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Morphogenesis and Renewal of Hair Follicles from Adult Multipotent Stem Cells

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Summary

The upper region of the outer root sheath of vibrissal follicles of adult mice contains multipotent stem cells that respond to morphogenetic signals to generate multiple hair follicles, sebaceous glands, and epidermis, i.e., all the lineages of the hairy skin. At the time when hair production ceases and when the lower region of the follicle undergoes major structural changes, the lower region contains a significant number of clonogenic keratinocytes, and can then respond to morphogenetic signals. This demonstrates that multipotent stem cells migrate to the root of the follicle to produce whisker growth. Moreover, our results indicate that the clonogenic keratinocytes are closely related, if not identical, to the multipotent stem cells, and that the regulation of whisker growth necessitates a precise control of stem cell trafficking.

Introduction

Skin appendages develop during fetal life following a precise spatio-temporal pattern involving complex interactions between the cells of the primitive epidermis, of ectodermic origin, and the underlaying mesenchymal cells of mesodermic origin (Hardy, 1992). Severe impairment of hair follicle morphogenesis results from mutations in developmentally regulated genes such as those encoding the transcription factors Lef-1 (Van Genderen et al., 1994) and p63 (Mills et al., 1999; Yang et al., 1999) or the signaling molecule Shh (St-Jacques et al., 1998; Chiang et al., 1999). Moreover, aberrant hair follicle morphogenesis has also been reported in mice with targeted epidermal overexpression of Lef-1 (Zhou et al., 1995), FGF-7 (Guo et al., 1993), or of a truncated β-catenin (Gat et al., 1998). Furthermore, hair follicles are constantly renewing, altering phases of growth (anagen), regression (catagen), and rest (telogen), and it is thought that the mechanisms involved during hair cycle reproduce some of the embryonic morphogenetic events (Montagna, 1962).

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Vibrissal follicles, or sinus hairs, are privileged models to study the cellular and molecular factors involved in stem cell behavior. These tactile follicles, which are the most evolved and the largest of all hair follicles, are present in many mammals and are crucial for behavior of the animal. They consist, as all hair follicles, of an epithelial core surrounded by a mesenchymal sheath, which is in continuity with the follicular papilla in the hair bulb (Figure 1A). The epithelial core is formed of several concentric layers including the outer root sheath (ORS), the inner root sheath (IRS), and the hair. Each layer itself consists of several individualized cell layers characterized by specific programs of differentiation. These layers are the outer root sheath and its companion cell layer, the IRS Henlé and Huxley's layers, the IRS cuticle, the hair cuticle, cortex, and medulla. However, vibrissal follicles differ from pelage hair follicles by the presence of vascular sinuses encompassed by a rigid capsula (Figure 1A), surrounded by bundles of striated muscle fibers (Dörfl, 1982). A whisker (or a hair) is formed of terminally differentiated hair cells generated by the proliferation of the germinative cells located in the hair matrix, i. e. in the lower part of the follicle (Figure 1B). In the rat, a whisker can grow to a length of 53 mm at a rate of 1.5 mm per day, which illustrates the extent of cell proliferation that occurs in the hair matrix (Ibrahim and Wright, 1982).

The cells located in the lowest part of the hair bulb, the so-called germinative cells, are thought to be stem cells (Reynolds and Jahoda, 1991; Hardy, 1992). However, this has been challenged (Cotsarelis et al., 1990) and there are now strong indications that stem cells reside in the upper portion of the follicle. Seminal experiments demonstrated that a new hair matrix regenerates from the upper portion of a vibrissal follicle after amputation of the follicle's lower third (Oliver, 1966). In the pelage follicle of the mouse, ^{3}HtdR- or BrdU-labeling experiments have revealed that slow-cycling cells (Label-Retaining Cells, LRC) reside in the bulge located at the level of the insertion of the arrector pili muscle (Cotsarelis et al., 1990; Taylor et al., 2000). Recently, it has been demonstrated that the progeny of the label-retaining cells of the mouse pelage follicles contribute to hair growth and participate in the closure of an epidermal wound (Taylor et al., 2000). In vibrissal follicles, the bulge region is located well below the sebaceous glands (Figure 1A) and it is not at all related to the arrector pili muscle (Vincent, 1913; Dörfl, 1982). It forms a small protuberance of the outer root sheath located in the upper portion of the follicle, easily recognizable by the presence of the sensory nerve endings. The basal layer of the bulge region rests on a thick basement membrane that separates it from the dermal sheath. The other part of the bulge region is constituted of cells forming a stratified layer that surrounds the remaining of the terminally differentiated inner root sheath and the whisker (Figure 1A). The cells located in the bulge region proliferate infrequently to the opposite of those located in the hair matrix (Lavker et al., 1991). Moreover, the bulge region contains 95% of the clonogenic keratinocytes

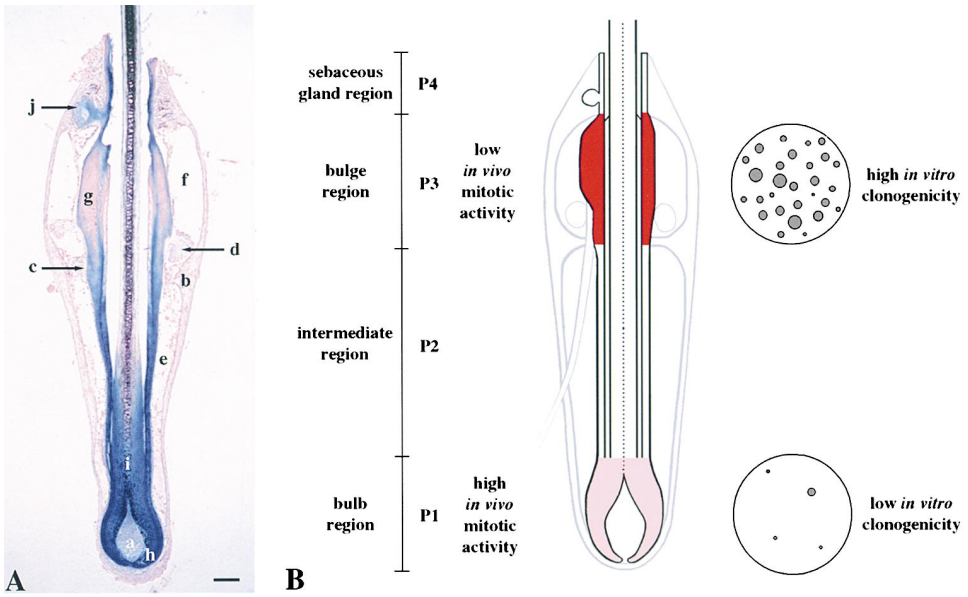

Figure 1. Location of Proliferative Cells in a Vibrissal Follicle

(A) β-galactosidase staining of a longitudinal section of a vibrissal follicle obtained from a Rosa 26 heterozygous mouse. a: follicular papilla, b: capsula, c: dermal sheath, d: ringwulst, e: lower vascular sinus, f: upper vascular sinus, g: bulge region, h: matrix, i: hair, j: sebaceous gland. Movement of the whisker results from the contraction of the bundles of the striated muscle fibers that envelop the follicle capsula (not shown on this section).

(B) Main locations of the clonogenic keratinocytes in an anagen vibrissal follicle. Note the inverse relation between mitotic activity in vivo and clonogenicity in vitro. Bar: 100 μm.

present in an anagen vibrissal follicle of the rat, whereas the hair bulb contains the remaining 5% (Kobayashi et al., 1993) (Figure 1B). Human hair follicles also contain hundreds of clonogenic keratinocytes with extensive growth potential, a characteristic of stem cells (Rochat et al., 1994), and with the capacity to form an epidermis (Lenoir et al., 1988; Limat et al., 1991). Thus, these bipotent clonogenic keratinocytes may participate, along with unipotent epidermal keratinocytes (Barrandon and Green, 1987; Jones and Watt, 1993; Jones et al., 1995), in the reconstitution of the epidermis when transplanted onto extensive third degree burn wounds excised to fascia (Gallico et al., 1984; Pellegrini et al., 1999; Ronfard et al., 2000).

