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Epithelial Stem Cells: A Folliculocentric View

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Putative epithelial stem cells were identified in the hair follicle bulge as quiescent "label retaining cells". The study of these cells was hindered until the identification of bulge cell molecular markers, such as CD34 expression and K15 promoter activity. This allowed for the isolation and characterization of bulge cells from mouse follicles. Bulge cells possess stem cell characteristics, including multipotency, high proliferative potential, and their cardinal feature of quiescence. Lineage analysis demonstrated that all epithelial layers within the adult follicle and hair originated from bulge cells. Bulge cells only contribute to the epidermis during wound healing, but after isolation, when combined with neonatal dermal cells, they regenerate new hair follicles, epidermis, and sebaceous glands. Bulge cells maintain their stem cell characteristics after propagation *in vitro*, thus ultimately they may be useful for tissue engineering applications. Understanding the signals important for directing movement and differentiation of bulge cells into different lineages will be important for developing treatments based on stem cells as well as clarifying their role in skin disease.

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Introduction

The field of epithelial stem cells is progressing rapidly largely because of technical advances in molecular and cellular biology. Many astute observations over the last century led to predictions about epithelial stem cells that only recently have been definitively addressed through new techniques. Here, I review the evolution of current thinking on epithelial stem cells, concentrating on those found in the hair follicle, and delineate future challenges for applying our knowledge to reap clinical benefits.

What are epithelial stem cells?

Self-renewing tissues, such as the epidermis and hair follicle, continuously generate new cells to replenish the dead squames and hairs, which are sloughed into the environment. Therefore, perhaps the simplest definition of an epithelial stem cell is based on lineage: *a stem cell is the cell of origin for terminally differentiated cells in adult tissues.* For example, tracing the lineage of a corneocyte or hair cell back to its ultimate source in the adult skin leads to a stem cell. However, because the tools required to perform lineage analysis have not been available until recently, investigators have principally adopted definitions from the hematopoietic system. In particular, stem cells were felt to be self-renewing, multipotent, and clonogenic, similar to stem cells in the hematopoietic system that can regenerate all of the blood lineages from one cell after transplantation. In contrast to the hematopoietic stem cell field, cutaneous epithelial stem cell biologists also relied heavily on quiescence as a major stem cell characteristic. This can be attributed to the pioneering work of Bickenbach and Mackenzie, who devised ''label-retaining cell'' methods for detecting quiescent cells in the epidermis (Bickenbach and Mackenzie, 1984), and Morris *et al.* (1986) who showed that these cells retained carcinogen and possessed proliferative characteristics of stem cells.

Localization of epithelial stem cells

With respect to the epidermis (which in this review refers to the interfollicular epidermis), we know that cells are generated through proliferation that occurs only in the basal layer; therefore, stem cells must be located there. Within the basal layer, however, keratinocytes display heterogeneous proliferative characteristics. In mouse skin, individual basal cells divide less frequently compared to their surrounding cells. The slower cycling central cell and more rapidly proliferating surrounding cells constitute approximately 10 basal cells and are roughly organized into a hexagonal unit, which lies beneath a single squame (Mackenzie, 1970). Based on these proliferative and morphological characteristics, Potten (1974) coined the term ''epidermal proliferative unit'' (EPU) to describe this architecture. Without the benefit of direct lineage analysis, it was assumed that the central cell within the EPU generates the rapidly proliferating cells, termed transient or transit amplifying (TA) cells, which move laterally and then differentiate and move upward (see article by Kaur, on page 1450). Thus, within the epidermis, the main source of cells, that is the stem cells,

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Abbreviations: BCC, basal cell carcinoma; EPU, epidermal proliferative unit; ORS, outer root sheath; TA, transit amplifying

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responsible for continual epidermal renewal appear to reside in the center of the EPU, and testing of this concept in unmanipulated pelage epidermis of the mouse supports this view (Ito *et al.*, 2005).

Similar to the epidermis, the hair follicle generates a terminally differentiated keratinized end product, the hair shaft, that is eventually shed. Tracing back a hair shaft cell to its origin in adult skin is not straightforward. In contrast to epidermis, the follicle undergoes cyclical regeneration, and has a more complicated proliferative profile and architecture with at least eight different epithelial lineages (Figure 1).

Hair is formed by rapidly proliferating matrix keratinocytes in the bulb located at the base of the growing (anagen) follicle. The duration of anagen varies drastically between hairs of differing lengths. For example, mouse hair follicles and human eyebrow hair follicles stay in anagen for only 2–4 weeks while scalp follicles can remain in anagen for many years. Nevertheless, matrix cells eventually stop proliferating, and hair growth ceases at catagen when the lower follicle regresses to reach a stage of rest (telogen). After telogen, the lower hair-producing portion of the follicle regenerates, marking the new anagen phase. Generally, in parallel to anagen onset in humans, the hair shaft is shed during the exogen stage (Milner *et al.*, 2002).

