

## Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation

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### SUMMARY

LEF/TCF DNA-binding proteins act in concert with activated  $\beta$ -catenin, the product of Wnt signaling, to transactivate downstream target genes. To probe the role of activated LEF/TCF transcription factor complexes in hair follicle morphogenesis and differentiation, we engineered mice harboring TOPGAL, a  $\beta$ -galactosidase gene under the control of a LEF/TCF and  $\beta$ -catenin inducible promoter. In mice, TOPGAL expression was directly stimulated by a stabilized form of  $\beta$ -catenin, but was also dependent upon LEF1/TCF3 in skin. During embryogenesis, TOPGAL activation occurred transiently in a subset of LEF1-positive cells of pluripotent ectoderm and underlying mesenchyme. Downgrowth of initiated follicles proceeded in the absence of detectable TOPGAL expression, even though LEF1 was still expressed. While proliferative matrix cells expressed the highest levels of *Lef1* mRNAs, LEF1 concentrated in the precursor cells to the hair shaft, where TOPGAL expression was co-induced with hair-specific keratin genes containing LEF/TCF-

binding motifs. LEF1 and TOPGAL expression ceased during catagen and telogen, but reappeared at the start of the postnatal hair cycle, concomitant with precortex formation. In contrast to hair shaft precursor cells, postnatal outer root sheath expressed TCF3, but not TOPGAL. TCF3 was also expressed in the putative follicle stem cells, and while TOPGAL was generally silent in this compartment, it was stimulated at the start of the hair cycle in a fashion that appeared to be dependent upon stabilization of  $\beta$ -catenin. Taken together, our findings demonstrate that LEF1/TCF3 is necessary but not sufficient for TOPGAL activation, revealing the existence of positive and negative regulators of these factors in the skin. Furthermore, our findings unveil the importance of activated LEF/TCF complexes at distinct times in hair development and cycling when changes in cell fate and differentiation commitments take place.

Key words: LEF/TCF, Hair,  $\beta$ -catenin, TCF3, Keratin

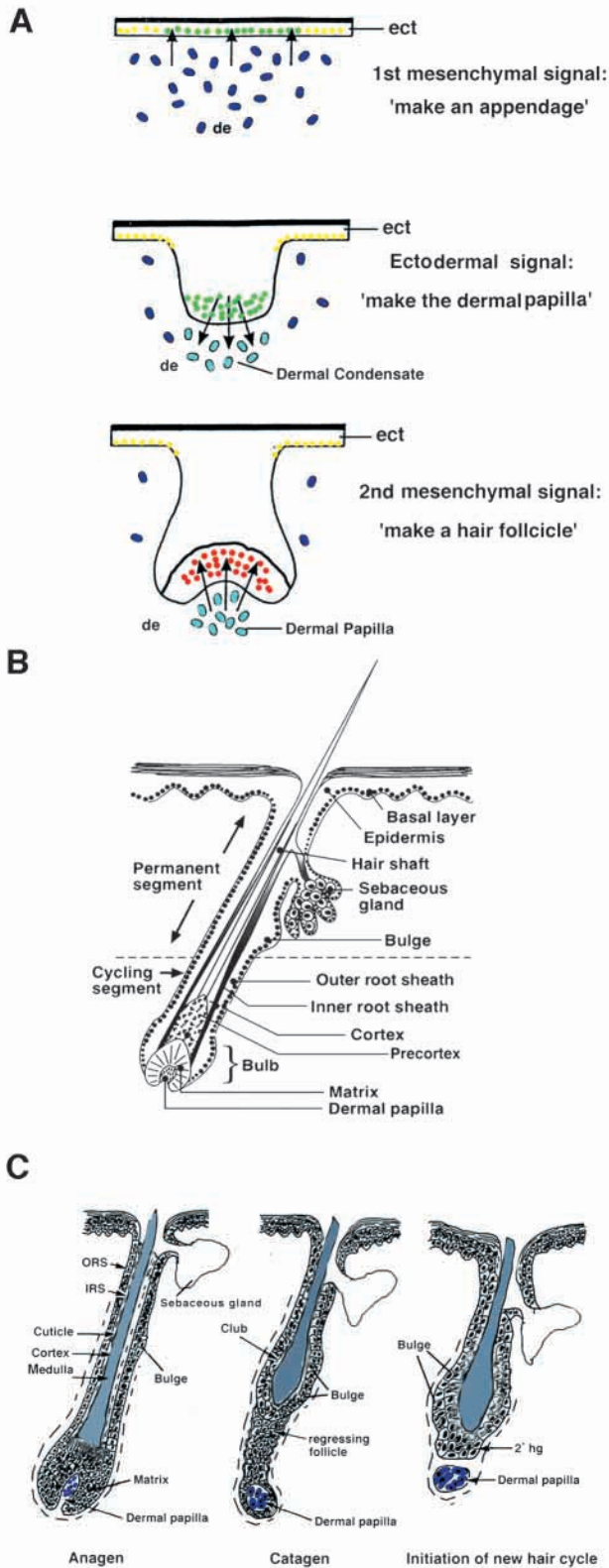
### INTRODUCTION

In mammals, embryonic skin epithelial cells are pluripotent, able to choose between epidermal and hair follicle cell fates. Commitment to follicle formation occurs when an underlying mesenchymal cue instructs overlying ectoderm to commit to forming an appendage (Sengel, 1976; Hardy, 1992; Fig. 1A). As the epithelium thickens, forming first a placode and then a small downgrowth or germ, it transmits a message to the underlying mesenchyme, stimulating their condensation into a dermal papilla. A second dermal message is then transmitted back to the adjacent epithelial cells, instructing them to proliferate. As development proceeds, the epithelial cells differentiate, producing first an outer and inner root sheath (ORS and IRS, respectively), and then near or at birth, a hair shaft at the center of the follicle (Fig. 1B).

The epithelial cells forming a cloak surrounding the dermal papilla are called matrix cells, which proliferate transiently. As matrix cells withdraw from the cell cycle, they differentiate into upwardly moving cells. At the center, matrix cells

differentiate into precortical cells, which subsequently give rise to the cortex, medulla and cuticle of the hair shaft. A surrounding concentric ring of matrix cells gives rise to the IRS, which in turn is surrounded by the ORS. Near the skin surface, the IRS degenerates, freeing the hair shaft to push outward as matrix cells proliferate and differentiate at the base (Fig. 1B; for review, see Hardy, 1992).

Once established, follicles proceed through cycles of active periods of hair growth (anagen), regression and shortening (catagen) and rest (telogen) (Fig. 1C). Only the lower epithelial portion of the follicle actually cycles. At the base of the permanent portion of the follicle is a region known as the bulge, thought to contain a population of self-renewing epithelial stem cells (for review, see Lavker et al., 1991). During the hair cycle, when matrix cells lose their proliferative capacity, the follicle ceases growth and the lower epithelial portion regresses, bringing the dermal papilla cells upward to the bulge. At the transition between telogen and the initiation of the next hair cycle, a signal, perhaps from the dermal papilla, converts one or more epithelial stem cells to proliferating



matrix and ORS cells, which now move downward and differentiate. Once the lower follicle is fully formed, proliferation becomes restricted to the base where matrix cells maintain contact with the dermal papilla.

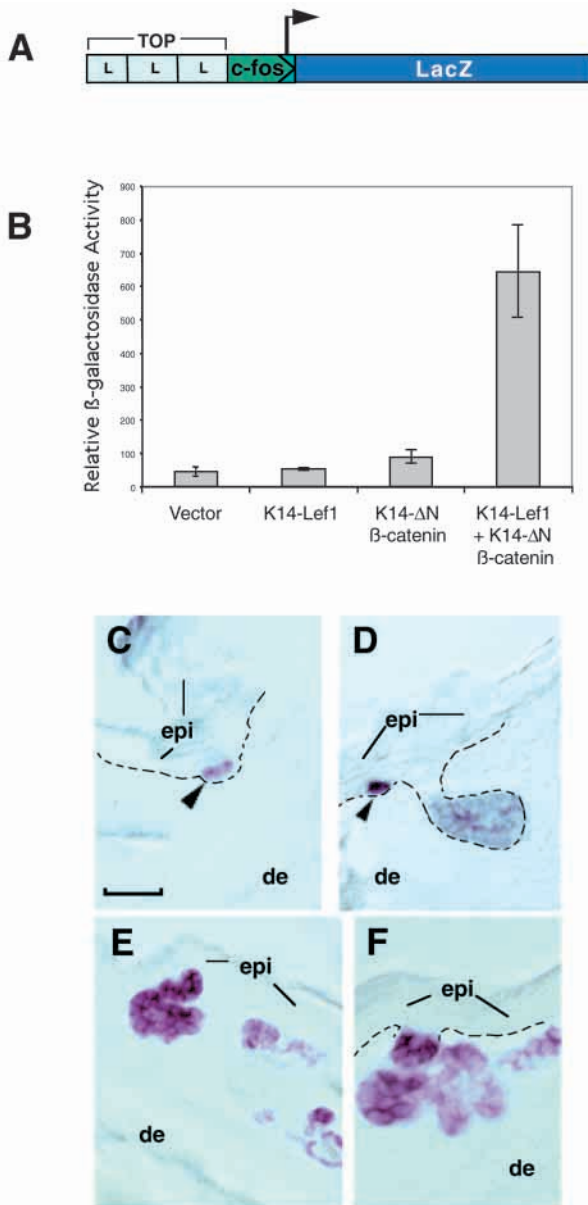
**Fig. 1.** Hair morphogenesis, hair structure and hair cycling. (A) Hair follicle morphogenesis. The hair follicle is derived from a series of mesenchymal and epithelial cues transmitted in early embryonic development (for review, see Hardy, 1992). These signals have been predicted on the basis of tissue recombination experiments conducted by early developmental biologists (Sengel, 1976; for review, see Hardy, 1992). Ect, ectoderm; de, dermis. (B) Structure of a hair follicle. Shown is a schematic of the components of the hair follicle (for review, see Hardy, 1992). (C) The postnatal hair cycle and initiation of the next cycle. Shown are diagrams of a follicle through periods of growth (anagen), rest (catagen) and regression (telogen). The transition from telogen to the new cycle is thought to depend upon a stimulus from the dermal papilla to the bulge, a permanent compartment of epithelial stem cells surrounding the dead hair club from the prior cycle. This results in epithelial proliferation to form the secondary hair germ (2°hg). ORS, outer root sheath; IRS, inner root sheath. Fig. 1C patterned after Ebling (1987).

