

Evaluation of Animal Models for the Hair-Inducing Capacity of Cultured Human Dermal Papilla Cells

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Key Words

Dermal papilla cell · Folliculogenesis · Chamber model · Sandwich model · Hair induction

Abstract

Background: The dermal papilla (DP) interacts with epithelial cells for folliculogenesis. For translational research on cell therapies for hair regrowth with cultured human DP cells (hDPCs), a model to evaluate the capacity of hDPCs to induce hair formation is inevitable. **Methods:** Chamber models were constructed by transplanting 4 different combinations of mouse or human epithelial and mesenchymal cells into a silicone chamber implanted onto the back of nude mice. In parallel, 3 types of sandwich constructs were created by inserting hDPCs or human DP tissue between the epidermis and dermis of isolated rat footpad skin or human facial skin, and subcutaneously transplanting the constructs into the back of nude mice. Four to six weeks later, skin sections of each model were histologically examined. **Results:** Folliculogenesis was detected in both chamber and sandwich models, although the induction rate and maturity of the hair follicles varied among cell combination subgroups in each model. The difference in hair induction rate was not statistically significant between 2 representative chamber and

sandwich subgroups using cultured hDPCs. The sandwich model, however, required fewer hDPCs, did not require human keratinocytes, and exhibited a higher rate of successful sample collection. **Conclusions:** Although there is no significant difference in hair induction rate, the sandwich model using cultured hDPCs and the rat sole skin is more feasible than the chamber model using human cultured keratinocytes and hDPCs as a tool to evaluate the hair-inducing capacity of cultured hDPCs.

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Abbreviations used in this paper

DP	dermal papilla
DPC	dermal papilla cell
DPT	dermal papilla tissue
FBS	fetal bovine serum
fmDC	fetal mouse dermal cell
fmEC	fetal mouse epithelial cell
hDPC	human dermal papilla cell
hDPT	human dermal papilla tissue
hKC	human epidermal keratinocyte
KC	epidermal keratinocyte

Introduction

Dermal papilla cells (DPCs) are mesenchymal cells in the mammalian hair follicles that induce differentiation of follicular or epidermal stem cells into hair [Cohen, 1961; Oliver, 1966; Jahoda et al., 1984; Stenn and Cotsarelis, 2005]. Optimization of DPC transplantation and expansion methods without compromising the capacity for hair induction are 2 key requirements for establishing a cell therapy targeting hair regrowth. For optimization of the expansion of hair-inducing human DPCs (hDPCs), a tool to evaluate the capacity of cultured hDPCs to induce hair growth is necessary.

Several growth factors such as insulin growth factor-1 [Itami et al., 1995] and fibroblast growth factor-7 [Rosenquist and Martin, 1966] have been suggested to mediate the hair induction signaling pathways from DPCs to follicular epithelial cells. Wnt signaling has been shown to maintain the hair-inducing activity of DPCs [Kishimoto et al., 2000]. Versican-positive cells were shown to induce hair formation, implicating an important role for versican in hair induction [Kishimoto et al., 1999]. Transforming growth factor- β 2 was expressed in hair-inducing DPCs but not in dermal fibroblasts [Chiu et al., 1996]. Alkaline phosphatase activity was highly maintained in hair-inducing DPCs [McElwee et al., 2003], and both versican expression [Kishimoto et al., 1999] and alkaline phosphatase activity [McElwee et al., 2003] were gradually lost with culture expansion of DPCs. Despite these findings, however, there is still an absence of a reliable biomarker for the hair-inductive capacity of hDPCs and, therefore, *in vivo* animal models are the only confirmative way to evaluate the hair-inductive capacity of hDPCs at present.

A number of animal models for hair regeneration have been reported thus far. The so-called chamber (or cabin) model was first introduced by Lichti and Weinberg [Lichti et al., 1993; Weinberg et al., 1993] and is one of most established methods to study hair regeneration [Kamimura et al., 1997; Kishimoto et al., 1999; Ehama et al., 2005; Rendl et al., 2005]. In the replacement model, the dermal papilla (DP) of the rat vibrissa was replaced with cultured DPCs [Jahoda et al., 1984]. In the ear wound model, cultured DPCs were subcutaneously transplanted through a small incision on the rat auricle skin to observe influences of the transplanted DPCs on existing villus hairs [Jahoda et al., 1993; McElwee et al., 2003]. In addition, a subcutaneous injection of a mixture of epithelial cells and DPCs (the so-called hair patch assay) into the auricular skin or the footpad in mice has also been developed [Morris et al.,

2004; Zheng et al., 2005]. In the sandwich model, first reported by Reynolds and Jahoda [1992], DPCs were inserted between the epidermis and dermis of the footpad skin and transplanted into a skin wound [Reynolds and Jahoda, 1992], under the kidney capsule [Inamatsu et al., 1998] or under the skin [Inamatsu et al., 2006].

