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Expression of ΔNLef1 in mouse epidermis results in differentiation of hair follicles into squamous epidermal cysts and formation of skin tumours

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SUMMARY

To examine the consequences of repressing β-catenin/Lef1 signalling in mouse epidermis, we expressed a ΔNLef1 transgene, which lacks the β-catenin binding site, under the control of the keratin 14 promoter. No skin abnormalities were detected before the first postnatal hair cycle. However, from 6 weeks of age, mice underwent progressive hair loss which correlated with the development of dermal cysts. The cysts were derived from the base of the hair follicles and expressed morphological and molecular markers of interfollicular epidermis. Adult mice developed spontaneous skin tumours, most of which exhibited sebaceous differentiation, which could be indicative of an origin in the upper part of the hair follicle. The transgene continued to be expressed in the tumours and β-catenin signalling was still inhibited, as evidenced by absence of cyclin D1 expression. However, patched mRNA expression was upregulated, suggesting that the sonic hedgehog pathway might play a role in tumour formation. Based on our results and previous data on the consequences of activating β-catenin/Lef1 signalling in postnatal keratinocytes, we conclude that the level of β-catenin signalling determines whether keratinocytes differentiate into hair or interfollicular epidermis, and that perturbation of the pathway by overexpression of ΔNLef1 can lead to skin tumour formation.

Key words: β-catenin, Lef1, Epidermis, Transdifferentiation, Hair follicles, Tumours, Mouse

INTRODUCTION

The Wnt signalling pathway controls many morphogenetic events during embryonic development (Miller and Moon, 1996). Secreted Wnt proteins bind to transmembrane receptors of the frizzled family to initiate a signalling cascade that results in regulation of Wnt target genes (Nusse, 1999). Ligation of frizzled leads to activation of the cytoplasmic effector Dishevelled and prevents degradation of β-catenin in the cytoplasm. β-catenin translocates to the nucleus and binds transcription factors of the TCF/LEF family (comprising four members: Lef1, Tcf1, Tcf3, Tcf4) (Clevers and van de Wetering, 1997), thereby modulating gene transcription (Behrens et al., 1996). In addition to the pool of free β-catenin that is rapidly degraded in the absence of a Wnt signal, cells have a pool of β-catenin that is stably complexed with the cytoplasmic domain of cadherins, thereby linking cadherins to the actin cytoskeleton (Cowin and Burke, 1996).

Activation of Wnt signalling is strongly implicated in tumorigenesis (Roose and Clevers, 1999; Polakis, 2000; Taipale and Beachy, 2001). Mutations in the N terminus of β-catenin that make the protein refractory to degradation have been found in a wide range of human tumours and contribute to neoplasia in a variety of animal tumour models. β-Catenin can also be upregulated through inactivating mutations in the tumour suppressor gene, *APC*, which encodes part of the β-catenin degradation machinery, and this is a particular feature of colorectal cancer.

One of the organs in which β-catenin is known to regulate normal morphogenesis and to be subverted in neoplasia is the skin (Oro and Scott, 1998). The epidermis contains pluripotential stem cells, which are responsible for generating the interfollicular epidermis, hair follicles and sebaceous glands (Al-Bawari and Potten, 1976; Reynolds and Jahoda, 1992; Taylor et al., 2000; Watt, 2001; Oshima et al., 2001). Whereas interfollicular epidermis is renewed continuously, the hair follicles undergo cycles of growth and regression, reflecting cyclical activation of the follicle stem cells.

It has been previously shown that *Lef1* null mice lack whiskers and have a reduced number of body hairs (van Genderen et al., 1994; Kratchowil et al., 1996) and that overexpression of Lef1 in the basal layer of the epidermis via the keratin 14 promoter perturbs the orientation of whiskers and hairs (Zhou et al., 1995). Upstream of LEF/TCF transcription factors, overexpression of dishevelled 2, Wnt3 (Millar et al., 1999) or the β-catenin related protein plakoglobin (Charpentier et al., 2000) results in a short hair phenotype by disturbing differentiation of hair shaft precursor cells (Millar et al., 1999) or perturbing the hair growth cycle (Charpentier et al., 2000). Deletion of β-catenin in embryonic or postnatal epidermis results in failure of hair follicle development or maintenance (Huelsken et al., 2001). When

stabilised N-terminally truncated β-catenin is overexpressed from the keratin 14 promoter in transgenic mice, hair follicle formation during embryonic development is normal, but in postnatal life there is unscheduled de novo formation of hair follicles; proliferation continues unchecked and hair tumours (trichofolliculomas and pilomatricomas) develop (Gat et al., 1998). Stabilising mutations in β-catenin have been found in the equivalent tumours of human hair follicles (Chan et al., 1999). Interestingly, some individuals with familial adenomatous polyposis caused by germline mutations in *APC* have extra-colonic abnormalities, including benign epidermoid cysts (Gardner, 1962; Narisawa and Kohda, 1995; Polakis, 1997).

We have shown previously that β-catenin may act to regulate stem cell fate in cultured human keratinocytes. Keratinocytes that express high levels of β1 integrins, a marker of human epidermal stem cells (Jones et al., 1995; Jensen et al., 1999), have higher levels of non-cadherin associated β-catenin than keratinocytes with lower self-renewal capacity and higher differentiation probability (transit amplifying cells), and expression of N-terminally truncated β-catenin increases the proportion of putative stem cells in culture (Zhu and Watt, 1999). Conversely, overexpression of the cytoplasmic domain of E-cadherin stimulates human keratinocytes to undergo terminal differentiation (Zhu and Watt, 1996) by reducing the size of the β-catenin pool available to activate TCF/LEF (Zhu and Watt, 1999).

Our demonstration that β-catenin regulates the size of the putative epidermal stem cell compartment in vitro (Zhu and Watt, 1999) and a recent report that perturbation of Wnt signalling results in transdifferentiation of one cell type into another (myoblasts into adipocytes) (Ross et al., 2000) raise interesting questions about the phenotype of transgenic mice that overexpress β-catenin in the epidermis (Gat et al., 1998). Does it reflect an overall increase in the number of pluripotential epidermal stem cells or does it reflect stimulation of hair-type differentiation at the expense of interfollicular epidermal differentiation? A further question is how to interpret the tumour data, given that overexpression of stabilised β-catenin is not sufficient to cause transformation or a block in terminal differentiation of human keratinocytes in culture (Zhu and Watt, 1999) and that more than one genetic change is necessary for human cells to become neoplastic (Hahn et al., 1999). Single oncogenic mutations in keratinocytes that are undergoing terminal differentiation will be innocuous, as the cells are rapidly cleared from the epidermis. By contrast, stem cells, as permanent tissue residents, have the potential to accumulate multiple deleterious mutations and thus undergo neoplastic conversion (Jensen et al., 1999; Taipale and Beachy, 2001). Are the tumours induced by activating β-catenin a consequence of stimulating hair follicle formation or are the two phenomena distinct?

In order to address these issues we have generated transgenic mice that express N-terminally deleted Lef1, which lacks the β-catenin binding site, under control of the keratin 14 promoter. We show that this construct acts in a dominant negative fashion by repression of β-catenin signalling and has two distinct effects. Keratinocytes at the base of hair follicles differentiate into cysts comprising interfollicular epidermis. Keratinocytes that we believe originate in the upper region of the follicle give rise to tumours, the types of tumours being different from those observed when the pathway is activated (Gat et al., 1998). Thus, either activating or repressing β-catenin signalling can lead to epidermal neoplasia.

MATERIALS AND METHODS

Generation of K14ΔNLef1 transgenic mice

An N-terminally deleted mouse Lef1 cDNA (lacking amino acids 1 to 32, which contain the β-catenin-binding site) was Myc-tagged at the N terminus and inserted into the blunt ended *Bam*HI site of the keratin 14 expression cassette (kindly provided by Dr Elaine Fuchs, University of Chicago) (Vasioukhin et al., 1999). The resulting construct was designated K14ΔNLef1.

Transgenic mice were generated by pronuclear injection of the purified K14ΔNLef1 construct into fertilised oocytes from (CBA×C57BL/6) F_1 mice. Animals were screened for transgene expression and copy number by Southern blotting, performed on genomic DNA isolated from tail snips. To determine transgene copy number the intensity of the radioactive signal for the Lef1 probe was compared with the endogenous interleukin 2 receptor α signal (Carroll et al., 1995) using a Storm 860 phosphoimager (Molecular Dynamics) and an Image Quant program.

Western blotting

Pieces of skin from wild-type and transgenic mice (3-4 cm^2) were flash frozen in liquid nitrogen. The epidermis was scraped from the frozen skin and transferred into 10 mM sodium phosphate homogenisation buffer containing 1% Triton X-100, 0.1% SDS, 0.1% sodium azide, 0.15 M NaCl, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonyl fluoride and 3 μM aprotinin. The samples were homogenised on ice and centrifuged at 100,000 ***g*** for 60 minutes at 4°C. The supernatants were diluted in PAGE sample buffer, separated on 12.5% SDS-PAGE gels, electroblotted and probed with antibodies against the Myc-epitope (9E10, Santa Cruz) and actin (AC-40, Sigma), as described by Zhu and Watt (Zhu and Watt, 1999). Immunoreactive proteins were visualised by chemiluminescence (ECL, Amersham).

Proteins were extracted from primary keratinocytes of wild-type and K14ΔNLef1 transgenic mice using Triton X-100 buffer or SDS buffer, as described previously (Zhu and Watt, 1999). Proteins were separated on SDS-PAGE, electroblotted and probed with a pan-cadherin antiserum (FWCAD) (Zhu and Watt, 1999), and monoclonal antibodies against β-catenin (clone14, Transduction lab), plakoglobin (clone15, Transduction lab) and actin (Sigma).

Keratinocyte culture and reporter gene assays

Keratinocytes were isolated from transgenic and wild-type newborn mouse skin and cultured for up to six passages on collagen coated dishes (Becton Dickinson) in low Ca^{2+} FAD medium (one part Ham's F12 medium plus three parts DMEM, supplemented with 1.8×10^{-4} M adenine) containing 10% chelated FCS and a cocktail of hydrocortisone (0.5 μg/ml), insulin (5 μg/ml), cholera toxin (10^{-10} M) and EGF (10 ng/ml), as described previously (Carroll et al., 1995).