The inductive signals exchanged among epithelial components within the follicle and between follicular epithelia and mesenchyme are largely unknown; however, recent studies suggest that Wnt signalling pathways might be involved (van Genderen et al., 1994; Zhou et al., 1995; Gat et al., 1998; Millar et al., 1999). Wnt genes encode a large family of secreted, signalling proteins that specify various cell lineage pathways in development (for review, see Nusse, 1999). In response to a Wnt stimulus, cytoplasmic  $\beta$ -catenin is stabilized, enabling its interaction with members of the LEF/TCF family of DNA-binding proteins to form an active transcription factor complex (Nusse, 1999). This, in turn, allows transcriptional activation of target genes containing consensus binding sites for LEF/TCF. Regulation of downstream target genes is complex, given that the GROUCHO/TLE family of repressors can also interact with nuclear TCF/LEF proteins to negatively influence activity (Roose et al., 1998; Cavallo et al., 1998; Levanon et al., 1998).

Various lines of evidence suggest the importance of LEF1 and  $\beta$ -catenin in hair follicle organogenesis. *Lef1* mRNAs appear in epithelial and mesenchymal compartments as early as the ectodermal placode stage (Zhou et al., 1995), and mice lacking LEF1 display a significant reduction of hair follicles and complete lack of whiskers (van Genderen et al., 1994). In vitro tissue recombinations of ectoderm and mesenchyme from *Lef1* knockout and wild-type embryos reveal a role for mesenchymal LEF1 in whisker follicle formation (Kratchowil et al., 1996), and transgenic mice overexpressing LEF1 or a constitutively activated form of  $\beta$ -catenin in the ectoderm display signs of de novo hair follicle morphogenesis (Zhou et al., 1995; Gat et al., 1998). Finally, LEF/TCF-binding motifs exist in a group of hair-specific promoters active in the cortex and cuticle of postnatal hair follicles (Zhou et al., 1995; Dunn et al., 1998).

While these findings strongly implicate LEF1 and  $\beta$ -catenin in hair follicle development and/or differentiation, they do not provide a clear picture of how these factors might regulate such processes. Moreover, at least one additional member of the LEF/TCF family, TCF3, is also expressed in hair follicles, raising the likelihood that the regulatory pathways mediated by *Lef1/Tcf3* in the hair follicle may be complex (Barker et al., 1999).

In the present report, we elucidate the relative patterns of LEF1 and TCF3 expression and localization during hair follicle



development and in the postnatal hair cycle. In addition, by engineering mice expressing a transgene whose expression is dependent upon activated LEF/TCF and  $\beta$ -catenin, we identify which cells receive and respond to a Wnt or equivalent signal at specific times within the developing and postnatal hair follicle. We examine transgene promoter activity in mice that are otherwise genetically wild type and in mice that express a constitutively active form of  $\beta$ -catenin. Our results reveal some very surprising findings as well as major new insights into the regulation of cell fate commitments during hair follicle morphogenesis and differentiation. Our findings directly implicate transient LEF/TCF target gene activation in the initiation step of hair follicle morphogenesis in embryonic development, and also in the conversion of a subset of proliferating matrix cells to postmitotic hair-shaft-forming cells. Finally, we provide evidence that  $\beta$ -catenin stabilization can result in the activation of TCF3-positive cells within the putative stem cell compartment of the hair follicle.

**Fig. 2.** Activation of the LEF/TCF-dependent TOPGAL transgene in keratinocytes and in transgenic mice that also express a stabilized form of  $\beta$ -catenin. (A) TOPGAL construct. The promoter contains three consensus LEF-1/TCF-binding motifs (L) and a minimal *c-fos* promoter to drive transcription of the *lacZ* gene encoding  $\beta$ -galactosidase. (B) pTOPGAL reporter assays. The mouse keratinocyte line, UG1, was transfected with pTOPGAL, pCMV-luciferase (as an internal control gene) and equimolar amounts of plasmids pK14- $\Delta$ N87 $\beta$ cat (K14- $\Delta$ N $\beta$ cat), pK14-Lef1 or empty expression vector, as indicated (see Gat et al., 1998 for method). 48 hours later, cells were lysed and protein extracts were assayed for  $\beta$ -galactosidase activity (test) and luciferase (to correct for transfection efficiency). Normalized activities represent an average of three experiments, with variations shown by error bars. FOPGAL had no activity (not shown). (C-F) TOPGAL expression in skin from transgenic mice which were also positive for K14- $\Delta$ N87 $\beta$ cat. The K14- $\Delta$ N87 $\beta$ cat transgene and its behavior in mice has been described (Gat et al., 1998). Sections (10  $\mu$ m) of the toe skin from 28-day-old double transgenic mice were assayed for  $\beta$ -galactosidase activity using the X-gal assay. Note: At all ages, control skins were negative for blue stain. (C) Two epidermal basal cells (arrowhead) have activated the TOPGAL transgene; (D) a de novo hair germ (hg), induced artifactually by  $\Delta$ N87 $\beta$ cat expression, along with one epidermal cell (arrowhead), have activated the TOPGAL transgene. (E,F)  $\Delta$ N87 $\beta$ cat-induced epithelial invaginations (brackets) expressing TOPGAL. de, dermis; epi, epidermis. Dotted lines denote epithelial/mesenchymal boundaries. Bar in C represents 100  $\mu$ m in C-E; 60  $\mu$ m in F.

## MATERIALS AND METHODS

### Generation of transgenic reporter mice

Plasmid pTOPGAL was engineered by replacing the luciferase reporter gene of pTOPFLASH (Korinek et al., 1998a and references therein) with the bacterial *lacZ* gene. The TOP promoter consists of three multimerized LEF/TCF consensus binding sites and the *c-fos* minimal promoter (*Xba*I fragment) with the promoter-less pNASS(m) (*lacZ*) plasmid (linearized with *Spe*I). Plasmid pK14- $\Delta$ N87 $\beta$ cat was engineered previously (Gat et al., 1998). Transgenic mice harboring K14- $\Delta$ N87 $\beta$ cat and TOPGAL or TOPGAL alone were made as previously described (Vassar et al., 1989), and transgene integration was verified using PCR analysis of tail DNAs. Transgene expression was verified by X-Gal staining of tail samples ( $\beta$ -galactosidase; Byrne et al., 1994) and by phenotype ( $\Delta$ N87 $\beta$ cat; Gat et al., 1998).

### X-Gal assays on frozen tissue sections

Frozen sections (10  $\mu$ m) were fixed with 0.2% glutaraldehyde for 2 minutes. After washing 7-8 times in 1 $\times$  PBS, slides were then transferred into Tissue Stain Base solution (Specialty Media) with X-Gal substrate at a final concentration of 2 mg/ml. Staining was performed in the dark at 37°C for 6-8 hours and, after mounting in 80% glycerol, samples were visualized by Nomarski optics with a Zeiss axiophot microscope. After photographing slides, coverslips were removed and slides were treated sequentially with Hematoxylin for 2 minutes, water rinse, eosin for 5 minutes and a final water rinse. Slides were then remounted and tissue sections were photographed as before.

