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Human Hair Follicle Pluripotent Stem (hfPS) Cells Promote Regeneration of Peripheral-Nerve Injury: An Advantageous Alternative to ES and iPS Cells

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ABSTRACT

The optimal source of stem cells for regenerative medicine is a major question. Embryonic stem (ES) cells have shown promise for pluripotency but have ethical issues and potential to form teratomas. Pluripotent stem cells have been produced from skin cells by either viral-, plasmid- or transposon-mediated gene transfer. These stem cells have been termed induced pluripotent stem cells or iPS cells. iPS cells may also have malignant potential and are inefficiently produced. Embryonic stem cells may not be suited for individualized therapy, since they can undergo immunologic rejection. To address these fundamental problems, our group is developing hair follicle pluripotent stem (hfPS) cells. Our previous studies have shown that mouse hfPS cells can differentiate to neurons, glial cells in vitro, and other cell types, and can promote nerve and spinal cord regeneration in vivo. hfPS cells are located above the hair follicle bulge in what we have termed the hfPS cell area (hfPSA) and are nestin positive and keratin 15 (K-15) negative. Human hfPS cells can also differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro. In the present study, human hfPS cells 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 generation. The regenerated nerve recovered function and, upon electrical stimulation, contracted the gastrocnemius muscle. The hfPS cells can be readily isolated from the human scalp, thereby providing an accessible, autologous and safe source of stem cells for regenerative medicine that have important advantages over ES or iPS cells. J. Cell. Biochem. 107: 1016–1020, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: HAIR FOLLICLE; STEM CELLS; PLURIPOTENCY; NESTIN; TRANSPLANTATION; PERIPHERAL NERVE; REGENERATION; REGENERATIVE MEDICINE

The optimal source of stem cells for regenerative medicine is a major question. Embryonic stem cells have shown promise for pluripotency but have ethical issues and potential to form teratomas [Thomson et al., 1998]. Pluripotent stem cells have been produced from skin cells by either viral-, plasmid- or transposon-mediated gene transfer. These stem cells have been termed induced pluripotent cells or iPS cells [Takahashi and Yamanaka, 2006]. iPS cells may also have malignant potential and are inefficiently produced. Embryonic stem cells may not be well suited for individualized therapy, since they can undergo immunologic rejection as well as form tumors. To address these fundamental problems, our group is developing pluripotent stem cells from the hair follicle (hfPS cells).

The intermediate filament protein, nestin, marks progenitor cells of the central nervous system (CNS). By placing green fluorescent protein (GFP) under the control of nestin regulatory sequences in nestin-driven GFP transgenic mice (ND-GFP), neural stem cells are labeled [Sawamoto et al., 2001; Mignone et al., 2004]. Our

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laboratory has observed that during early anagen or growth phase of the hair follicle, nestin-expressing cells, marked by GFP fluorescence in the ND-GFP transgenic mice, appear in the permanent upper hair follicle immediately below the sebaceous glands above the follicle bulge. The relatively small, oval-shaped, ND-GFP cells in the bulge area surround the hair shaft and are interconnected by short dendrites. Nestin expression in these hair follicle stem cells was the first suggestion that they may be pluripotent [Li et al., 2003].

We subsequently demonstrated that the ND-GFP hair follicle stem cells can differentiate into neurons, glia, keratinocytes, smooth muscle cells, and melanocytes in vitro. These pluripotent ND-GFP hair follicle stem cells are positive for the stem cell marker CD34, as well as keratin 15-negative, suggesting their relatively undifferentiated state. The apparent primitive state of the ND-GFP hair follicle stem cells is compatible with their pluripotency [Amoh et al., 2005a].