The following reporter constructs were used for transient transfection: pTOPtkLuciferase (TOPFLASH), pFOPtkLuciferase (FOPFLASH) (kindly provided by H. Clevers) (van de Wetering et al., 1997) and pJ7lacZ (control for transfection efficiency). The reporter constructs were co-transfected with cDNAs for a β-catenin/Lef1 fusion protein, *Xenopus* Tcf-3 that had been subcloned into pCMV5 (Olah et al., 1992) or the T2 mutant of *Xenopus* β-catenin that lacked the N-terminal 147 amino acids (Zhu and Watt, 1999). Transient transfection experiments were performed as described previously (Zhu and Watt, 1999). Cells were extracted using a kit (Promega) that permitted luciferase and β-galactosidase measurements to be performed on the same extracts. β-galactosidase

and luciferase activity were determined using a BioOrbit 1251 luminometer.

Newly confluent cultures of keratinocytes were labelled for 1 hour with BrdU and fixed with 3.7% paraformaldehyde in order to determine the proportion of cells in S-phase of the cell cycle. Cells were permeabilised with 0.5% Triton in 2 M HCl for 10 minutes at room temperature followed by an incubation in 100 mM NH_4Cl for 15 minutes. Keratinocytes were blocked in 5% skimmed milk powder, 10% FCS, 0.5% BSA, and 0.1% Triton for 1 hour at room temperature and then incubated with an anti-BrdU antibody (Becton Dickinson).

To determine the proportion of involucrin-positive cells in cultures of primary keratinocytes, single cell suspensions were air-dried at 37°C on microscope slides and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were permeabilised for 6 minutes at –20°C in methanol and afterwards incubated with an anti-involucrin antiserum (ERLI3) (Li et al., 2000) in 5% FCS. Secondary antibodies were conjugated with AlexaFluor 488 (Molecular Probes).

Preconfluent cultures of primary keratinocytes from wild-type and K14ΔNLef1 mice were harvested with trypsin/EDTA and labelled with an anti-β1 integrin antibody (MBI.2, kindly provided by B. Chan, University of Western Ontario) (von Ballestrem et al., 1996). The cells were incubated with the secondary antibody (AlexaFluor 488 conjugate) and analysed by flow cytometry, essentially as described previously (Zhu and Watt, 1999).

Semi-quantitative RT-PCR was performed on primary keratinocytes from newborn wild-type and K14ΔNLef1 mice in order to analyse expression of endogenous Lef1 and expression of the ΔNLef1 transgene. Primers were generated as follows: wild-type sense (Wts), 5′ ATGCCCCAACTTTCCG 3′; transgenic sense (Tgs), 5′ ATCA-GCGAGGAGGACCT 3′; and anti-sense (as), 5′ TGCTTTCCT-TCATCAGGGTGTTC 3′. The sense primers were selected to be as similar in length and melting temperature as possible. The sizes of the expected PCR products were: Wts + as=281 bp and Tgs + as=218 bp. The PCR products were analysed by agarose gel electrophoresis after 10, 25, 35 and 60 amplification cycles. Primers for the house keeping gene HPRT were included as a positive control and H_2O (H) instead of RNA was added to the reactions as a negative control.

Histology, immunohistochemistry and in situ hybridisation

Frozen sections of skin were subjected to indirect immunostaining (Carroll et al., 1995) using anti-keratin 1 (Babco), AE13 (Lynch et al., 1986) (a gift from T.-T. Sun, New York University Medical School), anti-Ha5 (kindly provided by L. Langbein, DKFZ, Heidelberg), anti-profilaggrin (Biogenesis), anti-laminin (Sigma), anti Myc-tag (Cell Signalling) and anti-β-catenin (Sigma) as primary antibodies. Secondary antibodies were conjugated with AlexaFluor 488 (Molecular Probes). In some experiments, the mouse on mouse kit (MOM, Vector Laboratories) was used according the manufacturer's protocol and the sections were counterstained with DAPI.

Some specimens of skin and tumours were fixed overnight in neutral buffered formalin and paraffin embedded. Sections (5 μm) were either stained with Haematoxylin and Eosin for histological analysis or processed further for immunostaining. Staining for Ki67 (polyclonal rabbit antibody, Novacastra) was performed using the ABC staining kit (Vector Laboratories) according to the manufacturer's recommendations. Before indirect immunofluorescence staining for keratin 10 (Babco), keratin 8 (Troma-1), β-catenin (clone14, Transduction lab), cyclin D1 (kindly provided by Martine Roussel) (Vallance et al., 1994), β1-integrin (MB1.2, kindly provided by B. Chan) (von Ballestrem et al., 1996) and Lef1 (clones C-19 and N-17, Santa Cruz), paraffin sections were microwaved in antigen retrieval solution (Bio Genex) for approximately 4 minutes and incubated for another 15 minutes with the retrieval solution. For immunostaining of cyclin D1 and Myc-epitope in spontaneous sebaceous adenomas and sebeomas of K14ΔNLef1 mice, chemically induced tumours (papillomas and squamous cell carcinomas) of wild-type animals were included as controls.

In situ hybridisation was performed on paraffin sections of adult skin and tumours. Antisense transcripts for Shh (nucleotides 120-760) and patched 1 (nucleotides 134-1008) were generated and probes were DIG labelled (Roche). After hybridisation, sections were washed in 50% formamide, 5× SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0) and 0.1% Tween at 70°C, and nonspecifically 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).

Electron microscopy

For transmission electron microscopym skin from wild-type and transgenic animals was fixed in 2.5% glutaraldehyde in Sorensen's buffer (pH 7.4) and post fixed in osmium tetroxide. Tissues were then embedded in araldite resin and 100 nm sections were cut on a Reichert ultracut S ultramicrotome. Sections were stained with uranyl acetate and lead citrate and viewed on a JEOL 1010 electron microscope.

For scanning electron microscopy hairs were plucked from wild-type and transgenic animals and fixed in 4% paraformaldehyde, washed in PBS and dehydrated through ascending grades of ethanol before being washed twice in hexamethyl-disilazane and air dried. Hairs were coated with platinum in a Polaron sputter coater and examined in a JEOL 5600 scanning electron microscope.

Analysis of wound healing and the mouse hair cycle

Transgenic and wild-type mice were wounded under general anaesthetic (Halothane-Vet, Merial, UK) with a 3 mm biopsy punch (Stiefel, Waechtersbach, Germany) and sacrificed 4, 7 or 14 days later. Two wild-type and two K14ΔNLef1 mice (10 and 18 weeks old) with two punch biopsies each were analysed for each time point.

To monitor the first postnatal hair cycle, hair was removed from a region of the dorsal skin by shaving when animals reached 3 weeks of age. Mice were checked every 1-2 days for new hair growth in the denuded area. If hair growth was observed, the area was shaved again. Mice were monitored in this way until they had completed their second postnatal hair cycle. At various times, individual animals within each experimental group were sacrificed for histological confirmation of hair stage.

RESULTS

Generation of K14ΔNLef1 transgenic mice

We introduced an N-terminally deleted Lef1 cDNA (ΔNLef1) into the keratin 14 transgene cassette (Fig. 1A). The deletion removed the β-catenin binding site of Lef1 (Behrens et al., 1996). Deletion of the equivalent regions of Tcf3 and Tcf4 has been shown to act as a dominant negative mutation (Kolligs et al., 1999; Roose et al., 1999; Ross et al., 2000). The transgene was Myc tagged to facilitate detection.

Transgenic lines from three founder animals (T, L and G) were established. Mice from all lines had the same phenotype, ruling out possible effects of a unique chromosomal integration site. The severity of the phenotype correlated with transgene copy number. Southern analysis of genomic DNA showed that transgenic lines T and G carried seven and 11 copies of the transgene, respectively. The L founder gave rise to animals carrying either two or 25 copies and must therefore have been mosaic; only those progeny carrying 25 copies of the transgene were subjected to detailed analysis. Immunoblotting with an antibody to the Myc epitope showed an immunoreactive band

