Embryonic dermal condensation and adult dermal papilla induce hair follicles in adult glabrous epidermis through different mechanisms

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Running title: Hair induction by dermal compartment

Abstract

Hair induction in the adult glabrous epidermis by the embryonic dermis was compared with that by the adult dermis. Recombinant skins composed of the adult sole epidermis and the embryonic dermis containing dermal condensations (DCs) was transplanted onto the back of nude mice. The epidermis of transplants formed hairs. Histology on the induction process demonstrated the formation of placode-like tissues, indicating that the transplant produces hair follicles through a mechanism similar to that underlying the hair follicle development in the embryonic skin. An isolated adult rat sole skin piece was inserted with either an aggregate of cultured dermal papilla (DP) cells or an intact DP between its epidermis and dermis was similarly transplanted. The transplant produced hair follicles. Histology showed the epidermis in both cases surrounded the aggregates of DP cells. The epidermis never formed placode-like tissues. Thus, it was concluded that the adult epidermal cells recapitulate the embryonic process of hair follicle development when exposed to DCs, whereas they directly get into the anagen of the hair cycle when exposed to DPs. The expression pattern of *Edar* and *Shh* genes, and P-cadherin protein during the hair follicle development in the two types of transplants supported the above conclusion.

Key words: placode, embryonic hairs, hair germs, EGFP-transgenic rats, hair cycle, hair follicles, recombinant skin, skin transplantation, nude mice

Introduction

Embryonic hair follicles develop from hair germs that have generated through a series of interactions between the embryonic ectoderm and mesoderm (Hardy 1992). The earliest histological sign of the development is the thickening of the epidermis, which leads to the formation of a unique histological entity called placode (Philpott & Paus 1998) at stage 1 of embryonic hair follicle formation (Hardy 1992; Philpott & Paus 1998). In response to the formation of placode, the adjacent subepidermal mesenchymal cells start to aggregate and form a unique histological entity called the dermal condensation (DC), the precursor of the dermal papilla (DP) (Philpott & Paus 1998). A pair of the placode and the DC constitutes the hair anlage (Philpott & Paus 1998) that is formed at stage 2 and develops to the hair peg at stage 3. A DC is ensheathed by epidermal components at stage 4, which results in the formation of the DP. The hair follicle starts to form at stage 5 and its development completes at stage 8. Thereafter, the hair follicle undergoes cyclic morphological changes known as the hair cycle consisting of growth (anagen), regression (catagen), and termination (telogen) through life. Hair cycle is driven by the reciprocal interactions between the dermal papilla and the follicular epidermis (Stenn & Paus 2001).

The hair development in the embryonic skin and the new hair formation in the adult skin are regulated by signaling molecules sequentially secreted by epidermal and dermal components (Botchkarev & Paus 2003). Especially, the dermal component is considered to play a leading role in the hair follicle induction, because both DCs and DPs are capable of inducing hair follicles in

the adult epidermis (Ferraris *et al.* 1997; Ferraris *et al.* 2000; Osada & Kobayashi 2000 for DCs; Reynolds & Jahoda 1992; Inamatsu *et al.* 1998; Xing & Kobayashi 2001; Miyashita *et al.* 2004 for DPs). However, there have been no studies hitherto that comparatively characterize the induction processes by DCs and DPs morphologically and at the expression level of genes and proteins.

The present study compared the hair-induction process by DCs and DPs using the adult glabrous epidermis as a common responder. As the source of DP, we tested two adult DP preparations, isolated intact DPs and aggregates of cultured DP cells. The adult rat sole skin epidermis was combined with either DC-containing embryonic rat dermis or adult rat DPs. The recombinant skins were transplanted to the back of BALB/c nu/nu mice (nude mice) and allowed to develop hairs. Hair developmental processes induced by DC and DP were histologically compared. The processes were also compared regarding the expression pattern of genes of ectodysplasin receptor (*Edar*) and sonic hedgehog (*Shh*), and P-cadherin protein. *Edar* is expressed in the early placode (Headon & Overbeek 1999; Millar 2002). *Shh* is expressed in the middle placode (Bitgood & McMahon 1995; Iseki *et al.* 1996; Millar 2002) and in the hair matrix of the anagen hair follicle (Gat *et al.* 1998; Sato *et al.* 1999). P-cadherin protein is distributed at the tip of the late placode (Hardy & Vielkind 1996; Muller-Rover *et al.* 1999) and in the hair matrix surrounding the DP of adult anagen hair follicles (Muller-Rover *et al.* 1999). As a result, we were able to demonstrate that these two types of dermal compartments induce hair follicles through different mechanisms. DC and DP induce the adult epidermis to form the follicular epidermis via the formation of epidermal placode-like tissues and via

the formation of early anagen phase-like tissues, respectively.

Materials and Methods

Animals and materials

Rats of Fischer 344 (F344) and Wistar, and male BALB/c nu/nu mice were purchased from Charles River (Yokohama, Japan). Enhanced green fluorescent protein (EGFP)-transgenic Wistar rats (EGFP-rats) were obtained from Health Science Research Resources Bank of Japan Health Sciences Foundation (Osaka, Japan). EGFP-transgene was driven by the CAG (cytomegarovirus enhancer-chicken ß-actin promoter-rabbit ß-globin poly A) promoter (Hakamata *et al.* 2001; Takeuchi *et al.* 2003; Miyashita *et al.* 2004). Dispase was obtained from Sanko Junyaku Co., Ltd. (Tokyo, Japan). Collagenase, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI), and diaminobenzidine were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Nitroblue tetrazolium, 5-bromo-4-chloro-3-indolylphosphate, and anti-digoxigenin (DIG) antibodies conjugated with alkaline phosphatase were products of Roche Diagnostics GmbH (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM) was a product of Invitrogen Corporation (Carlsbad, CA). Fetal bovine serum (FBS) and fibrin glue (BOLHEAL®) were from Hyclone Laboratories, Inc. (Logan, UT) and Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan), respectively. Hoechst 33258 (Hoechst) and fibroblast

growth factor 2 (FGF2) were obtained from Sigma-Aldrich Co. (St. Louis, MO) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Antibodies against cytokeratin (CK) 14, CK10, and P-cadherin were from Lab Vision Corporation (Fremont, CA), and those against loricrin and green fluorescent protein (GFP) from Covance Research Products, Inc. (Princeton, NJ) and BD Biosciences (Clontech, Palo Alto, CA), respectively. ABC system was a product of DakoCytomation Denmark A/S (Glostrup, Denmark). Nuclear Fast Red was a product of Vector Laboratories Inc. (Burlingame, CA). Nuclepore® filters were purchased from Corning Incorporated (Corning, NY).

Transplantation of rat embryonic skin to nude mouse

Pieces of skin (about 2 x 1 cm^2 at most) were isolated from F344 rat embryos at embryonic day (E) 14 to E20 and were divided along the median line into two halves. One half was grafted on the back of 4-week-old male nude mice with an aid of fibrin glue. The transplants were dissected from the animals 3, 8, and 12 weeks later and processed to 5-μm-thick serial paraffin sections for hematoxylin & eosin (H&E) staining. The other half was directly processed for immunohistochemistry whose procedure is described below.

Construction and transplantation of recombinant rat skin of adult epidermis and embryonic dermis

A skin piece (0.8 x 0.8 cm) was isolated from the sole of 10-week-old wild Wistar rats, incubated in 6 mL of DMEM supplemented with 10% FBS

(DMEM/FBS) containing 1,000 U/mL dispase at 37°C for 15 min, and separated into the epidermis and the dermis. Concurrently, a skin piece (0.8 x 0.8 cm) was isolated from EGFP-rat embryos at E17 (EGFP-E17-rat embryos) and incubated at 4°C for 2 h in 6 mL of DMEM/FBS containing 2,000 U/mL dispase and 0.1% collagenase. After removing the solution, 1 μL of 100 mg/mL DNase I was dropped on each of the skins. The skins were separated into the epidermis and the dermis. Then, the above adult sole epidermis and the EGFP-E17-dermis were recombined on Nuclepore filters using a pair of fur needles under a binocular microscope. The recombined skins were grafted on the back of 4-week-old male nude mice with an aid of fibrin glue. The transplants were dissected at 10, 14, and 21 days post-transplantation, and sectioned to H&E staining as above and also to immunohistochemistry of P-cadherin and *in situ* hybridization of *Edar* and *Shh* as described below.

