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At the Roots of a Never-Ending Cycle

Review

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Recent studies have yielded a number of important insights into the mechanisms of hair follicle development and cycling and have highlighted the particularly important roles played by stem cells and Wnt signaling pathways.

Across the eukaryotic kingdom, labyrinthine body surface appendages have evolved with extraordinary rapidity, leaving nature with a fascinating display of diversity ranging from the wings of a butterfly to the scales of a chameleon to the feathers of an ostrich to the fur of mammals. Given the magnificence and splendor of most epidermal appendages, it is no wonder that we have been fascinated with our own hair as well as the appendages of our furry, scaly, and feathery ancestors. From Delilah's snips of Samson's locks to Rapunzel's unfurling of her long tresses from the castle tower, our fascination with hair has evolved into a love affair turned obsession that has fueled a multibillion dollar industry. Among the most remarkable findings of recent years is that despite the striking phenotypic differences in epidermal appendages, the molecular features of these appendages are startlingly similar from feathers to scales to hair. This has edged us ever closer to uncovering the secrets of hair growth. This review will focus on mammalian hair follicle morphogenesis and cycling but will draw upon data from other organisms when appropriate.

Hair Follicle Morphogenesis and the Hair Cycle

After gastrulation, a single layer of pluripotent ectoderm covers the embryo surface. Soon after mesenchymal cells populate the skin, the epidermis and its appendages begin to form. The skin epithelium is separated from its underlying mesenchyme by a basement membrane of extracellular matrix. This feature has enabled developmental biologists to isolate and recombine epithelium and mesenchyme from different regions of the skin of a developing mouse or chick embryo and analyze the roles of the epithelium and mesenchyme in epidermal appendage morphogenesis. These studies have underscored the importance of the mesenchyme in influencing what type of epidermal appendage will develop. Thus, for instance, when embryonic chick wing dermis is grafted beneath chick leg epidermis, feathers develop on the leg, whereas when embryonic leg dermis is grafted under the chick wing epidermis, scales form on the wing (Sengel, 1975, 1990). Similarly, if mouse vibrissae dermis is grafted below the epidermis of a mouse backskin, whiskers form on the back. Even when dermis from follicle-containing skin is transplanted to nonhaired regions, e.g., plantar or palmar skin, follicles will develop. These findings led biologists to conclude that the initial signal in specifying an appendage is a mesenchymal cue (for review see Hardy, 1992).

Once the dermal cue signals to the overlying epithelium, the epithelium thickens to form a placode, which then grows downward to form a cup of uniform cells, or hair germ, that opens to the ectoderm (Figure 1). In turn, these epithelial cells appear to cue the mesenchymal cells underlying it to organize into a dermal condensate, or dermal papilla, which will become the permanent mesenchymal portion of the hair follicle. Mouse dermal tissue can stimulate epidermal appendage formation in other species; however, primordial chick epithelium still produces feather buds, and lizard epithelium still gives rise to scales when exposed to mouse dermal cues (Garber et al., 1968). In this way, the epithelium possesses the knowledge as to what type of appendage to make, while the mesenchyme presents the stimulus to make it.

The bulb of the hair germ epithelium forms a cloak about the demal papilla, and this close encounter stimulates the epithelium to proliferate and produce the hair follicle (Figure 1). As the follicle grows downward, the developing hair germ cells that lose contact with the dermal papilla become the outer root sheath (ORS) of the follicle. The ORS is contiguous with the developing epidermis, which has now begun to stratify. Those cells that maintain contact with the dermal papilla are called matrix cells. As matrix cells withdraw from the cell cycle, they move upward in concentric cylinders of cells, each of which adopts a morphologically distinct differentiation program. The three cylinders that compose the inner root sheath (IRS) form first, and then finally the hair appears at the center of the developing follicle. Shortly thereafter, a sebaceous gland emerges as an appendage to the upper segment of each hair follicle. The center of the sebaceous gland is open to and secretes lipids (sebum) into the hair canal at the skin surface. The mature hair is composed of an inner cylinder of ghost-like cells surrounded by the cortex and the cuticle of the hair shaft (Figure 2).

Once the hair follicles are fully mature, the matrix cells at the base of the follicle continue to proliferate and differentiate, yielding a period of active hair growth known as anagen (Figure 2). However, matrix cells have a finite proliferative capacity, setting a biological timer to the number of hair or IRS cells that a matrix cell can generate (reviewed by Hardy, 1992; Paus and Cotsarelis, 1999). The timer appears to be genetically determined, but is influenced by the size and stimulatory output of the dermal papilla as well as other factors in the surrounding environment. When the proliferative capacity of the matrix cells is exhausted, a destructive phase ensues. Known as catagen, this period is typified by an apoptotic cell death of the lower two-thirds of the hair follicle (Weedon and Strutton, 1981). As the follicle regresses, the dermal papilla cells maintain contact with

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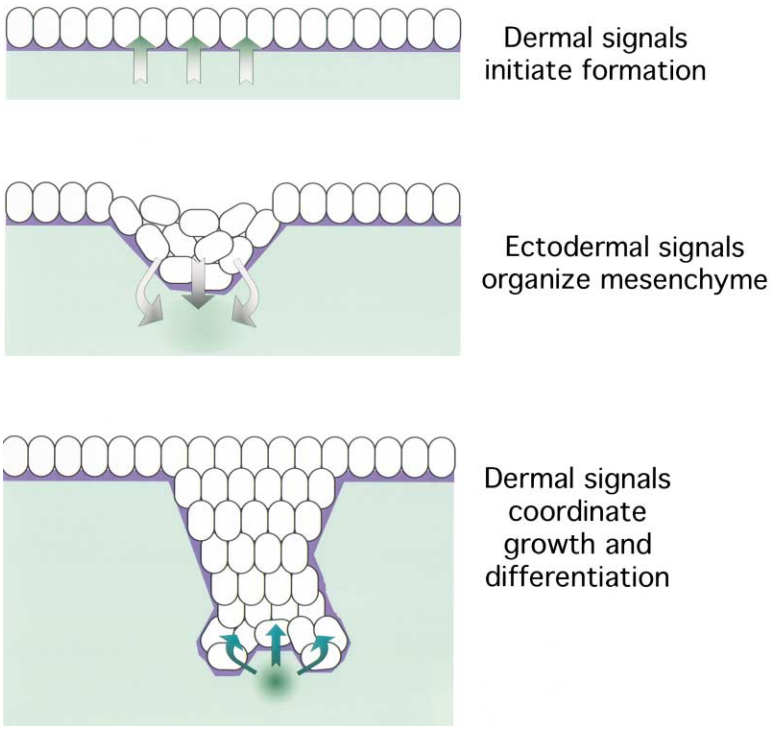

Figure 1. Epithelial-Mesenchymal Interactions Govern Hair Follicle Morphogenesis

In the early embryo, the skin begins as a single layer of ectodermal cells from which the epidermis and hair follicles are derived. In response to a mesenchymal cue, an ectodermal cell will choose to form a hair follicle rather than epidermis and begin to proliferate and grow downward. In response to a subsequent ectodermal message, the underlying mesenchymal cells organize and become the dermal papilla of the hair follicle. Finally, in response to a message from these dermal papilla cells, the developing hair follicle now grows and differentiates (for review see Hardy, 1992).

the basement membrane that separates the epithelium and mesenchyme. This draws the dermal papilla up to the base of the permanent epithelial portion of the follicle, a region known as the bulge. At this point, the follicle enters a resting phase known as telogen. Telogen can last for months in humans or barely at all, as in the case of rodent whisker follicles. The old hair shaft, left without anchorage, becomes fragile and can be liberated from its site when physically stressed.

