Cell, Vol. 105, 533–545, May 18, 2001, Copyright ©2001 by Cell Press

β-Catenin Controls Hair Follicle Morphogenesis and Stem Cell Differentiation in the Skin

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Summary

β-Catenin is an essential molecule in Wnt/wingless signaling, which controls decisive steps in embryogenesis. To study the role of β-catenin in skin development, we introduced a conditional mutation of the gene in the epidermis and hair follicles using Cre/loxP technology. When β-*catenin* is mutated during embryogenesis, formation of placodes that generate hair follicles is blocked. We show that β-*catenin* is required genetically downstream of *tabby*/*downless* and upstream of *bmp* and *shh* in placode formation. If β-*catenin* is deleted after hair follicles have formed, hair is completely lost after the first hair cycle. Further analysis demonstrates that β-catenin is essential for fate decisions of skin stem cells: in the absence of β-catenin, stem cells fail to differentiate into follicular keratinocytes, but instead adopt an epidermal fate.

Introduction

The hair follicle is generated during development in a complex morphogenetic process that relies on a reciprocal exchange of signals between epithelial and mesenchymal cells in the forming of hair placodes (for reviews, see Oro and Scott, 1998; Barsh, 1999). Fields of hexagonally ordered placodes are established in several waves by local activators and inhibitors that act over different ranges. Additional epidermal and dermal signals organize the final follicle structure and orientation. In the adult, hair follicles undergo continuous self-renewal and cycling, which depend on pluripotent stem cells that reside in the bulge of the hair follicles, a portion of the outer root sheath (Cotsarelis et al., 1990, 1999; Fuchs and Segre, 2000; Watt and Hogan, 2000; Lavker and Sun, 2000; Oshima et al., 2001). Recent findings revealed that these follicular stem cells are bipotent, as they give rise to both keratinocytes of the hair follicle and the interfollicular epidermis (Taylor et al., 2000). Hair follicles undergo periods of active growth (anagen phase), regression and shortening (catagen phase), and rest (telogen phase; Paus and Cotsarelis, 1999). At the onset of anagen, follicles grow from the dermis into the subcutis and contain at their tips transiently dividing epithelial cells, called matrix cells. These give rise to the inner root sheath and hair and engulf specialized mesenchymal cells, called the dermal papilla. In catagen, regression of the hair follicle drags the dermal papilla upward into the dermis toward the bulge. At the initiation of a new anagen stage, follicular stem cells appear to respond to signals from the dermal papilla, and give rise to the next generation of proliferating matrix cells. The bulge also delivers progenitor cells to the basal layer of the epidermis. Follicular stem cells and the different keratinocyte lineages express distinct proteins that can be used to distinguish the different cell types (Heid et al., 1988; Fuchs, 1995; Langbein et al., 1999). Little is known about how stem cells of the skin are maintained and how their commitment to different keratinocyte lineages is controlled. Recently, the P53 homolog P63 has been implicated in the regenerative aspect of stem cell division (Yang et al., 1999; Mills et al., 1999).

Wnt/β-catenin signaling, among other signaling systems, was implicated in the development of skin, hair, and the related appendages in birds, the feathers (Noramly et al., 1999; Widelitz et al., 1999, 2000). Many components of the Wnt signaling system have been identified: extracellular Wnt/wingless ligands activate Frizzled receptors and, through Dishevelled, induce an increase in cytoplasmic β-catenin by preventing its degradation in proteasomes (for reviews, cf. Nusse, 1999; Huelsken and Behrens, 2000; Barker et al., 2000). Axin/Conductin, in cooperation with the tumor suppressor gene product APC, promote β-catenin degradation, which involves serine-threonine phosphorylation of the N terminus of β-catenin by GSK3β and subsequent ubiquitination (Rubinfeld et al., 1996; Behrens et al., 1998; Aberle et al., 1997; reviewed by Polakis, 2000). Upon Wnt signaling, β-catenin accumulates in the cytoplasm and is transported to the nucleus, where it interacts with members of the LEF/TCF family of transcription factors and activates gene expression (Behrens et al., 1996; Molenaar et al., 1996; He et al., 1998; Tetsu and McCormick, 1999; cf. Barker et al., 2000; for a review). Mutations in *apc* that interfere with Axin/Conductin and β-catenin interactions, or mutations of β-*catenin* in the N-terminal phosphorylation sites, interfere with β-catenin degradation. These mutations result in accumulation of cytoplasmic and nuclear β-catenin, and in constitutive signaling and gene activation. Such mutations are causally involved in tumorigenesis, and are frequently observed in human tumors (Morin et al., 1997; Korinek et al., 1997; Rubinfeld et al., 1997; cf. Polakis, 2000; for a review). For instance, in human skin tumors, pilomatricomas, β-*catenin* is mutated in a high percentage of the analyzed cases (Chan et al., 1999).

Several Wnt family members are expressed in distinct patterns and stages in the developing skin of mammals and birds, among them Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt10a, and Wnt10b (Nusse and Varmus, 1992; Tanda et al., 1995; Wang and Shackleford, 1996; Chuong et al., 1996; Saitoh et al., 1998; St. Jacques et al., 1998; Millar et al., 1999; Kishimoto et al., 2000). It has been demonstrated that specific Wnts maintain anagen gene

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expression in vitro and mediate hair inductive activity in organ culture (Kishimoto et al., 2000). Mice lacking *LEF-1*, which is a downstream mediator in the Wnt signaling pathway, lack whiskers and have a reduced number of body hairs (van Genderen et al., 1994). Coculture of ectoderm and mesenchyme that derive from *LEF-1*$^{-/-}$ and wild-type embryos revealed a role of mesenchymal LEF-1 in whisker formation (Kratochwil et al., 1996). Interestingly, the transcription factor TCF3 has recently been localized in the nuclei of bulge cells, the ultimate stem cells of both hair follicles and epidermis (DasGupta and Fuchs, 1999). In addition, overexpression of oncogenic β-catenin that mimics an activated Wnt signaling pathway in the skin of transgenic mice induced de novo hair follicles (Gat et al., 1998).

Several additional signaling pathways have been implicated in mammalian hair follicle morphogenesis and in feather formation. These include fibroblast growth factors (FGFs), the TNF-like Tabby, and the TNF receptor-like Downless, bone morphogenetic proteins (BMPs), Sonic Hedgehog (SHH) and Notch (for reviews, cf. Oro and Scott, 1998; Barsh, 1999). Initiation of feather placodes is controlled by positive and negative signals mediated by FGFs and BMPs, respectively (Song et al., 1996; Jung et al., 1998). Subsequently, Notch/Delta signals refine the patterning of feather placodes (Chen et al., 1997; Crowe et al., 1998). Mutations in human and mouse genes that abrogate hair follicle development have been identified. In mice and man, mutations in *tabby* and *downless* (*ectodysplasin/edar* in humans) interfere with placode formation at an early stage (Kere et al., 1996; Srivastava et al., 1997; Monreal et al., 1999; Headon and Overbeek, 1999). In contrast, a mutation in *shh* affects the morphogenesis of the follicles at a later stage in hair follicle development (St. Jacques et al., 1998; Chiang et al., 1999).

β-Catenin is also located in adherens junctions where it binds cadherins through the armadillo repeats and establishes a link to the cytoskeleton via N terminally bound α-catenin (Huelsken et al., 1994; Aberle et al., 1996; Nieset et al., 1997). Plakoglobin (γ-catenin), the closest relative of β-catenin in vertebrates, can also bind cadherins and α-catenin and, in addition, mediates the interaction between desmosomal cadherins and the intermediate filament system (Palka and Green, 1997; Fuchs and Cleveland, 1998; and references therein). Deletion of the *plakoglobin* gene in mice leads to adhesion defects which cause heart rupture and skin blistering (Ruiz et al., 1996; Bierkamp et al., 1996). Similarly, mutations in the human *plakoglobin* gene have been identified in naxos disease, which leads to ventricular cardiomyopathy, palmoplantar keratoderma, and, interestingly, woolly hair (McKoy et al., 2000). We previously generated β-catenin-deficient mice and observed a defect in early embryogenesis, i.e., a block in anterior-posterior axis formation at E5.5 (Huelsken et al., 2000). In the absence of β-catenin, no mesoderm and head structures were generated subsequently. However, intercellular adhesion was maintained since Plakoglobin substitutes for β-catenin (Huelsken et al., 2000).

