GFP under the control of the chicken β-actin promoter and cytomegalovirus enhancer. Vibrissae (whiskers) from the GFP-transgenic mice were used as the source of GFP-expressing hfPS cells in the present study.

### TRANSPLANTATION OF UNCULTURED hfPSCA BETWEEN THE SEVERED SCIATIC NERVE FRAGMENTS IN C57BL/6 IMMUNOCOMPETENT MICE

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. All surgical procedures were made under a sterile environment. The upper part of the hair follicle containing the hfPSCA was isolated from the rest of the vibrissa.

C57BL/6 immunocompetent mice (6–8 week; Harlan Laboratories, Tokyo, Japan) with a severed sciatic nerve [Amoh et al., 2005b] were anesthetized with tribromoethanol. The upper part of hair follicle containing the hfPSCA was transplanted between the severed sciatic nerve fragments. The incision was closed with nylon sutures (6–0).

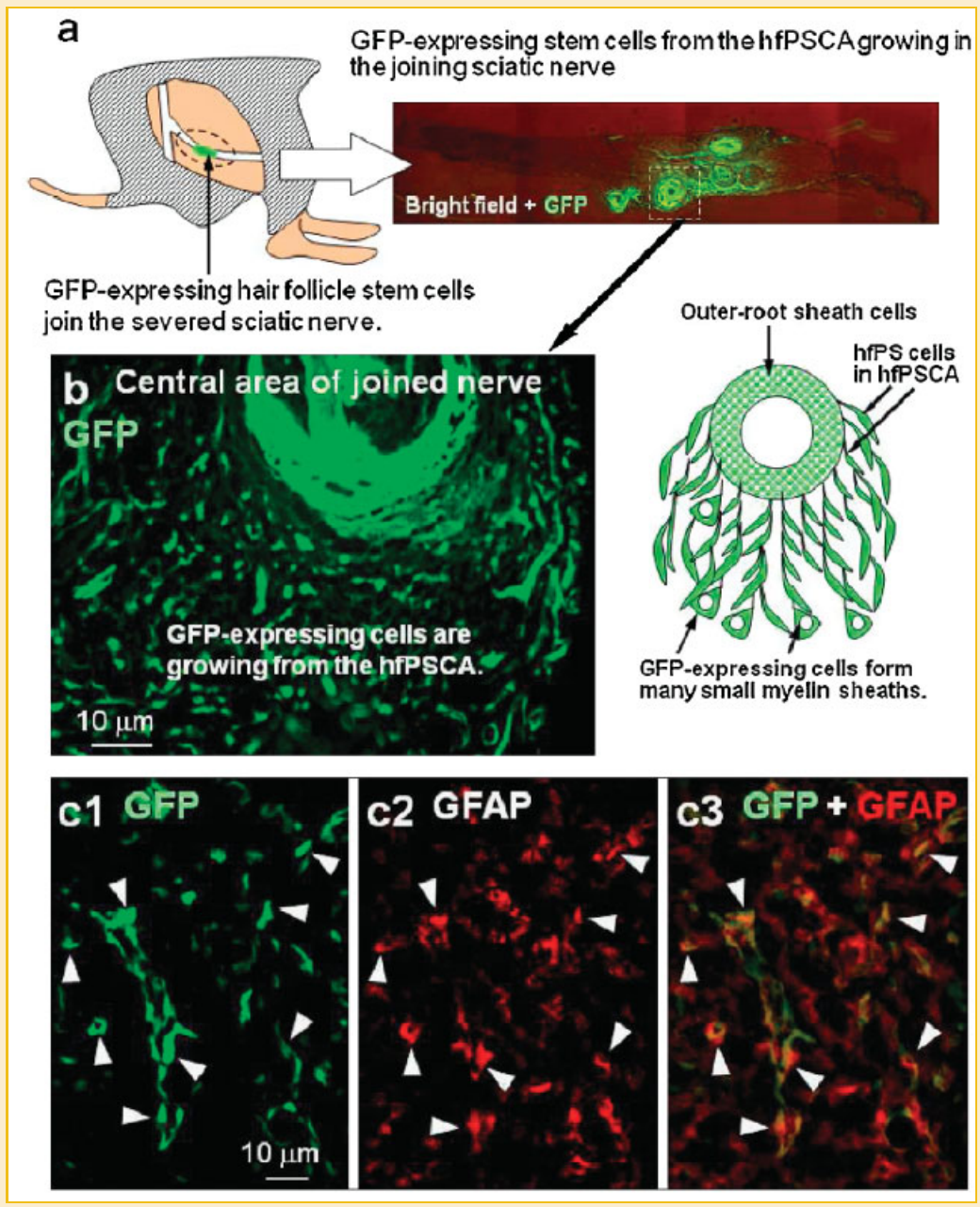


Fig. 2. Cell types growing in the area of the severed sciatic nerve joined after transplantation of the whisker GFP–hfPSCA b. (a) The GFP-expressing hfPS cells were growing in the joined sciatic nerve. (b) Transverse section of the central area of the joined nerve with transplanted GFP-expressing hfPS cells from the hfPSCA. Most of the GFP-expressing hfPS cells differentiated to Schwann cells and formed myelin sheaths. (c) The GFP-expressing hfPS cells differentiated to GFAP-positive Schwann cells in the rejoining sciatic nerve.

