Transient activation of β-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours

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Summary

When β-catenin signalling is disturbed from mid-gestation onwards lineage commitment is profoundly altered in postnatal mouse epidermis. We have investigated whether adult epidermis has the capacity for β-catenin-induced lineage conversion without prior embryonic priming. We fused N-terminally truncated, stabilised β-catenin to the ligand-binding domain of a mutant oestrogen receptor (ΔNβ-cateninER). ΔNβ-cateninER was expressed in the epidermis of transgenic mice under the control of the keratin 14 promoter and β-catenin activity was induced in adult epidermis by topical application of 4-hydroxytamoxifen (4OHT). Within 7 days of daily 4OHT treatment resting hair follicles were recruited into the hair growth cycle and epithelial outgrowths formed from existing hair follicles and from interfollicular epidermis. The outgrowths expressed Sonic hedgehog, Patched and markers of hair follicle differentiation, indicative of de novo follicle formation. The interfollicular epidermal differentiation program was largely unaffected but after an initial wave of sebaceous gland duplication sebocyte differentiation was inhibited. A single application of 4OHT was as effective as repeated doses in inducing new follicles and growth of existing follicles. Treatment of epidermis with 4OHT for 21 days resulted in conversion of hair follicles to benign tumours resembling trichofolliculomas. The tumours were dependent on continuous activation of β-catenin and by 28 days after removal of the drug they had largely regressed. We conclude that interfollicular epidermis and sebaceous glands retain the ability to be reprogrammed in adult life and that continuous β-catenin signalling is required to maintain hair follicle tumours.

Key words: Stem cells, Differentiation, Trichofolliculoma, β-catenin

Introduction

In mammals the interface between the body and the external environment is the epidermis, comprising a multilayered epithelium (the interfollicular epidermis) and associated appendages: hair follicles, sebaceous glands and sweat glands (Watt, 2001). It is not only a complex, but also a very dynamic, tissue because there is rapid cell turnover in the interfollicular epidermis (IFE) and hair follicles undergo cyclic phases of growth (anagen), regression (catagen) and rest (telogen). Maintenance of the epidermis depends on a population of multipotential stem cells that have the ability to generate all of the differentiated epidermal lineages (Niemann and Watt, 2002). The epidermis is a frequent target for oncogenic mutations and it is thought that stem cells are responsible for the production of most epidermal tumours because, as long-term tissue residents, they can accumulate multiple mutations and undergo clonal expansion (Perez-Losada and Balmain, 2003; Owens and Watt, 2003).

In recent years there has been tremendous progress in identifying the genetic and molecular changes occurring during malignant transformation. Abnormal β-catenin signalling, resulting from genetic alterations that act by stabilising β-catenin, have been implicated in many cancers, including colorectal and hepatocellular cancer, melanoma and tumours of hair follicles (reviewed by Polakis, 2000). β-catenin is a structural component of adherens junctions, linking cadherins to the actin cytoskeleton, and is also the key effector of Wnt signalling, which plays a role during development of many tissues (Peifer and Polakis, 2000). In the absence of Wnt, the cytoplasmic pool of β-catenin that is not complexed with cadherins is rapidly phosphorylated at the N terminus by glycogen synthase kinase 3β (GSK-3β) and ubiquitinated, resulting in its degradation. Wnt, through its receptor frizzled, inhibits GSK-3β, so that β-catenin accumulates in the cytoplasm and eventually translocates to the nucleus (Henderson and Fagotto, 2002). There it interacts with the N terminus of transcription factors of the Tcf/Lef family and regulates transcription of target genes (Huelsken and Birchmeier, 2001; Brantjes et al., 2002; Moon et al., 2002).

Several Wnts and their Frizzled receptors are expressed in the epidermis in a highly dynamic and complex pattern (Reddy

et al., 2001). In postnatal epidermis two members of the Tcf/Lef family are expressed: Tcf3 in the bulge and outer root sheath (ORS) of the hair follicle, and Lef1 in the ORS and matrix cells (DasGupta and Fuchs, 1999; Merrill et al., 2001). Using an artificial promoter constructed of multimeric Lef1/Tcf binding sites as a reporter of Wnt responsive cells, promoter activity is observed during hair follicle formation in embryonic skin and postnatally, both in the hair follicle bulge, a reservoir of stem cells, and in hair shaft precursor cells (DasGupta and Fuchs, 1999).

There is considerable evidence that β-catenin signalling is important in epidermal development, homeostasis and disease (reviewed by Fuchs et al., 2001). Mice expressing stabilised, N-terminally truncated β-catenin under the control of the keratin 14 (K14) promoter produce an excess of hair follicles and develop tumours similar to human pilomatricomas and trichofolliculomas (Gat et al., 1998). In humans these benign tumours are associated with activating mutations of β-catenin (Chan et al., 1999). Conversely, if β-catenin is conditionally deleted in the skin during embryogenesis there is no hair formation, and if the deletion occurs after birth the hair is lost after the first hair cycle (Huelsken et al., 2001). Hair follicle development is also prevented when Dickkopf 1, an inhibitor of Wnt action, is ectopically expressed (Andl et al., 2002) or when the Lef1 gene is ablated in the epidermis (van Genderen et al., 1994). Transgenic mice in which the Wnt pathway is blocked by expressing N-terminally truncated Lef1 in the epidermis have progressive hair loss and develop epidermal cysts with interfollicular and sebocyte differentiation (Merrill et al., 2001; Niemann et al., 2002). When a stabilised form of β-catenin lacking the C-terminal transactivation domain is expressed in the epidermis hair differentiation is promoted in the interfollicular epidermis and hair follicles develop cysts of interfollicular epidermis; this appears to reflect the status of the endogenous β-catenin/Tcf/Lef complexes in the cells (DasGupta et al., 2002).

These observations clearly establish that β-catenin plays a fundamental role in morphogenesis of the hair follicle and strongly suggest that the level of β-catenin signalling determines whether keratinocytes differentiate along the hair or interfollicular/sebocyte lineages. However, the keratin 14 and keratin 5 promoters used for many of the transgenic mouse studies are active during embryogenesis and indeed K5 and K14 expression is detected at E9.5, as early as the single layered ectodermal stage (Byrne et al., 1994). This raises the possibility that the epidermis is only competent to respond to altered β-catenin signalling in postnatal life if it has undergone some form of reprogramming during embryogenesis, analogous to T cell priming (Melief, 2003). Indeed the major effect of transient activation of β-catenin in adult epidermis appears to be to promote anagen (growth) phase of the hair cycle (Van Mater et al., 2003). In addition, experiments with cultured human interfollicular epidermis suggest that β-catenin may act primarily to alter stem cell number rather than to direct lineage commitment: the putative stem cells express high levels of cytoplasmic β-catenin, and expression of stabilised β-catenin expands the stem cell compartment, while inhibition of β-catenin signalling promotes exit from the stem cell compartment (Zhu and Watt, 1996; Zhu and Watt, 1999). The concept that β-catenin might act primarily to control stem cell number has recently received support from studies on haemopoiesis (Reya et al., 2003).

The goal of our experiments was to establish whether activation of β-catenin signalling exclusively in adult epidermis is sufficient to induce de novo hair follicle formation and hair follicle tumours. In addition we wanted to obtain information as to the timing and duration of β-catenin signals required for these effects. We used the K14 promoter to drive expression of a chimeric protein in which N-terminally truncated β-catenin is fused with an engineered form of the ligand binding domain of the oestrogen receptor (ER), which is insensitive to endogenous oestrogen (Littlewood et al., 1995). In this way, the site and timing of β-catenin activation is easily controlled by topical application of the synthetic steroid 4-hydroxytamoxifen (4OHT) to the skin of transgenic mice (Arnold and Watt, 2001; Van Mater et al., 2003).

Materials and methods

Transgene construction

The ΔNβ-cateninER construct was generated by in frame insertion of N-terminally truncated β-catenin cDNA (nucleotides 715-2604, T2) (Funayama et al., 1995; Zhu and Watt, 1999) into the *Bam*HI site of the retroviral vector pBabepuro (Morgenstern and Land, 1990) containing the hormone-binding domain of a mutant murine oestrogen receptor (Littlewood et al., 1995).

To generate the transgene construct, ΔNβ-cateninER was excised from pBabepuro, blunt-ended with Klenow DNA polymerase and cloned into the blunt-ended *Bam*HI site of a K14 expression cassette generously provided by E. Fuchs, Howard Hughes Medical Institute, Rockefeller University, New York (Vasioukhin et al., 1999). The K14 expression cassette (Fig. 1A) contains a 2100 bp *Ava*I fragment of the keratin 14 promoter/enhancer, a rabbit β-globin 5′ untranslated region (UTR), together with an intronic sequence upstream of a *Bam*HI site, and the K14 3′ UTR, followed by a polyadenylation site 3′ downstream of the *Bam*HI site. The transgene construct was excised from the pBabe vector as an *Eco*RI/*Hin*dIII fragment, gel purified (Geneclean, Stratech Scientific), further purified with an elutip column according to the manufacturer's instructions (Schleicher and Schuell), and then resuspended at a concentration of 5 μg/ml in sterile injection buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) for pronuclear injection.

