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ORIGINAL ARTICLE

Single follicular unit transplantation reconstructs arrector pili muscle and nerve connections and restores functional hair follicle piloerection

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ABSTRACT

The autologous transplantation of hair follicles that have been separated into single follicular units is an accepted treatment for androgenetic alopecia. Recent studies demonstrate that the multiple stem cell populations and surrounding cutaneous tissues coordinately regulate the hair follicle functions and skin homeostasis. Therefore, the critical issues for consideration regarding functional hair restoration therapy are reproduction the correct connectivity and cooperation with host cutaneous tissues, including the arrector pili muscle (APM) and nerve system. We report successful establishment of mouse single follicular transplantation model and autonomous restoration of transplanted hair follicle piloerection in mouse skin. Transplanted hair follicles were responsive to the neurotransmitter acetylcholine and formed proper connections with surrounding host tissues such as APM and nerve fibers, which in turn connect with not only the hair follicle bulge region but also the APM. These results demonstrate that the piloerection ability of transplanted hair follicles can be estimated quantitatively. This study makes a substantial contribution towards the development of transplantation therapy that will facilitate future functional regeneration therapy for skin and skin appendages.

Key words: **arrector pili muscle, functional regeneration, hair follicular unit transplantation, piloerection.**

INTRODUCTION

Transplantation therapy has been utilized for the treatment of patients with organ dysfunction following disease, injury or aging. In the field of dermatology, optimal improvement of a patient's quality of life relies on engrafted skin becoming re-innervated by recipient nerve systems.[1] Furthermore, autologous single follicular unit transplantation (FUT) has been adopted for the treatment for androgenetic alopecia based on successful hair restoration nearly equivalent to a normal scalp condition.[2] Additional criteria for fully successful hair restoration include whether the transplants regenerate normal inherent traits and proper physiological functions, including the correct connectivity and cooperation with host cutaneous tissues such as to the arrector pili muscle (APM) and nerve system.[1] Therefore, the use of FUT methods can successfully transplant both a hair follicle and their surrounding microenvironment, including APM, ineffective nerve fibers, minimum dermis and subcutaneous tissues.[1,3] However, the details of structural and functional restoration of transplanted hair follicle, and the precise mechanisms by which connections are formed between a hair follicle and host nerve system, are poorly understood.

Basically, the hair coat plays important roles in thermoregulation, physical protection, sensory activity to contact stimulations and social communication.[4] These roles function coordinately with peripheral nerves and APM consisting of smooth muscle muscle.[5,6] In the rodents, a vibrissa-type follicle is a specialized sensitive sensory organ that is connected to dense sensory nerve fibers and voluntary muscle, which is innervated by motor neurons.[6,7] Activation of sympathetic nerves stimulates the APM and initiates hair follicle piloerection in almost all human hairs and rodent coat hairs.[8,9] The APM, which is an involuntary smooth muscle, is immunopositive for α-smooth muscle actin, α8-integrin and calponin.[10,11] The APM connects the bulge region of a permanent portion of the hair follicle to the dermis and is innervated by adrenergic neurons, which are classified as sympathetic neurons.[12,13] Recently, it was reported that the embryonic hair follicle bud could function as a guide for

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migration of sense neurons *in vivo* and *in vitro* and that nephronectin-expressing follicular epithelial stem cells provide the niche for APM development and define the positioning of the follicular muscular junction.[10,14] However, functional restoration and re-innervation of APM of adult hair follicles are poorly understood.

In this study, we report the autonomous restoration of the pilo-erection ability of engrafted hair follicles in mouse skin by FUT. The transplanted hair follicles formed proper connections with surrounding tissues such as the APM and nerve fibers. These connections were not only in the hair follicle bulge region but also in the APM and were responsive to the neurotransmitter acetylcholine (ACh).[8–10] These findings and methods are a substantial contribution to improvement of ongoing skin grafting therapy.

METHODS

Animals

C57BL/6 and BALB/c nu/nu mice were purchased from Japan SLC (Shizuoka, Japan). C57BL/6-TgN (act-EGFP) OsbC14-Y01-FM131 (EGFP) mice were obtained from Japan SLC and the RIKEN Bioresource Center (Tsukuba, Japan). Mouse care and handling conformed to the National Institutes of Health guidelines and the requirements of the Tokyo University of Science Animal Care and Use Committee.