The mouse pluripotent hair follicle stem cells were implanted into the gap region of the severed sciatic nerve of mice. The transplanted hair follicle cells greatly enhanced the rate of nerve regeneration and the restoration of nerve function. The pluripotent hair follicle stem cells transdifferentiated largely into Schwann cells, which are known to support neuron regrowth. Function of the rejoined sciatic nerve was confirmed by contraction of the gastrocnemius muscle upon electrical stimulation of the regenerated nerve [Amoh et al., 2005b].

Subsequently, we severed the thoracic spinal cord of C57BL/6 immunocompetent mice and transplanted mouse pluripotent 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 re-established extensive hind-limb locomotor performance. These results suggest that pluripotent hair follicle stem cells can promote the recovery of spinal cord injury [Amoh et al., 2008].

We have previously shown that in the intact human hair follicle dissected from the scalp, the cells immediately below the sebaceous glands, just above the bulge area, are nestin-positive, K15-negative. In contrast, the hair follicle bulge itself contained nestin-negative, K15-positive cells [Amoh et al., 2009] (Fig. 1). These nestin-positive keratin-negative human hair follicle cells formed spherical colonies in culture, termed hair spheres by Yu et al. [2006]. Incubation of hair spheres in RPMI 1640 containing 10% FBS resulted in differentiated cells including β3-tubulin-positive neurons, S-100-positive, and GFAP-positive glial cells, K15-positive keratinocytes, and SMA-positive smooth muscle cells [Amoh et al., 2009]. The plucked scalp hair follicle, in contrast to the intact hair follicle obtained by dissection of the scalp, did not contain sebaceous glands or nestin-positive, K15-negative cells above the bulge. The upper part of the plucked scalp hair follicle, contained only the bulge area and nestin-negative, keratin-positive cells, which also formed hair spheres. After switching the medium to RPMI 1640 containing 10% FBS, keratinocystes were formed but neurons and other non-follicular differentiating cell types were not observed [Amoh et al., 2009]. Thus, the hair follicles of mice and human appear to have two populations of stem cells: a pluripotent type and an apparent unipotent type.

In the present study, the nestin-expressing human hair follicle stem cells were transplanted between severed sciatic fragments of the mouse, where they differentiated into glial fibrillary acidic protein (GFAP)-positive Schwann cells and apparently promoted the growth of pre-existing axons, resulting in nerve functional nerve regeneration. We have termed these hair follicle pluripotent stem (hfPS) cells.

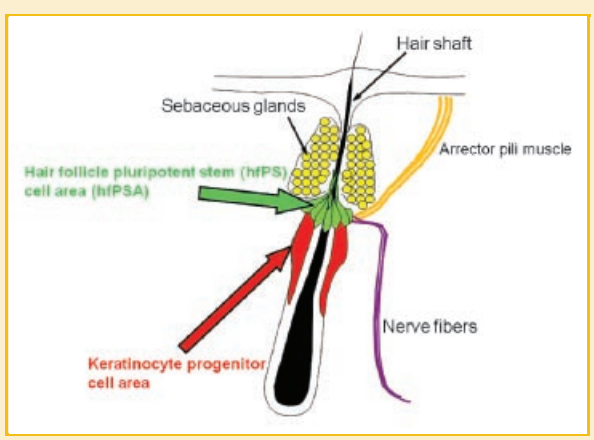

Fig. 1. Location of hair follicle pluripotent stem (hfPS) cells in the hair follicle stem cell area (hfPSA).

MATERIALS AND METHODS

ISOLATION OF HAIR FOLLICLES FROM HUMAN SCALP SKIN SAMPLES AND CULTURE OF hfPS CELLS

Human scalp skin samples were obtained from surgical specimens of normal human scalp skin obtained with informed consent of Kitasato University Medical School. All the experiments were performed according to Helsinki guidelines, in compliance with regulations for the experimental use of human material. To isolate the whole intact hair follicle, the scalp skin containing the hair follicle pad was cut and its inner surface was exposed.