As the lower portion of the follicle cyclically regenerates, hair follicle stem cells were thought to govern this growth. Historically, hair follicle stem cells were assumed to reside exclusively in the ''secondary germ'' (Figure 2), which is located at the base of the telogen hair follicle. It was felt that the secondary germ moved downward to the hair bulb during anagen and provided new cells for production of the hair. At the end of anagen, the secondary germ was thought to move upward with the dermal papilla during catagen to come to rest at the base of the telogen follicle. This scenario of stem cell movement during follicle cycling was brought into question when we identified a population of long-lived presumptive stem cells, using label retaining cell methods, in an area of the follicle surrounding the telogen club hair, and not in the hair bulb (Cotsarelis *et al.*, 1990). That the presumptive stem cells localized to a previously defined area called the bulge was not appreciated until I read a description of the human embryonic follicle by Pinkus (1958)

> This *bulge,* often the most conspicuous detail of the young germ, is as large as the bulb.... The function of

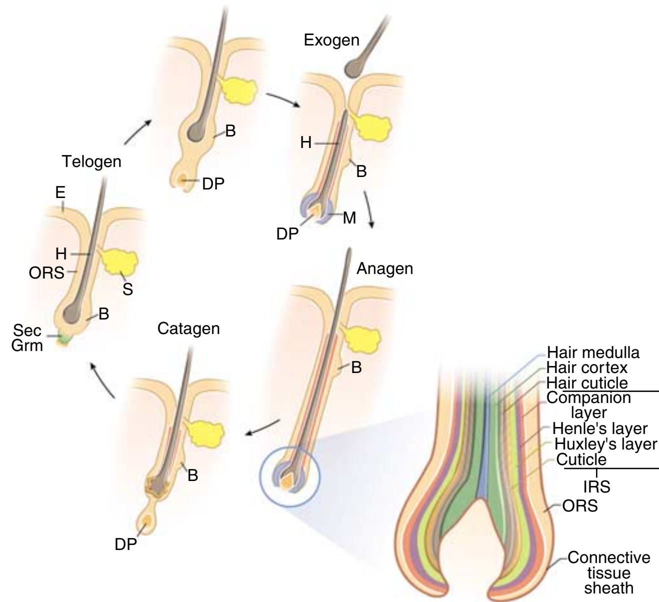

Figure 1. Hair follicle cycle and anatomy. The hair follicle cycle consists of stages of rest (telogen), hair growth (anagen), follicle regression (catagen), and hair shedding (exogen). The entire lower epithelial structure is formed during anagen, and regresses during catagen. The transient portion of the follicle consists of matrix cells in the bulb that generate seven different cell lineages, three in the hair shaft, and four in the inner root sheath (IRS). Abbreviations: B, bulge; DP, dermal papilla; H, hair; IRS, inner root sheath; M, matrix; ORS, outer root sheath; S, sebaceous gland; Sec Grm, secondary germ.

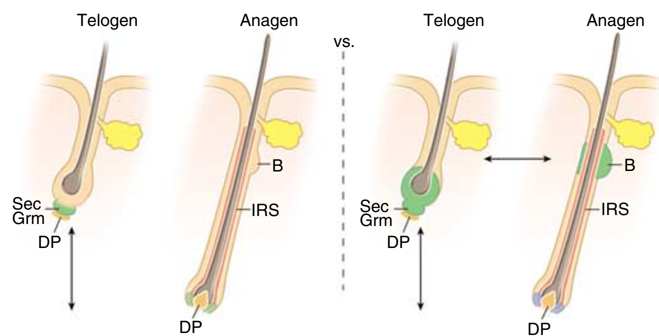

Figure 2. Location of hair follicle stem cells: two models. In one view, the ''secondary germ cells'', named for their similarity to primary germ cells present during development, were thought to contain the stem cells for the follicle. It was assumed that these cells migrated from the base of the telogen follicle to the bulb during anagen onset, and then migrated back up during catagen. To date, no direct evidence (e.g. label retaining cell studies or lineage analysis) has been presented that any epithelial cells in the bulb survive during catagen. The secondary germ cells found at the base of the telogen follicle appear to arise from the lowermost portion of the bulge at the end of catagen (Ito *et al.*, 2004). Based on morphology, the telogen secondary germ generates the new hair and inner root sheath (IRS), although this needs to be addressed experimentally. The origin and fate of the matrix keratinocytes in the bulb, which possess an undifferentiated phenotype is an area worthy of exploration. It is not known whether bulge cells migrate down the follicle during anagen to continuously supply the matrix cells with new cells, or whether the matrix cells self-renew throughout anagen. Abbreviations: B, bulge; DP, dermal papilla; IRS, inner root sheath; Sec Grm, secondary germ.

the bulge is obscure. While it serves as the point of insertion of the arrector muscle later in life, it develops much earlier than the muscle and the latter seems to originate quite independently in the skin near the sebaceous gland, and in many instances streaks by the bulge before approaching the lower follicle below it. Unna (1876) named the bulge area of the adult follicle the *hair bed* (Haarbett) believing that the club hair became implanted there and derived additional growth from it. Stöhr gave it the neutral name ''Wulst'' (bulge or swelling). Some texts state that this is an area of marked proliferative activity, but no mitotic figures were observed in the bulge even if other parts of the follicle contained them. Whatever its function, the bulge marks the lower end of the ''permanent follicle'' later in life. Everything below it is expendable during the hair change (cycle)''.

This remarkable description, based purely on morphological observations, portended the characterization of the bulge cells in both human and mouse follicles as an area containing quiescent cells important for hair follicle cycling (Cotsarelis *et al*., 1990; Lyle *et al*., 1998; Taylor *et al*., 2000; Oshima *et al*., 2001; Morris *et al*., 2004). In the mouse pelage follicle, the area analogous to the human bulge becomes morphologically apparent in the postnatal period during the first telogen stage at the site of arrector pili muscle attachment. The shape of the bulge in the mouse follicle results from displacement of the outer root sheath (ORS) by the club hair. In the human follicle, the bulge appears as a true thickening of the ORS, but generally becomes much less apparent with age (Figure 3).

