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The multipotency of adult vibrissa follicle stem cells

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ABSTRACT

Several studies focused on the characterization of bulge keratinocytes have proved that they are multipotent stem cells, being recruited not only to regenerate the hair follicle itself, but also the sebaceous gland and the epidermis. However, due to the difficulty in preparing transplantable cell sheets harvested with conventional enzymatic digestion, there is still no direct evidence of the bulge stem cells' multipotency. Whether they can respond to adult dermal papilla (DP) signals in recombination experiments also remains unclear. In this study, we addressed this problem by culturing and detaching intact bulge keratinocyte sheets from thermo-responsive culture dishes, only by reducing its temperature. When sheets of mass cultured bulge keratinocytes isolated from rat vibrissa follicles were recombined with fresh adult DPs and sole skin dermis *in vivo*, regeneration of epidermis and sebaceous gland-like structures, and formation of hair bulb with differentiating inner root sheath and hair cuticle were observed within 3 weeks. However, regardless the expression of stem cells markers like CD34, SA1004 and SA1006, no structures were observed when cloned bulge keratinocytes were used to prepare cell sheets and recombinants, revealing the possible existence of monoclonal stem cells within the bulge region. This report is the first to succeed in harvesting adult bulge keratinocyte sheets. Using these sheets it is demonstrated that bulge stem cells directly respond to adult DP signals to induce hair bulb formation *in vivo*.

1. Introduction

Hair follicles are specialized mammalian skin appendages responsible for hair fiber production. Thanks to their accessibility, many studies have focused and eventually succeeded in the localization, isolation and characterization of hair follicle stem cells. Like other adult stem cells, hair follicle stem cells are assumed to be slow cycling and have superior self-renewal and multipotency abilities (Cotsarelis, 2006). Being able to be detected as label-retaining cells (LRC) (Cotsarelis et al., 1990; Taylor et al., 2000), many studies have demonstrated that in rodent hair follicles, most stem cells are located in the bulge, a swelling in the upper part of the outer root sheath (ORS), with very few in the hair bulb (Kobayashi et al., 1993; Taylor et al., 2000; Oshima et al., 2001; Claudinot et al., 2005). Bulge keratinocytes isolated from vibrissa follicles show high clonogenecity and proliferative capacity *in vitro* (Kobayashi et al., 1993; Oshima et al., 2001; Claudinot et al., 2005) and their multipotency have been demonstrated by studies that followed the fate of LRCs during hair morphogenesis and through the hair growth cycle (Cotsarelis et al., 1990), as well as during skin wound-healing processes (Taylor et al., 2000).

Different tissue recombination and transplantation models using adult bulge keratinocytes have also shown the hair follicle stem cells' multipotency, proving that they are involved in the epidermis and sebaceous gland formation (Oshima et al., 2001; Morris et al., 2004; Blanpain et al., 2004; Claudinot et al., 2005). All these works, however, have utilized embryonic or neonatal cells or tissues in their models, demonstrating only that hair follicle stem cells can be recruited by an environment that is able to develop skin appendages, but showing no direct proof of their ability to reconstruct skin and form its appendages *de novo*.

One of the reasons for this lack of direct evidence is the difficulty in preparing transplantable bulge keratinocyte sheets harvested with conventional enzymatic digestion. In the present study, this problem was addressed by culturing and detaching bulge keratinocyte sheets using a technology that does not require the use of proteolytic enzymes (Yamato et al., 2001). Keratinocytes are plated in culture dishes coated with poly (*N*-isopropylacrylamide), a thermo-responsive polymer that becomes hydrophobic above 32 °C and can reversibly change to hydrophilic below this temperature. Using this technology, multilayered keratinocyte

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sheets can be detached intact, simply by reducing the dishes temperature to 20 °C. Using this method, we succeeded in demonstrating that cultured adult bulge keratinocytes can construct the epidermis and interact with adult dermal papilla (DPs), forming sebaceous glands and the hair follicle itself *in vivo*. These data indicated that the use of cultured bulge keratinocyte sheets is effective in hair follicle induction experiments and also strongly support the concept that hair follicle stem cells are multipotent, providing direct evidence of this feature.

2. Material and methods

2.1. Animals

Fisher F344/Jcl rats were obtained from Nihon SLC Co. Ltd. (Hamamatsu, Japan) and Japan Laboratory Animals Inc. (Tokyo). SD-Tg (CAG-EGFP) rats and BALB/c Slc-nu/nu mice were from Nihon SLC Co. Ltd. (Hamamatsu, Japan). The donor rats were 34–56 days old (for anagen follicles), and the host rats and mice were 6–8 weeks of age. All animals were fed and watered *ad libitum*.