In the present work, we have ablated the β-*catenin* gene in the skin of mice using the *keratin 14* driven Cre/loxP system. We observed two phenotypes, lack of placode formation during embryogenesis and loss of hair in the first hair cycle. Our data suggest that fate decisions of stem cells in the skin depend on β-catenin function: in wild-type mice, β-catenin controls differentiation of stem cells into follicular or epidermal lineages. In the absence of β-catenin, keratinocytes can only adopt an epidermal phenotype.

Results

Skin-Specific Ablation of the β-*Catenin* Gene in Mice

The β-catenin protein is stabilized by activation of the Wnt pathway in many tissues and organs (cf. Wodarz and Nusse, 1998, for a review). We also found that the β-catenin mRNA is upregulated in developing hair follicles during embryogenesis (Figures 1a–1d). Similar upregulation was also observed during feather formation of the chick and suggests a new mechanism of β-catenin regulation (cf. Widelitz et al., 2000). The upregulation of β-catenin in mouse embryos can be seen both by whole-mount in situ hybridization using a β-catenin cDNA probe (Figure 1a) as well as by β-galactosidase staining of mice in which a lacZ cDNA is integrated into the β-*catenin* locus (Figure 1b; cf. also Huelsken et al., 2000). The typical hexagonal pattern of forming hair follicles was seen, larger spots representing later stages and smaller spots earlier stages of follicle morphogenesis. The activation of the β-galactosidase reporter revealed that upregulation of β-catenin is due to activation of the β-*catenin* promoter at these stages of hair follicle morphogenesis. We found that this upregulation occurs in the epithelial part of the forming hair placodes, as revealed by sectioning of β-galactosidase stained embryos (Figures 1c and 1d).

To investigate the role of β-catenin in skin and hair development, we used the Cre/loxP technology to introduce conditional mutations of the β-*catenin* gene in mice. LoxP sites were introduced into the second and sixth intron of the β-*catenin* gene by homologous recombination in embryonal stem (ES) cells (Figures 1e and 1f; for structure of the β-*catenin* locus cf. Huelsken et al., 2000). Upon Cre-mediated recombination, a mutant allele of β-*catenin* is created in which the exon encoding the first four amino acids of β-catenin is fused to the seventh exon. Thus, a deletion/frame-shift mutation is introduced, which creates an allele that cannot encode a functional protein. A shortened mRNA, but no shortened protein, was produced by the mutated allele, as shown by Western blotting using antibodies against β-catenins N or C terminus (Figures 1g, 1h, and 2h). Mice generated from ES cells containing the floxed β-*catenin* locus (β-*cat* lox) were bred to homozygosity. They were viable, fertile, and did not display an overt phenotype (Figure 1i, and data not shown). To introduce the mutation of β-*catenin* into cells of the epidermis and hair follicles, we generated mice that express Cre under the control of the *keratin 14* gene (*K14*). The cDNA of Cre-recombinase was introduced into the *K14* locus by homologous recombination in ES cells (Figures 2a and 2b). Two *keratin 14*-Cre alleles were produced, one that retains the neomycin resistance cassette, *K14*-Cre(neo), and a second

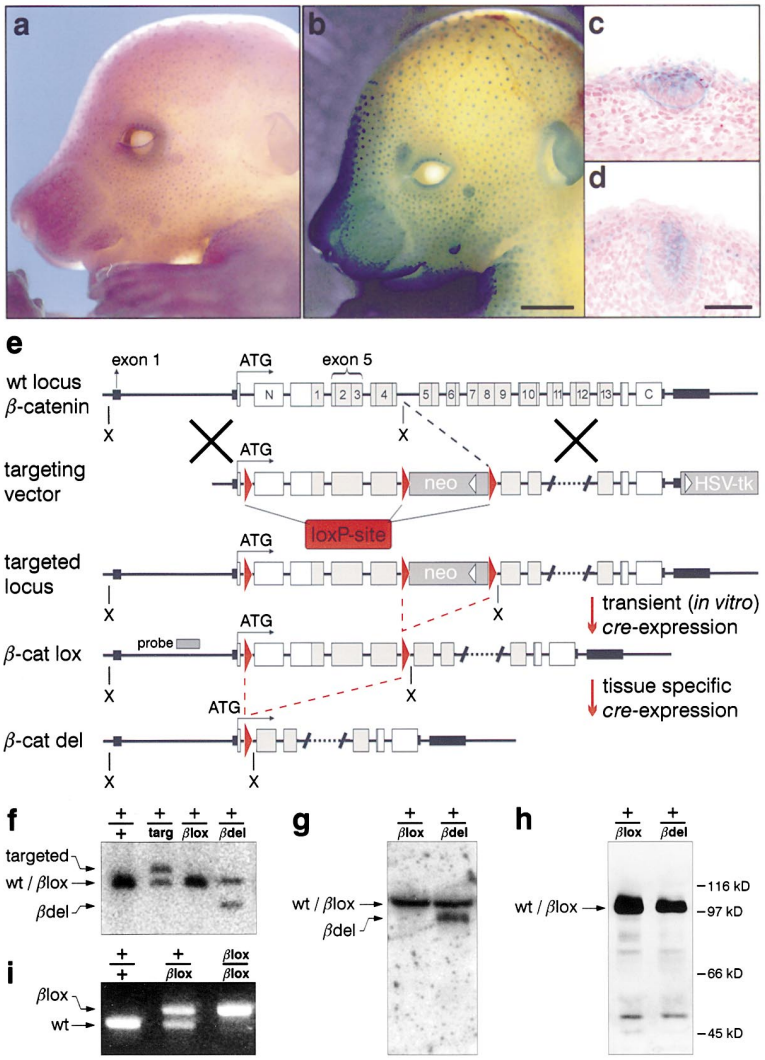

Figure 1. Upregulation of β-Catenin Promoter Activity at Early Stages of Hair Placode Formation and Generation of Mutated Alleles for Tissue-Specific Deletion of the β-*Catenin* Gene in Mice

(a) Whole-mount in situ hybridizations with β-catenin cDNA at E15.5. (b) Whole-mount β-gal staining, at E15.5, of mice in which a lacZ cDNA is integrated into the β-*catenin* locus (cf. Huelsken et al., 2000). Sections of the embryo shown in (b) reveal β-gal staining and thus activation of the β-*catenin* promoter in the epithelial part of the placode at stage 1 (c) and stage 2 (d) of follicle morphogenesis. Bars: (a and b, shown in [b]) 1 mm; (c and d, shown in [d]) 50 μm. (e) Scheme of the genomic structure of β-*catenin*, the targeting vectors, and the targeted alleles. Large boxes indicate coding exons of β-*catenin* (e.g., exon 5); small black boxes indicate noncoding exons (e.g., exon 1). The encoded β-catenin protein consists of central armadillo repeats (1–13) that are flanked by unique N- and C-terminal domains. In the targeting vector, loxP sites (red triangles) were introduced in introns flanking exon 3 and exon 6, which encode the N-terminal domain (N) and armadillo repeats 1 to 4. After homologous recombination in ES cells, the loxP-flanked neomycin resistance cassette (neo) was removed by transient expression of Cre-recombinase, and the resulting ES cells (β-*cat* lox) were used to generate mice. After tissue-specific, Cre-mediated deletion, a frame shift results from splicing together exon 2 and exon 7. The probe used for Southern analysis is indicated; X, XhoI; HSV-tk, herpes simplex virus thymidine kinase. (f) Southern and (g) Northern blots of heterozygous ES cell lines containing the mutated alleles. Equal amounts of RNA were loaded for Northern blotting, and full-size β-catenin cDNA was used as a probe. Note the shortened mRNA produced from the β-*cat* del locus. (h) Western blot of heterozygous ES cell lines with an antibody directed against the C terminus of β-catenin. Note that no shortened protein is produced from the β-*cat* del locus. (i) PCR genotyping of newborn mice from a cross of heterozygous β-*cat* lox mice.