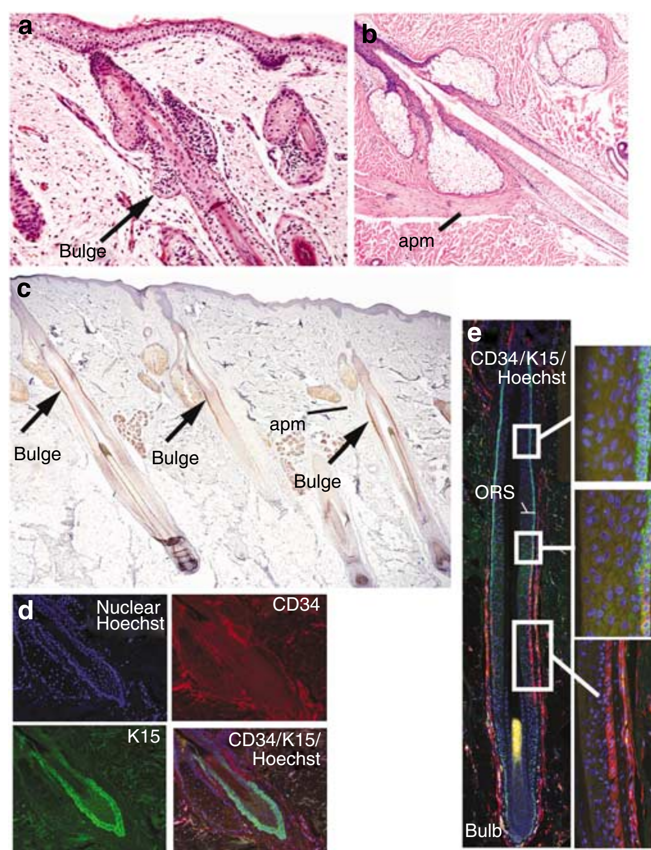

Figure 3. Human bulge cell morphology and markers. (**a**) The bulge is a prominent structure in fetal skin, but (**b**) generally is not morphologically distinct in the adult. (**c**) Immunostaining for K15 expression in scalp preferentially detects bulge cells. (**d**) Although CD34 is an excellent marker for mouse bulge cells, in human scalp, CD34 is not expressed by bulge cells, which do express K15, as shown in this telogen follicle. (**e**) CD34 is expressed by a population of cells in the lower outer root sheath (ORS) of the human anagen follicle. The staining patterns for K15 and CD34, which include a double-positive cell population (**e**, right middle panel), suggest that CD34-positive cells may be immediate descendents of K15-positive stem cells in the bulge. (magnification, (**a**, **b**, **d**, **e**): $\times 200$, (**c**): $\times 20$).

The lack of markers for bulge cells hindered the study of this area. To date, probably the best (most specific) marker for mouse hair follicle bulge cells is CD34 expression as first defined by Trempus *et al*. (2003). CD34, which interestingly is also a hematopoietic stem cell marker in the human, but not the mouse, highlights the bulge cells specifically within the cutaneous epithelium. Although it is also expressed by cells in the dermis, it is a cell surface protein, and antibodies recognizing CD34 were used to collect viable bulge cells by fluorescent activated cell sorting (Trempus *et al*., 2003; Blanpain *et al*., 2004). Keratin 15 expression in human bulge cells was first described by Lyle *et al*. (1998) (Figure 3). K15 mRNA and protein are reliably expressed at high levels in the bulge, but lower levels of expression can be present in the basal layers of the lower follicle ORS and the epidermis, thus the use of K15 expression as the sole criterion for defining a bulge cell is not advisable (Lyle *et al*., 1998; Waseem *et al*., 1999). K15 expression in the epidermis is prominent in neonatal mouse and human skin but decreases with age (Liu *et al*., 2003; Webb *et al*., 2004). However, a K15 promoter used for generation of transgenic mice possesses a pattern of activity restricted to the bulge in the adult mouse (Liu *et al*., 2003). This proved to be a powerful tool for studying bulge cells, and will be discussed below.

A salient feature of the bulge cells is their quiescence. In both adult mouse and human skin grafted to immunodeficient mice, the administration of nucleoside analogs, such as tritiated thymidine or bromodeoxyuridine, which are taken up by cells in S-phase, does not result in labeling of the bulge cells except at anagen onset (Ito *et al*., 2002, 2004). Once labeled as either neonates or during anagen onset, when stem cells are proliferating, bulge cells can remain labeled for 14 months in the mouse (Morris and Potten, 1999)

and at least 4 months (the longest period examined) in the human (Lyle *et al.*, 1998). This prolonged quiescence is remarkable given that the surrounding cells proliferate at a much higher rate, and suggests that bulge cells persist for the lifetime of the animal.

Once the bulge cells were identified as presumptive epithelial stem cells based on their ability to retain label, it was necessary to evaluate whether these cells possessed other expected characteristics important for stem cells. In particular, did they exhibit a high proliferative potential? Were they multipotent, generating all epithelial cell types within the follicle, as well as epidermis and sebaceous gland? Did they have a ''molecular signature of stemness'' shared with other stem cell populations? These questions were important not only from a biological perspective but also from a therapeutic one. A better understanding of the role of bulge cells in renewal of the epithelium should provide insights into wound healing, gene therapy, aging, and carcinogenesis.