Following this resting period, a new hair cycle is seemingly spontaneously initiated, as a fresh hair germ-like structure streams down from the bulge (Figure 2). In collaboration with the dermal papilla, the hair germ now blossoms into a fresh new follicle, retracing many of the same morphological steps that occurred during embryogenesis. The mesenchymal-epithelial cross-talk that led to the production of matrix cells, IRS, and hair during the early stages of embryonic morphogenesis appears to be similar to the initiation of anagen in the postnatal hair cycle.

Hair growth continues in this cyclic fashion throughout postnatal life. In rodents, the completion of the first two hair cycles is largely synchronous, although progressive asynchrony develops with subsequent cycles. This is likely attributable to the environmental impact on the telogen phase of the hair cycle. Plucking can shorten the duration of telogen, and many other factors influence this phase, including the nutrients, steroids, neurotransmitters, cytokines, and growth factors that are either delivered by the vasculature surrounding each follicle or by other cell types in the neighborhood. Because hair follicle cells can produce autoregulatory factors in response to various external cues, the follicle cells themselves can have an impact on telogen length. Taken together, these environmental factors can make the length of telogen highly variable.

The Bulge: The Skin Epithelial Stem Cell Niche

The ability of the epidermis, sebaceous gland, and hair follicle to constantly renew is a special feature of skin epithelium. Self-renewing tissues are able to do so by virtue of a compartment of stem cells, which have the unique capacity to divide in a fashion such that some of the daughter cells are stem cells, able to sustain the compartment of multipotent cells, while other progeny are biologically distinct in some way from their parent stem cells (Figure 3; reviewed by Fuchs and Segre, 2000; Watt and Hogan, 2000). It is not yet clear whether this heterogeneity arises from the ability of a stem cell to divide asymmetrically or from an environmental asymmetry that exposes some daughter cells to different external transducing signals. Despite its relatively small size, the bulge has been purported to be the residence of the self-renewing stem cells of the skin (Cotsarelis et al., 1990; Taylor et al., 2000; Oshima et al., 2001). Cells emanate from this compartment to regenerate the lower two-thirds of the hair follicle at the initiation of each new cycle. This compartment also seems the likely source for the hair follicle cells that regenerate the epidermis during wound healing (Potten and Morris, 1988).

Like other stem cells, bulge cells are slow cycling, and when labeled with a tracer nucleotide these cells will retain the label over extended chase periods (Bickenbach and Mackenzie, 1984; Morris and Potten, 1999; Wilson et al., 1994; Potten and Morris, 1988). Thus, after several weeks of chase, the transiently amplifying matrix compartment retains little or no label, whereas the bulge cells retain $>$95% of the remaining label (Cotsarelis et

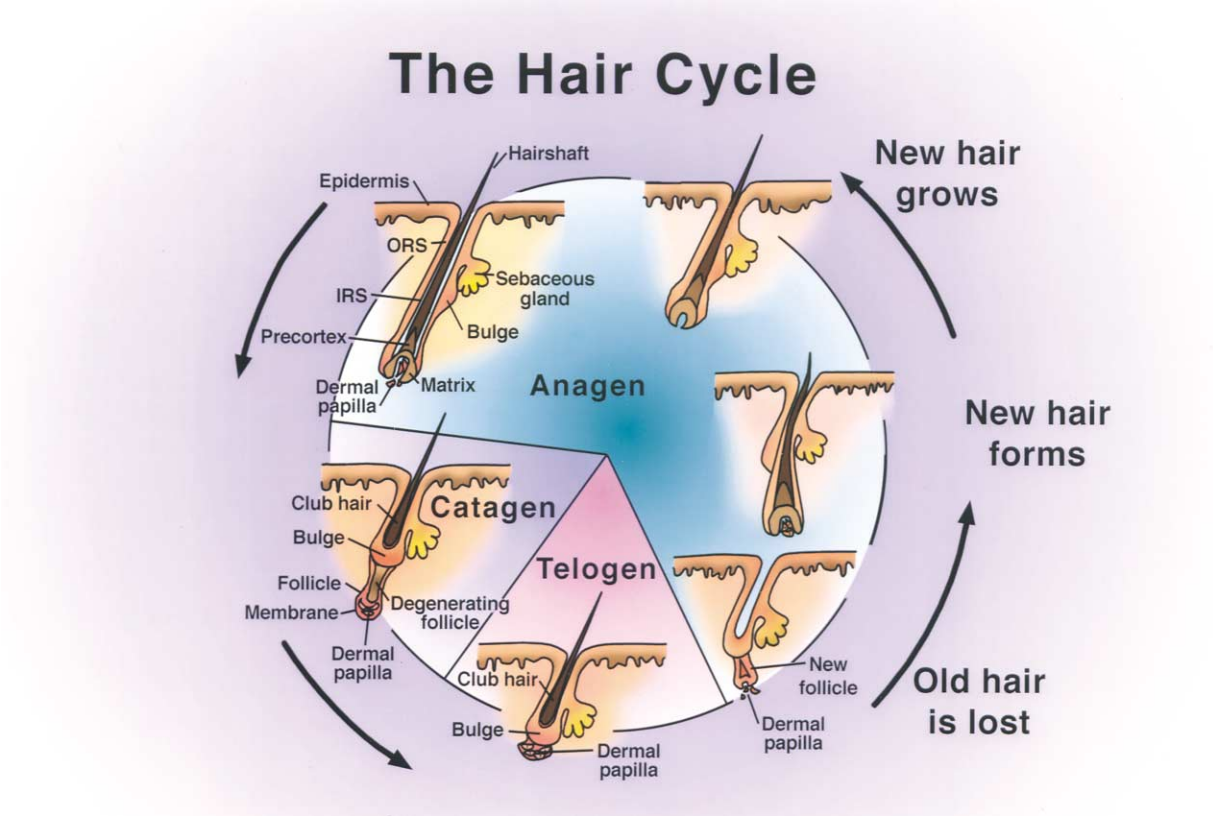

Figure 2. The Anatomy of the Mature Hair Follicle and Its Cycle

The mature follicle consists of an outer root sheath contiguous with the basal layer of the epidermis, an inner root sheath that serves as the channel from which the hair exits the skin surface, and the hair shaft itself. The hair is derived from the precortical cells, which along with the inner root sheath are descendents of proliferating matrix cells at the bulb of the follicle. Matrix cells are thought to maintain their proliferative state through sustained association with dermal papilla, encloaked by the matrix.