in which the neo cassette was removed by Flp-recombinase, *K14*-Cre(Δneo). The two alleles differed in the onset of Cre-mediated recombination during development. Intercrossing strain *K14*-Cre(neo) with Cre-inducible lacZ reporter mice (Thorey et al., 1998) revealed strong Cre-mediated recombination in a mosaic, patchy pattern at E15 (Figure 2c; the pattern of patches differed between individual embryos). At this age, recombination by *K14*-Cre(Δneo) was much weaker (Figure 2d). However, both strains of *K14*-Cre showed ubiquitous recombination in hair follicles and basal cells of the epidermis after birth (shown for *K14*-Cre(neo) in Figure 2e). Crosses of the *K14*-Cre strains with β-*cat* lox mice resulted in deletion of the β-*catenin* gene mainly in skin, but also in eye, tongue, and esophagus, which are tissues of known Keratin 14 expression (Figure 2f; cf. Fuchs, 1995). Deletion of the β-*catenin* gene in skin was virtually complete, as revealed by Southern and Western blotting (Figures 2g and 2h).

Keratin 14-Cre-Induced Early Deletion of the β-*Catenin* Gene Abrogates Hair Follicle Morphogenesis

Mutant mice of the genotype *K14*$^{+}$/Cre(neo) and β-*cat* lox/null (for the β-*catenin* null allele, cf. Huelsken et al., 2000) were born and showed reddish patches of hairless skin at P8 (Figure 3a). The pattern of patches differed between individual mice. Sectioning of mutant skin at P8 revealed that hairless patches result from a complete lack of hair follicles in these area (Figure 3d, right side), whereas a neighboring area contained normal numbers of fully developed follicles (Figure 3d, left side). The *K14*-Cre(Δneo) allele did not produce hairless patches after birth (not shown). Both *K14*-Cre alleles

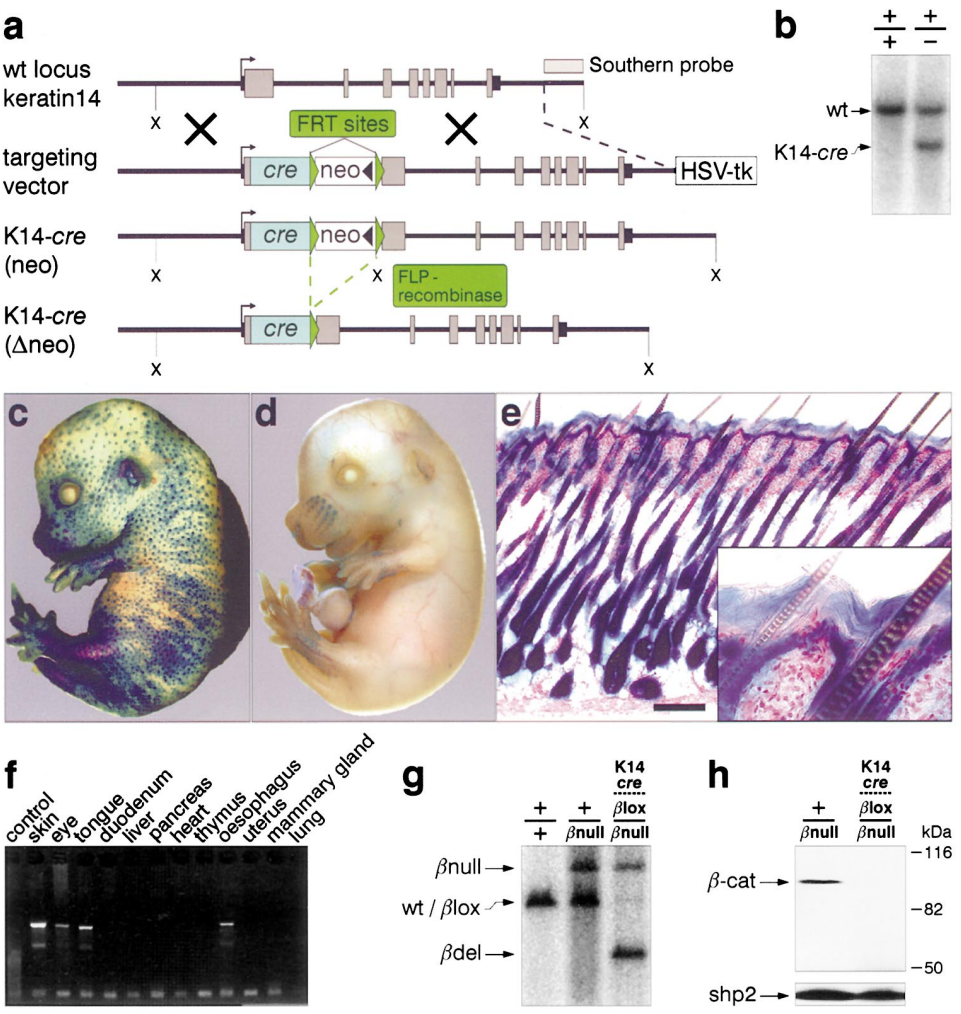

Figure 2. Analysis of Mice Expressing Cre-Recombinase under the Control of the *keratin 14* Locus

(a) Scheme of the genomic structure of *keratin 14*, the targeting vector, and the targeted alleles. Large boxes indicate coding, small black boxes indicate noncoding exons. In order to generate the *K14*-Cre(neo) allele, the cDNA of Cre-recombinase was introduced in-frame after the endogenous ATG start codon and was followed by a neomycin resistance (neo) cassette flanked by FRT sites (green triangles). A second locus, *K14*-Cre(Δneo), was generated following homologous recombination in ES cells by transient expression of flp-recombinase to remove the neo cassette. The probe used for Southern analysis is indicated; X, XbaI; HSV-tk, herpes simplex virus thymidine kinase. (b) Southern blot of wild-type and heterozygous ES cell lines containing the *K14*-Cre(neo) allele.

(c, d, and e) Cre-mediated activation of an inducible lacZ reporter by (c) *K14*-Cre(neo) and (d) *K14*-Cre(Δneo) on whole mounts at E15, and by (e) *K14*-Cre(neo) on skin sections at P12. β-Gal staining is found in the epidermis and hair follicles (see also insert). Bar: 200 μm.

(f) Tissue-specific recombination of the β-*catenin* gene was detected by PCR in skin, eye, tongue, mammary gland, and oesophagus.

(g) Southern blot of epidermis of wild-type and mutant mice at P42 showing virtual loss of the β-*catenin* wild-type allele following Cre-mediated recombination.

(h) Western blot of epidermis at P42 showing absence of β-catenin protein following Cre-mediated recombination, as determined by using an antibody directed against the N terminus of β-catenin. Western blot of Shp2 was used as a control.

allowed the development of shaggy fur during the initial hair growth phase (until P16, Figure 3b), although a reduced number of zigzag hairs were seen (data not shown). However, hairs were lost within the following two weeks, and the skin remained naked afterwards (Figure 3c). Mutant mice were viable and fertile for more than one year. These data indicate that β-catenin plays a role in two steps of hair formation, (1) during hair follicle morphogenesis (absence leads to hairless patches) and (2) during hair cycling (absence leads to complete hair loss after the first catagen phase).