Preparation and transplantation of adult rat sole skin containing adult rat dermal papilla

Two types of sole skin containing rat DP were prepared and transplanted to nude mice as Experiment I and II. In Experiment I cultured DP cells were used as the dermal component. DP cells were obtained from vibrissae of 6-week-old F344 rats and propagated by serial subculturing as previously reported (Inamatsu *et al.* 1998). DP cells (about 5 x 10^6 cells) at passage 20 under confluence in a 100-mm tissue culture dish containing 10 mL of DMEM/FBS and FGF2 (5 ng/mL) were gently scraped off in 5 mL of phosphate-buffered saline (PBS)

containing 0.02% EDTA and centrifuged at 210 g for 5 min at 4°C. The cell pellets were suspended in 5 mL of DMEM/FBS containing 40 μg/mL DiI and incubated for 5 min at room temperature. The DiI-labeled DP cells were distributed in wells of a 96-well plate (5 x 10^3 cells/well) and centrifuged at 1,370 g for 10 min. The pellets were treated with fibrin glue to make them solid aggregates. Concurrently, skin pieces (about 0.4 x 0.8 cm) was removed from the sole of 8-week-old female F344 rats, mildly digested at 37˚C for 3 min with 500 U/mL dispase in 6 mL of DMEM/FBS, and given a pocket-like space between the epidermis and the dermis. Three pieces of the above DP cell-aggregates were inserted into the space made in one piece of the above sole skin. Skin pieces inserted with the DP cell-aggregates were grafted on the back of 4-week-old nude mice as above. In Experiment II, intact DPs from EGFP-rats (EGFP-DPs) were used as the dermal component. EGFP-DPs from vibrissae of 6-week-old EGFP-rats were inserted in the space of isolated sole skin pieces of wild Wistar rats and transplanted to nude mice as in Experiment I. Transplants were removed weekly or at 10 days after transplantation in Experiment I and II, and serially sectioned for histology, immunohistochemistry, and *in situ* hybridization whose procedures are described as below.

Determination of cell number in a DC and a vibrissal DP

Serial sagittal 5 μm-thick sections of E17-skin and cheek skin of 6-week-old rats were prepared, and stained with H&E. We assumed that the steric shape of a DC and a vibrissal DP is hemisphereic and oval, respectively. Ten sections were selected that dissected the central region of DCs and DPs, where they showed

the largest area. On these sections we determined the diameter of the DCs as 44.3 ± 5.7 μm (the average ± SD), and the major and the minor axes of DPs as 464.2 ± 129.1 and 315.6 ± 46.4 μm, respectively, which enabled us to calculate their volumes. The number of nuclei in the DCs and the DPs on the sections was counted and converted to the number per volume of a DC and a DP.

Immunohistochemistry

Embryonic and adult skin Pieces of skin (about 1 x 1 cm^2 at most) were dissected from embryos at E14 to E17 and adults of F344 rats, fixed in 10% formalin in PBS, and processed to 5-μm-thick serial paraffin sections for immunohistochemistry. The sections were pretreated with 10 mM citrate buffer for 40 min at 95°C and treated with antibodies at 4˚C overnight against CK14, CK10, and loricrin. The bound antibodies were visualized with an ABC system using diaminobenzidine as substrate. Sections were counterstained with hematoxylin.

Transplants Transplants containing dermal constituents of EGFP-rats shown in Fig. 6 and 8 were fixed in 4% paraformaldehyde in PBS at 4˚C overnight, and processed to 7-μm-thick serial paraffin sections. The sections were pretreated with 4 mg/mL proteinase K for 7 min at room temperature and treated overnight with antibodies against GFP at 4˚C. The bound antibodies were visualized with fluorescent isothiocyanate (FITC)-labeled second antibodies. Sections were counterstained with Hoechst. The serial sections of transplants were pretreated with 10 mM citrate buffer for 40 min at 95°C and treated with antibodies against

P-cadherin at 4˚C overnight. The bound antibodies were visualized with an ABC system using diaminobenzidine as substrate. Sections were counterstained with hematoxylin.

In situ hybridization

Transplants in experiments shown in Fig. 6 and 8 were removed and fixed in 4% paraformaldehyde in PBS at 4˚C overnight, and processed to 7-µm-thick serial paraffin sections. The sections were pretreated with 5 µg/mL proteinase K in PBS for 10 min at 37˚C and refixed with 4% paraformaldehyde in PBS for 20 min. Hybridization was performed overnight at 68˚C with a DIG-labeled cRNA riboprobe under stringent conditions. DIG-labeled riboprobe templates for ectodysplasin receptor (*Edar*) and sonic hedgehog (*Shh*) were generated by the polymerase chain reaction of E17 rat skin cDNA, using the following primers: nt 431-1290 for *Edar* (NCBI/GenBank accession number, AF160502) and nt 137-780 for *Shh* (X76290). The sections were rinsed, probed with anti-DIG antibodies conjugated with alkaline phosphatase. The hybridization was visualized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate as alkaline phosphatase substrate. The slides were counterstained with Nuclear Fast Red and mounted.

Results

Hair formation potential of embryonic skin

Developmental changes of the embryonic skin were examined in relation to the formation of histological structures of hair follicles (Fig. 1). There were no obvious morphological signs of the hair germ formation at E14 (Fig. 1A) that corresponds to “before pre-hair germ” stage. The epidermis was one cell-layered. The epidermis at E15 (pre-hair germ stage) became 3 cell-layered and thickened at the sites of presumptive hair germs and was about to start to form the placode (Fig. 1B). Placodes were morphologically discernible at E16 (hair germ stage, Fig. 1C). The placode at E17 (dermal condensation stage) further thickened, whose tip was associated with condensed dermal fibroblasts (Fig. 1D). E17-skin was still embryonic because it lacked the granular layer and showed parakeratosis. Hair anlagen made further down-growth, and developed to hair pegs at E19 (data not shown) and to hair follicles at E20 (hair follicle stage, Fig. 1E).

To know when the embryonic skin acquires the ability to spontaneously form hair follicles without supports from embryonic environments, pieces of skin were isolated from embryos at each stage shown in Fig. 1 and transplanted onto the back of nude mice. The transplants were allowed to develop on the host animals. All the E14-skin grafts (n = 11) did not develop any macroscopical structures of hairs even at 12 weeks after transplantation (Fig. 1F). Thin hairs were sparsely formed in some of E15-skin grafts around 4 weeks after transplantation (Fig. 1G). The rate of hair formation (the ratio of the number of transplants with hairs to the number of tested transplants) was 42% (n = 12) at 8

weeks after transplantation. E16-skin transplants yielded dense and thicker hairs at 3 weeks after transplantation (Fig. 1H) at a rate of 78% (n = 9). All the tested E17- (n = 10) and E20-transplants (n = 10) developed normal hairs at 3 weeks after transplantation (Fig. 1I and J, respectively).

We examined whether the transplants had actually developed hairs composed of epidermal and dermal compartments of rat origin. Hoechst-staining makes intranuclear bodies in mouse cells brightly fluorescent, but rat cells do not contain such bodies (Cunha & Vanderslice 1984). In addition, rat nuclei are more uniform than mouse ones. These differences permitted us to distinguish donor rat cells from host mouse cells. Skins were isolated from embryos at E14 and E15, and divided into two halves, one half for transplantation experiments and the other half for immunohistochemistry of CK10. E14-skin transplants were removed at 12 weeks post-transplantation and processed to H&E histology (Fig. 2A). There were no hair-related structures in the transplants. Its serial Hoechst-section showed that dermal cells of the graft site were of rat origin as expected, but the epidermal cells were of mouse origin (Fig. 2B), indicating that the rat epidermis was replaced with the host epidermis. Histology was similarly made on serial sections of an E15-transplant that had not produce hairs at 8 weeks post-transplantation, and shown in Fig. 2D for H&E stain and in Fig. 2E for Hoechst-stain. Its dermal cells were rat cells, but its epidermis was replaced with mouse epidermis. Histology of an E15-transplant that had produced hairs at 8 weeks post-transplantation is shown in Fig. 2G for H&E stain, and in Fig. 2H and I for Hoechst-stain. Hair follicles were often observed (Fig. 2G), whose epidermal cells were rat cells (Fig. 2H). Interfollicular epidermal cells were also

rat cells (Fig. 2I). These results clearly show that the development of hair follicles in embryonic transplants depends on the engraftment and the survival of the epidermis of transplants. The E14-skin did not have the ability to form hairs in an adult nude mouse. The E15-skin started to gain it and the E17-skin acquires its full ability. The inability of the E14-skin might be due to the lack of differentiation potential or due to the structural instability or susceptibility to the host circumference. The differentiation state of E14- and E15-epidermis (Epi) was examined by detecting CK10, a marker of suprabasal epidermal cells (Fig. 2C, F, J). There were no CK10-positive cells in E14-skin (Fig. 2C). The positive epidermal cells were rarely seen in the E15-skin (Fig. 2F) that had failed to develop hair follicles when transplanted (Fig. 2D). In contrast, CK10-positive cells were frequently observed in the E15-skin (Fig. 2J) that had formed hairs when transplanted (Fig. 2G). Suprabasal cells of the E17-skin were all CK10-positive (Table 1). Similarly, we investigated the expression of additional two marker proteins, CK14, a marker of basal cells, and loricrin, a marker of cornified cell envelopes. The results are summarized in Table 1. CK14-expression was observed in basal layers of E14- to E17-skins. CK10- and loricrin-expression began at E15 and E17, respectively. These results strongly suggested that the acquisition of the ability of an embryonic epidermis to differentiate into adult epidermis" is closely associated with the expression of CK10 and loricrin.