Generation of transgenic mice and determination of copy number

The transgene construct was injected into the male pronucleus of day-1 fertilised (CBA × C57BL/6) F_1 embryos. Founders were backcrossed to establish lines of animals. Animals were screened for the presence of the transgene by PCR of tail DNA with primers specific for the transgene (one primer specific for ER, GCACACAAACTCTTCACC; the other specific for β-catenin, ATGCTGCTGGCTGGCTATGGTCAG). To determine transgene copy number Southern blotting was performed on genomic DNA isolated from tail snips and digested overnight with *Eco*RV. Blots were probed with a radiolabelled 1.9 kb β-catenin cDNA probe and with a probe to the single copy gene interleukin 2 receptor α, as described previously (Carroll et al., 1995).

Experimental treatments of mice

At the start of every experiment all the mice were 6-8 weeks old, and therefore in the resting phase of the hair cycle (Stenn and Paus, 2001). The ΔNβ-cateninER transgene was activated by topical application of 4-hydroxytamoxifen (4OHT; Sigma) to a clipped area of dorsal skin (4OHT was dissolved in acetone; dose: 1 mg per mouse per day).

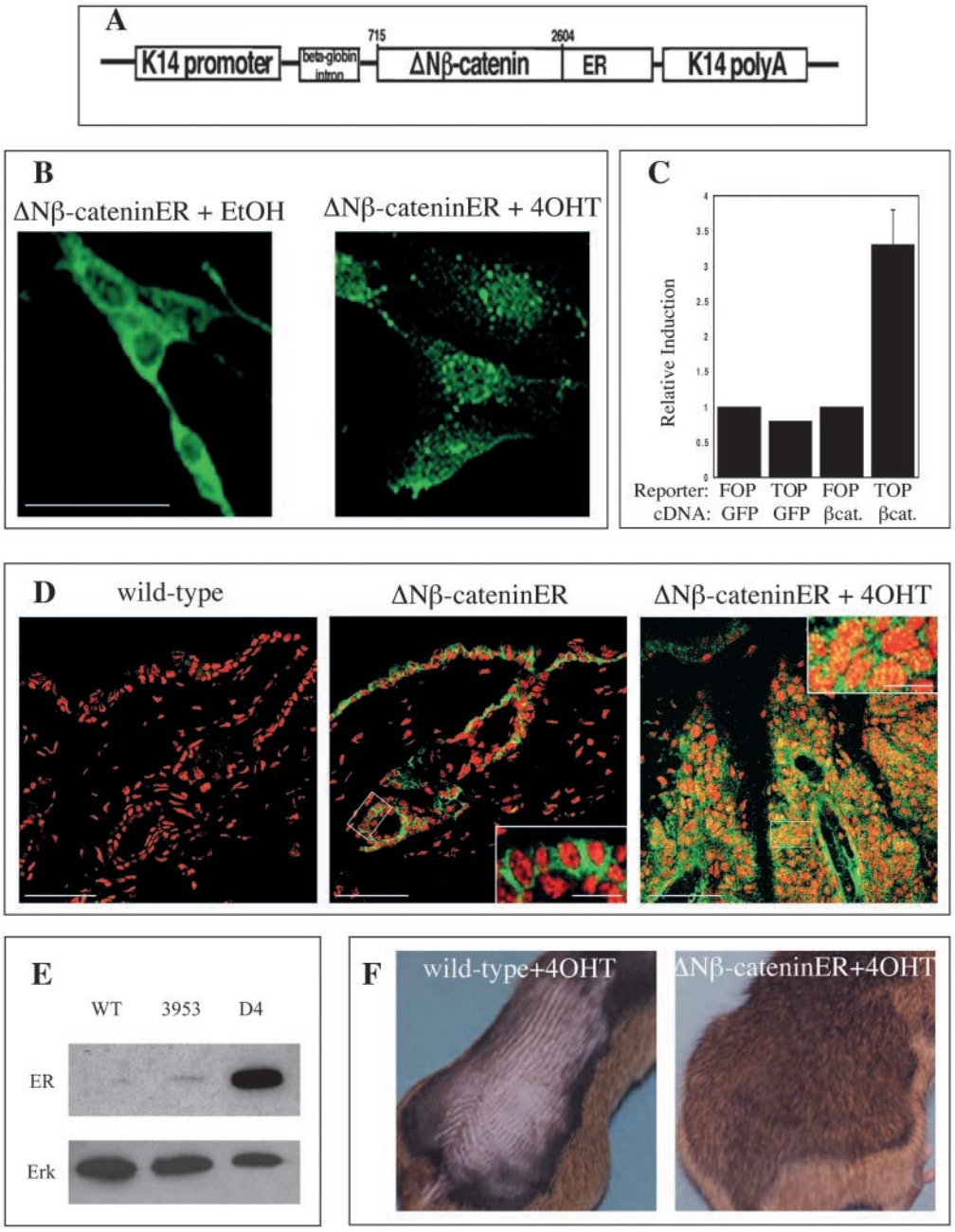


Fig. 1. Characterisation of the ΔNβ-cateninER fusion protein and generation of transgenic mice. (A) Schematic diagram showing the K14 expression cassette and ΔNβ-cateninER transgene. (B) Anti-ER immunofluorescence staining of 3T3 cells expressing ΔNβ-cateninER following treatment with ethanol or 4OHT for 24 hours. (C) The induction of luciferase activity after 4OHT treatment. 3T3 cells were transduced with GFP or ΔNβ-cateninER and transiently transfected with the luciferase reporters FOPFLASH or TOPFLASH. The luciferase activity of each reporter was measured in triplicate and the s.d. is shown. (D) Anti-ER immunofluorescence staining (green) of wild-type and transgenic K14ΔNβ-cateninER (line D4) mouse back skin, untreated and after 7 days treatment with 4OHT. Nuclei were stained with propidium iodide (red). Insets show higher magnification views of boxed areas. (E) Western blot of primary keratinocytes cultured from wild-type (WT) and transgenic (lines 3953 and D4) mice, probed with anti-ER (top panel) or, as a loading control, anti-Erk MAPK (bottom panel) antibodies. (F) Gross phenotype of wild-type and ΔNβ-cateninER mice (line D2) treated daily for 14 days with 4OHT, showing dramatic stimulation of hair growth in the transgenic mouse. Scale bars: (B) 50 μm, (D) 100 μm (main pictures), 25 μm (inserts).

Wild-type littermates were used as controls. No sex-specific effects of 4OHT were observed. In some experiments mice received an intraperitoneal injection of BrdU (0.1 mg/g body weight) 1 hour prior to sacrifice.

Cell culture

Keratinocytes were isolated from transgenic and wild-type newborn mouse skin and cultured at 32°C in a humidified atmosphere with 5% CO_2 in low calcium FAD medium containing 10% chelated FCS and a cocktail of 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^{-10} M cholera toxin and 10 ng/ml EGF. The methods were essentially as described previously (Carroll et al., 1995; Roper et al., 2001), except that the skin trypsinisation procedure was carried out at 4°C and disaggregation of keratinocytes was performed without stirring.

Phoenix and AM12 retroviral packaging lines and NIH 3T3 cells were cultured in DMEM containing 10% donor calf serum at 37°C in a humidified atmosphere with 5% CO_2.

Retroviral infection and luciferase assays

Retroviral vectors encoding ΔNβ-cateninER or GFP (Lowell et al., 2000) were packaged using a two step procedure involving transient transfection of ecotropic Phoenix cells (Swift et al., 1999) and infection of amphotropic AM12 cells with viral supernatant from the Phoenix cultures (Zhu and Watt, 1999; Watt et al., 2004). NIH 3T3 cells were infected overnight at 32°C with supernatant from transduced AM12 cells and selected with puromycin (2.0 μg/ml) to achieve close to 100% transduction efficiency.

The following Promega luciferase reporter constructs were used: pRL (Renilla luciferase control), TOPFLASH (firefly luciferase) and FOPFLASH (firefly luciferase) (van de Wetering et al., 1997). Transient transfections of NIH 3T3 cells transduced with ΔNβ-

cateninER or GFP retroviral vectors were performed using Superfect Transfection Reagent (Qiagen). Cells were extracted using Passive Lysis Buffer (Promega), enabling both firefly and Renilla luciferase measurements to be performed on the same extracts. Luciferase assays were performed according to the manufacturer's instructions using the Dual-Luciferase Reporter Assay kit (Promega) on a BioOrbit 1251 luminometer. All measurements were made in triplicate and corrected for transfection efficiency.

Antibodies

Antibodies against the following antigens were used: β-catenin (clone 15B8; Sigma), BrdU (Becton Dickinson), CCAAT displacement protein (CDP; a gift from M. Busslinger) (Ellis et al., 2001), cyclin D1 (Zymed), oestrogen receptor (MC-20; Santa Cruz Biotechnology or HL-7) (Arnold and Watt, 2001), Erk2 (sc-1647; Santa Cruz Biotechnology), fatty acid synthase (IBL), keratin 1 (Covance), keratin 10 (Covance), keratin 17 (a gift from P. Coulombe) (McGowan and Coulombe, 1998), Lef1 (Upstate) and trichohyalin (AE15; a gift from Tung-Tien Sun) (O'Guin et al., 1992). Additionally, we generated the polyclonal antibody ERP2 by injecting rabbits with a peptide (AHSLQTYYIPPEAEGFPNTI) corresponding to the C terminus of the murine oestrogen receptor, which was previously used to generate the HL-7 antibody (Arnold and Watt, 2001). Goat anti-rabbit and anti-mouse Alexa 488 conjugated IgG (Molecular Probes) and biotinylated goat anti-rabbit IgG (Vector Laboratories) were used as secondary antibodies.