Preparation of single follicular units for transplantation

Full thickness dorsal skin was obtained from 8-week-old C57BL/6-TgN (act-EGFP) OsbC14-Y01-FM131 (EGFP) mice that were depilated of dorsal hairs at 5 days before dissection.[15] Subcutaneous adipose and connective tissues were immediately removed and hair follicular units were dissected using a surgical knife (No. 14 blade; Feather, Osaka, Japan) as shown in Figure 1(a). Dissected follicular units were floated on cold Dulbecco's modified Eagle's medium (Kohjin Bio, Sakato, Japan) containing 10% fetal calf serum (Gibco, Carlsbad, CA, USA), 1% antibiotics (Gibco) and 1% HEPES (Gibco), until transplantation.

Transplantation of single follicular unit

Six-week old BALB/c nu/nu mice were anesthetized with an i.p. injection of pentobarbital. Using a 20-G Ophthalmic V-Lance (Alcon Japan, Tokyo, Japan), shallow stab wounds were made in the back skin of nude mice that were nearly parallel to the host pelage and approximately equal in length to that of the separated pelage follicle from the needle-stick point. The separated hair follicle was implanted into the wound intracutaneously using a pair of micro-forceps.[2] The transplantation sites were then covered with surgical bandage tape (Nichiban, Tokyo, Japan). The explants were followed up for 50 days after transplantation.

Follow up of transplanted hairs

To examine the transplant engraftment and the hair cycles of the transplanted hair follicles, all transplanted sites were observed at 3–5-day intervals using a SteREO Lumar V12 and AxioCam fluorescent stereoscopic microscope system (Carl Zeiss, Oberkochen, Germany).

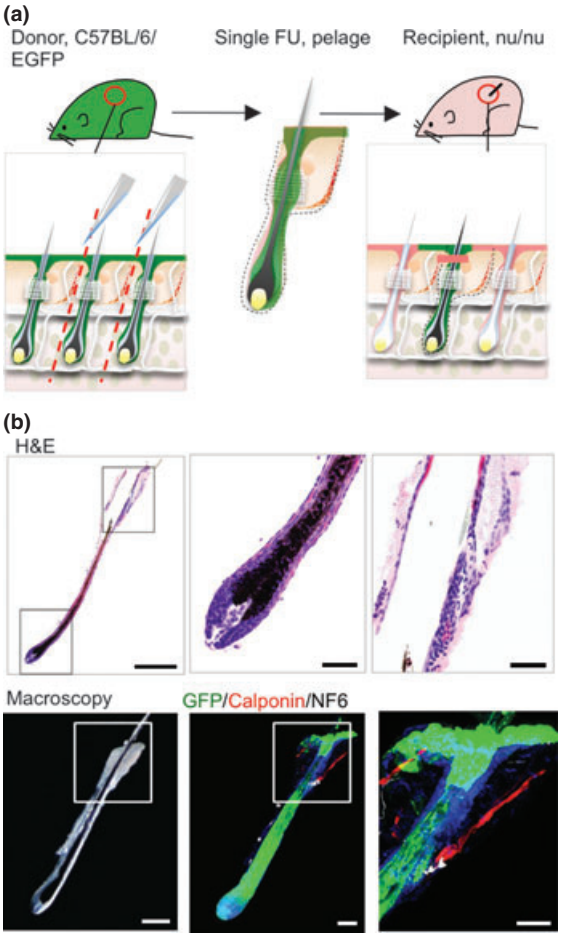

Figure 1. Single follicular unit transplantation model using mouse dorsal skin. (a) Schematic representation of the preparation of single follicular units and transplantation processes. (b) Histological (upper) and immunohistochemical (lower) analyses of a separated hair follicle. Boxed areas in the left and center panels are shown at a higher magnification in the right panels. Scale bars, 50 μm. EGFP, enhanced green fluorescent protein; FU, follicular unit; GFP, green fluorescent protein; H&E, hematoxylin–eosin.