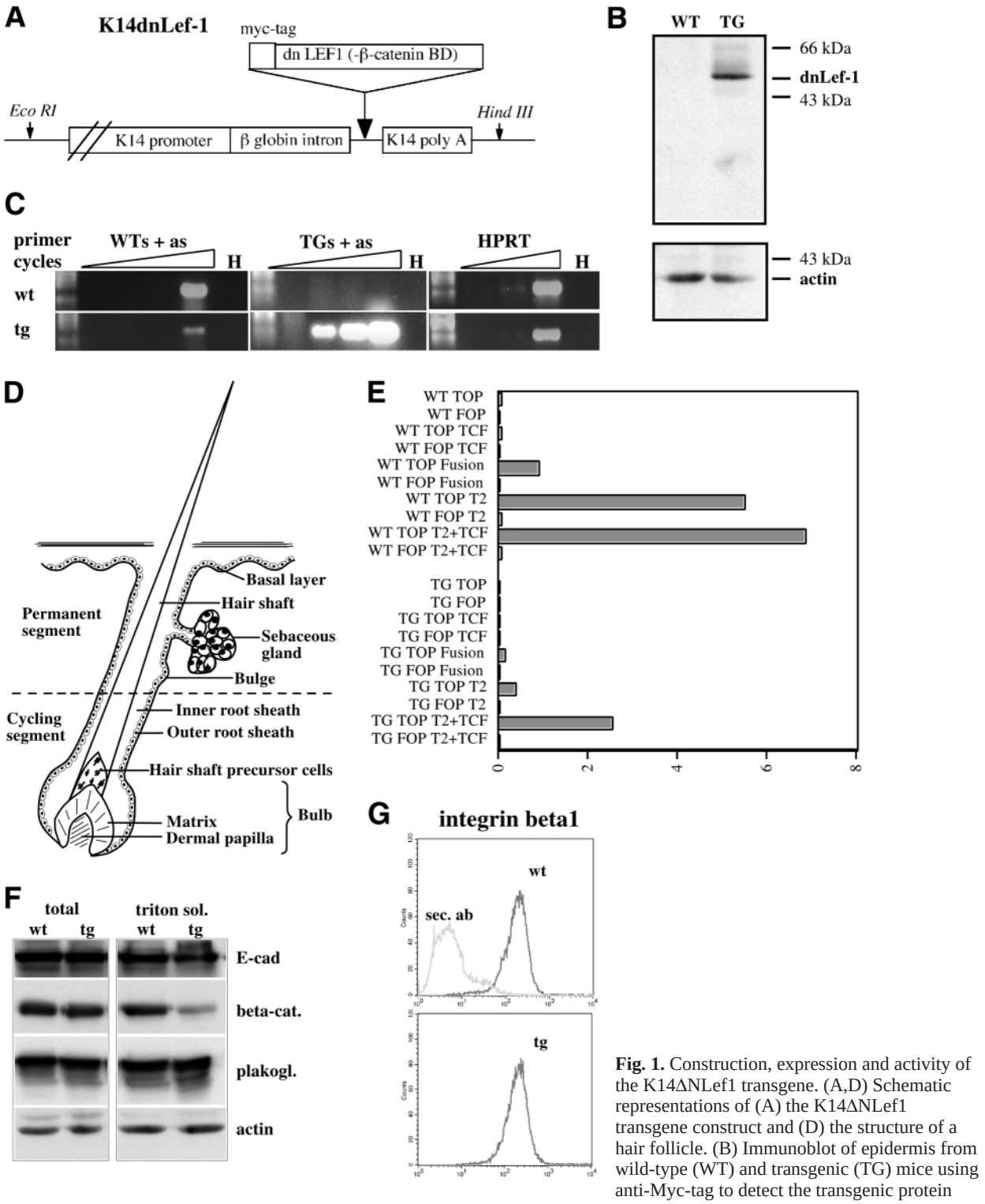


Fig. 1. Construction, expression and activity of the K14ΔNLef1 transgene. (A,D) Schematic representations of (A) the K14ΔNLef1 transgene construct and (D) the structure of a hair follicle. (B) Immunoblot of epidermis from wild-type (WT) and transgenic (TG) mice using anti-Myc-tag to detect the transgenic protein and anti-actin as a loading control. (C) Semi-quantitative RT-PCR for endogenous Lef1 (WTs + as), transgenic ΔNLef1 (TGs + as) and HPRT of RNA isolated from primary wild-type (wt) or transgenic (tg) keratinocytes. Left-hand lane in each gel shows molecular weight markers and lanes labelled H show controls in which no RNA was added. PCR reactions were analysed after (left to right) 10, 25, 35 or 65 (65 not shown for HPRT) rounds of amplification. (E) Luciferase reporter assay. Keratinocytes were isolated from wild-type (WT) and transgenic (TG) animals and transfected with pTOPFLASH (TOP) or pFOPFLASH (FOP). In some cases, cells were co-transfected with the (βcat/Lef1 fusion construct (Fusion), N-terminally truncated β-catenin (T2) or Tcf-3 (TCF). Luciferase activity values are shown in light units and represent the average of triplicate determinations, corrected for transfection efficiency. (F) Immunoblot of total and Triton-soluble protein lysates of primary keratinocytes isolated from wild-type (wt) and K14ΔNLef1 (tg) mice with antibodies to E-cadherin, β-catenin, plakoglobin and actin (loading control). (G) Flow cytometry of primary keratinocytes from wild-type (wt) and K14ΔNLef1 (tg) mice labelled with anti-β1 integrin antibody or secondary antibody alone.

of approximately 50 kDa in epidermis of transgenic animals, but not wild-type littermates (Fig. 1B).

In order to compare the relative abundance of ΔNLef1, endogenous Lef1 and TCF-3, semi-quantitative RT-PCR was performed on RNA isolated from cultures of primary transgenic and wild-type keratinocytes (Fig. 1C). Endogenous Lef1 mRNA was slightly downregulated in the transgenic cells relative to wild-type cells (left-hand panel). In transgenic cells, the level of ΔNLef1 was approximately 100-fold higher than endogenous Lef1 (middle panel). No difference was seen in the level of TCF-3 mRNA in wild-type and transgenic cells (data not shown). As a negative control H_2O was substituted for RNA in the tracks labelled 'H' in Fig. 1C. HPRT was used as a control for RNA loading (right-hand panel).

In postnatal life mammalian hair follicles undergo cycles of active hair growth (anagen), regression (catagen) and rest (telogen) (Hardy, 1992). The hair follicle stem cells are believed to reside in the permanent, upper portion of the hair follicle, in a region known as the bulge (Taylor et al., 2000; Oshima et al., 2001) (Fig. 1D). The lower part of the follicle undergoes the cyclical changes: it is hypothesised that on initiation of anagen, signals from specialised mesenchymal cells at the base of the follicle (dermal papilla cells) stimulate the transient proliferation of stem cells in the bulge, whose progeny differentiate into cells of the inner root sheath, cortex, medulla and cuticle (Fig. 1D). At the onset of catagen, matrix cells cease to proliferate; the follicle stops growing and the lower part of the follicle regresses. In wild-type epidermis Lef1 expression was confined to the upper outer root sheath cells (permanent segment) and matrix cells of the hair follicle, as reported previously (data not shown) (DasGupta and Fuchs, 1999). In transgenic epidermis ΔNLef1, detected with an antibody to the Myc tag, was uniformly expressed in the nuclei of all basal cells of interfollicular epidermis and along the entire length of the outer root sheath (Fig. 2G-I), consistent with the known pattern of activity of the keratin 14 promoter (Vassar et al., 1989; Byrne et al., 1994; Wang et al., 1997).

K14ΔNLef1 represses β-catenin dependent transcription

To investigate whether expression of the transgene inhibited β-catenin-dependent transcription we carried out transient transfection experiments on primary keratinocytes isolated from neonatal wild-type and K14ΔNLef1 mice (Fig. 1E). A luciferase gene driven by an enhancer containing multiple Lef1/TCF-binding sequences (TOP) (van de Wetering et al., 1997) was activated in wild-type keratinocytes when the cells were transfected with N-terminally truncated β-catenin (T2) or a Lef1/β-catenin fusion construct (Fusion), consisting of the N-terminally deleted Lef1 fused to the C-terminal transactivation domain of β-catenin (Fig. 1E). This fusion protein activates transcription of Wnt target genes containing Lef1/TCF-binding sites in their promoter sequences. Co-transfection of β-catenin (T2) and wild-type Tcf-3 (TCF) activated the luciferase reporter gene more than transfection of β-catenin alone. A reporter gene with mutated Lef1/TCF binding sites (FOP) (van de Wetering et al., 1997) served as a negative control.

Transcriptional activation of the TOP luciferase reporter by the Lef1/β-catenin fusion or β-catenin was greatly reduced in keratinocytes from K14ΔNLef1 transgenic mice (TG) in comparison with wild-type keratinocytes (Fig. 1E). This demonstrates that overexpression of ΔNLef1 in keratinocytes led to inhibition of β-catenin-dependent transcription. Transfection of K14ΔNLef1 keratinocytes with wild-type TCF-3 in combination with the β-catenin construct partially restored activation of the luciferase reporter, presumably by competing out the inhibitory effect of the ΔNLef1 transgene (Fig. 1E).

In cultured human keratinocytes, elevated expression of β1 integrins is a marker of clonogenic cells that are proposed to be stem cells (Watt, 2001). These cells express higher levels of Triton-soluble β-catenin than basal cells enriched for transit amplifying cells and the activity of the TOP reporter construct is greater when transfected into cultured human epidermal stem cells than transit amplifying cells (Zhu and Watt, 1999). Furthermore, expression of a dominant negative β-catenin construct that lacks armadillo repeats stimulates exit from the stem cell compartment in culture (Zhu and Watt, 1999). We therefore examined whether expression of the ΔNLef1 transgene altered the in vitro behaviour of mouse keratinocytes. As mouse keratinocytes are not as amenable to clonogenic analysis as human or rat keratinocytes (Oshima et al., 2001), we examined newly confluent polyclonal cultures. The percentage of S phase cells, as measured by incorporation of a 1 hour pulse of BrdU, was 20.8 for transgenic cultures and 21.8 for wild-type cultures (mean of two independent experiments). The proportion of cells expressing the terminal differentiation marker, involucrin, was 9.8% in transgenic cultures and 10.6% in wild-type cultures (mean of two independent experiments). No differences were observed in the total or Triton-soluble pools of E-cadherin or plakoglobin or in the level of total β-catenin; however, transgenic cells had a reduced amount of Triton-soluble β-catenin (Fig. 1F). Surface levels of β1 integrins, determined by flow cytometry, were the same in transgenic and wild-type keratinocytes (Fig. 1G). We conclude that the suppression of LEF/TCF-mediated transcription shown in Fig. 1E is not an indirect consequence of effects of the ΔNLef1 transgene on proliferation or terminal differentiation of keratinocytes in culture and that there is no compensatory upregulation of β-catenin.

Transgenic mice show dramatic changes in skin and hair

In wild-type mice, the first coat of hair appears at postnatal day 4.5 and results from growth of the hair follicles formed during embryogenesis. The follicles enter a synchronised resting phase (telogen) at day 16 and the first postnatal cycle begins with the onset of anagen at 4 weeks of age. No skin abnormalities were detected in the transgenics before the first postnatal hair cycle, even though there is keratin 14 promoter activity from E14.5 of development (Byrne et al., 1994; Wang et al., 1997). Differences between wild-type and K14ΔNLef1 skin were first observed at about 6 weeks of age, when the hairs and whiskers of transgenic animals appeared progressively sparser than littermate controls. With each successive hair cycle, the coat of K14ΔNLef1 mice became thinner and by 10 months of age the mice were almost completely bald (Fig. 2A).