Histochemistry, immunohistochemistry and in situ hybridisation

Tissue samples for immunohistochemistry were either fixed overnight in neutral buffered formalin and embedded in paraffin wax, or frozen, unfixed, in OCT compound (Miles, Elkhart, USA) on a frozen isopentane surface (cooled with liquid nitrogen). 5 μm sections were prepared and stained with Haematoxylin and Eosin.

Immunohistochemistry for CDP, fatty acid synthase and the keratins was performed on paraffin wax sections as described previously (Niemann et al., 2002). Briefly, antigen retrieval was performed by microwaving in 10 mM citrate buffer (pH 6) for 20 minutes and non specific binding was blocked by incubating the sections in 10% goat serum (Sigma) for 1 hour. Polyclonal primary antibodies were diluted in PBS or TBS containing 1% BSA (ICN) and detected using biotinylated secondary antibody (Vector Laboratories), streptavidin complex with horseradish peroxidase (Dako) and DAB (Sigma). For immunostaining of cyclinD1, trichohyalin and Lef1 the Mouse on Mouse kit (Vector Laboratories) was used following the manufacturer's instructions. Haematoxylin was used as a nuclear counterstain.

BrdU-labelled cells were detected in paraffin wax-embedded sections that had been treated sequentially with HCl and trypsin (Kim et al., 1997), using a mouse monoclonal antibody to BrdU (Becton Dickinson) and Alexa 488-conjugated goat anti-mouse IgG. To detect ER expression, 10 μm frozen sections were fixed in 3% paraformaldehyde (Sigma) for 10 minutes, permeabilized with 0.2% Triton X-100 (Sigma) for 5 minutes, incubated for 90 minutes in 10% goat serum (Sigma) in PBS, then for 40 minutes with the ER antibodies and finally with the Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes). β-catenin was visualised in the same way as ER, except that the Mouse on Mouse kit (Vector Laboratories) was used. Nuclei were labelled with propidium iodide and preparations were analysed on a Zeiss 510 confocal microscope.

Alkaline phosphatase activity was visualised in frozen sections with the NBT-BCIP method (Filipe and Lake, 1990). Briefly, the sections were incubated in NTMT buffer (100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Triton X-100) containing 4.5 μl/ml nitro blue tetrazoliun chloride (Roche) and 3.5 μl/ml 5-bromo-4-chloro-3-indolyl phosphate (Roche), counterstained with Fast Red and mounted in Permount (Fischer Scientific).

In situ hybridisation was performed as described previously (Poulsom et al., 1998), using ^{35}S-labelled riboprobes to Sonic hedgehog (Shh) and Patched (Ptc), kindly provided by B. Spencer-Dene (Revest et al., 2001). Hybridisation with a β-actin antisense probe served as a positive control.

Western blotting

Primary mouse keratinocytes were cultured to confluence, then lysed in RIPA buffer (50 mM Tris HCl pH7.4, 1% NP40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA). Protein concentrations were determined using a BCA protein assay kit (Pierce). Protein lysates (20 μg) were separated on a SDS-PAGE gel, transferred to a PVDF membrane (Amersham) by electroblotting, blocked in 5% non fat dried milk (Marvel, Cadbury), incubated with primary antibody and visualised with appropriate horseradish peroxidase-coupled secondary antibodies using enhanced chemiluminescence (ECL; Amersham).

Results

Generation of transgenic mice expressing ΔNβ-cateninER

In order to control the timing and duration of β-catenin activation we generated a construct in which a stabilised form of β-catenin was fused to the ligand binding domain of a mutant oestrogen receptor. β-catenin was stabilised by deletion of the N-terminal 147 amino acids, thereby removing the GSK-3β phosphorylation sites that target the protein for degradation (Clevers and van de Wetering, 1997; Zhu and Watt, 1999). The C terminus of ΔNβ-catenin was fused to the N terminus of a form of the ligand binding domain of the oestrogen receptor which is insensitive to endogenous oestrogen but responds to the synthetic steroid 4-hydroxytamoxifen (4OHT) (Littlewood et al., 1995; Gandarillas and Watt, 1997; Arnold and Watt, 2001).

β-cateninER chimeras have previously been shown to retain β-catenin activity (Weng et al., 2002; Kolligs et al., 2002; Van Mater et al., 2003). To establish that this was also the case for our construct we introduced ΔNβ-cateninER into a retroviral vector and transduced NIH 3T3 cells (Fig. 1B). As predicted, in the absence of 4OHT the chimeric protein was expressed and accumulated primarily in the cytoplasm, where it was detected with an antibody to the mutant oestrogen receptor (Fig. 1B, left hand panel). When cells were treated with 4OHT the cytoplasmic staining was reduced and the chimera was localised predominantly in the nucleus (Fig. 1B, right hand panel). This change in the expression pattern was expected, since in the absence of 4OHT the ER is complexed with heat shock proteins in the cytoplasm (Littlewood et al., 1995).

To examine whether ΔNβ-cateninER could activate transcription of Lef/Tcf target genes we retrovirally transduced 3T3 cells with the ΔNβ-cateninER construct or with GFP (Lowell et al., 2000) and we transfected them with a luciferase construct containing an enhancer with multiple Lef1/Tcf-binding sequences (TOP) (van de Wetering et al., 1997). As a negative control we used a reporter construct with mutated Lef1/Tcf binding sites (FOP) (van de Wetering et al., 1997). 4OHT treatment led to a three-fold increase in luciferase activity of the TOP construct in cells expressing ΔNβ-cateninER but had no effect on the FOP construct (Fig. 1C). 4OHT had no effect on TOP or FOP activity in cells transduced with GFP (Fig. 1C).

Having established that ΔNβ-cateninER was able to inducibly activate transcription in cultured cells we generated transgenic mice in which the construct was expressed under the control of the keratin 14 (K14) promoter (Fig. 1A). The K14 promoter is active in all the basal cells of interfollicular epidermis and along the length of the hair follicle outer root sheath (Vassar et al., 1989; Byrne et al., 1994; Wang et al., 1997). Four K14ΔNβ-cateninER founder lines were chosen for analysis, based on the range in transgene copy number that they represented. Line 3953 has a single copy of the transgene; line D2 has 12 copies; line C5 has 18 copies and line D4 has 21 copies. None of the mice exhibited any phenotype in the absence of 4OHT.

Expression of the transgene was examined by immunofluorescence labelling of skin sections (Fig. 1D) and by western blotting of cultured primary mouse keratinocytes (Fig. 1E), using antibodies to the oestrogen receptor. Epidermis from transgene-negative mice did not express any protein that could be detected with the anti-ER antibodies (Fig. 1D,E). In transgenic mouse skin that had not been treated with 4OHT, cytoplasmic staining for ER was detected in the basal layer of the interfollicular epidermis and along the outer root sheath of the hair follicle with no obvious cell to cell variation in levels (Fig. 1D, middle panel). In 4OHT-treated epidermis, nuclear staining was evident in all transgene-positive cells, although it was more intense in the hair follicles than in the interfollicular epidermis (Fig. 1D, right panel and data not shown). Nuclear staining for ER and β-catenin was similar (data not shown). The level of ΔNβ-catenin ER protein correlated with transgene copy number, with line 3953 keratinocytes expressing the lowest level of the protein and line D4 keratinocytes expressing the highest level (Fig. 1E and data not shown).