In vitro assessment of proliferative potential

As stem cells are responsible for continual renewal of the tissue, they should possess a high proliferative capacity. In keratinocytes, this proliferative capability can be assayed *in vitro* by examining the clonogenicity of individual cells through serial passage. Approximately 5% of adult epidermal basal cells possess a ''holoclone'' phenotype characterized by high reproductive capacity and low level of terminal differentiation, and these cells are thought to represent stem cells (Barrandon and Green, 1987). Another indicator of proliferative capability is colony-forming efficiency (CFE; colonies per number of cells plated) that is thought to correlate to the number of stem cells in a tissue (Jones and Watt, 1993; Kobayashi *et al.*, 1993). These types of analyses revealed that the epidermis possesses three major types of cells with respect to their proliferative capacity. Terminally differentiated cells have no proliferative capacity, TA cells have a limited capacity (forming abortive small colonies) and epidermal stem cells have clonogenic capacity (Jones and Watt, 1993; Jones *et al.*, 1995). For the epidermis, the number of colony-forming units is likely representative of the stem cell population, although direct proof, through lineage analysis, that all central cells within the EPU are holoclones is lacking.

Similar analyses have also been applied to the hair follicle epithelium with mixed and sometimes contradictory results. In rat vibrissae, CFE analysis localizes the large majority (>95%) of clonogenic cells to the hair follicle bulge (Kobayashi *et al.*, 1993). However, in human hair follicles, CFE was reported greatest outside of the bulge region, in the lower ORS (Rochat *et al.*, 1994). This was surprising since this portion of the follicle undergoes degeneration during catagen, and these data are not in line with *in vivo*, label-retaining studies that localize LRC to the human hair follicle bulge (Lyle *et al.*, 1998). Possible explanations for the culture results are that current culture conditions actually support propagation of TA cells in addition to hair follicle stem cells or that culturing keratinocytes leads to ''reprogramming'' events that overestimates the number of true stem cells within a tissue.

Some of the discrepancies are explained by studies on vibrissa follicles, which show that the stage of the follicle at the time of isolation influences how well cells grow in culture (Oshima *et al.*, 2001). Since human hair follicles are not synchronized in their growth relative to one another, and since the anagen stage in human follicles lasts for many years, each isolated anagen follicle that was analyzed for CFE may have yielded very different results (Rochat *et al.*, 1994). For example, a follicle that has been in anagen for only 1 month might produce a greater number of colonies from the bulge compared to one in anagen for 6 years, which may have more colonies from lower portions of the follicle.

Other types of *in vitro* studies based on epithelial outgrowths from explanted follicles have also produced conflicting results. Whole follicle explant cultures give rise to epithelial growths from the ORS (Yang *et al.*, 1993; Moll, 1996). In one study, the outgrowths were seen in the area believed to represent the bulge of human hair follicles (Yang *et al.*, 1993). In another, many outgrowths were seen in the upper central ORS, which was believed to be lower than the bulge, but which likely includes the lower portion of what is the bulge (Moll, 1996). Again, these differences may be related to the heterogeneity of anagen in human follicles, or simply to the inability to recognize the bulge in adult follicles. More recently, however, Roh *et al.* (2005) decreased the influence of the hair follicle cycle by concentrating on telogen follicles, which possess a well-defined bulge area. They demonstrated major differences *in vitro* between bulge cells isolated from telogen follicles and bulb cells isolated from anagen follicles. The bulge cells possessed a higher proliferative potential than the bulb cells, which possessed a very mobile phenotype demonstrated by time lapse photography (bulb keratinocytes migrated 0.7 μm/min compared to 0.1 μm/min for bulge cells). These data support the idea that bulb keratinocytes are TA cells and possess a finite proliferative potential. This concept was originally proposed as an explanation for why anagen follicles eventually enter catagen (Cotsarelis *et al.*, 1990), although this needs further testing.

To complicate matters, recent data using CFE and holoclone analysis remain somewhat mixed, although most of the data support the idea that the bulge cells possess a high proliferative potential. Isolated bulge cells grown on feeder cells *in vitro* formed more numerous colonies than non-bulge keratinocytes (Trempus *et al.*, 2003). Morris *et al.* (2004) showed that isolated bulge cells formed larger and more numerous colonies than non-bulge basal keratinocytes. Blanpain *et al.* (2004) reported that the large majority of holoclones originate from the bulge in the adult mouse, while basal epidermis did not form any holoclones, although both of these populations had similar CFE. Given that similar culture conditions were used for all three studies, these findings

may reflect the inherent variability and difficulty in interpreting *in vitro* proliferation studies (Joseph and Morrison, 2005). Furthermore, since the bulge cells are enriched for stem cells, while the basal epidermis to which bulge cells are compared, possesses stem, TA, and even differentiated cells, one would expect a higher percentage of holoclones from the bulge. Thus, the utility of this type of analysis seems limited.

Multipotent bulge cells?