Hair formation in transplants of recombined embryonic and adult skins

From the experiments described above, we concluded that E17-DC acquires the full potential to induce hairs in embryonic epidermis and, thus, we utilized E17- dermis (Der) as an inducer of embryonic hairs in the present study. The number of cells per DC was 78.8 ± 19.9 (mean ± SD). First we examined whether E17-DC can induce hair follicles in the adult glabrous epidermis. E17-Der was isolated from the skin of EGFP-rats, all the dermal cells of which were fluorescent under a fluorescent microscope (Fig. 3B). We prepared the recombinant skin (adult sole-Epi/E17-Der) by combining the epidermis obtained from the sole skin of wild-type Wister rats (Fig. 3D) with the above EGFP-E17-Der and transplanted it onto the back of nude mice. All the tested transplants produced hairs (Fig. 4A), Histology of the induced hairs is shown in Fig. 4B (H&E stain), 4C (through an EGFP filter) and D (nuclear stain). All the follicular epidermal cells were EGFP-negative, but all the DP and dermal sheath (DS) cells were EGFP-positive. These results indicated that the DC is capable of developing to DP and DS, and, as a result, is able to induce the adult glabrous epidermis to differentiate into the follicular epidermis. It can be also concluded that the adult sole epidermis can respond to the embryonic dermal message. In addition, the above result showed that E17-Der tissues were not contaminated with the E17-Epi and, thus, excluded the possibility that contaminated E17-Epi, but not sole-Epi, formed hairs in cooperation with E17-Der.

Embryonic dermal condensation induces hair follicles in adult epidermis through recapitulating embryonic processes

The present and our previous study (Inamatsu *et al.* 1998) showed that adult sole-Epi responds to DC and DP, respectively, and, as a result, is induced to develop hair follicle in both cases. We asked whether these dermal compartments induce follicular epidermis in the adult epidermis by the same mechanism. To answer this question the process of the hair follicle induction in both cases was compared. First, the process of hair induction in sole-Epi by DC was investigated utilizing recombined skins prepared from wild and EGFP-rats. Recombinants of adult sole-Epi/EGFP-E17-Der were prepared and transplanted onto the back of nude mice. These transplants were dissected at 10 and 14 days after transplantation for histological examinations (Fig. 5). Transplants at 10 days contained placode-like tissues (Fig. 5A). An EGFP-positive condensed dermis was associated with such a placode (Fig. 5E). There were regions in the transplants at 10 days, where hair germ- (Fig. 5B) and hair peg-like tissues (Fig. 5C) were developed, both of which were closely associated with EGFP-positive DCs (Fig. 5F and G, respectively). Well-developed hair follicles were observed at 14 days (Fig. 5D) whose dermal compartments were EGFP-positive (Fig. 5H).

Expression patterns of marker genes associated with hair development were investigated during the hair follicle induction. *Edar* was ubiquitously expressed in hair germ-like tissues of 10-day-transplants, although the intensity was not uniform (Fig. 6A, D), whereas *Shh* expression was polarized in a lateral side of their distal tips (Fig. 6B, E). These expression patterns of *Edar* and *Shh* were

similar to those in E17-hair germs (Fig. 6G and H for *Edar* and *Shh*, respectively). P-cadherin was detected in the whole region of hair germ-like tissues of the transplants (Fig. 6C, F) as in E17-hair germs (Fig. 6I). These results suggested that DC is capable of inducing hair follicles in the adult epidermis by compelling the adult tissue to recapitulate embryonic process of hair follicular development.

Adult dermal papilla induces hair follicles in adult epidermis by inducing hair cycle

We investigated the process of hair follicle induction in transplants of adult sole-Epi/adult DP. Two types of experiment (Experiment I and II) were made for this purpose, one with dissociated and cultured DP cells (Experiment I) and the other with intact DPs as the inducer of hair follicles (Experiment II). DP cells in Experiment I were obtained from adult Fischer rat vibrissae, propagated through serial subculturing (Inamatsu *et al.* 1998), and were labeled with DiI. Adult sole skins were stuffed with aggregates of 5 x 10^3 DP cells, and transplanted onto the back of nude mice. Histology of transplants was periodically examined (Fig. 7, Exp-I). The epidermis surrounded DiI-positive DP cell-aggregates (Fig. 7A, E) at 2 weeks after transplantation. Skin sections at 2 weeks also contained structures that completed the enclosure of DiI-positive DP cell-aggregates (Fig. 7B, F). Transplants developed follicular tissues at 3 weeks post-transplantation (Fig. 7C, G), and almost completed the formation of follicular tissues (Fig. 7D,

H) with hair shaft-like structures (Fig. 7I) at 4 weeks. The epidermis never formed placode-like tissues observed in transplants of adult sole-Epi/E17-Der.

Intact DPs in Experiment II were isolated from vibrissae of adult EGFP-rats shown in Fig. 3E-G and inserted into isolated adult wild sole skins shown in Fig. 3D. The number of cells per DP was 7,790.2 ± 2,097.3. These sole skins were transplanted onto the back of nude mice. Histological changes in the transplants were examined at 1, 2, and 3 weeks post-transplantation (Fig. 7, Exp-II). DPs induced hair follicles in transplants following a process similar to that observed in DP cell-aggregates. The epidermis began to surround EGFP-positive DPs (Fig. 7J, N) at 1 week after transplantation. The enclosure progressed further at 2 weeks (Fig. 7K, O). Some of transplants formed follicular tissues at 2 weeks (Fig. 7L, P). Transplants yielded almost complete follicular tissues with hair shaft-like structures at 3 weeks (Fig. 7M, Q). We noticed that the dermal sheath of the hair follicles induced by DP-cell aggregates and intact DPs was DiI- (Fig. 7G) and EGFP-positive (Fig. 7P), strongly suggesting that DP cells have a potential to differentiate into dermal sheath cells. From these two types of experiments we concluded that adult DP cells as a hair follicle inducer are not capable of inducing placodes in the adult epidermis. In these experiments, it took approximately 1 week for the transplants to be firmly engrafted in the transplantation sites and, thereafter, the events related to hair follicle induction started. Therefore, we examined in details the process of hair induction at 2 weeks and 1 week post-transplantation in Exp-1 and -II, respectively. The entire processes of the hair follicle induction in Exp-II proceeded approximately 1

week earlier than those in Exp-I. There was no possibility that the emergence of epidermal placodes was overlooked in the present study.

The transplants of adult sole skins stuffed with intact adult EGFP-DPs were characterized with respect to the expression pattern of markers as in sole-Epi/E17-Der. *Edar* was expressed in the epidermal cells (hair matrix cells) that enclosed the DPs of transplants at 10 days post-transplantation (Fig. 8A, D) as in the hair matrix of adult hair follicles in early anagen of hair follicle (Fig. 8G). *Shh* was expressed in lateral regions of hair bulbs in transplants (Fig. 8B, E) as in adult hair follicles at an early anagen (Fig. 8H). P-cadherin was localized in hair matrix cells that enclosed the DPs in transplants (Fig. 8C, F) as in adult hair follicles at an early anagen (Fig. 8I). These results supported the above conclusion obtained from histological examinations that the adult DP induces hair follicles in the adult epidermis by inducing hair cycle.

Discussion

The placode formation and the condensation in the epidermal and dermal compartment of the embryonic skin, respectively, are unique and key initial morphological events in the development of hair anlagen. In the present study, the developmental process of pelage anlagen was histologically examined in details using embryos of Fischer rats. Epidermal placodes were formed at E15, initiated down-growth at E16, and developed to DC-associated hair germs at E17. The formation of hair germs starts in the skin covered with morphologically homogeneous epidermis, which is defined as stage 0 of hair

follicle morphogenesis (Hardy 1992; Paus *et al.* 1999). The epidermis at the programmed sites thickens and develops to the placode at stage 1. Fibroblasts start to increase the number in close proximity to the epidermis thickening, a preparatory event towards their subsequent aggregation. The placode develops into a more enlarged and broad column of epidermal cells at stage 2 and into the hair peg with a concave basal border at stage 3, which is accompanied with rounded DCs that will later form the DPs. At stage 4, the hair peg become more elongated and displays a bulb-like thickening of its proximal portion. The DP is surrounded by the hair matrix and is almost completely enclosed at stage 5 (bulbous peg stage). From stage 6 to 8, the hair follicle continues to differentiate and elongate. Finally, the hair follicle acquires its maximal length and reaches the subcutaneous muscle layer, and the hair shaft emerges through the epidermis.