In this study, we selected 2 major animal models, the chamber model and the sandwich model, as the most practical tools to evaluate the hair-inducing capacity of cultured hDPCs, as these were the only 2 among many methodologies reported in the literature that had been successful in reorganizing hair follicles in animal tissues and that had been replicated by many groups [Reynolds and Jahoda, 1992; Lichti et al., 1993; Weinberg et al., 1993; Kamimura et al., 1997; Inamatsu et al., 1998; Kishimoto et al., 1999; Ehama et al., 2005; Rendl et al., 2005; Inamatsu et al., 2006]. We examined the reliability and practicality of the 2 model systems as well as various combinations of rodent or human epithelial and mesenchymal cells used as positive and negative controls.

Materials and Methods

Preparation of Human and Murine Cells

All experiments were conducted in accordance with the use of animals for scientific purposes and the Helsinki Principles. Human hair follicles were obtained from facelift operations with approval from the ethical committee. Under a microscope and microforceps and needles, human DP was dissected from the bulb portion of the follicle floating in a culture dish containing Hanks' balanced solution. The isolated DPs were transferred into a new 60-mm culture dish, attached to scratched scars on the dish and cultured in DMEM supplemented with 10% fetal bovine serum (FBS; fig. 1). After 2 weeks of the explant culture, hDPCs were subcultivated twice, and a cell sheet or a cell suspension of hDPCs was prepared for a transplant. To prepare the hDPC sheet, hDPCs were cultured until overconfluent, and hDPCs were harvested as a cell sheet by scraping (cell scraper; Sumitomo Bacrata, Tokyo, Japan). Human epidermal keratinocytes (hKCs) were isolated from a human skin fragment incubated overnight with DMEM containing 10% FBS and 1,000 U/ml dispase (Dispase I[®]; Sankyo, Tokyo, Japan) at 4°C; the skin sample was subsequently digested with 0.05% trypsin in phosphate-buffered saline at 37°C for 10–30 min. The isolated hKCs were cultured in DMEM containing 10% FBS on a human fibroblast feeder layer lethally preconditioned with mitomycin C (Nacalai Tesque, Tokyo, Japan), according to the method described by Rheinwald and Green [1975].

C57BL/6 pregnant mice were purchased from Japan CLEA Inc. (Tokyo, Japan). A dorsal skin fragment from E19.5 embryos was incubated with DMEM containing 10% FBS and 1,000 U/ml dispase (Dispase I) at 4°C overnight to separate the epithelial component from the dermal component. Each component was digested with 0.1% w/v collagenase (Wako, Osaka, Japan) at 37°C for 1 h to obtain fresh cell suspension of epithelial or dermal cells.

