β-Catenin and Hedgehog Signal Strength Can Specify Number and Location of Hair Follicles in Adult Epidermis without Recruitment of Bulge Stem Cells

Violeta Silva-Vargas,[1] Cristina Lo Celso,[1]
Adam Giangreco, Tyler Ofstad, David M. Prowse,[2]
Kristin M. Braun, and Fiona M. Watt*
Keratinocyte Laboratory
Cancer Research UK London Research Institute
44 Lincoln's Inn Fields
London WC2A 3PX
United Kingdom

Summary

Using K14ΔNβ-cateninER transgenic mice, we show that short-term, low-level β-catenin activation stimulates de novo hair follicle formation from sebaceous glands and interfollicular epidermis, while only sustained, high-level activation induces new follicles from preexisting follicles. The Hedgehog pathway is upregulated by β-catenin activation, and inhibition of Hedgehog signaling converts the low β-catenin phenotype to wild-type epidermis and the high phenotype to low. β-catenin-induced follicles contain clonogenic keratinocytes that express bulge markers; the follicles induce dermal papillae and provide a niche for melanocytes, and they undergo 4OHT-dependent cycles of growth and regression. New follicles induced in interfollicular epidermis are derived from that cellular compartment and not through bulge stem cell migration or division. These results demonstrate the remarkable capacity of adult epidermis to be reprogrammed by titrating β-catenin and Hedgehog signal strength and establish that cells from interfollicular epidermis can acquire certain characteristics of bulge stem cells.

Introduction

Mammalian epidermis consists of a multilayered epithelium, the interfollicular epidermis (IFE), with associated hair follicles (HFs), sebaceous glands (SGs), and sweat glands. Epidermal maintenance depends on stem cells. The best-characterized stem cell population is in the permanent part of the hair follicle outer root sheath (ORS), a region known as the bulge. Since epidermal stem cells tend to divide infrequently, they can be visualized as bromodeoxyuridine (BrdU) DNA label-retaining cells (LRC). LRC are concentrated in the bulge, and they express markers such as CD34 and keratin 15 (K15) (Morris et al., 2004; Trempus et al., 2003; Tumbar et al., 2004).

Bulge stem cells can give rise to all the differentiated lineages of the HF, IFE, and SG (Oshima et al., 2001;

Taylor et al., 2000). However, there are also stem cell populations in the IFE and SG (Ghazizadeh and Taichman, 2001), and it has been proposed that stem cells in a particular location normally feed a restricted number of lineages in response to local signals (Niemann and Watt, 2002). As in other tissues, it is the combination of the intrinsic characteristics of the stem cells and their microenvironment that shapes their properties and defines their fate (Fuchs et al., 2004; Wagers and Weissman, 2004).

A range of Wnts, their receptors, and antagonists are expressed dynamically within the epidermis (Millar, 2002). Epidermal expression of N-terminally truncated, activated β-catenin leads first to induction of hair growth (anagen), then to de novo HF formation from the IFE, SG, and preexisting HF (Gat et al., 1998; Lo Celso et al., 2004; Van Mater et al., 2003). Conversely, when β-catenin signaling is inhibited, new HF formation is prevented, growth of existing HF is disturbed, and follicles develop cysts of IFE with associated sebocytes (Niemann and Watt, 2002; Fuchs et al., 2004). These studies clearly show that β-catenin can control epidermal lineage commitment in the adult tissue. High levels stimulate HF formation, intermediate levels favor sebocyte differentiation, and low levels convert HF into IFE (Niemann and Watt, 2002).

In normal epidermis, Shh expression is restricted to the base of anagen hair follicles and inhibition of Shh blocks anagen (Oro and Higgins, 2003). When activated β-catenin is overexpressed in the epidermis, upregulation of Shh and Ptc occurs where new follicles form (Gat et al., 1998; Lo Celso et al., 2004). Whereas β-catenin regulates lineage choice, Shh is primarily a proliferative stimulus, mediated at least in part by direct induction of cell-cycle regulators such as cyclin D and cyclin E (Duman-Scheel et al., 2002).

The ability of β-catenin to induce ectopic hair follicles in adult epidermis raises a number of questions. Do cells exhibit a graded response to increasing β-catenin activation, or is HF induction a threshold response? Is responsiveness restricted to the stem cell compartment, or can committed progenitors be reprogrammed (Pearton et al., 2004) to enter the HF lineages? If stem cells are required to generate new HF, are IFE and SG stem cells involved, or does HF formation depend on preexisting bulge stem cells (Oshima et al., 2001)? Can β-catenin-induced HF, like normal follicles, provide a niche for melanocytes (Nishimura et al., 2002)? To explore these issues, we have used K14ΔNβ-cateninER mice in which the onset and duration of β-catenin activation in the basal layer of the epidermis are controlled by topical application of the drug 4-hydroxytamoxifen (4OHT) (Lo Celso et al., 2004; Van Mater et al., 2003).

Results

Measurement of β-Catenin Activation
Two lines of K14ΔNβ-cateninER mice were studied: D2, with 12 copies of the transgene, and D4, with 21 copies