### Immunohistochemistry

Frozen sections of tissues were subjected to indirect immunofluorescence (Gat et al., 1998 and references therein). For detection of TCF3, antigen unmasking was performed by autoclaving 4% paraformaldehyde-fixed tissue sections in 10 mM sodium citrate, for 2 minutes. Primary antibodies used were rabbit anti-LEF1 (van Genderen et al., 1994); mouse monoclonal anti-TCF3 (Barker et al., 1999); mouse monoclonal anti-trichohyalin (AE15; Manabe et al.,

1996) and anti-hair keratin (AE13; Lynch et al., 1986); guinea pig anti-K5 (Byrne et al., 1994); rabbit anti-Ki67 (proliferation marker). Fluorescence-conjugated secondary antibodies were obtained from Jackson Laboratories (West Grove, PA). Slides were mounted with antifade and visualized by Zeiss confocal microscopy.

### In situ hybridization

Digoxigenin-labeled cRNAs were synthesized according to the manufacturers' instructions (Boehringer Mannheim Biochemicals, Indianapolis, IN). A 517 bp mouse *Lef1* cDNA, lacking the conserved DNA-binding domain, was subcloned as an *EcoRI-BamHI* fragment into pCRII, and the cRNAs were made by either *EcoRV* linearization and SP6 RNA polymerase transcription, or *BamHI* linearization and T7 RNA polymerase transcription. An analogous vector was made using the unique sequences of the mouse *Tcf3* cDNA (Korinek et al., 1998a).

## RESULTS

### Generation of TOPGAL and K14- $\Delta$ N87 $\beta$ cat double transgenic mice capable of expressing $\beta$ -galactosidase in response to activated $\beta$ -catenin-LEF/TCF transcription complexes in skin

Previously, we generated transgenic mice expressing  $\Delta$ N87 $\beta$ cat, a constitutively activated form of  $\beta$ -catenin, under the control of the keratin 14 (K14) promoter which is upregulated at embryonic day 14.5 (E14.5) in the basal layer of epidermis, the ORS and the bulge (Gat et al., 1998). At the initiation of the first postnatal hair cycle of these mice, the skin produces interfollicular epithelial invaginations, which sometimes resemble primordial hair germs and develop into bona fide hair follicles (Gat et al., 1998).

To first assess whether this striking action of  $\Delta$ N87 $\beta$ cat in vivo is mediated through the activation LEF/TCF-responsive target genes, we engineered a second transgene, this one driving *lacZ* under the control of a promoter that contains multimerized LEF/TCF-binding motifs upstream from a minimal promoter (Fig. 2A). The transgene, referred to here as TOPGAL, is a derivative of TOPFLASH, whose expression is dependent upon stabilized  $\beta$ -catenin and is comparably responsive to TCF3 and LEF1 (Korinek et al., 1998a). In cultured keratinocytes, TOPGAL, but not a version with mutated LEF/TCF binding sites (FOPGAL), behaved similarly and was superactivated when co-transfected with vectors expressing  $\Delta$ N87 $\beta$ cat and hLef1, but not with either alone (Fig. 2B). With this verification, we then engineered mice transgenic for both K14- $\Delta$ N87 $\beta$ cat and TOPGAL. Two independently derived lines were generated and were bred to the F<sub>1</sub> stage to avoid the possibility of mosaicism. Both lines behaved similarly in all assays performed.

Transgenic mouse skin was analyzed for  $\beta$ -galactosidase activity at 28 days of age, when many hair follicles were in anagen, and when there was a prevalence of interfollicular epithelial invaginations caused by  $\Delta$ N87 $\beta$ cat expression (see Gat et al., 1998). As judged by X-gal staining,  $\beta$ -galactosidase activity was detected in a number of epithelial cells in the skin of these transgenic mice (Fig. 2C-F). No blue cells were seen in skin from either wild-type or K14- $\Delta$ N87 $\beta$ cat transgenic mice, indicating that blue staining was a faithful measure of TOPGAL reporter activity in these mice.

Approximately 5-10% of the basal epidermal cells of 28 day

K14- $\Delta$ N87 $\beta$ cat/TOPGAL transgenic mouse skin scored positive for  $\beta$ -galactosidase activity (Fig. 2C,D, arrowheads). In addition, postnatal de novo hair germs, unique to  $\Delta$ N87 $\beta$ cat transgenic skin, stained blue (frame D), as did the flower-like epithelial invaginations that often followed the initial signs of  $\Delta$ N87 $\beta$ cat-induced hair germ formation (Fig. 2E,F). TOPGAL activities in epidermis and in postnatal interfollicular epidermal invaginations were not seen when mice expressed only TOPGAL and not  $\Delta$ N87 $\beta$ cat (see below). These results provide strong evidence that transactivation of LEF/TCF-dependent target genes is induced in the epithelial invaginations and de novo hair follicles that arise as a consequence of K14- $\Delta$ N87 $\beta$ cat expression. Additionally, the data suggest that an endogenous LEF/TCF family member combined with  $\Delta$ N87 $\beta$ cat to activate TOPGAL in these invaginations.

It was intriguing that only a subset of K14- $\Delta$ N87 $\beta$ cat-expressing basal cells stained blue. This suggests a heterogeneity in the potential for basal cells within the epidermis to transactivate LEF/TCF-regulated promoters. This heterogeneity could stem from differences in endogenous LEF/TCF levels and/or their phosphorylation states (Ishitani et al., 1999), or differences in the levels of inhibitory factors such as the GROUCHO/TLE, CBP and C-terminal binding protein (CtBP), all of which interact and interfere with the action of TCF/LEF family members (for review, see Bienz, 1998; Brannon et al., 1999). We begin to distinguish between these possibilities in some of the experiments presented below.

### Transgenic mice harboring TOPGAL alone reveal that TOPGAL is active in a subset of *Lef1*-expressing embryonic skin cells at the start of hair follicle morphogenesis

Previously, we reported that *Lef1* mRNAs are expressed early in embryonic mouse skin in both the ectodermal placodes and in the underlying dermal condensates (Zhou et al., 1995), and recently it was reported that of the Tcf family members, TCF3 is also expressed in hair follicles (Barker et al., 1999). *Lef1* and *Tcf3* behave very similarly in TOP promoter assays in at least some cells in vitro, and both are activated by the presence of stabilized  $\beta$ -catenin (Korinek et al., 1998a). Thus, we next turned to using TOPGAL activation as an in vivo assay to understand how LEF1 and TCF3, presumably in conjunction with endogenously activated  $\beta$ -catenin, might be involved in normal hair follicle morphogenesis during embryonic skin development. To this end, we engineered mice harboring only the TOPGAL and not the K14- $\Delta$ N87 $\beta$ cat transgene. The TOPGAL transgenic mice were used for the remaining studies reported here. Two lines were generated, and they behaved similarly in all assays.

We first examined the patterns of LEF1 in embryonic skins of F<sub>1</sub> transgenic mice and correlated this with TOPGAL promoter activity. Indirect immunofluorescence revealed anti-LEF1 antibody staining throughout the basal layer and developing pregerms (placodes) of embryonic skin epithelium (Fig. 3A). Staining was consistently more intense in the pregerms than in other areas of the ectoderm (arrows in Fig. 3A,B). Staining was also seen in the underlying dermal condensates. The distinction between epithelium and mesenchyme was best visualized by co-staining tissue sections with anti-K5, specific for the cytoplasmic keratin network of the epithelial cells (Fig. 3B). Moreover, from this double

labeling, it was clear that the majority of anti-LEF1 staining was nuclear, at least in the epithelial component of the pregerm. Finally, no anti-LEF1 staining was seen in control skin (Fig. 3C) from the *Lef1* knockout mouse (van Genderen et al., 1994), verifying the specificity of the antibody. This was also confirmed by immunoblot analysis (data not shown; see also Kratchowil et al., 1996). Overall, these data were consistent with our prior in situ hybridizations showing *Lef1* mRNA expression in the pluripotent ectoderm, and in both epithelium and mesenchyme of early hair germs (Zhou et al., 1995).

Interestingly, as judged by  $\beta$ -galactosidase activity, TOPGAL activation occurred in both epithelium and dermal condensates of hair pregerms (Fig. 3D,E, respectively). Blue-stained cells were quite evenly distributed at these two sites in the embryonic basal layer, and correlated well with the frequency and location of sites where anti-LEF1 antibody staining was pronounced. These data suggested that LEF/TCF was able to act as a transcriptional activator at a time when both epithelium and mesenchyme were being programmed to form a hair follicle. Interestingly, within a pregerm, TOPGAL expression was often more prominent in either ectoderm or mesenchyme, raising the possibility that activation in one cell might promote activation of the other. This notion was attractive, given the documented series of mesenchymal and epithelial cross-signaling that occurs at these early stages of follicle commitment.

While  $\beta$ -galactosidase activity seemed to be restricted to a subset of pluripotent embryonic basal cells, LEF1 was readily detected in the nuclei of most if not all cells within the basal layer (Fig. 3A,B). Moreover, nuclear LEF1 could be seen in these basal cells throughout embryonic development, i.e. encompassing the time when ectoderm was pluripotent and able to choose between epidermal and follicle cell fates. This finding suggested that either the threshold levels of LEF/TCF factors were not sufficient to activate TOPGAL in many of these cells, or that an additional signal, perhaps a Wnt, was necessary to stabilize  $\beta$ -catenin and enable downstream target genes to be expressed.