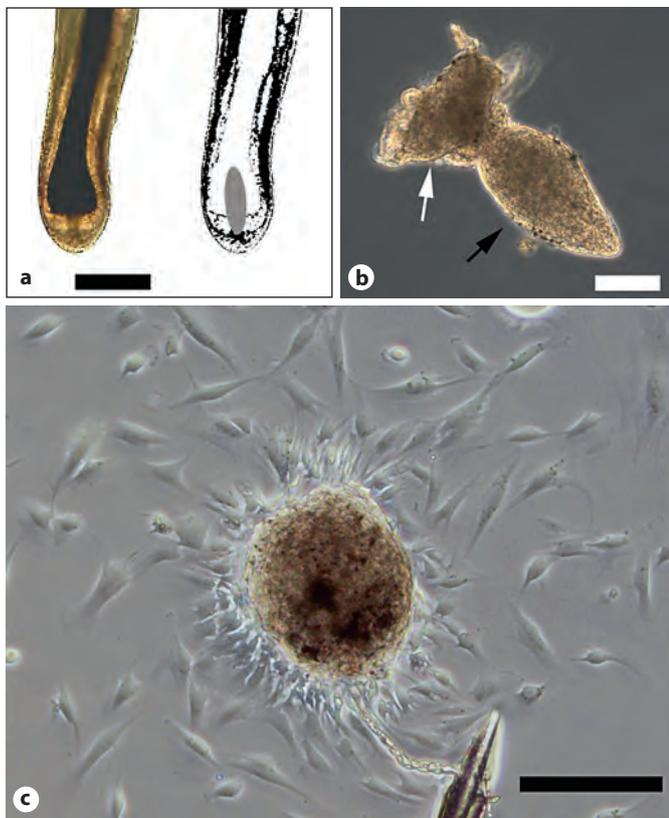
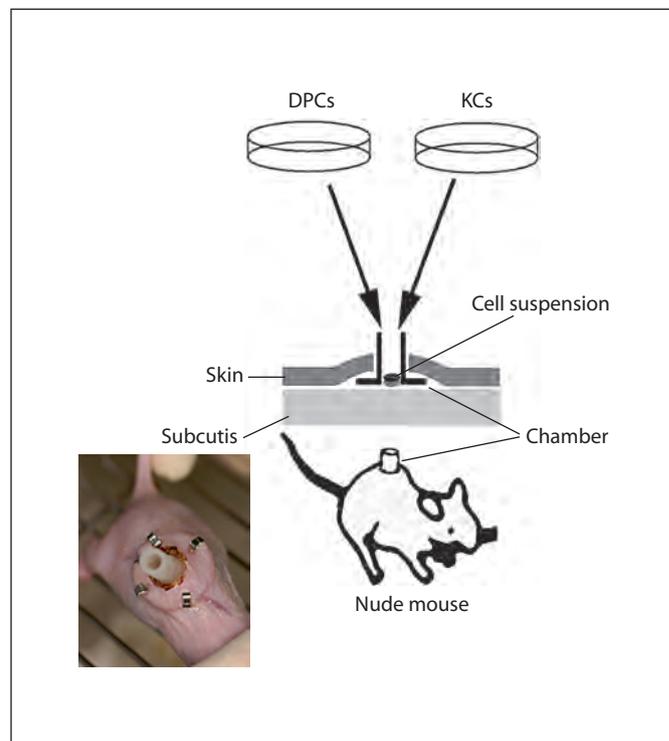


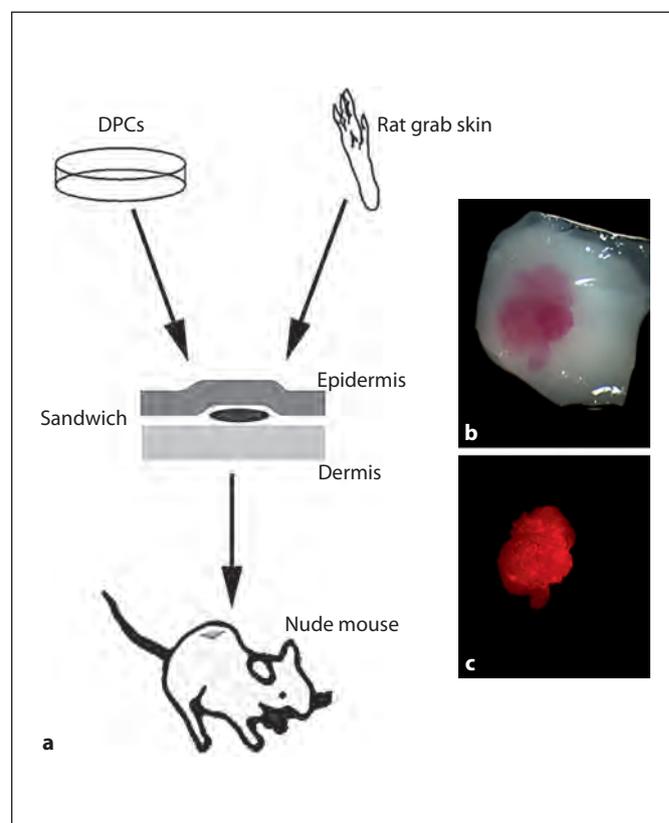
Fig. 1. Human DP and explant culture of DPCs. **a** Human anagen hair follicle and the position of DP (gray area). Scale bar = 1 mm. **b** DP and dermal sheath microscopically harvested from an anagen hair follicle. DP (black arrow), dermal sheath (white arrow). Scale bar = 200 μm . **c** Explant culture of hDP at 1 week. hDPCs proliferated and migrated around DP. Scale bar = 100 μm .

