Cell, Vol. 102, 451–461, August 18, 2000, Copyright ©2000 by Cell Press

Involvement of Follicular Stem Cells in Forming Not Only the Follicle but Also the Epidermis

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Summary

The location of follicular and epidermal stem cells in mammalian skin is a crucial issue in cutaneous biology. We demonstrate that hair follicular stem cells, located in the bulge region, can give rise to several cell types of the hair follicle as well as upper follicular cells. Moreover, we devised a double-label technique to show that upper follicular keratinocytes emigrate into the epidermis in normal newborn mouse skin, and in adult mouse skin in response to a penetrating wound. These findings indicate that the hair follicle represents a major repository of keratinocyte stem cells in mouse skin, and that follicular bulge stem cells are potentially bipotent as they can give rise to not only the hair follicle, but also the epidermis.

Introduction

The skin surface is covered by the epidermis, a stratified squamous epithelium dedicated to the formation of a highly impermeable stratum corneum (Fuchs, 1990; Suter et al., 1997). The epidermis is contiguous with the outer root sheath of the hair follicle, which is a complex structure specialized in making a hair shaft (Lavker et al., 1999). At present, the extent of cellular trafficking between the epidermis and hair follicle is uncertain. There are data indicating that, when the entire epidermis is lost, such as in severely burned patients, keratinocytes can emigrate from the hair follicle to reestablish the entire epidermis (Argyris, 1976). However, the precise origin of such hair follicle–derived cells has never been established. Moreover, it has been suggested that under normal circumstances, the epithelia of the epidermis and hair follicle represent two distinct, self-sufficient entities that are governed by their own stem cells, with no trafficking of cells from one compartment to another (Rochat et al., 1994; Watt, 1998).

One of the most reliable ways to identify the keratinocyte stem cells takes advantage of the fact that these cells are normally slow-cycling, hence can be identified experimentally as the so-called "label-retaining cells" (LRCs) (Bickenbach, 1981; Cotsarelis et al., 1989, 1990; Morris and Potten, 1994, 1999; Wei et al., 1995; Bickenbach and Chism, 1998; Lehrer et al., 1998). In this approach, one labels all the cells in the epithelium by a repeated or continuous supply of tritiated thymidine, followed by a long chase period during which the label is lost from all the cycling, transit amplifying (TA) cells—so that only cells that cycle slowly (the stem cells) retain the label. Using this approach, we found that corneal epithelium, which had been traditionally regarded as a self-sufficient tissue, contained no LRCs; such cells were found exclusively in the peripheral cornea in a previously little studied area called the limbus (Cotsarelis et al., 1989). That the limbal zone is the exclusive site of corneal epithelial stem cells is now well established, based on the fact that the limbal basal cells: (1) are biochemically primitive, lacking a differentiation-dependent keratin K3 (Schermer et al., 1986; Rodrigues et al., 1987; Chen et al., 1997); (2) have a superior proliferative capacity both in vivo and in vitro (Ebato et al., 1988; Cotsarelis et al., 1989; Lindberg et al., 1993; Wei et al., 1993; Lavker et al., 1998; Pellegrini et al., 1999); (3) represent the predominant site of corneal neoplasm (Russell et al., 1956; Pizzarello and Jakobiec, 1978); (4) are absolutely essential for the long-term maintenance of the corneal epithelium (Tseng, 1989; Kruse et al., 1990; Huang and Tseng, 1991); (5) give rise to TA cells that undergo centripetal migration (Davanger and Evensen, 1971; Buck, 1985; Auran et al., 1995); and (6) can rescue/reconstitute severely damaged or completely depleted corneal epithelium upon transplantation (Kenyon and Tseng, 1989; Tsai et al., 1990; Tsubota et al., 1995; Holland and Schwartz, 1996; Pellegrini et al., 1997). These data have led to the wide acceptance of the limbal epithelial stem cell concept (Schermer et al., 1986; Dua, 1995; Hodson, 1997; Voelker, 1997; Tseng and Sun, 1999), and strongly support the validity and reliability of identifying keratinocyte stem cells experimentally as the "label-retaining cells."

When we used this long-term labeling approach to localize the slow-cycling cells of the hair follicle, we found that all the follicular label-retaining cells were exclusively confined to a previously ignored area called the bulge (Cotsarelis et al., 1990; Lavker et al., 1993). The bulge is the part of the outer root sheath marking the lowest point of the upper, permanent portion of the follicle, as well as the attachment site of the arrector pili muscle (Unna, 1876; Cotsarelis et al., 1990; Figure 1a). The realization that the putative follicular epithelial stem cells reside in the bulge helps to explain several paradoxical observations, including the dispensability of the hair bulb (that contains the highly proliferative matrix keratinocytes) in hair reconstitution assays (Oliver, 1966); the cyclic nature of hair growth (Hardy, 1992; Lavker et al., 1999); and the hair cycle–dependent generation of skin tumors in skin chemical carcinogenesis (Stenback et al., 1981; Miller et al., 1993a). A curious outcome of our initial label-retaining cell study was that most of such cells were found to be associated with the bulge area of the hair follicle, with very few in the epidermis (Cotsarelis et al., 1990). A similar study by Morris and Potten (1999) also showed that the label-retaining cells were exclusively bulge associated. These