The scalp hair follicles were dissected from scalp skin under a binocular microscope and removed from the pad by pulling them gently by the neck with fine forceps. The follicles were washed in DMEM-F12 (Gibco-BRL, Grand Island, NY) containing B-27 (Gibco-BRL) and 1% penicillin/streptomycin (Gibco-BRL). All surgical procedures were performed in a sterile environment. The area, which is located immediately below the sebaceous glands and above the hair follicle bulge area, contains the nestin-positive and K15-negative hfPS cells [Amoh et al., 2009]. This area, termed the hair follicle pluripotent stem cell area (hfPSA), was then isolated under a binocular microscope, and was suspended in 1 ml DMEM-F12, containing B-27 with 1% methylcellulose (Sigma-Aldrich), for culture. Basic FGF (Chemicon, Temecula, CA) (20 ng ml^{-1}) was added every 2 days. 24-well tissue culture dishes (Corning, Aliso Viejo, CA) were used in a 37°C, 5% CO_2/95% air tissue-culture incubator. After 4 weeks, the nestin-positive hfPS formed cell colonies (spheres).

IMMUNOFLUORESCENCE STAININGS AND QUANTIFICATION

The primary antibodies that were used in immunofluorescence staining were: anti-βIII-tubulin monoclonal (1:500, Tuj1 clone; Covance Research Products, Inc., Berkeley, CA); anti-nestin polyclonal (1:200; Chemicon); anti-glial fibrillary acidic protein (GFAP) monoclonal (1:200; Lab Vision, Fremont, CA); and anti-K15 monoclonal (1:100; Lab Vision). Secondary antibodies were fluorescein-conjugated sheep anti-mouse IgG (1:10; Chemicon) and tetramethylrhodamine-conjugated swine anti-rabbit IgG (1:80; Nordic Immunological Laboratories, Tilburg, Netherlands). DAPI (Invitrogen, Carlsbad, CA) was used for nuclear counterstaining.

TRANSPLANTATION OF hfPS CELLS BETWEEN SEVERED SCIATIC NERVE FRAGMENTS IN NUDE MICE

A 2-mm section of the central area in the sciatic nerve in nude mice was cut. Three-to-four human hfPS colonies from intact scalp hair follicles were transplanted between the severed sciatic nerve fragments of the nude mice under tribromoethanol anesthesia. The skin incision was closed with nylon sutures (6–0). After 8 weeks, the rejoined sciatic nerves of the transplanted mice were dissected under anesthesia.

The sciatic nerve samples were put in 10% formalin medium and embedded in paraffin. Sections were made for hemotoxylin and eosin (H&E) staining, and for immunofluorescence staining of βIII-tubulin and glial fibrillary acidic protein.

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 ms, was used to stimulate control mice, mice with severed sciatic nerves, and mice that had transplanted hfPS cells. The difference of the gastrocnemius muscle lengths (from lateral epicondyle of femur to heel) before and after contraction stimulated by the electric stimulator was calculated. Each experimental group consisted of five mice.

STATISTICAL ANALYSIS

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

RESULTS AND DISCUSSION

GROWTH OF HUMAN hfPS CELLS IN VITRO FOR TRANSPLANTATION IN SEVERED NERVES

Human hfPS cells were grown for two months in DMEM-F12 containing B-27, 1% methylcellulose, and basic FGF, where they formed spheres. The spheres were used for transplantation in the severed sciatic nerve of nude mice (Fig. 2).

HUMAN hfPS CELLS PROMOTE THE RECOVERY OF PERIPHERAL NERVE INJURY

Eight weeks after transplantation of human hfPS cells between the severed sciatic nerve fragments of nude mice, many spindle cells grew in the severed part of the sciatic nerve (Fig. 3a1, 3a2). In the untransplanted control mice, however, only small numbers of spindle cells grew in the severed part of the sciatic nerve (Fig. 3b1, 3b2). The human hfPS cells differentiated to glial fibrillary acidic protein–positive Schwann cells after transplantation. The newly differentiated Schwann cells formed myelin sheaths that surrounded β3-tubulin-positive axons in the rejoining nerve (Fig. 4a). In the untransplanted control mice, small numbers of glial cells grew in the severed sciatic nerve and formed a glial scar (Fig. 4b). The glial cells in the untransplanted mice did not surround β3-tubulin-positive axons.