Fig. 2. Schematic of the chamber model. Both mesenchymal cells (for example, DPCs) and epithelial cells (for example, KCs) were transplanted as a cell suspension into the silicone chamber, which was implanted into the skin of the back of nude mice 1 week before cell transplantation. One week later, the silicon chamber was removed from the skin.

Fig. 3. Schematic of the sandwich model. **a** Mesenchymal cells (for example, DPCs) were inserted between the epidermis and dermis of the rat plantal skin. The sandwich construct was transplanted into the subcutis of nude mice. **b, c** In the sandwich construct, a fragment of DPC sheet was labeled by CM-DiI and sandwiched with the epidermis and dermis of the rat grab skin fragment. Ste-reomicroscopic view (**b**) and fluorescence microscopic view (**c**).



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Table 1. Summarized data of 4 chamber subgroups (chamber 1, P1, P2 and N1) and 3 sandwich subgroups (sandwich 1, P1 and P2)

Model subgroups	Epithelial cells	Mesenchymal cells	Number of transplanted samples	Number of collected samples	Number of hair-induced samples	Hair induction rate, %
Chamber 1	cultured hKCs	cultured hDPCs	25	17	5	29
Chamber P1	fresh fmECs	fresh fmDCs	6	6	6	100
Chamber P2	fresh fmECs	cultured hDPCs	3	3	1	33
Chamber N1	none	cultured hDPCs	3	2	0	0
Sandwich 1	rat sole epidermis	cultured hDPC sheet	38	34	14	41
Sandwich P1	rat sole epidermis	hDPT	5	5	2	40
Sandwich P2	human face epidermis	hDPT	4	2	2	100

Combination patterns of epithelial cells and mesenchymal cells and results of hair induction in each subgroup are summarized. The induction rate of each subgroup is calculated by dividing the number of hair-induced samples by the number of collected samples.

Animal Models

Fisher 344 rats (6 weeks old, male) and Balb/c nude mice (6 weeks old, male) were purchased from Japan CLEA Inc.

For the chamber models, a silicon chamber (cylindrical shape, 5 mm inner diameter and 15 mm height) was subcutaneously inserted into the back of nude mice (fig. 2). Total number of mice used in the chamber group was 38. One week later, 1 of the following 4 combinations of cell mixtures (epithelial cells and mesenchymal cells; 2×10^7 cells) was transplanted into the chamber: cultured hKCs and cultured hDPCs (chamber 1), fresh fetal murine epithelial (fmECs) and dermal cells (fmDCs; chamber P1: positive control 1), fresh fmECs and cultured hDPCs (chamber P2: positive control 2), and cultured hDPCs alone (chamber N1: negative control 1). The top of the chamber was left open to prevent excessive fluid accumulation effused from the base wound as a modification of the previously described method [Yuspa et al., 1970]. The chamber wound was kept wet enough to keep the transplanted cells alive. The chamber was removed 1 week after cell transplantation. Four (chamber P1) or 6 weeks (other chamber subgroups) later, the skin at the transplantation site was harvested and processed for histological examination.

For the sandwich models, a 5-mm square skin fragment was obtained from the rat sole or human face, and incubated with DMEM containing 10% FBS and 1,000 U/ml dispase (Dispase I) at 37°C for 20–30 min to dissect the epidermis from the dermis. A sandwich-like construct was prepared by inserting a fragment of the cultured hDPC sheet or fresh human dermal papilla tissue (hDPT) between the epidermis and the dermis. hDPT was microscopically dissected as a tissue fragment and used as an insert. Three combinations of epithelial and mesenchymal cells were prepared as follows: the rat sole epidermis and cultured hDPC sheet (sandwich 1), the rat sole epidermis and hDPT (sandwich P1: positive control 1), and the human facial epidermis and hDPT (sandwich P2: positive control 2). The sandwich construct was then transplanted into the subcutis of the back of nude mice (fig. 3), and 4 weeks later the construct was harvested and processed for histological examination. The total number of mice used in the sandwich group was 47.

Cell Labeling

To clarify the origin of cells, cell trafficking was performed using a fluorescent cell-labeling agent, CM-DiI (CellTracker®; Molecular Probes-Invitrogen Japan, Tokyo, Japan), which is a cell membrane-permeable fluorochrome. DPCs were incubated with DMEM containing 5 µg/ml CM-DiI at 37°C for 24 h before transplantation.