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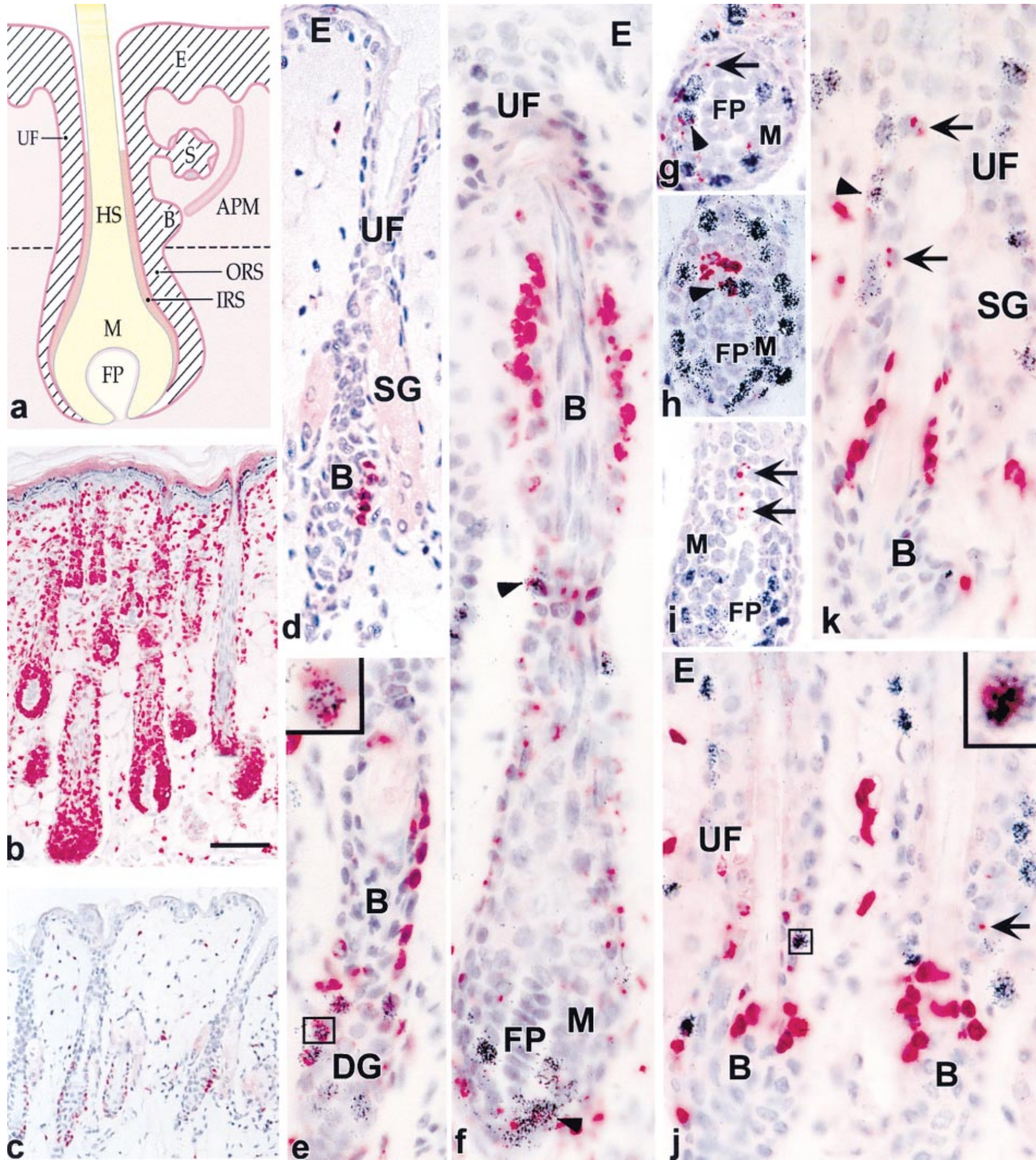

Figure 1. The Follicular Bulge as the Main Site of Label-Retaining Keratinocytes

(a) A schematic representation of the hair follicle showing the bulge (B) region of the outer root sheath—the site of follicular stem cells, arrector pili muscle (APM), epidermis (E), follicular papilla (FP), hair shaft (HS), inner root sheath (IRS), matrix (M), outer root sheath (ORS), sebaceous gland (S), and upper follicle (UF). (b) A paraffin section of the skin of a 5-day-old mouse that had been injected subcutaneously with BrdU twice daily for three days; note the uniform labeling of all basal epidermal cells as well as the entire hair follicle. (c) A paraffin-section of the skin of a mouse that had been labeled as described in (b) followed by a chase of 8 weeks; note the selective retention of BrdU (red staining) by the bulge cells. (d) A higher magnification of one of the follicles in panel (c); note the intensely red LRCs in the bulge (B), and the absence of such cells in the upper follicle (UF), sebaceous gland (SG), or the epidermis (E). (e) A paraffin section of the skin of a mouse that had been labeled as described in (b), followed by a chase of 10 weeks, by which time some of the follicles had entered the growth phase. The mouse was injected intraperitoneally with ^{3}H-TdR and was sacrificed 1 hr later. Note the concentration of red BrdU-labeled LRCs in the bulge (B), and the double-labeled cells (containing both red BrdU and silver grains due to ^{3}H-thymidine) in the newly formed downgrowth (DG). Boxed area shows a double-labeled cell at a higher magnification. (f–i) Anagen follicles from mice treated as described in panel (e) showing double-labeled cells (arrowheads). Note keratinocytes containing red-speckled nuclei (arrows) in different parts of the newly formed follicle, including matrix (M; panels f–h) and medulla (panel i; arrows). The BrdU in these cells proves their bulge origin; the reduced BrdU intensity reflects the dilution of the label by cell division. FP, follicular papilla. (j) The upper follicular epithelium (UF) of a resting hair follicle from a mouse treated

Table 1. The Distribution of BrdU-Positive Cells in Telogen and Anagen Follicles

Stage	No. of Follicles[a]	BrdU (+) Follicles[b]	BrdU (+) cells/Follicle[c]	Distribution of BrdU (+) Cells[d]				
				B	B + ORS	B + ORS + Mt	B + ORS + Mt + UF	B + Md
Telogen[e]	132	107	6.5 ± 2.4	107	na	na	na	na
Anagen[f]	54	38 (100%)	10 ± 4	5 (13%)	2 (5%)	18 (47%)	10 (26%)	3 (8%)

[a] Total number of follicles evaluated.
[b] Number of follicles with nuclei having two or more red-speckles; in nonlabeled follicles, additional sectioning may reveal the presence of BrdU-labeled cells.
[c] The average number of BrdU-labeled cells ± standard deviation.
[d] Abbreviations: bulge (B), matrix (Mt), medulla (Md), outer root sheath (ORS), and upper follicle (UF).
[e] Follicles from six mice that had been labeled with BrdU twice daily for 3 days and then chased for 8 weeks.
[f] Same as footnote (e) but had been chased for 2 additional weeks.

observations raise several important questions. If label retention is indeed a reliable feature for keratinocyte stem cells, why are such cells so scarce in the epidermis? What is the relationship between the "stem cells" of the epidermis and the follicle? Do the anatomically contiguous epithelia of the epidermis and hair follicle represent two distinct and self-sufficient compartments, or do they share a common stem cell population? Could it be possible that the follicle is a repository of the ultimate epidermal stem cells?

In this paper, we demonstrate that the follicular bulge stem cells are responsible for forming not only the lower hair follicle, but also the upper follicular epithelium. Our data reveal that in normal neonatal mouse skin the hair follicle provides a flow of proliferating keratinocytes into the epidermis. Such an emigration also occurs in adult mouse skin, in response to a relatively discrete, small, penetrating skin wound. The emigration of bulge-derived upper follicular epithelial cells into normal epidermis suggests a much closer relationship between the follicle and the epidermis than was previously appreciated. Since the bulge stem cells are also responsible for forming the lower hair follicle, we hypothesize that these cells are bipotent, i.e., capable of undergoing two distinct differentiation pathways leading to the formation of either cornified epidermal cells, or matrix keratinocytes that form the hair shaft. Our findings suggest that the hair follicle is a major repository of epidermal stem cells and have implications regarding the follicular origin of certain skin tumors.

