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RESEARCH ARTICLE

Clinically applicable transplantation procedure of dermal papilla cells for hair follicle regeneration

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Abstract

Dermal papilla cells (DPCs) interact with epithelial stem cells and induce hair folliculogenesis. Cell-based therapies using expanded DPCs for hair regeneration have been unsuccessful in humans. Two major challenges remain: first, expanded DPCs obtained from adult hair follicles have functional limitations; second, a clinically applicable method is needed for transplanting DPCs. This study aimed to identify an efficient, minimally invasive and economical DPC transplantation procedure for use in clinical settings. Five clinically applicable transplantation procedures were tested, termed the Pinhole, Laser, Slit, Non-vascularized sandwich (NVS) and Hemi-vascularized sandwich (HVS) methods. Labelled rat dermal papilla tissue was transplanted into rat sole skin, and hair follicle regeneration was evaluated histologically. Regenerated follicles and labelled DPCs were detected for all methods, although some follicles showed abnormal growth, i.e. a cystic or inverted appearance. The HVS method, pioneered here, resulted in significantly larger number of regenerated follicles that were more mature and regular than those observed using the other methods. Moreover, hair growth was detected after expanded adult-derived DPC transplantation using the HVS method. These results suggest that direct contact of epithelial and dermal components and better vascularization/oxygenation of the recipient site are critical for hair regeneration in cell-based therapies.

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Supporting information may be found in the online version of this article.

Keywords **dermal papilla cells; hair regrowth; epithelial-mesodermal interaction; epithelial stem cells; follicular stem cells; transplantation procedures**

1. Introduction

There is a huge demand for an effective treatment for hair thinning and baldness. A number of pathogenic mechanisms can lead to baldness, including genetic, hormonal, traumatic and iatrogenic events. Examples of these events include androgenetic alopecia, female androgenetic alopecia, injuries such as burn and traffic accidents, and side-effects from anticancer drugs and irradiation. There are several ways for patients to deal with baldness, such as wearing a wig, using oral or topical medicines or surgical management. Although autologous single follicle or follicular unit transplantation is a reliable surgical option (Barrera, 2003; Bernstein and Rassman, 1997; Choi and Kim, 1992), the limited number of donor follicles is a big disadvantage. Cell-based hair regeneration therapy using expanded dermal papilla cells (DPCs) has been investigated as a possible treatment for balding; tremendous demands for this non-life-threatening condition exist, as well as those for other epidermal or dermal skin regeneration (Gwak *et al.*, 2005; Liu *et al.*, 2006; Vriens *et al.*, 2008).

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Skin comprises both epidermal and dermal cells, and reciprocal signalling between the epithelial and mesodermal components plays critical roles in hair folliculogenesis and in the hair cycle (Hardy, 1992; Millar, 2002). In the 1960s, dermal papilla tissue (DPT) was identified as a mesodermal component with the potential to induce hair follicles (Cohen, 1961; Oliver, 1967). Since that time, several trichogenic assays have been reported, which are described briefly below. A wound assay using a small incision in the skin was used successfully by Jahoda (1992) to reconstitute hair follicles. In this method, freshly prepared or cultured DPCs are inserted into the incision with forceps or are injected under the epidermis at the wound site (Jahoda, 1992; Jahoda *et al*., 1993; McElwee *et al*., 2003; Reynolds *et al*., 1999). The chamber assay, first reported by Lichti *et al*. (1993) and Weinberg *et al*. (1993), used suspensions of fresh or cultured DPCs plus neonatal rat epidermal cells, which were grafted into a round chamber that was inserted in the dorsal skin of nude mice. Because hair shaft growth is apparent upon visual inspection, the chamber assay has been widely used to examine the ability of epithelial and/or mesenchymal cells to induce hair growth (Ehama *et al*., 2007; Kamimura *et al*., 1997; Kishimoto *et al*., 1999; Rendl *et al*., 2008). In the sandwich assay, a sandwiched complex is prepared by inserting DPCs between enzymatically-treated fragments of epidermis and dermis; this complex is subsequently transplanted into subcutaneous tissue (Inoue *et al*., 2009; Reynolds and Jahoda, 1992) or under the kidney capsule (Inamatsu *et al*., 1998; Inamatsu *et al*., 2006; Osada and Kobayashi, 2000). In the flap assay, a recent modified version of the sandwich assay, a sandwiched construct is prepared using an epidermis fragment obtained through digestion of embryonic mouse skin and the dermal side of temporally elevated skin flap (Qiao, 2008). Using this assay, cultured human DPCs at eight passages were shown to have the potential to induce regenerated hair follicles (Qiao *et al*., 2009). In the hair patch assay system, a mixture of epithelial cells and DPCs is injected subcutaneously or under the kidney capsule (Ito *et al*., 2007; Morris *et al*., 2004; Osada *et al*. 2007; Zheng *et al*., 2005). Although some of these assays are easy to perform in animal models and are relatively reliable if embryonic cells are used, none are practical for routine use in clinical settings. Some assays result in hair growing under the skin, and other assays are too surgically invasive or complicated to be used to treat a non-life-threatening condition.

In the present study, we designed five transplantation methods that we considered clinically applicable in terms of invasiveness, cost, technical complexity and reproducibility. These methods were termed the Pinhole, Laser, Slit, Non-vascularized sandwich (NVS), and Hemi-vascularized sandwich (HVS) methods. We evaluated the efficacy of the five procedures to assess whether any was suitable as a clinically applicable transplantation method. We first used freshly dissected rat DPT as transplants because we thought fresh DPT was a reliable grafting construct to induce hair follicle. Regenerated hair follicles were evaluated by histological observation to determine the most efficacious transplantation procedure. We then used a cultured rat DP cell-sheet fragment as a transplant, using the method that produced the best hair growth in order to test the method's potential as a cell-based therapy using expanded DPCs.

2. Materials and methods

2.1. Preparation of DPT and cultured DPC sheets as transplants

All animal experiments were performed under approval from the Institutional Animal Care and Use Committee of University of Tokyo.

For preparation of fresh DPT, the whisker pads of male Fisher 344 rats (6 weeks old) were harvested. DPT was freshly isolated from the end bulb portion of the hair follicles using micro-scissors and micro-forceps, and placed in phosphate-buffered saline (PBS) in a culture dish (Figure 1). For preparation of cultured DPC sheets, isolated DPT was transferred to a clean 60 mm culture dish, attached to scratches on the bottom of the dish, and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS). After 2–3 weeks of the explant culture, the DPCs were subcultivated twice and cultured in over-confluent conditions until a multilayered cell sheet formed. The DPC sheet was cut into round-shaped fragments (4 mm diameter) with a biopsy punch scarpel, scraped and used as a transplant. The average cell number of each DPC sheet fragment was 2775 ($n = 3$, data not shown).