Concomitant with the onset of hair loss in 6 week old transgenic animals, small white lumps were observed on the skin of K14ΔNLef1 animals. Over time, their number and size increased rapidly. The lumps were present all over the body,

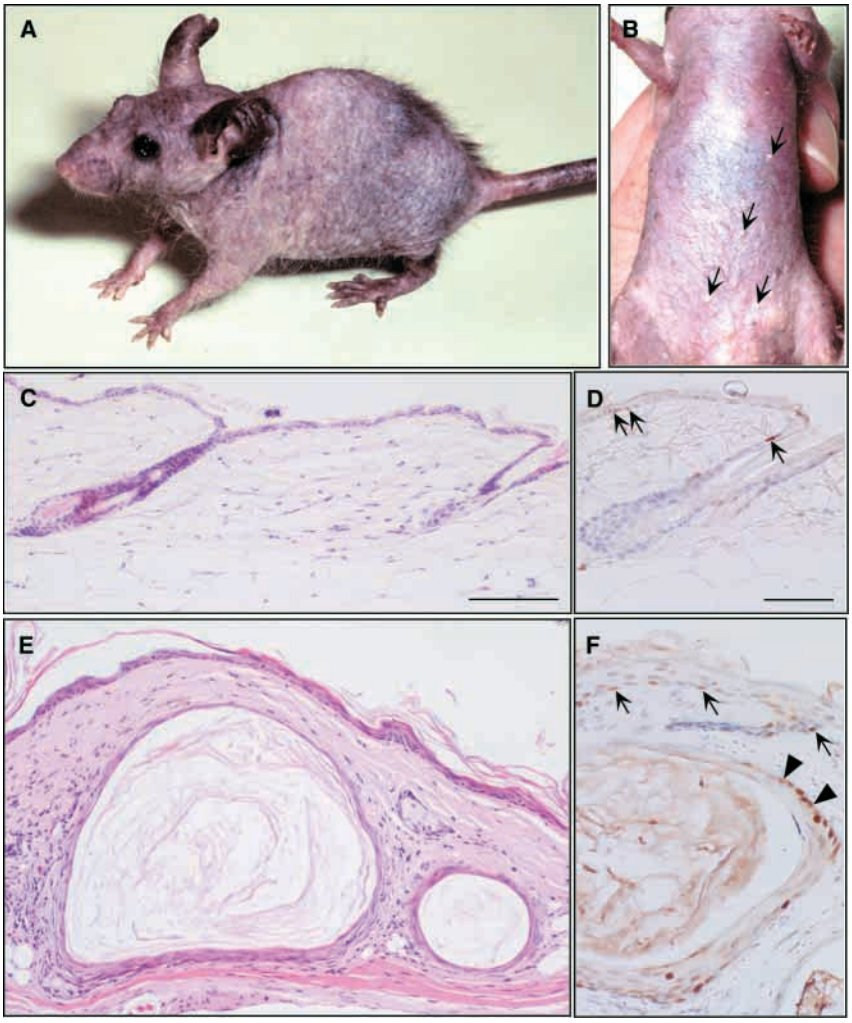


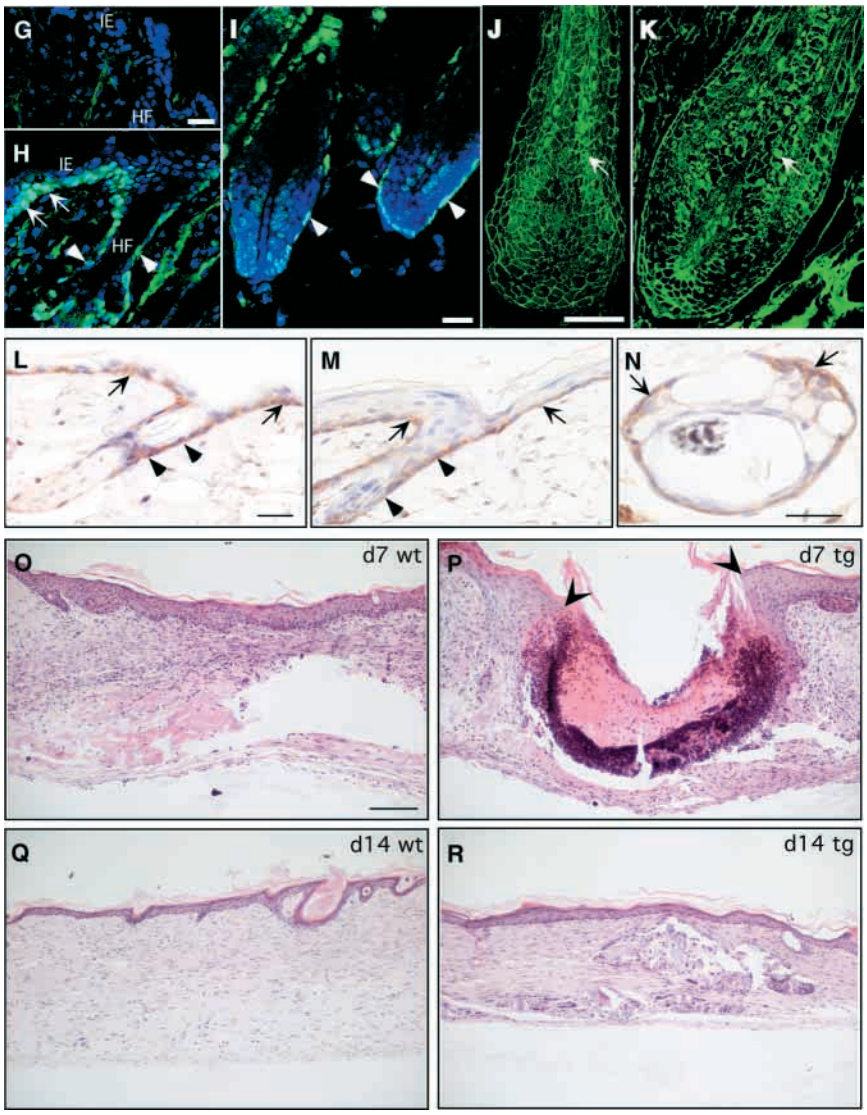
Fig. 2. Phenotype of the K14ΔNLef1 mice. (A) 10-month-old K14ΔNLef1 transgenic mouse (line L). (B) Belly of transgenic animal shown in A with small white lumps indicated (arrows). (C-F) Sections of skin from 3.5-month-old wild-type (C,D) and transgenic (E,F) animals. Sections in C,E were stained with Haematoxylin and Eosin. Sections in D,F were labelled with anti-Ki67; arrows, Ki67-positive cells in hair follicles and interfollicular epidermis; arrowheads, Ki67-positive cells in peripheral layer of cyst. (G-I) Expression of the ΔNLef1 transgene (green in G-I) and β-catenin (green in J,K) in wild-type (G,J) and transgenic (H,I,K) skin. ΔNLef1 was detected with an antibody to the Myc-tag in all cells of the basal layer of the interfollicular epidermis (arrows in H) and the outer root sheath (arrowheads in H,I) of K14ΔNLef1mice (H,I) but not in the skin of wild-type animals (G). Nuclei in G-I were visualised with DAPI (blue). (J,K) β-catenin antibodies detected the protein at cell-cell borders in hair follicles of wild-type and transgenic animals. In addition, β-catenin was detected in the nucleus of precortex cells of the hair follicles (arrows in J,K) in wild-type (J) and transgenic mice (K). (L-N) Immunostaining of wild-type skin (L) and skin from K14ΔNLef1 mice (M,N) with anti-β1 integrin antibody. Positive staining was detected throughout the basal layer of the interfollicular epidermis (arrows in L,M), the outer root sheath of hair follicles (arrowheads in L,M) and the outer layer of epidermal cysts (arrows in N). (O-R) Sections of skin from wild-type (O,Q) and K14ΔNLef1 mice (P,R) 7 days (O,P) and 14 days (Q,R) after wounding. Arrowheads in P show edges of wound where re-epithelialisation has not occurred. IE, interfollicular epidermis; HF, hair follicle. Scale bars: 100 μm (C,E,O-R); 50 μm (D,F,J,K); 25 μm (L-N); 20 μm (G-I).

but were first detected on the face, ears and belly and these areas were also the most severely affected (arrows in Fig. 2B).

Histological analysis showed that the lumps corresponded to large epithelial cysts within the dermis of K14ΔNLef1 mice. The cysts were multilayered, the centre of each cyst containing accumulated keratinised, anucleate cells (Fig. 2E). Such cysts were never observed in the skin of wild-type mice (Fig. 2C). In wild-type animals staining for Ki67, a marker of proliferating cells, was confined to basal keratinocytes of the interfollicular epidermis and cells of the outer root sheath of hair follicles (arrows in Fig. 2D) as reported previously (Carroll et al., 1995). In K14ΔNLef1 mice proliferating keratinocytes were located within the basal layer of the epidermis and the outer root sheath (arrows in Fig. 2F), and in the peripheral layer of the epithelial cysts (arrowheads in Fig. 2F). The majority of cells in the peripheral layer of the cysts were Ki67 positive. There were twice as many Ki67-positive cells in the interfollicular basal epidermal layer of transgenic as of wild-type mouse skin.

The interfollicular epidermis of the K14ΔNLef1 animals was slightly thicker than the epidermis of wild-type mice (Fig. 2C,E). The number of viable suprabasal cell layers was increased by one or two layers and the number of cornified layers was also increased. Although the epidermis was slightly thickened, the cornified layers were anucleate, as in wild-type epidermis, and a normal granular layer was present. Epidermal thickening was not confined to the regions overlying the cysts and was not, therefore, an indirect consequence of the skin being stretched as the cysts enlarged. Staining for β-catenin was largely confined to cell-cell borders in both wild-type and transgenic epidermis and in transgenic cysts; however, nuclear staining was observed in the precortex cells of anagen follicles of both wild-type and transgenic mice (Fig. 2J,K) (Merrill et al., 2001). β1 integrins were expressed by all basal keratinocytes of the interfollicular epidermis (arrows) and outer root sheath (arrowheads) in transgenic (Fig. 2M) and wild-type mice (Fig. 2L) and by the basal cells at the periphery of transgenic cysts (arrows in Fig. 2N).

Wound healing studies were performed as a measure of whether epidermal self-renewal was compromised by expression of the ΔNLef1 transgene (Fig. 2O-R). Seven days after creating a 3 mm diameter full thickness wound in the back skin of wild-type animals the wound was completely covered by a thickened, hyperproliferative epidermis (Fig. 2O). Wound healing was delayed in K14ΔNLef1 mice and by day 7 the wounds had not re-epithelialised completely (Fig. 2P). However, by day 14, wild-type and transgenic wounds were indistinguishable: in each case the overlying epidermis had



returned to close to normal thickness (Fig. 2Q,R). We conclude that any inhibitory effect of ΔNLef1 on epidermal self-renewal is mild and that there is therefore no evidence for significant depletion of the stem cell compartment.

Epithelial cysts show characteristics of interfollicular epidermis

The preliminary histological analysis of the cysts (Fig. 2E,F) revealed a resemblance to interfollicular epidermis. This was examined further by performing transmission electron microscopy on cysts (Fig. 3A-C). The cell layers comprising both small (Fig. 3B) and large (Fig. 3C) cysts showed remarkable similarities to the interfollicular epidermis of wild-type mice (Fig. 3A). Thus, moving inwards from the proliferative peripheral cell layer there were several viable cell layers with prominent desmosomes (equivalent to spinous cells in normal epidermis), then a layer filled with cytoplasmic granules (keratohyalin granules; asterisks in Fig. 3A,C) and finally electron dense, anucleate squames forming the innermost cell layers and detaching into the centre of the cysts. There was no evidence of extensive sebaceous differentiation within the cysts.