Results

Direct Evidence that the Bulge Keratinocytes Give Rise to the Lower Follicle

We and others have previously shown that slow-cycling cells, which retain ^{3}H-thymidine (^{3}H-TdR) as detected by autoradiography, are located predominantly in the bulge region of the upper follicle (Cotsarelis et al., 1990; Morris and Potten, 1999; Figure 1a). No direct evidence exists, however, that the bulge cells actually give rise to the lower follicle. To study this problem, we repeated the label-retaining experiment using bromodeoxyuridine (BrdU), instead of ^{3}H-TdR. Since BrdU can be easily detected as an intense, red (nuclear)-stain with an extremely low background, this affords a higher sensitivity not only for the identification of the label-retaining cells, but also for the detection of cells that are derived from the LRCs even after several rounds of cell division. Thus, we labeled newborn mouse skin with twice daily (subcutaneous) injections of BrdU for three days, which generated intense, uniform (nuclear) labeling of virtually all proliferating skin epithelial cells, including those of the bulge (Figure 1b). After an 8-week chase, the only labeled cells that remained were located in the bulge area of the follicles, with none detected in the upper follicle, epidermis, or sebaceous gland (Figures 1c and 1d; Table 1). These findings are consistent with earlier data using ^{3}H-TdR (Cotsarelis et al., 1990; Morris and Potten, 1999). After this 8-week chase, all the follicles were in the telogen or resting phase (Figures 1c and 1d). However, if the chase was extended to 10 weeks, some of the resting follicles had spontaneously entered into the anagen (growing) phase of the hair cycle (Figures 1e–1i). Many of the cells located in the newly formed lower follicle contained red-speckled nuclei (Figures 1f–1h; Table 1), thus providing direct evidence that the BrdU-labeled bulge cells not only were viable, but also gave rise to lower follicular cells. These bulge-derived, red-speckled cells were detected in many compartments of the lower follicle (Table 1), including outer root sheath (Figure 1f), matrix (Figures 1f–1h), and medulla (Figure 1i).

Selective Tagging of Upper Follicular Epithelial Cells in Neonatal Mouse Skin

Based on the fact that LRCs are found almost exclusively in the follicular bulge, we speculated in our original "bulge activation hypothesis" that the progeny of bulge stem cells may also migrate into the upper follicle and play a role in the long-term maintenance of the epidermis (Cotsarelis et al., 1990; Lavker et al., 1993; Miller et al., 1993). We tested this hypothesis by two independent approaches. In the first, we found that the bulge cells that were exclusively tagged with BrdU after long-term

as described in panel (e). Note some cells with red-speckled nuclei (arrow) indicating the bulge-origin of such upper follicular cells. Some of these cells have in addition incorporated ^{3}H-thymidine thus becoming double labeled (box). Inset shows a higher magnification of the boxed area, in which the silver grains were intentionally slightly underfocused to show the red-BrdU staining of the nucleus. (k) Another example of a telogen follicle showing some upper follicular (UF) cells containing red-speckled (arrows) or double-labeled nuclei (arrowhead). Bar in panel (b) equals: 80 μm (b and c); 50 μm (d); 28 μm (g–i); 25 μm (e, f, j, and k); 9 μm (inset e); and 6 μm (inset j).

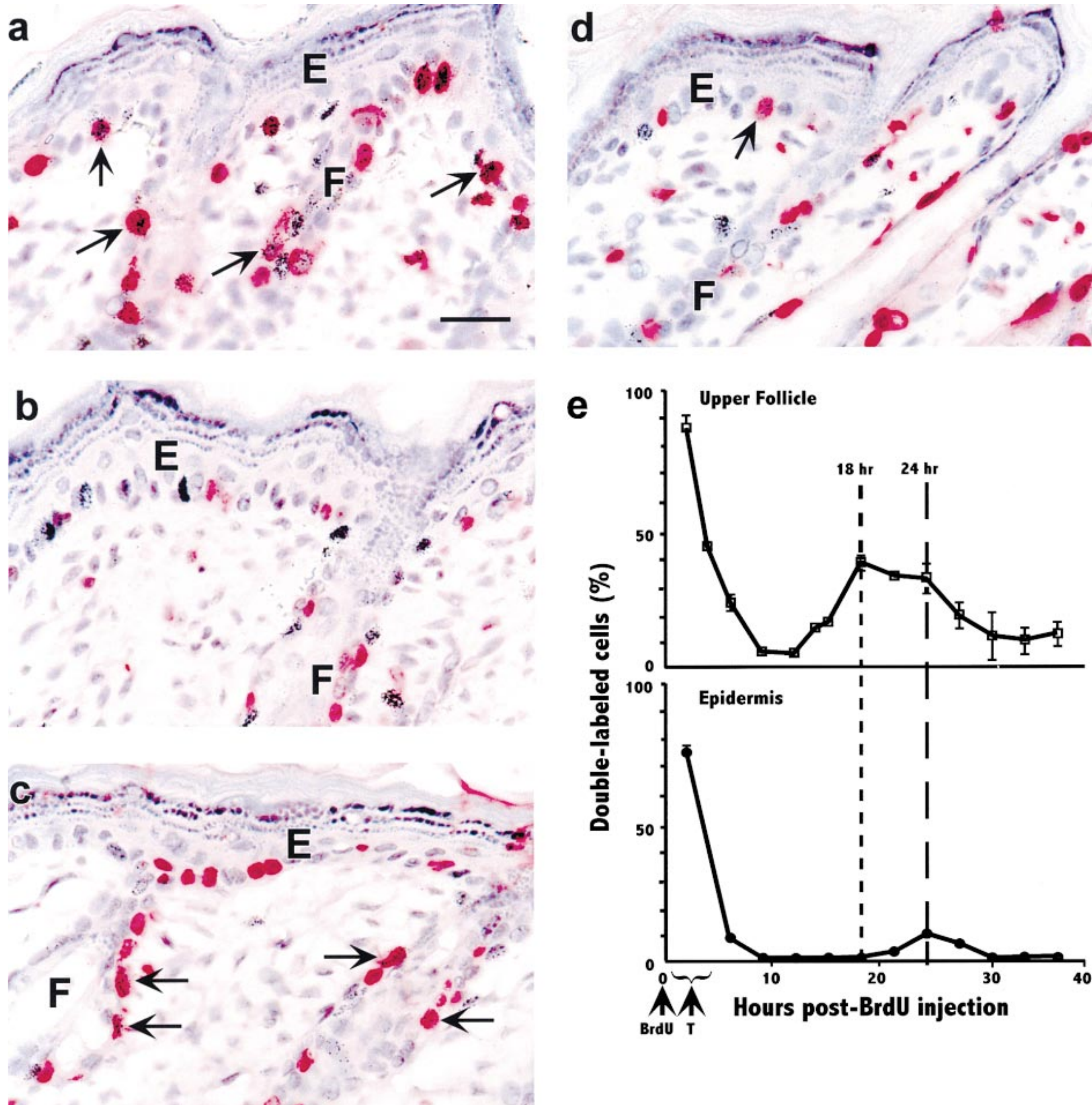

Figure 2. Upper Follicular Keratinocytes of Neonatal Mice Cycle Faster Than Epidermal Keratinocytes