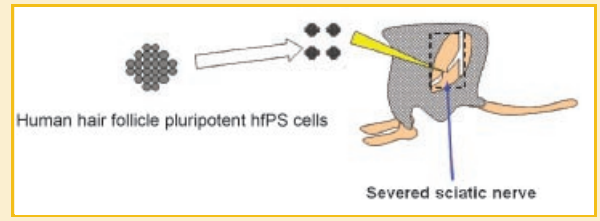

Fig. 2. Transplantation of human hfPS cells to the severed sciatic nerve. Human hfPS cells, grown for two months in DMEM-F12 containing B-27, 1% methylcellulose, and basic FGF, were transplanted in the severed sciatic nerve fragments in nude mice (blue arrow).

Eight weeks after human hfPS cell transplantation, the gastrocnemius muscle contracted upon electrical stimulation of the rejoined nerve. The sciatic nerve was stimulated above where the nerve was severed. The extent of gastrocnemius muscle contraction in transplanted mice was much greater in the transplanted mice than the untransplanted control mice. $^{*}P < 0.05$ versus control (Fig. 5a,b) (Supplemental Movie 1).

In a recent study, we observed that the intact anagen hair follicle of the human scalp contained nestin-positive, K15-negative hfPS cells, which are located immediately below the sebaceous glands in the hfPSA, which is above the bulge area in the intact hair follicle

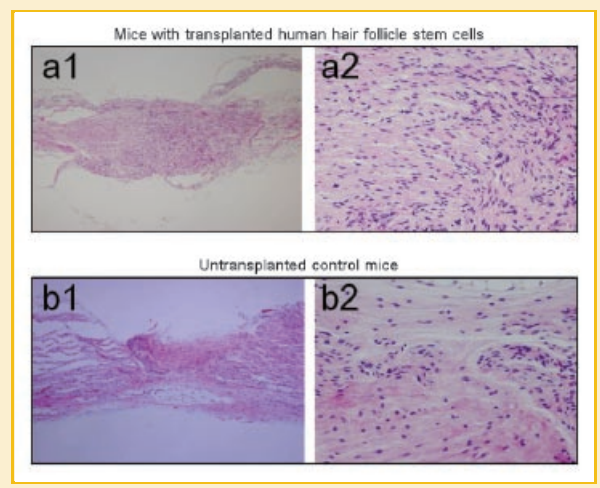

Fig. 3. H&E staining of the severed part of the sciatic nerve in transplanted and untransplanted control mice. Eight weeks after transplantation of human hfPS cells between the severed sciatic nerve fragments, many spindle cells grew in the severed part of the sciatic nerve (a1, a2). In the untransplanted control mice, small numbers of spindle cells grew in the severed part of the sciatic nerve (b1, b2). a2 and b2 are higher magnification of a and b, respectively.