2.2. Cell labelling

Cell tracing was performed using a cell membrane-permeable red fluorescence dye, CM-DiI (CellTracker®; Molecular Probes–Invitrogen Japan, Tokyo, Japan). DPT or DPCs were incubated with DMEM containing 5 μg/ml CM-DiI overnight at 37 °C before transplantation.

2.3. Preparation of graft site

To prevent activation of existing telogen follicles and confirm neogenesis of hair follicles, hairless region (rat sole skin) was employed as a recipient site. We prepared a sole skin transposition model in rats as follows. The right lower leg skin was tied to the back skin as the sole skin was exposed upwards under general anaesthesia (pentobarbital 30–50 mg/kg i.p.). After this procedure, the rats could walk using the remaining thigh and the healthy lower leg.

2.4. Transplantation procedures

DPTs or DPC sheet fragments were grafted to rat sole skin using one of the five transplantation methods: Pinhole,

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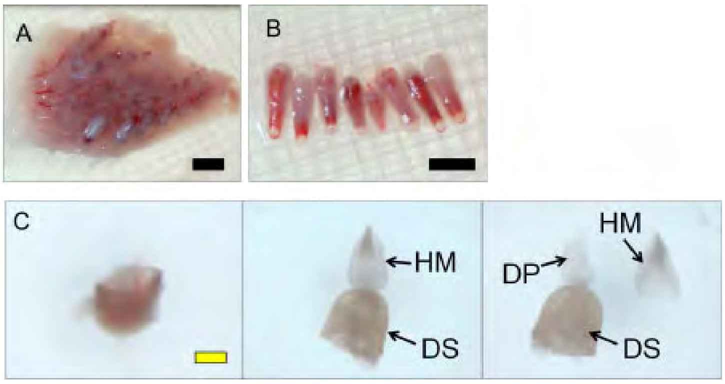

Figure 1. Preparation of dermal papilla for transplantation. (A) To prepare fresh rat dermal papilla (DP) for transplantation, the whisker fat pad was harvested from a 6 week-old rat. (B) The whisker pad was dissected into single follicles under a microscope. (C) The bulb portion of the follicle was isolated from the single follicle using micro-scissors and micro-forceps (left panel). The dermal sheath (DS) was turned over (middle panel) and the hair matrix (HM) was removed (right panel). Finally, a DP transplant was obtained by cutting the dermal sheath using a fine needle and forceps. All steps were performed in a culture dish containing PBS. Black bar = 2 mm; yellow bar = 250 μm

Laser, Slit, NVS or HVS (Figures 2, 3). For the Pinhole method, we made a small hole in the rat sole skin, using a pin (0.7 mm diameter). For the Laser method, we made a hole (0.9 mm in diameter, 15 J/cm^2 in laser output, two shots/one recipient site) using a CO_2 laser. For the Slit method, we made an incision (a slit approximately 200–400 μm in depth) using a round scalpel designed for use in skin biopsies (Kai industries, Gifu, Japan). Then, the three pieces of DPT were transplanted into the pinhole, laser hole or slit, respectively.

For the NVS method, a circle of full-thickness skin, 4 mm in diameter, was excised from the rat sole using a round scalpel. The round piece of skin was incubated in PBS plus 1000 U/ml dyspase (Dyspase II® Sankojunyaku, Tokyo, Japan) at 37 °C for 20 min to separate the epidermis from the dermis. Three pieces of DPT were sandwiched between the epidermal and dermal fragments, and the sandwiched construct was replaced in the donor site and sutured with two 8-0 nylon stitches. For the HVS method, a circular split-thickness of skin (4 mm in diameter) was excised from the rat sole by slicing with a scalpel after a round scalpel had been used to make a circular 150–300 μm deep incision. Three pieces of DPTs were sandwiched between the remaining dermal layer in the donor hole and the epidermal fragment separated from the dermis with treatment of dyspase, while the dermal fragment was discarded. The epidermis was then sutured as for the NVS method. For all methods, the wound was covered with an adhesive thin foam structured wound dressing (Hydrosite Usugata®; Smith & Nephew Wound Management KK, Tokyo, Japan).

In the first experiment, 24 DPTs in eight animals (three pieces of DPT per one sample) were used in each transplantation method. In the second experiment, 12 DPTs (two pieces per sample) were transplanted with the HVS method only in six animals. The third experiment was additionally performed using six cultured DPC sheet fragments in six animals; one cultured DPC sheet fragment per sample was transplanted using the HVS method only.

The summarized features of each method are shown in Table 3. For the first three methods, we expected epithelial cells migrate into the wound space and interact with transplanted DPCs; these methods are similar regarding the cell–cell contact status between dermal and epithelial components, while they are different in bleeding condition, burn injury and vascularity of the wound surface and dead space size. On the other hand, the two sandwich methods provide more reliable cell–cell contacts between dermal and epithelial cells, but the condition of vascularization/oxygenation of the recipient floor is different between the two methods. While the five transplant procedures have different characters, they have common limitations. We can adjust the amount of grafting dermal components easily but the amount of resident epidermal components is fixed.

2.5. Histological evaluation and scoring of regenerated hair follicles

Eight weeks after grafting the DPTs or DPC sheets, rat sole skin was harvested and subjected to histological examination. The skin samples were embedded in OCT compound (Tissue-Tek®; Sakura Finetechnical, Tokyo, Japan), frozen in liquid nitrogen and cut into 10 μm-thick sections. Every other section was processed for haematoxylin and eosin (H&E) staining, while the remaining sections were stained with Hoechst 33 342 (Dojindo, Kumamoto, Japan). Stained sections were observed and photographed under a fluorescence microscope (BioZero®, Keyence, Tokyo, Japan).

Hair follicle regeneration was evaluated histologically and classified into eight stages of developmental hair follicle maturation according to a previously described method (Paus *et al*., 1999) (see Supporting information, Figure S1). Although transplanted DiI-labelled DPT was detected in most of the samples, the tissue was not in its