Histology

Regeneration of hair follicles was evaluated by histological examination. The skin sample was dipped into the Tissue-Tek® OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen in liquid nitrogen, and serially sectioned (8 µm thick). Every other section was stained with hematoxylin and eosin staining for bright-field examination, and the other sections were stained with DAPI (Vectashield®; Vector Laboratories, Burlingame, Calif., USA) to visualize nuclei and were observed with a fluorescence microscope (BioZero®; Keyence, Tokyo, Japan).

Statistical Analyses

Significant differences in the hair induction rate among subgroups of each animal model were analyzed with Fisher's exact test. Significant differences between 2 representative subgroups of the chamber and sandwich models (chamber 1 and sandwich 1) were calculated with the Mann-Whitney U test. Values of $p < 0.05$ were considered significant.

Results

Hair Induction Rate

We defined 4 chamber model subgroups (chamber 1, P1, P2 and N1) and 3 sandwich model subgroups (sandwich 1, P1 and P2) using different combinations of epithelial and mesenchymal cells, and evaluated the induction of hair generation in each subgroup. The time points of harvest were determined based on the results of a pilot

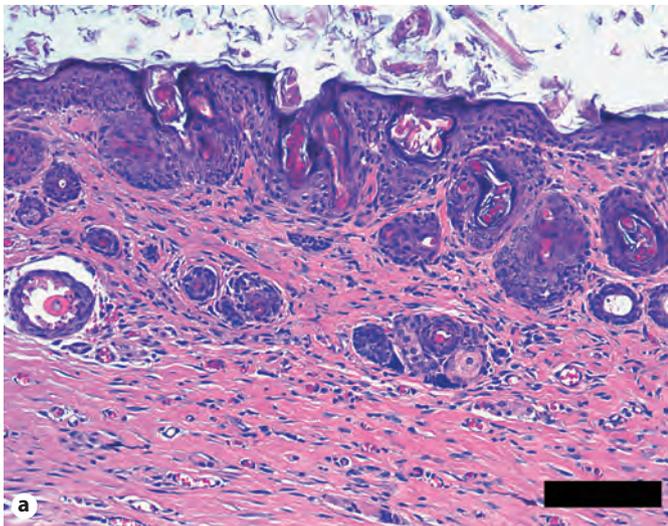


Fig. 4. A representative sample of the chamber P1 model (at 4 weeks). **a** Histology at 4 weeks demonstrated a number of regenerated hair follicles induced by transplantation of fmECs and fmDCs. Most hair follicles are highly matured and have hair shafts. H&E staining. Scale bar = 120 μm . **b** Small black hairs were detected at the chamber site on the back of nude mice.

prestudy; hair follicle formation was seen in 4 weeks in chamber P1 and all sandwich groups, while 6 weeks were appropriate for other chamber subgroups. Not all transplanted samples were collected due to chamber infection or animal death; 8 in chamber 1 samples were lost, 1 in chamber N1, 4 in sandwich 1 and 2 in sandwich P2. The results are summarized in table 1. Hair induction was detected in all subgroups except for chamber N1, which did not use epithelial cells and was considered a negative control. Chamber P1 generated 100% hair induction, and Fisher's exact test indicated statistically significant differences between chamber 1 and chamber P1 ($p = 0.0046$) as well as between chamber P1 and sandwich 1 ($p = 0.020$).

For the chamber 1 subgroup, 17 of the 25 prepared samples (using 11 cell strains of hDPCs) were collected, while for the sandwich 1 subgroup, 34 of the 38 transplanted samples (using 10 cell strains of hDPCs) were collected. The overall hair induction rate in the sandwich 1 subgroup was 41.1%, higher than that of the chamber 1 subgroup (29.4%), but the difference was not statistically significant. Hair was induced by hDPCs cultured prior to passage 3 in both groups, but hair induction was not observed by hDPCs cultured after passage 4 in either group.

