Hair Follicles, Stem Cells, and Skin Cancer

Stanley J. Miller, Tung-Tien Sun, and Robert M. Lavker

The epidermal portion of the skin consists of the epidermis and its appendages. These epithelia can give rise to a wide spectrum of tumors, naturally or experimentally; however, owing to the topographic proximity of the several normal skin epithelia (e.g., epidermis, sweat duct, hair follicle), the cellular origin of many of these non-melanoma skin cancers are unknown. In this report we examine the role of the hair follicle or ''pilosebaceous unit,'' as opposed to the interfollicular epidermis, in the development of non-melanoma skin cancer.

The majority of experimental skin carcinogenesis work has been performed in animals, especially mice. Early work used complete carcinogenesis protocols on strains of inbred Swiss mice; however, more recent studies have utilized two-stage carcinogenesis protocols and SENCAR mice that were bred for their extreme sensitivity to the initiator 7,12-dimethylbenz(a)antracene (DMBA) and the promoter 12-0-tetra-decanoylphorphol-13-acetate (TPA) [1,2]. Rabbits and rats have also been used. Because different results are frequently obtained from these different animals, it is important to bear in mind potential species differences.

Three broad theoretical constructs underlie modern thinking about skin carcinogenesis. The first is our current understanding of how organized populations of cells, such as the epidermis, reproduce themselves (reviewed in [3,4]), with ''stem cells,'' ''transient-amplifying cells,'' and ''terminally differentiated cells'' representing different stages in the mixed process of reproduction and differentiation. The second is our current concept of two-stage carcinogenesis (reviewed in [5–7]), which involves separate steps of initiation, promotion, and conversion. A third assumption in modern carcinogenesis thinking relates to the role of hair follicles in skin cancer formation. Work with two-stage chemical carcinogenesis protocols [8,9] has suggested that the act of initiation is irreversible. Initiated animals can be promoted months to years after the initial insult, and the yield of tumor production does not diminish significantly. This has led to the generally accepted notion that tumor initiation must involve primarily a population of long-lived cells (i.e., the stem cells). We emphasize, however, that this assumption, reasonable as it may seem, has not yet been rigorously proved, because we are unable to distinguish, morphologically or by any other means, the stem cells from cells that have already existed from the stem-cell compartment. In this regard, the question of the role of interfollicular epidermis versus follicular epithelium in skin cancer production becomes really a question of where the skin's stem cells are located and which ones are most sensitive to carcinogenic effects.

Much of the evidence suggesting a role for hair follicles in skin cancer formation comes from experiments performed in the 1950s and 1960s. Recent re-assessment of epidermal stem-cell location has raised new interest in this issue. Four lines of research suggest the importance of the hair follicle in skin carcinogenesis. Each is examined individually.

HAIRED AND HAIRLESS SKIN

Some of the earliest work suggesting a role for hair follicles in skin cancer development compares tumor development in haired versus hairless mouse skin. In a pioneering study in 1946, Lacassagne and Latarjet [10] created scars on the backs of newborn and adult mice with ultraviolet radiation or excision. In those scars that healed without hair follicles, tumors did not appear after treatment with 3-3 methylcholanthrene (MCA). Only the periphery of scars, where hair follicles were present, developed ulcerated tumors, which underwent histologically verified malignant transformation. Histologically, the border region from which the papillomas appeared to arise most frequently showed a persistent, marked epithelial and follicular hyperplasia. The authors concluded that hair follicles appear to play an important role in the genesis of skin tumors.

Similar studies over the subsequent 25 years examined tumor development in strains of haired and hairless mice and in newborn mice before and after hair development. Contradictory results were obtained, probably because rudimentary follicular structures were actually present in many of the hairless mice (reviewed in [11]). To circumvent earlier errors of experimental design, Giovanella *et al* [11] compared tumorigenesis in strains of hairless versus haired (Swiss) mice using a two-stage protocol of DMBA initiation to the backs of mice, followed by croton oil promotion at weekly or biweekly intervals. In newborn mice, when skin of the hairless strain still contains follicles, all treated animals developed papillomas and carcinomas: 70 haired mice developed 66 papillomas and 11 carcinomas, and 34 hairless mice developed 34 papillomas and three carcinomas. However, in adults a clear differential was noted in that 13 haired mice developed 87 papillomas and three carcinomas, whereas 44 ''hairless'' mice developed only seven papillomas and no carcinomas. Thus, Giovanella *et al* [11] suggested that hairless mice were refractory to chemical carcinogenesis. Iversen and Iversen [12] disputed this concept, as they demonstrated by using Oslo hr/hr hairless mouse that carcinomas could be induced by using a complete carcinogenesis protocol of a single application of MCA. Several factors should be considered in trying to evaluate these disparate findings: 1) different strains of hairless mice were used that might have different sensitivities to the chemical carcinogens; 2) a two-stage protocol was compared with a one-stage protocol; 3) Giovanella *et al* [11] used acetone as a vehicle, whereas Iversen and Iversen used benzene [12], which is itself a mild carcinogen; and, finally, 4) animals of different ages were used by these two groups.