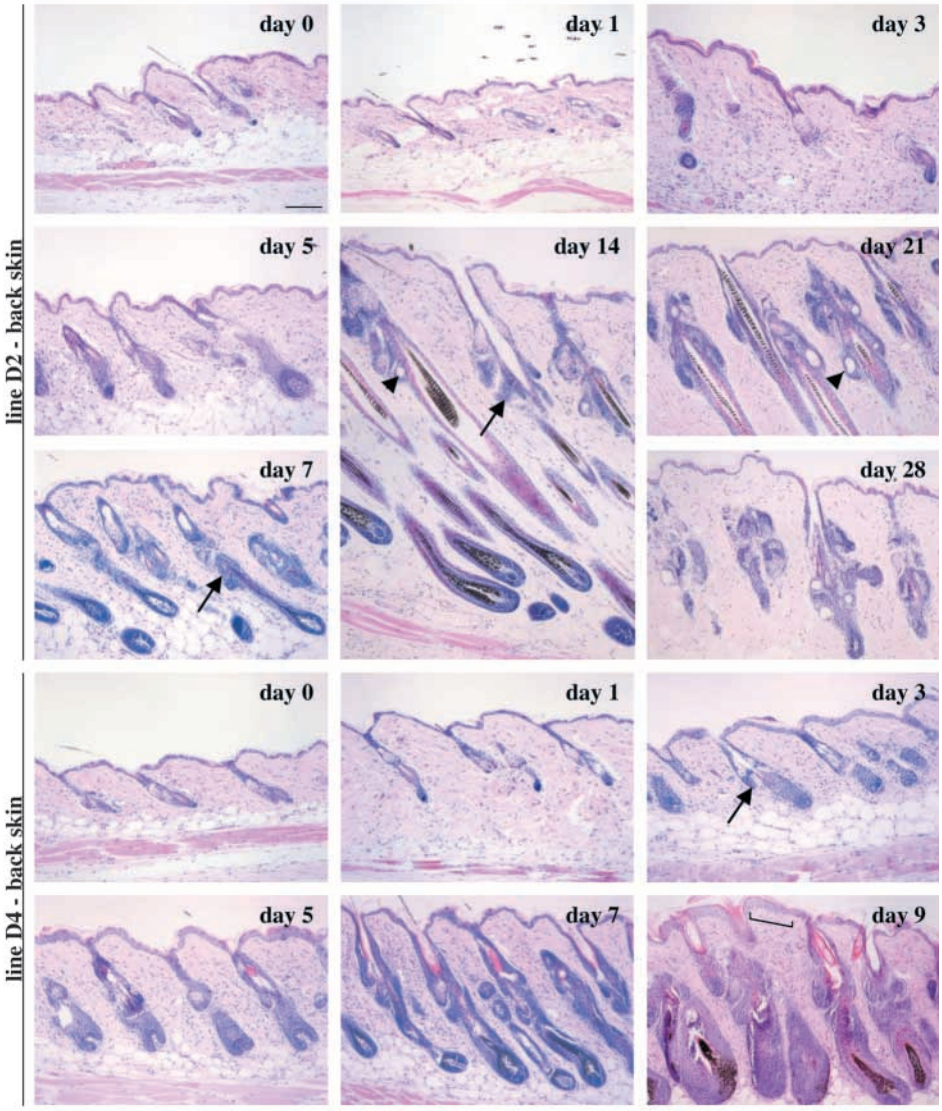


Fig. 2. Histological analysis of the effects of sustained β-catenin activation in back skin of transgenic mice (lines D2 and D4). Arrows indicate outgrowths from the permanent portion of hair follicles (days 7 and 14, line D2; day 3, line D4). Arrowheads indicate small empty cysts (days 14, 21, line D2). Bracket indicates a region of hyperproliferative IFE (line D4, day 9). Scale bar: 100 μm.

Consequences of activating β-catenin for different lengths of time

In pilot experiments we applied 4OHT topically to the back skin of transgenic and wild-type mice daily and assessed whether there was any macroscopic change in phenotype (Fig. 1F and data not shown). The mice were 6 weeks old at the start of treatment and were thus in the resting (telogen) phase of the hair cycle. As a consequence, clipped wild-type animals did not regrow hair, whether treated with 4OHT (Fig. 1F, left panel) or with acetone vehicle (data not shown). The D2 line (Fig. 1F, right panel) showed complete hair regrowth within 14 days. The 3953 line showed patchy hair regrowth (data not shown) and as the histological changes were similar to D2, only less pronounced, we did not study this line further. After 9 days of 4OHT treatment the skin of D4 transgenic mice was very dark and wrinkled, and although new hair growth was stimulated the hairs failed to fully elongate (data not shown). The C5 line showed the same gross phenotype as line D4, although the onset of the 4OHT-induced changes was slightly later (data not shown).

We next performed a time course experiment in which we analysed sections of back skin from D2, D4 and littermate control mice at intervals for up to 14 days of daily 4OHT treatment (Fig. 2). No effects of 4OHT on the back skin of transgene-negative mice were seen (Arnold and Watt, 2001)

(data not shown). 4OHT did not cause any changes in the histology of the interfollicular epidermis in D2 mice, while in the D4 line we observed occasional patches of hyperproliferative epidermis (e.g. Fig. 2, day 9).

In the D2 line the first changes were visible at day 5 and corresponded to an elongation and thickening of the hair follicles. By day 7 there was pronounced elongation of the follicles and by day 14 they were in full anagen (growth phase) (Fig. 2). The main difference between the D2 follicles and normal anagen follicles was the appearance of additional epithelial outgrowths extending from the permanent portion of the outer root sheath, usually below the level of the sebaceous glands (Fig. 2, arrows, day 7, day 14).

In D4 transgenics an elongation of the hair follicles was already visible 1 day after the beginning of 4OHT treatment (Fig. 2). By day 3, epithelial outgrowths were developing from the sebaceous glands (arrow in Fig. 2) and from the permanent portion of the outer root sheath below the sebaceous glands. Between day 5 and day 9 the follicles became progressively thicker, with the outgrowths becoming larger. In contrast to the hair follicles of D2 mice the D4 follicles tended not to produce normal hair shafts and this probably accounts for the abnormal macroscopic appearance of the pelage.

The D4 mice had to be sacrificed by day 11 and the C5 mice by day 17 because they developed thickening of the lips and tongue which affected their ability to feed. The D2 line could, however, be maintained indefinitely with 4OHT treatment. By day 21 the outgrowths from D2 hair follicles had become very pronounced and small cyst-like structures, corresponding to empty hair canals, had appeared (arrowhead, Fig. 2). The histology of each D2 follicle from day 21 onwards resembled a trichofolliculoma, a hair follicle tumour that has previously been reported to be induced by constitutive overexpression of stabilised β-catenin (Gat et al., 1998). At day 28, the follicles of D2 epidermis were entering catagen (regression phase) but the tumours were still enlarging and thus the epithelial outgrowths accounted for most of the mass of the follicles.

β-catenin induces de novo hair follicle formation in postnatal interfollicular epidermis

In addition to the changes in existing hair follicles (Fig. 2), de novo hair follicle formation was

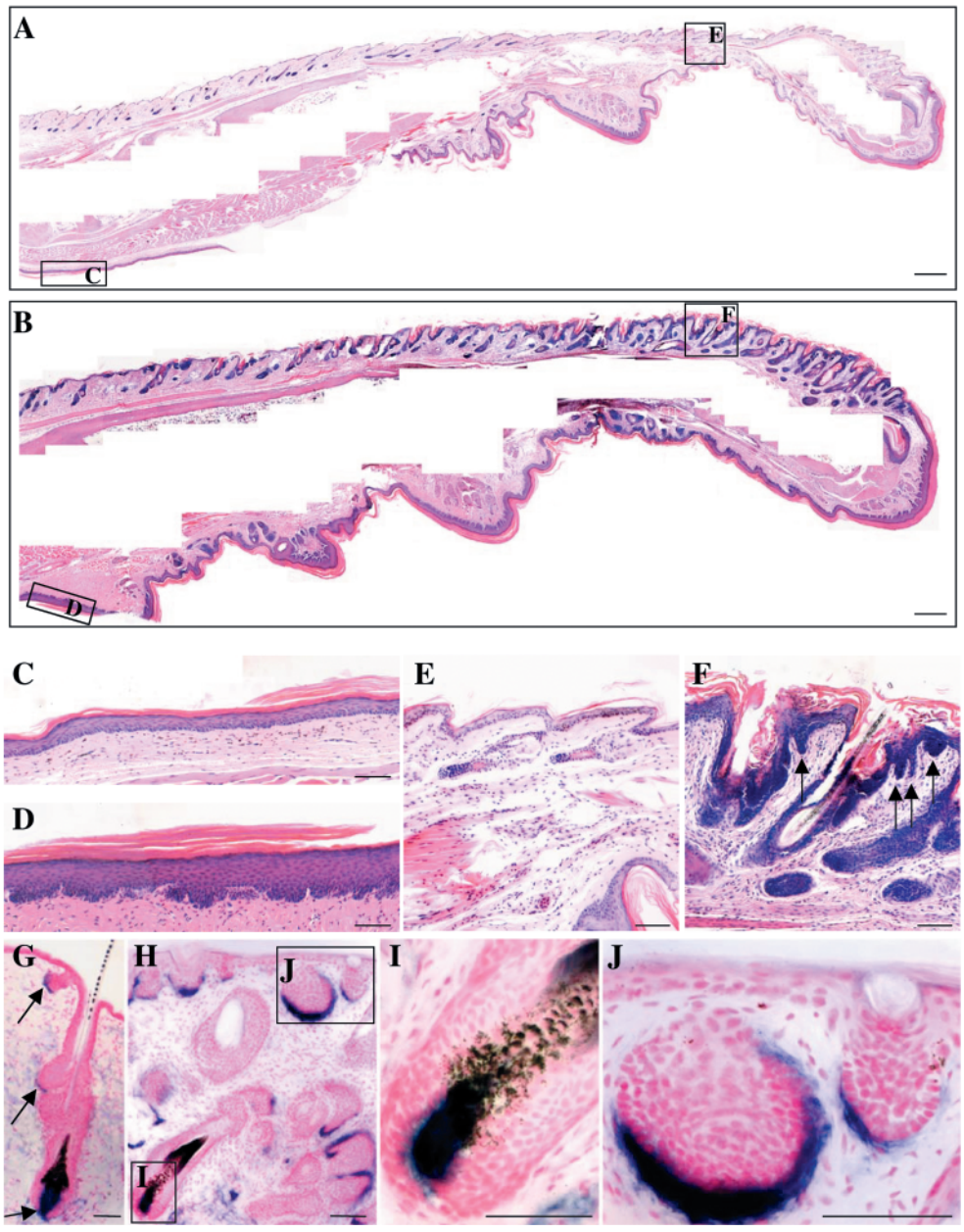


Fig. 3. De novo hair follicle formation in interfollicular epidermis. (A-F) Histology of the paw skin of (A,C,E) wild-type and (B,D,F) transgenic (line D4) mice treated daily with 4OHT for 1 week. A,B are montages reconstructed from images of sections covering the entire circumference of the paw. The boxed regions in A,B are shown at higher magnification in C,E and D,F respectively. Arrows in F indicate new follicles arising from interfollicular epidermis. (G-J) Visualisation of alkaline phosphatase activity (blue) as a dermal papilla marker in back skin (G) and paw (H-J) of transgenic (line D4) mice 8 days after a single application of 4OHT. Arrows in G show alkaline phosphatase-positive cells associated with the original dermal papilla (lowest arrow) and with new follicles arising from interfollicular epidermis (top arrow) and outer root sheath (middle arrow). The boxed regions in H are shown at higher magnification in I,J. (I) Original dermal papilla; (J) Two new rudimentary follicles. Scale bars: 500 μm (A,B) 100 μm (C-J).