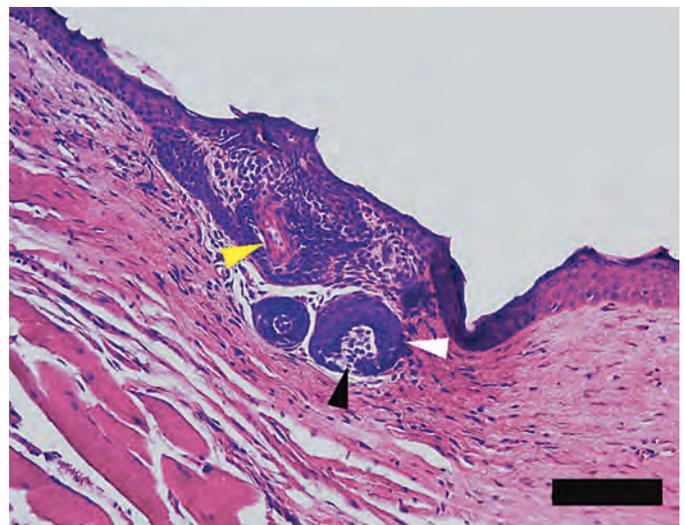


Fig. 5. A representative histology of the *Chamber 1* model (at 6 weeks). Cultured hKCs and cultured hDPCs were used for folliculoneogenesis. H&E staining demonstrated that a regenerated hair follicle was incomplete but has all major components of hair follicle, such as the hair matrix and outer root sheath (white arrowhead), the DP (black arrowhead) and a hair shaft (yellow arrowhead). Scale bar = 100 μm .

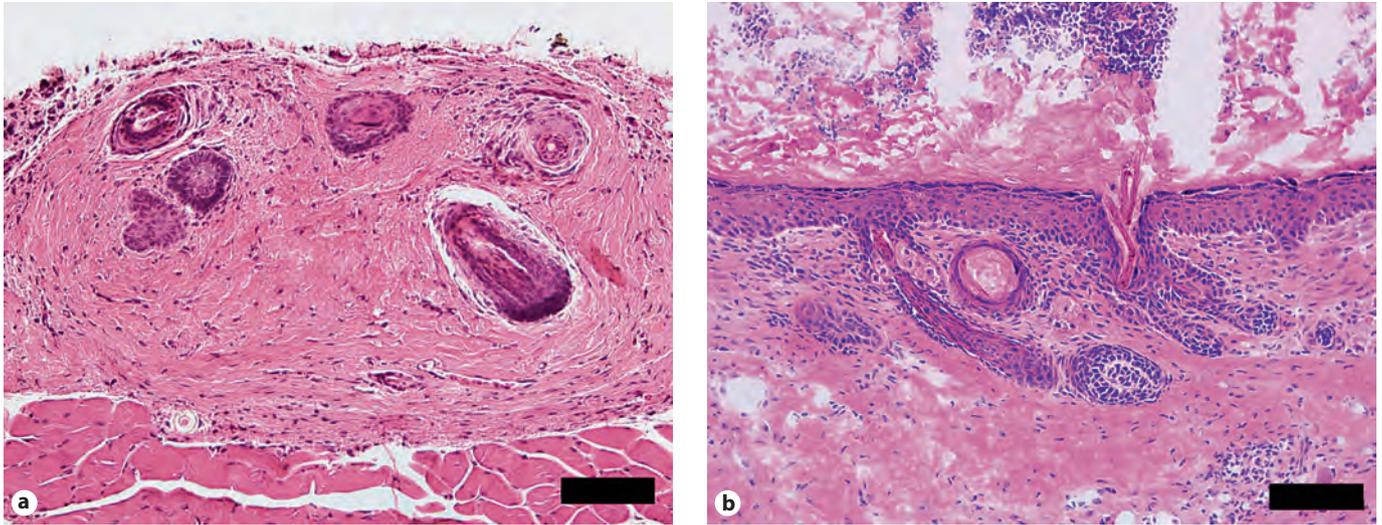
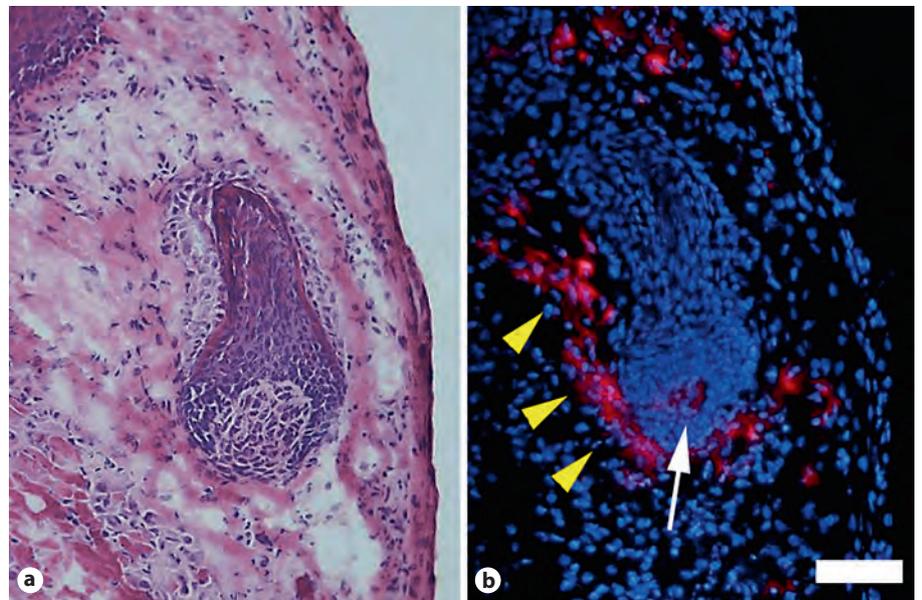