To further investigate the fate of the cells located in the upper region of a hair follicle, we implanted individual fragments of bulges obtained from vibrissal follicles of adult Rosa 26 mice onto the back of mouse embryos. We report here that the upper region of the vibrissal follicle of the adult mouse contains stem cells that respond to morphogenetic signals by generating hair follicles, sebaceous glands, and epidermis. To further evaluate the role of these stem cells and that of the clonogenic keratinocytes in hair renewal, we performed clonal analyses and transplantation of different parts of vibrissal follicles obtained at different phases of the hair cycle. We demonstrate that the distribution of the clonogenic keratinocytes is greatly influenced by the hair cycle, and that the lower part of a vibrissal follicle can respond to morphogenetic signals only when it contains a significant number of clonogenic keratinocytes. Our results demonstrate that multipotent stem cells migrate to participate in whisker renewal and strongly suggest that the clonogenic keratinocytes and the multipotent stem cells are closely related, if not identical.

Results

Generation of Chimeric Follicles

Rosa 26 transgenic mice constitutively express a *lacZ* reporter gene under the control of "SV40" promoter (Friedrich and Soriano, 1991). *LacZ* expression is maintained in tissues of adult heterozygous or homozygous Rosa 26 mice, which makes them ideal donors for chimeric analyses. Therefore, we partially amputated the bulge region of vibrissal follicles of wild-type adult mice and replaced them with identical bulge regions obtained from Rosa 26 adult mice. Chimeric follicles were then transplanted under the kidney capsula of athymic mice, harvested at regular intervals during the following weeks, and stained for β-galactosidase (Figure 2A). β-galactosidase-positive cells were observed solely in Rosa 26 chimeric follicles and never in mock-transplanted or wild-type follicles, thus demonstrating that the β-galactosidase-positive cells originated from the Rosa 26 bulge regions. New whiskers grew in most chimeric follicles indicating that hair renewal was not severely impaired by our transplantation procedures (Figure 2B, panels a and c). Nevertheless, whiskers were usually small or growth aborted when the amputated bulge regions were not replaced ($n = 12$), suggesting that the integrity of the upper portion of the follicle was necessary for normal hair growth.

Cell Migration in the Outer Root Sheath

During the first four weeks following transplantation, β-galactosidase-positive cells were observed solely in the upper region of the follicles (Figure 2B, panels a and d), but during the following weeks, positive cells were also observed below the transplant. With time, positive

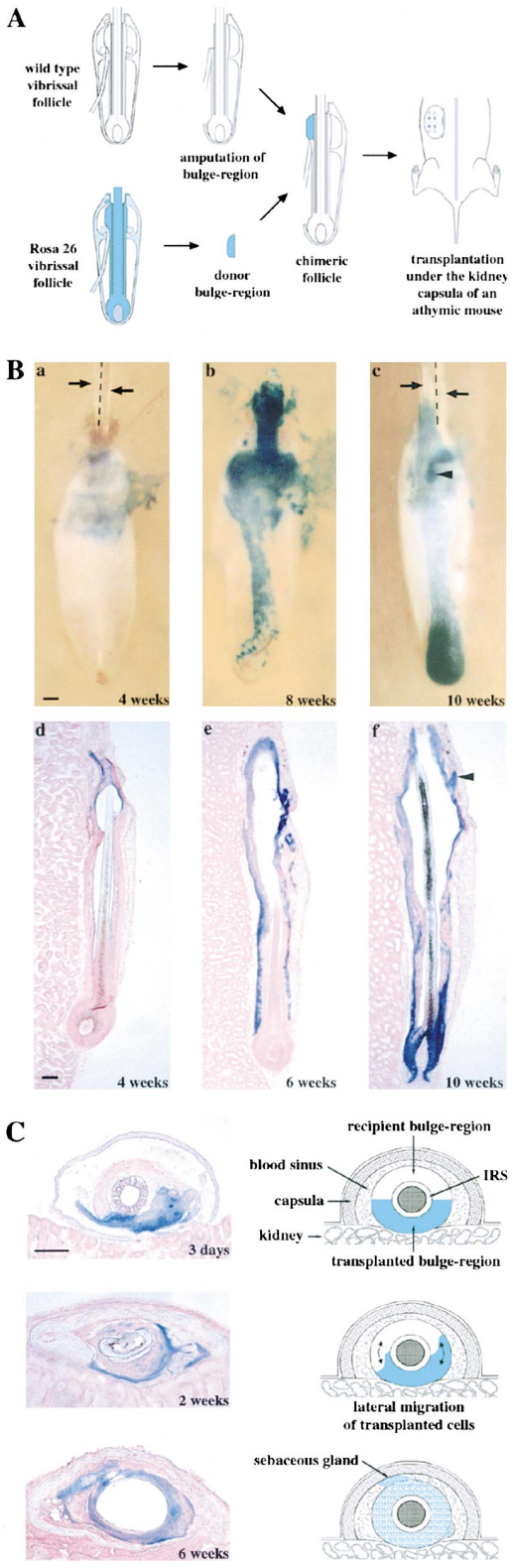

Figure 2. Pattern of Migration of Transplanted Cells

(A) Schematic representation of the transplantation of a chimeric vibrissal follicle.

(B) Longitudinal migration. Chimeric vibrissal follicles were harvested at various times after transplantation and stained for β-galactosidase. Cells originating from the Rosa 26 donor stain blue. The transplanted cells migrate to the upper and lower regions of the follicle within a four weeks period. Note that the transplanted cells migrated only to a small extent on the surface of the kidneys. (a–c) General aspect of the stained chimeric follicles still in place under the kidney capsula. (d–f) Longitudinal sections of the follicles shown in (a)–(c), except for follicles (b) and (e) which are different. Note the presence of two whiskers in follicles (a) and (c) (arrows, dot line delineates the boundary between the whiskers) and that of sebaceous glands in the upper part of the follicles (c) and (f) (arrow heads).

(C) Lateral migration. Transverse sections of chimeric follicles were obtained at the level of the implant. Cells migrated laterally from the implant and progressively mixed with the cells of the recipient follicle. Bars = 100 μm.

cells were found lower and lower in the hair follicle and, by six to eight weeks, they were present in the hair bulb (Figures 3B–3E). Once the cells had initiated their migration, they covered the 2 mm distance separating the bulge region from the hair bulb in about two to three weeks. Their rate of migration was thus 70–100 μm per day. The β-galactosidase-positive cells were often evenly distributed along the follicle length (Figure 2B, panels b and e), suggesting that there was a continuous flux of migrating cells from the bulge region to the hair bulb. These results unambiguously demonstrate that cells migrate in the basal layer of the ORS from the upper region to the hair bulb in an adult vibrissal follicle. Moreover, β-galactosidase-positive cells were constantly observed opposite to and above the transplant (Figures 2C and 2B, panel b), indicating that cells had moved laterally and to the upper region of the follicle. The cells located in the bulge region of the ORS can thus migrate to any part of the follicle. This is specific of bulge cells as it was not observed in follicles transplanted with hair bulbs (n = 12) or with glabrous epidermis of the footpad (n = 12). In the latter follicles, the β-galactosidase-positive cells remained localized within the transplanted area (data not shown).

Generation of Hair Follicle Lineages, Sebaceous Glands, and Epidermis

During the first weeks following transplantation, the migrating β-galactosidase-positive cells were mostly in the basal layer of the outer root sheath and less frequently in its differentiated suprabasal layers (Figure 3A). With time, β-galactosidase-positive cells were observed closer and closer to the proximal end of the follicle (Figure 3B). Once the cells had reached the tip of the hair bulb, they started to move upwards to participate, along with the cells of the recipient follicle, in the formation of the matrix, and later of the IRS and of the hair (Figure 2B, panels c and f, and Figures 3C–3F). This was observed in all transplanted chimeric follicles (n = 75). Moreover, a variety of mosaic patterns was observed (Figures 3C–3E), indicating that the respective contribution to the matrix of the donor cells and of the recipient cells varied. These results unambiguously demonstrate that the transplanted cells can contribute to all the epithelial lineages involved in the formation of a hair follicle, i.e. the outer root sheath, the inner root sheath, and the hair shaft. These results demonstrate that the bulge region contains stem cells and/or progenitor cells whose migration is indispensable for the proper growth of a whisker.