observed in D2 and D4 transgenics (Fig. 3). This was particularly striking in paw skin. On the dorsal surface of the paw the density of follicles was higher in transgenics treated with 4OHT for 7 days (Fig. 3B,F) than in littermate controls (Fig. 3A,E). In the transgenic skin many downgrowths of interfollicular epidermis into the underlying dermis were observed, which had the appearance of rudimentary hair follicles (arrows in Fig. 3F). On the dorsal region of the digits of D4 mice the interfollicular epidermis was hyperproliferative, with an excess of cornified material extending into the neck of pre-existing hair follicles (Fig. 3F). The induction of new rudimentary and mature hair follicles was not confined to the paws, since new hair follicle formation was observed in dorsal skin of D2 and D4 mice (Fig. 3G, and data not shown).

On the ventral surface of the paw (the foot pad) there are regions of wild-type skin that are devoid of hair follicles and the dermal/epidermal boundary is flat (Fig. 3A,C). In the equivalent region of treated transgenics the boundary was more complex, as a result of multiple epithelial projections into the dermis, which had the appearance of rudimentary hair germs (Fig. 3B,D) (Byrne et al., 1994). Similar alterations at the dermal-epidermal boundary were seen also in the internal surface of the ear of transgenic mice (data not shown).

At the base of wild-type follicles the epithelial cells ensheath specialised mesenchymal cells, known as dermal papilla cells, which express high levels of alkaline phosphatase (Van Mater et al., 2003) (Fig. 3G,H,I). The mesenchyme adjacent to the epithelial projections, both from existing follicles and the interfollicular epidermis, had high alkaline phosphatase activity (Fig. 3G,H,J). It thus appears that keratinocytes expressing high levels of β-catenin are able to induce dermal papillae.

One of the earliest features of de novo hair follicle formation is upregulation of Sonic hedgehog (Shh) and its receptor Patched (Ptc) (St-Jacques et al., 1998; Chiang et al., 1999), which are required for the maturation of the hair germ at the base of the follicle. We therefore used in situ hybridisation to examine whether Shh and Ptc expression was increased in response to activation of ΔNβ-cateninER (Fig. 4). In wild-type anagen hair follicles expression of Shh was confined to a group of matrix cells on one side of the bulb and Ptc was expressed in all the adjacent cells (Fig. 4A-D, arrows) (Oro and Higgins, 2003). In 4OHT-treated D2 (Fig. 4E-H) and D4 (Fig. 4I-L) back skin, Shh and Ptc were upregulated in the epithelial outgrowths from existing hair follicles. In D4 back skin follicles Shh expression was no longer asymmetric but rather

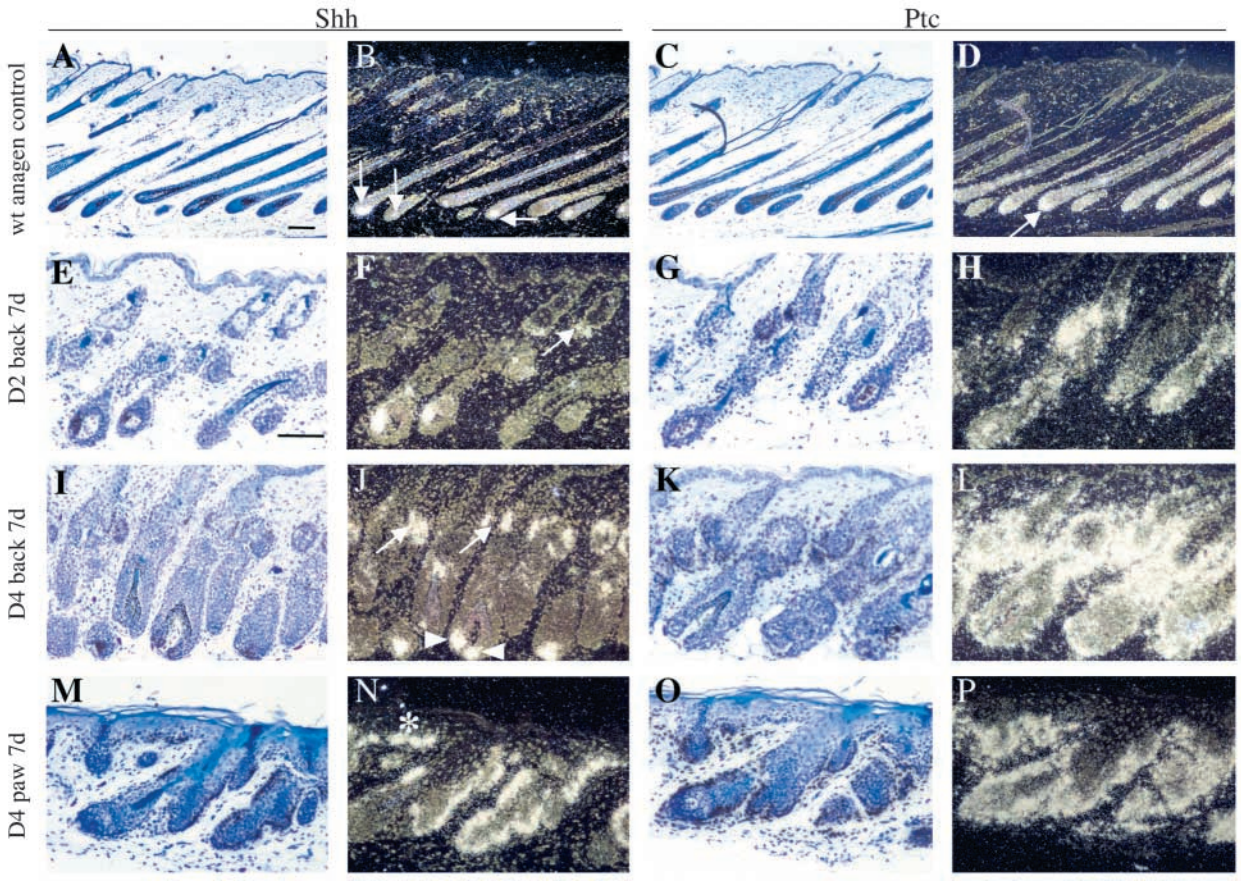


Fig. 4. Shh and Ptc expression. In situ hybridisation was performed on the dorsal skin of (A-D) untreated wild-type mouse with anagen follicles, (E-H) D2 or (I-L) D4 transgenic mice treated with 4OHT for 7 days, and (M-P) on the dorsal paw skin of D4 transgenic mice treated with 4OHT for 7 days. (B,F,J,N) Dark-field views of Shh in situ hybridisations in A,E,I,M, respectively. (D,H,L,P) Dark-field views of Ptc in situ hybridisations in C,G,K,O, respectively. Arrows in B indicate asymmetric Shh expression in the bulb of the hair follicle, while the arrow in D indicates more uniform Ptc expression in the bulb. Arrows in F,J show Shh expression in the outgrowths arising from the permanent portion of the follicles. Arrowheads in J show abnormal symmetrical expression of Shh in the bulb of the hair follicle and the asterisk in N indicates Shh expression in epithelial downgrowths in the interfollicular epidermis. Scale bars: 100 μm.

was observed on both sides of the hair bulb (Fig. 4I,J). Shh and Ptc were also expressed in the downgrowths of interfollicular epidermis on the dorsal surface of the paws of D4 mice (Fig. 4M-P). The induction of Shh and Ptc in all the observed epithelial outgrowths supports the conclusion that they are rudimentary hair follicles.

Effects of β-catenin on proliferation and differentiation

The proliferative status of the epidermis was examined by BrdU incorporation into S phase cells (Fig. 5A-D) and by expression of cyclin D1 (Fig. 5E,F), a known transcriptional target of β-catenin signalling (Tetsu and McCormick, 1999). By 5 days of 4OHT treatment there was increased proliferation in the epidermis of transgenic mice, but no effect on proliferation in the epidermis of wild-type littermate controls (Fig. 5 and data not shown). In D2 and D4 mice the increase in BrdU-positive cells occurred along the ORS, at the base of the hair follicles and in the epithelial outgrowths from the interfollicular epidermis and existing hair follicles, but not in the interfollicular epidermis itself (Fig. 5B-D). In D4 mice the increase in BrdU incorporation was more pronounced than in D2 mice and BrdU-positive cells were often found in more than one cell layer, reflecting the thickening of the ORS (Fig. 5B). Except in the epithelial downgrowths there was no increase in BrdU incorporation in the phenotypically normal interfollicular epidermis of treated transgenics (Fig. 5B,C) compared to wild-type mice (Fig. 5A). The expression of cyclin D1 mirrored BrdU incorporation, with increased labelling in the ORS, the epithelial outgrowths from the hair follicles and interfollicular epidermis, and at the base of follicles (Fig. 5E,F and data not shown).