In this study we observed that the epidermis of E14-transplants was replaced with the host epidermis, which is most likely to be a reason why the E14-transplants are not able to yield hair follicles. E14-Epi was one-cell layered, indicating that it is histologically quite immature. Studies hitherto have accumulated protein makers for the differentiation of epidermal cells (Koster & Roop 2004), such as CK5 and CK14 for basal cells, and CK1 and CK10 for spinous cells, and loricrin and profilaggrin for granular cells. The present study showed the E14-Epi does not express both CK10 and loricrin, indicating again its immaturity. When E14-skin is transplanted to adult host, such immature epidermis might not survive and, as a result, is replaced with the host epidermis. E17-skin is also still embryonic because it lacks the granular layer and shows

parakeratosis and on the way to full differentiation. However, this skin expresses both CK10 and loricrin, indicating that it is closer to the maturity. We consider that this progression allowed the E17-skin that had been transplanted on the back of nude mice to survive and even further develop to the terminal differentiation state.

Adult sole-Epi/EGFP-E17-Der transplanted in nude mice developed hairs following the above-described embryonic process of hair formation. Thus, we concluded that the adult epidermal cells retain a potential through life to respond to embryonic DC. They recapitulate the embryonic process of hair follicle development when exposed to stimuli of DC. The research group led by Dhoually constructed a heterospecific and heterochronic recombinant composed of adult rabbit corneal epithelium and E14.5-mouse dorsal dermis, and grafted it onto nude mice (Ferraris *et al.* 2000). The transplant was able to develop the hair follicle and the pilosebaceous gland. Together with expression profiles of specific marker proteins the cited research group concluded that the corneal epithelium cells of the transplant reprogram to epidermal cells, which differentiate into hair follicular cells under the influence of embryonic dermal messages. In the present study, we constructed a recombinant composed of adult plantar epidermis and embryonic dermis, and showed that adult epidermal cells retain the capacity to recapitulate the embryonic process of hair follicle formation. Osada & Kobayashi (2000) also demonstrated that the embryonic DC is able to exert its hair-inducing effect on adult epidermal cells. However, the cited authors did not make detailed histological examinations on the process of hair induction.

Anagen is divided in 6 phases, I-VI (Muller-Rover *et al.* 2001). One of the characteristic events in anagen is the enclosure of an enlarged DP with proliferating keratinocytes of the developing hair matrix, which takes place at anagen II. We also observed as a prominent event such an epidermal enclosure at an early phase of hair follicle induction in both adult sole-Epi/adult DP cell-aggregate and adult sole-Epi/adult DP. The epidermis never formed placode-like tissues in this type of the recombinant skin. Thus, we concluded that adult DP cells induce hair follicles in the adult epidermis by inducing a new hair cycle. This conclusion supports a previous histological observation by Xing & Kobayashi (2001). The cited authors made a recombinant composed of the adult rat sole epidermis with an adult rat DP, and transplanted it underneath the dermis of the dorsal skin of syngeneic rats or athymic mice. The epidermis of the transplant invaginated downward and surrounded the adult rat DP, and, as a result, induced hair follicles.

Not only the morphological features, but also the expression pattern of some of marker molecules associated with the development and formation of hair follicles supports the above conclusion concerning the difference of hair follicle induction by DC and DP. Eda/Edar was identified as a signaling unit for initiating the development of hair follicles (Kere *et al.* 1996; Ferguson *et al.* 1997; Srivastava *et al.* 1997; Headon & Overbeek 1999; Monreal *et al.* 1999). *Edar* is expressed in the hair placode of embryonic skin (Headon & Overbeek 1999; Huelsken *et al.* 2001; Andl *et al.* 2002). Later, *Edar* is expressed in the bulb of maturing hair follicles of newborn mice (Laurikkala *et al.* 2002). *Shh*, another proposed signaling molecule for hair development, is first detected in

the epidermal thickening at the stage 1 of placode formation (Bitgood & McMahon 1995; Iseki *et al.* 1996). *Shh* expression continues there and becomes polarized to the anterior epidermis of the hair bulb. In hair follicles at anagen, *Shh* expression is limited to the anterior side of the matrix of the hair bulb (Gat *et al.* 1998; Sato *et al.* 1999). The expression patterns of *Edar* and *Shh* genes in transplants of adult sole-Epi/E17-Der and those of adult sole-Epi/adult DP were similar to those in the hair germ formed in the embryonic skin and those in anagen hair follicles reported as above, respectively. P-cadherin is prominently expressed in embryonic placodes as compared to the "inter-placodal" epidermis (Hardy & Vielkind 1996; Muller-Rover *et al.* 1999), and was also expressed in early anagen hair follicles, its expression being high in the innermost portion of the hair matrix (Muller-Rover *et al.* 1999; Muller-Rover *et al.* 2001). P-cadherin expression patterns in transplants of adult sole-Epi/E17-Der and in transplants of adult sole-Epi/intact adult DP were similar to those of hair germs in the embryonic skin and those of adult hair follicles at early anagen as cited above, respectively.

We showed that although transplants of adult sole-Epi/E17-Der and those of adult sole skin inserted with adult DPs both developed hairs, the mechanisms of these two hair-inductions were different because E17-Der, but not the adult DP, was able to induce placodes. There is the possibility that the induction of placodes is dependent upon the number of the transplanted dermal cells. To test this possibility the cell number of DCs and DPs was counted on histological sections. The number of cells per DC of the transplanted E17-Der was much smaller than that per transplanted DP cell aggregate and the latter was much

smaller than that per transplanted adult DP. Therefore, it is concluded that adult DPs or DP cell aggregates are not capable of inducing the placode. This inability is not due to the shortage of cell number as compared to DCs. There is a possibility that the difference between the DC and the DP regarding the placode-inductive ability might be explained by the different procedures for preparation of the adult sole epidermis: the embryonic dermis containing DCs was recombined with the adult epidermis, whereas adult dermal counterparts were isolated and inserted in a pocket-like space between the adult epidermis and the dermis. However, this possibility seems to be unlikely, because, firstly, the procedure of the isolation in the form of DPs or DP cells did not affect their inherent hair follicle-inductive ability and, secondly, both procedures equally resulted in a proper recombination of dermal constitutes with the epidermis. In addition, in the present study, we did not perform the experiments using isolated DCs, because their isolation was technically impossible. It might be technically possible to examine the effects of DCs by inserting E17-Der in such a pocket-like space as in adult dermal experiments. However, we did not attempt such experiments, because we considered that DCs are not able to contact to the epidermis and, thus, can not exert their effects on it, because abundant dermal constituents surround DCs.

It can be said that the adult glabrous epidermis has the capacity to respond to both of embryonic and adult dermal messages, and to differentiate into the follicular epidermis. The adult epidermis receiving the former message reprograms embryonic processes and that receiving the latter message gets into the hair cycle. Accordingly, it seems that the message from the DC (the first

dermal message) is different from that from the DP. At the initiation of a new anagen stage, signals from the DP are thought to instruct pluripotent stem cells in the bulge region of the hair follicles to transiently proliferate (Cotsarelis *et al.* 1990; Wilson *et al.* 1994; Lyle *et al.* 1998). Stem cell progenies migrate to the base of the hair follicle and surround the dermal papilla, giving rise to the next generation of hair matrix (Taylor *et al.* 2000; Oshima *et al.* 2001). We showed that epidermal cells of the glabrous skin in association with a DP are able to differentiate into the hair matrix cells, which strongly suggests that the adult epidermal cells contain a population of such stem cell- or stem cell progeny-like cells that can respond to the DP signals.

Acknowledgement

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Figure legends

Fig. 1. Hair formation potential of embryonic skin. Skins were isolated from embryos at E14, E15, E16, E17, and E20, subjected to H&E histology, and shown in A-E, respectively. Pieces of skin were isolated from embryos at each of these stages and were grafted on the back of nude mice. A photograph was taken at 12 weeks post-transplantation for E14-skin (F), at 8 weeks for E15-skin (G), and at 3 weeks for E16- (H), E17- (I), and E20-skin (J). Arrows indicate the graft site. d, dermis; dc, dermal condensation; dp, dermal papilla; e, epidermis; hf, hair follicle; hg, hair germ; p, placode. Bars, 50 μm in A-E, 1 cm in F-J.

Fig. 2. Histology of transplants of E14- and E15-skin. Skins were separated from E14- and E15-embryos, and were divided into two halves. One half was transplanted (TP: A and B for E14-skin; D, E, and G-I for E15-skin) and the other half was for immunohistochemistry for CK10 (IH/CK10: C, F, J). The E14-transplant (A, B) was removed at 12 weeks post-transplantation and processed to serial sections. Similarly, serial sections were prepared from E15-transplants at 8 weeks post-transplantation (D, E, G-I). The sections were stained with H&E (A, D, G) and Hoechst (B, E, H, I). The E15-transplant shown in D and E did not formed hairs, but the E15-transplant shown in G, H, and I formed hairs. Regions enclosed with the rectangles indicated with H and I in G are magnified and are shown in H and I, respectively. Dotted lines in B, E, H, and I indicate the boundary between the epidermis and the dermis. E14- (C) and E15-skins (F, J) for IH/CK10 were fixed and sectioned for

immunohistochemistry of CK10. Some suprabasal cells at E15 were CK10-positive. d, dermis; dp, dermal papilla; e, epidermis; hm, hair matrix. Bars, 50 μm.