During the next phase of follicle development, as the cells proliferated to form the hair germs (more advanced germs are sometimes referred to as plugs or pegs), TOPGAL expression was no longer detected (Fig. 3F). Interestingly, anti-LEF1 staining was weak or absent not only in the stalk of the growing hair germ, but also in the basal epidermal cells adjacent to it (Fig. 3G). Instead, LEF1 seemed to be concentrated at the leading edge of the growing follicle, where it was detected both the developing matrix cells and dermal papilla (Fig. 3H). This was best visualized by double immunofluorescence labeling with anti-K5, restricted to the epithelium. This said, because K5 expression is downregulated at the leading edge of the hair germ and in the matrix cells of the follicle (Kopan et al., 1989), this distinction was still more difficult than it was at the pregerm stage (e.g. compare Fig. 3B with inset in H). Overall, our findings agreed with our prior in situ hybridization studies, reporting expression of *Lef1* mRNAs in both epithelium and mesenchyme at the leading edge of developing follicles (Zhou et al., 1995). We later provide additional evidence to further confirm mesenchymal and epithelial expression of *Lef1*.

Concomitant with the completion of follicle morphogenesis, we noted a marked decline in anti-LEF1 antibody staining in postnatal basal epidermal cells (data not shown), a finding consistent with *Lef1* mRNA expression patterns (Zhou et al.,

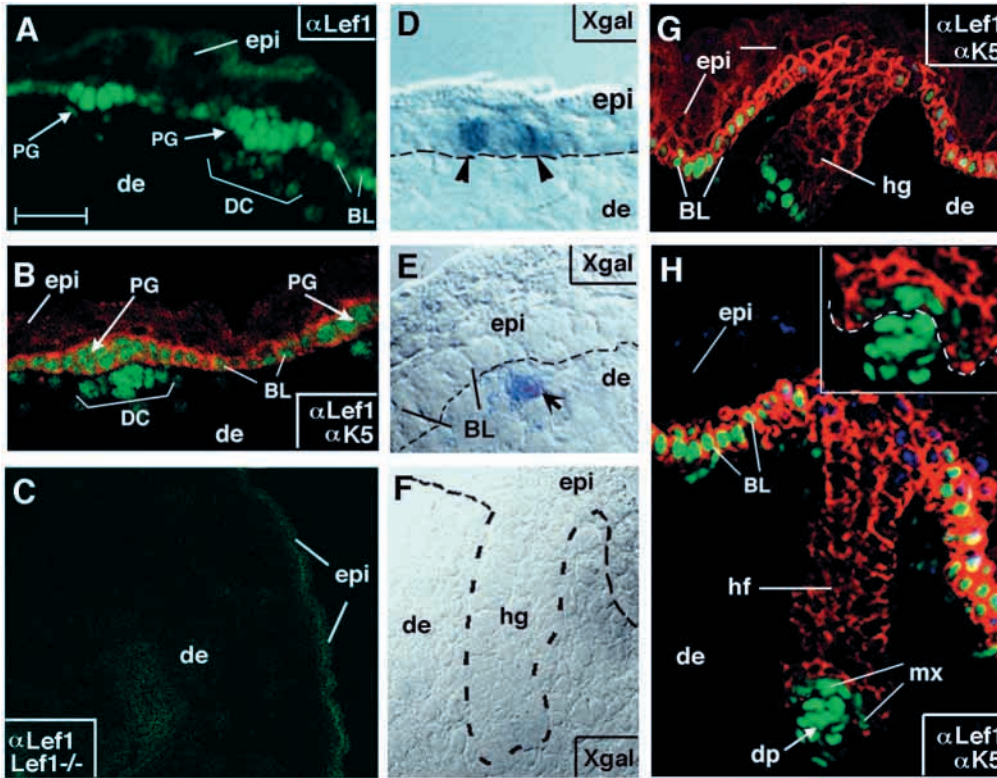
1995). Thus, a correlation seemed to exist between loss of pluripotency and a loss of *Lef1* expression in the basal layer. The downregulation of *Lef1* in postnatal epidermis provided further evidence that developmentally regulated TOPGAL expression appeared to be associated with events relating to hair follicle morphogenesis and not epidermal differentiation.

#### **In late embryonic and early postnatal hair follicles, *Lef1* mRNAs are primarily expressed in the follicle bulb, while TOPGAL is induced in the differentiating hair shaft precursor cells**

TOPGAL expression reappeared in transgenic follicles beginning at E16.5, and was dramatically upregulated in both whisker and body hair follicles after birth (Fig. 4). By this time, *Lef1* mRNA expression was beginning to weaken in the basal layer of epidermis, but increased in the hair bulbs, where it concentrated in the matrix and precortex (Fig. 4A; for earlier embryonic *Lef1* mRNA expression data, see Zhou et al., 1995). Intriguingly, appreciable  $\beta$ -galactosidase activity was detected only in a subset of these *Lef1* mRNA-expressing cells (Fig. 4A'). This triangular-shaped pocket of blue-stained cells appeared to be the precortex, i.e. the precursor cells to the hair shaft. In postnatal skin, TOPGAL expression was strongly upregulated in the postnatal anagen follicles. Interestingly, the difference between *Lef1* mRNA localization (Fig. 4B-D) and TOPGAL expression (Fig. 4B'-D') became even more pronounced by 6-9 days, when the well-established zone of transcriptional activity in the follicle broadened to encompass the upwardly migrating, differentiating cells (Kopan et al., 1989; Zhou et al., 1995; Dunn et al., 1998). At these times, the majority of skin *Lef1* mRNAs concentrated in the follicle bulbs, although two lines (e.g. a concentric ring in the three-dimensional follicle) of *Lef1* mRNA-expressing cells were seen extending from the bulb upward (Fig. 4D, arrows). In contrast, the strongest TOPGAL expression was detected in the differentiating cells above each hair bulb (Fig. 4C',D'). This said, the two lines of cells expressing *Lef1* mRNAs also expressed TOPGAL, creating a narrow zone of overlap between the two patterns.

We later provide biochemical evidence to establish the identity of the differentiating cells that express both TOPGAL and *Lef1* mRNAs. However, at this point, we suspected that the differentiating cells at least included the precursor cells of the hair shaft, since clipped hairs of postnatal TOPGAL transgenic animals stained faintly blue when subjected to  $\beta$ -galactosidase activity assays (not shown). This blue staining was not seen in hairs from control mice that lacked the transgene, indicating that in the TOPGAL mice, it must have stemmed from residual  $\beta$ -galactosidase activity produced at a time when the differentiating cells of the developing hair shafts were still metabolically active. An extrapolation from this result is that the LEF1-binding motifs previously noted in the hair-keratin-specific promoters (Zhou et al., 1995) are likely to be functionally relevant to the expression of the hair keratin genes in the precortex, cortex and cuticle. Moreover, the expanding pattern of  $\beta$ -galactosidase activity seen in the 9 day postnatal follicle (Fig. 4D') was remarkably similar to the pattern of hair-specific keratin mRNA expression in anagen-stage rodent follicles (Kopan et al., 1989; Zhou et al., 1995).

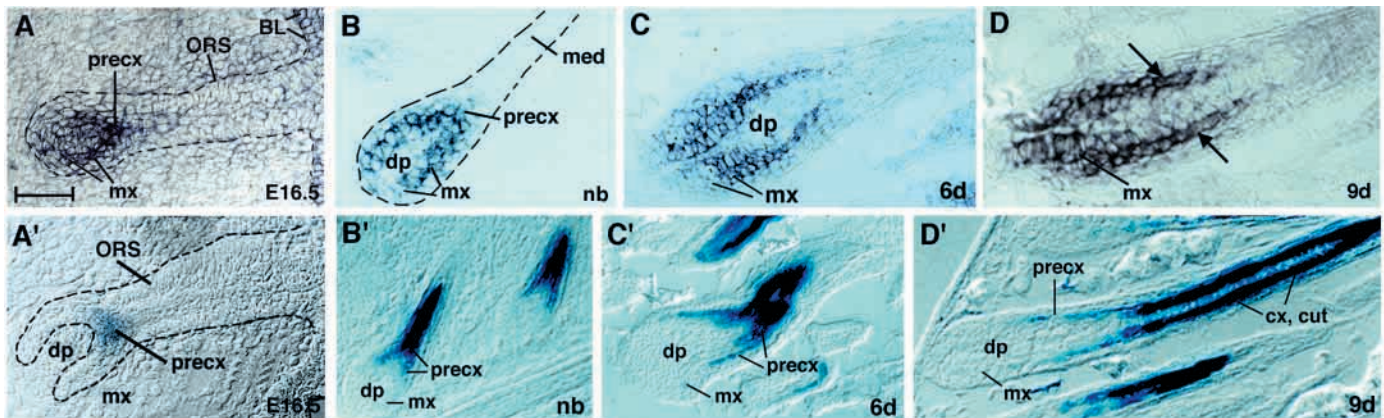
*Lef1* mRNA expression persisted throughout anagen, but then declined dramatically at catagen and telogen, as hair