SKIN GRAFTING

A second line of evidence implicating hair follicles in skin carcinogenesis comes from skin-grafting work with mice performed in the 1950s and 1970s. Billingham *et al* [13] in 1951 performed an interesting series of experiments transplanting skin grafts of several thicknesses from carcinogen-treated sites on mice to untreated sites on the same mice, and vice versa. Epidermal grafts contained 1) epidermis only; 2) thin ''Thiersch grafts'' that included epidermis and papillary dermis but rarely sebaceous glands; 3) thick Thiersch grafts that included sebaceous glands but no hair bulbs; or 4) pinch grafts that included epidermis and dermis as well as underlying adipose and muscle layers. Carcinogen treatments consisted of 0.3% MCA applied once weekly for 12 weeks, followed by a

Department of Dermatology (SJM, RML), University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; and The Ronald O. Perelman Department of Dermatology and Pharmacology (T-TS), New York University School of Medicine, New York, New York, USA

Reprint requests to: Dr. Robert M. Lavker, Department of Dermatology, University of Pennsylvania School of Medicine, Clinical Research Building, Room 235A, 422 Currie Boulevard, Philadelphia, PA 19104.

Abbreviations: BCC, basal cell carcinoma; BP, benzo(a)pyrene; DMBA, 7,12-dimethylbenz(a)anthracene; LRCs, label-retaining cells; MCA, 3-methylcholanthrene; SCC, squamous cell carcinoma; TdR, [^{3}H]thymidine; TPA, 12-O-tetradecanoylphorbol-13-acetate

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2-week rest before grafting. When normal epidermis was grafted onto carcinogen-treated dermis (the recipient site being treated with carcinogen followed by removal of its epidermis by the taking of a thin Thiersch graft), 13 of 21 animals developed eight papillomas and six carcinomas at the grafted sites. When the reverse was performed (i.e., when carcinogen-treated epidermis was grafted onto untreated dermal beds), none of the 28 animals developed tumors at the site of grafting; however, at the carcinogen-treated donor sites, 14 of the 28 animals developed six papillomas and 16 carcinomas. These first two experiments by Billingham *et al* [13] strongly implicate the lower half of the follicle (below the level of a thin Thiersch graft) as a primary source of epidermal-derived cancers.

Two additional sets of experiments attempted to further localize the sites of cancer origin. Carcinogen-treated, thick Thiersch grafts were transplanted onto untreated fascial beds devoid of epidermis, dermis, or fat. Small but equal numbers of animals developed tumors at both recipient and donor sites. At the grafted recipient sites, five of 30 animals developed three papillomas and two carcinomas, whereas at the donor sites, five developed one papilloma and five carcinomas. When an identical experiment was performed using pinch grafts, similar results were obtained. At the graft sites, three of 23 animals developed tumors, and at the donor sites, three of 23 developed tumors. It is difficult to interpret the latter pinch-grafting experiment because pinch grafts are usually thinner peripherally and thicker centrally and thus contain varying amounts of skin elements present at different depths. However, the thick Thiersch-graft work suggests that the level at which these Thiersch grafts are taken – between the sebaceous gland and the hair bulb – is a primary site for tumor origin because division at this level leads to equal numbers of tumors in the donor and recipient sites. The lower absolute numbers of tumors produced may simply reflect cellular damage, by incision at this level, to stem cells located there. This localization of tumor origin is especially interesting in light of recent work by Cotsarelis *et al* [4] in which follicular stem cells were found by the tritiated thymidine-label – retaining technique to be located in the bulge of the follicle, a structure located between the sebaceous gland and the hair bulb [14]. This subject is discussed in more detail in a following section.

An elegant recreation of the first experiment of Billingham *et al* [13] was performed by Steinmuller [15] in 1971. In this instance, untreated epidermis from one strain of mice was grafted onto previously carcinogen-treated dermis of another strain of mice. As before, 53 of 63 animals developed carcinomas at the recipient site of grafting. Histocompatibility studies of 14 of these tumors revealed that all of them had arisen from the recipient as opposed to the donor mouse strain. These results again strongly implicate follicular structures as a primary source of epidermal tumor origin. *In toto,* skin-grafting studies support a primary role for the hair follicle as a source from which the chemically induced skin tumors arise.