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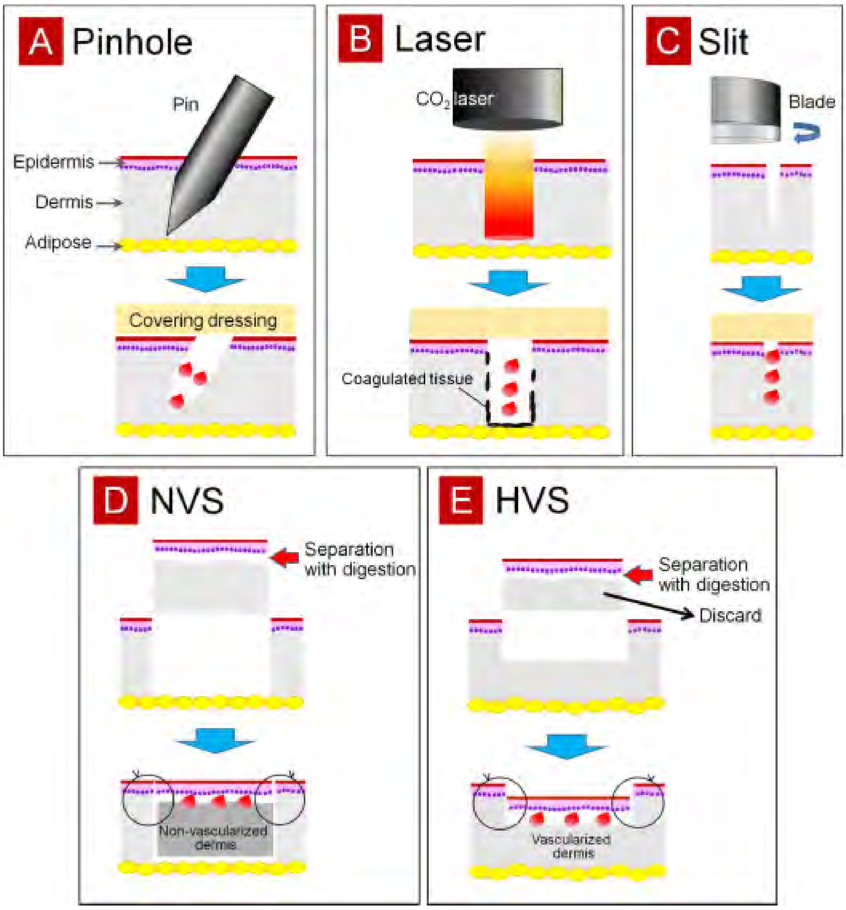

Figure 2. Schema showing the five transplantation procedures. Five clinically applicable transplantation procedures were designed and their efficacy for hair regeneration tested. (A) Pinhole method: a single hole was made on the skin with a pin (0.7 mm diameter) and dermal papillae were grafted into the hole. (B) Laser method: a single hole (0.9 mm diameter) was made with a carbon dioxide laser and dermal papillae were grafted into the hole. (C) Slit method: an incision (a 200–400 μm deep slit) was made with a device for skin biopsy and dermal papillae were grafted into the slit. (D) Non-vascularized sandwich (NVS) method: a circle of full-thickness skin was removed and digested to separate the epidermis from the dermis. Dermal papillae were sandwiched between the epidermis and dermis and the sandwiched construct was replaced in the original circular hole. (E) Hemi-vascularized sandwich (HVS) method: a circular split-thickness skin (150–300 μm thickness) was sliced off and digested to separate the epidermis from the dermal compartment. The epidermis was replaced on the remaining deep dermis in the donor hole after dermal papillae were grafted

original shape but rather appeared in small scattered clusters. In addition to regenerated follicles showing normal developmental stages (regulated hair follicles with an ‘R’ noted after the maturation grade, e.g. Stage 5R), there were a number of regenerated follicles with atypical morphology, such as cysts, inverted follicles and others. The atypical regenerated follicles, such as multiply-fused follicles, were termed dysregulated hair follicles and had a ‘D’ noted after the maturation grade, e.g. Stage 4D. Examples of representative regenerated follicles with atypical morphology are shown in the Supporting information (Figure S2).

2.6. Statistical analysis

Using a simple linear regression analysis, ‘estimate’ values [for numbers of total, mature (stage $\geq$ 6) and regulated follicles per graft sample] were calculated from the data of 24 DPTs in eight animals for each method (using only the first experiment data in the HVS method). The estimate follicle number for each method was compared for statistical significance by simple linear regression analysis, which was performed with statistical software (S-PLUS® v 8.0; Insightful Corp., Seattle, WA, USA) and the difference was evaluated as significant when $p < 0.05$ (two-sided).

3. Results

3.1. Macroscopic evaluation 8 weeks after transplantation

Each of the five experimental methods was tested in rats ($n = 46$), which were sacrificed 8 weeks after transplantation. All scars were unremarkable. Notably, only the HVS method resulted in hair growth from the skin, although the hair was curled (Figure 3,

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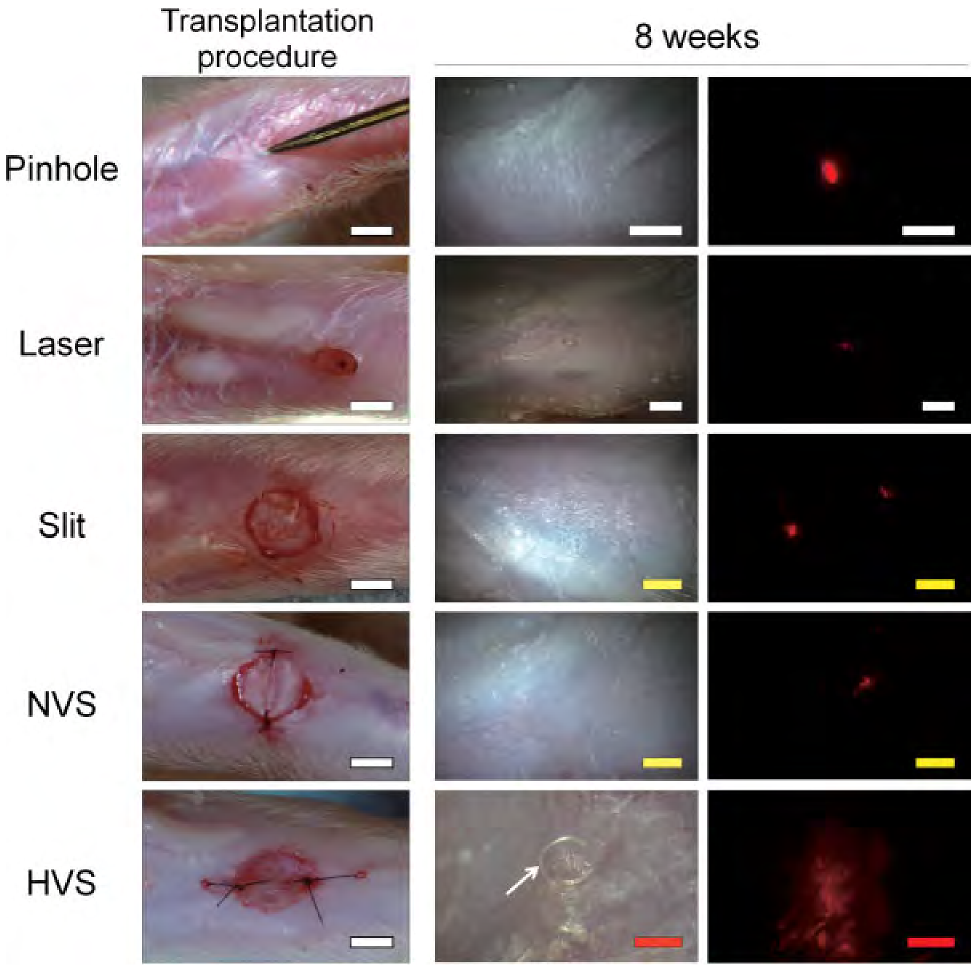