**Fig. 3.** Lef1 protein and  $\beta$ -galactosidase activity assays in skin from mouse embryos expressing the TOPGAL transgene on its own. Timed pregnancies of F<sub>1</sub> mice harboring only the TOPGAL transgene were made to compare Lef1 protein with TOPGAL promoter activity during hair follicle development. Shown are E16.5 transgenic skin sections depicting the pregerm (PG) stage, where the follicles are merely ectodermal placodes and underlying dermal condensates (A,B,D,E; whisker follicles), the hair germ/plug stage (hg), where epithelial downgrowth has been initiated (F,G; ventral torso) and the early developing hair follicle stage (hf), where a pocket of dermal papilla cells has been encased by matrix cells to form a bulb-like structure (H; inset shows high magnification of bulb; ventral torso). (C) Skin of a *Lef1* knockout newborn, used as a control for the antibody stainings. As indicated on each frame, sections were assayed for anti-K5 (K5) and/or anti-Lef1 (Lef1) immunofluorescence or  $\beta$ -galactosidase activity. Additional abbreviations: epi, epidermis; de, dermis; DC, dermal condensate; BL, basal layer; dp, dermal papilla; mx, matrix. Dotted lines denote epithelial-mesenchymal boundaries. Arrowheads in D and E denote blue cells. Note (1) sites of blue ectodermal and mesenchymal cells corresponded roughly in frequency and distribution to Lef1-intense sites (see A), and (2) absence of blue staining in developing hair germ/plug (F). Bar in A represents 120  $\mu$ m in C; 60-70  $\mu$ m in all other panels.

growth ceased and follicles regressed.  $\beta$ -galactosidase activity persisted in the hair shaft cells into telogen but, by 18 days postnatally, only faint staining was detected. Similar patterns of blue staining were seen in postnatal follicles at different body sites, including body, tail and whisker follicles. This was somewhat surprising, given that whiskers are completely

lacking in *Lef1* knockout mice, whereas body hairs were only partially compromised (van Genderen et al., 1994). Whether this difference might stem from variations in the importance of Lef/TCF activation in the waves of embryonic follicle development that give rise to different hair types as recently postulated (Headon and Overbeek, 1999) is a possibility that



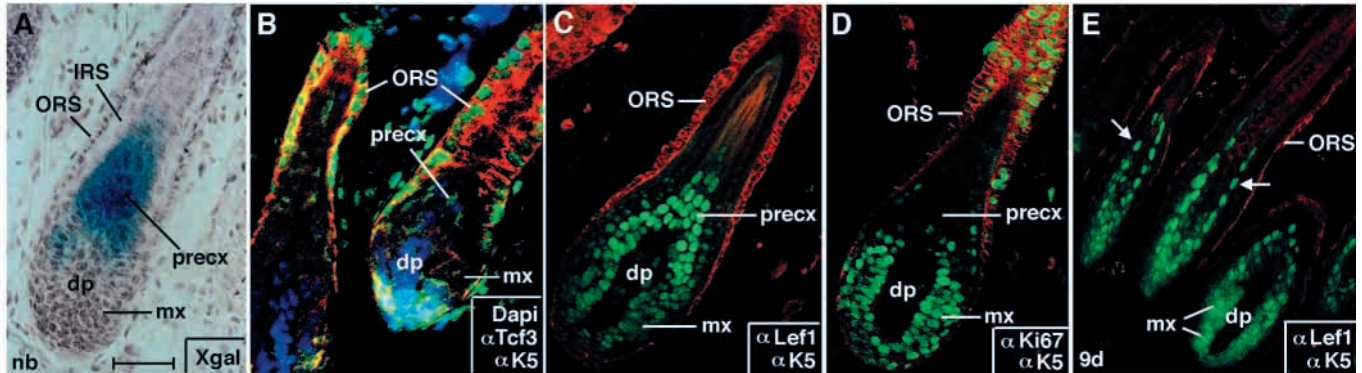
**Fig. 4.** Expression of *Lef1* mRNAs and TOPGAL in developing follicles that have formed the precursor cells of the hair shaft. Skin sections were obtained from F<sub>1</sub> generation TOPGAL transgenic mice at the developmental ages indicated on each frame (E16.5 to postnatal day 9). Sections were either hybridized with digoxigenin-labeled antisense *Lef1* cRNAs (A-D) or processed for  $\beta$ -galactosidase activity assays (A'-D'). Abbreviations: dp, dermal papilla; BL, basal epidermal layer; mx, matrix of the hair follicle; precx, precortex; cx, cortex; cut, cuticle; med, medulla of the hair shaft; ORS, outer root sheath. Note (1) E16.5 skin is from ventral torso; rest is from dorsal torso and (2) no differences in *Lef1* mRNA or TOPGAL expression were observed in whisker and body hair follicles at equivalent stages of morphogenesis. Bar in A represents 60  $\mu$ m in C,D; 90-100  $\mu$ m in A,B,A',C',D'; 120  $\mu$ m in B'.

cannot be tested rigorously until we improve the limits of sensitivity of our reporter assay.

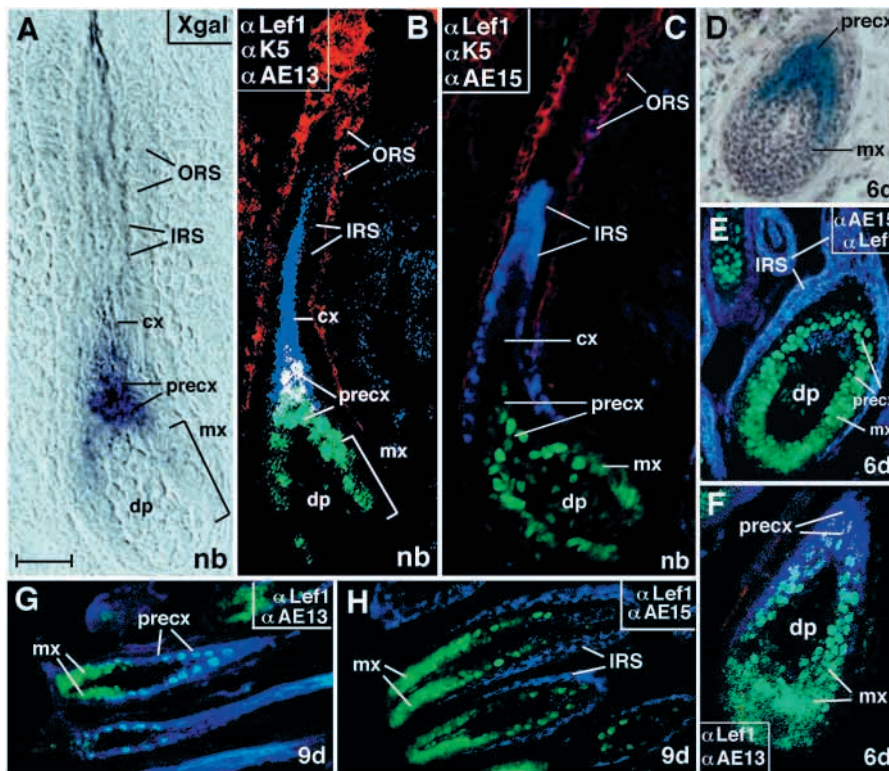
**LEF1/TCF3 protein in the follicle: nuclear LEF1 appears to accumulate in the differentiating hair shaft precursor cells, where TOPGAL is active, rather than proliferating matrix cells where TOPGAL appears to be silent**

Given the notable difference between *Lef1* mRNA and

TOPGAL expression in the postnatal hair follicle, we first wondered whether TCF3 protein, known to be expressed in skin (Barker et al., 1999), might be present in the precortex and account for the activation of TOPGAL in these cells. To address this question, we subjected sections of newborn TOPGAL transgenic mouse skin to  $\beta$ -galactosidase activity assays, and anti-TCF3 immunofluorescence (Fig. 5). In contrast to TOPGAL, which was expressed in the precortex of newborn tailskin follicles (Fig. 5A), TCF3 was confined to the



**Fig. 5.** TOPGAL expression occurs as matrix cells differentiate, concomitant with an increase in intensity of nuclear anti-LEF1 immunofluorescence and in the apparent absence of TCF3. Skins were taken from TOPGAL transgenic mice at  $t=0$  (A-D; tail skin), or 9 days (E; dorsal torso) and processed for TOPGAL expression (X-gal; counterstained with Hematoxylin and Eosin) or indirect immunofluorescence using the reagents indicated in the lower right corner of each frame. Antibodies used were: TCF3 (green), LEF1 (green), K5 (red), Ki67 (green), Dapi (blue). K5 is a marker of the outer root sheath (ORS); Ki67 is a nuclear marker of proliferating skin cells. (A,C,D) Serial sections of the same follicle. (E) Serial section of the same follicle shown in Fig. 4D'. Abbreviations: mx, matrix; precx, precortex; dp, dermal papilla. Arrows in E point to brightly stained LEF1 flat nuclei; note more diffuse LEF1 staining of matrix and also the immunofluorescence negative zone that separates the K5-positive ORS cells from the LEF1-stained cells. This zone is likely the inner root sheath (IRS), which can be distinguished morphologically from the precortex (see A). Bar in A represents 60  $\mu$ m in A-D; 100  $\mu$ m in E.