HISTOLOGIC STUDIES

A third way in which investigators have attempted to determine the site of origin of skin cancers has been to microscopically look for that site of origin in very early tumors in animals and in humans. Zackheim performed careful histologic analyses of chemically induced skin cancers in rats, and noted that basal cell carcinomas (BCCs) arose from follicular appendages [16], whereas squamous cell carcinomas (SCCs) arose primarily from interfollicular epidermis [17]. In studies in humans, Zackheim observed early BCCs developing from both follicular structures and interfollicular epidermis [18]. Pinkus [19] noted that actinic keratoses, a precursor lesion of SCCs, form initially in interfollicular regions and then enlarge to include follicular epidermis as well. These microscopic studies thus suggest that SCCs arise primarily from interfollicular regions, whereas BCCs develop in follicular sites, although in humans, BCCs may arise from interfollicular sites as well [20]. It is interesting to note here the difference that host species and specific carcinogenic exposure make in analyzing ''skin cancer production.'' How these differences relate to the role of pilosebaceous structures in skin cancer development is still unclear. In humans, the great majority of ''spontaneous'' skin cancers that occur are BCCs [20]. In these instances, the common carcinogenic exposure is probably to ultraviolet light from the sun; however, following chemical carcinogen exposure, tumors that subsequently develop are almost exclusively of the SCC variety [5].

In mouse skin, chemical carcinogenesis produces almost exclusively squamous papillomas and carcinomas [21]. In rats, the most common spontaneous skin tumor is the SCC [22]. Tumor development in rats following chemical exposure depends on the specific carcinogen used and, in fact, its route of application. Zackheim [22] noted that topical application of 2-aminoanthracene to rats produced primarily BCCs: 87% of animals developed BCCs, whereas only 36% developed SCCs. Topical application of MCA produced similar results: 90% developed BCCs, but only 26% developed SCCs. Topical application of DMBA, however, resulted in a majority of SCCs: 73% of animals developed SCCs, whereas only 32% developed BCCs [22]. Zackheim also observed the histologic changes that followed topical application of each carcinogen [24]. He noted that only DMBA produced a scarring alopecia. He suggested that this follicular loss might be a reason for SCCs predominating following DMBA application, as the source of origin for BCC development was gone [16]. Of note, Bennington *et al* [23] found that with intravenous DMBA application, six of 70 rats developed only tumors with pilosebaceous differentiation. Here, no scarring alopecia resulted.

Finally, in rabbits [25], investigators have noted a preponderance of keratoacanthoma-like lesions following carcinogen application. Further advances in our knowledge of stem-cell types and location will hopefully explain some of these interspecies and specific carcinogen-related differences in cancer production.

HAIR CYCLE

Clinical Results A fourth way that the possible role of hair follicles in skin cancer development has been studied is the application of carcinogens to animals during discrete phases of their hair cycle. In many animals, unlike humans, hair cycles are largely synchronous. Growth (anagen phase) occurs in waves [26–29] that spread rapidly in a head-to-tail fashion; a similar transition into the involu-tional (catagen) and resting (telogen) phases is noted. The hair ''cycle'' is repeated several times during the life of an individual animal. This synchrony makes hair cycle-dependent carcinogenesis experiments possible; however, because variations exist in the cycles of individual animals based on species, gender, litter, and age, accurate determination of phase at the time of carcinogen application is essential for appropriate interpretation of the data.

It has been known for many years that application of carcinogen to animals during different phases of their hair cycle (anagen versus telogen) leads to differences in tumor development [30]. Mice and, to a lesser extent, rabbits have been most extensively studied. Andreasen and Engelbreth-Holm [31] found a striking increase in papilloma formation when complete carcinogen was applied to the backs of mice during telogen as opposed to anagen phase. A single 0.05-ml dose of 0.5% DMBA in benzene was applied to 164 Swiss mice after determining each mouse's hair-cycle phase by skin biopsy (this often can result in a wound-healing reaction that can greatly complicate the interpretation of these results). The animals were followed for 10 weeks. Of those in telogen at the time of carcinogen application, 37 of 68 developed papillomas; of those in anagen, only six of 56 did. In addition, ''pre-cancerous lesions,'' appearing to represent regions of epilation and ulceration that were never biop-sied, developed in 87% of the telogen animals but in only 16% of the anagen ones. Borum [32] used a similar protocol with 100 Swiss mice and obtained similar results. Hair dyeing was used instead of skin biopsy to determine hair-cycle phase, and all animals were followed for 13 weeks. Forty of 45 animals in telogen phase at the time of carcinogen application developed papillomas; none of 55 animals in anagen phase did. Prolonged epilation and ulceration with subsequent scar formation following carcinogen application in telogen but not anagen phase was

again noted. Within the telogen tumors, 10 were noted to regress, and two developed into SCCs.