Neonatal mice (4 days old) received a subcutaneous injection of BrdU, followed by a second subcutaneous injection of ^{3}H-TdR after (a) 2 hr, (b) 12 hr, (c) 18 hr, and (d) 24 hr. Panels show paraffin sections of the skin that were stained for BrdU followed by the detection of ^{3}H-TdR by autoradiography. (e) Quantification of the double-labeled cells in upper follicle versus epidermis. Note in (a) numerous double-labeled cells (arrows) in both epidermis (E) and upper follicle (F), that were in the same S phase of the cell cycle during the two pulse labels; in (b) a scarcity of such double-labeled cells when the two injections were 12 hr apart due to the exit of BrdU-labeled cells from the cell cycle; in (c) the reappearance of double-labeled cells (that have traversed into the second cell cycle) in upper follicular epithelium, but not in the epidermis; and in (d) the appearance of double-labeled cells in both upper follicle and epidermis. These results indicate that upper follicular cells cycle faster than epidermal cells, and that the upper follicular cells can be selectively tagged by a BrdU injection followed 14–18 hr later by a ^{3}H-TdR injection. All panels (a–d) are of the same magnification (bar in panel a = 25 μm).

labeling and an 8-week chase give rise later (after an additional 2-week chase) to red-speckled cells not only in the lower follicle, as noted above, but also in the upper follicle (Figures 1j and 1k). Some of these red-speckled cells also incorporated a pulse of ^{3}H-TdR thus demonstrating their ability to divide.

In another approach, we sought to selectively tag some of the cells in the upper follicle in order to follow their fate. To do this, we exploited the fact that keratinocytes have a heterogeneous cycle time (Lavker and Sun, 1982; Potten et al., 1982; Lehrer et al., 1998). To assess the cell cycle time in different parts of the hair follicle and the epidermis in newborn mouse skin (4–5 days postnatal), we used a double-labeling technique in which we first pulse-labeled the proliferating TA cells with BrdU, followed, after an interval of 1–36 hr, by an-

other pulse with ^{3}H-thymidine (^{3}H-TdR). Short intervals of <8 hr resulted in the double-labeling of cells that had not yet completed their initial S phase (Figures 2a and 2e). Intervals of 8–12 hr yielded no double-labeled cells due to the exit of all the BrdU-labeled cells from S (Figures 2b and 2e). Intervals of >14–16 hr were long enough for some of the BrdU-labeled cells to traverse into a second S phase, hence incorporating a second label of ^{3}H-TdR (double-labeled; Figures 2c and 2e). Such an analysis of newborn mouse skin established that the upper follicular cells in the infundibulum zone had a cell cycle time of about 16–28 hr, with a peak around 18 hr. In contrast, epidermal cells cycled significantly slower, with no double-labeled cells appearing until 22–24 hr (Figures 2d and 2e).

Taken together, these results established that the bulge gave rise to a population of upper follicle cells; that the upper follicle contained a population of TA cells that cycled more rapidly than those of the epidermis; and that a time period of 18 hr between the two deoxynucleoside-pulses resulted in the exclusive tagging of some of the upper follicular TA cells (Figure 2e). This last finding allowed us to analyze the trafficking of upper follicular cells and their possible contribution to the epidermis (see below).

Emigration of Upper Follicular Epithelial Cells into Normal Neonatal Epidermis

Using the double-labeling approach as described above, we generated a group of newborn mice whose upper follicular epithelial cells were specifically tagged, and followed the migration pattern of such upper follicular cells. Immediately after the ^{3}H-TdR pulse (18 hr post-BrdU labeling), many (38.5%) of the BrdU-labeled infundibular cells became double-labeled with thymidine (Figures 3a and 3d). The "background" of double-labeled cells in the epidermis was extremely low, i.e., less than 1.2% of the BrdU-labeled epidermal cells were double-labeled (Figures 3a and 3d). Since the labeling index of the two tissues was about the same (13%–15%), there was a large number of double-labeled cells in the upper follicle, with scarcely any in the epidermis (Figure 3a). We then followed the fate of the double-labeled follicular cells after chase periods of 18, 36, and 54 hr (Figures 3b–3d). A progressive decrease in the number of double-labeled cells in the upper follicle, accompanied by a dramatic increase of such cells in the epidermis (Figure 3d), clearly demonstrated an emigration of the upper follicular cells into the epidermis. The fact that the increase in the number of double-labeled epidermal cells was greater than the decrease in such cells in the upper follicle can be explained, if one assumes that some of the follicle-derived, double-labeled cells underwent one or two more rounds of cell division during or after their migration to the epidermis. These results clearly demonstrate that populations of follicular keratinocytes contribute to the normal neonatal epidermis during a time of tissue expansion.

Contribution of the Upper Follicular Epithelium to Adult Epidermis after a Small Full-Thickness Wound

To address whether follicular cells contribute to adult epidermis, we used a wound repair model—for two reasons. First, normal adult mouse epidermis has a low proliferative index (2%–4%) and a relatively long cell