If the stem cells of the hair follicle are located in the bulge, then these cells should give rise to all of the lower hair follicle epithelial cell types. Early evidence supporting the concept that bulge cells generate the lower follicle includes proliferation studies showing that bulge cells preferentially proliferate at anagen onset (Wilson *et al.*, 1994; Ito *et al.*, 2004). More convincing evidence suggesting that bulge cells are the origin of the lower follicle arose from *in vivo* labeling studies and transplantation studies. Taylor used a double-labeling technique to trace the progeny of bulge cells in intact pelage follicles (Taylor *et al.*, 2000). Faint labeling evident as a ''speckled'' pattern was found in some cells of the lower follicle, suggesting that these cells had indeed originated in the bulge. Similarly, Tumbar *et al.* (2004) used persistence of GFP label as an indication that lower epithelial cells were progeny of the bulge cells. Neither study provided convincing evidence that all hair matrix keratinocytes in the bulb originated from bulge cells, and both suffered from inability to permanently mark bulge cells and their progeny. Oshima *et al.* took a different approach and transplanted bulge regions from vibrissa follicles isolated by dissection from ROSA26 mice into non-ROSA follicles that were then grafted under the kidney capsule of an immunocompromised mouse. ROSA26 mice express *lacZ* under the control of the ubiquitous ROSA promoter, thus the fate of the transplanted cells could be followed. After several weeks, the labeled bulge cells had migrated down the vibrissa follicle and at later time points, some follicles expressed *lacZ* in all epithelial cell layers of the lower follicle. These elegant studies were limited because of the unclear starting cell population, the manipulation performed during grafting, and the use of vibrissa follicles, which are markedly different than other mouse and human follicles.

More definitive evidence for bulge cell multipotency *in vivo* was reported using the K15 promoter to target these cells with an inducible Cre (CrePR1) construct. CrePR1 is a fusion protein consisting of Cre-recombinase and a truncated progesterone receptor that binds the progesterone antagonist, RU486 (Berton *et al.*, 2000). In *K15-CrePR1* transgenic mice, CrePR1 remains inactive in the cytoplasm of the K15-positive cells except during RU486 treatment, which permits CrePR1 to enter the nucleus and catalyze recombination. *K15-CrePR1* mice were crossed with *R26R* reporter mice that express *LacZ* under the control of the ubiquitous ROSA26 promoter after Cre-mediated removal of an inactivating sequence. Transient treatment of adult *K15-CrePR1;R26R* mice with RU486 results in permanent expression of *LacZ* in the bulge cells and in all progeny of the labeled bulge cells (Morris *et al.*, 2004). From this approach, it is clear that cells originating in the bulge generated all epithelial cell types in the lower hair follicle (Morris *et al.*, 2004).

Although these studies convincingly showed that the bulge cells as a whole gave rise to the lower follicle, none addressed the question of how many stem cells participate in the formation of the new anagen follicle or at what point bulge cells or their progeny are committed to specific lineages (Kamimura *et al.*, 1997; Ghazizadeh and Taichman, 2001; Kopan *et al.*, 2002). Several studies have examined lineage within the follicle using reconstitution assays in which isolated keratinocytes are combined with neonatal dermal cells and grafted onto nude mouse skin (Weinberg *et al.*, 1993). The mixture then forms hair follicles. By using a combination of keratinocytes labeled with a retrovirus encoding alkaline phosphatase plus nonlabeled keratinocytes for the epithelial component of the assay, then examining the proportion of labeled cells within the follicle, an indication of the number of clones within a follicle can be inferred (Kamimura *et al.*, 1997; Topley *et al.*, 1999). Dotto and co-workers suggested that the majority of reconstituted follicles arose from three different clone types: one each for the ORS, inner root sheath and hair shaft. Other combinations were also present, including some follicles with partial labeling within one of the layers. Only rarely were entire follicles labeled indicating that they may have arisen from single cells. The limitations of the reconstitution studies include the manipulation of the cells required for the reconstitution of the follicle, which may lead to ''reprogramming'' events; the resulting follicles lack normal orientation and often grow at right angles to each other – this limits the ability to accurately orient the follicles in tissue sections and makes analysis of clones difficult; the growth cycle of the follicles is not synchronized making the evaluation of the hair cycle almost impossible. This especially limits evaluation of the bulge, which is most evident during telogen. Lastly, silencing of the reporter construct or its inadequate expression in follicle subpopulations may complicate matters as well.

Kopan and *et al.* (2002), using different strategies involving chimeric mice, presented evidence for approximately four different clones within a follicle, but each with the ability to generate all follicular epithelial cell types. These chimeric studies were also limited by manipulation during embryogenesis. Related studies by the same group looked at X-inactivation in adult mice, and the findings support the concept that hair follicle stem cells are multipotent, but these studies were limited by the use of vibrissa follicles during the neonatal time period. Another recent study, examining Cre mice exhibiting recombination events of low frequency within their cells, also recognized several different types of clones responsible for different follicle layers (Legue and Nicolas, 2005). None of these three studies assessed clones within the bulge during telogen, thus the question of how many and which

bulge cells participate in hair follicle formation at anagen onset remains unanswered.

Ultimately, formal lineage analysis, perhaps using techniques as in the brain (Zong *et al*., 2005), will be needed to elucidate the exact contribution of individual bulge cells to the different lineages within the follicle. Questions remaining include: At what point, if any, are stem cells ''set aside'' during development? When are hair follicle lineages established? That is, are individual bulge cells committed to a specific lineage or is each bulge cell multipotent? How many bulge cells are required to generate a new lower anagen follicle, and is the number different based on the size and type of follicle? The answers may be important for designing cell-based treatments for alopecia (Stenn and Cotsarelis, 2005).

Plasticity of bulge cells?