A third and very interesting series of experiments with mice was performed by Berenblum *et al* [33]. In two experiments, DMBA was applied as a complete carcinogen to the backs of Swiss mice, and results were similar to previous work. Seventeen of 24 animals with carcinogen applied during telogen developed papillomas after 10 weeks, whereas none of 34 anagen-applied animals did. Prolonged ulceration leading to scarring was again noted clinically only in the telogen group. In the second experiment, DMBA was applied at the onset of telogen in one group of animals and near the end of telogen in another. Differential tumor production resulted; nine of 11 early telogen applications but only two of 32 late telogen applications resulted in papilloma formation. This again suggested an important role for telogen as opposed to anagen phase in tumor formation.

These investigators performed a second set of experiments that utilized for the first time a cycle-specific, two-stage carcinogenesis protocol. Of interest, in these experiments, no difference in tumor development was seen after telogen versus anagen initiation. Hair phases were again verified by a hair-dyeing technique. In the first experiment, animals were initiated by a single application of 0.5% DMBA in benzene (a treatment that can in itself produce cycle-specific differences in papilloma formation) and were promoted 10 weeks later. After 10 weeks of promotion, using biweekly application of 0.5% croton oil in liquid paraffin, papilloma formation was 100% in both groups. In a second, similar study, 0.3% DMBA in liquid paraffin (a topical solution that is in itself unable to produce tumors after solitary application in previous experiments) was applied during a specific hair phase as an initiator and followed 2 weeks later with 20 biweekly croton oil applications for promotion. Again, 100% papilloma formation occurred in both telogen and anagen animal groups.

Superficially, Berenblum's series of experiments [33] suggest that although complete (one-step) carcinogen protocols are hair-cycle dependent, as previous studies have indicated, two-stage carcinogenesis protocols are not. However, the difference in the results of the two protocols might be interpreted in several ways. First and most simply, it is possible that the differences between the anagen and telogen initiation were obscured (considering that both animals yielded 100% papilloma formation). Second, owing to an overdose of carcinogens, none of the above-noted investigators, including Berenblum *et al* [33], followed their complete carcinogen-protocol animals for 20 weeks. Thus, one cannot rule out the possibility that tumor development in animals with anagen-applied carcinogen might occur more slowly. In other words, this equalization of tumor formation between anagen and telogen groups of animals might have occurred later, even in the one-stage protocol, but was missed because of insufficient observation time.

Experiments by Whiteley suggest that hair-cycle phase in rabbits influences not only the number of tumors that develop but their type as well. In one study [34], Shope papilloma virus was inoculated onto 14 chinchilla rabbits that had half of their quiescent back hairs artificially stimulated to enter anagen phase by hair plucking. Contrary to the mouse results, numerous rapidly growing tumors developed in anagen skin areas, but only a few slowly growing ones occurred in telogen regions. In a second experiment [35], 2% DMBA in lanolin was applied weekly for 5 months to the backs of 18 rabbits in different stages of the hair cycle. After 7 months of observation, 598 papillomas had developed, again almost all within regions of anagen skin, and 11 became SCCs. During the same time period, 296 keratoacanthoma-like ''self-healing tumors'' developed, primarily in regions of telogen follicles, and approximately 10 of these also became invasive. These data suggest that during different phases of the hair cycle, either the cell type being stimulated or the character of the stimulation itself is different.

A final caveat with regard to the influence of hair cycles on skin tumorigenesis is that growth cycles have been noted to morphologically alter not only follicular cells but also interfollicular skin cells [27,36]. In fact, the overall thickness of skin increases during anagen phase due primarily to a marked increase in the thickness of the subcutis, with a slight thinning of the epidermis and dermis; the skin thickness decreases during telogen. This means that stem-cell changes may occur in both follicular and interfollicular regions. Therefore, conclusions focusing exclusively on the role of follicles during changes in the hair cycle must be viewed with caution.