Throughout the entire life of most mammals, hairs undergo periods of growth (anagen), destruction (catagen), and rest (telogen). In order to constantly initiate a new round of follicle growth to replace the hair that degenerated and was released, it requires an inexhaustible supply of stem cells, which are located in the bulge. Periodically, upon initiation of a new anagen, division of stem cells provides proliferative progeny that are directed by the dermal papilla to differentiate and form a new hair shaft.

al., 1990; Morris and Potten, 1999; Taylor et al., 2000). Recently, Robert Lavker and his colleagues devised a double-label technique, first labeling most of the epithelial skin cells with bromodeoxyuridine (BrdU), then chasing until only the bulge cells retained this label, and then finally labeling with tritiated thymidine to follow the subsequent migration of the now double-labeled bulge stem cells (Taylor et al., 2000). Interestingly, cells emanating from the bulge migrated both upward to populate the basal layer of the epidermis as well as downward to populate the cells of the growing hair follicle (Figure 4B). Oshima et al. (2001) used a slightly different approach to tackle the same problem. They transplanted follicle segments dissected from a genetically engineered, β-galactosidase-expressing mouse into the bulge region of a normal vibrissae follicle. By tracking the migration of these tagged cells at times after transplantation, the researchers confirmed that indeed, the bulge cells are multipotent and can differentiate along multiple lineages, including those of the sebaceous gland as well as the epidermis and hair follicle (Figure 4A). These simple but elegant experiments provide compelling evidence that the bulge cells are the multipotent stem cells, replenishing the skin's epithelial cells as needed.

The Bulge Activation Hypothesis versus the Stem Cell Migration Hypothesis: Activating Stem Cells to Restart the Hair Cycle

The bulge is an attractive location for the resident compartment of the hair follicle epithelial stem cells because it resides at the base of the permanent epithelial portion of the hair follicle, i.e., as deeply tucked away as possible in the skin. While bulge stem cells are now known to be utilized in replenishing the epidermis and sebaceous glands, these cells were initially investigated for their ability to generate a new follicle at the start of each hair cycle.

At the end of each hair cycle, each dermal papilla of pelage follicles conveniently comes to rest at the base of the bulge, rendering it in the right place to have a close encounter with a stem cell. We know from transplantation studies that without a dermal papilla, a new round of hair cycling becomes an impossible feat (Oliver, 1970; Jahoda et al., 1984; Hardy, 1992). Coining the term

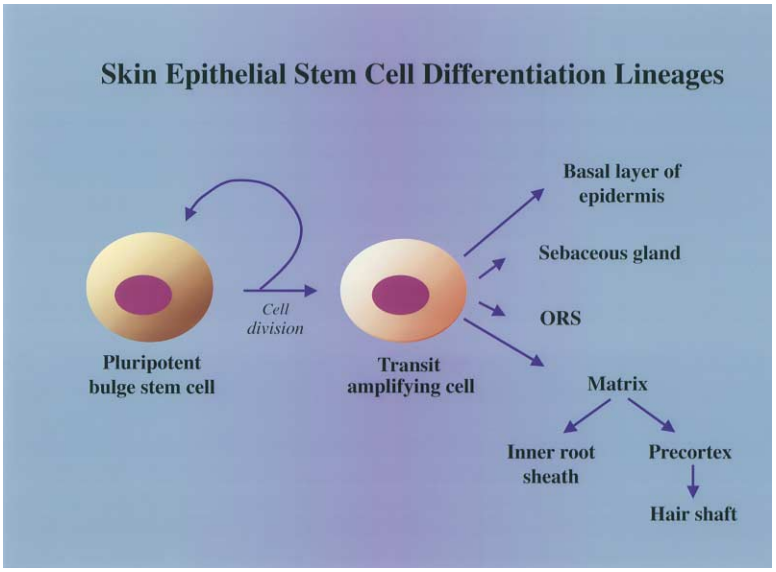

Figure 3. Lineage of the Epidermis and Its Appendages

The bulge is the epithelial compartment in which the stem cells of the epidermis and hair follicle reside. In response to various environmental signals, a stem cell divides to give rise to one daughter that is a stem cell and another which is in some way distinct from its parent. The multipotent daughter cell acquires the ability to exit this specific niche and, depending upon the signal(s) received, contributes to the maintenance of the sebaceous gland, outer root sheath, and epidermis or the development of a new hair follicle by development of transforming into matrix cells which subsequently differentiate into the hair shaft and inner root sheath.

"bulge activation hypothesis," Lavker and Sun postulate that some key signal from the dermal papilla triggers the transition from a resting to a cycling follicle (Cotsarelis et al., 1990 and references therein). While the dermal papilla is in the right place and time to activate bulge stem cells, a couple of observations suggest that it may not provide the signal that initiates stem cell division. First, the dermal papilla can remain perched next to the bulge for extended periods of time (months in some human follicles) without activating a new hair cycle. The long period of inactivity complicates the bulge activation hypothesis by requiring an explanation for how the bulge stem cells can remain quiescent for extended periods despite their contact with dermal papilla. In addition, in *hairless* mutant mice dermal papillae are stranded deep in the dermis during a defective catagen stage, and yet bulge cells continue to proliferate, suggesting that stem cells can divide even in the absence of dermal papilla signals (Panteleyev et al., 1999).

In addition, it would seem at first glance that the bulge activation hypothesis does not apply to the large rodent whisker follicles. In the rat, these specialized tactile organs differ from pelage follicles in that they grow at a whopping rate of 1.5 mm per day and have a short catagen and almost no telogen (Ibrahim and Wright, 1982). Consequently, these follicles seem to begin the next hair cycle before they have fully finished the previous one, and thus never reach a stage where the dermal papilla comes into contact with the bulge. Barrandon and colleagues have recently suggested that the basic principles of the bulge activation hypothesis (derived to explain cycling in pelage follicles) can remain intact if the stem cells of the whisker follicles migrate down to the dermal papilla rather than bringing the dermal papilla to the bulge (Oshima et al., 2001).

Seemingly consistent with this notion, 3.5% of the keratinocyte colony-forming cells of the skin reside within the hair bulb rather than the bulge (Rochat et al., 1994), and early transplantation experiments were also suggestive that some stem cells might reside within the bulb (Reynolds and Jahoda, 1991). In the Barrandon transplantation experiments, β-galactosidase-tagged cells appeared to exit the bulge and migrate downward along the outer surface of the developing new follicle (secondary hair germ) on a journey taking approximately 3–4 weeks time. Upon reaching the bulb, the tagged cells then appeared to move inward and upward to form the whisker. Oshima et al. (2001) interpreted these results to suggest that the growth of the whisker begins when stem cells reach the follicle bulb, where they stop migrating and are converted to proliferating matrix cells. The researchers further posited that growth (anagen) stops when the bulge cells are instructed by some unidentified signal to stop generating new stem cells. To account for all of their clonogenic data, Barrandon and coworkers suggest that stem cells temporarily lose the ability to exert their proliferative potential when excised from the follicle while in the act of migration, but they regain this capacity when they slow down near the base of the bulb. A depiction of their model is shown in Figure 4A.