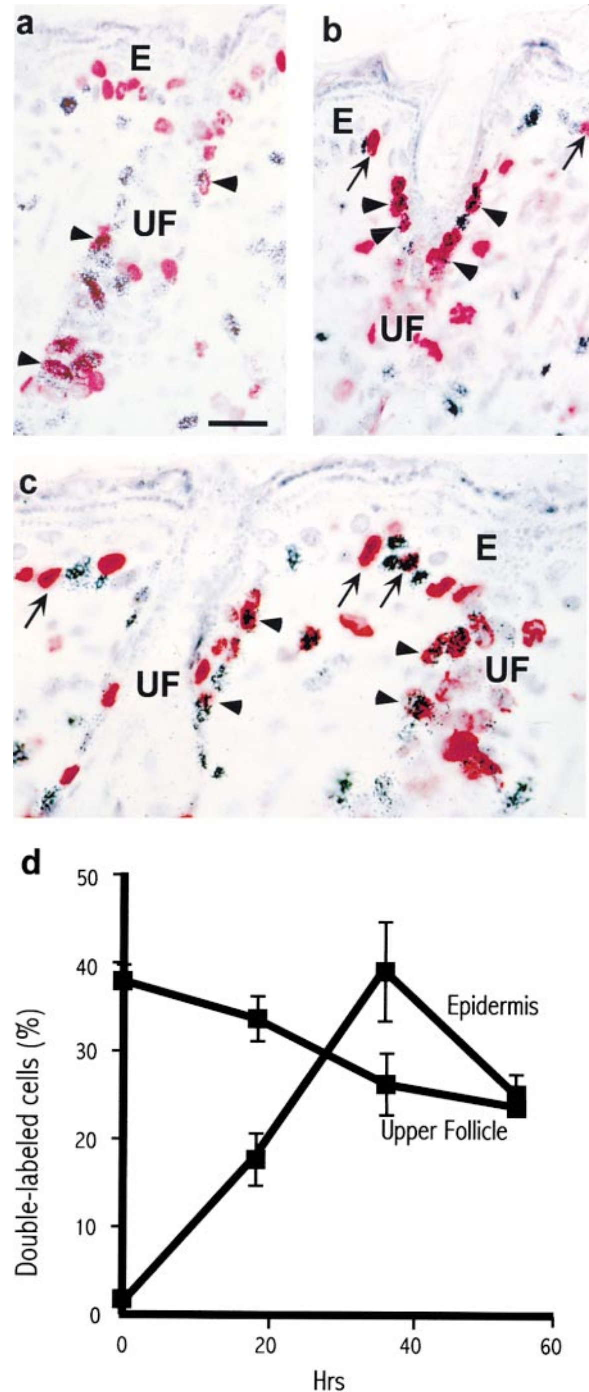

Figure 3. Emigration of Upper Follicular Keratinocytes into Normal Neonatal Mouse Epidermis

Neonatal mice (4 days old) received a subcutaneous injection of BrdU, followed 18 hr later by a subcutaneous injection of ^{3}H-TdR (time zero; selective double-labeling of upper follicular cells). These mice were then sacrificed after (a) 1 hr, (b) 18 hr and (c) 36 hr. Paraffin-sections were stained for BrdU followed by autoradiography. (d) Quantification of double-labeled cells in upper follicle versus epidermis. Note in (a) numerous double-labeled, upper follicle (UF) cells (arrowheads), but none in the epidermis; in (b) the appearance of some double-labeled cells (arrows) in the epidermis (E) near the opening of the follicle, and in (c) the appearance of numerous follicle-derived, double-labeled, epidermal cells (arrows). Panels (a) to (c) are of the same magnification (bar in panel a = 20 μm).

cycle time (72–100 hr), making it difficult to study its cell cycle (Potten and Loeffler, 1987). This problem can be alleviated in wounded epidermis, which has a shortened cell cycle time (Lehrer et al., 1998). Second, although it has been well established that follicular cells can emigrate to regenerate the epidermis after it is totally destroyed by mechanical abrasion or burn (Argyris, 1976), it is unclear whether this would also occur in response to a small, limited skin wound. We therefore created full-thickness wounds on the backs of 7-week-old mice when their hair follicles were in the telogen or resting phase of the hair cycle (Wilson et al., 1994). We administered the first BrdU injection at 21 hr post-wounding, when it was the peak of epidermal and upper follicular keratinocyte proliferation. This was followed by, after several intervals of 1–18 hr, a second, ^{3}H-TdR injection. We found again that the infundibular keratinocytes cycled faster than epidermal cells; however, in the wounded skin a 10 hr interval between the BrdU- and ^{3}H-TdR-pulses resulted in the predominant (double-labeled) tagging of the upper follicular keratinocytes (data not shown). Subsequent chases of 10, 20, and 30 hr again resulted in the progressive decrease in the double-labeled upper follicular cells, and a marked increase in such cells in the epidermis (Figure 4). This result clearly indicates that follicular epithelium contributes to the repair of epidermis even when the wound is relatively small and well defined.

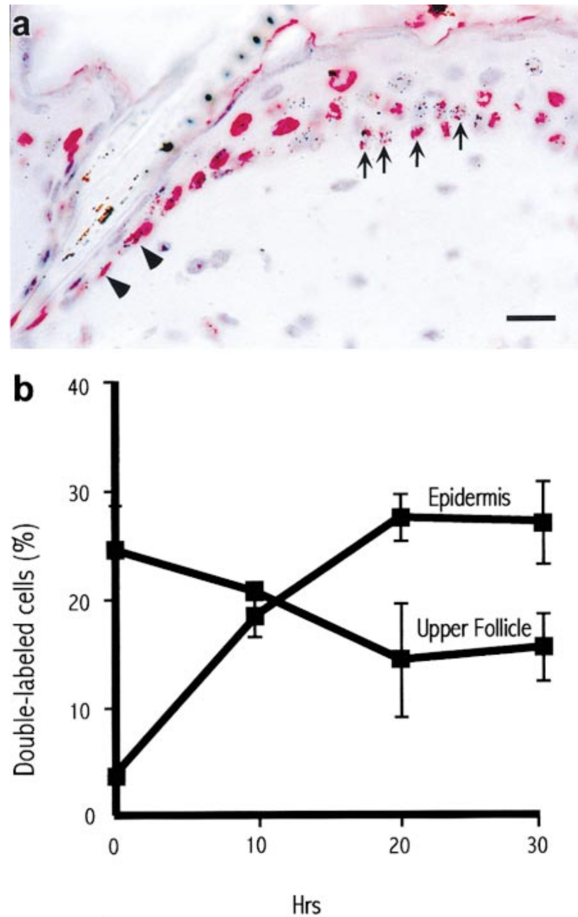

Figure 4. Emigration of Upper Follicular Keratinocytes into Wounded Adult Mouse Epidermis

Penetrating wounds were created on the back skin of adult mice. Twenty-one hours later BrdU was injected intraperitoneally, followed 10 hr later by an intraperitoneal injection of ^{3}H-TdR in order to tag selectively the upper follicular cells. These mice were then sacrificed after 1 hr, 10 hr, and (a) 20 hr, and the skin paraffin sections were processed for BrdU immunocytochemistry and autoradiography. (b) Quantification of the double-labeled cells in upper follicle versus epidermis. Numerous double-labeled cells were present in the epidermis (a; arrows) that must have originated from the upper follicle (arrowheads). The bar in panel (a) equals 16 μm.