Intriguing results suggest that cells isolated from the mouse hair follicle bulge area have the potential to differentiate into multiple different nonepithelial tissues, including Schwann cells and neurons (Amoh *et al*., 2005a, b). These cells were isolated from nestin-eGFP transgenic mice (Li *et al*., 2003). Nestin is a neural stem cell marker. The exact origin of the nestin positive cells, and whether they represent a single or mixed population of cells, needs to be addressed. As nestin was not differentially expressed in mouse or human bulge cells (Morris *et al*., 1986; Tumbar *et al*., 2004; Ohyama *et al*., 2006), it is unlikely that these cells represent keratinocytes. However, regardless of their origin, nestin positive cells from the skin possess remarkable regenerative capabilities when used in a nerve injury model (Amoh *et al*., 2005a). Similar *in vitro* plasticity findings using cells isolated from human follicles were also reported (Yu *et al*., 2006); thus, the follicle could serve as a source of cells for a variety of tissue engineering applications in the future.

Bulge cells: the ultimate cutaneous epithelial stem cells?

In addition to the role of the bulge cells in the formation of the hair follicle at anagen onset, there is a question of whether bulge cells are necessary for the homeostasis of the epidermis and sebaceous gland. One view is that bulge cells continuously provide progeny that repopulate these tissues (Lavker and Sun, 2000; Taylor *et al*., 2000). If true, then ablation of the bulge should lead to failure of epidermal renewal. Recent experiments show that loss of bulge cells does not lead to loss of the epidermis, suggesting that the epidermis possesses stem cells capable of renewing itself for long periods (Ito *et al*., 2005). Other studies have shown long-term persistence of clones within the epidermis. These studies utilized reconstitution assays (Kamimura *et al*., 1997; Ghazizadeh and Taichman, 2001). Some evidence was provided that hair follicle derived cells did move into the epidermis, especially adjacent to the hair follicle, but it was not clear that these cells were bulge derived. Morris *et al*. (2004) and Tumbar *et al*. (2004) did find that bulge cells can move into the epidermis, but this was not systematically studied using lineage analysis. Recent work in which isolated bulge keratinocytes were injected into neonatal mice, then followed, showed that bulge cells contributed to the epidermis after engrafting, but eventually no cells arising from the injected cells were found in the epidermis implying lack of movement of bulge cells to the epidermis (Claudinot *et al*., 2005).

Studies directly addressing the question of whether and when bulge cell progeny migrate to the epidermis have recently been accomplished (Ito *et al*., 2005). Using the K15CrePR;R26R transgenic mouse, bulge cells were labeled in 3-week-old mice. Mice were followed for 6 months to determine the movement of cells derived from the bulge into the epidermis. At the end of the experiment, there was no evidence that bulge derived cells had migrated to epidermis. These findings were confirmed using a different (sonic hedgehog) promoter active in the bulge and lower follicle (Levy *et al*., 2005). Thus, under homeostatic conditions, the epidermis alone is responsible for its continual renewal. These findings are important for gene therapy since it will be important to target stem cells within the epidermis to correct genetic disorders such as epidermolysis bullosa.

Role of bulge cells in wound healing

The contribution of the hair follicle to healing of the epidermis following wounding has been appreciated for decades by investigators working with mice and rabbits (Argyris, 1976; Taylor *et al*., 2000; Tumbar *et al*., 2004). Clinicians are also well aware that keratinocytes emerge from the follicle to repopulate wounds. However, the role of the bulge cells in wound healing has only recently been characterized (Ito *et al*., 2005).

Bulge cell progeny migrate to the epidermis after different types of wounding. Using the K15CrePR;R26R transgenic mouse, bulge cells were labeled in adult mice (Ito *et al*., 2005). Excisional wounding with a 4 mm (punch) trephine resulted in the migration of bulge cell progeny into the healing epidermis. At least 25% of the newly formed epidermis originated from the bulge cells. Bulge cells were also stimulated to move into the epidermis following incisional wounds and after tape stripping, indicating that bulge cell activation plays a role in replenishing lost cells from the epidermis after wounding. Surprisingly, however, despite the presence of bulge-derived cells in the basal layer of the reepithelialized epidermis, the majority of the bulge-derived cells did not persist in the regenerated epidermis. This suggests that bulge cells and epidermal stem cells are intrinsically different in that epidermal-derived cells seem better suited for establishing long-term EPUs (Figure 4).

Role of bulge cells in tumorigenesis

Since epithelial stem cells are thought to have a lifespan at least as long as that of the organism, they are thought to be susceptible to multiple genetic ''hits'', which cumulatively may result in tumor formation (Perez-Losada and Balmain, 2003). A great deal of evidence in the mouse system points to hair follicles and stem cells as the origin of many skin tumors (Stenbach, 1980; Morris *et al*., 1986). Keratinocyte stem cells appear to be the target of