In certain ways, the stem cell migration hypothesis provides a resolution to many seemingly controversial findings. If correct, it would explain why Oliver and Jahoda's transplantation studies focused on the hair bulb as the source of the stem cells and why Watt and coworkers found evidence that stem cells also reside in the basal layer of human epidermis (Jones and Watt, 1993; Jones et al., 1995). However, the hypothesis as it stands is counter to existing dogma that stem cells remain in their niche and that heterogeneity in stem cell progeny provides the driving force that compels cells to exit the bulge. If stem cells exit the bulge and migrate, then in order to maintain homeostasis and prevent depletion of stem cells in the bulge, skin epithelial stem cells would either have to home back to their niche or they would have to be able to divide symmetrically to give rise to two daughter stem cells, neither of which has yet been demonstrated experimentally. The stem cell migration hypothesis also lacks a molecular explanation for what prompts a stem cell to exit the bulge, why stem cell character is lost when the cell is in the act of migrating,

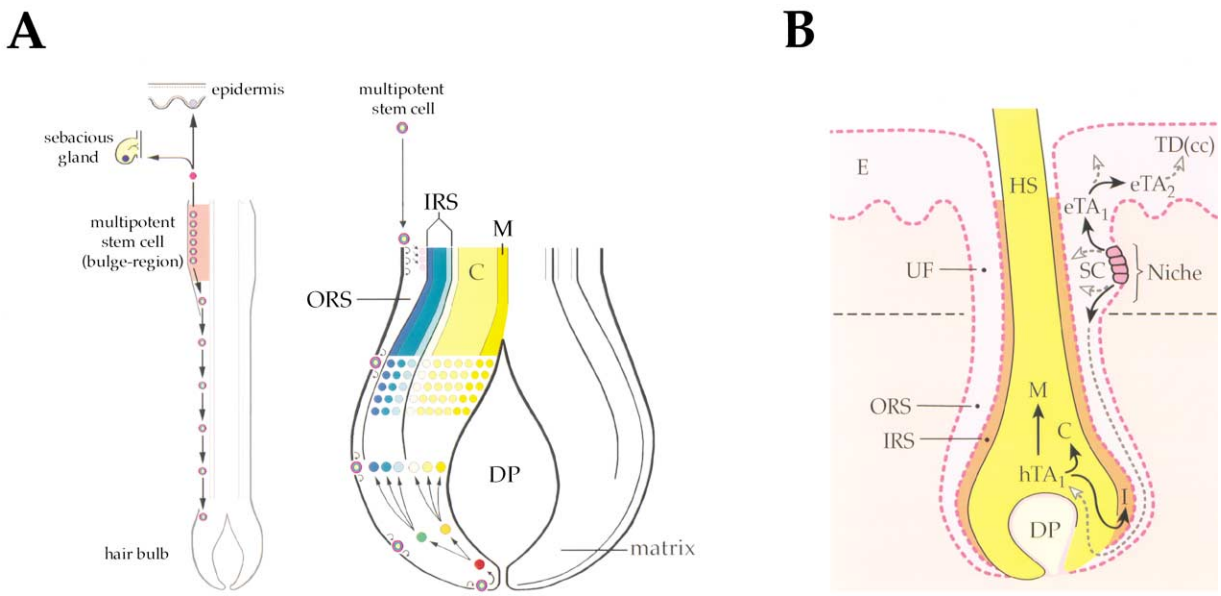

Figure 4. Models of Stem Cell Activation and Differentiation

(A) After conducting transplantation experiments on rodent whisker follicles, Barrandon proposed a model whereby stem cells are not confined to their niche, but rather can migrate to populate various epithelial compartments in the skin (Oshima et al., 2001). They then adopt their character according to the environmental cues provided by the selected migratory pathway (from Oshima et al., 2001). The migrating cells retain multipotency which is influenced by the different environments to which each stem cell is exposed. According to this model, stem cells migrate to the dermal papilla, rather than requiring the dermal papilla to migrate to the bulge.

(B) Lavkar and Sun propose that stem cells (SC) are converted to transient amplifying (TA) cells that exit their niche and migrate upward from the bulge to populate the basal layer of the epidermis; basal cells then undergo additional cues to commit to terminally differentiate (TD). When stem cells receive a signal from the dermal papilla, they convert to TA cells which migrate down and repopulate the lower portion of the new follicle. These cells differentiate into cortex (C) and inner root sheath (I) cells. According to this model, in order for a stem cell to choose the hair cell fate, the bulge must be in close proximity to the dermal papilla (Taylor et al., 2000).

and why this property is not rejeuvenated upon transplantation or culture.

Seeking a Resolution to Stem Cell Lineages

In reviewing the often beautiful and compelling experimental data that have been amassed from the labs of stem cell biologists, a few modifications would seem to resolve several of the apparent controversies that remain in understanding the behavior of the stem cells in the skin. The simplest explanation to account for most of the data at hand is that the decision of a cell to leave the bulge is based upon the ability of stem cells to generate two types of daughter cells. Irrespective of the molecular cues that drive this step, stem cell division rates could be highly variable across different species and for different follicle types. The generation of non-stem cell progeny might depend upon certain various environmental changes, e.g., hair plucking or the degree to which the bulge region may be innervated and/or vascularized. Differences such as these are known to influence telogen phase, which is the step in the hair cycle that is most highly variable. When the cell cycle controlling division is fast, a short telogen would ensue, and when the division cycle is slow an extended telogen would be the consequence. The non-stem cell daughters of these divisions could possess features that render their surfaces distinct from those of stem cells, enabling a natural mechanism for facilitating their detachment and exit from the niche at rates that would then differ for different follicles.

According to this model, the pluripotent skin epithelial stem cells remain within the niche of the bulge, neatly tucked out of harm's way and protected from receiving external cues that may coax them down differentiation lineages. In contrast, the non-stem cell progeny of stem cell divisions would encounter new stimuli as they migrate along basement membranes into new territories. In doing so, they would acquire additional characteristics that might then direct them along specific lineages. Thus, for instance, encounter with the dermal papilla could convert the progeny to matrix cells, able to produce the hair shaft and inner root sheath. What governs the decisions to become the transiently amplifying cells of the epidermis, sebaceous glands, or outer root sheath is largely unknown.

Controlling the Hair Follicle Cell Fate: A Role for Wnts

A main question in the field of hair cycling and follicle morphogenesis is what is the stimulus that a dermal papilla cell emits that instructs the bulge progeny to become matrix cells? Forays into the molecules that govern hair follicle morphogenesis came initially from explorations of how hair keratin genes are regulated. Transcriptional control regions of a group of hair-specific keratin genes each possess a sequence motif that

binds the LEF1/TCF family of HMG box DNA binding proteins (Zhou et al., 1995). Originally identified as a transcriptional regulator of T-lymphocyte differentiation (Travis et al., 1991; van de Wetering et al., 1991; Waterman et al., 1991), LEF1 (lymphoid enhancer factor 1) is also expressed in hair follicles, where its mRNA is highest in the matrix cells (Zhou et al., 1995; DasGupta and Fuchs, 1999). While LEF1 is found in both nuclei and cytoplasm of matrix cells, it concentrates in the nuclei of the hair precursor cells (precortex) at a time corresponding to the induction of the hair-specific keratin genes (DasGupta and Fuchs, 1999). LEF1 is also expressed much earlier in embryogenesis, in the ectodermal placodes as well as the underlying dermal condensates (Zhou et al., 1995; DasGupta and Fuchs, 1999). The physiological relevance of LEF1 was illuminated by gene targeting, which resulted in mice with a paucity of hair follicles and a complete loss of whisker follicles (van Genderen et al., 1994), and by transgenic expression of LEF1 in interfollicular epithelium, which led to invaginations resembling hair germ formation (Zhou et al., 1995).

Several other groups soon realized that LEF1 and its relatives are not ordinary transcription factors but rather DNA binding proteins which function primarily when coupled with β-catenin, providing the transactivation domain of the complex (Behrens et al., 1996; Molenaar et al., 1996). The β-*catenin* gene is expressed in both mesenchymal and epithelial cells. Only in epithelial cells is β-catenin detectable at the membrane, where it participates in intercellular adhesion. In both mesenchymal and epithelial cells, β-catenin not utilized for cell-cell adhesion is normally phosphorylated by a protein complex containing the GSK-3 kinase, and this targets the protein for further modification and degradation by the 26S proteosome (for review see Nusse, 1999; Mao et al., 2001 and references therein).