Fig. 3. Histology of embryonic and adult skins used for constructing recombinant skins. Recombinant skins were prepared by combining sole-Epi of adult rats with either of E17-Der of EGFP-rats or DP of vibrissal follicle of EGFP-rats. Sections of the E17-skin of EGFP-rats (A, B, C), the sole skin of adult rats (D), and the vibrissal follicle of EGFP-rats (E, F, G) were stained with H&E (A, D, E) and Hoechst, and viewed through a fluorescent microscope for EGFP (B, F) and for nuclei (C, G). Note that the hair matrix and inner root sheath were EGFP-negative. d, dermis; dc, dermal condensation; dp, dermal papilla; ds, dermal sheath; e, epidermis; hg, hair germ; hm, hair matrix; irs, inner root sheath; ors, outer root sheath. Bars, 50 μm.

Fig. 4. Hair formation in adult sole-Epi/E17-Der. Adult sole-Epi/EGFP-E17-Der was transplanted onto the back of nude mice and allowed to develop for 3 weeks. (A) A macroscopic view of the graft site at 3 weeks post-transplantation. The arrow points to the site of transplantation. Fraction in A indicates the number of hair-produced transplants over the number of tested transplants. (B-D) Histology of the transplants at 3 weeks post-transplantation. Serial sections were stained with H&E (B), or stained with Hoechst and viewed through a fluorescent microscope for EGFP (C) and for nuclei (D). dp, dermal papilla; hf, hair follicle. Bars, 1 cm in A; 50 μm in B-D.

Fig. 5. Process of hair follicle induction in transplants of adult sole-Epi/E17-Der. Recombinants of adult sole-Epi/EGFP-E17-Der were transplanted on the back of nude mice, and removed at 10 days (A-C, E-G) and 14 days (D, H) after transplantation. The transplants were processed for H&E (A-D) and Hoechst staining (E-H), the sections of the latter being viewed through a fluorescent microscope for EGFP and nuclei. The whitish vacuole-like spots often found in the epidermis in A-C were artifacts that yielded in the process of H&E staining, most probably due to air expelled from tissues upon freezing. d, dermis; dc, dermal condensation; dp, dermal papilla; e, epidermis; hf, hair follicle; hg, hair germ; hp, hair peg; p, placode. Bars, 50 μm.

Fig. 6. Expression patterns of markers in transplants of adult sole-Epi/E17-Der. Recombinants of adult sole-Epi/EGFP-E17-Der were transplanted on the back of nude mice, and removed at 10 days after transplantation. The transplants were processed for *in situ* hybridization of *Edar* (A) and *Shh* (B), and for immunohistochemistry of P-cadherin (P-cad, C). Serial sections were stained with anti-GFP antibodies and Hoechst, and viewed through a fluorescent microscope for EGFP and nuclei (D-F). E17-skin was processed for *in situ* hybridization and immunohistochemistry in the same way as above (G-I). The *in situ* hybridization with the sense probes for each gene did not show significant signals. d, dermis; dc, dermal condensation; e, epidermis; hg, hair germ. Bar, 50 μm.

Fig. 7. Developmental process of hair follicles in transplants of adult sole-Epi/adult DP. [Experiment I] Isolated adult sole skins were stuffed with adult DiI-labelled DP cell-aggregates, transplanted onto the back of nude mice, and were removed at 2 weeks (A, B, E, F), 3 weeks (C, G), and 4 weeks (D, H, I) after transplantation. The transplants were subjected to histological examinations for H&E (A-D), for fluorescence microscopy of DiI (red) and Hoechst (blue) (E-I). [Experiment II] Isolated adult wild sole skins were stuffed with intact adult EGFP-DPs, transplanted as above, removed at 1 week (J, N), 2 weeks (K, L, O, P), and 3 weeks (M, Q) after transplantation, and processed for histology as in Fig. 5. J-M, H&E; N-Q, fluorescent views for EGFP and Hoechst. The red (E-I) and green fluorescence (N-Q) distributed in the dermis surrounding the induced hair follicles were signals of the DiI- and EGFP-positive cells derived from the transplanted DiI-labeled DP cell aggregates and EGFP-labeled DPs, respectively. The red (E, F, I) and green fluorescence (O, P) observed in the epidermis were background signals. d, dermis; dp, dermal papilla; dpc, dermal papilla cells; e, epidermis; hf, hair follicle; hm, hair matrix; hs, hair shaft. Bars, 50 μm.

Fig. 8. Expression patterns of hair follicle-markers in transplants of adult sole-Epi/adult DP. Isolated adult wild sole skins of Wistar rat were stuffed with adult EGFP-DPs of Wistar rat, transplanted onto the back of nude mice, and were removed at 10 days after transplantation. The transplants were processed for *in situ* hybridization of *Edar* (A) and *Shh* (B), and processed for immunohistochemistry of P-cadherin (P-cad, C). Serial paraffin sections were stained with anti-GFP antibodies and Hoechst, and viewed through a fluorescent

microscope for EGFP and nuclei (D-F). Sections of 26-day-old rat skin with hair follicles at early anagen stage were processed for *in situ* hybridization and immunohistochemistry in the same way as above (G-I). The *in situ* hybridization with the sense probes for each gene did not show significant signals. d, dermis; dp, dermal papilla; e, epidermis; hf, hair follicle; hm, hair matrix; hs, hair shaft. Bars, 50 μm.

Table 1. Hair formation-potential of embryonic epidermis in relation to its differentiation status

		Development						
		E14	E15	E16	E17	Adult		
	Differentiation markers					HF	IF	S
Epidermis	CK14	+	+	+	+	+	+	+
	CK10	-	+/-	+/-	+	-	+	+
	Loricrin	-	-	-	+	-	+	+

HF, Hair follicle; IF, Interfollicular skin; S, Sole skin; -: Immunologically negative for antibodies against the indicated markers; +/-: Some epidermal cells are positive. +: All epidermal cells are positive.