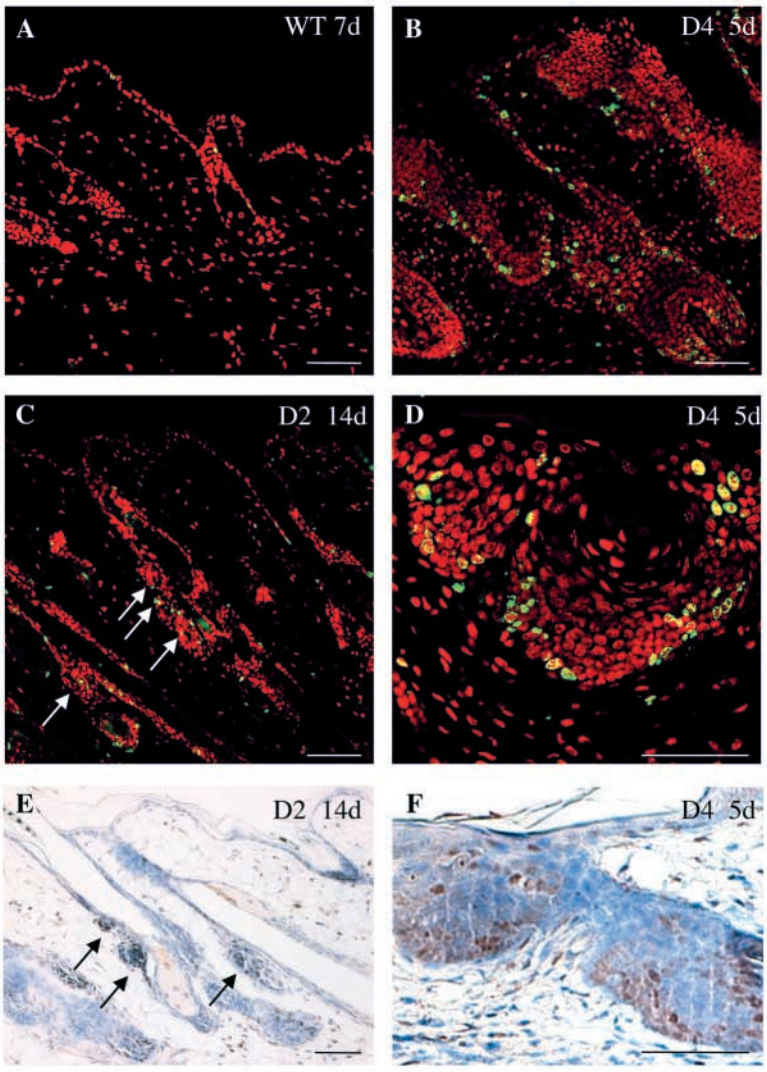


Fig. 5. Analysis of proliferation in back skin of (A) wild-type or (C,E) D2 transgenic mice and in (B,D,F) paw skin of D4 transgenic mice. (A-D) BrdU labelling (green) with propidium iodide nuclear staining (red). Green fluorescence of hair shafts is non specific. (E,F) Cyclin D1 immunohistochemistry (brown) with Haematoxylin counterstain (blue). Mice were treated with 4OHT for the periods indicated. Arrows in C and E indicate outgrowths arising from the hair follicles that are positive for BrdU and Cyclin D1, respectively. Scale bars: 100 μm.

The histological appearance of the epiderrmis and induction of Shh and Ptc strongly suggested that ΔNβ-catenin was inducing new hair follicle formation. For further confirmation, we examined expression of a range of markers of hair follicle differentiation (Fig. 6). In wild-type follicles keratin 17 is expressed along the outer root sheath (Fig. 6A) (McGowan and Coulombe, 1998), while the transcriptional repressor CCAAT displacement protein (CDP), essential for normal hair follicle inner root sheath formation, is confined to the bulb (Fig. 6B) (Ellis et al., 2001). Trichohyalin is a terminal differentiation marker expressed in cells that are producing the hair shaft (O'Guin et al., 1992) (Fig. 6C) and Lef1 is localised in the matrix (Fig. 6D) (DasGupta and Fuchs, 1999). In 4OHT-treated D2 transgenics expression of keratin 17 (Fig. 6E), CDP (Fig. 6F) and Lef1 (Fig. 6H) was observed in the outgrowths from the hair follicles, but the outgrowths did not express trichohyalin (Fig. 6G). In D4 mice all four markers were expressed in the new follicles forming from interfollicular epidermis and pre-existing follicles (Fig. 6I-L). These results support the conclusion from the histological examination of the skin (Figs 2, 3) that β-catenin promotes hair lineage differentiation. The increase in proliferation (Fig. 5) coincided with, rather than preceded, the ectopic expression of hair differentiation markers (Fig. 6).

We also examined markers of interfollicular epidermal differentiation and sebocyte differentiation (Figs 7, 8). No changes in the morphologically normal interfollicular epidermis were observed using antibodies to keratin 1 and 10 and the small cysts in D2 follicles did not express these keratins (data not shown; see also Fig. 8K). Using fatty acid synthase as a marker of sebaceous differentiation (Kusakabe et al., 2000) (Fig. 7A,B) we frequently observed duplication of the sebaceous glands in D2 and C5 epidermis during the first 15 days of 4OHT treatment (data not shown and Fig. 7C). At later time points there was loss of morphologically identifiable sebaceous glands in D2 skin and sebocytes were found only in small clusters in a scattered distribution in the trichofolliculomas (Fig. 7D). In the D4 line sebaceous gland differentiation was lost within 9 days (Fig. 7E).

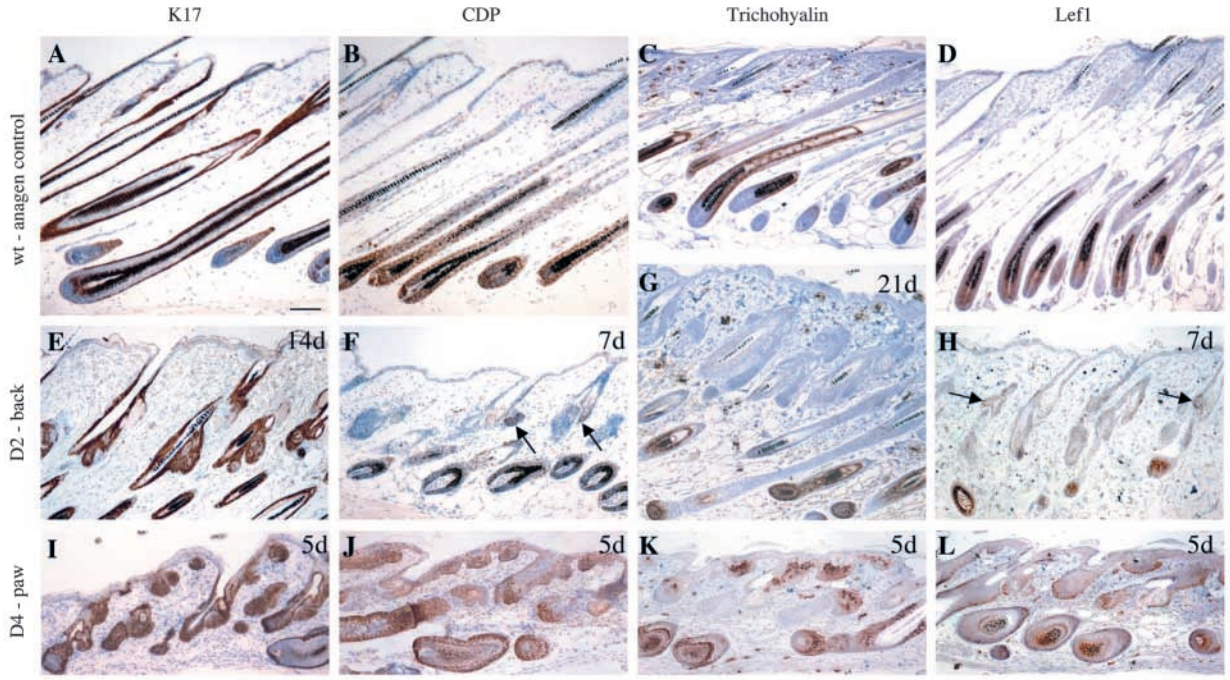


Fig. 6. Expression of hair follicle differentiation markers. (A-D) Untreated wild-type anagen follicles. (E-H) Dorsal back skin of D2 transgenic mice treated with 4OHT for the periods indicated. (I-L) Dorsal paw skin of D4 transgenic mice treated with 4OHT for 5 days. Paraffin sections were immunostained with (A,E,I) anti-keratin 17, (B,F,J) anti-CDP, (C,G,K) anti-trichohyalin, or (D,H,L) anti-Lef1 antibodies and counterstained with Haematoxylin. Arrows in F and H indicate positive staining in the epithelial outgrowths from pre-existing follicles. Scale bar: 100 μm.