Histologic Changes Histologic changes of mouse skin after carcinogen application differ when application is made during different phases of the hair cycle. Studies of the effects of MCA application [36,37] reveal degeneration of both interfollicular epidermis and follicles in the central region of carcinogen application, with an accompanying hyperplasia of these structures peripherally. In the central region, persistent lower portions of damaged follicles develop into proliferating, cyst-like structures. Peripherally, hyper-plastic interfollicular epidermis and atypical follicular patterns involving enlargement and fusion of neighboring follicles with loss of sebaceous glands are seen. With time and especially after repeated carcinogen applications, irregularities in the maturation of the surrounding hyperplastic areas are noted. Papillomas and carcinomas develop within these hyperplastic regions [38].

Studies of hair-cycle-specific carcinogen application reveal significant differences in the ensuing histologic changes. Liang and Cowdry [36] observed that follicular alterations following an MCA application on mouse back skin are much more frequent with hairs in telogen as opposed to anagen phase. Montagna and Chase [39] found that sebaceous gland regrowth after a single MCA application occurs much more slowly when the follicles are in telogen as opposed to anagen phase. Andreasen and Borum [40] noted distinct histologic differences in mouse skin following DMBA application in telogen as opposed to anagen phase. With telogen application, a central degeneration with peripheral hyperplasia, like that described for non-cycle-specific carcinogen application above, was noted. Following anagen application, however, interfollicular epidermis and follicular outer root sheaths thickened, and sebaceous glands disappeared, but no other structural change to the follicles was noted. Argyris [41] showed that following MCA application, telogen-phase skin accumulates glycogen, but anagen-phase skin does not. *In toto,* these histologic studies indicate that carcinogen application is clinically and histologically much more destructive when applied during the telogen rather than the anagen phase. A possible explanation for this is that carcinogens are accumulated for a longer period of time in telogen follicles owing to a lack of inner root sheath, which normally seals the hair canals (see below).

Carcinogen Localization Several experimental techniques have been used to localize carcinogen within the skin immediately following topical or systemic application. Most reveal a diffuse presence that involves both follicular and interfollicular skin. Only a few studies have compared application during different hair-cycle phases.

Fluorescence studies of hair follicles [42] show MCA immediately after application to be most prominent in the sebaceous glands and in the lipid layers of keratinized epithelium, including the follicles. Over the next several days, excretion of carcinogen-containing sebum onto the surface of the skin occurs, and this is ultimately exfoliated. Berenblum *et al* [33] noted a clear difference in the persistence of DMBA when it was applied during the anagen versus telogen phases of the hair cycle. Application during the anagen phase disappeared over 1–4 d, whereas application during telogen persisted for 10 d. Berenblum *et al* [33] theorized that inactivity of sebaceous glands during telogen, with a resulting lack of a sebum flow, allows DMBA persistence during this phase and contributes to the increased tumor production with telogen application of carcinogen that they observed.

Autoradiographic localization of tritiated DMBA [43] and 3,4-benzopyrene (BP) [44] 1–72 h after application reveals a diffuse presence in the epidermis and sebaceous glands and in the follicle, above the level of the sebaceous duct. Electron microscopic examination of BP [44] and MCA [45] reveals carcinogen to be diffusely present in the nucleus and

cytoplasm along nuclear and cytoplasmic membranes and at the dermoepidermal junction. Dermal mast cells in skin to which DMBA has been applied are also labeled [46]. In an early study, Borum [47] compared the application of ^{14}C labeled DMBA during anagen and telogen phases of hair growth. A diffuse presence was noted in both instances. A very interesting, more recent, autoradiographic study was performed by Morris *et al* [48]. They observed labeled BP in mouse skin up to 1 month after application. Initially, a diffuse epidermal and sebaceous gland presence was noted; however, 1 month after application, only 2% of interfollicular epidermal cells and 4% and 5% of infundibular and external root sheath cells, respectively, were still labeled. Seventy percent of the interfollicular epidermal cells were located at the center of the so-called epidermal proliferative units, where epidermal stem cells are said to reside [3,49]. Additional application of TPA to the skin resulted in an increased mitotic rate in these labeled cells, with no displacement from the basal layer. This, too, reflects a stem-cell -like activity. When a double-labeling experiment was performed to compare BP retention with the thymidine retention (indicative of stem cells), double-labeling of nuclei in some cells in both interfollicular and follicular locations was noted. This study suggests that carcinogen is retained by slow-recycling stem cells, although a specific comparison of follicular and interfollicular sites was not made [49]. Furthermore, distribution of the BP at different phases of the hair cycle was not investigated.