*Correspondence: fiona.watt@cancer.org.uk
[1]These authors contributed equally to this work.
[2]Present address: Centre for Cutaneous Research, Institute of Cell and Molecular Science, Barts and The Royal London School of Medicine and Dentistry, 4 Newark Street, London E1 2AT, United Kingdom.
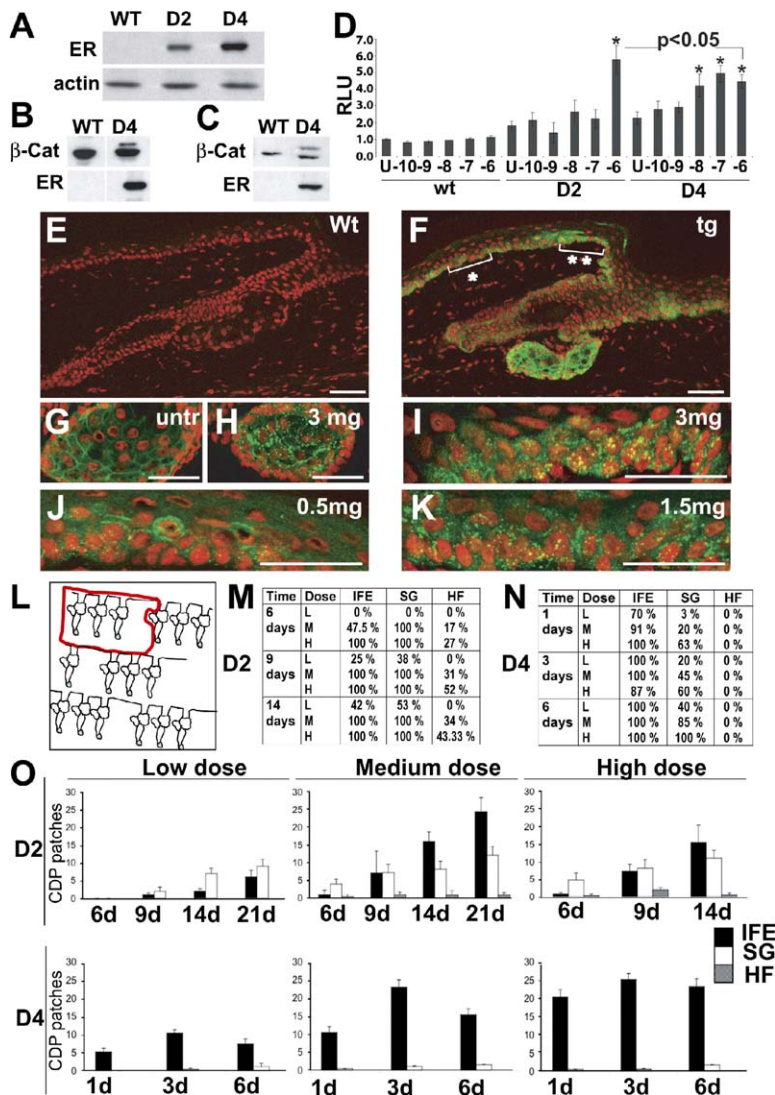
Figure 1. Titration of β-Catenin Signaling In Vitro and In Vivo

(A–D) Cultured keratinocytes from wild-type (wt), D2, and D4 mice were examined by Western blotting (A–C) or transient transfection with a TOPFLASH luciferase reporter (D).

(A) Comparison of relative levels of ΔNβ-cateninER by immunoblotting with anti-ER or, as a loading control, anti-actin antibody.

(B and C) Comparison of relative levels of endogenous β-catenin and ΔNβ-cateninER in total Triton X-100 soluble lysates (B) or the supernatant from hypotonic cell lysates (C). Upper band detected with anti-β-catenin is ΔNβ-cateninER as it was also detected with anti-ER.

(D) Cells were untreated (U) or treated with increasing 4OHT concentrations, from 2×10^{-10} (−10) to 2×10^{-6}(−6) M, for 24 hr. Relative light units (RLU) are shown. Bars represent means of at least three replicates in up to four experiments ± SEM. Asterisks indicate significant increase relative to untr.

(E–K) Sections of wild-type (E) and D4 transgenic (F–K) tail skin stained with anti-ER. Skin was either untreated (E–G) or treated for 24 hr with the 4OHT doses shown. (G and H) SG; (I–K) IFE. Brackets in (F) indicate patches of higher (**) or lower (*) transgene expression. Scale bars equal 100 μm in (G) and (J), 50 μm in (E) and (F).

(L) One unit of tail epidermis is outlined in red, corresponding to three HF with associated SG and IFE.

(M and N) % units with ectopic CDP expression in D2 (M) and D4 (N) epidermis treated with low (L), medium (M), or high (H) 4OHT dose.

(O) Average number of CDP-positive patches per unit. Mean ± SEM of three experiments shown in (M) and (N). d, days.

(Lo Celso et al., 2004). In previous work, we used a single dose of 4OHT, 1 mg per mouse (Lo Celso et al., 2004). For the present experiments, we used three concentrations: 0.5 (low dose), 1.5 (medium dose), or 3 mg (high dose) per mouse, applying 4OHT every second day.

To determine the effects of transgene copy number and 4OHT concentration on β-catenin signaling, we generated spontaneously immortalized lines of keratinocytes. D4 keratinocytes expressed approximately 2-fold more ΔNβ-cateninER protein than D2 keratinocytes (Figure 1A). ΔNβ-cateninER ran as a band of approximately 110 kDa, slightly higher than endogenous β-catenin. In lysates of total Triton soluble proteins, ΔNβ-cateninER was considerably less abundant than endogenous β-catenin (Figure 1B). In extracts depleted of the pool of β-catenin bound to E-cadherin (Zhu and Watt, 1999), ΔNβ-cateninER and endogenous β-catenin were similar in abundance (Figure 1C). Thus, in transgenic epidermis the amount of β-catenin available for signaling is approximately 2-fold that in wild-type epidermis.

To examine β-catenin-mediated transcriptional activation, we transfected wild-type, D2, and D4 keratinocytes with a luciferase construct containing an enhancer with multiple Lef1/Tcf binding sites (TOPFLASH) or, as a negative control, one with mutated Lef1/Tcf binding sites (FOPFLASH). 4OHT treatment of cells expressing ΔNβ-cateninER transfected with FOPFLASH did not result in induction of luciferase (Lo Celso et al., 2004, and data not shown). TOPFLASH activation occurred within 8 hr of addition of 4OHT and was greater at 24 hr (data not shown).

We compared TOPFLASH activation in wild-type, D2, and D4 keratinocytes 24 hr after adding a range of concentrations of 4OHT (Figure 1D). There was no transcriptional activation at any concentration of 4OHT in wild-type cells. In D2 cells, there was activation only at the highest 4OHT concentration. In D4 cells, there was significant activation at concentrations of 2×10^{-8} M and higher. The maximum activation achieved in D2 and D4 cells was the same, even though D4 cells expressed more ΔNβ-cateninER protein.

We next examined whether there was regional variation in transgene expression in vivo. While staining with an antibody to the mutant ER confirmed that all basal cells of the IFE, ORS, and periphery of the SG expressed the transgene (Lo Celso et al., 2004), there was heterogeneity in expression levels, with higher levels being detected in the sebaceous gland than the IFE or ORS (Figures 1E and 1F). IFE expression was heterogeneous, with patches of cells at the junction between the ORS and the IFE expressing higher levels than cells in other regions (Figure 1F). The same expression pattern was observed in both transgenic lines and also in K14MycER mice (data not shown; Arnold and Watt, 2001).