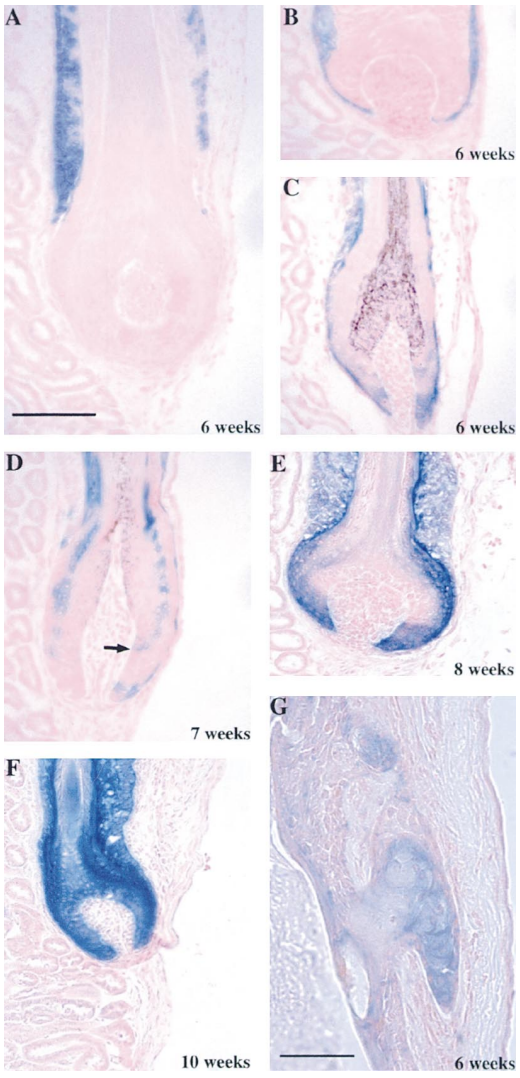

Figure 3. Adult Bulge Cells Generate All the Epithelial Lineage of a Hair Follicle

Rosa 26 chimeric vibrissal follicles were transplanted as described in Figure 2, harvested at various times, and stained for β-galactosidase. (A and B) The transplanted cells contribute to the differentiated layers of the outer root sheath while progressively migrating down to the hair bulb. (C–F) After reaching the lowest part of the follicle, they contribute to the formation of the hair matrix, of all the IRS layers (Henlé and Huxley's layers and IRS cuticle), and of all layers forming the hair (cuticle, cortex, and medulla). Note the mosaic aspect of the matrix in (D), indicating that the cells originated from both the donor and the recipient. Arrow points to IRS. (G) The transplanted cells can generate sebaceous glands. Thus, the cells of the bulge region can generate all the epithelial lineages of a vibrissal follicle. Bars = 100 μm (A–F) and 50 μm (G).

Moreover, similar results were obtained when the transplanted bulge regions (n = 16) were from the opposite side to the nerve endings, indicating that stem cells and/or progenitors cells are distributed all over the bulge region.

Transplanted Rosa 26 cells also migrated to the upper part of the follicle (Figure 2B, panel b). There, one or two clusters of β-galactosidase-positive cells with a foamy cytoplasm and an eccentric nucleus were observed. The cells were arranged in lobular glands whose ducts opened in the hair canal. This is typical of sebaceous glands (Figure 3G). After the fourth week of transplantation, sebaceous glands were constantly observed. They could be located on either side of a follicle but never in the follicle lower part, indicating that the signals required for sebaceous gland morphogenesis are present only in the upper portion of the follicle. β-galactosidase-positive sebaceous glands were occasionally associated with β-galactosidase-negative sebaceous glands, which likely originated from recipient cells. These results demonstrate that the transplanted ORS cells have the capacity to generate the sebaceous gland lineage. Epidermis and sebaceous glands were generated when the bulge regions were implanted into dermal incisions made onto the back of adult athymic mice (3 transplants out of 3, data not shown). Collectively, these results demonstrate that the bulge region contains a population of multipotent stem cells and/or progenitor cells that can generate all the epithelial lineages present in hairy skin.

Morphogenesis of Hair Follicles

Individual bulge regions (n = 17) were obtained from adult Rosa 26 vibrissal follicles and implanted in utero onto the back skin of E14.5–E16.5 mouse embryos. Once the pups were born, the regions containing the implants were localized by the presence of charcoal and transferred onto the back of adult athymic mice (Figure 4A). This was necessary because the implants, if left in place on the pups, were lost, probably from graft rejection. Within days, the implanted bulge regions rearranged with the formation of cell down growths (Figure 4B). After a few days, they had considerably flattened and their original shape could not be recognized. An epidermis with developing hair germs then formed (Figure 4C). These germs were identical to those observed during hair follicle development. Mature follicles, to which sebaceous glands were annexed, later formed (Figure 4D). Multiple β-galactosidase-positive hair follicles were frequently observed, and up to 15 follicles were even observed in one transplant. Moreover, a hair shaft of significant length was present in most of the β-galactosidase-positive follicles (Figures 4D and 4E, arrows). Moreover, these follicles progressed normally through the hair cycle (Figure 4E), further demonstrating that they were functional. Similar results were also obtained when the implanted bulge regions were from the side opposite to the nerve endings.

Interestingly, the follicles generated from the implanted vibrissal cells had no capsula and closely resembled pelage follicles (Figure 4D). Moreover, they cycled as pelage follicles (Figure 4E) and not as vibrissal follicles (see below). Most importantly, this result indicates that the stem cells contained in these follicles are self renewing. It also indicates that the type of follicle formed (vibrissal or pelage) depends on the mesenchymal environment in which the stem cells reside. Interestingly, the cells of the follicular papilla were β-galactosidase-negative indicating that the papilla originated from the recipient. Only rarely, a faint β-galactosidase staining was observed in some papilla, suggesting that the dermal sheath, transplanted along with the bulge region, had contributed to the formation of the papilla (Matsu-

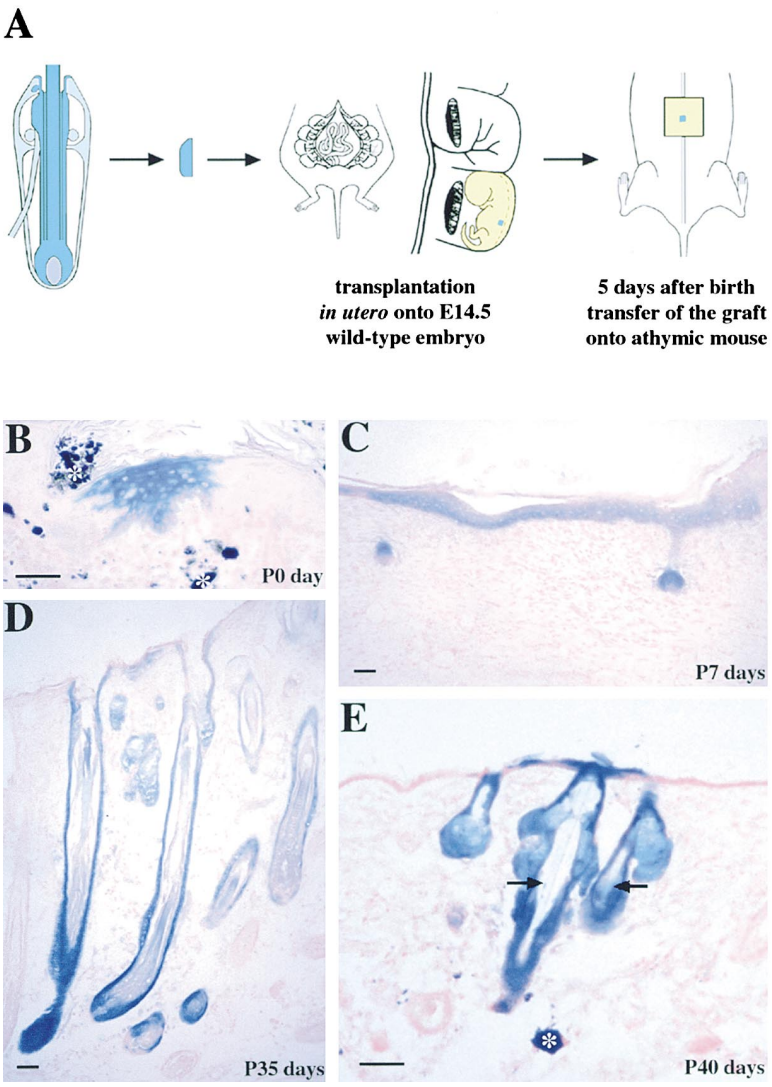

Figure 4. Hair Follicle Morphogenesis from an Implanted Bulge

(A) Transplantation scheme of a Rosa 26 adult bulge region onto the back of a wild-type E14.5 embryo. Implants, localized by the presence of charcoal, were transplanted five days after birth onto the back of athymic mice, harvested at various times, stained for β-galactosidase, and sectioned.