Figure 3. Representative images of the transplantation sites before and after each of the five transplantation procedures used: the pinhole method; the laser method; the slit method; the non-vascularized sandwich (NVS) method; and the hemi-vascularized sandwich (HVS) method. Panels on the left show the transplantation sites after each of the five procedures were performed. The middle and right panels show light and fluorescent microscopy images of the same fields of view 8 weeks later; a fluorescent stereomicroscope was used. In the right panels, DiI-labelled transplanted dermal papilla tissue was detected after 8 weeks in all five experimental methods. Note that only the HVS method resulted in hair growth from the skin; the arrow in the middle HVS panel shows a regenerated curly hair. White bar = 2 mm; yellow bar = 1 mm; red bar = 0.5 mm

central panel). The transplanted DiI-labelled DPT was detected in all experimental methods under a fluorescent stereomicroscope (Figure 3, right panels).

3.2. Analysis of hair folliculogenesis in the five DPT transplantation methods

Figure 4 shows representative histological images of skin samples after DPT transplantation, using each of the five transplantation procedures. The raw data of regenerated follicle scores are summarized in Table 1 and the analysed data are shown in Figure 5. The data of the second experiment in the HVS method (12 DPTs in six animals) are included in Table 1 and Figure 5D, E, but are not included in Figure 5A–C because statistical analyses were performed using data of the first experiment (with the same sample number of each group) after the raw data of regenerated follicle numbers were converted to the estimate values using the simple linear regression analysis.

Hair follicle regeneration was observed using all transplantation methods, but the estimate value of total regenerated follicles per sample (three DPT transplants) was significantly higher (2.25) using the HVS method compared to the other four methods (range 1.00–1.125; $p < 0.01$; Figure 5A). The HVS method also showed a significantly larger estimate number of mature (stage $\geq$ 6) follicles (0.5 mature follicles/sample) compared to the other methods (range 0–0.375; $p < 0.05$; Figure 5B). No mature follicles were observed using the Pinhole and Slit methods (Table 1). For regulated follicles, the estimate value per sample was also highest in the HVS method (0.75) compared to other methods (range 0.125–0.375; $p < 0.05$; Figure 5C).

Regulated follicles were seen most frequently when the HVS method was used (48.8%) compared to other methods (range 11.1–37.5%; Figure 5D). Most of the regenerated follicles (87.5–88.9%) observed using the Laser, Slit and NVS methods showed atypical morphology (i.e. dysregulated follicles) (Table 1). There were 0.250

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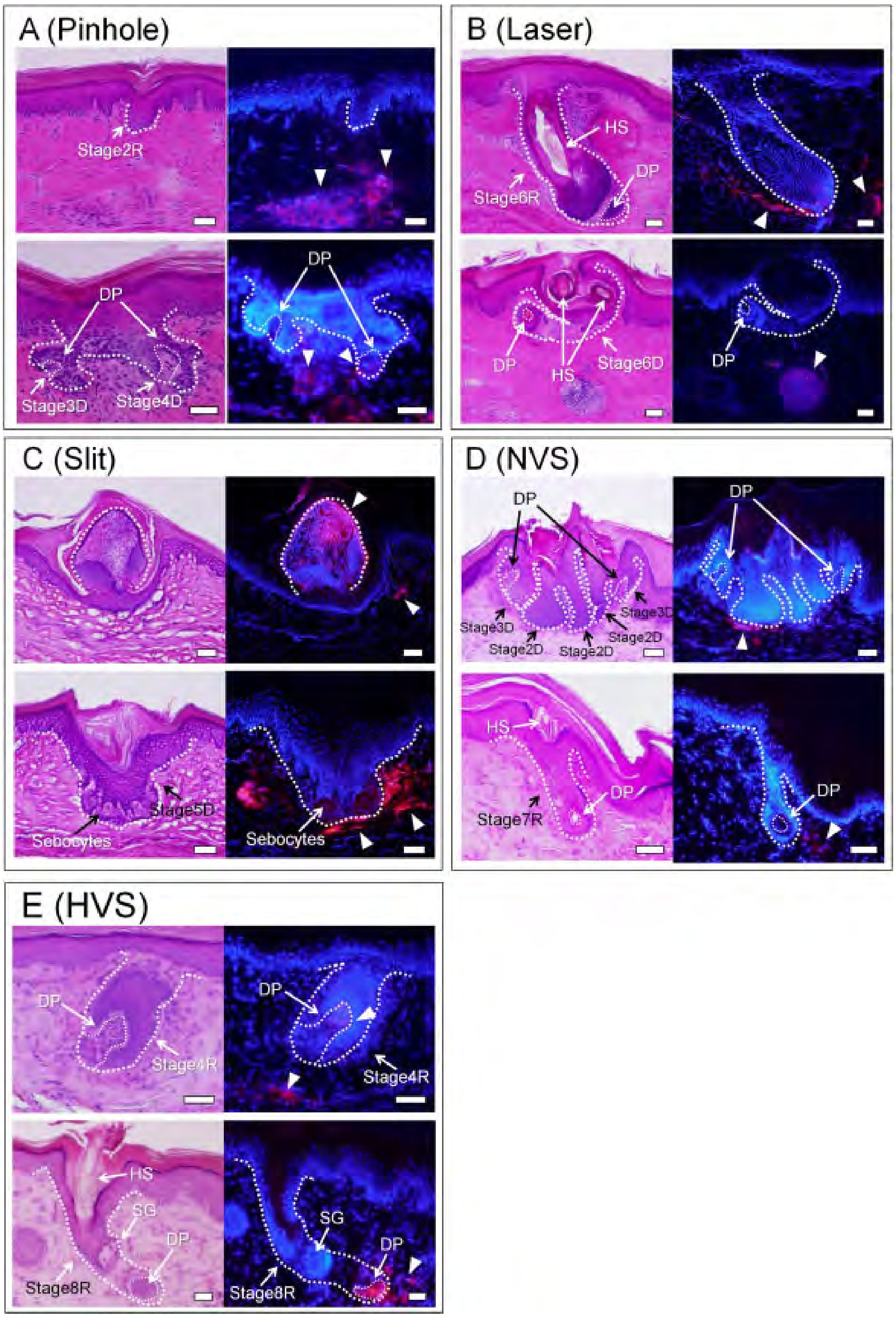