At the ultrastructural level, the two main differences between the cysts and normal interfollicular epidermis were that the nuclei (and indeed the cytoplasm also) of cyst cells were more flattened and elongated than normal, and that the basement membrane surrounding the cysts was poorly defined (arrowheads in Fig. 3B,C). Both of these changes might be attributable to stretching and expansion of the cysts due to accumulation of cornified cells in the centre.

We next investigated whether the cysts expressed molecular markers of interfollicular or hair keratinocytes. We observed strong staining with antibodies AE13 (Lynch et al., 1986) and anti-Ha5 (Rogers et al., 1996), which recognise hair-specific keratins, in cells of the hair matrix and cortex in wild-type anagen hair follicles, but no staining in the cysts (Fig. 3D,E and data not shown). Conversely, keratin 1 and keratin 10, markers of terminal differentiation in interfollicular epidermis that are not expressed in normal hair (Lynch et al., 1986) were detected in all the viable cyst cell layers except for the most peripheral layer (Fig. 3F and data not shown). The innermost viable cell layer of the cysts expressed profilaggrin, a marker of the interfollicular granular layer (Fig. 3G), consistent with the observation of cytoplasmic granules by electron microscopy (Fig. 3C). Keratinocytes in the central layers of the cysts expressed keratin 6, a keratin that is characteristic of both normal hair follicles and hyperproliferative interfollicular epidermis (Carroll et al., 1995) (Fig. 3H). Finally, laminin was detected in the basement membrane zone of interfollicular epidermis, beneath the outer root sheath of hair follicles and at the boundary between the peripheral cell layer of the cysts and the surrounding dermis (Fig. 3I). Together, these data demonstrate that the keratinocytes forming the layers of the epithelial cysts undergo a program of terminal differentiation similar to that of interfollicular epidermis.

Epidermal cysts of K14ΔNLef1 mice are derived from hair follicles

As the appearance of the cysts and onset of hair loss coincided with anagen of the first postnatal hair cycle we investigated whether hair follicles gave rise to the cysts. We analysed skin sections from animals that had just entered the first postnatal hair cycle. Small cysts were observed at the base of hair follicles in anagen phase (Fig. 4A). In some cases it was possible to see an abnormal anagen follicle in the process of developing a cyst, the remains of matrix cells and hair (arrows in Fig. 4A; see also cyst marked with two arrowheads in Fig. 4C) being clearly visible. In these developing cysts, a small number of sebocytes were observed (Fig. 4A); by contrast, sebocytes were not seen in mature cysts (Fig. 3). Immunofluorescence staining with AE13, an antibody highly specific for hair shaft keratins (DasGupta and Fuchs, 1999), or anti-Ha5 did not detect any

protein within the keratinocytes in developing cysts (data not shown). Instead we found expression of keratin 10 in hair follicle cells of K14ΔNLef1 animals (Fig. 4B) but not of wild-type mice (data not shown). Strong staining for keratin 10 was observed in matrix cells (arrowheads) and weaker staining was seen in outer root sheath cells of transgenic follicles (arrows) (Fig. 4B). The signalling molecule sonic hedgehog (Shh) and its receptor patched (Ptc1) were expressed in anagen follicles (St-Jacques et al., 1998; Chiang et al., 1999) (Fig. 4C, arrows; and data not shown). However Shh (arrowheads in Fig. 4C) and Ptc1 (data not shown) were not detectable in cysts developing during the first postnatal cycle.

To further compare the hair follicles from wild-type and K14ΔNLef1 transgenic animals, we performed scanning electron microscopy on plucked hairs (Fig. 4D,E). In comparison with hair follicles plucked from wild-type animals (Fig. 4D), the bases of follicles from transgenic animals were enlarged and swollen (Fig. 4E). The deformed follicles appeared to represent follicles that had begun to form epidermal cysts.

These data suggest that the cysts develop as a result of differentiation of hair follicles into interfollicular epidermis, a process initiated during anagen of the first postnatal hair cycle. The switch in gene expression from hair type (as exemplified by AE13 and anti-Ha5 staining and expression of Shh and Ptc) to interfollicular epidermal (keratin 1 and keratin 10) indicates that ΔNLef1 was able to change the entire program of differentiated gene expression within the keratinocytes.

Transgenic mice develop abnormal hair follicles and show changes in the postnatal hair cycle

Although cysts developed from 6 weeks after birth, hair loss was not complete until 10 months. We therefore examined whether the hair cycle was perturbed independently of cyst formation. We investigated the onset and duration of the first postnatal hair cycle in wild-type and K14ΔNLef1 transgenic animals by repeatedly shaving the back skin of the mice and monitoring hair regrowth (Fig. 5). Transgenic animals had a delay in the start of the first postnatal hair cycle: wild-type animals entered anagen between 4 and 6 weeks of age (Fig. 5A,G), whereas in K14ΔNLef1 mice it occurred between 6 and 8 weeks of age (Fig. 5B,D,G). In 7.5-week-old wild-type mice, the hair follicles were synchronously resting in telogen phase (Fig. 5C), while in K14ΔNLef1 animals of the same age, hair follicles were still in anagen (Fig. 5D). When hair growth following the first postnatal hair cycle was monitored, K14ΔNLef1 animals exhibited some growth at a time when wild-type mice remained in telogen (Fig. 5E,G, arrows in F). In addition to dysregulation of the hair cycle, K14ΔNLef1 mice exhibited disturbed orientation of hair follicles during anagen of the first postnatal hair cycle (Fig. 5D, arrowheads). These results demonstrate that in K14ΔNLef1 transgenic animals, the normal postnatal hair cycle was disturbed. In addition to causing transdifferentiation of individual follicles, the ΔNLef1 protein altered the timing of the onset of anagen throughout the skin.

K14ΔNLef1 mice develop spontaneous skin tumours

K14ΔNLef1 mice started to develop spontaneous skin tumours at about 3 months of age. The tumours developed quickly, the time between first observation and attainment of a size that required the animal to be sacrificed being 2-3 weeks. This was very surprising given that ΔNLef1 blocked β-catenin-dependent transcriptional activation (Fig. 1E) and that the CBA and C57BL/6 genetic backgrounds are inherently resistant to skin

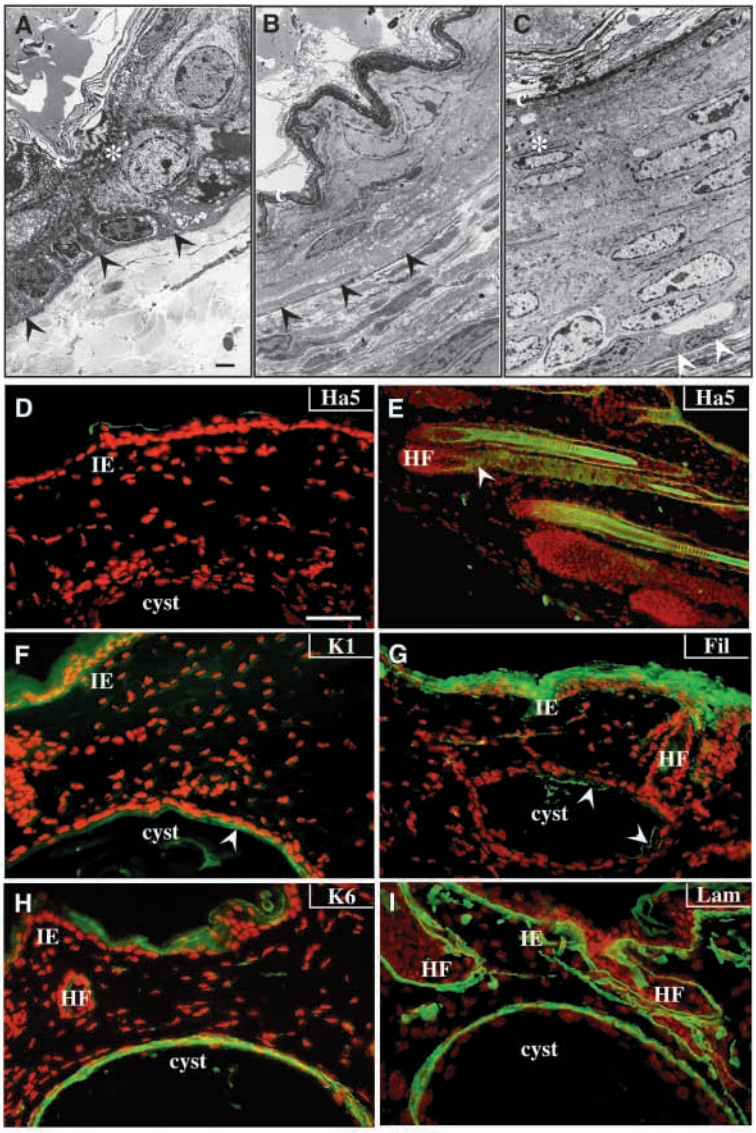


Fig. 3. Transmission electron microscopy of epidermal cysts and expression of markers of hair and epidermal differentiation in cysts. (A-C) Transmission electron microscopy of interfollicular epidermis of wild-type mouse (A) and cysts from transgenic mouse skin (B,C). Arrowheads indicate basement membrane in A and boundary between epithelial cells and dermis in B,C. c, cornified layers. Asterisks in A and C indicate cells with keratohyalin granules. Scale bar: 2 μm. (D-I) Expression of markers of hair and epidermal differentiation in transgenic skin (D,F-I) and wild-type skin (E). Red fluorescence: DAPI staining of cell nuclei. Green fluorescence: hair keratin Ha5 (D,E; note that staining of cornified layer of interfollicular epidermis is nonspecific); keratin 1 (F); filaggrin (G); keratin 6 (H); and laminin (I). IE, interfollicular epidermis; HF, hair follicle. Arrowhead in E indicates matrix cells. Arrowheads in F,G indicate positive staining in cysts. Scale bars: 50 μm in D,F-I; 100 μm in E.