We next examined sections of tail skin from mice that had been treated for 24 hr with three doses of 4OHT. In untreated epidermis, ER immunoreactivity was most abundant at cell-cell borders (Figure 1G). After treatment with 3 mg 4OHT, cell border staining was reduced (Figure 1H) and there was punctate staining in the nucleus and cytoplasm (Figure 1I; Lo Celso et al., 2004). In epidermis treated with 0.5 mg 4OHT, cell-cell border staining was predominant (Figure 1J), while with 1.5 mg, staining was primarily nuclear and cytoplasmic (Figure 1K). The same dose-dependent redistribution of ΔNβ-cateninER was observed in IFE, SG, and HF in both lines (data not shown). There was no evidence that activation of β-catenin by 4OHT triggered epidermal apoptosis, necrosis, or inflammation (data not shown).

We conclude that different concentrations of 4OHT result in different levels of β-catenin activation and that ΔNβ-cateninER is activated to approximately the same extent in cells of the SG, IFE, and HF ORS. Transgene copy number also affects activation, because D4 cells were more responsive than D2 cells to low concentrations of 4OHT.

Control of Hair Follicle Number and Location by Transgene Copy Number and 4OHT Dose

To evaluate ectopic hair follicle differentiation, we used an antibody to CCAAT displacement protein (CDP) that is expressed primarily in the base of the follicle, known as the bulb (Braun et al., 2003). Induction of CDP, together with Lef1 and keratin 17, is an early indicator of ectopic HF formation (Lo Celso et al., 2004).

We performed whole-mount labeling of 4OHT-treated mouse tail skin (Braun et al., 2003) to facilitate quantitation (Figures 1L–1O). Figure 2 shows the morphology of the ectopic follicles, both schematically (Figure 2A) and in representative CDP-labeled whole mounts (Figures 2B–2J).

In wild-type and untreated K14ΔNβ-cateninER tail skin, the hair follicles have prominent sebaceous glands and are clustered in groups of three. We defined one HF triplet and adjacent interfollicular epidermis as one epidermal unit (Figure 1L). The percentage of units in which ectopic expression of CDP occurred in the IFE (Figure 2A; arrowheads in Figure 2J), SG (arrows in Figure 2C), and HF ORS (arrow in Figure 2E) is shown for each 4OHT concentration and each transgenic line in Figures 1M and 1N. The number of CDP-positive patches per positive unit is shown in Figure 1O.

In D2 mice, the 4OHT dose affected the time of appearance of ectopic CDP expression, the proportion of units with ectopic CDP, and the number of patches of ectopic CDP per unit (Figures 1M and 1O). Ectopic CDP expression was first observed at day 6 with medium and high 4OHT concentrations and at day 9 with the low dose (Figures 2A and 2C). CDP was most readily induced in the SG (Figure 2C), while the HF was most refractory (Figure 1M). Medium-dose 4OHT induced a higher number of CDP-positive patches than low-dose 4OHT, and at all doses, the number of positive patches per unit increased over time (Figure 1O; compare Figures 2D and 2F). At the high 4OHT dose, the number of CDP-positive patches was lower than at the medium dose, but high-dose patches were larger (data not shown).

β-catenin activation in K14ΔNβ-cateninER mice induces the underlying mesenchyme to form a dermal papilla, surrounded by a cup-like sheath of keratinocytes (Figure 2D, arrow; Lo Celso et al., 2004; Van Mater et al., 2003). In D2 mice, formation of dermal papillae (red in Figure 2A) was 4OHT dose dependent and occurred after induction of ectopic CDP.

D4 epidermis responded more rapidly than D2 to 4OHT (Lo Celso et al., 2004), and by 1 day ectopic CDP expression was observed (Figures 1N, 2G, and 2H). In D4 mice, the IFE was most sensitive and the HF most refractory (Figures 1N and 2G). Although CDP expression was observed along most of the D4 ORS, distinct projections of CDP-positive epithelium and dermal papillae were hardly ever observed (Figure 2A; compare Figures 2J and 2E). The correlation between the number of CDP-positive patches and 4OHT dose and length of treatment was less pronounced in D4 than D2 mice; patch size tended to be greater and individual patches merged with one another (Figure 1O; compare Figures 2D and 2J).

We conclude that different regions of the epidermis differed in their responsiveness to β-catenin activation. SGs were most responsive and HFs most refractory. Regional variation in transgene expression did not appear to determine where ectopic HF formed in the IFE (data not shown; see Figure 1F). However, it might explain why D2 SG were most sensitive to HF induction (Figure 1M). HF morphogenesis progressed further in D2 than in D4 mice (Figure 2A).

Hedgehog Signaling Is Necessary for β-Catenin-Induced Formation of New Hair Follicles

To investigate how β-catenin induced new hair follicles, we isolated RNA from whole dorsal skin of triplicate 6-week-old female D2 mice, comparing untreated skin with skin treated either for 1 day with 1 mg 4OHT or harvested after seven daily treatments. Using Affymetrix chip technology, we analyzed >18,000 transcripts, representing >14,000 genes. Genespring software was used to normalize the raw data and identify genes with a t test p value of less than 0.05 and a change in relative expression levels of at least 3-fold in 4OHT-treated transgenics. 150 probe sets were identified as >3-fold upregulated and 13 probe sets >3-fold downregulated after 7 days of 4OHT treatment. The full list of known genes, as well as the normalization and filter parameters, are available as Supplemental Data with this arti-
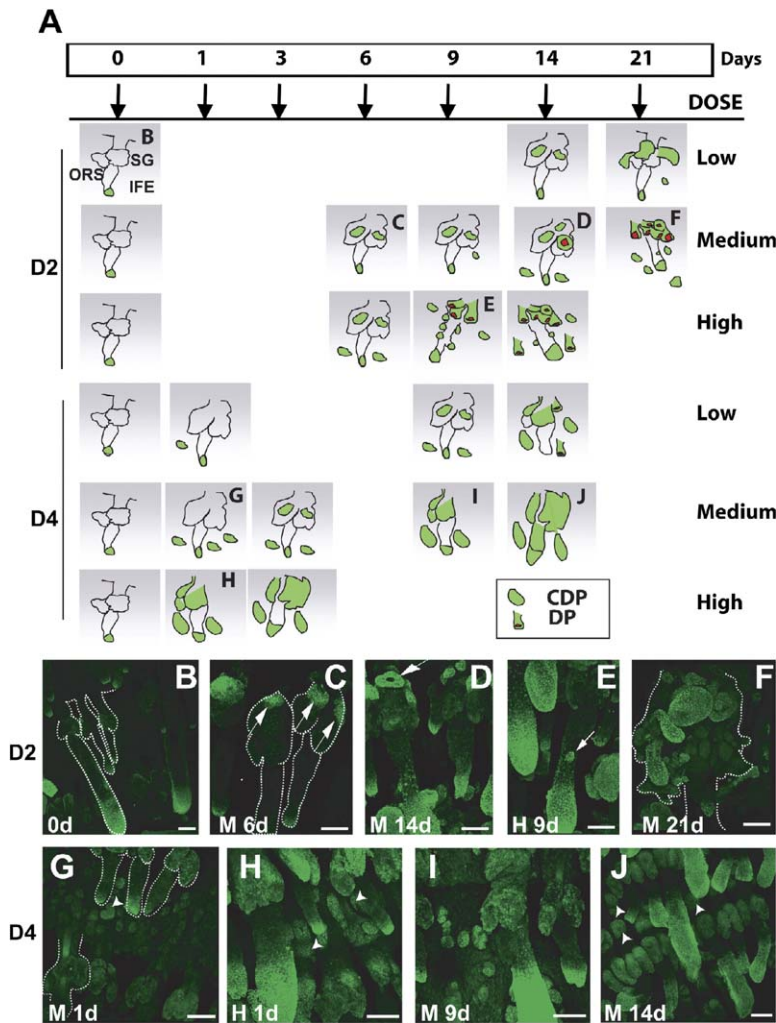
Figure 2. Effects on D2 and D4 Transgenic Epidermis of Activating ΔNβ-CateninER with Different Concentrations of 4OHT for Different Lengths of Time