Fig. 1

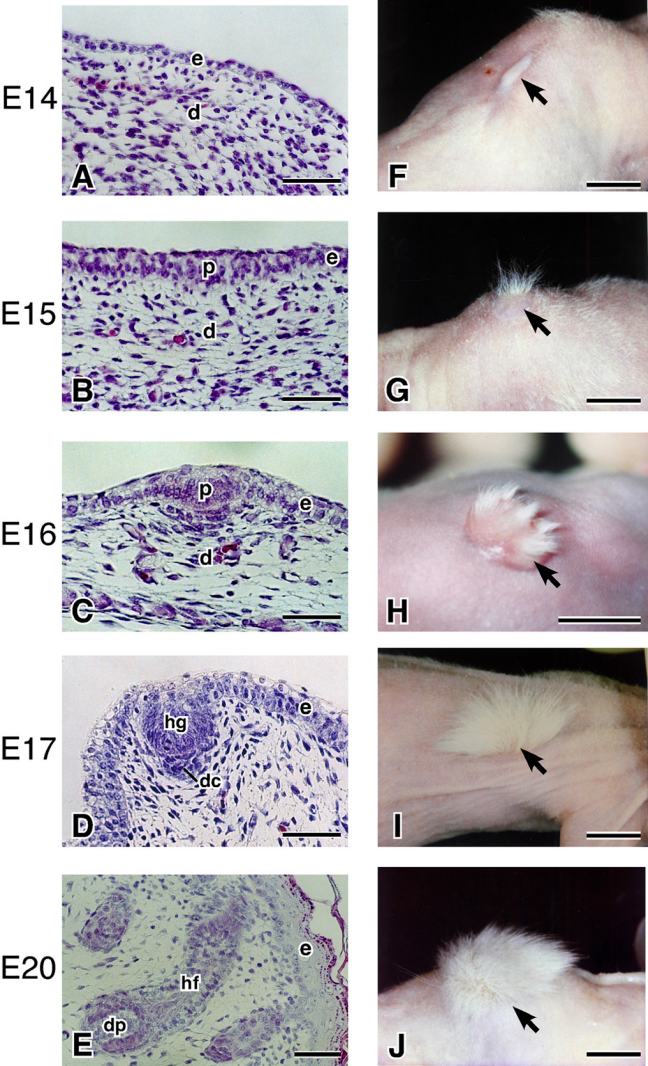


Fig. 2

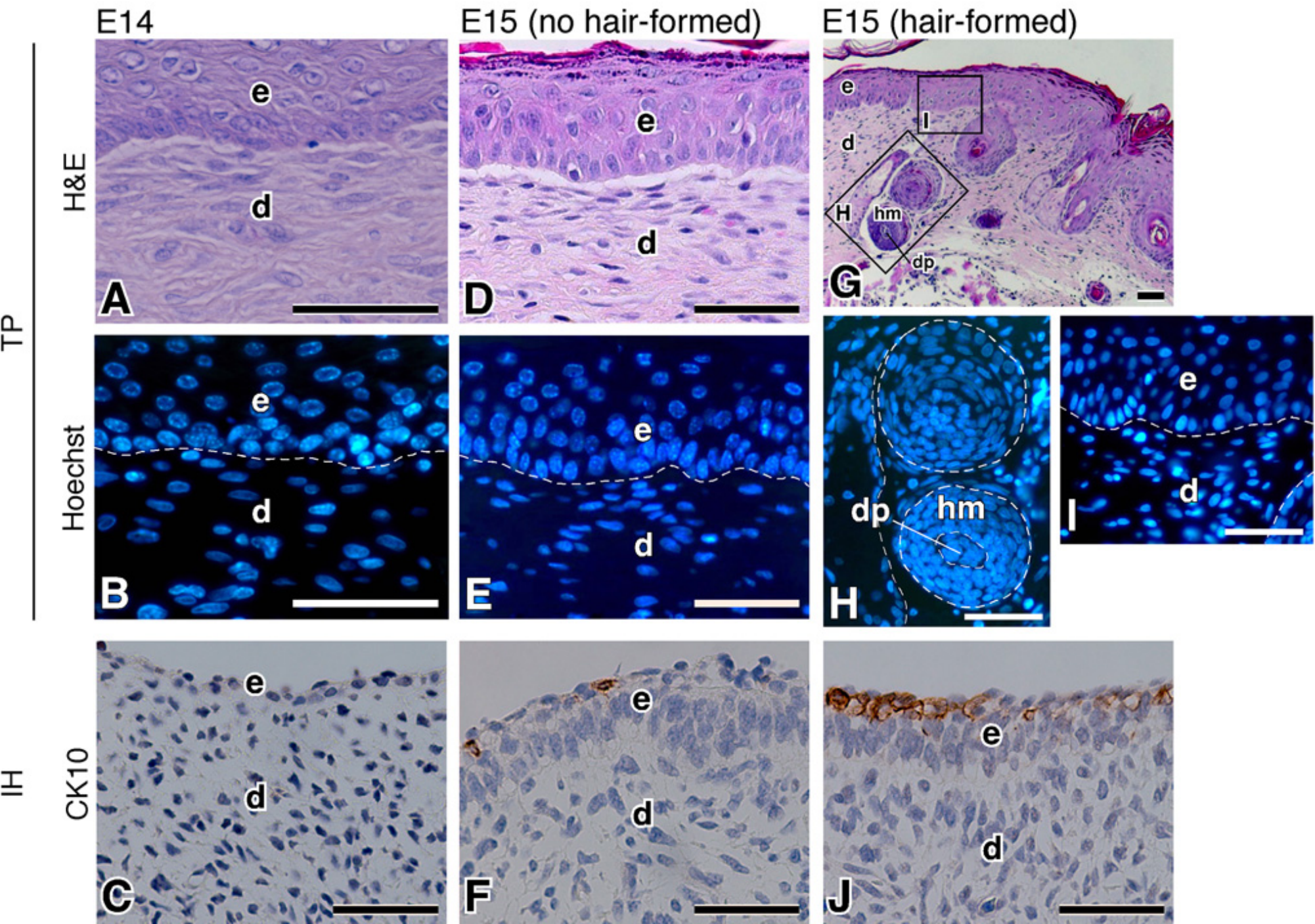
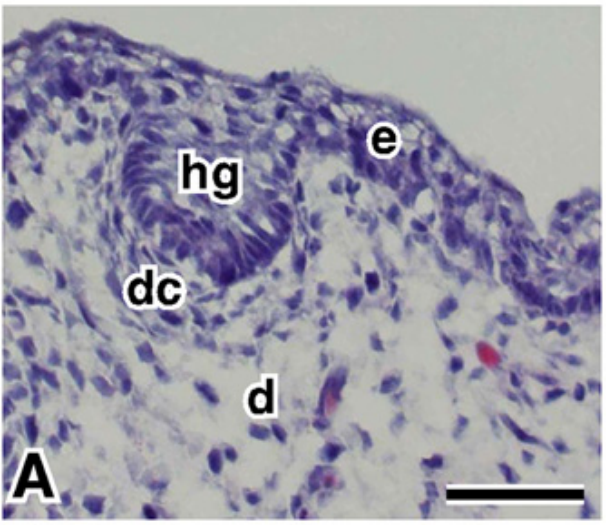
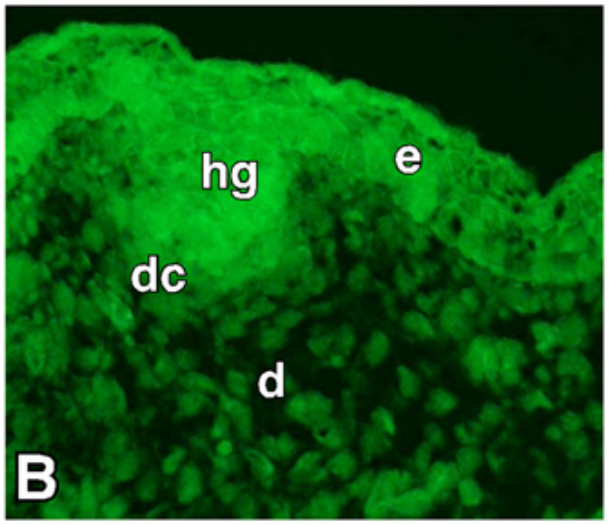
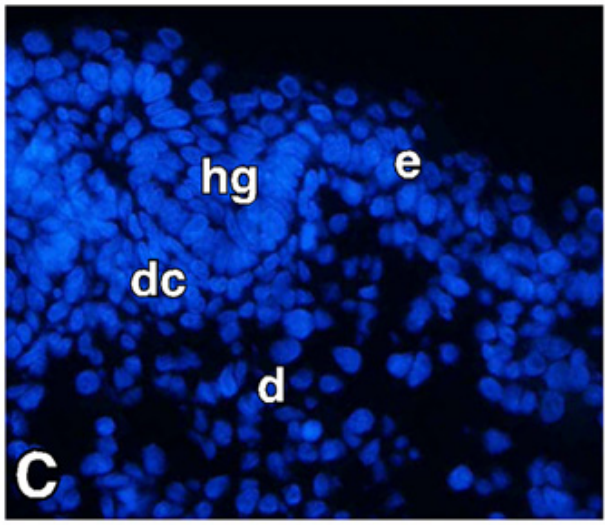

E14
E15 (no hair-formed)
E15 (hair-formed)
TP
IH
H&E
Hoechst
CK10
e
d
A
D
G
I
H
hm
dp
B
E
C
F
J