**Fig. 6.** Biochemical identification of the strongly LEF1-positive, TOPGAL-expressing cells as those that begin to express the hair-specific keratins and not inner root sheath markers. Backskins from TOPGAL transgenic mice were taken at the ages indicated, and tissues were processed for either  $\beta$ -galactosidase activity (X-gal, A,D; D was counterstained with hematoxylin and eosin), or indirect immunofluorescence (antibodies indicated on each frame). Antibodies used were: LEF1 (green), AE13 against hair-specific keratins (purple), K5 specific for the ORS of the follicle (red), AE15 specific for the IRS (purple). Abbreviations: mx, matrix; precx, precortex; cx, cortex; dp, dermal papilla; ORS, outer root sheath; IRS, inner root sheath. Notes (1) Serial sections in A-C are of the same follicle, as are those in D-F; serial sections in G and H are from same follicles as those in Figs 4D' and 5E. (2) A filter was applied across the images of all immunofluorescence frames shown. This distorts the actual coloring slightly, but allows the reader to visualize better the blue secondary antibody as purple and the overlap of blue AE13 and green LEF1 labeling as turquoise (more LEF than AE13) and white (more AE13 than LEF1). (3) Sections in A-C and G,H are through the heart of the follicles; those in D and E are through the heart of the follicle bulbs, but at a slight angle grazing through the IRS rather than the hair shaft. Bar in A represents 50  $\mu$ m in A-C; 70  $\mu$ m in D-F; 100  $\mu$ m in G,H.

follicle bulbs, but at a slight angle grazing through the IRS rather than the hair shaft. Bar in A represents 50  $\mu$ m in A-C; 70  $\mu$ m in D-F; 100  $\mu$ m in G,H.

outer root sheath (Fig. 5B). Double immunofluorescence staining with antibodies against the cytoplasmic keratin K5 (red) revealed that the anti-TCF3 staining (green) was clearly nuclear. Thus, despite the fact that TCF3 and LEF1 behave similarly with stabilized  $\beta$ -catenin in TOP-promoter assays in lymphocytes in vitro (Korinek et al., 1998a), TCF3 did not seem to coincide with TOPGAL expression in skin in vivo. Whether the inactivity of TCF3 is a reflection of its interaction with the CtBP repressor protein, known to act selectively on TCF3 and not LEF1 (Brannon et al., 1999) is an intriguing question, beyond the scope of the present study.

We next examined whether the pattern of LEF1 might provide us clues to help us understand the apparent differential expression of *Lef1* mRNAs and TOPGAL expression. Serial sections of the follicle shown in Fig. 5A were stained with anti-LEF1 antibodies. Close inspection revealed a surprising staining pattern of anti-LEF1 that differed considerably from the anti-LEF1 pattern seen in the embryonic follicle as well as the pattern seen by *Lef1* cRNA in situ hybridization (Fig. 5). In the dermal papilla, the intensity of anti-LEF1 staining seemed weaker than had been observed in embryonic dermal condensates (compare Figs 5C and 3B). Additionally, in the matrix cells of newborn follicles, where *Lef1* mRNAs were most strongly expressed (see Fig. 4), anti-LEF1 staining was weak and somewhat diffuse (Fig. 5C). However, in the precortex, anti-LEF1 staining was intense and more sharply nuclear (Fig. 5C). Thus, whereas *Lef1* mRNAs were more abundant in the undifferentiated matrix cells, LEF1 appeared to accumulate in the differentiating precortex cells. Moreover, whereas TOPGAL and *Lef1* mRNA expression patterns only overlapped in a narrow zone, TOPGAL expression correlated well with the pattern of intense nuclear anti-LEF1 staining. Taken together, it seems likely that some factor, possibly stabilized  $\beta$ -catenin, is responsible for the apparent accumulation of LEF1 and activation of TOPGAL in the precortex.

Interestingly, staining of a serial section of these follicles with antibodies against the proliferation-associated antigen Ki67 (Smith et al., 1995) revealed that the proliferating matrix cells were those that stained more faintly for anti-LEF1, whereas the precortex cells, which stained very strongly for anti-LEF1, did not stain with anti-Ki67 (Fig. 5D). These data are consistent with prior [<sup>3</sup>H]thymidine labeling (Wilson et al., 1994) and histone H3 in situ hybridizations (C. Byrne and E. F., unpublished data). The findings provide compelling evidence that the site of TOPGAL expression and intense nuclear LEF1 antibody staining is in the non-proliferative compartment at the follicle base. This finding was unexpected given recent reports that TCF/LEF and  $\beta$ -catenin transcription complexes can activate *c-myc* and *cyclin D* genes (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999), and the well-established role of activating  $\beta$ -catenin mutations and cancer (for review, see Clevers and van de Wetering, 1997; see also Chan et al., 1999 and references therein).

The distinction between diffuse anti-LEF1 staining in the matrix and more focused nuclear staining in the precortex became even more prominent at 6 and 9 days (Fig. 5E). During this time, nuclear LEF1 expanded to include one to two layers of cells above the bulb (Fig. 5E, arrows). Co-staining with anti-K5 revealed a zone of immunofluorescence negative cells between the K5-positive ORS cells and the LEF1-positive

layers (Fig. 5E). This negative zone adjacent to the ORS was likely the inner root sheath (IRS; see Fig. 5A), suggesting that the one to two layers of cells with flat nuclei were likely to be the differentiating cortical and/or cuticular layers of the hair shaft, previously found to express TOPGAL. Additional evidence in support of this identification is provided below.

### **Biochemical identification of the TOPGAL-expressing, strongly anti-LEF1 nuclear stained cells as hair-keratin-expressing cells and not inner root sheath cells**

To verify that the TOPGAL/strongly nuclear LEF1-positive cells in the mature follicle were also the ones that expressed the hair specific keratins, we used well-characterized monoclonal antibodies that are highly specific for either the hair shaft keratins (AE13) or the IRS marker trichohyalin (AE15) (Manabe et al., 1996; Lynch et al., 1986). As shown by the serial sections stained in Fig. 6A,B,  $\beta$ -galactosidase activity, intense nuclear anti-LEF1 staining and anti-hair keratin (AE13) staining were clearly overlapping in the precortex region of newborn mouse skin (Fig. 6A, TOPGAL expression; Fig. 6B, anti-LEF1 in green and AE13 in purple; turquoise blue to whitish cells represent cells that are double-labeled). In contrast, another serial section of the same newborn follicle stained with anti-trichohyalin (AE15) and anti-LEF1 revealed that these patterns were mutually exclusive (Fig. 6C; purple staining is AE15; green is anti-LEF1). The overlapping patterns of  $\beta$ -galactosidase activity, anti-LEF1 and AE13, but not AE15, were also seen later in anagen, as represented by the 6 and 9 day postnatal skin samples in Fig. 6D-H. Again, serial sections were taken so that the same follicle could be examined following various treatments. These data unequivocally identify the TOPGAL-expressing cells as precortex and not IRS, and further demonstrate that hair keratin expression initiates in the cells that express TOPGAL.

### **TCF3 nuclear localization in the bulge and TOPGAL activation at the initiation of the new hair cycle**

Contiguous with the ORS and localized at the transition zone between permanent and cycling portions of the hair follicle is the bulge, the putative compartment of follicle stem cells (see Fig. 1; Lavker et al., 1991). This compartment of epithelial cells is not needed for establishing the embryonic hair follicle, but it is required for the initiation of each new hair cycle, as stem cells are stimulated by an as yet unidentified signal, most likely from the dermal papilla (Lavker et al., 1991). We therefore wondered whether Wnt signaling and TCF/LEF transcription factors might be utilized at this stage of the hair cycle.

By 26-28 days, virtually all follicles in CD1 mice have entered their first postnatal hair cycle. Although we also examined follicles at earlier ages, we concentrated our study on this age to optimize the likelihood that a follicle would be activated rather than dormant, a distinction that is difficult to make morphologically when follicles are at telogen or the transition stage from telogen to anagen. Despite the quite prominent synchrony of the first postnatal hair cycle, there are some differences in the timing of initiation due to such factors as regional variations (e.g. whisker, torso, tail, limb skin), variations in the cycles of coat hair types, anterior-to-posterior waves of hair cycle initiation, and also the sex and birth time



( $\pm 0.5$  day) of the animals (Kobori and Montagna, 1975; Wilson et al., 1994; Headon and Overbeek, 1999).