The only mechanism currently known that leads to stabilization of extra β-catenin is through receipt of an external Wnt signal. Cells that possess Wnt membrane receptors (a combination of frizzleds, or Fzs, and LRP5/6; Mao et al., 2001 and references therein) can bind the appropriate Wnt and transmit a signal to inactivate the GSK-3 complex by a series of intermediate steps. With the kinase temporarily crippled, β-catenin begins to accumulate, and if LEF1 or one of its TCF cousins is present a transcription factor is produced which can then activate downstream target genes (Salic et al., 2000). Figure 5 (left) provides a simplified version of the Wnt signal transduction pathway as it was known five years ago, indicating the main steps of the pathway (Nusse, 1999).

There are at least 15 different genes encoding Wnts, 10 encoding frizzleds and 2 Wnt-regulated LRPs in the mouse and human genomes. Although their functional roles are still largely undetermined, a number of Wnts have been found to be expressed in skin (Millar et al., 1999; St-Jacques et al., 1998). While recent evidence has surfaced that not all Wnts and receptors act alike, many Wnt receptors,when activated, seem to result in β-catenin stabilization through mechanisms that are just beginning to emerge (see Mao et al., 2001 and references therein). The gene multiplicity enables different cells of the animal to express and receive Wnt signals at different times in accordance with the involvement of Wnt pathways in the development of many different organs in the body, including limbs, cartilage, kidney, and brain (reviewed by Cadigan and Nusse, 1997).

To demonstrate that Wnt-like signaling might be important in hair follicle development, DasGupta and Fuchs (1999) engineered mice expressing a transgene (TOPGAL) consisting of β-galactosidase coding sequence under the control of a minimal promoter and an enhancer containing multimerized LEF1/TCF binding sites. Under these circumstances, cells of the animals express β-galactosidase when a cell possesses a member of the LEF1/TCF family of DNA binding proteins and when it has also stabilized β-catenin (i.e., is receiving a Wnt or Wnt-like signal). Transgenic embryos harboring this gene display spots of blue cells in the skin at discrete times in development (Figure 6A; DasGupta and Fuchs, 1999). Just at/or prior to hair follicle formation, cells within the single layer of pluripotent ectoderm and the underlying dermal condensates express the gene (Figures 6B and 6C). Nuclear LEF1 and nuclear β-catenin also appear in these cells at this time, further confirming that Wnt signaling is taking place (Figure 6D; see also Merrill et al., 2001).

The next sign of transgenic β-galactosidase expression occurs just before birth, when the hair follicle is nearly fully formed. At this time, as matrix cells at the center of the hair bulb differentiate, the precortical cells activate the reporter gene (Figures 6E and 6F). These cells also concentrate LEF1 and β-catenin in their nuclei and induce the hair keratin genes that display LEF1/TCF binding sites in their 5′ regulatory domains (Zhou et al., 1995; DasGupta and Fuchs, 1999; Merrill et al., 2001).

The β-galactosidase-expressing cells in early skin embryonic epithelial and mesenchymal cells suggest that these cells talk to each other through Wnt signals. To demonstrate a functional role for β-catenin stabilization in hair follicle morphogenesis, Gat et al. (1998) engineered mice to express a hybrid transgene composed of an epidermal keratin promoter/enhancer driving expression of a constitutively stable β-catenin lacking the GSK-3 phosphorylation sites and thus unable to be rapidly degraded. Beginning with the first postnatal hair cycle, the transgenic mice become extremely furry, resulting from what appear to be new hair follicles within the interfollicular epidermis (Gat et al., 1998). This finding is striking, given that normally hair follicle morphogenesis occurs only in embryonic skin.

While vestiges of hair follicle development take place in adult skin expressing constitutively stable β-catenin, the new hair follicles seem to form only when the old hair follicles are stimulated to start a new hair cycle, suggesting that an additional signal is required to unleash the effects of β-catenin. Although the nature of this signal is still unknown, its timing is suggestive that it is derived from the stimulae occurring early in the anagen phase of the hair cycle. Another interesting feature of these animals as they age is that they develop benign tumors bearing a striking resemblance to a human scalp tumor, known as pilomatricoma (Gat et. al., 1998). This led to the identification of the origin of these tumors as well as their genetic basis: the tumors consist of pure matrix and hair cells, and in humans they arise from stabilizing mutations in the endogenous β-catenin (Chan et al., 1999).

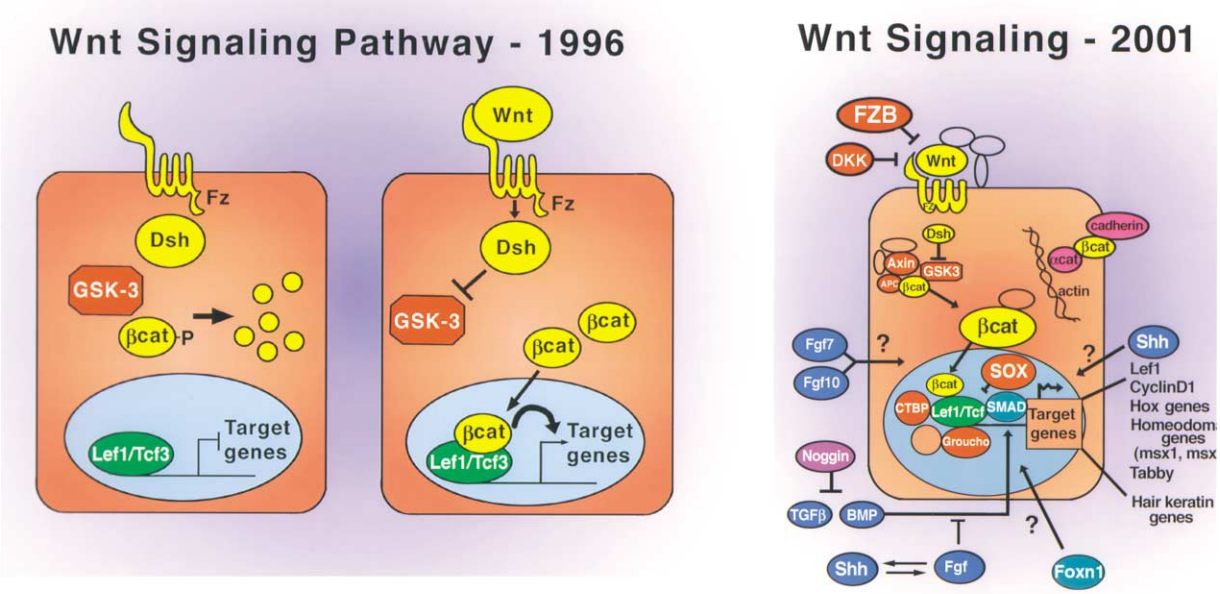

Figure 5. Models of the Wnt Signaling Pathway

On the left is the Wnt signaling pathway as it existed in 1996. In response to an external Wnt signal, a cell with a receptor, called frizzled or Fz, modifies another protein called disheveled (Dsh), which in turn inhibits a kinase called GSK-3. When β-catenin no longer becomes phosphorylated by GSK-3, it begins to accumulate where it can now make a complex with a member of the LEF1/TCF family of DNA binding proteins. An active transcription factor is generated which now activates target genes. In the precortex, these target genes are likely to include the hair keratin genes and other essential structural components of the hair shaft cells. On the right is a model depicting the Wnt pathway in skin epithelium as it is surmised in 2001. The Wnt receptor now appears to be comprised of at least two proteins: a frizzled and LRP5/6, and Wnt signaling can be intercepted and inhibited through other external signaling molecules. Wnt signaling may act through activated receptor-mediated association with axin, a protein required for GSK-3-mediated turnover of β-catenin. A myriad of additional positive- and negative-acting factors have been found to associate with β-catenin and/or TCF/LEF1 to influence the ability of the complex to activate downstream target genes. In addition, a number of external signals, including BMPs, TGFβs, FGFs, and Shh, are known to stimulate transduction pathways in some epithelial cells of the skin, and these pathways are known to intersect and influence Wnt pathways. Downstream from these various and complex interactions is the activation of genes encoding transcription factors such as hoxc13, msx1, msx2, and Foxn1 as well as other hair-associated proteins such as Tabby and the hair-specific keratins (see Nusse, 1999; Mao et al., 2001; Wilson et al., 2001).