This suggests that sustained activation of β-catenin signalling promotes differentiation along the hair lineages at the expense of sebaceous differentiation.

Transient activation of β-catenin is sufficient to induce anagen and de novo hair follicle formation but not to sustain trichofolliculomas

To investigate whether transient activation of β-catenin was sufficient to induce anagen in existing follicles, de novo follicle formation and hair follicle tumours, the three phenotypes caused by repeated 4OHT applications, we carried out kinetic experiments that are summarised in Fig. 8A. Each black spot represents a time point examined. Continuous lines represent continuous daily applications of 4OHT and the dotted lines represent periods when no 4OHT was given.

In D2 and D4 mice a single application of 4OHT induced the same changes as seven daily treatments (Fig. 8B-D; compare Fig. 2). One dose of 4OHT was sufficient to induce anagen and epithelial outgrowths from hair follicles in D2 mice (Fig. 8B; compare Fig. 2). One dose of 4OHT induced thickening of the hair follicles (Fig. 8C) and epithelial outgrowths from the outer root sheath (Fig. 8C) and interfollicular epidermis (Fig. 8D) in D4 skin (compare with Fig. 2). Similarly, mice treated for 5 days with 4OHT and then examined after 5 or 10 days (Fig. 8A) were phenotypically

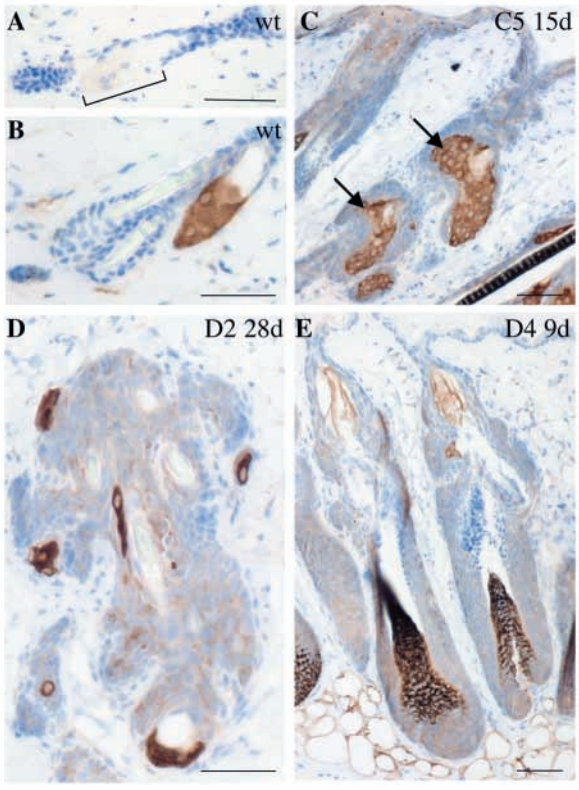
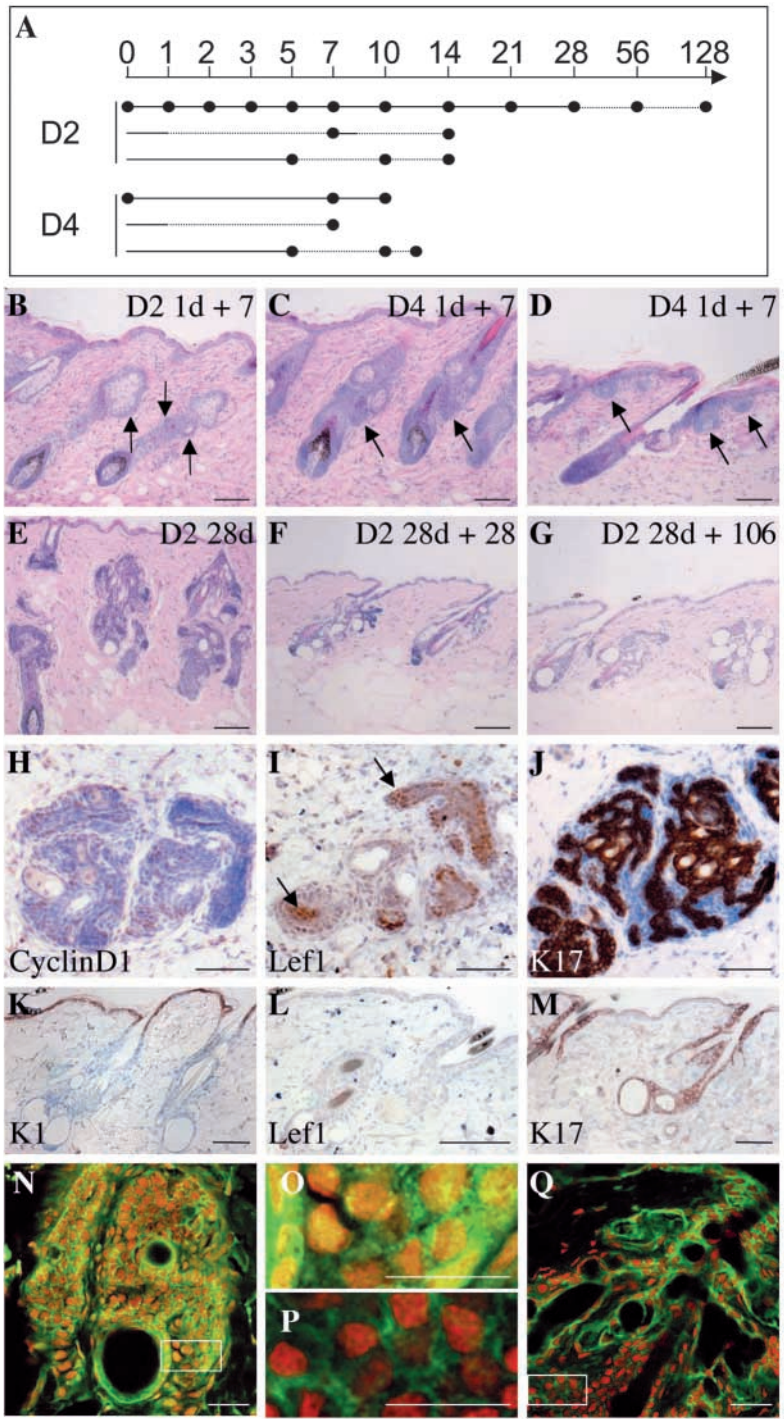


Fig. 7. Effect of β-catenin activation on sebocyte differentiation. Immunolocalisation of fatty acid synthase in dorsal back skin of (B) untreated wild-type mouse skin, (C) C5 transgenic treated with 4OHT for 15 days (arrows highlight duplication of sebaceous gland), (D) D2 transgenic treated with 4OHT for 28 days and (E) D4 transgenic treated with 4OHT for 9 days. A is wild-type follicle stained with secondary antibody only (bracket indicates the sebaceous gland). Scale bars, 100 μm.

indistinguishable from mice treated continuously for 10 or 15 days (data not shown).

In contrast to the ability of transient β-catenin activation to induce anagen and de novo follicle formation, continuous β-catenin expression was required to maintain hair follicle tumours (Fig. 8E-G). The tumours observed after 28 days of continuous β-catenin activation (Fig. 8E) started to regress after 4OHT treatment had ceased (Fig. 8F). The skin did not return completely to normal, however, as the hair follicles retained small epithelial outgrowths and cysts as late as 106 days after 4OHT treatment had stopped (Fig. 8G). Remarkably, in ear skin the tumours continued to expand for about 2 weeks in the absence of 4OHT before regression began (data not shown). While the tumours induced by continuous β-catenin activation were positive for cyclin D1 (Fig. 8H), Lef1 (Fig. 8I) and keratin 17 (Fig. 8J), during regression Lef1 and cyclin D1 expression were lost (Fig. 8L and data not shown). The residual tumour masses remained positive for keratin 17 (Fig. 8M) and negative for the interfollicular epidermal marker keratin 1 (Fig. 8K). Whereas the tumours showed strong staining for nuclear β-catenin (Fig. 8N,O) this was lost when the tumours regressed (Fig. 8P,Q).