Finally, detection of carcinogen-DNA adducts in mouse skin by ^{32}P- post-labeling analysis [50] reveals that 1) covalent binding of carcinogen to DNA occurs, and 2) although the majority of the adducts disappear in the 2 weeks following carcinogen application, a fraction persists for as long as 5 months. This longevity again suggests a presence in long-lived, non-differentiating cells (i.e., stem cells). Comparison of adducts by location or after phase-specific carcinogen application has not yet been done.

HAIR-FOLLICLE STEM CELLS

Experimental work thus suggests an important role for the hair follicle in skin cancer development. As mentioned previously, it is generally accepted that a subpopulation of cells having stem-cell characteristics are the target cells that interact with carcinogens and eventually become transformed [48,51–53]. Because the hair follicle is a self-renewing tissue, by definition it must contain stem cells (for a review of stem cells in general, see [54,55]). Thus, knowledge of the location and biologic properties of hair-follicle stem cells is crucial to our understanding of skin cancer/hair follicle interrelationships.

On the basis of our experience in studying epidermal [56,57] and corneal epithelial stem cells [58,59], we learned that one of the most diagnostic criteria for identifying keratinocyte stem cells is their slow cycling nature [54]. Because stem cells are slow cycling [55], a single pulse of [^{3}H]thymidine (TdR) will not be incorporated by these cells. Continuous exposure of TdR is necessary to ensure incorporation by stem cells, which rarely divide; however, tissue hyperproliferation can enhance the incorporation of TdR by stem cells [59]. Once labeled, cells that cycle slowly will retain the TdR for a long period of time and thus can be identified as label-retaining cells (LRCs) [49,60]. When we