(B) At birth, down growths of the implanted bulge region start to form. Note the presence of charcoal (white stars).

(C) At P7, an epidermis and several hair germs have formed.

(D) At P35, an epidermis, multiple sebaceous glands, and hair follicles were generated from the transplanted bulge region.

(E) At P40, generated follicles are renewing (catagen-telogen). Arrows point to club hairs and white star to charcoal. Bars = 100 μm.

zaki et al., 1996). This, however, awaits confirmation. Collectively, these results demonstrate that the bulge region of an adult vibrissal follicle contains multipotent stem cells with the capacity to respond to morphogenetic signals to generate all the epithelial structures of the hairy skin, and that these stem cells are distributed all over the bulge region.

Renewal of a Vibrissal Follicle

Whiskers, as pelage hairs, are periodically shed and replaced to maintain the integrity of the tactile function. Whisker growth during anagen (growth phase) results from the proliferation and the differentiation of matrix cells generated by precursor cells originating from the bulge region. As the hair bulb undergoes extensive reorganization during catagen (regression phase), a new population of precursor cells must rejoin the root of the follicle to reinitiate a growth phase. Therefore, we studied the influence of the hair cycle on the distribution of proliferative cells to further document the role of the bulge in whisker renewal.

Determination of the Growth Phase of a Living Vibrissal Follicle

The changes defining the various phases of the hair cycle are well characterized in pelage follicles (Chase et al., 1951; Straile et al., 1961; Montagna, 1962). During anagen, the keratinocytes located in the matrical part of the hair bulb actively proliferate and generate large numbers of differentiated cells that form the hair shaft and the inner root sheath. During catagen, the overall organization of the hair bulb breaks down. The matrix cells stop dividing, hair production ceases, and a club hair forms. Simultaneously, the follicular papilla dramatically decreases in size and the follicle shortens. This phase is then followed by a resting phase (telogen) during which the follicle remains quiescent. A new anagen phase is then initiated and hair growth resumes. However, the existing nomenclature cannot accurately accommodate the differences in the cycle of vibrissal follicles as they progress rapidly through the hair cycle. Therefore, we evaluated the progression of the hair cycle in vibrissal follicles by obtaining mystacial follicles occupying positions a3 and a4 from rats of various ages

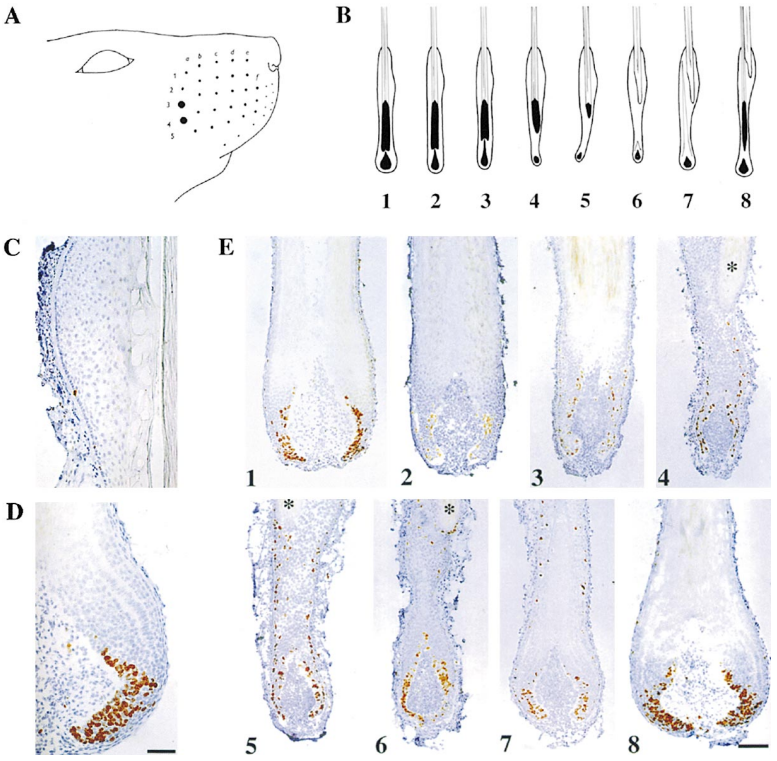

Figure 5. Location of Multiplying Cells in the Vibrissal Follicle of the Rat

(A) Location of vibrissal follicles of the mystacial pad of the rat (modified from Oliver, 1966). Vibrissal follicles are arranged in rows; the first follicles to cycle are those in the rows located the closest to the snout, and the last are those occupying the positions a3 and a4 (black dots).

(B) Schematic representation of the hair cycle phases of a vibrissal follicle of the rat (position a3) drawn to scale from photomicrographs taken at various times during the hair cycle. Progression through the hair cycle is given in successive stages because the different phases overlap in vibrissal follicles, making it difficult to strictly apply the criteria described for pelage follicles. Stage 1: mid-anagen; stage 2: late anagen; stages 3–5: catagen; stage 6: early anagen; stages 7 and 8: anagen. Note that the follicular papilla decreases in size and the follicle shortens after whisker growth has stopped (stage 4). The length of stage 1 follicle is 2.75 mm.

(C–E) Location of dividing cells in vibrissal follicles. Follicles at various stages of the hair cycle (1–8) were incubated with BrdU and immunostained. (C and D) A follicle in anagen. Very few BrdU-labeled cells are present in the bulge-containing region (C) whereas they are abundant in the matrix (D). Bar = 50 μm. (E) Numerous BrdU-labeled cells are present throughout the entire cycle in the lower outer root sheath and next to the follicular papilla. Black stars point to club hairs. No significant change was detected in the upper region of the follicle (not shown). Bar = 100 μm.

(Figure 5A). These follicles were microdissected, photographed, and processed for histology. Their cycle status was then determined from longitudinal sections. The photographs were examined to define morphological criteria typical of the growth states of living follicles (Figure 5B). Vibrissal follicles in anagen typically had an onion-shaped bulb containing a prominent follicular papilla and a growing hair with an extended dark zone that corresponds to the region where the hair shaft is not yet fully mature (Figure 5B, 1). Follicles in late anagen had a less prominent bulb and a smaller elongated follicular papilla (Figure 5B, 2). As the hair cycle progressed, the follicles became slightly shorter, thinner, and developed a rod-shaped bulb (Figure 5B, 3). The follicular papilla then became tiny, ball-shaped, and barely visible and the dark zone gradually shortened and the club hair started to form (Figure 5B, 4). At this stage, the production of hair fibers had definitely ceased. The dark zone kept shortening as the pointed club hair moved upward (Figure 5B, 5). A new growth phase was initiated without delay, involving an increase in the size of the follicular papilla and the appearance of a matrix cone (Figure 5B, 6). As the new anagen phase progressed, the size of the follicular papilla increased rapidly and a thin whisker was soon identified opposite the club hair (Figure 5B, 7). The tip of the new whisker reached the neck of the follicle before the club hair had reached its final position. The old whisker persisted for up to four weeks before being shed (Figure 5B, 8). Thus, the hair cycle status of living microdissected follicles could be reliably determined using a binocular microscope. These experiments confirm that the various phases of the hair cycle of vibrissal follicles are not as separated and clearly defined as those of pelage follicles (Young and Oliver, 1976), and that vibrissal follicles rapidly initiate a new anagen phase and have an extremely short or nonexistent telogen phase. For example, the anagen phase of the mystacial follicles occupying positions a3 and a4 lasted for 35 days and a new whisker started to grow within two days of the old one stopping growth. They also confirm that vibrissal follicles of the right and left mystacial pads cycle almost synchronously.