After 4 weeks, the sciatic nerve of the transplanted mice was directly observed by fluorescence microscopy, and the nerve samples of the transplanted mice were excised under anesthesia. The 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 directly observed by fluorescence microscopy and after that were used for the IF staining of anti-glial fibrillary acidic protein (GFAP), anti-2′-3′-cyclic nucleotide 3′-phosphodiesterase (CNPase), and βIII-tubulin.

### IMMUNOFLUORESENCE STAININGS

For immunofluoresence (IF), staining of the rejoined sciatic nerve was performed. The primary antibodies used were: anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; Covance Research Products, Inc., Berkeley, CA), GFAP monoclonal (1:200; Lab Vision, Fremont, CA), and CNPase monoclonal (1:50; Lab Vision). Secondary antibodies were Alexa Fluor® 568 goat anti-mouse (1:200; Molecular Probes, Eugene, OR).

### FLUORESCENCE MICROSCOPY

The rejoining sciatic nerve transplanted with GFP–hfPSCA 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).

### SCIATIC NERVE STIMULATION BY ELECTRIC STIMULATION

After 4 weeks, the rejoined nerve was stimulated upstream of the transplanted hfPSCA. The electric stimulator (FGK-1S, Medical Access Corp., Tokyo, Japan) delivered repetitious electric pulses of 0.05 mA, 10 Hz, with pulse widths of 0.5 m/s. Mice, with severed sciatic nerves, but without transplantation of GFP–hfPSCA, were used as controls. The gastrocnemius muscle lengths were measured