4OHT was applied at 0.5 mg (L), 1.5 mg (M), and 3 mg (H) doses.

(A) Schematic representation of results, showing outer root sheath (ORS) of a single HF with associated SG and IFE. Green, CDP expression; red, new dermal papillae (DP). Arrows: time points analyzed. Letters correspond to whole mounts in (B)–(J).

(B–J) Tail whole mounts immunolabeled for CDP. Transgenic lines, 4OHT doses, and time of treatments (days, d) are indicated. Dashed lines demarcate hair follicles and sebaceous glands in (B), (C), (F), and (G). Arrows in (C) indicate CDP expression in SG, in (D) a dermal papilla, and in (E) an outgrowth arising from ORS. Arrowheads in (G), (H), and (J) indicate new outgrowths arising from IFE. Scale bars equal 100 μm.

cle online. The raw chip data files are available at http://www.ncbi.nlm.nih.gov/geo/info/linking.html (accession number GSE1579).

Some of the upregulated genes corresponded to known β-catenin targets or markers of HF differentiation (see, for example, Gat et al., 1998; Lo Celso et al., 2004; Van Mater et al., 2003), including cyclin D1, hair keratins, CDP, and Lef1. The increase in cyclin D1 and other cell-cycle regulators is consistent with the local increase in proliferation that occurs in response to β-catenin (Lo Celso et al., 2004).

Some of the genes that were most highly induced were members of the Hedgehog signaling cascade: Shh was upregulated 4.8-fold, Ptch2 6.6-fold, Gli1 8.4-fold, and Gli2 3.6-fold. In addition, N-Myc, a target of Shh (Oliver et al., 2003), was upregulated 8-fold at 7 days (see Supplemental Data). Increased expression of Shh and Ptc has previously been observed when β-catenin is activated in the epidermis (Gat et al., 1998; Lo Celso et al., 2004). Since Shh drives proliferation during normal hair follicle anagen (Oro and Higgins, 2003) and β-catenin activation results in local increases in proliferation (Lo Celso et al., 2004), we investigated

whether Hedgehog signaling was required for ectopic HF formation.

K14ΔNβ-catenin transgenics were treated with 1 mg 4OHT ± cyclopamine to block Hedgehog signaling (Figures 3A–3H; van den Brink et al., 2004). In untreated and cyclopamine-treated epidermis, Gli1 was mainly cytoplasmic (Figures 3K and 3M; Niemann et al., 2003), whereas after 4OHT treatment alone, Gli1 was primarily nuclear (Figure 3L). Cyclopamine blocked the induction of anagen (Figures 3A and 3B) and reduced the appearance of ectopic HF (Figures 3E and 3F) in D2 mice, thereby converting the D2 phenotype to wild-type. Cyclopamine did not completely normalize the D4 phenotype, but converted it to a D2 phenotype (Figures 3C and 3D): the new follicles became more pronounced and dermal papilla formation was stimulated (Figures 3G and 3H).

The morphological observations were confirmed by staining whole mounts with a range of antibodies. Keratin 17 expression induced by β-catenin in the IFE and SG of D2 mice (Figure 3I) was largely inhibited, and keratin 17 expression was confined to the ORS (Figure 3J), as in wild-type epidermis (data not shown; Lo Celso et
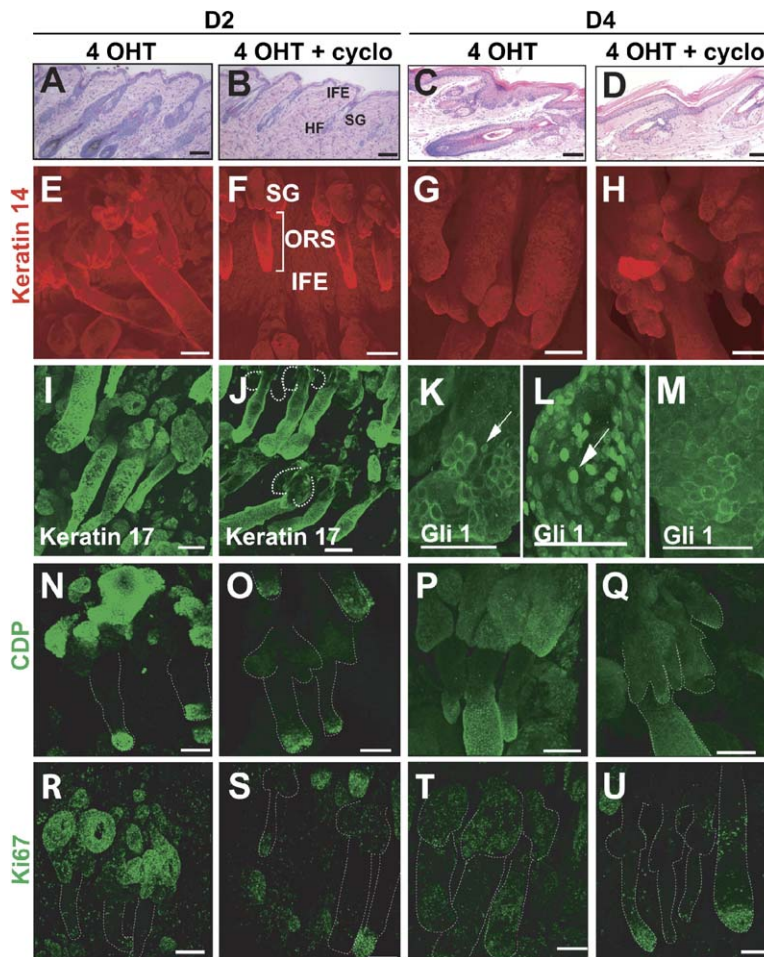
Figure 3. Hedgehog Signaling Is Required for β-Catenin-Induced Hair Follicle Formation