Location of Dividing Cells

We determined the location of the cells that multiplied during the various phases of the hair cycle by microdissecting vibrissal follicles and incubating them in medium containing BrdU. Frozen longitudinal sections were then obtained and stained with an anti-BrdU monoclonal antibody. A very small number of BrdU-labeled cells, scattered in the basal layer of the outer root sheath, were detected in the upper part of the permanent portion of the follicle containing the bulge throughout the hair cycle (Figure 5C). As expected, numerous BrdU-labeled cells were observed in the matrix next to the follicular papilla when the follicle was in anagen (Figures 5D and 5E, panel 1), but very few were detected in the portion of the follicle connecting the hair bulb to the bulge region. After hair growth had ceased, a small number of BrdU-positive cells were detected at the lower end of the follicle. They were mostly located in the outer root sheath, adjacent to the shrunken follicular papilla (Figure 5E, panels 2 and 3). More BrdU-labeled cells were detected in the outer root sheath of the lower end of the

Table 1. Skin Morphogenesis from Implants of Different Regions of Vibrissal Follicles

Origin of Implants and Hair Cycle Stage	Age of Recipient Embryos or Pups	Number of Implants	Number of Pups Born Alive	Number of Athymic Mice Transplanted	Hair Follicles	Sebaceous Glands	Epidermis
Bulge region All stages	E14–E16	17	13	9	7/9	7/9	7/9
Hair bulb Stage 1	E14–E16	6	4	3	0	0	0
Bulge region All stages	P0	8	–	8	6/8	6/8	6/8
Hair bulb Stage 1	P0	11	–	11	0	0	0
Hair bulb Stage 5	P0	6	–	6	3/6	3/6	3/6
Intermediate region Stage 1	P0	6	–	6	0	0	0
Intermediate region Stage 5	P0	6	–	6	4/6	4/6	4/6

Hair cycle stages were determined as described in Figure 5. Similar results were obtained when samples were implanted onto the back of embryos or newborns.
The lower region of a follicle responds to morphogenetic signals only when the follicle is cycling.

follicle as the follicle began to reorganize (Figure 5E, panel 4). BrdU-labeled cells were also observed just beneath the club hair as it moved upward (Figure 5E, panels 4–6). These cells were distributed along a line joining the tip of the club hair to the contralateral side of the follicle (Figure 5E, panel 6). Numerous labeled cells were also observed in the basal and suprabasal layers of the outer root sheath in the middle part of the follicle (Figure 5E, panel 7). The distribution of these cells reverted to normal as the follicle progressed through anagen (Figure 5E, panel 8).

The Hair Cycle Influences the Morphogenetic Capacity of Vibrissal Follicle Cells

To investigate the capacity of the cells contained in the different regions of vibrissal follicles to respond to morphogenetic signals, we obtained follicles at various stages of the hair cycle from Rosa 26 mice. Vibrissal follicles were microdissected and cut into several fragments which were then individually implanted onto the back of embryos or newborn pups. When donor follicles were in anagen, epidermis and hair follicles to which sebaceous glands were annexed, formed solely when the bulge region–containing fragments were implanted (Table 1). However, morphogenesis of hair follicles, sebaceous glands, and epidermis occurred when the fragments containing the hair bulb and the lower intermediate region were obtained from follicles in catagen (Figure 6) and early anagen (Table 1). This demonstrates that multipotent stem cells are present in the lower part of a follicle at the initiation of a new cycle and that multipotent stem cells have thus migrated from the bulge region to the root of the follicle.

The Clonogenic Capacity of Vibrissal Follicle Cells Is Influenced by the Hair Cycle

The poor performances of mouse keratinocytes in culture prevented us from performing reliable clonal analyses on the vibrissal follicles of the mouse. We thus determined the effect of the hair cycle on the distribution of clonogenic keratinocytes by obtaining vibrissal follicles ($n = 102$), occupying positions a3 and a4 on the mystacial pads, from rat female littermates of various ages. Follicles were microdissected and photographed to determine the phase of the hair cycle using the morphological criteria defined above. Follicles were then cut transversely into five fragments (Figure 7A). The fragment containing the matrix and the follicular papilla was called P1. The other fragments were designated in order as the lower-intermediate fragment P2, the mid-intermediate fragment P3, and the upper-intermediate fragment P4. The last fragment, which did not change during the cycle and contained the bulge and the sebaceous glands, was called P5. The fragments were arranged in their original order and photographed. Each fragment was incubated in collagenase-dispase to facilitate the dissociation of the mesenchymal sheath. Trypsin was then added to obtain a single-cell suspension. The cells were cultured for ten days in individual culture dishes containing lethally irradiated 3T3 cells to evaluate their colony-forming ability.

Many clonogenic keratinocytes (mean of 1040 cells per follicle, $n = 100$ follicles) were isolated from the bulge-containing fragment P5 over the entire hair cycle (Figure 7B). Few clonogenic cells (mean of 29 cells per follicle, $n = 27$ follicles) were isolated from the bulb-containing fragment P1 and almost none from the intermediate fragments P2–P4 when the follicles were in mid-anagen phase. However, a significant number of clonogenic keratinocytes (mean of 100 cells per follicle, $n = 29$ follicles) were isolated from the P1 fragment and from the lower-intermediate fragment P2 (mean of 136 cells per follicle, $n = 29$ follicles) at the very end of the anagen phase and over the entire catagen phase. Many clonogenic keratinocytes were also isolated from the mid-intermediate fragment P3 (mean of 232 cells per follicle, $n = 25$ follicles) and later from the upper-intermediate fragment P4 (mean of 187 cells per follicle, $n = 17$ follicles) as the follicle progressed through the cycle (Figure 7B, 4–8). Clonogenic keratinocytes were isolated from all fragments for the following two or three weeks. The distribution reverted to its usual pattern as the ana-

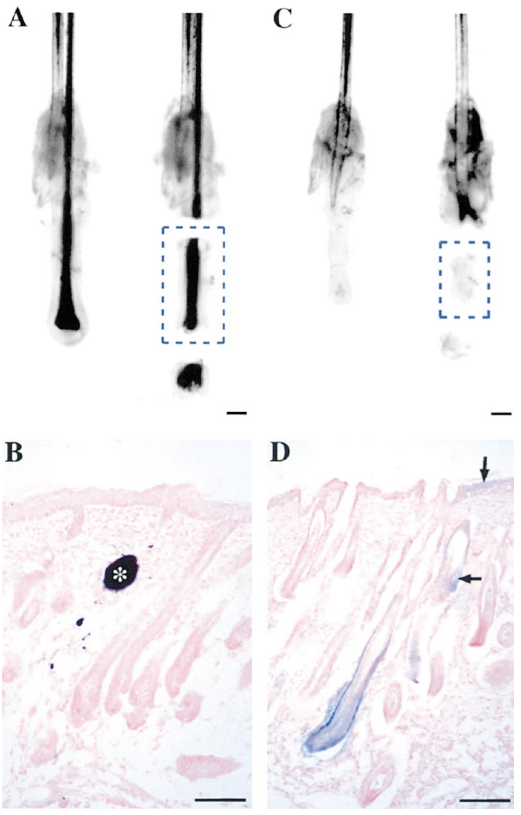

Figure 6. Morphogenesis from Implanted Length of Vibrissal Follicles

(A) A follicle at stage 1 of the hair cycle. (C) A follicle at stage 5. The intermediate region of each follicle (boxed) was implanted into the back of a newborn mouse. (B and D) Sections of the implant-containing regions 33 days after implantation on newborns. Skin morphogenesis was obtained only with the stage 5 follicle. White star points to charcoal. Black vertical arrow points to epidermis and horizontal one points to sebaceous gland. This demonstrates that the implanted length contained multipotent stem cells and thus that the latter had migrated from the bulge region. Bars = 100 μm.

gen phase progressed (Figure 7B, 1). A similar pattern was also observed if the cells were isolated from follicles obtained during the following cycle (n = 12) or from follicles occupying row b (n = 24 follicles) of the mystacial pads of rats from different litters (data not shown). Thus, the number of clonogenic keratinocytes dramatically increased in the lower portion of the follicle at the end of anagen, during catagen, and during the early part of the following cycle.