Discussion

We have devised a novel, double-labeling method that has allowed us to track the movement of the bulge-derived cells into various compartments of the hair follicle, as well as to observe the migration of follicular epithelial cells into the epidermis. Our findings strongly support our original "bulge activation hypothesis," in which we proposed that hair follicular epithelial stem cells are located in the bulge and that these stem cells give rise to all the epithelia of the follicle as well as potentially contribute to the epidermis (Cotsarelis et al., 1990; Lavker et al., 1993; Miller et al., 1993; also see Fuchs and Segre, 2000).

The Bulge Origin of the Lower Hair Follicle

Recently, issues have been raised concerning several elements of this hypothesis. For example, it has been questioned whether the LRCs are radiochemically damaged and thus become nonviable (Watt, 1998), and whether the bulge LRCs represent the true progenitors of the lower follicle. Our data, which show that keratinocytes of lower follicles in spontaneous early anagen contain red, speckled nuclei (Figures 1e–1g), provide direct proof that the BrdU-LRCs are viable and that cells of the lower follicle are bulge-derived (Figures 1e–1i). Morris and Potten (1999) reported recently that LRCs (that have survived a 14-month chase, instead of the 8- to 10-week chase used in the present study) are not the first cells to divide during early anagen induced by hair plucking, and that these LRCs appear to undergo only limited migration into the lower follicle. Based on these findings, they questioned whether LRC progeny constitute an important first wave of cells needed to form a new follicle (Morris and Potten, 1999). However, the finding that such long-term LRCs are not among the first cells to divide and migrate during early anagen could mean many things, none of them incompatible with the bulge origin of the lower follicle: (1) Some of the stem cells may have escaped detection in their studies due to the fact that only a small proportion of such cells are known to retain the label after such an extended chase period. (2) The few LRCs that have retained the label for 14 months are by definition more resilient to growth stimuli; such LRCs may therefore be the last ones among the stem cells to proliferate. (3) LRCs that have retained ^{3}H-TdR for such an extended period of time may have indeed become partially damaged, and therefore become somewhat sluggish in responding to growth stimuli. Finally, (4) one cannot rule out the possibility that the transit amplifying cells located in the vicinity of the bulge stem cells, instead of the stem cells per se, are the very first to divide during early anagen.

Hair reconstitution using retroviral-tagged mouse epidermal cells sometimes gave rise to follicles with selected tagging of distinct follicular cell layers, leading

to the suggestion that 2 or 3 separate progenitor cells may persist in adult skin to yield these follicular layers (Kamimura et al., 1997; also see; Ghazizadeh et al., 1999). It is unclear, however, (1) whether the expression of the reporter genes in these studies depended on the differentiation state of the follicular cells, and, more importantly, (2) where these separate progenitor cells are located in the follicle and how they maintain their spatial relationship during consecutive catagen phases when the lower follicle is destroyed and reorganized. Our observation of red-speckled cells in outer root sheath, matrix, and medulla (Figures 1f–1i) favors the simpler hypothesis that the bulge stem cells give rise to a population of multipotent matrix cells which in turn yield all components of the hair fiber (Figure 5).

The Bulge Origin of the Upper Follicle and Possibly the Epidermis

In our original hypothesis, we speculated that cells might move from the bulge into the upper follicle and then epidermis (Cotsarelis et al., 1990; Lavker et al., 1993; Miller et al., 1993). The fact that many of the upper follicular keratinocytes acquired red-speckled nuclei, after a chase, strongly suggests that such upper follicle cells are indeed, like the lower follicular keratinocytes, bulge-derived (Figures 1j and 1k). Moreover, by exploiting our finding that a population of upper follicular keratinocytes cycles faster than the epidermal keratinocytes (Figure 2), we were able to demonstrate that upper follicular cells emigrate into normal neonatal mouse epidermis (Figure 3), and into the adult mouse epidermis in response to a limited penetrating skin wound (Figure 4). Taken together, these results raise the possibility that the bulge stem cells are bipotent, as they give rise not only to the lower follicle, but also to the upper follicle and the epidermis (see below).

Relationship between the Follicular Stem Cells and Epidermal "Stem Cells": Members of the Same Hierarchy?

In previous studies of both mouse and human skin, the great majority of LRCs have been observed in the bulge region of the hair follicle, with very few if any in the epidermis (Cotsarelis et al., 1990; Morris and Potten, 1994; Lyle et al., 1998). These data, in conjunction with our present findings, raise the possibility that the bulge area of the hair follicle contains the ultimate stem cell of the epidermis/hair follicle system. This idea is strongly supported by some data, previously deemed paradoxical, indicating that the upper follicle–derived keratinocytes have a higher in vitro proliferative potential than the epidermal cells (Yang et al., 1993), and that the upper follicle contains more clonogenic keratinocytes than the epidermis (Rochat et al., 1994).

Whether there are separate populations of epidermal and follicular stem cells is to some extent a matter of definition (see below). In the scheme shown in Figure 5a, we propose that the progeny of hair follicular stem cells of the bulge can undergo two independent pathways of cell migration/specialization: moving upward they are involved in the long-term maintenance of the epidermis (the bulge–epidermal pathway), whereas moving downward during the early anagen of each hair cycle they give rise to the lower, dispensable portion of a new follicle (the bulge–hair pathway).