We first examined the function of β-catenin during hair follicle morphogenesis by immunofluorescence staining of β-catenin at E15. In wild-type embryos, the surface epithelium uniformly expressed β-catenin, and high levels of β-catenin were found in the epithelial part of the forming hair placodes (Figure 3e; cf. Widelitz et al., 2000). In *K14*-Cre(neo); β-*cat* lox/null mice, extended regions of β-catenin-negative epithelium were seen which did not contain hair placodes (Figure 3f). When placodes were present, these were always uniformly β-catenin positive (cf. also below). Several signaling pathways

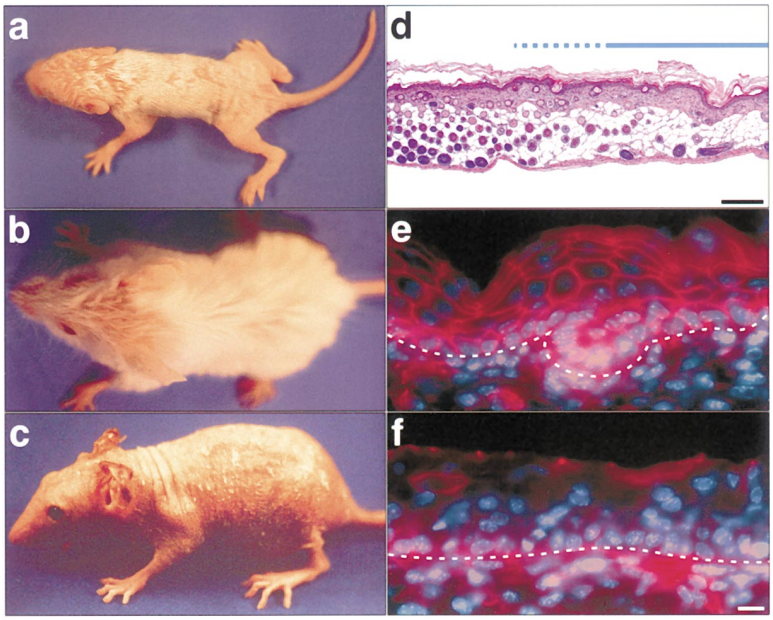

Figure 3. *Keratin 14*-Cre-Induced Deletion of the β-*catenin* Gene Abrogates Hair Follicle Morphogenesis and Maintenance of Hair

(a–c) Fur of *K14*-Cre(neo); floxed β-*catenin* mice at P8, P16, and P30, respectively.

(d) Hematoxylin/eosin-stained transversal sections of mutant back skin at P8; the blue line marks the area lacking hair follicles.

(e) Expression of β-catenin in wild-type and (f) in mutant skin at E15. Immunofluorescence analysis (red) was performed using an antibody against an epitope of the C terminus of β-catenin, DAPI-stained nuclei are shown in blue, a white dotted line marks the basement membrane between epidermis and dermis. Bars: (d) 200 μm; (e and f, shown in [f]) 10 μm.

have been implicated in early hair follicle morphogenesis (for reviews, cf. Oro and Scott, 1998; Barsh, 1999). The Tabby/Downless ligand and receptor system is essential for early placode formation. By whole-mount in situ hybridization, Downless expression in wild-type embryos was seen in a spot-like pattern in the epithelial placodes (for instance at E15.5, Figure 4a, cf. also section in the insert). Downless expression was not affected by *K14*-Cre(neo)-induced β-catenin ablation (Figure 4b), nor was the expression of Tabby (not shown). In wild-type embryos, BMP2 and BMP7 are expressed in the epithelial placodes (Figure 4c, and data not shown), and BMP4 is expressed in the underlying mesenchymal condensations (Figure 4e; cf. Botchkarev et al., 1999; Wilson et al., 1999). BMP2, BMP4, and BMP7 were absent in the β-catenin-negative patches in the mutant embryos (Figures 4d and 4f). Sonic hedgehog (SHH) and its receptor Patched-1 are expressed in wild-type mice in the epithelial placodes and the underlying mesenchymal condensations, respectively (Figures 4g and 4i; cf. St. Jacques et al., 1998; Chiang et al., 1999). Both SHH and Patched-1 were missing in β-catenin-negative skin (Figures 4h and 4j). In wild-type embryos, LEF-1 is expressed initially in the entire epithelium, is then upregulated in the epithelial placodes, and is later induced in the underlying mesenchyme (Kratochwil et al., 1996; Botchkarev et al., 1999). In the wild-type animals, spots and ring-like structures were detected at E15.5, which reflect earlier and later stages of follicle morphogenesis (Figure 4k). In β-catenin-negative skin, LEF-1-expressing spots were observed, although their number was reduced; however, the LEF-1-positive rings were absent (Figure 4l). Thus, β-*catenin* is required at an early step in the formation of epithelial placodes, and can be placed genetically downstream of *tabby/downless*. No placode formation was found in β-*catenin*-negative skin, and *bmp* and *shh* are not expressed, indicating that these are downstream events.

Late *Keratin 14*-Cre Induced Deletion of the β-*Catenin* Gene Blocks Hair Cycling

We have shown that hair of *K14*-Cre/floxed β-*catenin* mice initially grows, but that complete hair loss is observed four weeks after birth (Figure 3c), which is after the end of the first growing phase of hair in wild-type mice (cf. Paus et al., 1999). We therefore performed a detailed analysis of the morphological changes in the hair follicles of wild-type and *K14*-Cre(Δneo); β-*cat* lox/null animals, i.e., sections of skin were examined every second day between P13 and P35. During the anagen phase of the hair cycle (until P15), hair follicles of wild-type mice grow deeply into the subcutaneous fat, and both hair follicle and epidermal keratinocytes strongly express β-catenin (Figure 5a). In the skin of the conditional mutant mice, hair formation also progressed, resulting in deeply inserted, sometimes distorted, but β-catenin-positive hair follicles (Figure 5b). The upper part of the hair follicles and the epidermis were however β-catenin negative. In contrast, Plakoglobin, which can replace β-catenin in cell adhesion, was uniformly expressed at borders of β-catenin-negative cells (Figures 5i and 5j). In the following catagen and telogen phases (at P20 and P25), hair follicles in the wild-type skin retract into the dermis, maintaining close contact between the dermal papilla and the epithelial hair shaft (Figures 5c and 5e; arrows and arrowheads, respectively). However, in the β-catenin mutant skin, retracting epithelial hair shafts became separated from the dermal papillae and loss of β-catenin progressed (Figures 5d and 5f; arrows and arrowheads). A similar phenotype was recently observed in mice harboring a mutation of the *hairless* gene (Panteleyev et al., 1999). However, expression of Hairless was not affected in our β-catenin-negative skin (not shown). The hair was lost subsequently and the hair canals widened, giving rise to small cysts (Figure 5f, asterisks). In the skin of wild-type mice, a new anagen phase is initiated at the end of telogen (at P30), and new