Fig. 6. Representative histological views of the sandwich 1 model (at 4 weeks). A sandwich construct was prepared by inserting a fragment of the hDPC sheet between the epidermis and dermis of the rat grab skin. Regenerated hair follicles varied in size and maturity. Several new follicles with a hair shaft were buried in the dermis (a), while other samples demonstrated matured follicles penetrating the epidermis (b). H&E staining. Scale bars = 100 μ m.

Fig. 7. A representative histology of a regenerated hair bulb in the sandwich 1 model (at 4 weeks). CM-DiI-labeled hDPCs were sandwiched between the epidermis and dermis of the rat grab skin before transplantation. Serial sections showed that a matured hair bulb was observed in H&E staining (a) and the CM-DiI-labeled hDPCs localized at the dermal papilla and dermal sheath in the new hair bulb (b). Scale bar = 100 μ m.



Histological Features of Regenerated Hair Follicles

In chamber P1 models, the combination of fmECs and fmDCs generated a number of small black hairs growing from the skin, and matured hair follicle structures were histologically confirmed (fig. 4). No regenerated hair growth at the skin was observed in chamber 1, but histo-

logical examinations revealed that regenerated hair follicles had nearly mature follicle structures, such as spindle-shaped DP, inner root sheaths and hair shafts (fig. 5), though the maturity grades of regenerated follicles varied among samples. A CM-DiI-labeled hair follicle was not confirmed in chamber 1, suggesting that transplanted

hDPCs divided repeatedly before hair follicle formations. It was occasionally difficult to distinguish original hair follicles from regenerative ones at the border area between the chamber area and the surrounding skin.

In the sandwich 1 subgroup, various grades of maturity of regenerated hair follicles were seen in samples; some had the outer root sheath and DP-like structures, while others demonstrated a complete set of hair follicle components, including a hair shaft (fig. 6). Some samples showed a regenerated hair shaft growing through the rat plantar epidermis, although the size of the hair was small (fig. 6). As the rat plantar skin lacks hair follicles, all hair follicles observed in the sandwich samples were regenerated follicles derived from the mouse epidermal (stem) cells and hDPCs. This was confirmed by observations of labeled hDPCs in regenerated hair follicles that constituted a part of the DP and dermal sheath of a regenerated hair follicle in sandwich 1 (fig. 7).

Discussion

Many *in vivo* animal models for hair regeneration have been utilized, but almost all are unreliable and unpredictable due to technical difficulties, and require substantial time to complete evaluation of hair induction capacity of hDPCs. For example, in the model in which hDPCs were transplanted subcutaneously into the rat auricle through small skin incision [Oliver, 1966; Jahoda et al., 1984], it was difficult to distinguish original hairs from regenerated hairs, as the original hairs of the auricle skin exist and vary in size. Other reports employed a simple injection into the skin with a syringe [Morris et al., 2004; Zheng et al., 2005], and attempts to regenerate human hair follicles by these injection methods have been frequently unsuccessful thus far [Stenn and Cotsarelis, 2005]. Based on our experience, a combined injection of cultured hKCs and hDPCs frequently resulted in the formation of cystic lesions resembling epidermal cysts (data not shown). From a practical perspective, an evaluation model should ideally have high reproducibility, be readily prepared with a small number of cells, not generate false-negative or false-positive findings, and be evaluated in as timely a manner as possible. Epithelial cells used in evaluation models should be undifferentiated and maintain their capacity for hair differentiation. Histology was the method we employed for evaluation of regenerated hair maturity in this study, as most regenerated hair follicles were not fully matured and frequently buried in the dermal component.