Immunohistochemistry

Paraffin sections (5-μm thick) were stained with hematoxylin–eosin and observed using Axioimager A1 (Carl Zeiss) and AxioCAM MRc5 (Carl Zeiss) microscopes. For fluorescent immunohistochemistry, 100-μm thick frozen sections were blocked in Tris-buffered saline containing 1% bovine serum albumin, 0.5% and Triton X-100 (Sigma, St Louis, MO, USA) for 2 h at room temperature, and then incubated overnight with the following primary antibodies in blocking solution at 4°C: Neurofilament-H (rat, Chemicon, Billerica, MA, USA) and Calponin (rabbit, Abcam, Cambridge, UK). Bound primary antibodies were detected using the secondary antibodies in phosphate-buffered saline (PBS) (–) and were incubated for 3 h at room temperature. The secondary antibodies used were goat anti-immunoglobulin (Ig)G (H + L) Alexa Fluor 633 (Invitrogen, Carlsbad, CA, USA) and goat anti-IgG (H + L) Alexa Fluor 594

highly cross-absorbed (Invitrogen). Slides were counterstained with 4 μg/mL of Hoechst 33258 dye (Dojindo, Kumamoto, Japan) during secondary antibody incubation. All fluorescence microscopy images were acquired using an LSM 780 confocal microscope (Carl Zeiss).

Piloerection of a transplanted hair

To investigate whether piloerection could be demonstrated in transplanted hairs, hair responses to neurotransmitter agents and piloerection inhibitors were evaluated as reported previously.[8–10] Agents were injected i.d. into the close vicinity of the transplanted hair follicles using a 10-μL micro-syringe (Hamilton, Reno, NV, USA). To prevent possible cutaneous reflex movements, a 1 μL volume of PBS(–) was first injected i.d., followed by a 1 μL volume of 0.1, 1.0 or 10 mg/mL acetylcholine at the same position. The piloerection angle was then measured using microscopic image analysis. To determine the specificity of acetylcholine initiating the piloerection process, 1 μL of a 100 mg/mL solution of the acetylcholine inhibitor atropine sulfate (Sigma) was injected prior to acetylcholine.[8] Transplanted hairs were photographed as side views before and after this injection using a SteREO Lumar V12 and AxioCam fluorescent stereoscopic microscope system. Each hair's angle of change (radian) was measured using image analysis software. The hair angle before injection was defined as the baseline reference point.

Statistical analysis

Statistical significance was determined using the unpaired Student's *t*-test. All analyses were conducted using the Common Gateway Interface Program (twk, Saint John's University).

RESULTS

Single FUT model using mouse dorsal skin

To establish a mouse model of single FUT performed in the same manner as clinical therapy for androgenetic alopecia,[2,3] intact pelage follicular units with caudal dermis tissues were separated from the dorsal skin of EGFP-Tg mice and then transplanted onto the backs of nude mice (Fig. 1a). All transplanted pelage follicles were synchronized in their hair cycle using previously reported depilation methods.[15] The separated pelage follicles were at anagen phase and remained intact as verified by thin section histological analysis (Fig. 1b, upper panels). Whole separated pelage follicles were double immunostained with anti-calponin and anti-neurofilament-H (NF-H) antibodies, which recognize the APM components of smooth muscle and nerve fiber, respectively. Preservation of the APM and its connection between the bulge area of the separated pelage follicle and the dermal layer was confirmed by confocal laser scanning microscopy (Fig. 1b, lower panels). Nerve fiber shreds were observed to remain on the APM and surrounding outer root sheaf region bulge area (Fig. 1c). These results indicated that our preparation of pelage follicular units was similar to the human FUT method.[2,3]

Restoration of the transplanted pelage follicle and connection with surrounding tissues

We next investigated autonomous wound healing and restoration of a transplanted pelage follicle. Using a microsurgical knife, shallow stab wounds were made into the back skin of nude mice that were nearly parallel to the host pelage and at a length that was approximately equal to that of the separated pelage follicle from the needle-stick point (Fig. 1a). Individual follicular units were then implanted into each stab injury. Transplantation site wounds had visually healed and a follicular orifice was identified 10 days after transplantation, concurrently with the first hair shaft loss (Fig. 2a). The first growth phase of implanted hair shafts resumed at 14 days after transplantation (Fig. 2a). Hair growth and regression was repeated twice in the first 50 days after transplantation (Fig. 2a).