D2 (A, B, E, F, I, J, N, O, R, S) and D4 (C, D, G, H, K–M, P, Q, T, U) mice were untreated (K), treated with 4OHT alone (A, C, E, G, I, L, N, P, R, T), or treated with 4OHT and cyclopamine (B, D, F, H, J, M, O, Q, S, U).

(A–D) H&E-stained sections of dorsal (A and B) or tail (C and D) epidermis.

(E–U) Tail whole mounts labeled with antibodies to K14 (E–H), K17 (I and J), Gli1 (K–M), CDP (N–Q), or Ki67 (R–U). Arrows in (K) and (L) show nuclear Gli1 staining. Dashed lines outline SG (J), ORS (N, O, R), or HF and SG (Q, S–U). Scale bars equal 100 μm in (A)–(J) and (N)–(U) and 50 μm in (K)–(M).

al., 2004). Ectopic expression of CDP in D2 epidermis (Figure 3N) was also inhibited by cyclopamine (Figure 3O). In D4 epidermis, the size of the ectopic CDP patches was reduced (Figures 3P and 3Q). As expected, cyclopamine treatment led to a reduction in epidermal proliferation, as evaluated by Ki67 staining (Figures 3R–3U).

Lineage Reprogramming Occurs Independently of Bulge Stem Cells

We used two markers to examine the effect of β-catenin activation on the hair follicle stem cell compartment: retention of BrdU label and expression of K15. In wild-type mice and untreated transgenic mice, LRC were concentrated in the bulge, and there were scattered LRC in the sebaceous glands and IFE (Figure 4A; Braun et al., 2003). There was no evidence of LRC depletion in the bulge of D2 HF treated with 4OHT (medium dose) for up to 14 days (Figure 4B) or D4 mice treated for up to 9 days (Figures 4D and 4E). Thus, at times when ectopic hair follicle formation was well advanced in IFE and SG (Figure 2A), bulge LRC had not been lost and there was no evidence that they had migrated from their original location.

By 28 days of treatment of D2 mice (Figure 4C) and 15 days of treatment of D4 mice (Figure 4F), the number of LRC in the bulge had declined significantly. This did not correlate with increased apoptosis (data not shown) but with stimulation of LRC to divide. Some LRC became Ki67 positive, and lightly BrdU-labeled cells appeared in the bulge (Figure 4G; see also Braun et al., 2003).

K15 expression in the bulge of untreated HF (Morris et al., 2004) corresponded to the zone of LRC (Figures 4H and 4I). K15 expression in the bulge was preserved in both transgenic lines, even at high 4OHT concentrations and late time points (Figures 4J and 4K and data not shown) when LRC had been completely lost (Figure 4K). We conclude that β-catenin-induced HF formation was initiated without involvement of bulge LRC and that even when LRC divided and lost their label, the original bulge remained, as detected by K15 expression.

As further evidence that β-catenin induction of HF could occur independently of bulge stem cells, we used the K14 promoter for lineage tracing in order to provide a positive marker of IFE cell progeny. We generated triple transgenic mice by crossing D2 K14ΔNβ-cateninER mice with K14CreER mice (Vasioukhin et al., 1999; Hong et al., 2004) and Rosa 26 Cre-Reporter mice in which a stop codon flanked by LoxP sites has been inserted upstream of the β-galactosidase gene (Soriano, 1999). The mice were treated for 21 days with 1.5 mg 4OHT, and then the skin was harvested and exam-
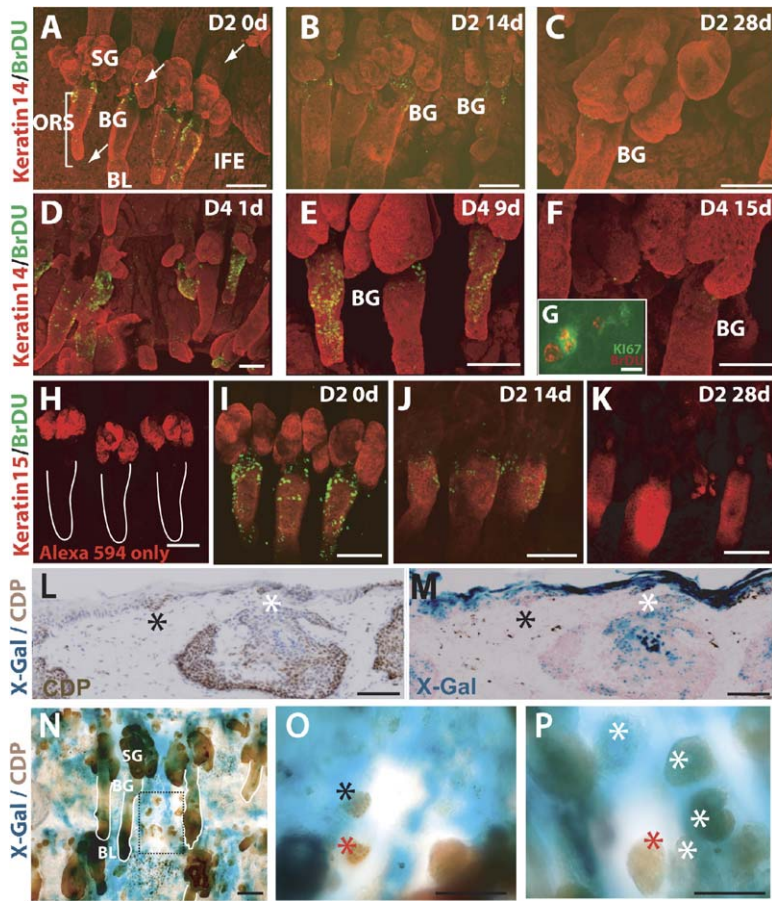
Figure 4. ΔNβ-CateninER-Induced Hair Follicles Are Not Derived from Existing Bulge Cells

(A–K) Transgenic line (D2 or D4) and days (d) of 4OHT treatment (medium dose) are shown.