Figure 4. Representative histological images of regenerated hair follicles for dermal papilla tissue transplantation with each of the five procedures. Two representative samples are shown for each transplantation procedure. Alternating serial sections were stained with H&E or Hoechst 33342 and are shown in the left and right panels, respectively. Dotted white lines indicate the basal lamina, while white arrow heads indicate DiI-labelled transplanted dermal papilla cells (DPCs). (A) Pinhole method. A large DiI-labelled DPC cluster can be seen below a stage 2R follicle (top). A fused follicle (stages 3D and 4D) was seen, along with scattered DPCs (bottom). (B) Laser method. Both samples showed stage 6 follicles, but one was a regulated follicle (top) and the other was a dysregulated one (bottom). The dysregulated follicle has a coiled shape and likely lacks vertical polarity. (C) Slit method. Inverted follicles were seen only using the slit method (top). In a stage 5D follicle, sebocytes were observed but were dislocated, and an inner root sheath (IRS), bulb, and dermal papilla (DP) were not detected (bottom). (D) Non-vascularized sandwich (NVS) method. A large follicular complex was seen that was the result of fusion of five follicles (top). A stage 7R follicle had a hair shaft (HS) growing to the level of the epidermis (bottom). (E) Hemi-vascularized sandwich (HVS) method. A stage 4R follicle showed a well-developed DP in a thickened bulb (top). Stage 8R follicles with a hair shaft emerging from the skin surface were observed only using the HVS method. SG, sebaceous gland. Bar = 50 μm

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Table 1. Comparison of hair follicle regeneration results using five transplantation procedures

							HVS		
Grafting method			Pinhole	Laser	Slit	NVS	Exp1	Exp2	Total
Grafted DPT, *n* per sample			3	3	3	3	3	2	N/A
Samples, *n*			8	8	8	8	8	6	14
Total grafted DPT, *n*			24	24	24	24	24	12	36
Failed sample, *n* (rate)			2 (25.0%)	2 (25.0%)	3 (37.5%)	5 (62.5%)	4	0	4 (28.6%)
RF-containing sample, *n* (rate)			6 (75.0%)	6 (75.0%)	6 (62.5%)	3 (37.5%)	4	6	10 (72.4%)
RF, *n* (per grafted DPT)	Immature [Stage 1–5]	R	3 (0.125)	0 (0)	1 (0.041)	0 (0)	3	7	10 (0.278)
		D	5 (0.208)	7 (0.292)	7 (0.292)	6 (0.250)	11	6	17 (0.472)
		Total	8 (0.333)	7 (0.292)	8 (0.333)	6 (0.250)	14	13	27 (0.750)
	Mature [Stage 6–8]	R	0 (0)	1 (0.041)	0 (0)	1 (0.041)	3	6	9 (0.250)
		D	0 (0)	1 (0.041)	0 (0)	2 (0.083)	1	2	3 (0.083)
		Total	0 (0)	2 (0.082)	0 (0)	3 (0.125)	4	8	12 (0.333)
	Regulated follicle rate		37.5%	11.1%	12.5%	11.1%	–	–	48.8%
	Mature follicle rate		0%	22.2%	0%	33.3%	–	–	38.1%
	Total		8 (0.333)	9 (0.375)	8 (0.333)	9 (0.375)	18	21	39 (1.083)
Cysts			0 (0)	0 (0)	1 (0.041)	0 (0)	2	0	2 (0.057)
Inverted follicles			0 (0)	0 (0)	3 (0.125)	0 (0)	0	0	0 (0)

Skin samples were harvested 8 weeks after rat whisker-derived fresh DPT was transplanted to rat sole skin, using one of the five tested methods. Regenerated hair follicles were evaluated histologically and divided into groups by maturation scores (immature, stages 1–5; mature, stages 6–8) and by their regularity (regulated or dysregulated). Cysts and inverted follicles were not regarded as regenerated follicles and were counted separately. RF, regenerated follicles; DPT, dermal papilla tissue; R, regulated; D, dysregulated; NVS, non-vascularized sandwich; HVS, hemi-vascularized sandwich.

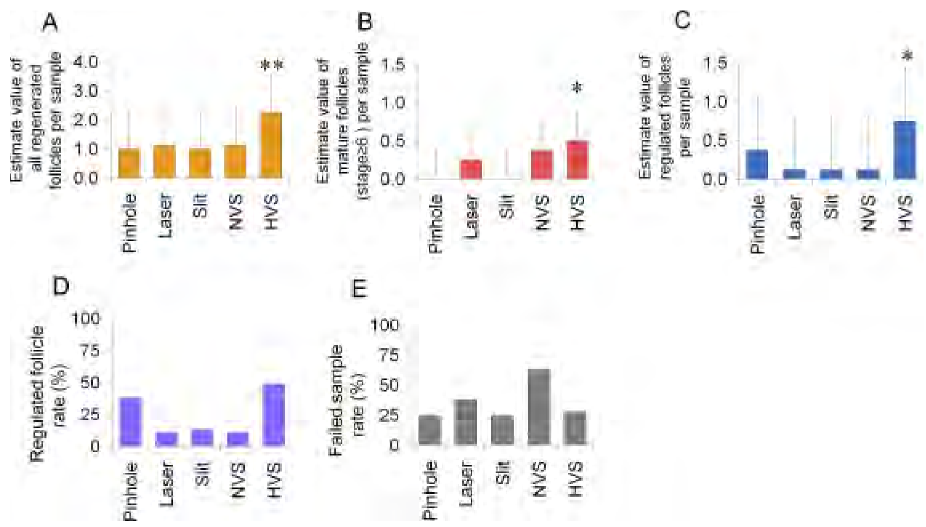

Figure 5. Quantitative analyses of hair regeneration for dermal papilla tissue transplantation with each of the five procedures. (A) The estimate number of total regenerated hair follicles per sample containing three transplanted dermal papilla tissues (DPTs). All regenerated hair follicles (follicles at any stages or both regulated and dysregulated follicles) were included; $^{**}p < 0.01$. (B) The estimated number of mature (stage ≥ 6) regenerated hair follicles per sample containing three transplanted dermal papilla tissues (DPTs). Immature (stage ≤ 5) follicles were excluded; $^{*}p < 0.05$. (C) The estimate number of regulated regenerated hair follicles per sample containing three transplanted dermal papilla tissues (DPTs). Dysregulated follicles were excluded; $^{*}p < 0.05$. (D) Regulated follicle rate for each transplantation method. The number of regulated follicles was divided by the total number of regenerated follicles (regulated and dysregulated follicles). The pinhole and HVS methods showed higher regulated follicle rates than the other methods, 38% and 49%, respectively. (E) Failed sample rate for each transplantation method. The number of failed samples, i.e. samples in which no follicle regeneration was detected, was divided by the sample number for each transplantation method. NVS showed the highest failed sample rate (63%), while the pinhole, laser and HVS methods showed relatively lower failed sample rates