Discussion

The keratin 14 promoter cassette developed by Fuchs and co-workers has been an immensely valuable reagent for directing transgene expression in the proliferative compartment of mouse interfollicular epidermis, sebaceous gland and hair follicle outer root sheath (Vassar et al., 1989; Byrne et al., 1994; Wang et al., 1997). It has been used to manipulate β-catenin signalling in several different transgenic models, thereby demonstrating that β-catenin plays a key role in epidermal lineage commitment (e.g. Gat et al., 1998; Merrill et al., 2001; Niemann et al., 2002; DasGupta et al., 2002). However, the endogenous promoter is first active at E9.5, before the hair placodes form (Byrne et al., 1994), and so it is possible that transgene expression during embryonic development is necessary to render the epidermis responsive to changes in β-catenin signalling in postnatal life. In order to establish whether the epidermis is truly capable of undergoing lineage conversion in adulthood we generated an inducible form of β-catenin under the control of the K14 promoter. This allowed us to activate β-catenin signalling exclusively in postnatal epidermis and to investigate the effects of activating β-catenin for different lengths of time. A similar approach has

Fig. 8. Effects of transient β-catenin activation in K14ΔNβ-cateninER transgenic mice. (A) Schematic representation of experiments performed with the D2 and D4 transgenic lines. The arrow represents time in days (not to scale). Dots are time points analysed. Continuous lines connecting dots represent periods of daily 4OHT treatment and dashed lines indicate no 4OHT treatment. (B-G) Mice received the number of doses (d) of 4OHT indicated and were kept without 4OHT for number of days shown (+). (B,C,E-G) Back skin, (D) paw skin. Arrows in B and C indicate epithelial outgrowths from the permanent portion of the hair follicles and from sebaceous glands. Arrows in D indicate new hair buds arising from interfollicular epidermis. (H-Q) expression of cyclin D1 (H), Lef1 (I,L), keratin 17 (J,M), keratin 1 (K) and β-catenin (green, N-Q; red fluorescence is propidium iodide counterstain) in D2 tumours, after 28 days of 4OHT treatment (H-J,N,O) or 28 days of treatment followed by 28 (P,Q) or 106 days (K-M) without 4OHT. Arrows in I highlight Lef1 expression. O,P are high magnification views of boxed regions in N, Q respectively. Scale bars: 100 μm (A-M), 50 μm (N-Q).

recently been described by Van Mater et al. (Van Mater et al., 2003) who found, as we did, that transient β-catenin activation is sufficient to induce hair follicles to enter the growth phase (anagen) of the hair cycle. In addition we show that β-catenin induces de novo hair follicle formation in interfollicular epidermis and can cause either duplication of the sebaceous glands or inhibition of sebocyte differentiation depending on the level of activation. These results reveal a remarkable plasticity in the differentiation potential of adult epidermis.

In all of the K14ΔNβ-catenin lines the first effect of applying 4OHT to skin with telogen (resting) hair follicles was to induce anagen. In D2 mice the anagen was essentially normal and the coat of fur that grew could not be distinguished macroscopically from a wild-type pelage. However, in D4 mice, which have a higher transgene copy number and express a higher level of ΔNβ-catenin, the degree of proliferation induced by 4OHT was greater than in normal anagen with the result that the follicles became abnormally thickened and failed to produce normal hair shafts. Anagen was induced as effectively by transient as by continuous activation of β-catenin (see also Van Mater et al., 2003). Conversely, anagen did not continue indefinitely, since follicles in epidermis treated with 4OHT for 28 days were shorter than follicles treated for 21 days, demonstrating that additional factors are required for maintenance of hair growth (Fuchs et al., 2001).

Shh is a target gene of β-catenin in the epidermis (Gat et al., 1998; Huelsken et al., 2001) and was induced by activation of ΔNβ-cateninER. Shh is expressed in the bulb of growing wild-type follicles and is known to initiate anagen (Oro et al., 1997; St-Jacques et al., 1998; Sato et al., 1999; Wang et al., 1997; Callahan and Oro, 2001; Stenn and Paus, 2001). It therefore seems likely that the reason for the entry of transgenic follicles into anagen was that Shh was induced by ΔNβ-cateninER. Levels of Shh and its receptor Ptc, itself a Shh target (Freeman, 2000), were higher in D4 than in D2 epidermis and whereas in wild-type and D2 follicles Shh expression was asymmetric, in D4 follicles expression was observed on both sides of the hair bulb. Thus, aberrant Shh levels and localisation may contribute to the abnormalities in D4 anagen follicles.

De novo follicle formation was a striking phenotype of K14ΔNβ-cateninER mice and established that β-catenin can induce new follicles even when expression is restricted to postnatal epidermis (cf. Gat et al., 1998). Rudimentary follicles developed as epithelial outgrowths from the permanent portion of the outer root sheath, from sebaceous glands and from interfollicular epidermis. Such outgrowths also formed on the ventral surface of the paw and in the internal surface of the ear, regions normally devoid of hair follicles. Dermal cells adjacent to the outgrowths had high alkaline phosphatase activity, a marker of dermal papilla cells. At later times the base of the new follicles was seen enveloping a dermal condensate that was morphologically indistinguishable from a dermal papilla (data not shown).

All of the outgrowths expressed Shh, Ptc, K17, CDP and Lef1, markers of hair follicle formation (Millar, 2002; McGowan and Coulombe, 1998; Ellis et al., 2001; Zhou et al., 1995). Whether the outgrowths progressed to form complete hair follicles with hair shafts and trichohyalin expression depended on the duration of β-catenin activation, complete follicles appearing from 14 days onwards. The level of β-catenin appeared to determine the number of new follicles developing from interfollicular epidermis: in the D4 transgenic line there were more new follicles and the initial outgrowths appeared in a shorter time than in the D2 line.

The formation of hair follicles during embryogenesis depends on a series of signals that are exchanged between the epidermis and the underlying dermis (Hardy, 1992; Millar, 2002). In the embryo the initiating signal comes from the dermis and involves activation of Wnt signalling (Kishimoto et al., 2000); the response in the overlying epithelium also involves activation of β-catenin. In K14ΔNβ-cateninER skin the dermal signal is not required and activation of β-catenin signalling in the epidermis leads to organisation of a dermal papilla (Fig. 3G-J). In each location where new follicles formed in K14ΔNβ-cateninER epidermis there was induction of Shh, Ptc and Lef1. Just as Shh drives anagen, Shh is downstream of Wnt signalling in hair follicle development (Fuchs et al., 2001; Millar, 2002): in mice lacking Shh hair follicle formation is initiated and the dermal condensate is formed but mature hair follicles fail to develop (St-Jacques et al., 1998; Chiang et al., 1999). Lef1 is a known transcriptional target of β-catenin (Filali et al., 2002), required for normal hair follicle formation (van Genderen et al., 1994; Zhou et al., 1995; Kratochwil et al., 1996). Although during normal hair placode formation expression of Lef1 is regulated by Noggin, produced by dermal cells (Jamora et al., 2003), in our system Lef1 upregulation is independent of a pre-existing dermal signal.

The effects of prolonged activation of ΔNβ-cateninER were not confined to hair follicle differentiation. While differentiation within the interfollicular epidermis was largely normal there were also some areas of hyperproliferation, leaving open the question of whether β-catenin can expand the size of the IFE stem cell pool (Zhu et al., 1999; Reya et al., 2003). The effects on sebocyte differentiation were even more puzzling, because there was initial duplication of sebaceous glands, but thereafter sebocytes were lost; in D4 epidermis no sebocytes were detectable by 9 days of 4OHT treatment. c-Myc is induced by β-catenin (He et al., 1998) and direct activation of c-Myc in adult epidermis stimulates sebocyte differentiation in both the sebaceous glands and interfollicular epidermis (Arnold and Watt, 2001; Braun et al., 2003). However, when β-catenin signalling is blocked with a ΔNLef1 transgene there is ectopic sebocyte differentiation and sebocyte tumours appear (Niemann et al., 2002; Braun et al., 2003). One interpretation of these results is that sebocyte differentiation is promoted by intermediate levels and duration of β-catenin signalling and blocked by higher levels and duration. The phenotype of epidermis expressing ΔNLef1 demonstrates that sebocyte differentiation can be promoted independently of β-catenin activation, presumably through the ability of ΔNLef1 to interact with other pathways (Labbe et al., 2000).

Just as β-catenin levels regulate lineage choice in the epidermis so they also influence the types of tumour that develop when signalling is deregulated (Owens and Watt, 2003). High β-catenin expression results in hair follicle type tumours (Gat et al., 1998; Chan et al., 1999) whereas inhibition of β-catenin signalling with ΔNLef1 leads to the formation of sebaceous tumours at high frequency (Niemann et al., 2002; Niemann et al., 2003). The tumours induced by sustained activation of ΔNβ-cateninER were highly differentiated and resembled human trichofolliculomas, consistent with the

earlier observations of Gat et al. (Gat et al., 1998) and Chan et al. (Chan et al., 1999). Every follicle in which ΔNβ-catenin was activated for 21 days developed into a tumour. However, once β-catenin activation ceased all the tumours regressed, and by three months what remained were remnants of the tumour mass that mainly consisted of empty hair shafts. Thus continued β-catenin activation was needed to maintain the tumours, and they could not grow autonomously. One reason may be that β-catenin induces p53, which serves to limit growth (Damalas et al., 2001). Additional oncogenic changes, such as mutation of p53 or Ras, would be necessary to convert the trichofolliculomas into malignant tumours capable of autonomous growth (Hahn and Weinberg, 2002; Perez-Losada and Balmain, 2003; Owens and Watt, 2003).

Considerable debate surrounds the degree of plasticity of adult stem cells (Alison et al., 2003; Goodell, 2003). While the current consensus is that the ability of adult stem cells to undergo conversion into unrelated cell types is limited (e.g. Wagers et al., 2002), interconversion between epidermal lineages can be readily induced. The present studies show that β-catenin can induce de novo hair follicle formation from existing follicles, interfollicular epidermis and sebaceous glands, and it has previously been reported that c-Myc induces sebocyte differentiation in interfollicular epidermis (Braun et al., 2003). Thus all regions of the epidermis are competent to undergo lineage conversion. We and others have argued that there are distinct stem cell pools in IFE, sebaceous glands and hair follicles (reviewed by Niemann and Watt, 2002). The challenge now is to find out whether only stem cells are capable of undergoing reprogramming or whether plasticity is also retained in committed progenitors (Braun et al., 2003).

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