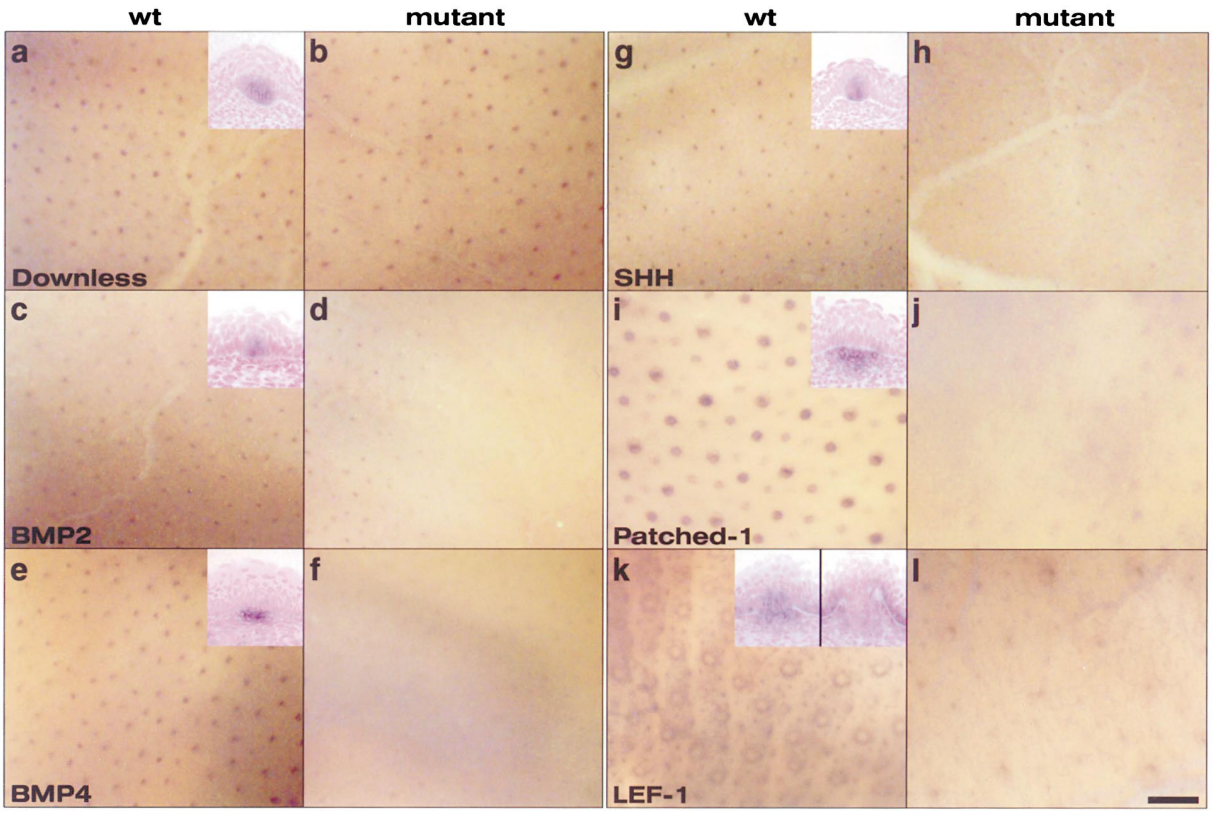

Figure 4. Expression of Signaling Molecules Implicated in Hair Placode Formation

In situ hybridizations of (a, c, e, g, i, and k) wild-type and (b, d, f, h, j, and l) β-catenin-negative area of embryonic skin at E15.5 are shown with cDNAs of (a and b) Downless, (c and d) BMP2, (e and f) BMP4, (g and h) SHH, (i and j) Patched-1, and (k and l) LEF-1. Inserts show sections of wild-type skin at stage 1 (in [k] also stage 2) of follicle morphogenesis. Bar: (a–l, shown in [l]) 250 μm.

hair follicles develop which grow down into the subcutis (Figure 5g). In contrast, no new hair follicles were formed in the mutant skin and, instead, cysts enlarged and became surrounded by a multilayered epithelium (Figure 5h, asterisks).

We studied retraction and regrowth of hair follicles by analyzing signaling molecules like BMP4 and SHH, which are expressed in the dermal papilla during catagen and in the hair matrix at the initiation of a new anagen phase, respectively (Figures 5k and 5m; arrows and arrowheads; cf. Wilson et al., 1999). In β-catenin mutant skin, BMP4 expression is lost in the dermal papilla, and SHH is not induced in the decaying hair follicle (Figures 5l and 5n; arrows and arrowheads). These data indicate that β-catenin is essential for regression of hair follicles during catagen and for initiation of a new anagen phase. Interestingly, expression of BMP4 and SHH appears to depend on β-catenin controlled events both during hair morphogenesis (cf. above) and during hair cycling.

Loss of β-Catenin Controls the Fate Decision between Keratinocyte Lineages

Recent results indicate that one ultimate stem cell population, which resides in the bulge of the hair follicle and gives rise to keratinocyte precursors of the hair follicle and of the epidermis, exists in the skin (Taylor et al., 2000; Lavker and Sun, 2000). We examined how the absence of β-catenin influences the differentiation of these stem cells. Cytokeratin 10 (K10) and Involucrin are markers of terminal differentiation of the epidermis of wild-type animals (Figures 6a and 6c; cf. Fuchs, 1995). Both K10 and Involucrin continued to be expressed in the epidermis of mutant skin and surprisingly, also in the new hair follicle-derived cysts (Figures 6b and 6d). In the wild-type hair follicle, Keratin 6hf (K6hf) and Hair keratin 2 (Ha2) are expressed in a layer between outer and inner root sheet and in the early cuticular cells of the lower hair bulb, respectively (Figures 6e and 6g; cf. Winter et al., 1998; Langbein et al., 1999). Neither K6hf nor Ha2 were expressed in the follicle-derived cysts of mutant skin (Figures 6f and 6h). Cytokeratin 17 (K17) and 6 (K6) in the wild-type skin are markers of the outer root sheet of hair follicles and of regenerating epidermis after wounding (Figure 6i, data not shown; cf. Fuchs, 1995; McGowan and Coulombe, 1998). K17 was strongly upregulated in mutant skin in both the epidermis and the follicle-derived cysts (Figure 6j), as was K6 (not shown). Ultrastructural analysis also revealed similarities between the multilayered epithelium of the cysts and the normal epidermis (Figures 6p and 6q): the cysts were surrounded by a basement membrane and several viable cell layers; the inner layers contained prominent desmosomes (arrowheads) and resembled the spinous layers of the wild-type epidermis. The innermost layers