Scientists in the field are trying to learn more about how the process of β-catenin stabilization and LEF1/TCF gene expression is regulated during embryonic development in the surface epithelium of animals. For mammalian skin, we must know the molecular nature of the dermal papilla signals that seem to be so important in triggering and maintaining matrix cell character during anagen. The biology of dermal papilla cells has eluded scientists, mostly because there are proportionately so few of these specialized fibroblasts in the skin. Although it is possible to isolate dermal papilla by dissection, the cells rapidly lose their inductive capacity in culture (Jahoda et al., 1984). A promising advance in this field comes from the laboratory of Bruce Morgan, who used fluorescence-activated cell sorting (FACS) to purify dermal papilla cells from a mouse expressing a versican promoter-driven green fluorescent protein (GFP) transgene specifically expressed in dermal papilla cells (Kishimoto et al., 2000).

The most surprising result from Morgan's study came when the authors cultured their pure dermal papilla cells in the presence of Wnt-expressing cells (Kishimoto et al., 2000). While normal dermal papilla cells rapidly lose their inductive powers in culture (Jahoda et al., 1984), the Wnt-bathed dermal papilla cells maintained their inductive capacity to generate hair follicles when transplanted back into mice. Although Wnt-activated gene expression has not been reported in adult dermal papilla cells, the results of Morgan are consistent with the notion that Wnt signaling plays an important role in converting mesenchymal embryonic skin cells to dermal papilla cells. Further characterization of the Wnts and frizzleds involved will be an important next step in understanding how Wnt cross-talk between epithelial and mesenchymal components of the hair follicle may orchestrate the generation of embryonic hair follicles and maintain dermal papilla and/or stem cell character in adult cycling hair follicles.

What's after Wnts? Tailoring a Wnt to Suit a Cell's Whims

Another issue presently unsolved is whether different Wnts and different Wnt receptors dictate the different responses that hair follicle cells make to the signals, or whether inherent differences inside the different follicle cells determine the output of a Wnt signal. For instance, Wnt signaling in the precortex seems to result in the activation of hair keratin genes and genes whose products are needed to make a hair shaft, whereas Wnt signaling to the dermal papilla cells seems to maintain the inductive capacity of the dermal papilla cell. What are the β-catenin/LEF1/TCF-regulated target genes that are activated to promote these various responses? The

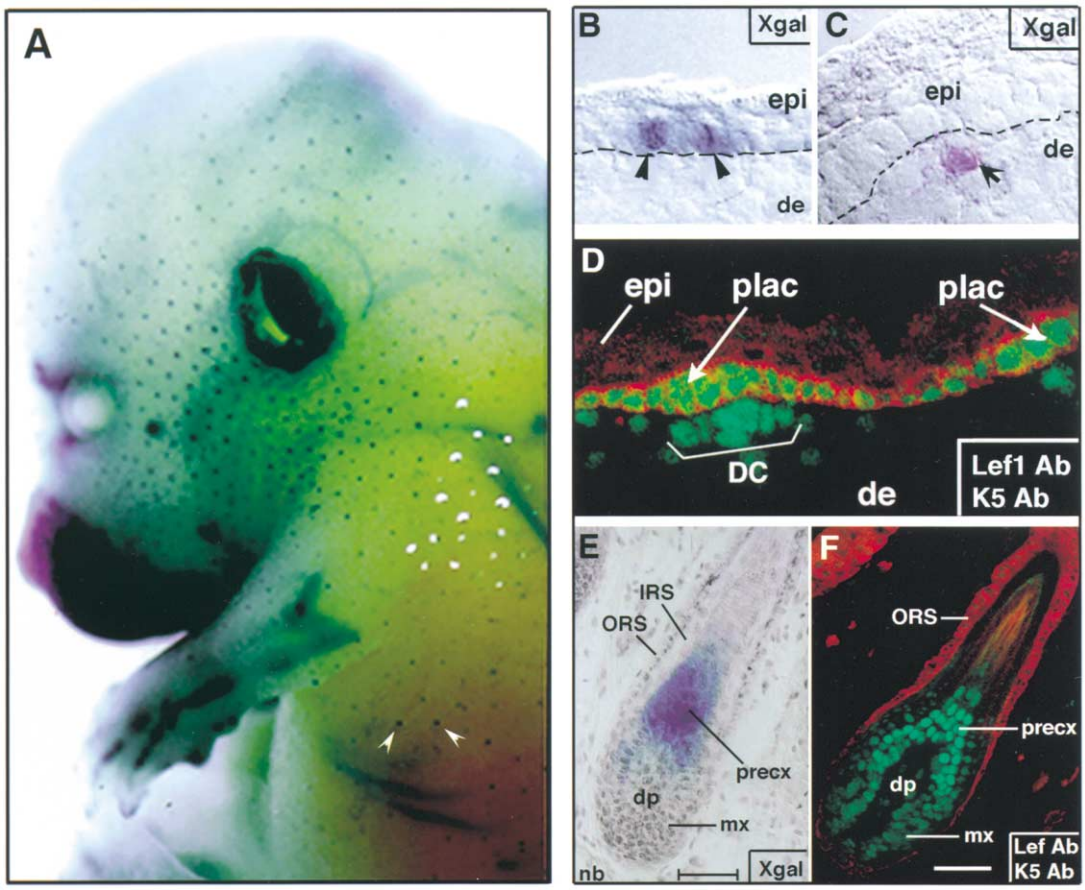

Figure 6. Assay for Putative Wnt Signaling in Mouse Embryos

A transgenic mouse was engineered to express β-galactosidase whenever a putative Wnt signal was received by a cell and an activated transcription factor composed of β-catenin and LEF1/TCF was produced (DasGupta and Fuchs, 1999). The colorless dye X-gal, which turns blue in the presence of β-galactosidase, was used as a detector.

(A) Transgenic embryo at 16.5 days of gestation (newborn = 19 days); spots correspond to developing hair follicles (arrowheads for examples).

(B and C) Skin from the sectioned embryo showing blue cells (arrowheads) in the epithelium (epi) and in the dermal mesenchyme (de).

(D) Skin from same embryo labeled with antibodies against LEF1 in green and antibodies against keratin K5 in red. The ectodermal placodes (plac) and the underlying dermal condensates (DC) both show nuclear LEF1 (and also nuclear β-catenin) at this early stage of hair follicle development.