(A–F) Green, BrdU; red, keratin 14. In (A), locations of sebaceous glands (SG), hair follicle outer root sheath (ORS), bulge (BG), and bulb (BL) are indicated; arrows show scattered LRC in SG and IFE.

(G) Double labeling for Ki67 (green) and LRC (red).

(H–K) K15 expression.

(H) Wild-type epidermis stained with secondary antibody alone, showing nonspecific SG labeling.

(I–K) Double labeling for K15 (red) and LRC (green). No SG remain in (J) and (K).

(L–P) Lineage tracing in K14CreER × R26R × K14ΔNβ-cateninER D2 transgenics treated with 1.5 mg 4OHT for 2 weeks. Blue, XGal; brown, CDP.

(L and M) Back skin.

(N–P) Whole mounts of tail epidermis. Position of HF ORS is indicated in (H) and (N). Ectopic HF in IFE, identified by CDP expression, were either completely XGal positive (white asterisks), completely negative (red asterisks), or mixed (black asterisks). Box in (N) shows region of IFE with ectopic CDP expression; similar regions of IFE from other whole mounts are shown at higher magnification in (O) and (P). Scale bars equal 100 μm in (A)–(H) and (J)–(N) and 50 μm in (I).

ined for expression of CDP and β-galactosidase in conventional sections (Figures 4L and 4M) and whole mounts (Figures 4N–4P).

The efficiency of K14CreER-mediated recombination depended on the dose of 4OHT (A.G. and F.M.W., unpublished observations). Under the 4OHT treatment conditions used, β-catenin was activated in all K14-positive cells (Figures 1F–1K), but there was patchy induction of β-galactosidase (Figures 4M and 4N). CDP-positive, ΔNβ-cateninER-positive epithelial outgrowths in the IFE were either uniformly β-galactosidase positive (white asterisks), uniformly negative (red asterisks), or a mixture of positive and negative cells when at the boundary between recombined and nonrecombined IFE regions (black asterisks) (Figures 4L, 4M, 4O, and 4P). The β-galactosidase status of ectopic follicles nearly always matched that of the surrounding IFE (Figures 4N–4P). This strongly suggests that the majority of new follicles were derived from immediately adjacent IFE rather than from neighboring hair follicles, supporting the conclusion that bulge LRC were not involved.

β-Catenin-Induced Follicles Can Undergo Cycles of Growth and Regression and Provide Niches for Neural Crest-Derived Cells

To evaluate whether ΔNβ-cateninER-induced follicles could undergo cycles of growth and regression, we induced new follicles in D2 epidermis with 1.5 mg 4OHT

for 3 weeks, left the skin untreated for 3 weeks, and then applied 4OHT for a further 1 or 2 weeks (Figures 5A–5F). Withdrawal of 4OHT caused preexisting follicles to cease the growth phase (anagen) of the hair cycle and enter the resting phase (Figures 5A and 5B) and caused ectopic follicles to regress (Figures 5A and 5B). When 4OHT was reapplied, the original follicles reentered anagen (Figure 5D); ectopic follicles regrew and were wider than before (Figures 5A, 5C, and 5D). Occasionally three distinct hair shafts were found associated with a single follicle, excluding the possibility that the regrowth phenotype was due simply to existing club hairs reentering anagen (Figure 5E).

On reapplication of 4OHT, the number of sites of ectopic CDP in the ORS returned to the same level as after the first treatment (Figure 5F), suggesting that the original ectopic follicles regrew. The reason why on regrowth the ectopic follicles were wider than before may be that dermal papilla cells were induced over a wider region than following the first treatment (data not shown). Although some β-catenin-induced follicles do form a mature hair shaft (Figure 5E; Gat et al., 1998; Lo Celso et al., 2004), the regrowth phenotype we observed could not be interpreted as a true hair growth cycle (Millar, 2002), since it was entirely 4OHT dependent, and the rudimentary outgrowths, while positive for CDP, Lef1, and K17, usually lacked the inner root sheath marker trichohyalin (Lo Celso et al., 2004).
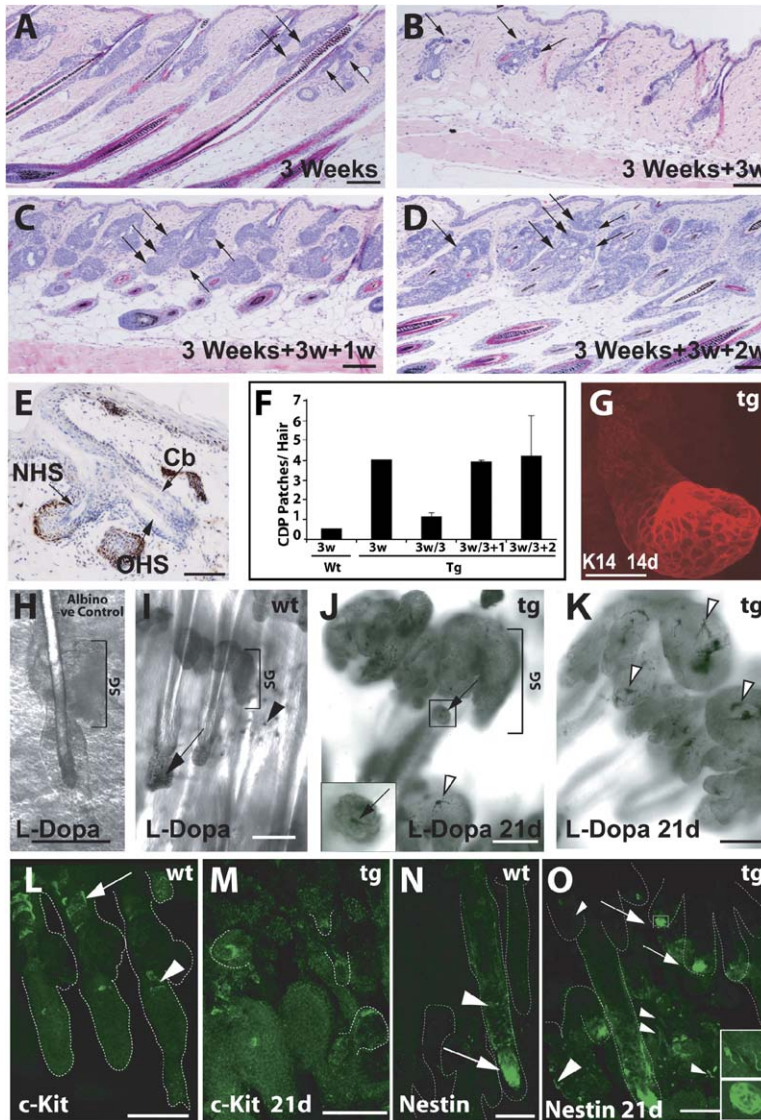
Figure 5. β-Catenin-Induced Hair Follicles Can Undergo Cycles of Growth and Regression and Provide a Niche for Melanocytes and Dermal Papilla Cells