Both the chamber and the sandwich model using human cells and tissues are xenograft models. Although the athymic nude mouse is not completely immunodeficient due to the residual immunoreactions by B cells and other cells, signs of rejections were not detected within 6 weeks after transplantation in this study. Indeed, many previous studies employed nude mice as recipient animals of DPC transplantation without immunological rejection [Weinberg et al., 1993; Kamimura et al., 1997; Kishimoto et al., 1999; Ehama et al., 2005; Inamatsu et al., 2006; Rendl et al., 2006].

In the present study, several combinations of murine and/or human epithelial and mesenchymal cells were included. We regarded fresh cells or fresh tissues as having strong inductive capacity compared with cultured cells, and those of mouse or rat cells also with stronger capacity compared with human cells. Thus, we included subgroups of positive or negative control in chamber and sandwich groups using fresh and/or animal cells or fresh human tissue. In the chamber group, we prepared chamber P1 and P2 as positive controls, and chamber N1 as a negative control. In the sandwich group, we prepared sandwich 2 and 3 as positive controls. Chamber P1 (combination of fmECs and fmDCs) generated a large number of well-matured hair follicles in each sample with a high success rate, suggesting that freshly isolated fmDCs have a much higher hair-inductive capability than adult hDPCs. Chamber 1 (combination of cultured hKCs and hDPCs) also generated a hair shaft, but the hair remained buried in the skin even in a successful sample, as shown in figure 5. In contrast, sandwich 1 produced hair shaft formation with hairs growing from the rat plantar epidermis in several samples, although the model employed cultured hDPCs. Thus, this indicates that the sandwich model is not inferior to the chamber model in regards to regenerated hair maturity.

The chamber model with a large-sized (for example, 10 mm) chamber requires a substantial amount of epithelial cells (at least $10^7/10$ -mm chamber) along with mesenchymal hair-inducing cells for wound healing and hair regrowth, while the chamber model with a small size is more likely to lead to false-positive results due to the contraction of the small skin wound and migration of the cells from the surrounding tissue into the wound. In addition, it is not easy to consistently prepare high-quality cultured hKCs, as required in chamber 1, and failure to do so can lead to a false-negative evaluation of DPCs. Infection of skin wounds and subsequent animal death were occasionally seen in the chamber models.

On the other hand, sandwich models require fewer hair-inducing mesenchymal cells (5×10^5 to 5×10^6 per model) and do not require the preparation of cultured epithelial cells, although technical problems such as dislocation of the overlaid epidermis could induce a false-negative result. If a human skin fragment is used as in the sandwich model, the original villus hair could be misevaluated as a regenerated hair; thus, use of the rat grab skin as sandwich material could avoid a false-positive result. Infection or necrosis of the grafted tissue was rarely observed in sandwich models and more than 90% of the grafts were successfully collected.

A number of groups have employed chamber models as experimental models for hair folliculogenesis, but have used murine DPCs as hair-inducing cells. To our knowledge, no reports employed hDPCs as hair-inducing cells due to unsuccessful or unpredictable results of folliculogenesis in chamber models, as previously discussed [Stenn and Cotsarelis 2005; Ehama et al., 2006]. In this study, we have confirmed that a chamber model (chamber 1) can be used as an experimental model for folliculogenesis using hDPCs, but the success rate is not superior to the sandwich model (sandwich 1). Chamber 1 requires cultured keratinocytes, and risks losing the differentiating capacity after culture expansion. On the contrary, in sandwich 1, fresh epidermis containing epidermal stem cells can be readily prepared, thus the quality of epidermal cells is controlled.

Taken together, we conclude that the sandwich model is superior to the chamber model as an in vivo evaluation model for the hair-inducing capacity of hDPCs. Although hair induction in the sandwich model can only be evaluated by histology at least 4 weeks after transplantation, viable and reliable epithelial cells (the rat grab epidermis) can be easily prepared, much less hDPCs are required per sample, reliable results can be obtained from a high rate of sample collection and a low rate of false-positive evaluation, and the efficacy and reproducibility of hair induction is comparable to the chamber model. Due to the absence of a reliable biomarker for hair-inductive capacity of hDPCs, histological evaluation in an animal model is the best method to assess the specific ability of hDPCs to induce hair growth. Although the assays for hair-inductive capacity in this study do not cover all conditions or styles of transplant constructs for therapeutic applications, the evaluation models will be helpful for the optimization of expansion and preparation processes of hDPCs, and facilitate the exploration of genes and factors contributing to hair regeneration.

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