Next, histological analyses were performed to determine whether the transplanted pelage follicles displayed restoration of APM and nerve connections. At 6 days after transplantation, transplanted pelage follicles, which were distinguishable from host hair follicles by the presence of melanin granules or enhanced green fluorescent protein fluorescence, were histologically at the telogen phase. The transplantation wounds had completely healed, although the transplanted pelage follicles were scarcely observed to connect to the APM or to the nerve fibers at the bulge region of the transplanted pelage follicle. However, during the second hair growth phase 40 days after transplantation (Fig. 2a), the transplanted pelage follicles were observed to clearly connect to both the APM and to the nerve fibers, which is green fluorescent protein-negative, at the bulge region (Fig. 2b). Similar profiles were also observed in the natural pelage follicles. Importantly, nerve terminals were observed in the reproduced APM (Fig. 2b). These findings indicated that transplanted pelage follicles could autonomously restore proper connections with the APM and nerve fibers and suggested that transplanted follicles should have piloerection abilities comparable to the normal functioning of natural follicles.

Reproduction of the piloerection ability of transplanted hair

Finally, to investigate whether the transplanted pelage follicles exhibited normal piloerection, ACh was administrated i.d. into the vicinity of the engrafted follicles at the second hair growth phase and the angle of each hair shaft was assessed before and after stimulation (Fig. 3). Exposure to 10 μg ACh led to a significantly increased angle of piloerection in the bioengineered pelage compared with the response to i.d. injection of PBS used as control (Fig. 3a). The angle of piloerection in the transplanted pelages following ACh administration increased dose-dependently (Fig. 3b). Furthermore, administration of atropine, an anti-cholinergic agent, prior to ACh significantly inhibited the piloerection response compared with pelages injected with ACh alone (Fig. 3c). These results indicate that the transplanted single follicular unit pelages have a piloerection ability that is comparable to natural hair follicles. After restoration, these follicles exhibit induced piloerection that is comparable to natural pelage follicles (Fig. 3c). These findings suggest that transplanted pelage follicles can autonomously induce restoration of hair follicle function and form proper connections with the APM at the appropriate location on the hair follicle.

DISCUSSION

This study demonstrates the autonomous restoration of piloerection ability through the formation of proper connections between the hair