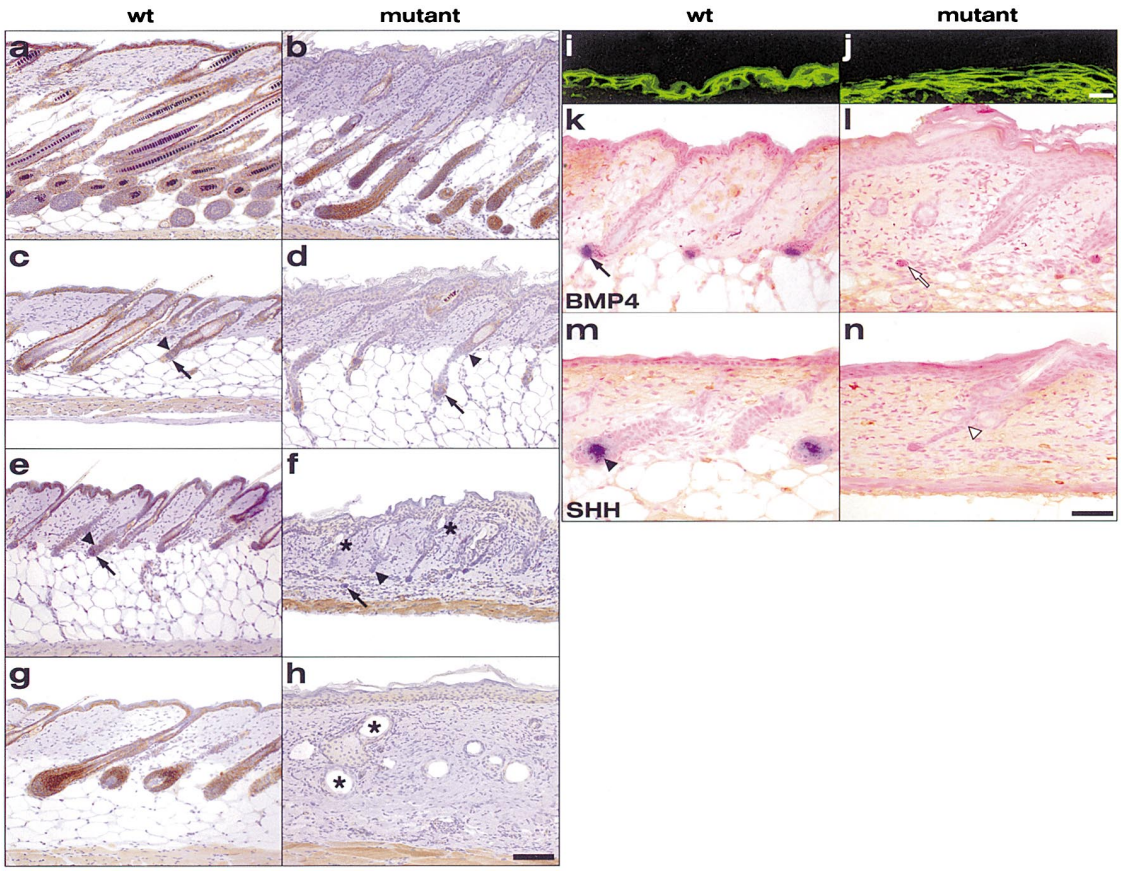

Figure 5. Lack of Hair Cycling in β-Catenin-Deficient Skin

(a–h) Sagital sections of wild-type (a, c, e, and g) and mutant (b, d, f, and h) skin were stained for β-catenin by immunohistochemistry (brown staining). (a and b) First anagen at P15, (c and d) catagen at P20, (e and f) telogen at P25, and (g and h) second anagen at P30.
(i and j) Expression of Plakoglobin in (i) wild-type and (j) mutant epidermis, as determined by confocal immunofluorescence microscopy.
(k–n) Sagital sections of wild-type (k and m) and mutant skin (l and n) were stained for signaling molecules by in situ hybridization. (k and l) BMP4 expression at telogen and (m and n) SHH expression at early second anagen. Arrows, arrowheads, and asterisks depict dermal papilla, base of epithelial hair shaft, and dermal cysts, respectively. Bars: (a–h, shown in [h]) 100 μm; (i and j, shown in [j]) 10 μm; (k–n, shown in [n]) 50 μm.

consisted of flattened, electron dense squames that were similar to the cornified layers of the normal epidermis. These data indicate that in the absence of β-catenin, cells of the hair follicle-derived cysts have lost their follicular characteristics and exhibit epidermal differentiation.

Finally, we analyzed the presence and distribution of stem cells in the mutant skin after the hair follicles are lost. Ultimate stem cells of the skin are thought to reside in the bulge of hair follicle (Lavker and Sun, 2000) and have been characterized by high expression level of β1-integrin and Cytokeratin 15 (K15, Figures 6k and 6m; arrows; cf. Lyle et al., 1998; Jones and Watt, 1993; Akiyama et al., 2000). Cells that express these markers were initially also present in bulges of mutant skin (data not shown), and, after the loss of hair follicles, they were confined to specific areas located close to the cyst epithelium (Figures 6l and 6n; arrows). However, cells expressing high levels of β1-integrin and K15 were not found in the epidermis of mutant skin, indicating that stem cells identified by these markers were not involved in the self-renewal of mutant epidermis. Instead, the proportion of basal cells in the mutants that incorporate BrdU was found to be increased more than 10-fold compared to wild-type (Figure 6o), and the number of cells that stained with antibodies against the proliferation-associated protein Ki67 was also strongly increased (not shown). These data suggest that bulge stem cells of the mutant skin may only contribute to the follicle-derived cysts, but not to the epidermis, and that the epidermis is renewed by rapidly dividing cells in the basal layer. It is known that these transiently amplifying cells have an impressive proliferative potential and may be considered progenitor cells (Lavker and Sun, 2000).

Discussion

We demonstrate here that β-catenin takes over a dual role in formation of hair follicles. First, β-catenin has an essential function for the formation of hair placodes during embryogenesis. Second, β-catenin is required for the differentiation of skin stem cells in the adult. In the absence of β-catenin, these stem cells are unable

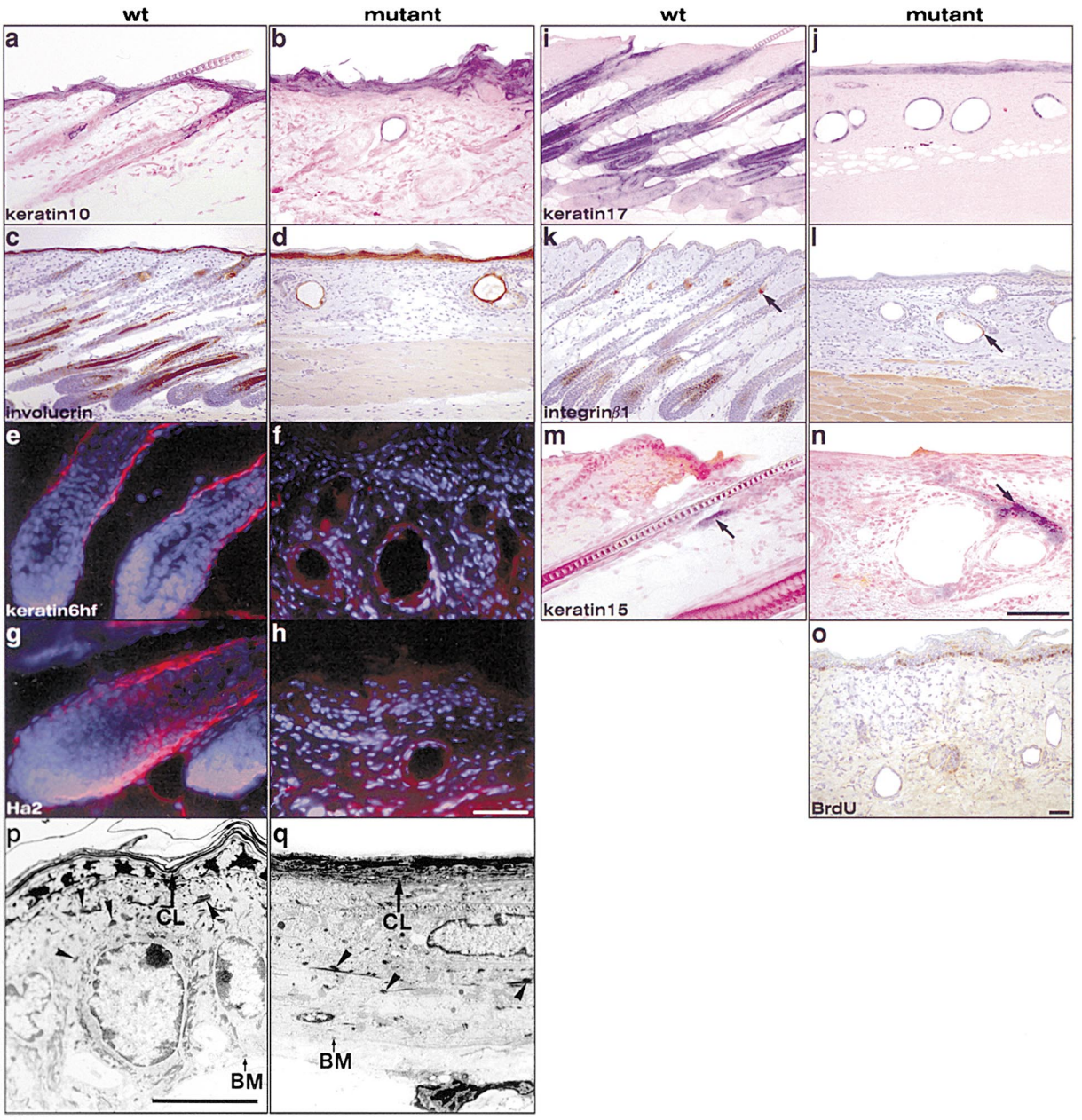

Figure 6. Differentiation of Stem Cells into Follicular Keratinocyte Lineages Is Disturbed in β-Catenin-Deficient Skin

Expression of (a and b) Keratin 10, (c and d) Involucrin, (e and f) Keratin 6hf, (g and h) Hair keratin 2, and (i and j) Keratin 17 was determined by immunostaining and in situ hybridization. Blue/black and brown/black staining in (a)–(d) were used for immunohistochemistry, red staining in (e)–(h) was used for immunofluorescence, and blue staining in (i) and (j) was used for in situ hybridization. Shown are sagital sections of wild-type at p15 (left columns) and mutant back skin at p40 (right columns). Stem cells are associated with dermal cysts in β-catenin-deficient skin: (k and m) wild-type and (l and n) mutant skin at p32 was stained by (k and l) immunohistochemistry for β1-Integrin and by (m and n) in situ hybridization using the cDNA of Keratin 15. Arrows indicate the bulges containing stem cells. (o) Rapidly cycling cells in the basal layer of mutant skin at p40 were marked by BrdU staining. (p and q) Electron microscopy of (p) wild-type epidermis and (q) epithelial cell layers of follicle-derived cysts in the mutant. BM, basement membrane; CL, cornified layers; arrowheads mark desmosomes in spinous layer. Bars: (a, b, i, j, m, and n, shown in [n]); (c, d, k, l, and o, shown in [o]); (e–h, shown in [h]) 50 μm; (p and q, shown in [p]) 500 nm.

to adopt the fate of hair keratinocytes and instead, differentiate into epidermal keratinocytes.