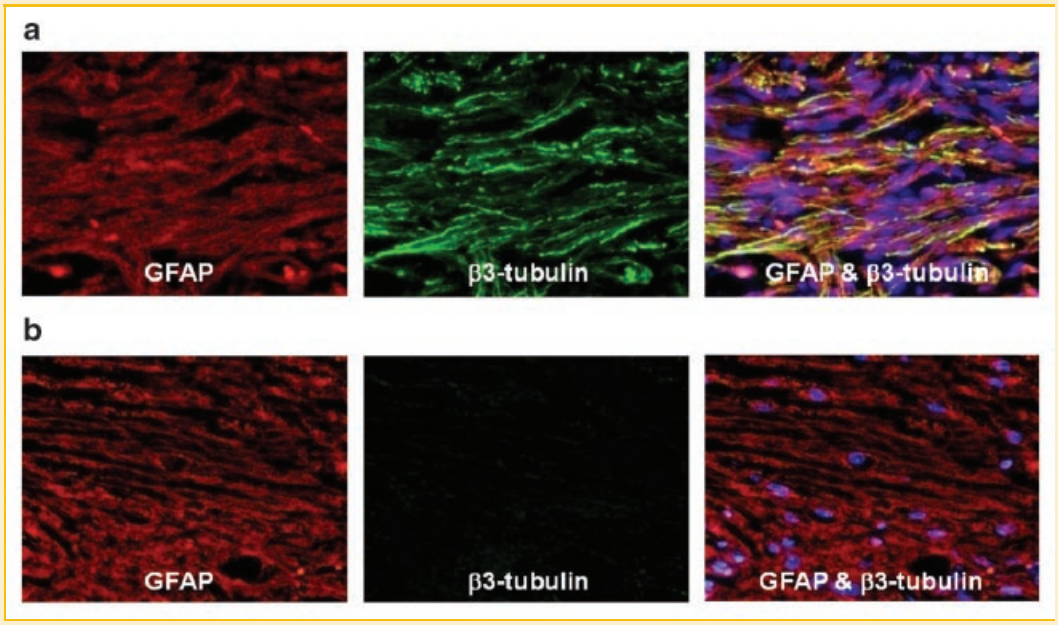

Fig. 4. Immunofluorescence staining. Human hfPS cells differentiated to glial fibrillary acidic protein–positive Schwann cells after transplantation between the fragments of the severed sciatic nerve in nude mice. The Schwann cells formed myelin sheaths which surrounded β3–tubulin–expressing axons (a). In the untransplanted control mice, small numbers of glial cells grew in the severed part of the sciatic nerve and formed a glial scar (b). Please see Materials and Methods section for details of procedures.

[Amoh et al., 2009]. The nestin-positive, K15-negative hfPS cells can differentiate into neurons, glial cells, keratinocytes, and smooth muscle cells. In contrast, plucked anagen hair follicles from the human scalp contained nestin-negative, K15-positive cells and not the nestin-positive, K15-negative hfPS cells. The upper part of the plucked hair follicle differentiated only into K15-positive keratinocytes. These results suggest that the nestin-positive, K15-negative hfPS cells in the hfPSA of the intact hair follicle are distinct from the nestin-negative, K15-positive cells in the bulge which are keratinocyte progenitor cells [Amoh et al., 2009].

Human pluripotent cell lines derived from blastocysts were described in 1998 [Thomson et al., 1998]. These cells have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize embryonic stem cells. These cells have the developmental potential to form trophoblast and derivatives of all three embryonic germ layers. The term "ES cell" was introduced for these embryo-derived pluripotent stem cells. ES cells, however, can term teratomas in mice [Thomson et al., 1998].

Differentiated cells can be reprogrammed to an embryonic-like state by transfer of their nuclei into oocytes or by fusion with ES