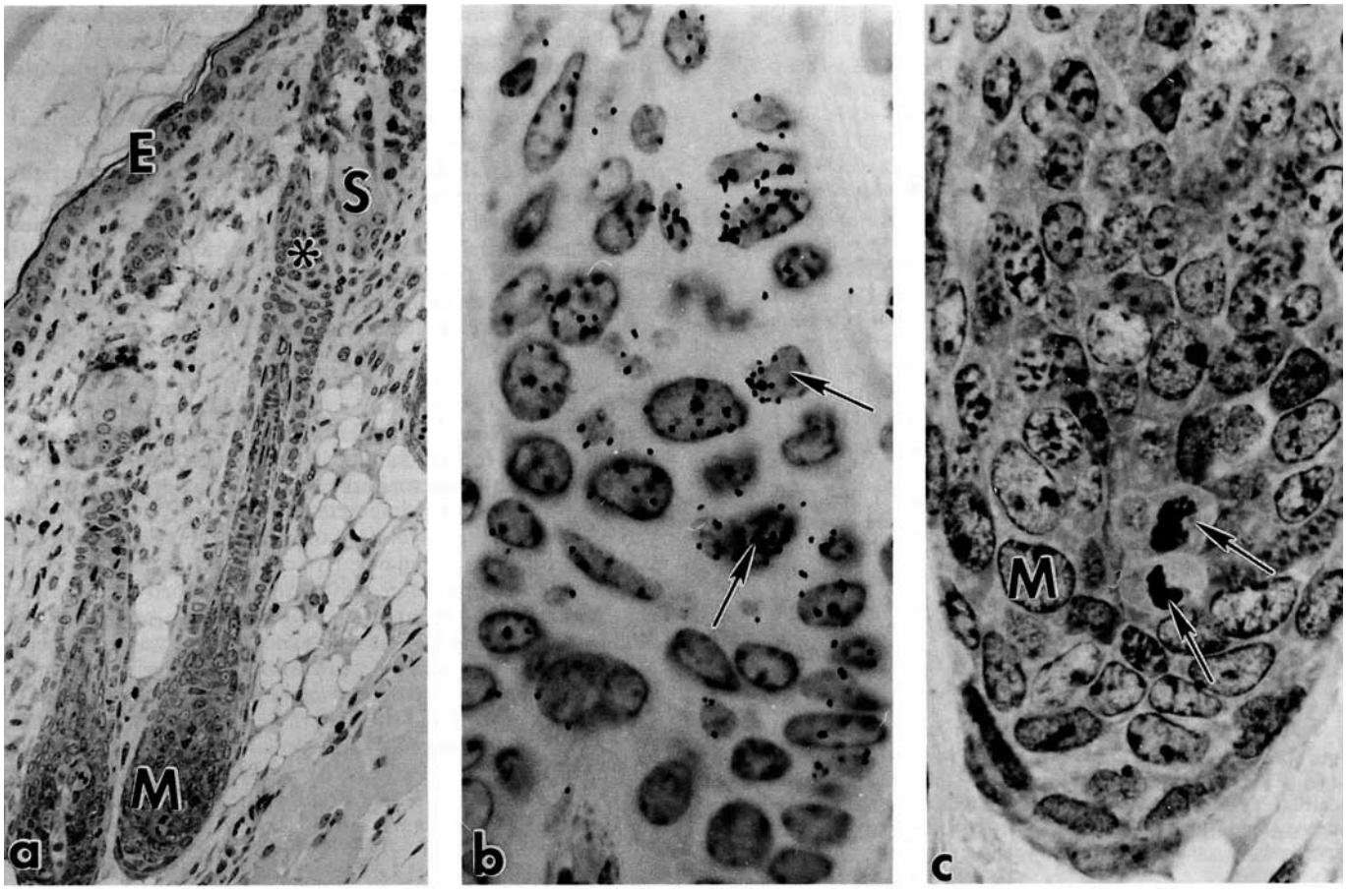

Figure 1. Localization of slow-cycling cells detected as LRCs in the mouse hair follicle, *(a)* Survey photomicrograph of murine hair follicles 4 weeks after a long-term (14 day) exposure to TdR, showing the relationship of the bulge region (*) to the matrix keratinocytes (M) of the bulb; (E) epidermis; (S) sebaceous gland, *(b)* Portion of the bulge region (shown in *a)* with cells having prominently labeled, irregularly shaped nuclei *(arrows), (c)* Portion of the bulb area (shown in *a)* with numerous mitotic figures *(arrows)* present in matrix (M) keratinocytes. Note absence of any labeled nuclei in this region.

located LRCs in the mouse pelage hair follicle, we were unable to find any slow-cycling cells in the matrix keratinocytes of the bulb (Figure la, c), which was thought to be the site of follicular stem cells [61,63]. Instead, we found a population of LRCs in the outer root sheath of the upper follicle in an area called *the bulge* (Fig 1a, b) [4].

Cells of the bulge area possess the following features: 1) this area is the insertion site of the arrector pili muscle [14], and it marks the lower end of the permanent portion of the hair follicle; 2) these cells have an ultrastructurally primitive cytoplasm [4]; 3) although they are normally slow cycling, they undergo a transient phase of cell proliferation during early anagen and can be stimulated to proliferate by hair plucking, retinoic acid, and TPA [64]; and 4) they are located in a physically well-protected and highly vascularized area. We recently obtained similar results from two specialized hairs – vibrissae and eyelash follicles [64]. A population of slow-cycling cells was localized in the outer root sheath of the vibrissa follicle at the level of the ringwulst and ring sinus; an area analogous to the bulge in pelage hairs. Similarly, in eyelash a population of LRCs was exclusively observed in the outer root sheath in a region corresponding to the bulge. Again, LRCs were not detected in the matrix keratinocytes comprising the bulb region of vibrissae or eyelash follicles, even though these specialized hairs have relatively long anagen phases. On the basis of these findings, we have proposed that the bulge was the site of the hair-follicle stem cells [4,64,65].

Additional support for this conclusion is derived from a critical reevaluation of some previous experiments whose biologic significance was unclear. For example, Oliver [66–68] demonstrated that the lower half of rat vibrissae hair follicles could be removed, and a new hair bulb could regenerate after implantation of a new follicular papilla. (Although this group of cells is customarily known as dermal papilla cells, it may be more precise to call them ''follicular papilla'' to distinguish them from the ''dermal papilla,'' which are the pegs of connective tissue that interdigitate with the epidermal rete ridges forming the dermal/epidermal junction (A. Bernard Ackerman, personal communication).) This indicates that the upper portion of the hair follicle is ''indispensible.'' Similarly, surgical removal of the lower half of human axillary hair follicles did not impede the formation of new follicles [69]. Taken together, these previous observations support our new model that the upper portion of the follicle, not the bulb region, is the site of the follicular stem cells.

The identification of putative stem cells in the upper portion of the hair follicle led us to develop a ''bulge-activation hypothesis,'' which provided for the first time a unifying concept that explains many paradoxical properties of the hair follicle. This model (Fig 2) takes into account many important events known to occur during various phases of the hair cycle [4,65]. The first tenent of this model is that during late telogen or early anagen, the normally slow-cycling stem cells of the bulge area are transiently activated, presumably by signals from the abutting follicular papilla, and proliferate. We recently showed that during the onset of early anagen in both the second and third hair cycles in SENCAR mice, cells in the bulge region indeed became ''activated'' and proliferated, as evidenced by the presence of TdR and the detection of mitotic figures in these cells. With the formation of the new epithelial downgrowth, only matrix keratinocytes were pulse labeled with TdR, whereas bulge cells returned to a quiescent state at this time [64]. We believe that the downward migration of follicular papilla cells away from the bulge area results in the return of the bulge cells to their normal quiescent state [4,65].