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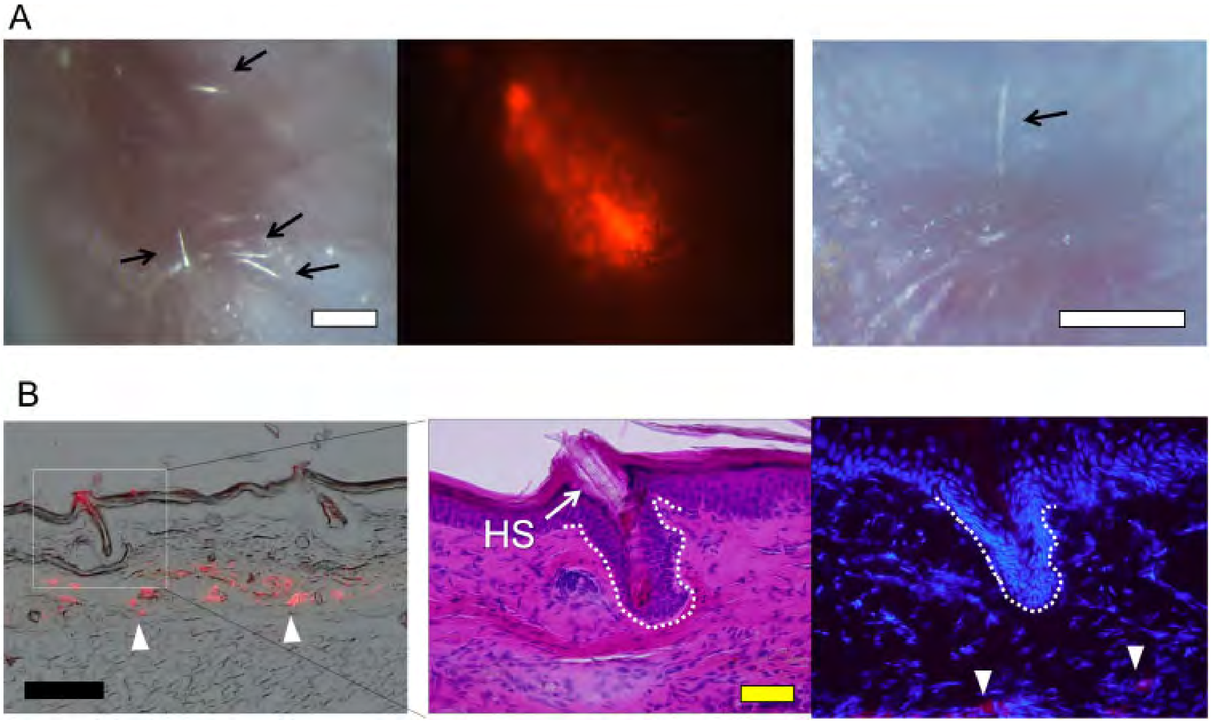

Figure 6. Representative images and histology after transplantation of a cultured dermal papilla cell construct using the HVS method. Fragments of cultured rat DPC cell sheets were DiI-labelled and transplanted to rat sole skin using the HVS procedure. (A) Macroscopic views 8 weeks after transplantation of the DPC construct at lower magnification (left panel) and higher magnification (right panel). Many hairs could be seen emerging from the skin (black arrows). Fluorescent stereomicroscopy revealed the transplanted DPC construct under the skin (middle panel; same field as in the left panel). (B) Histology 8 weeks after transplantation showing regeneration of a stage 8R follicle. An unstained section at low magnification is shown in the left panel. The middle (H&E staining) and right (Hoechst 33342 staining) panels show serial sections at higher magnification. A hair shaft (HS) emerged from the skin, and DiI-labelled transplanted DPCs (white arrow heads) were detected in the reticular dermis (left, right). White bar = 1 mm; black bar = 200 μm; and yellow bar = 50 μm

mature regulated follicles/graft using the HVS method, 0.041 follicles/graft using the Laser and NVS methods and none using the Pinhole and Slit methods (Table 1). Failed samples, i.e. skin samples containing no regenerated follicles, were seen most frequently when the NVS method was used (62.5%; Figure 5E).

3.3. Hair folliculogenesis in cultured DPC sheet transplantation using the HVS procedure

To test the efficacy of the HVS procedure for cultured DPC transplantation, we prepared multi-layered DPC sheet fragments that were grafted using the HVS method. The folliculogenesis results are shown in Figure 6 and Table 2. Eight weeks after transplantation, 23 hair follicles had regenerated from six transplanted DPC sheets, and grafted DiI-labelled cells were detected in the middle to deep layers of the dermis. Hairs were observed emerging from the surface of the skin, although the induced hairs were very small.

4. Discussion

Despite ongoing efforts by scientists and physicians, there is currently no established cell-based therapy for hair follicle formation. Several challenges remain to be overcome for clinical success. First, the cell preparation method must be optimized: This step is crucial and may represent the most difficult challenge. Epithelial stem cells and DPCs derived from adult tissue must be prepared while preserving their original functions, since the two cell populations interact with each other, form hair follicles and regulate the hair cycle. To overcome the challenge of limited donor tissue, culture expansion of DPCs is a necessity; Preservation of the hair-inducing capacity of expanded DPCs must be assured but this is known to be a big challenge. To overcome the loss of hair-inducing capacity of DPCs during expansion culture, supplementation of keratinocyte-conditioned media (Inamatsu M *et al.*, 1998) or basic FGF (Osada A *et al.*, 2007) have been utilized. Recently, it was reported that a supplementary grafting of dermal sheath cells combined with DPCs may enhance experimental hair growth (Yamao *et al.*, in press).

Construction/preparation of cells or tissues for transplant must also be optimized. Finally, the grafting procedure must be optimized to achieve maximal hair growth efficiency and be refined to be less invasive and more acceptable as a viable aesthetic treatment.

In this study, we focused on optimizing and refining the DPC grafting procedure by encouraging the involvement

Table 2. Hair follicle regeneration induced by expanded DPC transplantation using the HVS procedure

Grafting method			HVS
Grafted DPC sheet, *n* per sample			1
Samples, *n*			6
Total grafted DPC sheet, *n*			6
Sample failure, *n* (rate)			1 (16.7%)
RF-containing sample, *n* (rate)			5 (83.3%)
RF, *n* (per grafted DPC sheet)	Immature [Stage 1–5]	R	7 (1.167)
		D	1 (0.167)
		Total	8 (1.333)
	Mature [Stage 6–8]	R	10 (1.667)
		D	5 (0.833)
		Total	15 (2.500)
	Regulated follicle rate		73.9%
	Mature follicles rate		65.2%
	Total		23 (3.833)
Cysts			0 (0)
Inverted follicles			0 (0)

Expanded DPC sheet fragments were grafted to rat sole skin using the hemi-vascularized sandwich (HVS) method and harvested at 8 weeks. Regenerated hair follicles were evaluated histologically and divided into groups by maturation scores (immature, stages 1–5; mature, stages 6–8) and regularity (regulated or dysregulated). Unexpectedly, much better follicle regeneration was observed with this method than when freshly isolated DPT was used for the same transplantation method. Cysts and inverted follicles were not observed.
RF, regenerated follicles; DPT, dermal papilla tissue; R, regulated; D, dysregulated.