(E and F) Skin from a newborn transgenic mouse stained with X-gal in (E) (to test for Wnt signaling) and labeled with antibodies against K5 in red and LEF1 in green (to show that nuclear LEF1 is present in the precortex, which is also responding to a Wnt signal). ORS, outer root sheath; precx, precortex; mx, matrix; dp, dermal papilla.

answer to this question will likely be prerequisite to understanding how these gene regulatory complexes function to control cell fates in the skin.

From the myriad of studies conducted on Wnts in various systems and species, it is clear that Wnt signaling intersects with and impacts on many other signaling pathways in cells. These studies have led to a considerably more complex view of Wnt signaling than was apparent five years ago (Figure 5B, right). An important feature of this new body of knowledge is that there are many factors that associate with the LEF1/TCF transcription factor complex to alter its activity. Although LEF1/TCF proteins often function as transactivators, these proteins can also interact with and behave as transcriptional repressors. CtBP represses a Wnt/Wingless response and selectively interacts with the C-terminal domain of TCF3, missing in LEF1 (Brannon et al., 1999). In contrast, the Groucho family of repressor proteins associates with the proline-rich domain of all known TCF/LEF1 family members (Cavallo et al., 1998; Roose et al., 1998; Brantjes et al., 2001). When these proteins bind to TCF3, they can in turn bind and recruit histone deacetylases, thereby packaging the chromatin into an inactive state (reviewed by Bienz, 1998; Billin et al., 2000; Brantjes et al., 2001). Genetically, a *Drosophila* dTcf–Groucho interaction antagonizes Wnt/Wingless signaling (Cavallo et al. 1998), and expression of Groucho proteins in *Xenopus* embryos can block axis formation and inhibit activation of β-catenin target genes, such as siamois and xnr3 (Roose et al. 1998; Brannon et al. 1999). In zebrafish, embryonic TCF3 appears to function as a transcriptional repressor, as an N-terminally truncated TCF3 (missing its β-catenin binding domain) complements the headless phenotype caused

by loss of function of wild-type TCF3 (Kim et al. 2000). Thus, in the absence of a Wnt/Wingless signal, the association of repressor proteins with TCFs can in some instances repress genes regulated by TCFf/LEF1. In these cases, some as yet unidentified signal(s) besides or in addition to a Wnt may be needed in order to relieve their repression.

Once β-catenin has been stabilized through Wnt signaling, it too can associate with nuclear proteins in addition to LEF/TCF. One such group includes the HMG box-containing Sox proteins, which appear to inhibit LEF/TCF-mediated signaling activity through physical interactions with Wnt-stabilized β-catenin (Zorn et al., 1999). Other factors, such as SMAD factors, may enhance the ability of the β-catenin/LEF/TCF complex to activate genes, suggesting a physical interaction between TGF-β and Wnt signaling components (Nishita et al., 2000; Labbe et al., 2000). TGF-β can also negatively affect LEF1/TCF signaling by activating the kinase TAK1, which phosphorylates TCFs and prevents DNA binding (Ishitani et al., 1999). Taken together, the data in hand tell us that the mere presence of nuclear β- catenin and a member of the TCF/LEF family does not necessarily imply that target genes will be activated. Moreover, the ability of interacting molecules to recognize and associate with the complex may vary depending upon the specific LEF1/TCF family member expressed by a cell. In addition, the particular DNA sequence(s) in a target gene may influence which complex(es) bind. Thus, there may be conditions where irrespective of Wnt signaling LEF/TCF factors can act as repressors, as well as situations where Wnt signaling might act either to relieve repression or activate downstream target genes.

What does all this mean for the hair follicle? Although LEF1 appears to act primarily in conjunction with β-catenin to transactivate downstream target genes, TCF3 is expressed in the bulge of adult hair follicles, where it may act as a repressor protein. In TOPGAL reporter mice, β-galactosidase gene expression is only transiently activated in the TCF3-positive bulge cells at the start of each new hair cycle (DasGupta and Fuchs, 1999). Thus, in most phases of the hair cycle TCF3 might be acting as a repressor rather than a transcriptional activator. This notion is consistent with recent studies on lower vertebrate TCF3 forms which argue that TCF3 is a repressor protein that can act through association with the Groucho and CtBP families of transcriptional repressor proteins (Roose et al., 1998; Cavallo et al., 1998; Brannon et al., 1999).

The role of LEF1 in coaxing cells down the hair lineage suggests that analogously, TCF3 might function either to maintain stem cell character or to direct stem cell progeny along the outer root sheath program of differentiation, which is similar to that which exists in the bulge. Recent evidence suggests that this is the case (Merrill et al., 2001). It is also intriguing that the conversion of a stem cell to a matrix cell involves the apparent loss of TCF3 and the gain of LEF1 expression (DasGupta and Fuchs, 1999; Merrill et al., 2001). This switch, in conjunction with receipt of a Wnt signal, appears to be essential in the commitment of stem cells to a hair lineage fate. When the effect of Wnt signaling is blocked, either by conditional ablation of β-catenin or by expression of a truncated form of LEF1, hair formation is blocked. Intriguingly, cell proliferation is maintained and cells that normally adopt hair cell fates switch to adopt epidermal or sebocyte differentiation characteristics (Huelsken et al., 2001; Merrill et al., 2001).

We might predict that the signal necessary to relieve TCF3-mediated repression is the elusive "inductive signal" of the dermal papilla cells. While the putative Wnt-activated DP signal remains as yet unidentified, candidates include members of the fibroblast growth factor (FGF) family, which are made by dermal papilla cells but affect skin epithelial cells. Recent evidence indicates that when the receptor for FGF-7 and FGF-10 is genetically mutated, hair follicle morphogenesis fails, with the tissue undergoing extensive apoptosis (De Moerlooze et al., 2000). Although Wnt signaling is known to inhibit FGF signaling in certain situations (Wilson et al., 2001), the Wnt signal that might do this in the hair follicle might be expected to be the Wnt that prompts a matrix cell to differentiate to a hair cell.

Once TCF3 expression is suppressed and LEF1 gene expression is switched on, a number of additional factors appear to be required for the differentiation pathway that leads to hair production. Many of these insights come from analyses of mice and/or human genetic disorders that result in either perturbations in hair development or complete loss of hair. Those that have phenotypic similarities to the *Lef1* knockout mice include (1) mice deficient in Msx2 and its close relative Msx1, which are homeodomain transcription factors that are likely to be downstream target genes of LEF1/TCF-activated complexes (Satokata et al., 2000), and (2) the human genetic disorder X-linked anhidrotic ectodermal dysplasia (EDA) and its cousin the *tabby* mutant mouse, which are caused by respective mutations in genes encoding EDA, a member of the tumor necrosis factor family of growth factors, and its receptor (EDAr) (Monreal et al., 1999; Headon and Overbeek, 1999). The *tabby* gene has a LEF1/TCF binding motif in its promoter and *Lef1*$^{-/-}$ mice fail to express EDA, suggesting that these genes may be direct downstream targets of LEF1 (Laurikkala et al., 2001).