2.2. Mass culture of bulge cells

Vibrissa follicles were isolated from rats euthanized with chloroform and microdissected as described before (Kobayashi et al., 1993). Fragments containing only the bulge region were immersed in dispase solution (500 units/ml) and incubated for 30 min at 37 °C. The dermal sheath was teased away from the follicles, and the fragments were further incubated in a solution of 0.1% trypsin–0.02% ethylene diamine tetraacetic acid (EDTA) in calcium- and magnesium-free Dulbecco's PBS for 1 h at 37°C to completely dissociate the epithelial cells. These cells were centrifuged and re-suspended in a standard keratinocyte medium, a 3:1 mixture of Dulbecco modified Eagle's medium (DMEM), and Ham's F12 medium supplemented with 10% fetal bovine serum (Rheinwald and Green, 1975). The cells were then plated on a feeder layer consisted of 3T3 cells (Japanese Collection of Research Bioresources, Osaka, Japan), which had been lethally irradiated or treated with mitomycin C at 18 μg/ml for 4 h. The keratinocytes were cultured until semi-confluency and were passaged every week, being fed every 3 days. Human recombinant epidermal growth factor (EGF) was added at 10 ng/ml at the beginning of the first feed. All culture reagents were purchased from Gibco (Invitrogen Corp.; Carlsbad, CA), except for the mitomycin C (Sigma Chemical Co., St. Louis, MO) and the EGF (Invitrogen).

2.3. Cloning of bulge cells

Bulge cells were completely dissociated from vibrissa follicles as described, and transferred to a culture dish containing keratinocyte standard medium. Single cells were isolated with a Pasteur pipette by using an inverted microscope (Barrandon and Green, 1985) and individually cultured on a feeder layer of 3T3 cells lethally irradiated or treated with mitomycin C at 18 μg/ml.

2.4. Preparation of cell sheets and construction of recombinants

Bulge keratinocyte sheets were obtained from thermo-responsive dishes (CellSeed Inc., Tokyo, Japan). Mass cultured bulge cells at P2 and cloned bulge cells at P2 and P14 were plated on 3T3 feeder layers previously seeded on thermo-responsive dishes. After keratinocytes became confluent and formed a multilayered epithelium, approximately 10–14 days after plating, the culture medium was removed and the dishes were washed with DMEM. After incubation at 20 °C for 30 min, the multilayered keratinocyte sheets were peeled slowly from the edge of the dish with tweezers. The construction of recombinants (Fig. 1) was carried as described before (Xing and Kobayashi, 2001). Cell sheets were cut into 6 small pieces and a plate of sterilized silicon of 1 cm^2 was gently placed on the top of each epithelial fragment. The silicon plate with the cultured epithelium was then turned upside down so the basal layer side of the cell sheet faced up. Two to three DPs, freshly isolated from anagen vibrissa follicles, and sole dermis fragments were gently placed on the sheet and the peripheral cultured epithelium was gently wrapped back (represented in Fig. 1 by small arrows), so the DPs could be stably trapped between the tissues. Remaining pieces of the detached sheets were also fixed in neutral buffered formaldehyde for histological observations.

2.5. Construction of DP-upper follicle and DP-sole skin recombinants

To demonstrate that mechanically isolated DPs were devoid of matrix cells and consequently validate our tissue isolation technique and the results of induction experiments, DP-sole skin and DP-upper follicle recombinants containing DPs from EGFP-transgenic rats were used as control experiments. DP-upper follicle recombinants were prepared as described before (Oliver, 1966; Kobayashi and Nishimura, 1989). In brief, anagen vibrissa follicles isolated from donor rats were divided in two fragments by transection, and associated with a fresh DP at their amputated base. To prepare DP-sole skin recombinants, the glabrous region of sole skin fragments were treated with dispase (500 units/ml) for 30 min at 37 °C and rinsed with DMEM. The epidermis was partially detached from the dermis, creating a pocket where DPs were gently introduced.

2.6. Grafting

Rats were anesthetized by intramuscular injection of KETALAR® 50 (ketamine hydrochloride, 0.2–0.25 ml/6–8-week-old rats), the dorsal area of each rat was shaved and the required back skin was disinfected with 70% alcohol. A vertical incision was made in the back skin, a pocket was opened beneath it, where the recombinants were introduced onto the muscle fascia, with the dermis being in direct contact with the host rat hypodermis. The incision was closed with commercially available liquid adhesive. Nude mice were used as hosts for control grafts. DP-sole skin recombinants were transplanted as described above. DP-upper follicle recombinants were transplanted under the kidney capsule of nude mice as described before (Kobayashi and Nishimura, 1989).

2.7. Histology

The recombinants were harvested 3 weeks after transplantation, fixed in Bouin's solution or neutral buffered formaldehyde and embedded in paraffin. The sections (6 μm) were stained in a combination of Weigert's iron-hematoxylin, 1% Alcian blue and Curtis's Ponceau S solution.

2.8. Immunohistochemistry

Control grafts containing DPs isolated from EGFP-transgenic rats were fixed in 4% paraformaldehyde–PBS and embedded in paraffin. The sections (6 μm) were treated with rabbit anti-GFP serum (Molecular Probes Inc., Eugene, OR, USA) for 2 h at room