(A–E) H&E-stained sections of D2 transgenic dorsal epidermis treated with 4OHT for 3 weeks, then harvested immediately (A), left untreated for 3 weeks (+3w) and harvested (B), or treated with 4OHT for a further 1 (+1w) (C) or 2 (+2w) (D and E) weeks. CDP staining in brown (E). Arrows in (A)–(D) show ectopic follicles. Arrows in (E) show hair shaft formation; Cb, club hair; OHS, old hair shaft; NHS, new hair shaft.

(F) Number of ectopic CDP-positive outgrowths per existing HF in experiment illustrated in (A)–(E). Error bars correspond to standard deviation.

(G–O) Whole mounts of wild-type (wt) and D2 transgenic (tg) tail epidermis, untreated (H) or treated with a low (G) or medium (I–O) dose of 4OHT for number of days shown. Epidermis was labeled with L-Dopa (H–K) or with antibodies to the proteins shown. In (H), albino wild-type mouse is negative control for L-Dopa staining. Brackets in (H)–(J) indicate SG.

(G) Cuff of keratinocytes at base of ectopic follicle into which dermal papilla cells insert.

(I) Melanocytes in follicle bulb (arrow) and IFE (arrowhead).

(J and K) Melanocytes in HF induced from SG (white arrowhead) and ORS (arrow) are shown. Insert in (J) shows high-magnification view of ectopic HF arising from ORS, with melanocyte indicated by arrow.

(L) c-Kit-positive cells in infundibulum (arrow) and bulge (arrowhead) are shown; HF and SG are indicated by dotted line.

(M) Dotted lines demarcate new HF.

(N and O) Arrows indicate nestin-positive dermal papilla cells; arrowheads show scattered nestin-positive cells in ORS (N) and IFE (O). Upper inset in (O) shows dendritic nestin-positive cells in ORS and lower inset shows clustered nestin-positive cells in dermal papilla of an ectopic HF. Dotted lines demarcate HF and SG.

Scale bars equal 100 μm in (A)–(E) and (H)–(M) and 50 μm in (G).

Normal hair follicles provide a niche for neural crest derivatives such as melanocytes (Nishimura et al., 2002). We visualized the tyrosinase activity of differentiated melanocytes using L-Dopa as substrate. Albino mouse epidermis provided a negative control (Figure 5H). In wild-type tail epidermis, differentiated melanocytes were concentrated at the base of the hair follicles (Figure 5I, arrow), as reported previously (Nishimura et al., 2002), and small numbers were also scattered in the IFE (Figure 5I, arrowhead). L-Dopa-positive melanocytes were present in the ectopic follicles induced by β-catenin in SG (Figures 5J and 5K, arrowheads), IFE, and HF (insert) (Figures 5J and 5K).

Undifferentiated melanocytes (melanoblasts) were detected with anti-c-Kit antibody (Nishimura et al., 2002). In wild-type epidermis, there were c-Kit-positive cells in the HF infundibulum (arrow) and the bulge (arrowhead) (Figure 5L). In 4OHT-treated ΔNβ-cateninER

epidermis, c-Kit-positive cells appeared in IFE immediately adjacent to the HF after 9 days of 4OHT treatment (data not shown), and by 21 days they were found in ectopic HF induced in all regions of the epidermis (Figure 5M, dotted line indicates position of ectopic follicles).

In whole mounts of 4OHT-treated K14ΔNβ-cateninER epidermis, a cuff of keratinocytes surrounding an indentation at the base of the new follicles indicated dermal papilla formation (Figures 2D and 5G). Dermal papilla cells in wild-type and 4OHT-treated transgenic skin expressed not only alkaline phosphatase (data not shown; see Lo Celso et al., 2004) but also nestin, a marker of neural crest-derived stem cells (Figure 5N, arrow; Figure 5O, lower insert; Fernandes et al., 2004). In addition, small numbers of nestin-positive cells were scattered along the length of wild-type and induced follicles (Figures 5N and 5O, arrowheads; upper inset in
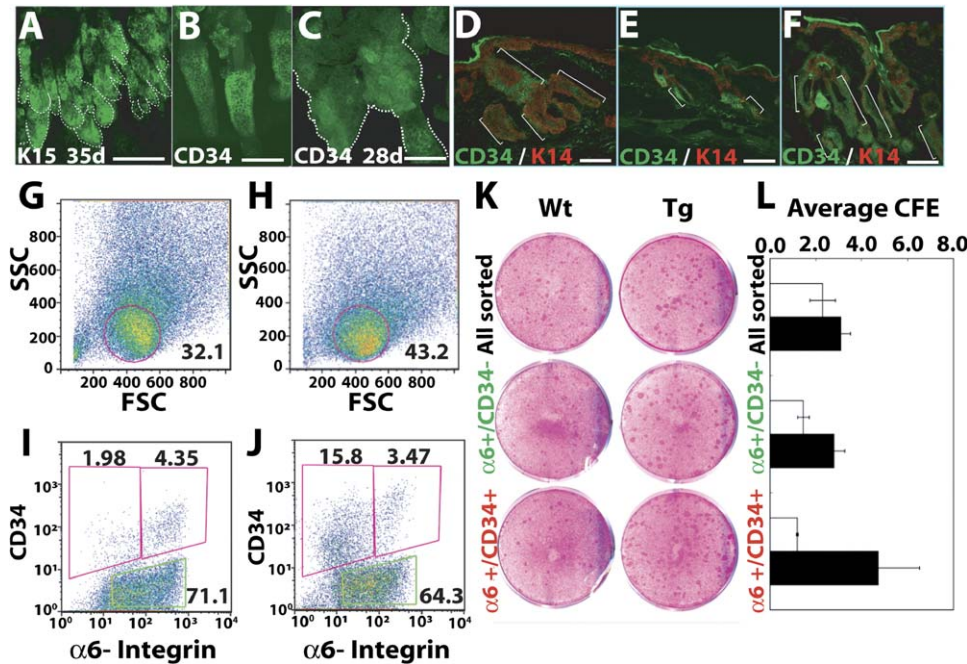
Figure 6. β-Catenin Activation Induces Formation of Keratinocytes that Have Characteristics of Bulge Stem Cells

(A–F) Whole mounts (A–C) and sections from tail (D) or back (E and F) epidermis of wt untreated (B and E) or D2 transgenic mice treated with 1.5 mg 4OHT for number of days shown or for 21 days. Ectopic follicles are demarcated with dotted lines in (A) and (C). Brackets indicate position of bulge in untreated follicle (E) and more extensive CD34 labeling following β-catenin activation (D and F). Scale bars equal 100 μm in (A)–(F).