Fig. 3

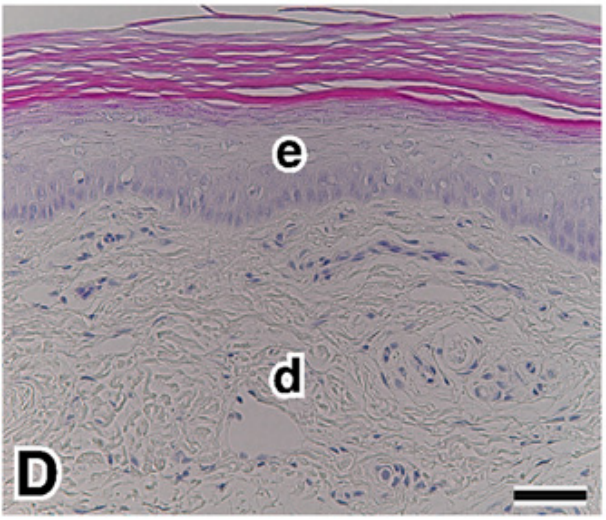
E17



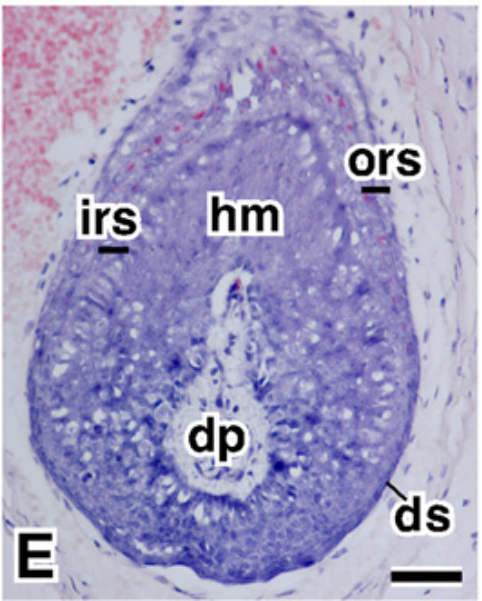


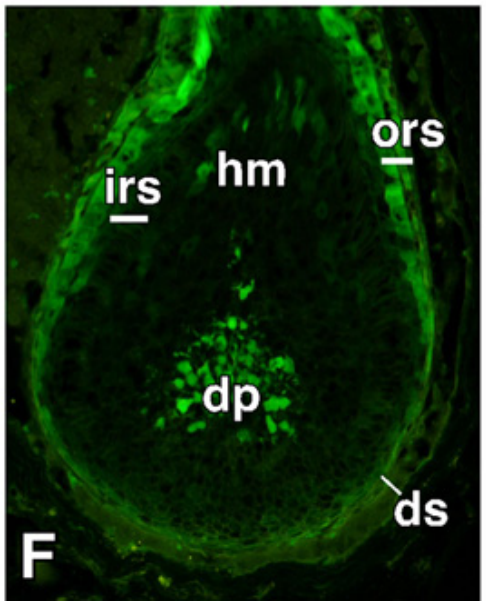


Adult-sole



Adult-vibrissa





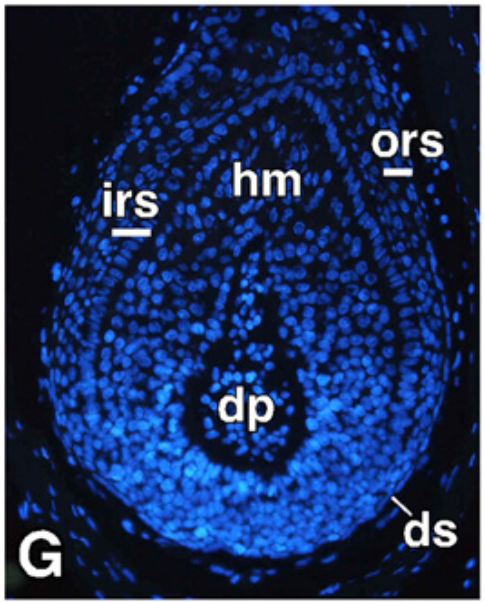


Fig. 4

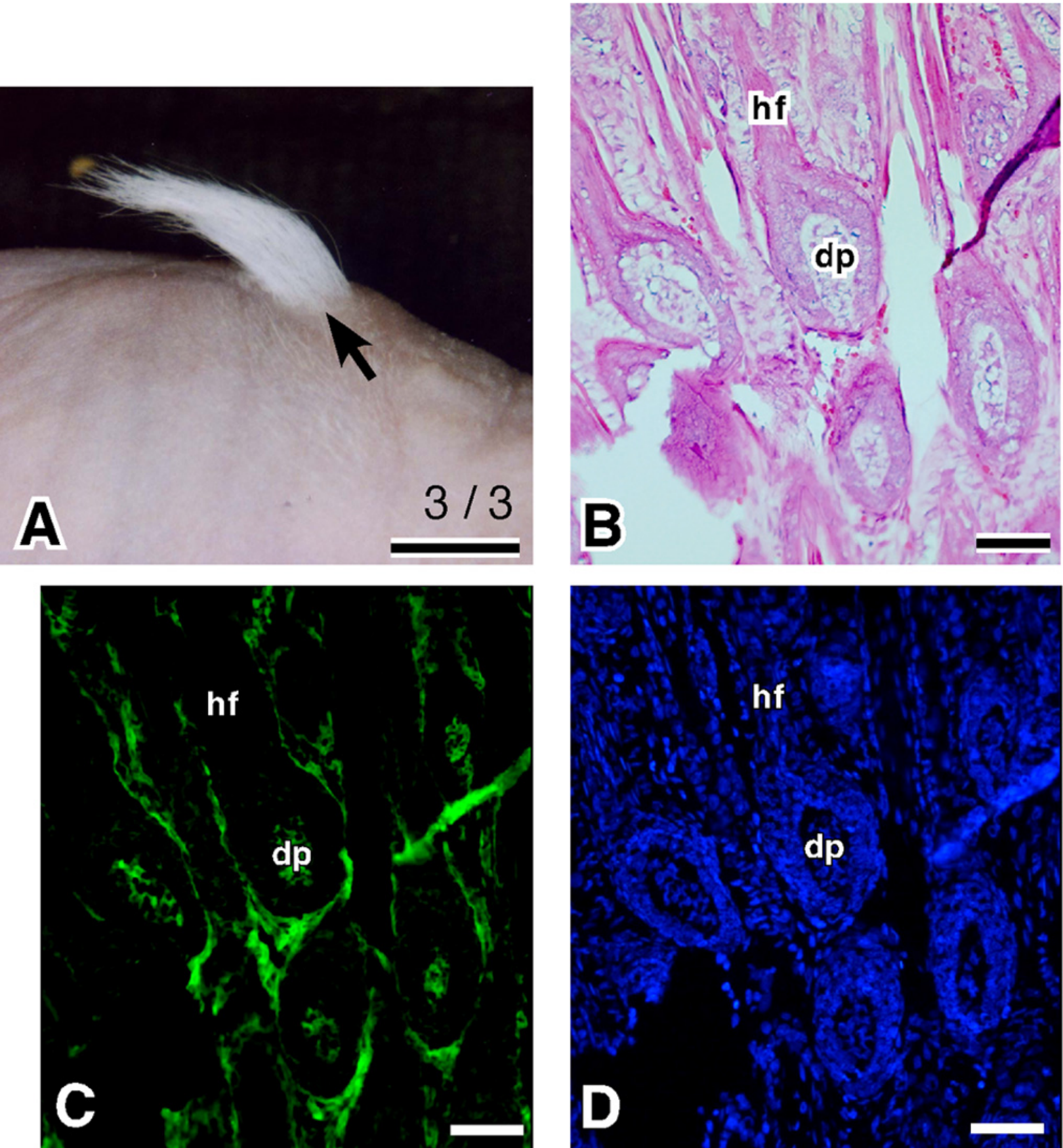
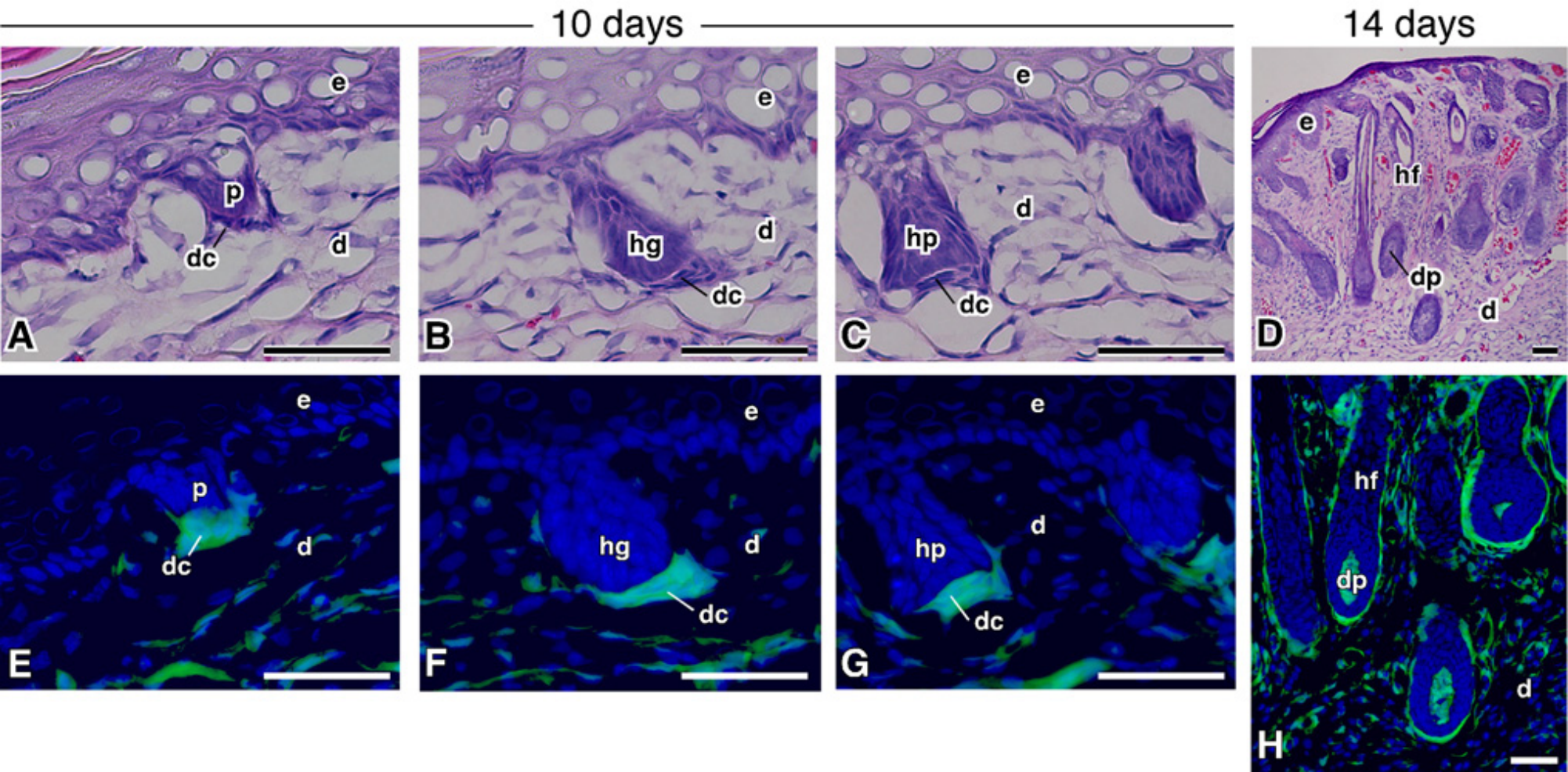