Discussion

The criteria defining a somatic mammalian stem cell are still a matter of discussion. The most reliable, and less controversial, criterion is the cell ability to self renew and to generate its target tissue for an extended period of time (van der Kooy and Weiss, 2000). Other criteria, including label-retaining ability, clone-forming ability, and multipotency are circumstantial and depend on the tissue. We demonstrate here that the upper region of the outer root sheath of vibrissal follicles of adult mice contains multipotent stem cells that can generate all the epithelial lineages of the hairy skin, and that these stem cells migrate to the root of the follicle to produce whisker growth. Moreover, our results indicate that the clonogenic keratinocytes are closely related, if not identical, to the multipotent stem cells, as the lower region of a follicle can respond to morphogenetic signals only when it contains a significant number of clonogenic keratinocytes. Collectively, our results strongly suggest that the regulation of whisker growth involves a precise control of stem cell trafficking.

Multipotent Stem Cells and Skin Morphogenesis

The multipotent stem cells present in mature follicles are most likely generated from more precocious stem cells. Indeed, the existence of stem cells that contribute to all epithelial lineages of the hairy skin, including the periderm, an epidermal layer present only during embryonic life, has been demonstrated by the retroviral labeling of individual epidermal cells in E.9 mouse embryos (I. Tong et al., our unpublished results).

The bipotent label-retaining cells recently identified by kinetic experiments in the pelage of the mouse (Kamimura et al., 1997; Taylor et al., 2000) are most likely related to the multipotent stem cells that we described here. That vibrissal cells generate self-renewing pelage follicles strongly supports this hypothesis. However, we were unable to induce hair follicle morphogenesis from individual bulge regions of adult pelage follicles. This could be because the cells of adult pelage follicles differ from those of vibrissal follicles in their capacity to respond to morphogenetic signals, or because pelage follicles contain a limited number of stem cells. That stem cells need to be in sufficient number for morphogenesis is supported by our transplantation experiments of cycling follicles. In addition, we have not observed the formation of sweat glands from the transplanted bulges. This is not surprising as sweat glands are only observed in the glabrous skin of the footpads in rodents. However, glands resembling nasal glands can be generated from E15 embryonic whisker germs treated with retinoic acid, indicating that embryonic vibrissal stem cells have the capacity to generate glands other than sebaceous (Hardy, 1968; Dhouailly et al., 1984). Whether adult vibrissal follicle stem cells have retained this capacity awaits further investigation.

A region of a hair follicle can respond to morphogenetic signals only if it contains multipotent stem cells and/or a significant number of clonogenic cells. In a vibrissal follicle, only the bulge region and the lower region at the time of initiation of a new hair cycle fulfill this requirement, strongly supporting the hypothesis that the multipotent stem cells and the clonogenic keratinocytes are closely related, if not identical. This further indicates that label-retaining ability, and clonogenic ability, in association with an extended growth potential, are the expression of a unique phenotype linked to a stem cell or its close progeny (Kobayashi et al., 1993; Morris and Potten, 1994).

We cannot formally exclude that different progenitor cells, committed to one or several epithelial lineages of the skin, reside along with the multipotent stem cells. Studies of the differentiation potentiality of individual

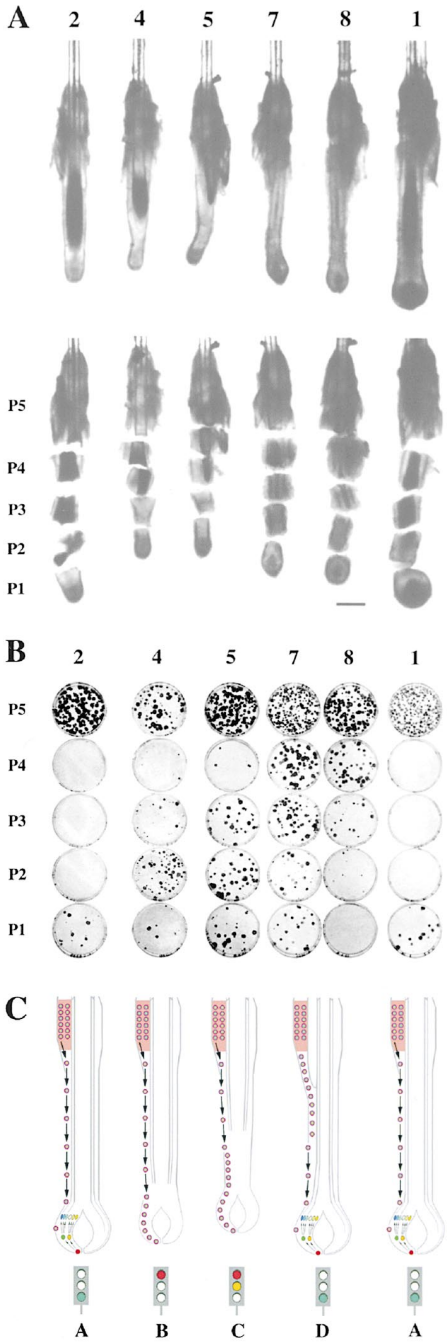

Figure 7. Distribution of Clonogenic Keratinocytes during Hair Cycle

(A) Aspect of microdissected vibrissal follicles. Upper panel: vibrissal follicles occupying position a3 on the right mystacial pad were microdissected from six littermate female rats of various ages. Stage 2 (35-day-old rat); stage 4 (37-day-old rat); stage 5 (39-day-old rat): stage 7 (41-day-old rat); stage 8 (42-day-old rat); stage 1 (71-day-old rat). Lower panel: the follicles after cutting into five fragments. P1, lower fragment containing the dermal papilla; P2, lower-intermediate fragment; P3, mid-intermediate fragment; P4, upper-intermediate fragment; P5, upper fragment containing the bulge. Note that the upper cut was always made at the same place and that the lengths of the P5 fragments were identical. Note also that four days separate the end of an anagen phase from the beginning of the next one. Bar = 400 μm.

(B) Distribution of clonogenic keratinocytes: keratinocytes were isolated from P1 to P5 fragments obtained from those six follicles shown in (A). Cultures were fixed after ten days and stained with 1% Rhodamine B. The number of clonogenic keratinocytes increased in the lower fragments as the follicles progressed through the hair cycle.

(C) Traffic light hypothesis. (Panel A) In anagen, stem cells are continuously leaving the bulge region and migrating to the hair bulb. (Panel B) When whisker growth is about to stop, the stem cells that have reached the hair bulb are instructed to stop migrating. (Panel C) With time, the incoming cells stop higher and higher in the follicle and pile up. (Panel D) After hair growth has resumed and a new matrix is initiated, the stem cells can move again. Note that mobile stem cells cannot initiate colonies.

cells should elucidate this point. Nevertheless, these committed progenitors share label-retaining ability and clone-forming ability with the multipotent stem cells (Lavker et al., 1991; Kobayashi et al., 1993) and should be orderly sorted from the bulge region before they migrate to their final destination. Consequently, sebaceous glands and epidermis should not form when the lower portions of cycling follicles are implanted in embryos. The fact that it is not the case strongly indicates that the implanted lower fragments contained multipotent stem cells that have migrated from the bulge. Therefore, it reinforces the hypothesis that committed progenitors are generated from stem cells solely in defined places, i.e., above the bulge region for sebaceous and epidermal progenitors and in the hair bulb for matrix progenitors (Figures 8A and 8B).