Interestingly, in those 28-day follicles that did not yet show signs of the secondary hair germ, a few cells within the bulge sometimes stained blue for TOPGAL expression (Fig. 7A,B). Our ability to detect a signal in this region was facilitated in mice transgenic for both TOPGAL and  $\Delta N87\beta$  catenin (examples shown). Curiously, we consistently observed only a few cells in each bulge area that stained blue (examples shown). While the interpretation of these findings must await more extensive biochemical characterization of follicle stem cells and identification of the signals that activate them, this result was similar to that obtained for the initiation stage of follicle morphogenesis in embryonic skin.

We next examined the bulge region for the presence of TCF3 and LEF1. Interestingly, anti-TCF3 staining was seen within the bulge, where it was localized in cell nuclei (Fig. 7C). Nuclear TCF3 persisted at this location throughout all stages of the hair cycle, even in telogen, when the ORS below the bulge was absent. In the upper portion of the follicle above the bulge, and in epidermis, anti-TCF3 labeling was dramatically diminished (Fig. 7D, arrows denote boundary between strong and weak zones of anti-TCF3 labeling in the ORS; inset shows adjacent epidermis, double labeled with anti-K5 in red and anti-TCF3 in green).

At the initiation of the next hair cycle, anti-TCF3 was detected in the K5-positive cells that grew downward to form the secondary hair germ (Fig. 7D,E). However, at the leading edge of the germ, cells were weak or negative for anti-TCF3 staining (Fig. 7E). In contrast to anti-TCF3, anti-LEF1 did not stain the bulge appreciably at any stage of the hair cycle (Fig. 7F). Just after the initiation of the next hair cycle, however, anti-LEF1 stained a subset of cells emanating down from the bulge to form the secondary hair germ (Fig. 7F,F'). These cells were clearly positive for K5, distinguishing them from the dermal papilla, which remained anti-LEF1 positive throughout the hair cycle. In this regard, and in their intense nuclear staining, these developing matrix cells of the secondary hair germ resembled those of primary hair germs formed during embryonic development.

A serial section from this follicle was stained with proliferation antigen anti-Ki67, revealing that the outer cell layer of the secondary hair germ was strongly positive (Fig. 7G,G'). In contrast, the dermal papilla were negative for Ki67. A few Ki67-positive cells were occasionally seen above the secondary hair germ at the base of the old follicle (arrow). The proliferative capacity of these cells at the base has been described by Wilson et al. (1994), who showed that putative stem cells transiently incorporate labeled thymidine at this early anagen phase of the new cycle.

We next examined TOPGAL expression in this follicle by staining another serial section of tissue. As shown in Fig. 7H (counterstained with Hematoxylin and Eosin in Fig. 7H'),  $\beta$ -galactosidase activity was not detected in the growing hair germ. It was found at the base of the old hair club and, while this was never seen in non-transgenic skin, it was most likely due to residual enzymatic activity in the dead hair cells rather than new induction of TOPGAL. At a slightly later stage of anagen, as the secondary follicle began to form a bulb,  $\beta$ -galactosidase was detected in a triangular zone of cells, which appeared to be the developing precortex (schematic in Fig. 7I;  $\beta$ -gal in Fig. 7J,J').

The pattern of anti-LEF1 staining at this stage of anagen was strikingly different from that at the slightly earlier stage of secondary hair germ formation (compare Fig. 7F and K). This was true not only for the epithelium but also for the dermal papilla. Thus, as the dermal papilla became engulfed by the follicle epithelium to make a bulb, the intensity of anti-LEF1 staining increased in the center where the precortex region developed, and decreased in both the developing matrix and the internalized dermal papilla (Fig. 7K). While we had observed these changes in the transition from embryonic to early postnatal development, these changes were particularly striking when observed within individual samples of 28-day skin.

The changes in TOPGAL and anti-LEF1 staining were accompanied by changes in anti-K5 and anti-Ki67 patterns (Fig. 7L; compare with Fig. 7G,G'). During this transition, K5 became restricted to the ORS and was not expressed in matrix. Both ORS and matrix cells maintained marked proliferative activity, but clear zones of Ki67-negative cells were now seen in the follicle.

## DISCUSSION

### **A role for the activation of LEF1 as a transcription factor in both ectoderm and mesenchyme at the initiation stage of hair follicle morphogenesis: implications for Wnt signaling**

Recent evidence has implicated LEF/TCF family members and activated  $\beta$ -catenin in hair follicle development and differentiation (van Genderen et al., 1994; Zhou et al., 1995; Kratchowil et al., 1996; Gat et al., 1998). In vitro, LEF/TCF family members interact directly with activated  $\beta$ -catenin to form a functional transcription factor complex (Behrens et al., 1996; Molenaar et al., 1996), suggesting that these two factors may function together to govern transcriptional events in hair development. In *Drosophila*, activated  $\beta$ -catenin (Armadillo) and dTcf are genetically within the Wnt (Wingless) pathway that influences epidermal appendage patterning and development (for review, see Clevers and van de Wetering, 1997), making it likely that Wnt signaling is similarly important for mammalian hair development. Our findings here provide the strongest evidence to date that implicates Wnt signaling in these processes.

Our study defines two distinct roles for LEF/TCF activation of downstream target genes in initiating hair follicle morphogenesis. Early tissue recombination experiments have long predicted that the first signal in hair follicle development stems from the mesenchyme that instructs the ectoderm to form a placode (Sengel, 1976; Hardy, 1992). Based on our ability to detect TOPGAL in ectoderm, this first mesenchymal cue could either be a Wnt directly, or alternatively, a signal such as BMP-4, known to induce *Lef1* gene expression and influence hair organogenesis in vitro (Kratchowil et al., 1996; Keranen et al., 1998). While there may be additional LEF/TCF factors in this process, we detected LEF1 but not TCF3 at this early stage of development.

We also observed TOPGAL expression in the developing dermal condensate and, since TOPGAL expression seemed to be predominantly in either ectoderm or dermal condensate, it seems that the two signals leading to LEF/TCF target gene

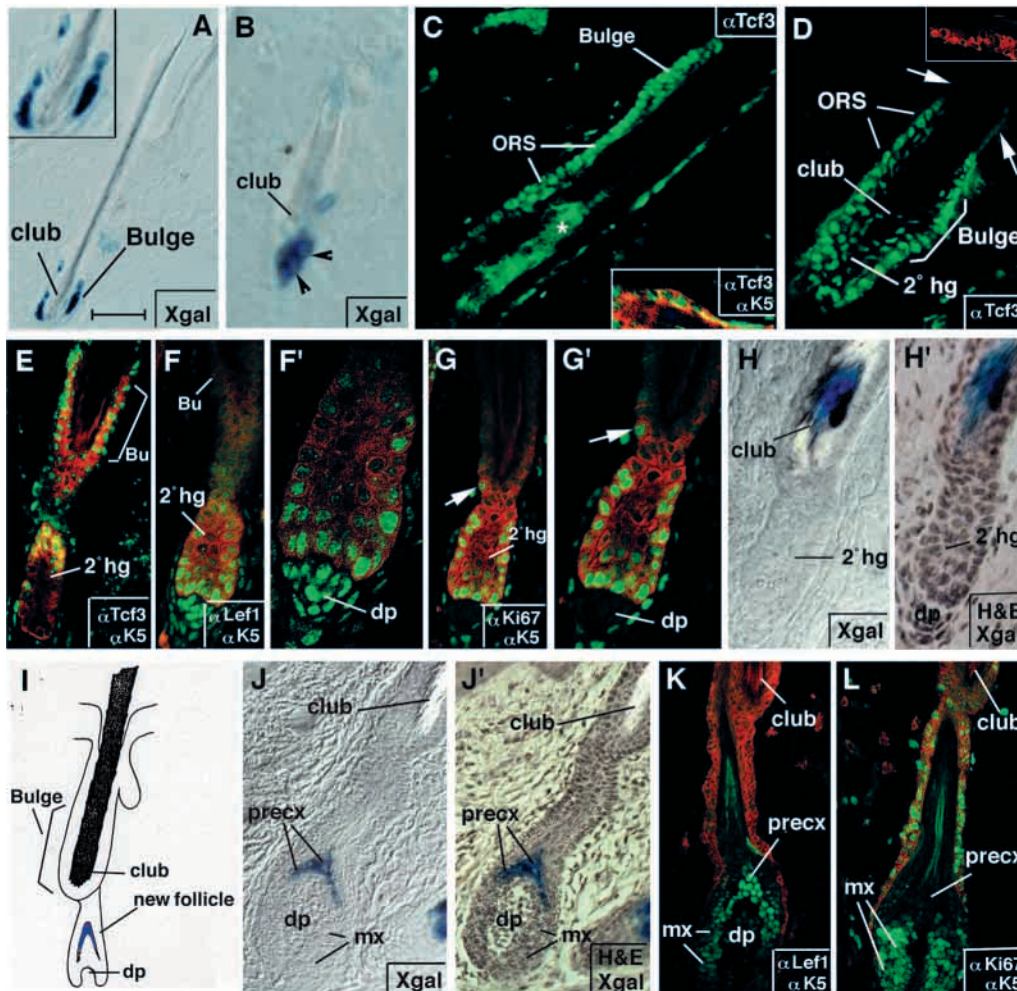
expression are sequential, rather than simultaneous. An attractive hypothesis consistent with our data and with developmental models is that one or more of the mesenchymal-ectodermal signals that lead to hair germ formation are Wnts. In contrast, our results suggest that the next dermal message, instructing committed epithelium to proliferate and form the hair follicle (Hardy, 1992), might not involve a Wnt, if indeed the TOP promoter provides a faithful and broad representation of LEF/TCF activation *in vivo*, as it is known to do in lymphocytes *in vitro* (Korinek et al., 1998a). Rather, based upon the block in the proliferation step of embryonic *Sonic hedgehog* null follicles (St. Jacques et al., 1998; see also Oro et al., 1997), *Shh* is a possible candidate for regulating this process. Future studies will be necessary to assess the extent to which these hypotheses might be correct, and to further define the mechanisms underlying follicle development.