The formation of hair or feather placodes is a complex process in which a hierarchy of signaling molecules cooperate (Oro and Scott, 1998; Barsh, 1999). We show that β-*catenin* acts genetically downstream of *tabby* and *downless* during placode formation since the expression of both genes was unchanged in the absence of β-*catenin* (cf. scheme in Figure 7a). In contrast, BMP and SHH expression was lost in β-catenin mutants, and therefore these genes are genetically located downstream of β-*catenin*. Consistent with such a genetic hierarchy in hair

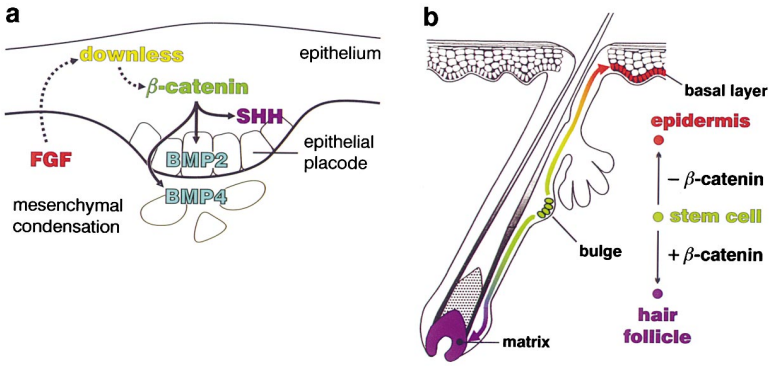

Figure 7. Scheme of the Possible Roles of β-Catenin in Hair Follicle Morphogenesis and Skin Stem Cell Differentiation

(a) The genetic placement of β-catenin in the ordered interplay of various signaling pathways mediated by FGF, Downless, BMP, and SHH is shown.

(b) The putative role of β-catenin in the fate decision of stem cells of the bulge between the follicular or epidermal keratinocyte lineages is shown. Modified from Fuchs and Segre, 2000 and Taylor et al., 2000.

placode development, β-catenin overexpression in the chick results in ectopic expression of BMP4 and SHH as well as in de novo formation of feather buds (Noramly et al., 1999). BMP4 and SHH expression was also lost in dermal papilla and hair matrix during the adult hair cycle in the conditional β-catenin mutants. Thus, the expression of BMP and SHH depends on β-catenin controlled events both during placode formation in development and during hair cycling. Whether *bmp* and *shh* are direct or indirect target genes of β-*catenin* signaling in hair or feather formation is presently unknown.

Classically, signaling via β-catenin is regulated by adjusting the stability of the protein, and β-catenin stability is increased in response to Wnt signals (Wodarz and Nusse, 1998). It is intriguing that spots of high concentrations of β-catenin and LEF-1 mRNA appear in the skin of mice during early hair follicle morphogenesis. We show here that the β-catenin mRNA level is controlled on the transcriptional level. The level of β-catenin mRNA in the early feather buds of the chick is also high (Widelitz et al., 2000). This high concentration of β-catenin and LEF-1 mRNA correlates with high protein levels (data not shown; cf. also van Genderen et al., 1994; Botchkarev et al., 1999). The two proteins together can form an active, bipartite transcription factor (Behrens et al., 1996; van de Wetering et al., 1997), and it is thus possible that β-catenin/LEF-1 signaling activity in hair follicle placodes is achieved by a Wnt-independent mechanism. Tabby/Downless signaling might control this local, high-level expression of β-catenin and LEF-1. Interestingly, in the chick mutant *scaleless*, spots of high concentration of β-catenin mRNA are not found in the forming placodes, but application of FGF restored this (Widelitz et al., 2000). Thus, FGFs might also act upstream of β-catenin in placode formation (Figure 7a).

A conspicuous second phenotype observed here is the complete loss of hair in β-catenin-deficient skin during the first hair cycle. In catagen, a separation of the hair follicle epithelium and the dermal papilla becomes apparent. Subsequently, we observed the formation of dermal cysts, which exhibited features of epidermal differentiation and possessed stem cells identified by the expression of Keratin 15 and high levels of β1-integrin. These results, together with the lack of hair shaft markers, suggest that stem cells in the cysts differentiate into epidermal keratinocytes in the absence of β-catenin (cf. scheme in Figure 7b). Cysts and associated stem cells remain observable for many weeks in the mutant skin. This implies that β-catenin is necessary for fate decisions of stem cells to form follicular keratinocytes, and that stem cells can only differentiate into a single (epidermal) lineage in the absence of β-catenin. β-catenin may however not be sufficient to induce follicular differentiation of keratinocytes, as additional mesenchymal signals may be required (cf. Introduction). Previous experiments have shown that overexpression of a stabilized form of β-catenin induced the de novo formation of hair follicles, but did not markedly disturb epidermal differentiation (Gat et al., 1998). Thus, the available experimental evidence points toward a role of β-catenin in the control of stem cell differentiation (Figure 7b).

It is well recognized that β-catenin, besides its role in transcriptional regulation, also functions in cadherin-mediated intercellular adhesion. Does β-catenin act as a signaling or as an adhesion molecule in hair follicle formation? We showed previously that cellular adhesion of epithelial cells is not disturbed in early mouse embryos that lack β-*catenin* (Huelsken et al., 2000). Epithelia in the mutant embryos are well developed, and the cells are connected by well-defined adherens junctions and desmosomes. Concomitantly, levels of Plakoglobin (γ-catenin) were increased in these mutants, and Plakoglobin appeared to compensate for β-catenin in cell adhesion. Here, we observe no overt cell adhesion defects in β-catenin-negative skin. In particular, skin blistering, a phenotype typically found in mice or patients with mutations in genes encoding cell adhesion components, was not observed (Bierkamp et al., 1996; Dowling et al., 1996; Koch et al., 1997; McGrath et al., 1997). Hyperproliferation and defects in epithelial polarity were observed upon conditional ablation of the α-catenin gene in skin, which is clearly different from our phenotypes (Vasioukhin et al., 2001). Furthermore, Plakoglobin is also present in the skin of the conditional β-catenin mutants and might compensate for adhesive, but not for signaling, functions of β-catenin. Indeed, Plakoglobin does not significantly participate in Wnt signaling in *Xenopus* (Miller and Moon, 1997; Kofron et al., 1997; Ben Ze'ev and Geiger, 1998).

Previous publications reported a role of LEF-1 in the development of epithelial appendages (a reduced number of hairs in LEF-1$^{-/-}$ animals) and increased numbers of hair follicles after overexpression of β-catenin (van Genderen et al., 1994; Gat et al., 1998). Thus, β-catenin signaling had been implicated in skin and hair follicle development, but neither the mechanisms nor the cell

types that require the signal had been identified. We show here that stem cells of the skin require β-catenin signaling in order to differentiate into hair follicles. By following the fate of the stem cells in the mutants, we show that they differentiate aberrantly into epidermal cysts. No other technique but conditional mutagenesis could determine this function of β-catenin. Stem cells in the skin were known to differentiate into several lineages, among them epidermis and hair. The fact that we have identified a specification signal for the hair follicle does not only increase our knowledge on skin development and function, but can be considered as a general advance in stem cell biology. Our paper sheds light on the molecular signals required for stem cell maintenance and hair follicle formation.