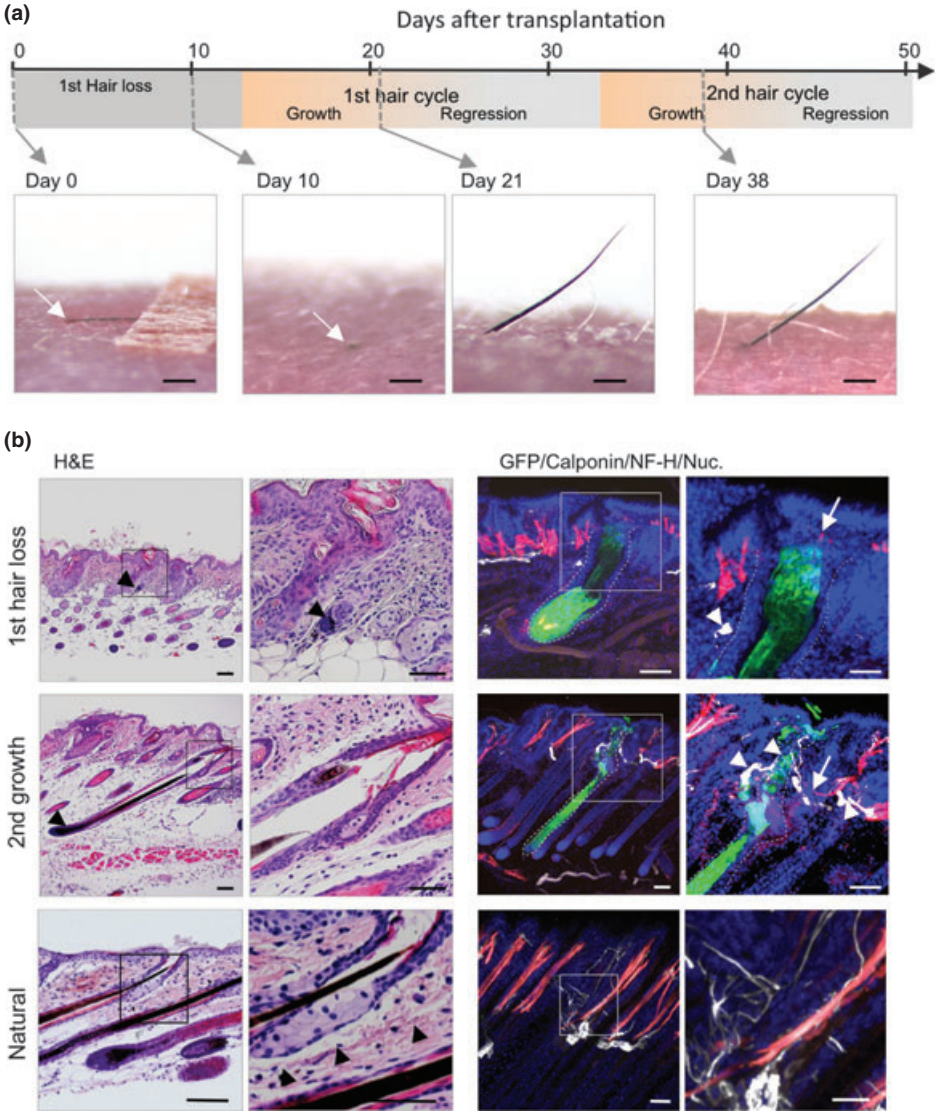

Figure 2. Restoration of transplanted pelage follicle and its connections with surrounding tissues. (a) The hair cycle of transplanted hair follicles as defined by macro-morphological observations of hair shaft. Arrows, transplantation site wounds and a follicular orifice. Scale bars, 1.0 mm. (b) Histological (left) and immunohistochemical (right) analysis of the transplanted pelage follicle at 6 days (first hair loss after) and 40 days (second hair growth). Wild-type mouse skin was used as a control (natural). Transplanted hair follicles were distinguishable from host hair follicles (black arrowheads). Transplanted hair follicles (green) connect the arrector pili muscle (red, arrows) and nerve fibers (white, white arrowheads). Boxed areas in the left and center panels are shown at a higher magnification in the right panels. Scale bars, 50 μm at low magnification and 25 μm at high magnification. GFP, green fluorescent protein; H&E, hematoxylin–eosin; NF-H, neurofilament-H.

follicle, the APM and nerve fibers in a mouse model. These findings will significantly advance transplantation therapy and have relevance to not only hair follicles, but also organ–nerve junctions in the skin and these appendages.

The peripheral nervous system has essential roles in cooperative functions of tissue and organ, such as voluntary and involuntary movement, regulatory external secretion of saliva, sebum and sweat, which are protection functions against the drying surface of mucosa and skin, and the perception of noxious stimulations, such as contact, warmth, pain and mechanical stresses.[7,16] Fully functional autologous or allogeneic skin grafting and artificial skin, which has no skin appendages, transplantation is clinically applicable for skin defects due to burn, injury and ulcer.[17,18] Reproducible sensory innervation of skin grafts and bioengineered skin equivalents could have been previously reported.[1,14,19] The sensory neuron, which is connected to the follicular bulge region, provide a epidermal stem cell niche in the adult skin.[20] The restoration of a structurally correct nervous