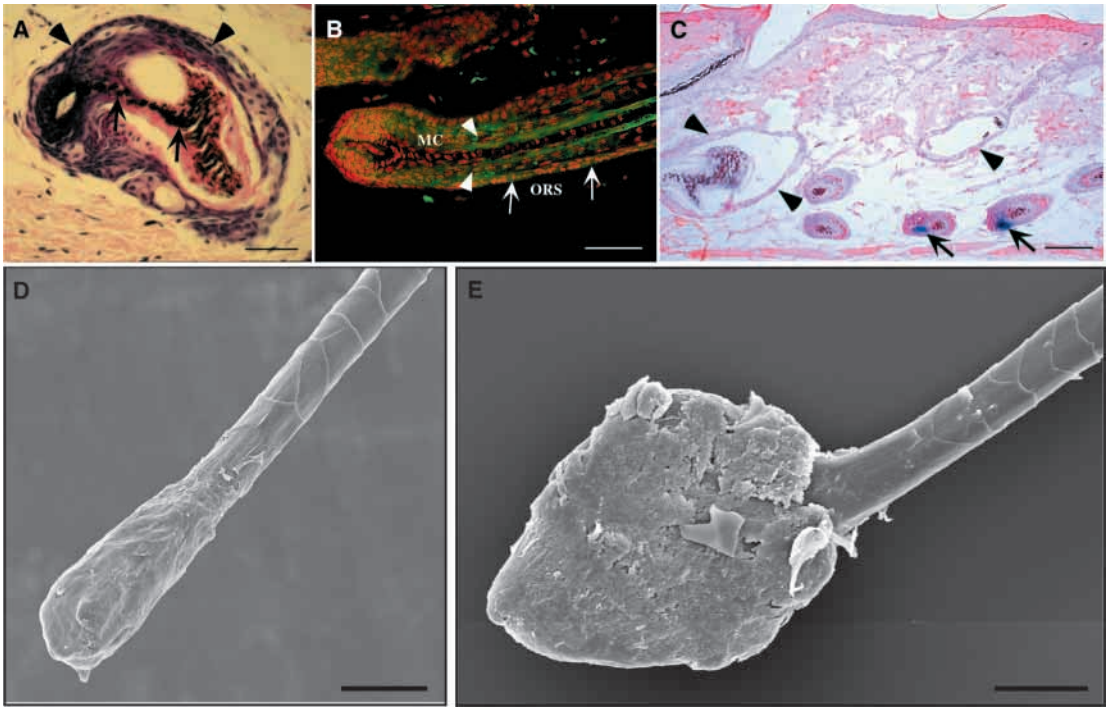


Fig. 4. Transdifferentiation of hair follicles in K14ΔNLef1 transgenic animals. Hair follicles from K14ΔNLef1 transgenic (A-C,E) and wild-type (D) mice during anagen of the first postnatal hair cycle. (A) Haematoxylin and Eosin staining; arrows indicate residual hair shaft; arrowheads indicate developing cyst. (B) Immunofluorescence staining with antibody to keratin 10 (green) and DAPI counterstain (red). MC, matrix cells; ORS, outer root sheath. Arrows and arrowheads show individual keratin 10-positive cells. (C) In situ hybridisation for Shh; arrows indicate positive signals in hair follicles and arrowheads indicate early cysts that do not express Shh. (D,E) Scanning electron micrographs of plucked hairs. Scale bars: 50 μm in A,B; 100 μm in C, 20 μm in D,E.

tumour formation (Ashman and Kotlarski, 1978; Hennings et al., 1993). No tumours were observed in any wild-type littermates. Animals from all three founder lines developed tumours, and in all lines males and females had an equal incidence of tumours. The age range at which animals had to be killed as a result of tumour growth was similar in each line: L, 12-48 weeks; T, 16-52 weeks; G, 17-36 weeks. The tumour incidence in mice over the age of 3 months in each line was as follows: L, 27% (12/44); G, 16% (7/45); T, 6% (3/49).

The histology of all the K14ΔNLef1 tumours is summarised in Table 1. The most

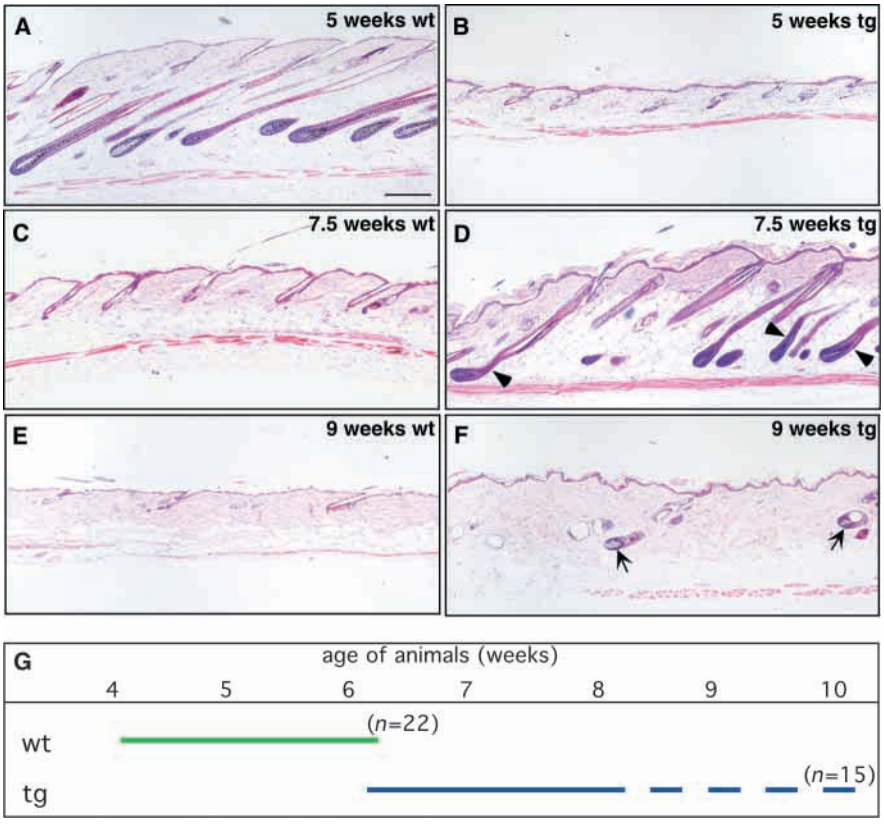


Fig. 5. Comparison of the first postnatal hair cycle in transgenic and wild-type mice. (A-F) Haematoxylin and Eosin stained sections of skin from 5- (A,B), 7.5- (C,D) and 9- (E,F) week-old wild-type (A,C,E) and K14ΔNLef1 transgenic animals (B,D,F). Scale bar: 200 μm. (G) Schematic representation of the time of onset (start of bar) and duration (bar length) of first postnatal hair cycle in wild-type (wt) and transgenic (tg) animals. The number of animals examined (*n*) is indicated. See text for explanation of features marked with arrows and arrowheads.

frequently observed tumour was a sebaceous adenoma (Table 1), which originates from the upper region of hair follicles (Bogovski, 1994). These tumours arose from underneath the epidermis (see, for example, Fig. 6A) and comprised a proliferative outer layer of keratinocytes (Fig. 6B,D) and an inner differentiation compartment of sebocytes (Fig. 6B,C). The sebaceous glands of normal hair follicles stain positive with Oil Red O, a histochemical dye specific for lipid-containing cells (arrow in Fig. 6C, insert). Cells in the centre of sebaceous adenomas were also positively stained with Oil Red O (Fig. 6C). Keratin 10 was either absent or confined to a few scattered cells (Table 1 and data not shown), consistent with the histological analysis that showed no squamous differentiation.

The second type of tumour we observed was a sebeoma (Table 1; Fig. 6E,F), a tumour with mixed elements of sebaceous (Fig. 6E, arrow) and squamous differentiation (Fig. 6F, arrows) that also contained areas of undifferentiated basaloid keratinocytes (Fig. 6E, arrowhead). The regions of squamous differentiation expressed keratin 10 (Fig. 6D, arrows) while the areas containing sebocytes stained positive with Oil Red O (data not shown). Even in the third category of tumour, squamous papillomas, which had the most extensive squamous differentiation and keratin 10 staining (Table 1 and data not shown), small areas of sebocytes were present (Fig. 6G, arrow).

The final type of tumour we observed was a single case of invasive squamous cell carcinoma (Table 1; Fig. 6H). In this tumour, small clusters of cells undergoing squamous differentiation were present (arrows in Fig. 6H) and they expressed keratin 10. However, the majority of the cells had a fibroblastic morphology and did not express keratins that were characteristic of keratinocytes (keratin 10) or simple epithelia (keratin 8), and were β-catenin-negative (Table 1 and data not shown). This was the most aggressive of the tumours we analysed and invaded the muscle fascia (Fig. 6H, arrowheads).

The Wnt pathway is repressed in ΔNLef1 tumours and Patched1 is upregulated

One potential explanation for the formation of tumours in K14ΔNLef1 mice is that the cells that gave rise to the tumours had lost expression of the transgene. As the

Table 1. Characterization of spontaneous tumours in K14ΔNLef1 mice

Tumor type	Frequency*	Keratin 10	β-Catenin	Cyclin D1	Patched
Sebaceous adenoma	10	5/8†	8/8	0/6	7/7
Sebeoma	5	5/5	5/5	0/4	3/3
Squamous papilloma	6	3/3	3/3	0/1	2/2
Invasive squamous carcinoma	1	1/1§	1/1§	n.d.	n.d.

Expression of keratin 10, β-catenin and cyclin D1 was examined by immunohistochemistry and that of patched 1 by in situ hybridisation in the numbers of tumours shown.

*Total number of each tumour type observed from all three founder lines (n=138 mice).

†Staining was restricted to a few scattered cells.

§Staining was restricted to small groups of cells showing squamous differentiation; the cells of fibroblastic morphology were keratin 10 and β-catenin negative.

n.d., not determined.