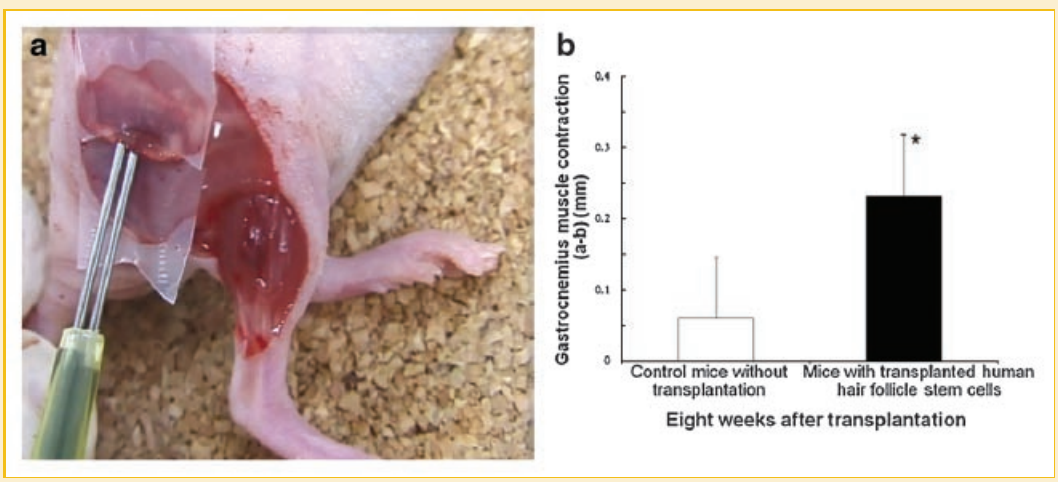

Fig. 5. The sciatic nerve, that was rejoined by transplantation of hfPS, contracted the gastrocnemius muscle upon electrical stimulation. The rejoined sciatic nerve was electrically stimulated above where the nerve was severed and caused gastrocnemius muscle contraction in mice eight weeks after human hfPS cell transplantation (a). Comparison of the extent of gastrocnemius muscle contraction in transplanted and untransplanted control mice showed a major increase in the hfPS–cell transplanted mice, $^{*}P < 0.05$ versus control (b).

cells. Pluripotent stem cells can also be derived from mouse embryonic or adult fibroblasts by introducing four genes, Oct3/4, Sox2, c-Myc, and Klf4. These cells were designated iPS (induced pluripotent stem) cells. iPS cells exhibit the morphology and growth properties of ES cells and express ES cell marker genes. However, subcutaneous transplantation of iPS cells into nude mice resulted in tumors containing a variety of tissues from all three germ layers [Takahashi and Yamanaka, 2006].

In iPS cells, reactivation of the c-Myc retrovirus increases tumorigenicity. A modified protocol for the generation of iPS cells has been developed that does not require the Myc retrovirus resulting in reduced tumorigenicity [Nakagawa et al., 2008].

In order to further reduce tumorigenicity, mouse iPS cells were made without viral vectors. Transfection of two expression plasmids, one containing the complementary DNAs (cDNAs) of Oct3/4, Sox2, and Klf4 and the other containing c-Myc cDNA, into mouse embryonic fibroblasts resulted in iPS cells without evidence of plasmid integration. Plasmid integration is known to result in tumorigenicity [Okita et al., 2008].

Very recently, non-viral transfection of a single multigene expression vector, which comprises the coding sequences of c-Myc, Klf4, Oct4, and Sox2 linked with 2A peptides, has been shown to reprogram both mouse and human fibroblasts. Moreover, the transgenes could be removed once reprogramming has been achieved. The iPS cells produced with this removable non-viral vector are pluripotent. Such iPS cells may have reduced tumorigenicity [Kaji et al., 2009].

In addition, very recently reprogramming of murine and human embryonic fibroblasts was achieved using doxycycline-inducible transcription factors including C-MYC, Kf4, Oct4, and Sox2 delivered by the *piggyBac* (PB) transposon. Stable iPS cells thus generated express characteristic pluripotency markers and differentiate to many cell types. Individual PB insertions could be removed from established iPS cell lines [Woltjen et al., 2009].

Also very recently, fibroblasts from patients with Parkinson's disease were reprogrammed with the above transcription factors and then differentiated into dopaminergic neurons. The transcription factors were removed using Cre-recombinase excisable viruses. The factor-free reprogrammed cells maintained their pluripotent state [Soldner et al., 2009].

In contrast to ES and iPS cells, hfPS cells do not require any genetic manipulation, are readily accessible from any patient, do not form tumors, and can be used for nerve and spinal cord generation. hfPS cells, therefore, have important potential for regenerative medicine and provide an advantageous and attractive alternative to ES and iPS cells.

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