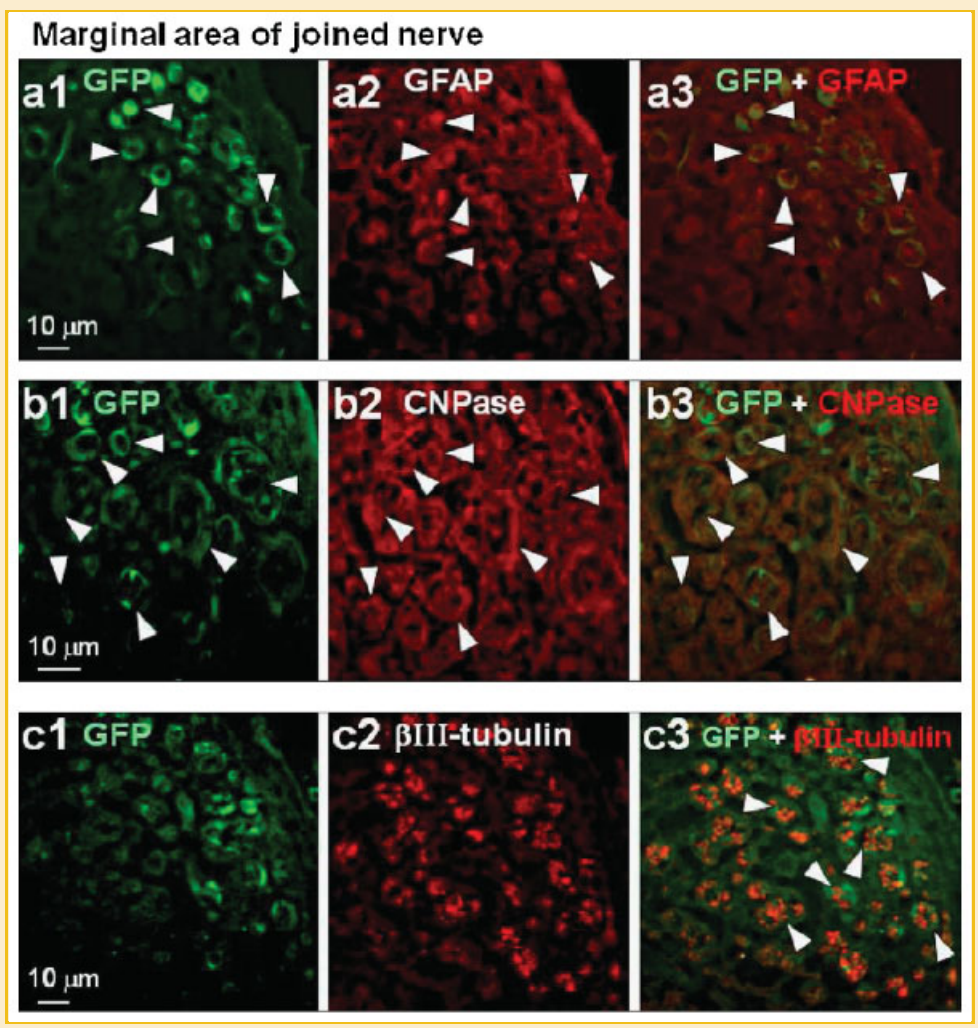


Fig. 3. Cell types growing in the rejoined region of the sciatic nerve after transplantation of hfPSCA. Most of the GFP hfPS cells differentiated to Schwann cells and formed myelin sheaths surrounding axons. (a–c) Transverse section of a rejoined sciatic nerve after transplantation of the hfPSCA. (a,b) The GFP-expressing hfPS cells differentiated to GFAP- and CNPase-positive Schwann cells in the rejoined sciatic nerve. (c) GFP-expressing Schwann cells formed myelin sheaths and surrounded βIII-tubulin-positive axons.

before and after contraction due to electric stimulation. Each experimental group consisted of five mice.

### STATISTICAL ANALYSIS

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

## RESULTS AND DISCUSSION

### TRANSPLANTATION OF THE hfPSCA PROMOTES JOINING OF THE SEVERED SCIATIC NERVE IN C57BL/6 IMMUNOCOMPETENT MICE

Vibrissa hair follicles from GFP transgenic mice were separated into three parts. The upper part containing the hfPSCA was transplanted to the severed sciatic nerve in C57BL/6 immunocompetent mice (Fig. 1a,b).

Four weeks after transplantation of the GFP–hfPSCA, the sciatic nerve rejoined and GFP cells were observed in the rejoined area (Fig. 1b1). A network of blood vessels around the joined sciatic nerve was observed (Fig. 1b2,b3).

### GFP-EXPRESSING hfPS CELLS DIFFERENTIATE INTO SCHWANN CELLS AFTER TRANSPLANTATION TO THE SEVERED SCIATIC NERVE

The GFP-expressing hfPS cells were growing in the joined sciatic nerve (Fig. 2a,b). Most of the GFP-expressing hfPS cells differentiated into Schwann cells (Fig. 2c). Transverse sections of the joined nerve were made. In the marginal area of the joined nerve, GFP-expressing cells formed GFAP- and CNPaes-positive myelin sheaths. GFP-expressing Schwann cells formed myelin sheaths, which surrounded βIII-tubulin-positive axons (Fig. 3).

### THE REJOINED SCIATIC NERVE CONTRACTED THE GASTROCNEMIUS MUSCLE UPON ELECTRICAL STIMULATION

Four weeks after transplantation of the GFP-expressing hfPSCA between the severed sciatic nerve fragments, the sciatic nerve was