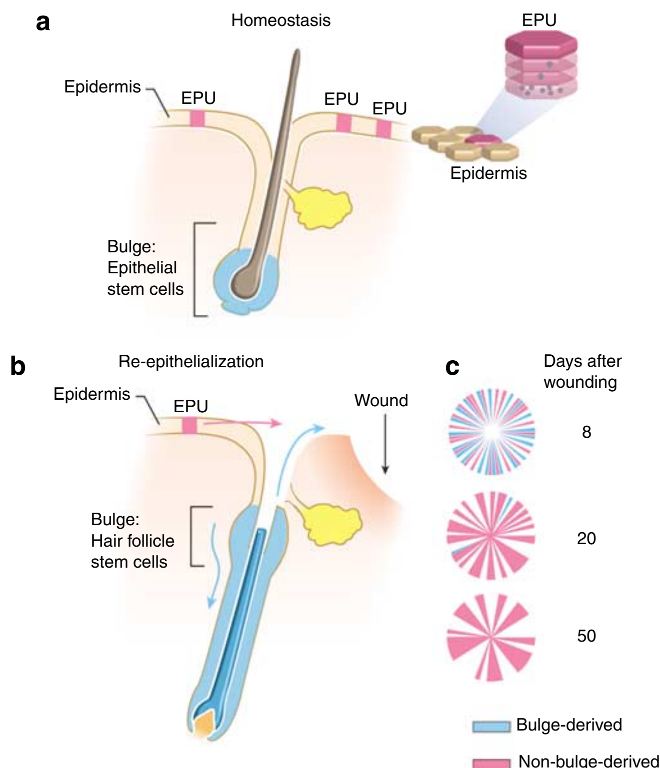

Figure 4. Model for relationship between epidermal and hair follicle stem cells during homeostasis and after wounding. (**a**) During normal conditions, epidermal renewal is dependent on cell proliferation within EPUs, which are clonal populations of cells roughly arranged in hexagonally shaped columns that produce a single outer squame. Epithelial stem cells in the hair follicle bulge do not contribute to epidermal renewal. (**b**) Following full thickness wounding, bulge cells contribute cells to the epidermis for immediate wound closure (blue upward arrow). Bulge cells also are required for hair follicle cycling (blue downward arrow) (**c**). Over time, bulge-derived cells diminish while nonbulge derived cells appear to predominate in the re-epithelialized wound.

cutaneous carcinogens, and since they are the slowest cycling cells, hair follicle stem cells also retain carcinogens for extended periods and are thus more susceptible to tumor promotion (Morris, 2000).

Since human basal cell carcinomas (BCCs) are slowly growing tumors, composed of poorly differentiated cells with the ability to differentiate into various adnexal structures, it has been suggested that the cell of origin is a multipotent stem cell that is slowly cycling and has a high proliferative potential (Cotsarelis *et al*., 1990). There is considerable evidence that BCCs may arise from stem cells within the hair follicle (Hutchin *et al*., 2005). BCCs have been shown to express bcl-2 (Verhaegh *et al*., 1997), a marker of the permanent portion of the hair follicle, including the bulge (Stenn *et al*., 1994). Furthermore, the overexpression of sonic hedgehog (*Shh*) in mouse epidermis causes BCC-like tumors from invaginating hair follicles, but has little effect on interfollicular epidermis (Oro *et al*., 1997), again suggesting a follicular origin for BCCs (Adolphe *et al*., 2004). The *Shh* signaling pathway, including *ptc*, is active in hair follicle morphogenesis during fetal development (Millar, 2002).

As trichoepitheliomas (TEs), a benign hair follicle tumor, and BCCs have a similar clinical and histological appearance, and may develop concurrently in some patients, it has been proposed that these tumors arise from a common precursor cell type within the hair follicle (Headington, 1976). Biochemically, both TEs and BCCs express cytokeratins 5, 6, 14, 17, and 19 (Schirren *et al*., 1997a, b); a profile found in the infrainfundibular ORS cells of the normal hair follicle, in fetal germinative cells, and in the fetal hair follicle bulge. In addition, in one study, 13 of 13 TEs and about one-third of BCCs expressed K15 suggesting they originate from bulge cells (Jih *et al*., 1999). The findings that both sporadic TEs and BCCs contain mutations of the *patched (ptc)* gene (Gailani *et al*., 1996; Johnson *et al*., 1996) provide compelling evidence that these tumors share a common tumorigenic mechanism and are linked to the hair follicle. The normal expression of *ptc* in the developing hair follicle and the presence of *ptc* mutations in sporadic TEs as well as both hereditary and sporadic BCCs suggest that these tumors arise from a common cell type within the hair follicle. The examination of TEs, BCCs, and other hair follicle tumors with additional markers for hair follicle stem cells should lead to insights into the role stem cells play in formation of these tumors.

Stem cells and alopecia

Alopecias can be classified into cicatricial and non-cicatricial types (Olsen *et al*., 2003). The localization of hair follicle stem cells to the bulge area may explain why some types of inflammatory alopecias cause permanent follicle loss (such as lichen planopilaris and discoid lupus erythematosis), while others (such as alopecia areata) are reversible (Paus and Cotsarelis, 1999). In cicatricial alopecias, inflammation involves the superficial portion of the follicle, including the bulge area, suggesting that the stem cells necessary for follicle regeneration are damaged (Mobini *et al*., 2005). The inflammatory injury of alopecia areata, however, especially in early lesions, involves the bulbar region of the hair follicle that is composed of bulge cell progeny (Whiting, 2003). Because this area is immediately responsible for hair shaft production, its destruction leads to hair loss. However, the bulge area remains intact, and a new lower anagen follicle and subsequent hair shaft can be produced. Even patients with alopecia areata for many years can regrow their hair either spontaneously or in response to immunomodulation.

The bulge may actually be targeted by inflammation in androgenetic

alopecia (common baldness) as well. Jaworsky *et al*. (1992) showed that in patients with early androgenetic alopecia, inflammatory cells localize to the bulge (Jaworsky *et al*., 1992). Over time, this damage could contribute to the irreversible nature of androgenetic alopecia as well. The bulge area appears specifically attacked in early graft-versus-host disease, which can cause alopecia (Murphy *et al*., 1991). These findings are also consistent with the idea that the bulge and lower hair follicle are ''immune privileged'' since they express low levels of MHCI.