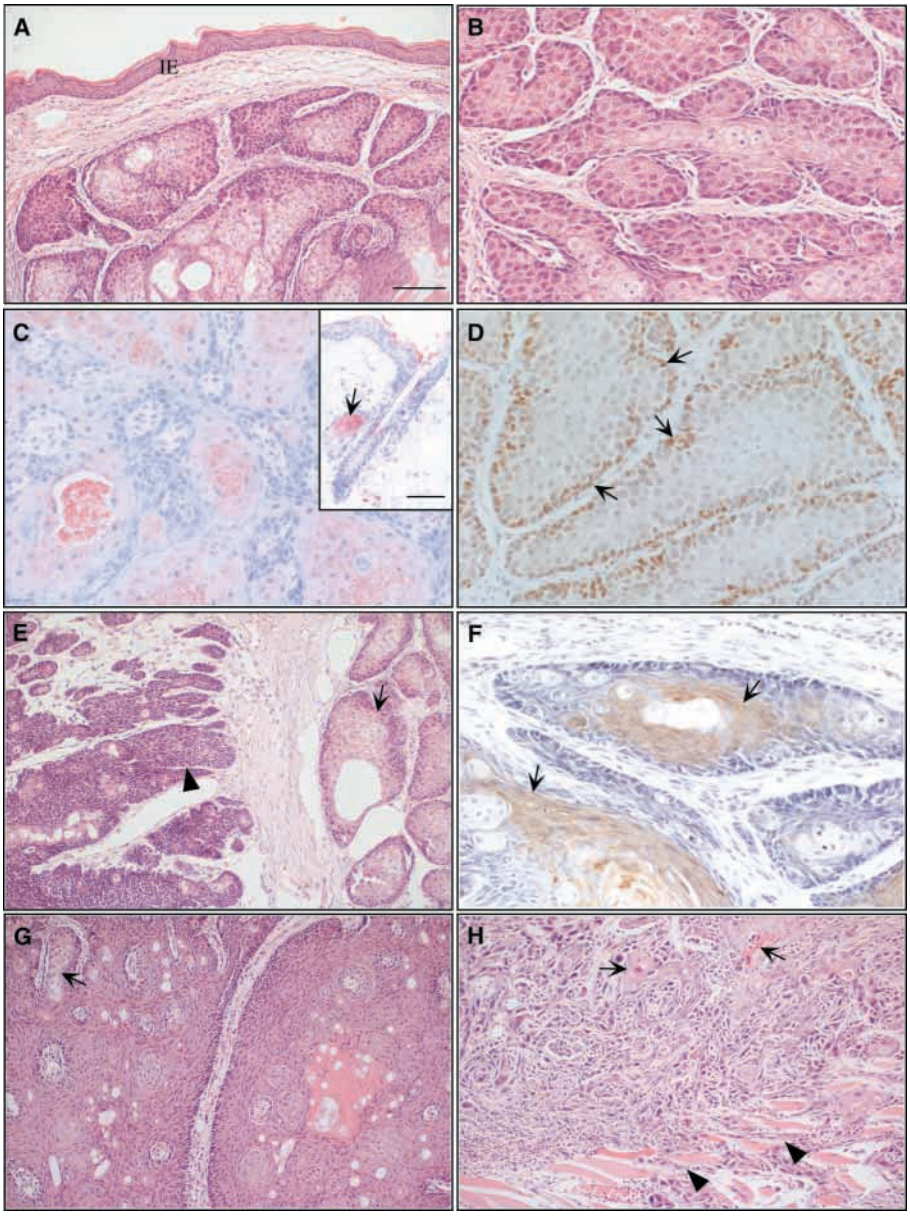


Fig. 6. Tumours in the skin of adult transgenic mice. Paraffin-embedded sections were stained with Haematoxylin and Eosin (A,B,E,G,H), Oil Red O (C), Ki67 (D) or keratin 10 (F). Tumours shown are sebaceous adenoma (A-D), sebeoma (E,F), squamous papilloma (G) and invasive squamous carcinoma (H). IE, interfollicular epidermis. Scale bars: 100 μm in A,F-H; 50 μm in B-D,F. See text for explanation of features marked with arrows and arrowheads.

transgene was tagged with a Myc epitope (Fig. 1A; Fig. 2G-I), we investigated this possibility by staining tumours with an anti-Myc antibody. Squamous cell carcinomas induced in wild-type mice with a DMBA/TPA chemical carcinogenesis protocol (Owens et al., 1999), which were examined for comparison, did not show any positive nuclear staining (data not shown). The anti-Myc antibody stained the cells at the periphery of the K14ΔNLef1 tumours and did not stain the differentiated cells in the tumour centres (Fig. 7A). The antibody also labelled cells in the basal layer and outer root sheath of unaffected epidermis of K14ΔNLef1 mice (Fig. 2G-I), consistent with the known sites of expression of the K14 promoter. We conclude that transgene expression had not been lost in the tumours.

To examine expression and localisation of β-catenin in the tumours we stained tumours with antibodies to β-catenin (Table 1). In all tumours examined, the protein was concentrated at cell-cell borders (Fig. 7B, arrows) and nuclear staining was not observed. Nuclear staining in the precortex cells of wild-type and anagen follicles served as a positive control (Fig. 2J,K). Therefore, upregulation of endogenous β-catenin signalling was unlikely to be responsible for the emergence of spontaneous tumours in K14ΔNLef1 mice.

We stained tumours with antibodies to cyclin D1, a well characterised target gene in the LEF/TCF pathway (Tetsu and McCormick, 1999; Shtutman et al., 1999). Cyclin D1 was not detectable in normal or hyperproliferative wild-type epidermis, but was upregulated in 3/3 benign papillomas and 3/3 squamous cell carcinomas induced in the skin of wild-type mice by a two-stage chemical carcinogenesis protocol (Fig. 7C, arrows, and data not shown), consistent with the observations of Robles and Conti (Robles and Conti, 1995). By contrast, 11/11 tumours from K14ΔNLef1 mice showed no cyclin D1 staining (Fig. 7D; Table 1). The absence of cyclin D1 in K14ΔNLef1 tumours demonstrates that the transgene repressed β-catenin signalling in vivo, as observed in vitro (Fig. 1E).

Given that ΔNLef1 was expressed and functional in the tumours, we conclude that activation of β-catenin signalling did not contribute to tumorigenesis in K14ΔNLef1 mice. A second pathway that is strongly implicated in the development of epidermal tumours is via Shh and its receptor, Ptc: inactivating mutations in Ptc are found in hereditary and sporadic human basal cell carcinomas (Hahn et al., 1996; Johnson et al., 1996) and overexpression of Shh leads to the formation of basal cell carcinomas in transgenic mice (Oro et al., 1997). Because Ptc controls its own transcription, inactivating Ptc mutations lead to overexpression of Ptc mRNA in skin tumours (Undén et al., 1997; Vorechovsky et al., 1997). We examined representative examples of sebaceous adenomas, sebeomas and papillomas from K14ΔNLef1 mice for expression of Shh and Ptc1 mRNA by in situ hybridisation (Table 1, Fig. 7E and data not shown). None of the tumours had detectable Shh mRNA, but 12/12 showed scattered cells expressing Ptc1 (arrowheads in Fig. 7E; Table 1). This demonstrates that at least one other pathway that is implicated in epidermal neoplasia is altered in K14ΔNLef1 tumours.

DISCUSSION

Expression of the ΔNLef1 transgene in the epidermis has two striking effects. The first is to reprogram lower hair

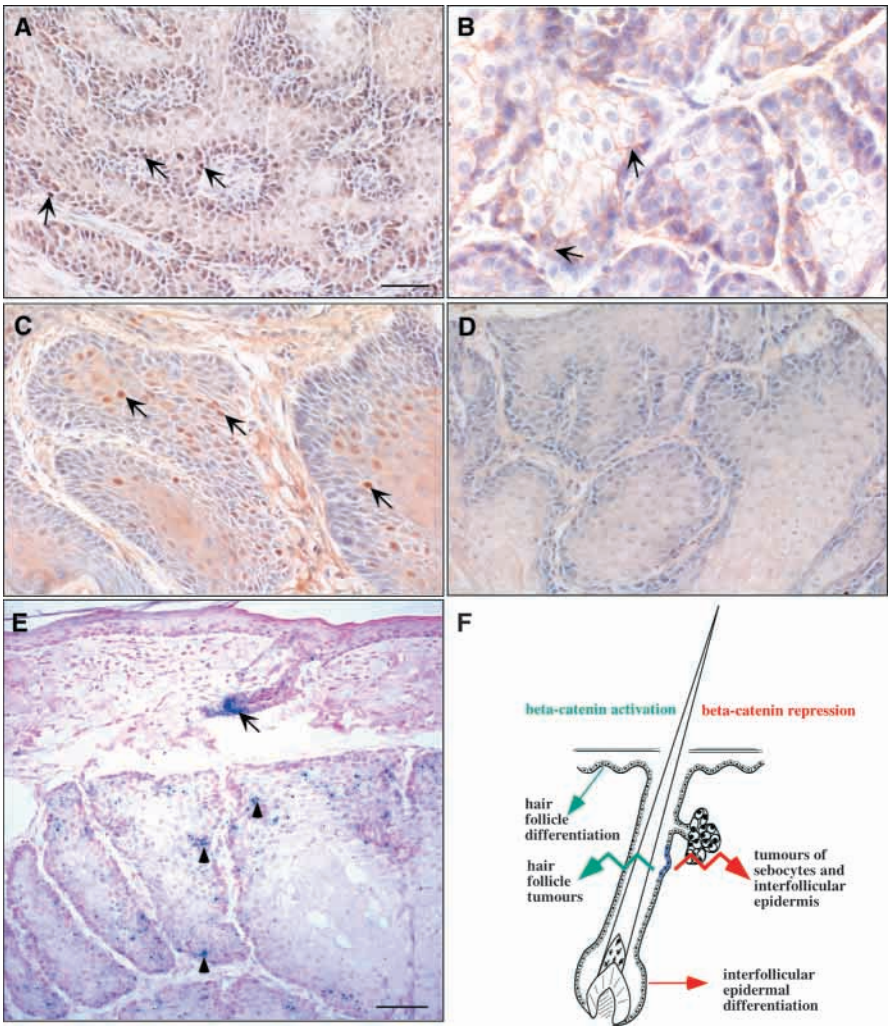


Fig. 7. Characterisation of spontaneous tumours of K14ΔNLef1 mice and model of their development. Paraffin-embedded sections of sebaceous adenoma from K14ΔNLef1 mice (A,B,D) and chemically induced squamous carcinoma of wild-type mouse (C) were immunostained with anti-Myc epitope (A), anti-β-catenin (B), or anti-cyclin D1 (C,D) antibodies. Diffuse staining of stroma and sebocytes in C,D is nonspecific. (E) In situ hybridisation for Ptc in sebaceous adenoma (arrowheads) and overlying interfollicular epidermis (arrow indicates expression in hair follicle). (F) Schematic model of consequences for differentiation and tumorigenesis of activating (green; Gat et al., 1998) or repressing β-catenin signalling (red) in mouse skin. Scale bars: 50 μm in A,C,D; 25 μm in B; 100 μm in E.

follicle keratinocytes to undergo interfollicular epidermal differentiation and the second is to induce sebaceous tumours. The phenotype of our mice is a mirror image of mice overexpressing β-catenin in the epidermal basal layer (Gat et al., 1998), as illustrated schematically in Fig. 7F. Thus, activation of the pathway promotes de novo formation of hair follicles, whereas inhibition promotes conversion of hair follicles into interfollicular epidermis; activation and suppression both result in tumours, but the type of tumour is dictated by the level of β-catenin signalling activity.