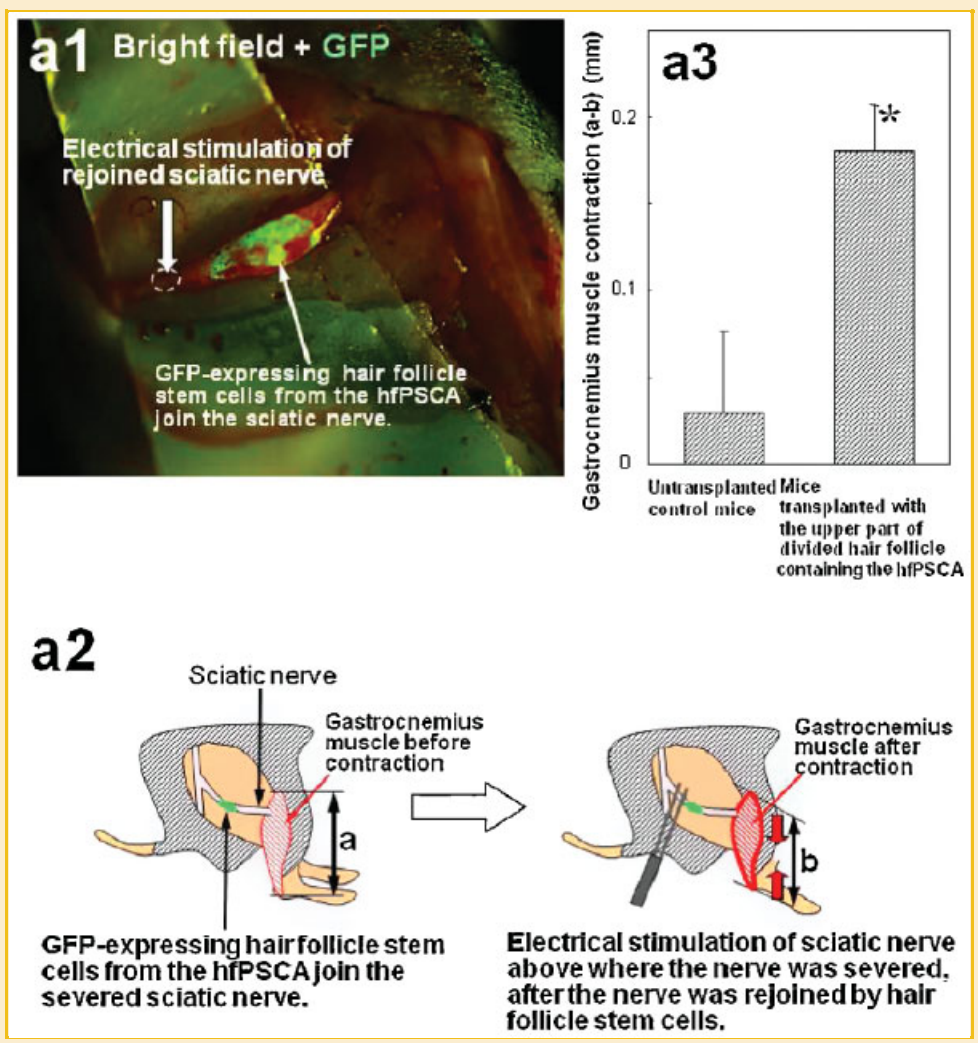


Fig. 4. Electrical stimulation of the rejoined sciatic nerve after transplantation of the hfPSCA. (a1) Four weeks after transplantation, electrical stimulation was carried out upstream of where the nerve was severed, and rejoined after hfPSCA transplantation. (a2) In the transplanted mice, the gastrocnemius muscle contracted upon electrical stimulation of the rejoined nerve. (a3) The transplanted mice had a greater degree of contraction of the gastrocnemius muscle compared to the untransplanted control mice. *$P < 0.001$ versus control (without transplantation).

electronically stimulated upstream of the rejoined area. The gastrocnemius muscles contracted upon electrical stimulation in the transplanted mice but not the untransplanted mice. These results suggest that transplantation of the upper part of the hair follicle containing the hfPSCA promoted nerve rejoining and functional recovery to effect gastrocnemius muscle contraction upon electrical stimulation, compared to severed nerves without transplantation (Fig. 4).

The fact that hair follicle nestin-expressing stem cells can be obtained from an autologous, accessible source, and do not form tumors, provides solutions to problems with using other sources of cell therapy, including fetal cells, ES cells, and induced pluripotent stem cells (iPS).

We have previously shown that the hfPS cells can regenerate peripheral nerve and spinal cord injuries [Amoh et al., 2005b, 2008]. If we use hfPS cells for nerve regeneration therapy, we should transplant the hfPS cells right after patients sustain injury. Previous methods used cultured hfPS cells from the mouse or human. However, clinically, it is detrimental to wait the time it takes to culture hfPS cells. Thus, in this study, we transplanted the uncultured hfPSCA, which resulted in rejoining and recovery of the severed nerve, as demonstrated by nerve rejoining and contraction of the gastrocnemius muscle upon electrical stimulation of the rejoined nerve. The results in the present report suggest that direct hfPSCA transplantation promotes axonal growth and functional recovery after peripheral nerve injury. The hfPSCA, as a direct source of pluripotent cells for nerve regeneration therapy, has important clinical potential.

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## REFERENCES

Amoh Y, Li L, Yang M, Moossa AR, Katsuoka K, Penman S, Hoffman RM. 2004. Nascent blood vessels in the skin arise from nestin-expressing hair-follicle cells. Proc Natl Acad Sci USA 101:13291–13295.

Amoh Y, Li L, Katsuoka K, Penman S, Hoffman RM. 2005a. Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. Proc Natl Acad Sci USA 102:5530–5534.

Amoh Y, Li L, Campillo R, Kawahara K, Katsuoka K, Penman S, Hoffman RM. 2005b. Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves. Proc Natl Acad Sci USA 102:17734–17738.

Amoh Y, Li L, Katsuoka K, Hoffman RM. 2008. Multipotent hair follicle stem cells promote repair of spinal cord injury and recovery of walking function. Cell Cycle 7:1865–1869.

Amoh Y, Kanoh M, Niiyama S, Hamada Y, Kawahara K, Sato Y, Hoffman RM, Katsuoka K. 2009. Human hair follicle pluripotent stem (hfPS) cells promote regeneration of peripheral-nerve injury: An advantageous alternative to ES and iPS cells. J Cell Biochem 107:1016–1020.

Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 1997. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407:313–319.