Bulge cells for tissue engineering

An exciting approach for the use of hair follicle stem cells in the treatment of alopecia includes tissue engineering (Stenn and Cotsarelis, 2005). In one scenario, isolated hair follicle stem cells could be used for generating new follicles in bald scalp. For this to occur, isolated bulge cells must be capable of generating new hair follicles. At least two groups have shown that freshly isolated bulge cells from adult mice, when combined with neonatal dermal cells formed hair follicles after injection into immunodeficient mice (Blanpain *et al*., 2004; Morris *et al*., 2004). These studies provided proof of concept that isolated stem cells could be a part of tissue-engineering approaches for treating alopecia.

A goal for treating alopecia with cell-therapy approaches includes increasing the number of existing follicles, for example, by amplifying keratinocyte and dermal papilla cell numbers *in vitro* prior to transplantation. Cultured keratinocytes from neonatal epidermis have been used for many years to generate hair follicles in reconstitution assays. More recently, freshly isolated bulge cells from adult mice were shown to form hair follicles in skin reconstitution assays (Morris *et al*., 2004). Importantly, cultured, individually cloned bulge cells from adult mice also were shown to form hair follicles in skin reconstitution assays (Blanpain *et al*., 2004). However, the ratio of new follicles formed from the number of donor follicles, and whether non-bulge keratinocytes also possessed these properties were not analyzed.

The use of hair follicle stem cells for tissue engineering approaches will depend on isolation and characterization of human hair follicle stem cells. A major advance in this direction was reported by Ohyama *et al*. (2006). In this work, cell surface markers, including CD200 and FRIZZLED receptor were identified on human bulge cells by using laser capture microdissection and microarray analysis for gene expression. A cocktail of antibodies against cell surface proteins was devised allowing for isolation of living hair follicle bulge stem cells, thus the stage is set for isolating human hair follicle stem cells to address biological questions relevant to diseases of the follicle, and eventually for using these cells for therapeutic purposes.

Molecular profile-stem cell phenotype

Defining the stem cell phenotype at the molecular level is important for several reasons. Quiescence is a hallmark of these cells. Understanding the genes that distinguish bulge cells from proliferating TA cells, as well as the genes that convert resting bulge cells to growing cells, bodes well for gaining insights into the uncontrolled proliferation in cancer cells as well as the precisely orchestrated events of hair follicle formation at anagen onset. With the advent of microarrays, large-scale comparisons of gene expression in bulge cells versus non-bulge basal keratinocytes could be performed (Morris *et al*., 2004; Tumbar *et al*., 2004). These studies, in which bulge cells were isolated by two very different techniques, resulted in reassuringly similar results. At least 60% of the genes reported as differentially expressed by the two groups were the same. Both studies found that genes involved in activation of the WNT pathway were generally decreased, while inhibitors of this pathway were increased relative to non-bulge basal keratinocytes. These findings are in line with studies indicating the importance of WNT activation for anagen onset (Millar, 2002; Van Mater *et al*., 2003). It is worth noting that large-scale gene expression studies have been successful in transgenic mice; however, the relevance of the findings to human hair and stem cell biology has only recently been tested (Ohyama *et al*., 2006). These investigators found many similarities to the mouse studies, thus validating the mouse as a useful model for studying human hair growth. However, very important differences were also described (Cotsarelis, 2006). In particular, CD34, which serves as a mouse bulge cell marker, is not expressed by human hair follicle bulge cells (see also Figure 3). These studies are a major step in our understanding of human hair follicle bulge cells.

Regarding genes that may maintain the stem cell phenotype, functional studies demonstrate that Rac-1 plays an important role in the self-renewal of the epidermis and hair follicle (Benitah *et al*., 2005; Dotto and Cotsarelis, 2005). Loss of Rac-1 causes a burst of proliferation in epidermal keratinocytes and then synchronized differentiation and loss of proliferative capability resulting in thinning of the cutaneous epithelium. Thus, this gene suppresses proliferation and differentiation, and seems important for the switch from stem cell to TA cell.

The bulge as stem cell niche

Keratinocytes within the bulge area may depend on their environment or niche for maintaining their stem cell characteristics. The bulge area also houses melanocyte stem cells, which are normally quiescent but proliferate at anagen onset to repopulate the new lower anagen hair follicle with melanocytes that generate melanin leading to pigmentation of the hair (Nishimura *et al*., 2002). Intriguingly, there is evidence that the follicle, if not the bulge, serves as a reservoir for immature Langerhans cells as well as other immunocytes (Gilliam *et al*., 1998; Kumamoto *et al*., 2003). The role of the bulge environment in maintaining cells of different lineages in a relatively undifferentiated state or instructing (educating?) immune cells needs further study.

Summary

The bulge begins as a prominent structure during human fetal develop-

ment, but then becomes less obvious; thus hindering its study. Markers for bulge cells, such as CD34 expression and K15 promoter activity, allowed for the isolation and characterization of these cells in mouse and human follicles. Important parallels and differences between mouse and human bulge cell gene expression validate the use of the mouse as a model for studying human hair follicle stem cells, but also indicate the need to study human hair follicles and disease directly. Propagation of bulge cells *in vitro* maintains their stem cell characteristics; although whether this is an exclusive property of bulge cells remains a question. Under normal homeostatic conditions, epidermal stem cells and hair follicle stem cells constitute two distinct populations. Understanding the differences between these two types of stem cells, as well as the signals important for directing differentiation of these cells into different lineages will be important for developing treatments based on stem cells as well as clarifying their role in skin disease.

CONFLICT OF INTEREST

The author states no conflict of interest.

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