Stem cells are pluripotent cells that can adopt various cell fates. When exposed to growth factors and cytokines, stem cells can generate progenitors, which transiently amplify and then withdraw from the cell cycle to terminally differentiate (Fuchs and Segre, 2000; Watt and Hogan, 2000). Currently, extensive research is directed to the identification of molecules that maintain stem cells and that control their commitment to particular lineages. In the bone marrow, stem cell factor (SCF) and its receptor c-Kit are essential for maintenance of stem cells of the hematopoietic lineage (Nocka et al., 1990; Toksoz et al., 1992). Later, transcription factors of the GATA family are responsible for proliferation of progenitor cells and their maturation into erythroid and megakaryocyte lineages (Tsai et al., 1994; Weiss and Orkin, 1995). In the intestine, the transcription factor TCF-4 is essential for maintaining the stem cell compartment and for their differentiation into the entero-endocrine lineage, and might require β-catenin for this function (Korinek et al., 1998). Our data indicate a role of β-catenin in the differentiation of stem cells of the skin, where it might collaborate with LEF-1 and/or TCF-3 (van Genderen et al., 1994; DasGupta and Fuchs, 1999). Thus, the function of β-catenin/Wnt signaling in maintenance and differentiation of stem cells deserves attention.

Experimental Procedures

Generation of Mutant Mice

For construction of the targeting vectors, genomic fragments isolated from a λGEM-12 129/Ola library were introduced into the pTV0 vector (Huelsken et al., 2000). The targeting vector for the generation of the floxed β-*catenin* allele contained at the 5′ arm genomic sequences from the end of the first intron to an XhoI site in the sixth intron. LoxP sites (oligo nucleotide 5′-ATA ACT TCG TAT AAT GTA TGC TAT ACG AAG TTA T-3′) were inserted into a SacI site of the second intron, and 5′ and 3′ of the neomycin resistance cassette (neo). The 3′ arm of the vector contained exons 7 to 16 of the β-*catenin* gene. The targeting vector for the generation of the *keratin 14*-Cre allele contained at the 5′ arm genomic sequences from the end of the first intron to the start codon of *K14*, followed by an in-frame fusion with the cDNA of Cre-recombinase (Gu et al., 1993). Two FRT sites were introduced 5′ and 3′ of the neo cassette which were isolated from pMS101 (Snaith et al., 1995). The 3′ arm of the vector contained exons 1 to 8 of the *K14* gene. Following homologous recombination in E14 ES cells, expression vectors of Cre-recombinase (Gu et al., 1993) or flp-recombinase (Dymecki, 1996) were transiently transfected to delete the corresponding neo cassettes. Recombined loci were analyzed by Southern blotting, and Northern and Western blotting were used to demonstrate that the β-*cat* del locus produced a null allele. Two independent heterozygous ES cell clones of each mutated allele were used to generate chimeric mice by blastocyst injection (Huelsken et al., 2000) and mutant animals were bred on a mixed 129×C57Bl6 background. PCR genotyping was performed using the primers ACT GCC TTT GTT CTC TTC CCT TCT G and CAG CCA AGG AGA GCA GGT GAG G (for β-*cat* lox), ACT GCC TTT GTT CTC TTC CCT TCT G and CAG ACA GAC AGC ACC TTC AGC ACT C (for β-*cat* del), or AGG GAT CTG ATC GGG AGT TG and CTT GCG AAC CTC ATC ACT CG (for *K14*-Cre). A Cre-inducible lacZ reporter mouse line (Thorey et al., 1998) was used to detect *K14*-Cre activity. For β-galactosidase staining, embryos or frozen sections were fixed in 0.2% glutaraldehyde in 100 mM potassium phosphate buffer, (pH 7.4), permeabilized in 0.01% sodium desoxycholate, 0.02% NP40, 5 mM ethylene glycol-bis-(β-amino ethyl ether) (EGTA) and 2 mM $MgCl_2$ in potassium phosphate buffer, and stained in 0.5 mg/ml X-gal, 10 mM potassium hexacyanoferrate(III), 10 mM potassium hexacyanoferrate(II), 5 mM EGTA, and 2 mM $MgCl_2$ in potassium phosphate buffer. Counterstaining of sections was performed using 0.01% pyroninG.

Immunostaining and BrdU-Labeling

Immunofluorescence analysis of embryonic skin and electron microscopy were performed as described (Huelsken et al., 2000). For the detailed morphological analysis of hair follicles during cycling, back skin samples of three wild-type and mutant animals each were taken every second day between P13 and P35, fixed in Bouin's (70% picric acid, 8% formaldehyde, 5% acetic acid), and embedded in paraffin. Immunohistochemistry was performed on sections, which were steamed in 10 mM sodium citrate, (pH 6.0), for 15 min prior to incubation with primary antibody. We used antibodies directed against β-catenin (polyclonal serum, Huelsken et al., 1994, and 7D11, Nanotools, Teningen), Plakoglobin (15F11, Sigma, St. Louis), Cytokeratin 6 (MK6, Babco, Richmond), Cytokeratin 10 (K8.60, Sigma), Involucrin (Babco), Cytokeratin 14 (LL002, Biotrend, Koeln), Keratin 6hf and Hair keratin 2 (L. Langbein, Heidelberg, Germany; Langbein et al., 1999), β1-integrin (M-106, Santa Cruz Biotechnology), Ki67 (Dianova, Hamburg), and BrdU (BU-33, Sigma). Primary antibodies were detected by biotinylated secondary antibodies plus streptavidin-peroxidase complex and brown FAST DAB staining (Sigma) or by secondary antibodies directly coupled to alkaline phosphatase and blue FAST NBT/BCIP (Sigma). Counterstaining of sections was performed using hematoxylin for DAB-stained sections or pyroninG for NBT/BCIP-stained sections. For BrdU-labeling experiments, animals were injected with 0.1 mg/g body weight of BrdU in PBS, and skin samples were taken after 3 hr, fixed in metharcarne (60% methanol, 30% trichloromethane, 10% acetic acid), and embedded in paraffin, followed by immunohistochemistry.

In Situ Hybridization

In situ hybridizations were performed on paraffin sections of adult skin (Huelsken et al., 2000) or on whole-mount embryos up to E15. Embryos were fixed in 4% formaldehyde in phosphate-buffered saline (PBS), permeabilized by proteinase K digestion in 0.3% Tween in PBS, and refixed in 0.2% glutaraldehyde, 4% formaldehyde in PBS prior to hybridization (Huelsken et al., 2000). Probes used were DIG-labeled (Roche), anti-sense transcripts of mouse cDNAs of β-catenin (nt 150–540, NM007614), Downless (nt 302–1038, AF160502), BMP2 (nt 825–1524, NM007553), BMP4 (nt 290–1780, X56848), BMP7 (nt 191–881, NM007557), SHH (nt 120–760, X76290), Patched-1 (nt 134–1008, NM008957), LEF-1 (nt 1805–2659, NM010703), Cytokeratin 15 (nt 540–1130, D16313), Cytokeratin 17 (nt 940–1530, AB013608), and Hairless (nt 3148–3830, Z32675). After hybridization, embryos were washed in 50% formamide, 5× SSC (0.75 M NaCl, 7 5 mM sodium citrate, [pH 7.0]), 0.1% Tween at 70°C, and unspecifically bound probe was digested by RnaseA treatment, followed by extensive washing in 50% formamide, 2× SSC, 0.1% Tween at 65°C. The DIG-label was detected by an anti-DIG Fab (Roche) coupled to alkaline phosphatase using FAST NBT/BCIP (Sigma). Subsequently, areas with altered patterning of hair placodes were cryosectioned and analyzed by immunohistochemistry to demonstrate absence of β-catenin.

Acknowledgments

We thank Carmen Birchmeier and Allistair Garrat, Berlin, for critical reading of the manuscript and helpful discussions, Lutz Langbein,

Heidelberg, for antibodies against Keratin 6hf and Hair keratins, and Alistair Garrat, Berlin, for providing mouse genomic DNA of the *keratin 14* gene. The work was supported by a grant of the Volkswagen-Stiftung.

Received November 6 2000; revised April 5, 2001.

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