Multipotent Stem Cells Reside in a Niche

Epithelial stem cells, as hematopoïetic stem cells and neural stem cells, necessitate a privilege environment to survive, migrate, proliferate, and differentiate (Rochat et al., 1994; Slack, 2000; Watt and Hogan, 2000). Such an environment, including nurse cells and a suitable extracellular matrix, must be present in the entire bulge region of the vibrissal follicle, as the multipotent stem cells are distributed all over it. The location of the stem cells in the upper region of the follicle then neatly preserves them from receiving signals that may inadequately instruct them to generate IRS and hair progenitors. The fact that a hair matrix is formed when a follicular papilla is implanted in the upper region of the outer root sheath of a vibrissal follicle further supports this hypothesis (Matsuzaki et al., 1996; and our unpublished results). In pelage follicles, the proliferation of stem cells located in the bulge, identified by the insertion of the arrector pili muscle, is thought to reconstitute the pool of matrix cells at the end of telogen. The influence of the follicular papilla, which then physically approach the stem cells, appears determinant. This mechanism is known as the bulge activation hypothesis (Cotsarelis et al., 1990; Lavker et al., 1991). However, it does not apply to vibrissal follicles because the follicular papilla cells never come into close contact with the cells located in the bulge region, even though vibrissal follicles do shorten slightly during catagen. Consequently, the stem cells and/or the committed progenitors must leave their niche, and migrate the entire follicle length (up to 2 mm) before they receive the appropriated signals from the follicular papilla to contribute to whisker growth.

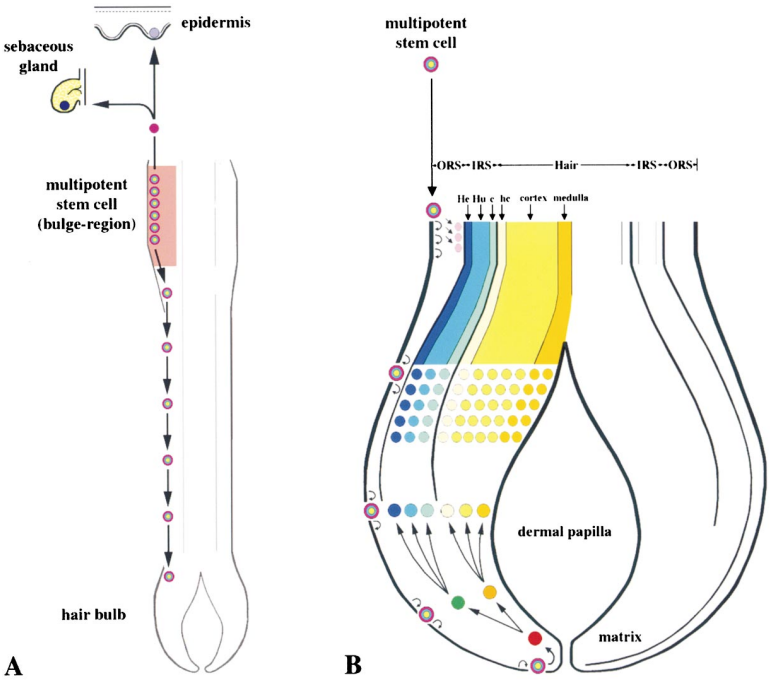

Figure 8. Fate of a Multipotent Stem Cell in a Vibrissal Follicle

(A) Multipotent stem cells are located in the upper-region of the vibrissal follicle. They (or their committed progenitors) may migrate to the upper part of the follicle to generate sebaceous glands and epidermis and to the hair bulb to generate the hair lineages.

(B) Multipotent stem cells migrate in the basal layer of the ORS to the hair bulb while contributing to the ORS differentiated layers. They are then committed to IRS- and hair-forming progenitors in the matrix.

Arrest of Whisker Growth Does Not Result from a Shortage of Stem Cells

There are two possible reasons why hair growth stops. First, the production of hair fibers ceases because the matrix cells have exhausted their proliferative capacities (Lavker et al., 1993). This assumes that the proliferative capacity of matrix cells is determined once for all at the initiation of a new hair cycle, and that new matrix cells are not generated for the entire growth phase. Second, stem cells may continuously generate new matrix cells, with the production of hair fibers ceasing only when the stem cells are instructed to stop generating new progeny. Our clonal analyses demonstrate that cells with significant growth potential are present in the hair bulb throughout the entire anagen phase, and surprisingly that their number increases when hair production is about to stop. Moreover, our transplantation experiments demonstrate that multipotent stem cells are present in the lower end of a cycling follicle. Thus, the cessation of hair production in vibrissal follicles is unlikely to result from a shortage of stem cells. Rather, our results support the hypothesis that the production of hair fibers ceases when the stem cells are instructed to stop generating new matrix cells.

Control of Stem Cells Trafficking: the Traffic Light Hypothesis

Most importantly, our results emphasize the control of stem cell mobility as a means to regulate the duration of hair growth. One would expect clonogenic cells to appear first in the upper-intermediate part (P4), then in the mid-intermediate part (P3), and finally in the lower-intermediate part of the follicle (P2) as they progressively migrated down to the hair bulb. We observed exactly the opposite: clonogenic cells increased in number first in P2, then in P3, and finally in P4. The usual distribution of clonogenic cells resumed only after the next anagen phase was well engaged. These results are unlikely to reflect a flux of clonogenic cells from the bulb toward the upper follicle, but they rather reflect the events taking place in the lower part of the follicle at the time when hair growth ceases. The rate of cell migration in the outer root sheath is about 70–100 μm per day. Therefore, a stem cell takes three to four weeks to cover the 2.0 mm distance separating the bulge region from the tip of the hair bulb, i. e., the duration of an anagen phase. It remains to explain why no colony-forming cells are isolated from the intermediate region of a follicle in mid-anagen. One explanation is that stem cell mobility is inversely related to the ability to initiate a colony in vitro. Consequently, only nonmobile stem cells can initiate colonies. Thus, the distribution of the clonogenic cells in the intermediate region of the follicle neatly reflects the migrating state of the stem cells (Figure 7C). During anagen, stem cells are continuously leaving the bulge region to migrate to the hair bulb (Figure 7C, panel A). The migrating stem cells cannot initiate colonies (Figure 7B, stages 1 and 2). When whisker growth is about to cease, the stem cells that have reached the hair bulb are instructed to stop migrating (Figure 7C, panel B). They are now nonmobile and thus can initiate colonies (Figure 7B, stage 4). With time, the incoming stem cells stop higher and higher in the follicle (Figure 7C, panel C). This is why the clonogenic cells in the lower portion of the follicle follow an upward distribution (Figure 7B, stages 4–7). After a new matrix is initiated and hair growth resumes, stem cells can move again (Figure 7C, panel D). Consequently, the number of clonogenic cells diminishes first in the hair bulb and later in the upper portions of the follicle (Figure 7B, stages 7–8). The distribution of the clonogenic keratinocytes then resumes to an anagen pattern (Figure 7B, stage 1)

That the pattern of BrdU-positive cells follows that of the clonogenic cells indicates that cell multiplication

may also contribute to increasing the population of clonogenic cells/stem cells in the lower part of a cycling follicle. However, cell multiplication cannot account by itself for the quick and dramatic increase in the number of clonogenic keratinocytes that most likely results from a combination of two events, the key one being the arrest of stem cell migration, which then permits cell proliferation.

However, the mechanism by which the flux of stem cells is regulated remains elusive. The shape of the hair bulb may affect the fluidity of the cell traffic, as the pronounced angle of the lower curvature of the bell-shape follicular papilla likely slows down the migrating stem cells. This may explain why the cells located at the lowest extremity of an anagen hair bulb are clonogenic (Reynolds and Jahoda, 1991; Reynolds et al., 1993).