The development of epidermal cysts and progressive hair loss seen in our K14ΔNLef1 mice is also seen with a further truncation of Lef1 (N-terminal 62 amino acids) (Merrill et al., 2001) and when β-catenin is deleted from the epidermis via K14 Cre-mediated recombination (Huelsken et al., 2001). This strongly suggests that hair loss and cyst formation are due to inhibition of β-catenin signalling, rather than to any effects of β-catenin ablation on intercellular adhesion. What is less clear is why our mice develop tumours whereas those of Merrill et al. (Merrill et al., 2001) and Huelsken et al. (Huelsken et al., 2001) do not, and why sebaceous differentiation predominates in the cysts reported by Merrill et al. (Merrill et al., 2001), but not in the other models. Possibilities that remain to be explored are the contribution of the different genetic backgrounds of the mice and the significance of the number of amino acids deleted from Lef1. It is also unclear why hair loss is gradual in all three models.

Although Lef1 activation plays a role in hair follicle morphogenesis during embryonic development (DasGupta and Fuchs, 1999) and the keratin 14 promoter is active from E14.5 (Byrne et al., 1994) the formation of epidermal cysts was not observed before the first postnatal hair cycle. This is probably because expression of the keratin 14 transgene is not high enough during development to perturb β-catenin-dependent transcription (Huelsken et al., 2001) and is in keeping with the finding of Gat et al. (Gat et al., 1998) that overexpression of β-catenin does not start to affect hair follicle formation until the first postnatal cycle. Although Lef1 is clearly implicated in inductive events in embryonic skin, the phenotype we observed in K14ΔNLef1 mice must be attributed to events in postnatal life, and this probably explains why the phenotype of the K14ΔNLef1 mice is different from that of the Lef1 knockout (van Genderen et al., 1994; Kratchowil et al., 1996).

Expression of the ΔNLef1 transgene in the basal layer of interfollicular epidermis resulted in a two-fold stimulation of proliferation and an increase in the number of differentiated cell layers. ΔNLef1 inhibited transcription in cultured keratinocytes (Fig. 1E), which express Lef1, Tcf3 and Tcf4 (Zhou et al., 1995) (C. N. and F. M. W., unpublished observations), and is therefore a general inhibitor of TCF/LEF factors rather than specific for Lef1. The ΔNLef1 transgene was also effective at inhibiting signalling in vivo, as evidenced by the lack of expression of the β-catenin target gene cyclin D1 in the tumours. Lef1 is not expressed in postnatal interfollicular epidermis (Zhou et al., 1995; DasGupta and Fuchs, 1999), but Tcf-3 is a potential target of the ΔNLef1 transgene (DasGupta and Fuchs, 1999; Barker et al., 1999). There may also be additional, as yet unidentified, β-catenin interacting factors in interfollicular epidermis (Gat et al., 1998) and it will clearly be of great interest to discover what they are.

In cultured human keratinocytes, the clonogenic stem cells have a larger pool of Triton-soluble β-catenin than the transit amplifying cells and expression of a dominant-negative β-catenin mutant, lacking most of the armadillo repeats, stimulates exit from the stem cell compartment and reduced expression of β1 integrins (Zhu and Watt, 1999). Although keratinocytes cultured from K14ΔNLef1 transgenics had a reduced pool of Triton-soluble β-catenin compared with wild-type keratinocytes and LEF/TCF-mediated transcription was inhibited, the proportion of cells in S-phase or undergoing terminal differentiation was the same as in cultured wild-type keratinocytes and β1 integrin expression was unaltered. Wound healing in the transgenics was delayed, but not inhibited. These results suggest that there is no significant depletion of the stem cell compartment in K14ΔNLef1 mice and therefore that either human cells in vitro and mouse cells in vivo respond differently to inhibition of β-catenin signalling or that ΔNLef1 and dominant-negative β-catenin have different effects on keratinocytes, probably via interaction with additional, as yet unidentified, molecules.

There are putative TCF/LEF binding sites in the hair-specific keratin genes expressed in the cortex of the hair follicle (Zhou et al., 1995) and mutation of these sites can reduce promoter activity (Dunn et al., 1998). However, the skin phenotype we observed reflected more than a simple repression of hair keratin genes. There was de novo expression of keratin 1 and keratin 10, which is characteristic of interfollicular epidermis, in matrix and outer root sheath cells. There was loss of Shh and Ptc expression, and execution of the complete morphogenetic program of interfollicular epidermis, which culminated in accumulation of cornified cells.

The cells that gave rise to the cysts were outer root sheath cells in the lower, cycling part of the hair follicle (Fig. 1D). The differentiation of hair follicles into interfollicular epidermal cysts correlated with the anagen growth phase of the hair cycle and epidermal cysts arose from the bottom of the cycling follicles. Dermal papilla cells are maintained in an anagen promoting state by a Wnt signal emanating from the hair follicle keratinocytes (Kishimoto et al., 2000); we speculate that cyst formation either reflects a failure of outer root sheath keratinocytes to respond to dermal papilla signals or a failure to secrete the Wnt proteins that dermal papilla cells respond to normally. In addition to inducing cysts, suppression of β-catenin/Lef1 signalling in K14ΔNLef1 animals delayed the start of the first postnatal hair cycle by up to two weeks (Fig. 5) but did not affect the length of the hair cycle. Thus, once a hair had entered anagen, expression of ΔNLef1 in the outer root sheath cells did not interfere with the completion of the hair cycle; nuclear β-catenin staining in precortex cells (which are themselves transgene-negative) of transgenic anagen follicles suggests that the responsiveness of those cells to Wnt signals was unimpaired.

Epidermal stem cells are pluripotential, with interfollicular keratinocytes being able to undergo hair differentiation (Reynolds and Jahoda, 1992) and hair stem cells able to give rise to interfollicular epidermis and sebaceous glands (Al-Bawari and Potten, 1976; Taylor et al., 2000; Watt, 2001; Oshima et al., 2001). At the onset of the hair cycle stem cells or their progeny in the bulge region of the upper part of the vibrissae follicle migrate to the base of the follicle and produce daughter cells that differentiate along the hair lineages (Oshima et al., 2001). Cyst formation could either be due to hair follicle

stem cells selecting the interfollicular epidermal pathway because of their inability to respond to Wnt proteins or, alternatively, cells in the lower outer root sheath that are already committed to a hair fate might be reprogrammed to differentiate into interfollicular epidermis. In addition to the newly recognised plasticity of stem cells (Watt and Hogan, 2000), there are recent examples of neuronal and haemopoietic cells changing fate after commitment to a particular program of differentiation (Ross et al., 2000; Kondo and Raff, 2000; Kondo et al., 2000). Further experiments will be required to distinguish whether the cysts in K14ΔNLef1 mice arise from stem cell reprogramming or transdifferentiation of cells that had been committed to the hair lineages. Interestingly, epidermal cysts with evidence of hair differentiation are a feature of several mouse models with mutations in APC that prevent it from downregulating β-catenin (Shoemaker et al., 1995; Fodde et al., 1999).

The most striking feature of the K14ΔNLef1 phenotype was the spontaneous development of skin tumours, because to date only mutations that activate the β-catenin/Lef1 signalling pathway have been shown to induce tumours in any organ (Roose and Clevers, 1999; Polakis, 2000). $Tcf1^{-/-}$ mice develop adenomas, but as Tcf1 represses Tcf4 the net result of Tcf1 ablation is to activate β-catenin dependent transcription (Roose et al., 1999). Although the K14ΔNLef1 tumours were hair follicle derived, they had different characteristics from the tumours that resulted from activating mutations in β-catenin (Gat et al., 1998; Chan et al., 1999) because they showed sebaceous and interfollicular epidermal differentiation, whereas when β-catenin is activated the tumours (pilomatricomas and trichofolliculomas) are characterised by densely packed, misangled hair shafts (Gat et al., 1998; Chan et al., 1999). Thus, in the tumours as in normal skin, the degree of activation of β-catenin determined the differentiation programme (Fig. 7F).

The formation of tumours in K14ΔNLef1 mice did not reflect loss or inactivation of the transgene. The transgene continued to be expressed in the undifferentiated cells of the tumours and the LEF/TCF target gene cyclin D1 (Tetsu and McCormick, 1999; Shtutman et al., 1999) was undetectable, even though it is commonly upregulated in epidermal squamous cell carcinomas (Robles and Conti, 1995). β-catenin was expressed in the tumours, where it was localised to cell-cell contacts and not in the nucleus. All of these observations strongly suggest that activation of β-catenin signalling did not contribute to tumour formation.

We propose that in normal skin, the degree of β-catenin activation determines whether, on commitment to terminal differentiation, keratinocytes differentiate into hair (strong activation) (Gat et al., 1998), interfollicular epidermis or sebocytes (weak or no activation) (Huelsken et al., 2001) (our data) (Fig. 7F). Local environmental factors can profoundly influence cell fate decisions (Watt and Hogan, 2000) and this is certainly true in the epidermis (Oshima et al., 2001). Thus, the environment at the base of the follicle may not be conducive to sebocyte differentiation, while the upper part of the follicle clearly is. This would explain why sebocytes are such a prominent feature of the differentiation within the tumours, but not in the cysts.

The cells that give rise to tumours are presumably stem cells that lie in the upper part of the hair follicle, which as permanent tissue residents have the potential to accumulate more than one genetic lesion (Hahn et al., 1999; Taipale and Beachy, 2001). We speculate that tumours originate from stem cells that have sustained mutations rendering their progeny resistant to entering any of the differentiation pathways controlled by β-catenin. One possibility is that mutations in the Ptc tumour suppressor gene are involved (Undén et al., 1997; Vorechovsky et al., 1997), as Ptc mRNA was detected in all the tumours we examined. This would be of interest because β-catenin is normally required for Shh and Ptc expression in the epidermis (Huelsken et al., 2001) and because of the importance of Ptc in the genesis of skin tumours (Oro and Scott, 1998; Taipale and Beachy, 2001). Clearly, in trying to understand how the β-catenin/Lef1 pathway controls both differentiation and tumorigenesis, interest must now lie as much in the target genes that are normally repressed as in the genes that are activated (He et al., 1998; Tetsu and McCormick, 1999; Shtutman et al., 1999).

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