A second aspect of the hypothesis concerns the activation of the normally quiescent follicular papilla by the matrix keratinocytes during mid-anagen. Although the follicular papilla appears to be dormant during most phases of the hair cycle, Pierrard and de la Brassinne [70] showed that during mid-anagen, follicular papilla cells undergo transient proliferation, resulting in the formation of new blood vessels and the ''loosening up'' of the mesenchymal cells. Because matrix keratinocyte proliferation clearly precedes that of the follicular papilla, we hypothesized that matrix keratinocytes stimulate follicular papilla cells to undergo transient proliferation.

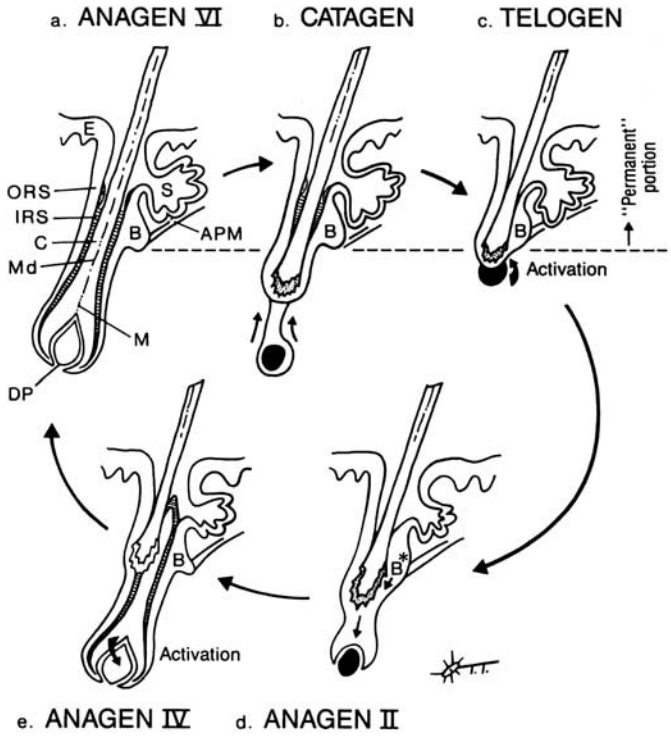

Figure 2. Bulge-activation hypothesis: (APM) arrector pili muscle, (B) bulge, (C) cortex, (DP) dermal papilla, (E) epidermis, (IRS) inner root sheath, (M) matrix, (Md) medulla, (ORS) outer root sheath, and (S) sebaceous gland. (B) and (B*) denote quiescent and activated bulge cells, respectively. (From [4]; reproduced with permission.)

Third, matrix keratinocytes are capable of TdR incorporation after a single-pulse exposure, which indicates that these cells have a relatively rapid cycle time; a feature consistent with transient amplifying cells [4,52], Because such transient amplifying cells, by definition, have only a limited proliferative potential, they eventually exhaust their replicative ability and undergo terminal differentiation. This results in the bulb undergoing a degenerative or apoptotic phase (catagen).

The fourth feature concerns the follicular papilla-epithelial interaction during catagen, when the papilla condenses and is connected to the degenerating matrix by a ''connective tissue sheath.'' The connective tissue sheath contracts during catagen, and the follicular papilla is pulled upward and eventually comes to rest in the vicinity of the bulge. A default in this upward movement can result in a failure of subsequent hair cycles, presumably due to the inability of the bulge cells to become activated [71].

CONCLUSIONS AND PERSPECTIVES

The data that we have summarized here clearly indicate that there is a close relationship among the hair cycle, skin carcinogenesis, and follicular stem cells. Many issues centering around the cellular origin and clinical relevance of experimentally induced skin tumors need to be resolved. Although it seems clear that the hair cycle (anagen versus telogen) can influence the outcome of tumor initiation and subsequent tumor yield, how this occurs is unclear. Furthermore, do tumors arising from one-stage carcinogenesis protocols behave (progress) differently than tumors developing from a two-stage protocol when the stage of the hair cycle is considered? Most important, what is the cellular origin of the tumors produced by these different protocols? Is it possible that some tumors are follicle-derived, whereas others are derived from the interfollicular epidermis? A systematic and critical reexamination of the role of the hair follicle in skin-tumor formation, using experimental approaches, is needed to answer these fundamental questions of cutaneous carcinogenesis.

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