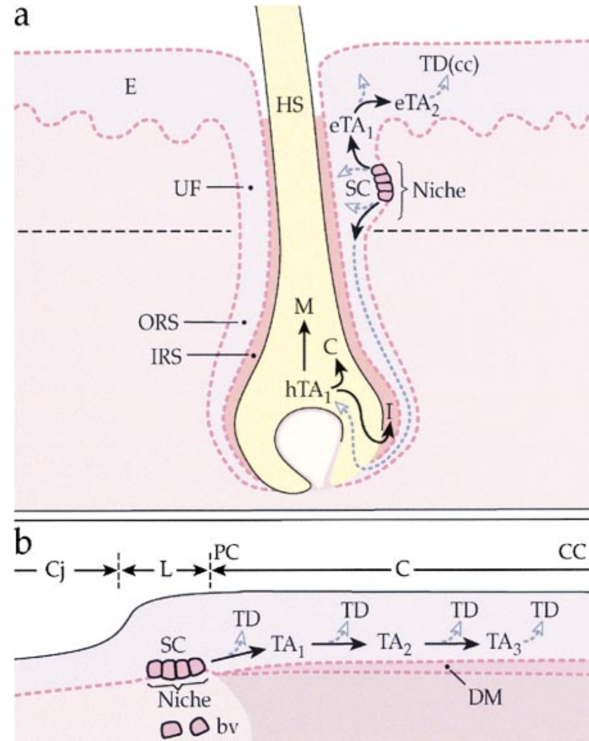

Figure 5. Keratinocyte Lineages in Skin and Cornea

(a) A schematic diagram showing the location of stem cells (SC) in the bulge area of the hair follicle in close contact with the arrector pili muscle, which may provide a specialized "niche" known to be crucially important in maintaining the in vivo stem cell features. The division of these normally slow-cycling stem cells gives rise to a hierarchy of transit amplifying (TA) cells with progressively less proliferative potential and more restricted differentiation flexibility (TA_1, TA_2,...TA_n). It is hypothesized that the bulge stem cells are bipotent, as they can undergo two distinct differentiation pathways. The bulge–hair pathway: When the bulge-derived TA cells migrate downward during early anagen they give rise to a population of young TA cells (hTA_1,hTA_2,...hTA_n) specialized in making various hair compartments including inner root sheath (I), cortex (C), and medulla (M). These hTA cells undergo apoptosis when they have exhausted their proliferative potential, thus triggering the onset of the catagen of the hair cycle (Cotsarelis et al., 1990). The bulge–epidermis pathway: When the bulge-derived, young TA cells migrate upward into the epidermis (eTA_1, eTA_2, ... eTA_n), they may be regarded as the "epidermal stem cells." These cells are specialized in making various epidermal products culminating in the formation of the terminally differentiated, cornified cells (TDcc). Solid arrows indicate hypothesized ("horizontal") path of keratinocyte migration along a basement membrane, while the dashed arrows indicate the ("vertical") path of cell migration into the suprabasal layers forming terminally differentiated cells (TD). Other abbreviations: E (epidermis), HS (hair shaft), IRS and I (inner root sheath), ORS (outer root sheath), UF (upper follicle). (b) A schematic diagram showing the location of corneal epithelial stem cells (SC) in the basal layer of the peripheral corneal zone called the limbus (Schermer et al., 1986). These stem cells overlie a mesenchyme (niche?) that is distinctively more cellular and blood vessel (bv)–rich than the corneal stroma. Solid arrows denote the well-established centripetal ("horizontal") migration of limbal-derived TA cells (Davanger and Evensen, 1971; Buck, 1985; Auran et al., 1995) which progressively lose their proliferative potential; dashed arrows denote the ("vertical") migration of cells into the suprabasal compartment to become terminally differentiated (TD). Other abbreviations: C (cornea), CC (central cornea), Cj (conjunctiva), DM (Descemet membrane), L (limbus), and PC (peripheral cornea). See text for details.

An important feature of the "bulge–epidermal" pathway is that the bulge stem cells give rise to a hierarchy of transit amplifying cells (TA_1, TA_2, TA_3...TA_n), with a continuous spectrum of progressively less proliferative potential. Some of the early transit amplifying cells in this hierarchy, e.g., the TA_3 or TA_4, that have arrived in the epidermis, may still have a significant proliferative potential (Kamimura et al., 1997; Mackenzie, 1997; Ghazizadeh et al., 1999)—and therefore may be justifiably thought of as epidermal stem cells. However, to incorporate the idea of hierarchy and to avoid confusion, it may be helpful to adopt the nomenclature of the hematopoietic system in which the term "stem cell" is reserved for the ultimate, pluripotent precursor cells residing in the bone marrow (Quesenberry and Levitt, 1979). These hematopoietic stem cells give rise to daughter cells with progressively more restricted differentiation flexibility and less proliferative potential. Although these progeny cells still have a significant proliferative potential and can give rise to neoplasm, such cells are called "progenitor cells," instead of stem cells. Following this convention, one may consider the label-retaining cells of the bulge the "stem cells," and the (early) TA cells of the epidermis the "progenitor cells."

While we have demonstrated that the follicular stem cells are actively involved in supplying additional progenitor cells to the epidermis during times of need, including neonatal expansion of the skin and adult wound repair, the progenitor cells of the epidermis, under normal circumstance, can apparently be quite self-sufficient for a long period of time (Kamimura et al., 1997; Mackenzie, 1997; Bickenbach and Chism, 1998; Li et al., 1998; Ghazizadeh et al., 1999). However, one should note that although corneal epithelium had been regarded, for decades, as a self-sufficient, self-renewing epithelium (Buschke et al., 1943; Scheving and Pauly, 1967; Burns and Scheving, 1975; Fogle et al., 1980), it is now well accepted that corneal epithelial homeostasis depends on a supply of limbus-derived TA cells (Schermer et al., 1986; Cotsarelis et al., 1989; Kenyon and Tseng, 1989; Dua, 1995; Hodson, 1997; Voelker, 1997; Tseng and Sun, 1999). Therefore it seems likely that a similar situation exists in the follicle/epidermis system, in that there exists a flow of the bulge-derived (early) TA cells into even the normal, adult epidermis. Additional data are needed to test this possibility.

An important element of our hypothesis, as shown in Figure 5a, is that follicle-derived TA cells must be able to migrate "horizontally" along the basement membrane for a fair distance. Our data clearly indicate that this can occur (Figures 3 and 4). Moreover, it has been well established that cells of several other epithelia can achieve a significant "horizontal" migration. For example, the enterocytes of intestinal epithelium, originating from stem cells located in the crypt (Cheng and Leblond, 1974), migrate along the basement membrane for 0.5 mm toward the tip of the villi at an estimated rate of 200–400 μm/day (Kaur and Potten, 1986). Corneal epithelial cells, derived from the limbal stem cell zone, are known to migrate centripetally at a rate of 32 μm/day toward the central cornea that can be up to 5.5 mm away (Buck, 1985; Auran et al., 1995; Figure 5b). With a hair density of 0.6–5 follicle/mm^2 of glabrous skin (Szabo, 1967), the maximal distance that a follicle-derived TA cell has to travel (from the bulge to midway between two hair follicles) is $\sim$3 mm, which is about half of the distance that needs to be covered by the limbal-derived corneal epithelial TA cells. Therefore, the physical migration that is required for the follicle-derived TA cells to cover the entire skin surface does not seem to be an insurmountable task.