Table 3. Summarized features of each transplanting procedure

	Pinhole	Laser	Slit	NVS	HVS
Contact	±	±	±	+	+
Bleeding	±	−	+	−	+
Burn	−	+	−	−	−
Vascularity	+	±	+	−	+
Dead space	+	+	−	−	−

'Contact' means a condition of the cell–cell contact between epidermal (resident epithelial stem cells) and dermal (grafted DPTs or DPCs) components. 'Bleeding' means the amount of haemorrhage, while 'Burn' means the existence of burn damage to the tissue. 'Vascularity' means a condition of blood flow in the graft bed tissue. 'Dead space' means the size of excessive space around the grafted DP construct. These indices were displayed as +, ± or −.
NVS, non-vascularized sandwich; HVS, hemi-vascularized sandwich.

of resident epithelial stem cells in the recipient site, rather than transplanting epithelial stem cells. We also used fresh DPTs as transplants. Preparation of functional epithelial stem cells is critical but challenging because the differentiation potential of keratinocytes in the follicular epithelium is easily lost, e.g. after the second culture passage (Ehama *et al*., 2007). Our intent was that the transplanted DPCs would interact with resident epithelial stem cells that were activated by surgical wounding in the grafting procedures. Injury-initiated microenvironments stimulate epithelial stem cells to proliferate, migrate and differentiate in order to repair wounds and reconstitute the skin (including the hair follicles).

Three of the five transplantation procedures evaluated in this study, i.e. the Pinhole, Laser and Slit methods, involved simple insertion of DPCs into a wound space. The wound surface was relatively or very fresh for the Pinhole and Slit methods, and was coagulated with burning for the Laser method. The Pinhole and Laser methods resulted in a small hole-shaped wound space, while the Slit method has a plane-shaped wound space. For these three methods, keratinocytes, including epithelial stem cells, were expected to migrate into the wound, but direct attachment of epithelial stem cells to the transplanted DPCs was not guaranteed. In contrast, epithelial stem cells that were resident in the basal layer were expected to communicate with transplanted DPCs via direct physical contact when the other two methods, NVS and HVS, were used. The epidermis was detached at the level of the basal membrane by enzymatic digestion and then replaced on the dermis (or dermal fragment), with the DPC constructs (DPT or DPC sheet fragment in this study) on the upper surface. As for vascularization and oxygenation, transplanted DPCs had direct contact with well-vascularized tissue on both sides for the Pinhole, Laser and Slit methods, on one side for the HVS method, and on neither side for the NVS method.

Although the covered epidermis was sutured to the dermis in the NVS and HVS methods, the transplanted DPC constructs could still be dislocated. Such displacement would result in a lack of folliculogenesis. We speculate that this may underlie the higher rate of failed samples observed for the NVS and HVS methods, and it is an issue that must be addressed in future studies. DiI-labelled DPCs were not well preserved in their original form; rather, they often spread into the recipient site. DPCs were particularly scattered when the Slit, NVS and HVS methods were used. DPT transplanted using the Slit

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method could dislocate upward, resulting in inverted follicles, which were detected only in this method. Thus, the size and shape of the prepared recipient space may affect the spread of DPCs, which is undesirable.

Glabrous sole skin was chosen as the recipient site for testing these methods. Thus, any follicle-like structures were regenerated hair follicles induced by the interaction between the resident epithelial stem cells and transplanted DPCs. This comparison of five grafting procedures revealed that epithelial–mesenchymal interaction occurred using all five methods. The HVS method was clearly superior to the other four methods in terms of hair regeneration efficiency, as shown by the rates of regulated and mature follicles and by the number of regenerated follicles per DPC graft. This indicated that direct contact of DPCs with the basal layer and sufficient vascularization of the recipient bed (which determines the oxygenation) are requisites for the induction of hair formation by DPC transplantation. In addition, it was clear that transplantation of DPCs without epithelial cells is sufficient for inducing hair formation *in vivo*, presumably by activating resident epithelial stem cells.

We also investigated the hair regrowth induced by HVS transplantation of expanded DPCs. Unexpectedly, many more regenerated hair follicles, including stage 8 hairs, were obtained this way than with DPT transplantation using the same procedure. DPT, which should be fully differentiated and functional, may be less desirable for transplants than cultured DPCs, which have been suggested not to be fully functional. Instead, spontaneous functional differentiation of DPCs may be induced after transplantation in response to signals from epithelial cells. This indicates that the HVS method can be used as a transplantation method using expanded DPCs and shows potential for therapeutic use.

Although hair folliculogenesis was achieved at a high frequency and quality using the new HVS method, the quality of the hair shaft is not yet acceptable in terms of use as a clinical treatment and must be improved. The quality of the transplanted DPCs should also be improved, since it is well known that expanded DPCs lose, at least in part, their hair-inducing capacity. The optimal number of DPCs for use in the transplantation construct is still unknown, and the best preparation method (e.g. aggregates, spheres, sheets or use of a bioscaffold) for the DPC construct must still be determined, since this affects the DPC function. Recently, microencapsulated cultured human DPCs which were transplanted subcutaneously into rat ear were reported to produce hairs at high density (Lin *et al*, 2009), although further examination is needed to determine whether microencapsuled DPCs induced neogenesis of hair follicles or activated existing telogen stage follicles.

In conclusion, this study showed that DPC transplantation alone can induce hair folliculogenesis without including epithelial stem cells in the transplant. The HVS procedure was more efficient than the other procedures in inducing hair regeneration with freshly-isolated DPT transplantation. When the HVS method was used for transplantation of expanded adult-derived DPCs, it resulted in hair regrowth that emerged from the skin. This holds promise as the basis of an effective, clinically applicable method for cell-based hair regrowth. The results also suggested that direct contact of DPCs with activated epithelial stem cells and vascularization of the recipient bed are determining factors for DPC transplantation efficacy for hair regrowth.