### A role for the activation of the LEF1 transcription factor in commitment of matrix cells to hair shaft differentiation

A fascinating feature of hair matrix cells is that they are able

to select morphologically and biochemically distinct differentiation pathways leading to the IRS and hair shaft (see Hardy, 1992). How matrix cells select these programs of differentiation has always been a mystery. Our present studies provide among the first biochemical insights into this process. Thus, while most if not all matrix cells express *Lef1* mRNAs and some LEF1 protein, only cells at the center of the bulb appear to accumulate LEF1 and activate downstream target genes concomitant with commitment to differentiate. The TOPGAL-expressing cells give rise to the hair shaft, while the surrounding cells choose an alternative pathway of differentiation and give rise to the IRS. Our findings suggest that induction of *Lef1* expression is a characteristic of undifferentiated matrix cells, and that utilizing LEF1 to activate downstream target genes is a feature of matrix cells that become committed to a hair shaft differentiation program.

The finding that LEF1 activation plays a role in hair shaft differentiation provides an explanation for why so many hair-specific keratin genes possess LEF1-binding motifs in their upstream regulatory sequences (Zhou et al., 1995). Testing the role of LEF1 in hair-specific keratin gene expression has been



**Fig. 7.** Activation of TOPGAL and expression of LEF1 and TCF3 at the start of the new hair cycle. Tail skin or the haired portion of hindfoot skin from 28 days TOPGAL/K14- $\Delta$ N87 $\beta$ cat mice (A,B), 28 days TOPGAL mice (D-J) or a newborn TOPGAL mouse (C) were sectioned and processed for either  $\beta$ -galactosidase activity (X-gal in blue) or indirect immunofluorescence with anti-TCF3 (green), anti-LEF1 (green) anti-Ki67 (green) or anti-K5 (red) as indicated on each frame. While we detected no major differences in the two skin regions of 28-day mice with regards to the biochemical assays employed here, some regional differences in the timing of the second hair cycle were observed. Thus, follicles in A and B showed no outgrowth, the follicle in D and the one in E-H' (serial sections of the same follicle) had produced a secondary hair germ (2°hg), and the follicle in J-L (serial sections of the same follicle) had progressed further into anagen, already producing a precortex (schematic of this cycle stage depicted in I). Several cells (arrowheads in B) in the bulge region surrounding the old hair club are stained blue (higher magnification in inset in A).

Asterisk in C denotes autofluorescence of hair shaft; arrows in D denote boundary of upper region of ORS, where anti-TCF3 is largely negative; arrow in G and G' denotes a Ki67-positive cell in the lower portion of the primary hair follicle. Blue staining of the old hair club in H and H' may reflect residual galactosidase activity persisting in the dead hair cells; this section was stained overnight to verify the lack of staining in the 2°hg. Additional abbreviations: Mx, matrix; precx, precortex; dp, dermal papilla; ORS, outer root sheath; Bu, bulge. Bar represents 150  $\mu$ m in A; 40-50  $\mu$ m in B,F',G',H,H'; 70-90  $\mu$ m in C-G;J-L.

complicated by the lack of a suitable cell culture system for hair matrix cells, the cell type that gives rise to the hair shaft. In vivo studies in transgenic mice have revealed that mutation of the LEF1-binding site in one of these hair-specific promoters results in an apparent reduction in promoter activity (Dunn et al., 1998). While promising, interpretation of such negative results are problematic, particularly when transgene integration site alone can often lead to dramatic variations in promoter activities. Our finding that TOPGAL is expressed in precortical cells that give rise to the hair shaft provides compelling evidence that activated LEF1 is important in regulating genes that specifically give rise to the hair shaft.

Several Wnt mRNAs have been found in mature hair follicles. Of these, *Wnt-3* is of special interest in that its mRNAs are expressed in the precursor cells of the hair medulla (Millar et al., 1999). Future experiments will be necessary to assess the extent to which WNT-3 or other Wnts might be important for signaling to the precortex and inducing LEF1 transcriptional activity.

### **A possible role for TCF3 or LEF1 in activating follicle stem cells at the start of a new hair cycle?**

An attractive model to explain the hair cycle is that stem cells in the bulge become activated at the start of each new cycle, when they receive an as yet unidentified signal transmitted by dermal papilla cells that have retracted upwards at the end of the previous cycle (for review, see Hardy, 1992). Our studies here have shown that nuclear TCF3 resides continually in the putative stem cell compartment within the bulge. In addition, although co-expression of  $\Delta N87\beta$  cat was needed to detect strong TOPGAL activation in the bulge, a few cells within the bulge area of both TOPGAL and TOPGAL/ $\Delta N87\beta$  cat follicles expressed  $\beta$ -galactosidase at or around the initiation of the first postnatal hair follicle. LEF1 also appeared to be activated at or soon thereafter, as judged by the fact that intense nuclear staining was concentrated in the secondary hair germ epithelial cells maintaining direct contact with the dermal papilla. This evidence suggests the possibility that TCF3/LEF1-mediated target genes may be expressed as an important step in stem cell activation. Additional studies using factors such as plucking to stimulate bulge cells (see Wilson et al., 1994), as well as measurements of TCF3's transactivation potential in ORS keratinocytes and/or bulge cells, will be necessary to explore this intriguing notion further.

While our studies on stem cell activation of TOPGAL in the bulge are still preliminary, several recent findings suggest that TCF/LEF and/or activated  $\beta$ -catenin may be key components of epithelial stem cells. Korinek et al. (1998b) ablated *Tcf-4* gene expression in mice and discovered that the epithelial stem cell compartment of small intestine was depleted in these animals. Additionally, Zhu and Watt (1999) found that cultured keratinocytes with a high proliferative capacity possess a high level of cytoplasmic  $\beta$ -catenin, leading them to hypothesize that this might be a characteristic of epidermal stem cells. Furthermore, our ability to induce de novo hair morphogenesis in postnatal mice expressing a constitutively activated form of  $\beta$ -catenin in the epidermis and ORS is suggestive that these adult cells had acquired the characteristics of pluripotent ectoderm (Gat et al., 1998). All of these findings support the view that the expression of TCF3 in the bulge may be more than coincidental, and merits further investigation in the future.

### **Proliferation, tumorigenesis and activation of TCF/LEF1**

A curious finding was the lack of appreciable TOPGAL expression in proliferating cells within the skin. This was surprising, given the well-documented presence of activating  $\beta$ -catenin mutations and APC mutations in a number of human cancers, including pilomatricomas, relevant to this study (see Chan et al., 1999 and references therein). Indeed, the only known mammalian target genes presently known for activated  $\beta$ -catenin/LEF/TCF complexes are *cyclin D1* and *c-myc*, both protooncogenes important for cell proliferation (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999).

One possibility is that activation of proliferation-associated target genes by activated LEF/TCF factors may occur only when the natural mechanisms governing LEF/TCF/ $\beta$ -catenin regulation are subverted, as in the case of  $\beta$ -catenin activating mutations. In this regard, it is relevant that the interfollicular downgrowths induced by our constitutively active  $\beta$ -catenin transgene, K14- $\Delta N87\beta$ cat, displayed strong TOPGAL activation in the double transgenic mice. These invaginating epithelial pockets grow very rapidly and ultimately lead to pilomatricoma tumorigenesis (see Gat et al., 1998 for details). When taken together with our finding that in normal postnatal follicles, activation of LEF/TCF transcription factor complexes seems to arise to change an epithelial cell's fate or differentiation state, we speculate that different threshold levels of LEF1/TCF activation may explain how TCF/LEF-mediated activation can induce a terminal differentiation program on the one hand, and tumorigenesis on the other.

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