Molecular Control of Hair Follicle Lineage

A large variety of molecules may affect the production of hair fibers by acting on stem cell migration, or the commitment, the proliferation, and the differentiation of progenitors. There is compiling evidence that developmentally regulated molecules are crucial for hair follicle morphogenesis and renewal (Oro and Scott, 1998; Fuchs and Segre, 2000). These molecules include the transcription factors Lef-1 (van Genderen et al., 1994; DasGupta and Fuchs, 1999), p63 (Mills et al., 1999; Yang et al., 1999), Whn (nude) (Segre et al., 1995; Brissette et al., 1996), and hairless (Cachon-Gonzalez et al., 1994), members of the Wnt (Gat et al., 1998; Millar et al., 1999; Kishimoto et al., 2000), Notch (Kopan and Weintraub, 1993), or Shh (St-Jacques et al., 1998; Chiang et al., 1999; Wang et al., 2000) signaling pathways. They also include EGF/TGF-α (Green and Couchman, 1984; Luetteke et al., 1993; Mann et al., 1993; Philpott and Kealey, 1994) and members of the TGF-β (Blessing et al., 1993; Bitgood and McMahon, 1995; Kratochwil et al., 1996; Gambardella et al., 2000) and of the FGFs growth families (du Cros, 1993; Hébert et al., 1994). For instance, FGF-5, a member of the FGF superfamily, appears to have a major role in the regulation of hair growth as FGF-5-deficient mice have long hair (angora phenotype) as a result of an extended anagen phase (Hébert et al., 1994). Undoubtedly, many of these molecules may act on the stem cells and the progenitors. Unravelling the cellular events involved in stem cell and/or progenitor behavior and in the dynamic of a hair follicle should help to comprehend the molecular control of hair lineage.

Experimental Procedures

Animals

Mice and rats were maintained and operated according to French government guidelines. Fisher 344 (inbred) rats were obtained from the Curie Institute animal facility or from Iffa Credo (France). Wild-type OF1 (inbred), B6/D2F1/JIco (inbred) mice, and athymic (Swiss-nu/nu, inbred) mice were from Iffa Credo (France). Rosa 26 mice (Friedrich and Soriano, 1991) were from the Jackson laboratory and bred in our animal facility.

Transplantation of Mouse Chimeric Vibrissal Follicles

Male or female mice were 8–12 weeks old at the beginning of the experiments. Mice were anesthetized with a mixture of ketamine (0.104 mg/g body weight) and xylazine (0.033 mg/g body weight) in phosphate buffered saline (PBS). Pregnant mice were anesthetized with a mixture of flunitrazepan (0.315 μg/g body weight), ketamine (0.158 mg/g body weight), and xylazine (0.004 μg/g body weight) in PBS. All surgical procedures were made under a sterile environment. The upper lip containing the vibrissal pad was cut and its inner surface was exposed. The follicles were dissected under a binocular microscope and plucked from the pad by pulling them gently by the neck with fine forceps as described (Kobayashi et al., 1993). All samples were then kept on ice in Dulbecco's Vogt modified Eagle medium supplemented with 10% fetal calf serum. Two longitudinal incisions were made in the collagen capsula of each recipient follicle with a 26G needle (Terumo) and the resulting slice of capsula was discarded. Special care was taken to completely remove the sebaceous glands and not to damage the follicle core. The bulge region, whose location was easily identified by the presence of the nerve endings, was then amputated from the upper-part of the follicles using a fine needle. Each amputated bulge region was replaced by a bulge region cut off from a Rosa 26 vibrissal follicle (Figure 2). All chimeric follicles were kept in refrigerated medium until they were transplanted underneath the kidney capsula of an athymic mouse. Briefly, an incision was made on the left flank of the recipient mouse and the kidney was exposed. Three to five follicles were inserted side by side beneath the kidney capsula. The kidney was then brought back into place and the incision was closed with nylon sutures (6–0, Ethicon). Grafts were harvested at various times.

Transplantation in Embryos and Newborns

Wild-type females (strain OF1) at 13 to 16 days of gestation were anesthetized as described above. A vertical incision was made in the abdomen of the mouse and the two horns of the uterus were gently pulled out with the help of forceps. The uterus was laid out onto the abdominal wall and each embryo was examined under a surgical microscope (Leica). The uterus and the amniotic membranes were incised using fine scissors as not to injure the embryo (Salaün et al., 1986). A small incision was made on the back skin of the embryo with the tip of a 26G needle. A fragment, obtained from a Rosa 26 vibrissal follicle, as described above, was then carefully implanted in it. Sterile charcoal was placed next to the implant to facilitate its future localization. The amniotic membranes and the uterus were then closed quickly by a nylon suture (7–0, Ethicon). The uterus was then gently replaced back and the abdominal incision was closed with nylon stitches (6–0, Ethicon). Operated pups, when born alive, were observed until they were five days old. At that time, the regions containing the bulge implants, identified by the presence of charcoal, were harvested and transplanted onto the back of athymic mice. This avoided rejection and allowed the development of the grafts for several weeks. Alternatively, samples were implanted in the back skin of newborns instead of that of embryos. After five days, implants were processed as described.

Cell Culture

Keratinocytes were isolated from microdissected vibrissal follicles as described (Kobayashi et al., 1993) and cultured on a lethally irradiated feeder layer of 3T3-J2 cells as described (Rheinwald and Green, 1975; Rochat et al., 1994) using a single batch of fetal bovine serum (FBS, Hyclone). 3T3-J2 cells were cultured in Dulbecco-Vogt modified Eagle medium (DMEM) containing 10% calf serum (Hyclone).

Histology

Dissected follicles were fixed in 3.7% formaldehyde in phosphate buffered saline (PBS), or in Bouin's solution. They were embedded in paraffin and 7 μm-thick longitudinal sections were cut and stained with Meyer's hematoxylin. Alternatively, the dissected follicles were snap frozen in liquid nitrogen and stored at -80°C until required. Frozen longitudinal sections (8 μm-thick) were cut with a Cryostat (Leica), whose cutting chamber temperature was set at -30°C.

X-Gal Staining

Several weeks after transplantation, mice were anesthetized as described above and perfused in the abdominal aorta with a 4% paraformaldehyde solution in phosphate buffered saline (PBS) at pH 7.4 for 10 min. Kidneys bearing the follicle transplants were harvested, rinsed three times in buffer, and stained in a 5-Br-4-Cl-3-indolyl-β-

D-galactoside (X-gal) solution according to standard procedures (Sanes et al., 1986). Samples were incubated in 25% sucrose solution overnight at 4°C, snap frozen in liquid nitrogen, and stored at −80°C before frozen sections (8 μm) were obtained. Sections were counterstained with nuclear fast red, dehydrated, and mounted in Eukitt.

Bromodeoxyuridine Incorporation

Rats were injected intraperitoneally with bromodeoxyuridine (BrdU; 3 mg/kg body weight; Sigma) in phosphate buffered saline and were killed 1 hr later. Selected vibrissal follicles were microdissected and washed in DMEM containing 10% calf serum for 1 hr at 37°C. Alternatively, vibrissal follicles from untreated rats were microdissected, incubated in culture medium containing 10 mM BrdU for 1 hr at 37°C, and washed in culture medium as described above. Both procedures gave similar results and follicles from untreated rats were routinely used. Microdissected follicles were snap frozen in liquid nitrogen and 8 μm-thick longitudinal sections were cut. Frozen sections were fixed in acetone/methanol (v/v) for 5 min at room temperature and incubated in 95% formamide in 0.15 M trisodium citrate buffer for 30 min at 70°C to partially denature the DNA. Activity of endogenous peroxidase was quenched by treating the samples with 5% hydrogen peroxide. Sections were then washed twice in PBS for 10 min each at room temperature, incubated for 1 hr at room temperature with a mouse monoclonal anti-BrdU antibody (Becton-Dickinson) diluted 1/20 in PBS, and stained using the peroxidase-antiperoxidase technique according to standard procedures and the manufacturer's guidelines. The rabbit anti-mouse antibodies and the PAP antibodies (Dako Corporation) were diluted 1/40 and 1/100 in PBS, respectively. Samples were counterstained with Meyer's hematoxylin, dehydrated, mounted in Eukitt, and examined with a Zeiss microscope.

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