Follicular Origin of Skin Cancers

Our demonstration that the upper hair follicle contains a population of young TA cells is relevant to the paradoxical observation that many of the experimentally induced skin tumors appear to be associated with the infundibulum of the upper follicle, instead of the bulge, suggesting that the infundibular cells may be an oncogenic target (Hansen and Tennant, 1994; Schmitt et al., 1996; Binder et al., 1998; Morris et al., 2000). This paradox can be explained if one assumes that the *early* TA ("progenitor") cells of upper follicle give rise to the nonmalignant tumors. This seems reasonable given the fact that such cells still have a significant proliferative potential (Figure 5a), and that they are highly proliferative and are thus particularly susceptible to DNA damage (Miller et al., 1993a). However, the bulge stem cells are almost certainly another major target of tumor initiating agents, conceivably giving rise to more malignant tumors, because we and others have shown earlier that (1) the skin tumor yield is higher if the initiation step of the two-stage carcinogenesis protocol is done during early anagen, i.e., when the bulge stem cells are proliferating (Miller et al., 1993a), and (2) damaging the epidermis and infundibulum with 5-fluorouracil, which kills specifically cycling (TA) cells but not quiescent (stem) cells, has no effect on skin tumor formation (Morris et al., 1997).

There are several limitations in our studies. Since our experiments are conducted using the fur-bearing mouse skin, some of our conclusions are obviously not applicable to nonhairy skin such as human foreskin or the palm/sole epithelium—where the stem cells reside at the bottom of the deep rete ridges (Lavker and Sun, 1982, 1983). In addition, although the bulge cells appear to be homogeneous raising the possibility that such stem cells may be bipotent capable of giving rise to both epidermis and hair follicle, additional studies testing a clonal population of bulge cells are needed to firmly establish this point. Finally, whether the emigration of follicle cells into the epidermis is continuous or hair cycle–dependent is currently unknown.

Similarities between the Limbal/Corneal and Follicular/Epidermis Stem Cell Systems

A comparison of the corneal epithelium and follicular/epidermis illustrates that keratinocyte stem cells of these two systems share several important attributes (Figure 5). They are slow-cycling and thus can be identified as label-retaining cells; they have a high proliferative potential; they give rise to TA cells which in some cases have to migrate along the basement membrane to cover a large area; they reside in a well protected and vascularized location; they are in contact with a highly specialized microenvironment; and they are frequently the dominant sites of tumor formation (this study and Miller et al., 1993).

Selective Tagging of TA Cells: A Useful Tool for Studying Cell Lineage and Trafficking

Our ability to tag selectively a subpopulation of proliferating cells in the follicle has enabled us to follow the trafficking of these cells, and to carry out a cell lineage

analysis of the cells in the follicle and epidermis. This approach should be generally applicable to many tissues that contain subpopulations of transit amplifying cells with heterogeneous cell cycle time. When coupled with the experimental identification of the slow-cycling stem cells (as the "label-retaining cells"), this approach permits a systematic analysis of the progenitor–progeny relationships, as well as the cellular migration patterns in many self-renewing tissues including the epithelia of the lung, prostate, and mammary gland as well as some neuronal tissues—systems that are, like the limbal/corneal epithelium and the follicular epithelium/epidermis, governed by stem cells.

Experimental Procedures

SENCAR (NCI) and C57Bl/6 mice were used in compliance with protocols established by the University of Pennsylvania Animal Care and Ethics Committee.

Detection of Slow-Cycling Cells

To label the slow-cycling cells, we injected subcutaneously (s.c.) neonatal SENCAR mice beginning at day 3 of life with 5-bromo-2-deoxyuridine (BrdU; 50μg/g body weight) twice daily (8 a.m. and 5 p.m.) for 3 days. Cells retaining the label after 8–10 weeks were identified as label-retaining cells (LRCs). To identify the rapidly cycling transit amplifying (TA) cells, we injected mice intraperitoneally (i.p.) with ^{3}H-TdR (10 μCi/g body weight), sacrificed the mice 1 hr later, processed the upper back (scapula) skin, and detected BrdU by immunohistochemistry and ^{3}H-TdR by autoradiography (Lehrer et al., 1998; Jensen and Lavker, 1999). A cell was considered to be double-labeled if it contained >5 silver grains over a bright red (BrdU) nuclear staining.

Cell Cycle Parameters of Keratinocytes in Normal Neonatal Mouse Skin

To determine the cell cycle time of mouse keratinocytes, we injected 3-day-old SENCAR mice with BrdU (50 μg/g; s.c.), followed at intervals of 2 to 36 hr by an injection of ^{3}H-TdR (10 μCi/g; s.c.). Skin samples from the upper back (scapula) taken 1 hr later were processed (Lehrer et al., 1998), and cells that were labeled by BrdU, ^{3}H-TdR, or both were counted. Only basal keratinocytes that were obviously interfollicular and clearly not associated with a follicular structure were counted as epidermal cells. In a control experiment, we pulse-labeled 4-day old mice with ^{3}H-TdR followed, 2 hr later, by an injection of cold-thymidine (1000-fold molar excess). The animals were sacrificed 1 hr later. The epidermal labeling index of such animals (15.7) was the same as the controls (15.8), thus ruling out a long-lived thymidine pool which could confound the interpretation of the labeling experiments.

Cell Cycle Parameters of Keratinocytes in Wounded Adult Mouse Skin

Mice (C57Bl/6; 7-week-old) with almost exclusively telogen (resting) hair follicles were anesthetized with gamma-hydroxybutyric acid (i.p. injection of 100 μl of 10% sol. in PBS). The hairs of the back skin were clipped carefully using a pair of scissors. The skin was folded and two neighboring, full-thickness wounds, ca. 15 mm apart, were made with a 2 mm biopsy punch. Twenty-one hours following wounding, BrdU was injected (50 μg/g; i.p.) and, after a period of 3 to 18 hr, ^{3}H-TdR (10 μCi/g; i.p.) was injected into three mice. The animals were sacrificed 1 hr later and the wound was excised and processed. The numbers of nuclei in upper follicular epithelium and epidermis that were labeled by BrdU, ^{3}H-TdR, or both, were counted.

Migration of Double-Labeled Infundibular Epithelial Cells

To tag selectively the upper follicle keratinocytes, we injected 3-day-old SENCAR mice with BrdU (s.c.) followed 18 hr later by an injection of ^{3}H-TdR (s.c.). Groups of three mice were sacrificed and their back skin tissues were excised and processed (Lehrer et al., 1998).

Acknowledgments

We dedicate this paper to Dr. Albert M. Kligman (UPenn) and Dr. Irwin M. Freedberg (NYU), two giants in the field of cutaneous biology, who infected us with enthusiasm, taught us to look far, and supported us so that we could persevere. We thank Ms. Dorothy Campbell for her excellent technical assistance, and Mr. David Totugul for help with the wounding experiments. This work was supported by National Institutes of Health Grants EY06769 (R. M. L.), AR42998 (P. J. J.), DK39753, DK52206, DK52634, DK57269 (T.-T. S.), and an educational grant from Galderma Laboratories (G. T.).

Received January 14, 2000; revised June 6, 2000.

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