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5. Supporting information on the internet

The following supporting information may be found in the online version of this article:

Figure S1. Maturation stage scoring of folliculogenesis: representative images of regulated follicles at stages 1R–8R

Figure S2. Representative histological images of abnormally regenerated hair follicles

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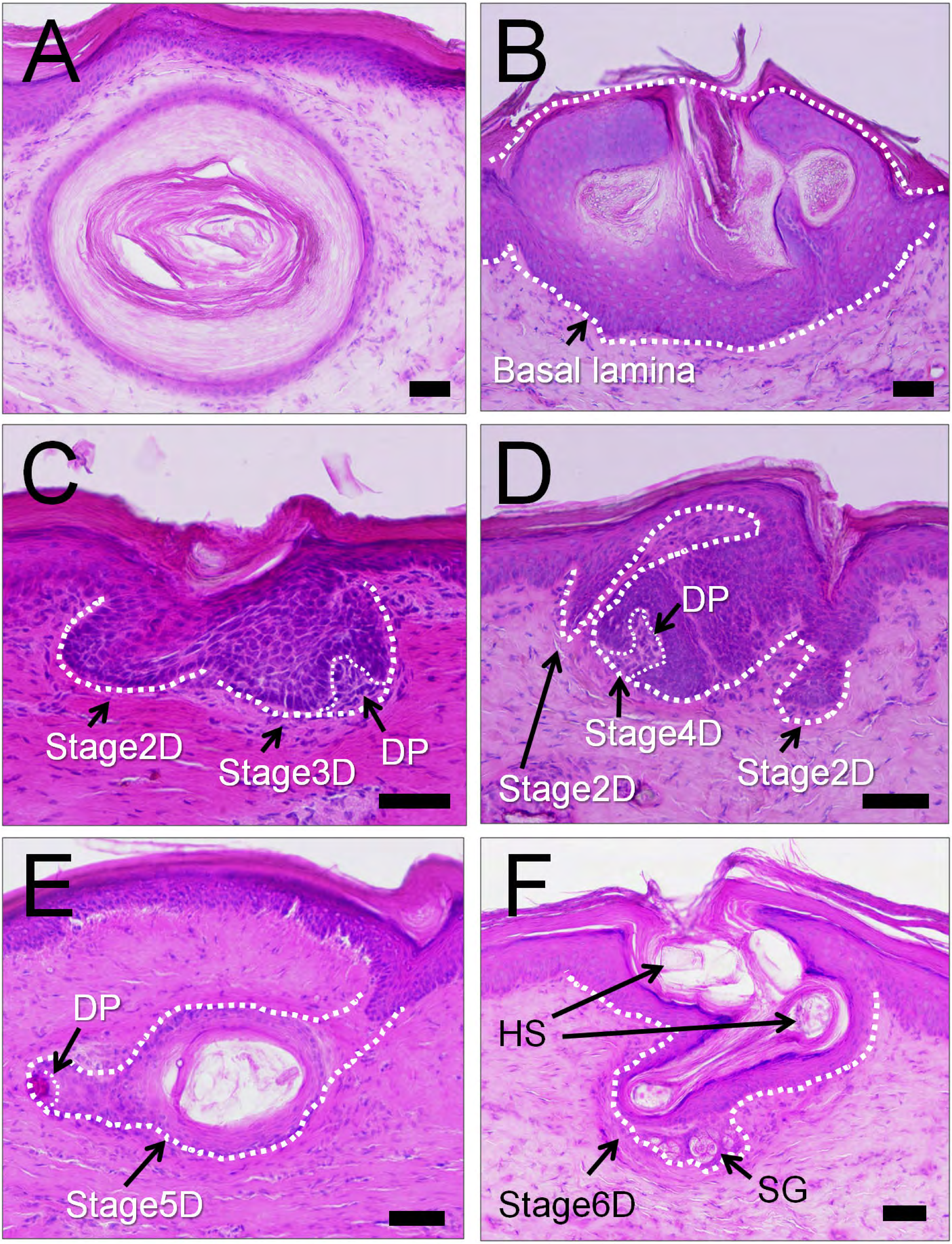
<Supporting information>

Figure S1. Maturation stage scoring of folliculogenesis: representative images of regulated follicles at stages 1R–8R. H&E-stained sections are shown in the left panels, while fluorescent images of adjacent sections stained with Hoechst 33342 are shown in the right panels. Regenerated follicles with normal developmental morphology were considered regulated follicles. The maturation stage was categorized as one of eight stages (1–8) according to Paus's (1999) classification. Regulated follicles were scored as stage 1R–8R, where 'R' denoted 'regulated'. The following features are typical of each stage: stage 1R, thickening of the epidermis (placode; PC); stage 2R, a hair germ (HG) can be observed; stage 3R, a hair peg (HP) can be seen that has a concave basal border and a dermal papilla (DP); stage 4R, core-like formation of the inner root sheath (IRS) is detected, the bulb is thickened and the DP is enclosed; stage 5R, sebocytes become visible and the IRS elongates; stage 6R, DP is enclosed by the hair matrix and the IRS contains a hair shaft (HS) fragment; stage 7R, a hair shaft grows up to the level of epidermis; stage 8R, the hair shaft emerges from the epidermis. Bar = 50 µm

Figure S2. Representative histological images of abnormally regenerated hair follicles. Several types of abnormally regenerated hair follicles were observed: a cyst (A); an inverted follicle (B); multiply-fused follicles (C, D); and follicles with abnormal morphology (E, F). We excluded cysts and inverted follicles from the regeneration scoring because they were considered adverse complications from a therapeutic viewpoint. Other atypical or abnormal follicles, such as multiply-fused follicles, were evaluated as dysregulated follicles and a 'D' was noted after the maturation score. Incomplete epithelial–mesenchymal interaction or simultaneous signalling from multiple mesenchymal fragments may result in the dysregulated follicle structures. (A) A cyst that contained horny material within a capsule with thin epithelium. (B) An inverted follicle had the basal lamina extended upward as though the follicle was regenerated above the epidermis. (C) A stage 3D follicle with a hair peg that has a concave basal border was fused to another stage 2D follicle. (D) This stage 4D follicle had a dermal papilla (DP) but showed atypical morphology and was fused with other follicles. (E) This stage 5D follicle had an oval-shaped DP and inner root sheath-like structure that was elongated about halfway up the follicle and contained a cyst-like structure. (F) This stage 6D follicle had a hair shaft (HS) and sebaceous gland (SG), but the follicle was twisted, the HS was disrupted and the SG was displaced. Bar = 50 µm

Stage1R
Placode
Placode
Stage2R
HG
HG
Stage3R
HP
DP
HP
DP
Stage4R
DP
IRS-cone
Bulb
IRS-cone
DP
Bulb
Stage5R
Sebocytes
IRS
Sebocytes
IRS
Stage6R
DP
HS
Stage7R
HS
DP
DP
Stage8R
DP
HS
HS
DP


A
B
Basal lamina
C
Stage2D
Stage3D
DP
D
DP
Stage4D
Stage2D
Stage2D
E
DP
Stage5D
F
HS
Stage6D
SG