Other interesting hair disorders that involve putative transcriptional regulators of the process include (1) the *ragged* mutant mouse, which was recently shown to arise from mutations in Sox18, a close cousin of the β-catenin negative regulator Sox17 (Zorn et al., 1999; Pennisi et al., 2000); (2) hair-deficient mice mutated for the gene encoding *Hoxc13* (Goodwin and Capecchi, 1998), which belongs to the homeobox (hox) family of transcription factors known to be influenced by Wnt signaling (for review see Bienz, 1998); (3) the *nude* mouse and corresponding rare human hair disorder, known to involve mutations in whn (winged- helix-nude; now known as Foxn1), a transcription factor expressed relatively late in development and involved in hair keratin gene expression (Nehls et al., 1994; Frank et al., 1999; Schlake et al., 2000); and (4) the *hairless* and RXRα conditionally mutant mice, which both display a normal first hair coat that is subsequently lost upon initiation of the next hair cycle (Mann, 1971; Li et al., 2001). Hairless and RXRα seem to be good candidates for interacting with one another to regulate genes involved in maintaining dermal papilla connections with the hair follicle (Panteleyev et al., 1999; Li et al., 2001).

Researchers in hair biology have begun to weave this new array of hair follicle factors into a more global view of follicle morphogenesis. An intriguing example of this is the recent study on noggin, a mesenchymally derived factor which interacts with and neutralizes the activity of bone morphogenic proteins (BMPs) made by both dermal papilla and hair shaft precursor cells. Although noggin knockout mice are defective in LEF1 expression and hair development (Botchkarev et al., 1999), misexpression of noggin in the proliferating hair matrix cells and differentiating hair precursor cells leads to hyperproliferation of the LEF1-expressing matrix cells and suppression of hair cell differentiation (Kulessa et al., 2000). These results suggest that contact with noggin-expressing dermal papilla might naturally prevent premature hair shaft formation and may function by governing the balance between matrix cell division and hair cell differentiation. In addition, expression of *whn (Foxn1)*, *hoxc13*, *Msx1*, and *Msx2* are strongly reduced or absent in the transgenic mice, suggesting that BMP-4 may be required to maintain expression of these transcription factor genes, which in turn may act cooperatively with LEF1 to promote hair shaft differentiation (Kulessa et al., 2000). In this regard, it is interesting that TGF-β/BMP and Wnt signal transduction pathways can intersect to enhance activation of downstream target genes (Nishita et al., 2000).

Interestingly, in early embryonic ectoderm, Wnt signaling appears to promote epidermal versus neuronal cell fate commitment by inhibiting FGF action and allowing BMP signaling (Wilson et al., 2001). In the hair follicle, this may also be happening, as evidenced by the fact that when β-catenin is conditionally ablated in the skin epithelium, BMP expression is impaired and hair follicle cycling is blocked (Huelsken et al., 2001). The phenotype of these mice is remarkably similar to that of the *hairless* and RXRα conditional null mice, providing further evidence that these pathways are interconnected to Wnt signaling. Plakoglobin appears able to compensate fully for the adhesive role of β-catenin but not for its transcriptional coactivator role, providing an important animal model for elucidating the role of Wnt signaling and excluding a transcriptional role for plakoglobin in hair follicle morphogenesis (Huelsken et al., 2001).

A discussion of the biochemical events involved in Wnt signaling and hair follicle formation would not be complete without further discussion of the Shh signal transduction pathway. In many developmental processes, including limb and neuronal development, Shh goes hand in hand with Wnt signaling, often but not always in complementary ways. In a concentration-dependent fashion, Shh produced and secreted by one cell type can alter the differentiation status and/or developmental fate of a neighboring target cell (Briscoe et al., 2000). To respond, the target cell must display the transmembrane receptor for Shh, called patched (Ptc), which appears to act by blocking a signal transduction pathway mediated by smoothened (Smo). Often when Shh relieves Ptc of their inhibitory duties, the target cell responds by proliferating. A good example of this in the skin comes from recent studies showing that genetically defective Ptc or mutations in downstream effector molecules result in basal cell carcinomas in humans (Hahn et al., 1996; Johnson et al., 1996; Dahmane et al., 1997; Xie et al., 1998). Overexpression of *Shh* and activating mutations in Smo also leads to basal cell carcinomas in mice (Oro and Scott, 1998; Xie et al., 1998), and conversely *Shh* knockout embryos display severe defects in hair follicle development (St Jacques et al., 1998). Shh is expressed in ectodermal placodes and mesenchymal condensates of embryonic skin, as is Ptc, while in the adult Shh appears to concentrate in bulb ORS cells with Ptc in the matrix just after the start of each new hair cycle. While we don't yet understand precisely how Shh controls hair follicle morphogenesis and cycling, it is likely to act downstream of Wnt signaling, since hair germs still develop in *Shh* knockout embryos and since *Shh* expression is absent in conditional β-catenin null skin (Huelsken et al., 2001). One possibility is that Shh participates with FGFs in stimulating follicle cells to proliferate to convert the hair germ to a fully grown hair follicle, and in this regard the interdependency of FGFs and Shh noted in embryonic epidermal precursor cells is intriguing (Wilson et al., 2001).

Summary

Our view of hair follicle morphogenesis and cycling is that it is orchestrated by changes in the status of the activity of TCF/LEF1-regulated genes, which are at least in part regulated by Wnt signaling. In turn, a number of other key signaling pathways, including those triggered by BMPs, FGFs, and Shh, participate in activating downstream nuclear regulatory factors, such as Hoxc13, Msx1, Msx2, Whn, hairless, RXRα, SMADs, and Soxes. Why would nature select Wnts to conduct this symphony? While the answer remains unknown, β-catenin has a Jeckyl and Hyde life—sometimes it functions in cell-cell adhesion, and other times it masquerades as a transcription factor. On the surface, it seems simpler to design a brand new gene encoding an entire transcription factor rather than to reuse β-catenin to provide a transactivation domain for LEF1.

A possible explanation for this enigma emerges when reflecting upon the three times during hair follicle morphogenesis where Wnts have thus far been shown to play a role: as single layered ectoderm remodels to form a hair germ, as mesenchyme assembles a dermal condensate to form the dermal papilla, and as matrix cells differentiate to produce the highly structured cells of the hair shaft (DasGupta and Fuchs, 1999). At all three of these times which rely upon signaling/communication between two distinct cell types, dramatic changes in intercellular adhesion accompany the changes in gene expression that impact on proliferation and differentiation. These adhesive changes are likely to be essential to the process, given that antibodies against cadherins impair intercellular junction formation and block hair follicle morphogenesis in organ culture (Hirai et al., 1989) and ablation of the α-*catenin* gene in skin inhibits hair follicle morphogenesis in mice (Vasioukhin et al., 2001). When taken together with a role for Wnt signaling in this process, we might now predict that when Wnt signaling activates β-catenin/LEF1/TCF transcription factor complexes, it might also influence the cadherin-β-catenin-α-catenin complexes involved in intercellular junctions,

perhaps facilitating remodeling of ectoderm and mesenchyme into a hair follicle.

In closing, it might seem that sifting through the factors involved in Wnt signaling and elucidating how Shh, FGF, and TGF-β/BMP signal transduction pathways influence Wnt signaling will be no less daunting than piecing together the mysteries of a 12th century B.C. Minoan civilization on an archeological dig. This said, technological advances in functional genomics and in methods to isolate pure populations of hair follicle cells will soon make it possible to contrast global programs of gene expression in different follicle cell populations and at different stages of the hair cycle. By elucidating the precise molecular differences that might impact on Wnt signaling and its converging pathways, such approaches should provide new insights into the relative importance of specific factors as follicle cells differentiate through their intricate lineages.

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