Fig. 5


10 days
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p
dc
d
E
e
hg
d
dc
F
e
d
hp
dc
G
hf
dp
d
H

Fig. 6

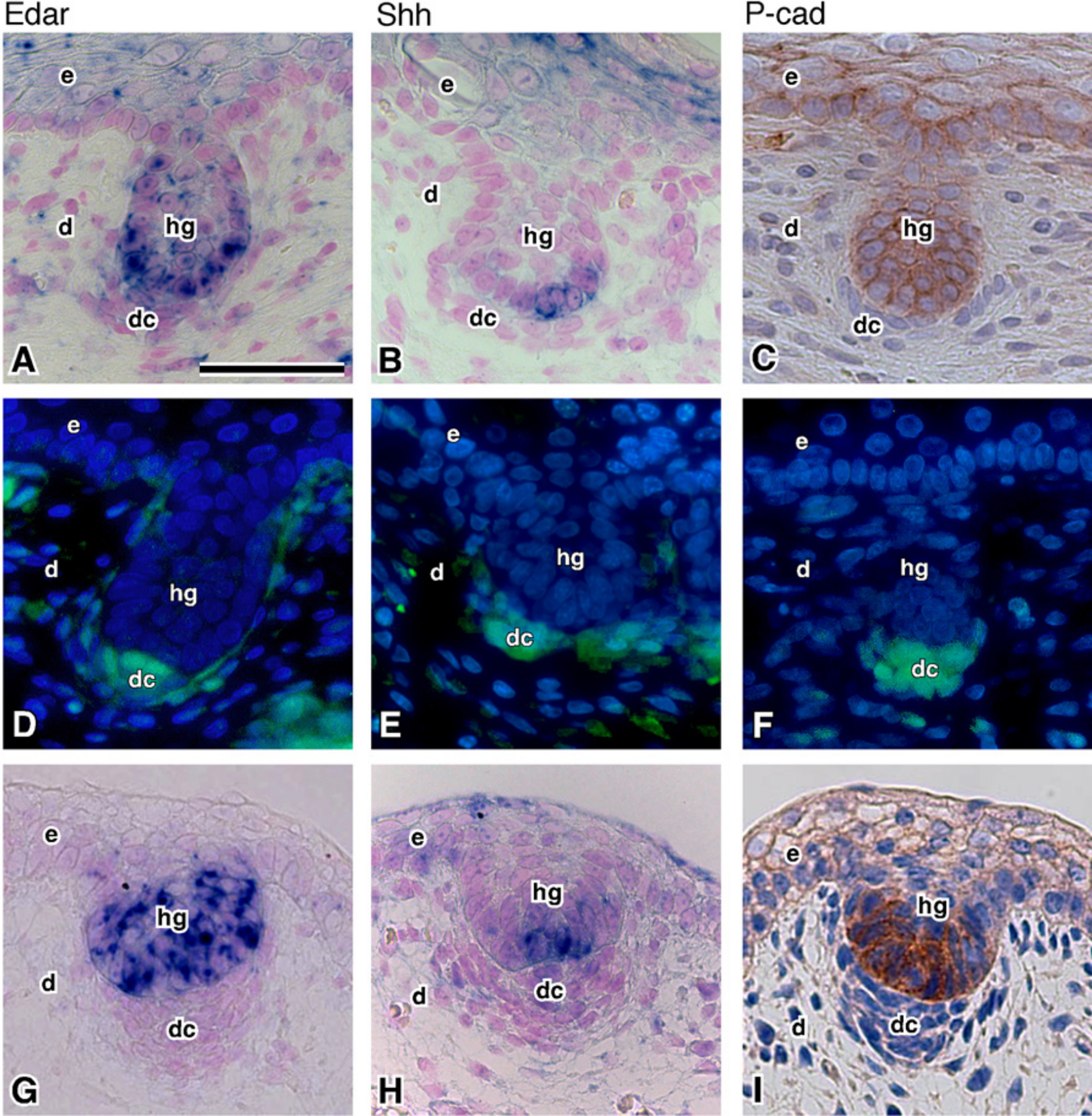

Fig. 7

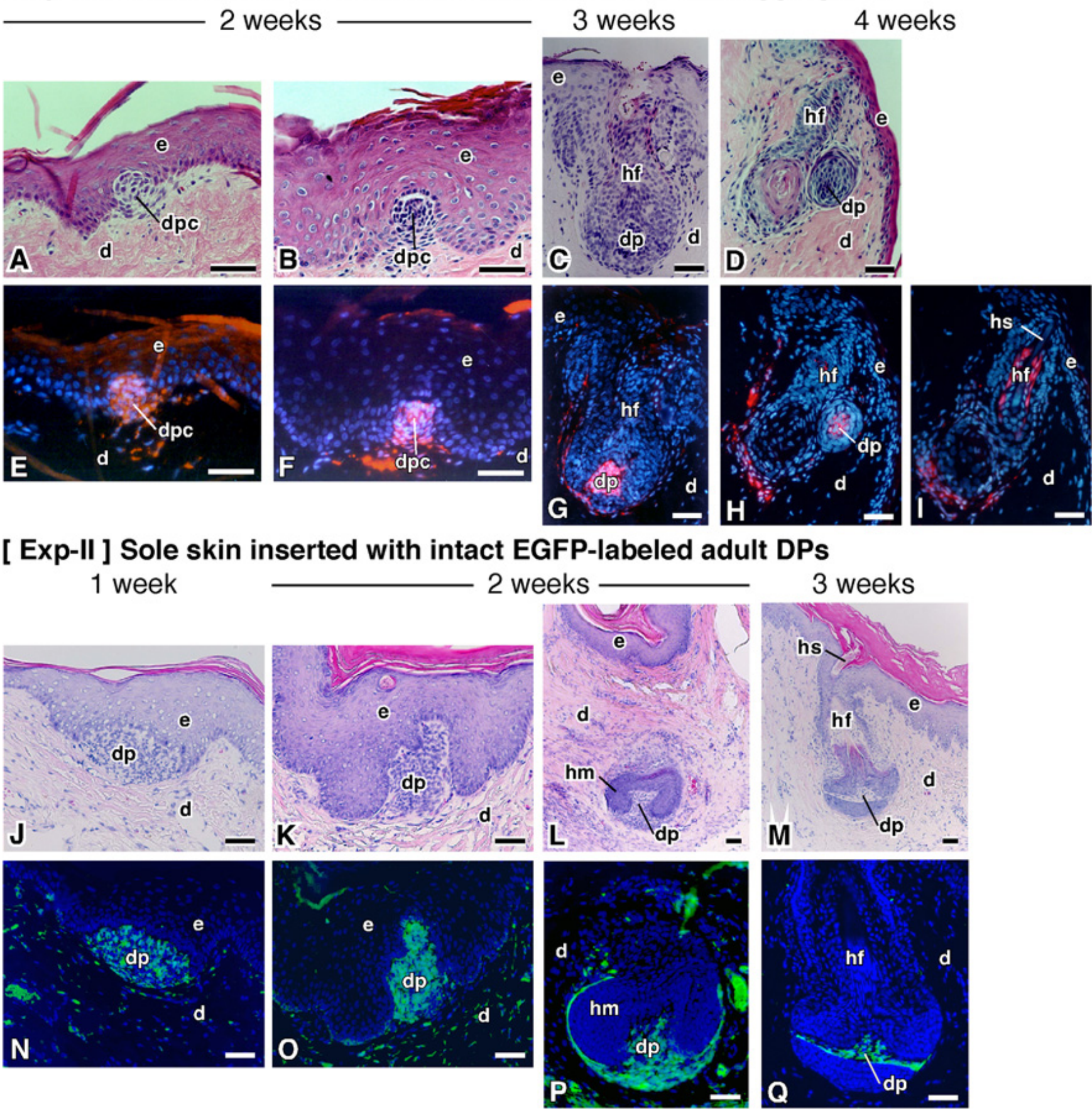
[Exp-I] Sole skin inserted with DiI-labeled adult DP cell-aggregates
2 weeks
3 weeks
4 weeks
e
dpc
d
A
B
C
hf
dp
D
E
F
G
H
hs
I
[Exp-II] Sole skin inserted with intact EGFP-labeled adult DPs
1 week
2 weeks
3 weeks
J
K
L
hm
M
N
O
P
Q

Fig. 8