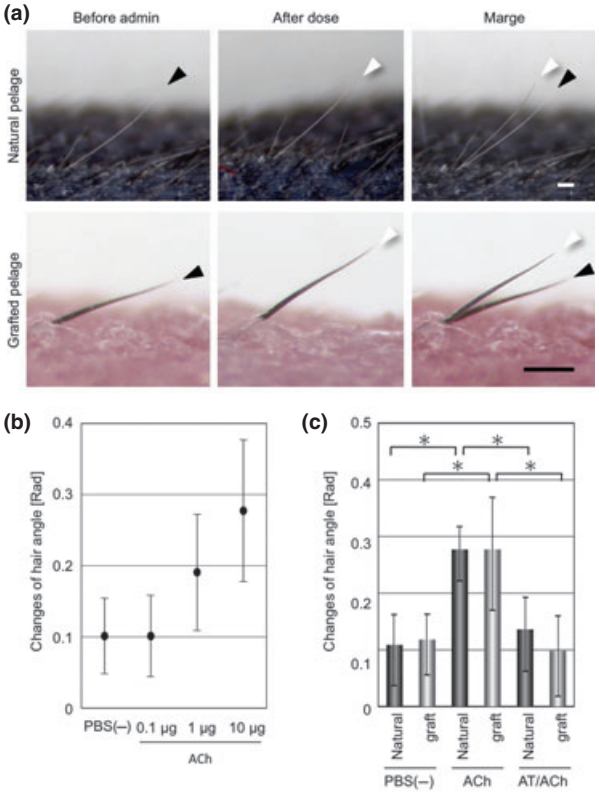

Figure 3. Quantitative assessment of transplanted pelage piloerection ability. (a) Analyses of the piloerection abilities of transplanted pelages following an i.d. injection of ACh. Hair shaft positions (black or white arrowheads) shifted after ACh treatment. Right, merged images. Scale bars, 1.0 mm. (b) Dose-dependent ACh stimulation of piloerection. (c) Assessment of changes in hair angle (radians) associated with transplanted and natural pelage before (PBS^-) and after the administration of ACh without (ACh) or with atropine (AT/ACh). Error bars show the standard deviation (n = 10). *Statistical significance was accepted when $P < 0.01$. ACh, acetylcholine; AT, atropine; PBS, phosphate-buffered saline.

system and functional innervation is thus one of the critical issues facing transplantation therapy at present and regenerative therapy in the near future.[21–23]

The hair follicle is a major component of the skin nerve network, including sensory and sympathetic nerves, and has a central function to modulate skin innervation.[5,14,20] It was recently reported that embryonic hair follicle buds could function as a guide for sense neuron migration.[14] Similarly, we reported a successful bioengineered tooth and hair follicle innervation.[21–23] However, the precise mechanism for the re-innervation of the autonomic nerve, which has essential roles in adult tissues, such as the arrector pili muscle, sweat glands and salivary glands, is poorly understood. In the present study, we demonstrate that cutaneous nerve fibers can connect autonomously to the histologically proper portions of the transplanted hair follicle, and their APM, which reproduces the piloerection ability. These results suggested that the transplanted adult hair follicle unit, including functional APM, autonomously induces not only the restoration of the neurofollicular junction but also the neuromuscular junction.

In the murine pelage, the neurofollicular junction is formed and positioned in the bulge region during late stages of folliculogenesis, and maintained through the lifetime of the animal.[5,24] The arrector pili muscle also develops at the same stages and is selectively connected to the bulge region, which is the niche of adult follicular epithelial stem cells, and also contains non-epithelial multipotent stem cells, which is nestin, neural progenitor marker-positive and can differentiate into many lineage cells, such as keratinocytes, smooth muscle cells, neurons and glial cells.[5,25,26] It was also reported that the adult skin dermis contains a precursor capable of generating neurons, Merkel cells and neural sensory receptors.[27,28] In this study, it was shown that the green fluorescent protein-negative neurons selectively connected with bulge regions of the transplanted follicle and APM, which is functionally restored. It is possible for the complex stem cell niches consisting of multiple stem cell populations to differentiate APM cells and induce the migration and connection of host mature neurons and/or the neuron differentiation from precursor and/or stem cells in adult dermis.[26–28]

In conclusion, this study provides the animal model for FUT utilizing analysis of the hair follicle functions, which are coordinately regulated with connecting tissues, such as hair eruption, hair cycle and piloerection. These results demonstrated that an autonomous restoration ability of the neurofollicular junction and novel evidence of single follicular transplantation resulting in autonomous restoration of the APM–neuron junction. Furthermore, these results demonstrate that the piloerection ability of transplanted hair follicles can be quantitatively determined using changes in the angle of piloerection. These findings and methods are a substantial contribution to not only the ongoing development of transplantation therapy that may enable future functional regeneration therapy for skin and skin appendages damaged by injury, burns and aging, but also investigation of stem cells and niches.

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