(G–L) FACS selection and culture of keratinocytes from dorsal epidermis of adult wild-type (G, I, K, L) and D2 transgenic (H, J–L) mice treated with 1.5 mg 4OHT for 3 weeks. Cells isolated from epidermis were double labeled for CD34 and α6 integrin. Cells with low forward and side scatter (circled in [G] and [H]) were either sorted directly onto culture dishes (All sorted, [K] and [L]) or further fractionated into α6 single positive (green boxes in [I] and [J]) or CD34/α6 double positive populations (red boxes in [I] and [J]). In (I) and (J), left red boxes show cells with lower surface α6 than in right red boxes.

(K and L) Colony-forming efficiency of FACS-sorted keratinocytes.

(K) Dishes stained with Rhodamine B.

(L) Representative of two separate experiments, in each of which cells from triplicate wild-type or transgenic mice were pooled. White bars, wild-type; black bars, transgenic cells. Standard deviations shown.

Figure 5O); these were dendritic in morphology and lacked the Merkel cell marker K18 (data not shown).

Cells in β-Catenin-Induced Hair Follicles Express Markers of Bulge Stem Cells

To investigate whether β-catenin-induced HF contained cells with characteristics of bulge stem cells, we stained whole mounts and histological sections of skin with antibodies to K15 and CD34 (Figures 6A–6F and data not shown). In wild-type follicles, expression of both markers was confined to the permanent portion of the follicle (Figures 4I, 6B, and 6E; Morris et al., 2004; Trempus et al., 2003). In tail and back epidermis from D2 and D4 mice treated with 4OHT for 28 days, expression of both markers was observed in all sites of new HF formation (Figures 6A, 6C, 6D, and 6F and data not shown), often extending along the length of the new follicles. CD34 staining in ectopic follicles was slightly weaker than in the preexisting bulge (Figures 6E and 6F).

We performed FACS of disaggregated keratinocytes from back (Figures 6G–6L) or tail (data not shown) skin, gating out the differentiated cells on the basis of high forward and side scatter (Figures 6G and 6H; Jones et al., 1995). The majority of undifferentiated cells (inside red circles in Figures 6G and 6H) expressed α6 integrin (Figures 6I and 6J and data not shown). In 4OHT-treated wild-type epidermis, approximately 4% of undifferentiated cells expressed high levels of both CD34 and α6 integrin, while 2% were CD34 positive and had low levels of α6 integrin (Figure 6I). In D2 epidermis, the proportion of cells with high CD34 and α6 integrin was similar (3.5% in the profile shown), but the CD34-positive/α6 integrin-low population was increased to about 15% (Figure 6J).

Three cell fractions were selected for in vitro clonal analysis (Figure 6K): the total low forward and side scatter population ("All sorted"), α6-positive/CD34-negative cells (green gates, Figures 6I and 6J), and α6/CD34 double-positive cells, irrespective of whether integrin expression was high or low (red gates, Figures 6I and 6J). After 14 days of culture without 4OHT, there was no significant difference between the colony-forming efficiency of each fraction in wild-type dorsal epidermis (Figures 6K and 6L). In transgenic cultures, the colony-forming efficiency of the total and the α6-positive/CD34-negative population was slightly increased, while

the double-positive population had twice the colony-forming efficiency of wild-type (Figure 6L). In all three fractions, the size of individual colonies was larger in transgenic compared to wild-type keratinocytes (Figure 6K). We conclude that β-catenin-induced HF contain cells with three characteristics of bulge stem cells: clonal growth in culture and expression of K15 and CD34.

Discussion

By expressing different levels of ΔNβ-cateninER in epidermal cells and applying different concentrations of 4OHT, we can control the level of β-catenin transcriptional activation. The concept that cells exhibit a graded response to a gradient of β-catenin regulatory activity was previously established in studies of ES cell lines with different APC mutations (Kielman et al., 2002). However, the ΔNβ-cateninER transgene has the advantage that transcriptional activity can be titrated within individual cells, both in vivo and in culture.

As Figure 2 illustrates, the response of adult epidermis to β-catenin activation was more complex than a simple switching on of de novo HF formation. With increasing β-catenin activation, the timing and location of ectopic HF formation were altered. In both transgenic lines, the ORS, including the bulge, of existing HF was most refractory to induction in tail epidermis. This is consistent with evidence that bulge cells are quiescent cells in an environment that makes them refractory to a range of signaling pathways (Tumbar et al., 2004). Factors that are upregulated in the bulge include the Wnt antagonist Dickkopf-3 (Morris et al., 2004; Tumbar et al., 2004) and Tcf3, which can repress Wnt signaling (Merrill et al., 2001).

During embryonic development, there is a complex interplay between Hedgehog and Wnt signaling (Taipale and Beachy, 2001), and inappropriate activation of either pathway is associated with a range of epidermal cancers (Oro and Higgins, 2003; Gat et al., 1998; Lo Celso et al., 2004; Niemann et al., 2003). Shh was upregulated in response to β-catenin activation in K14ΔNβ-cateninER epidermis, and inhibition of Shh with cyclopamine reduced β-catenin-induced HF formation in D2 transgenic mice.

In the absence of cyclopamine, 4OHT-induced HF morphogenesis progressed further in D2 than in D4 mice, resulting in induction of dermal papillae and epithelial outgrowths from the ORS. In D2 epidermis, lineage reprogramming was accompanied by only local increases in proliferation, whereas in D4 epidermis, proliferation was more widespread and the follicles became grossly thickened. The ability of cyclopamine to convert the D4 to the D2 phenotype suggests that the D4 phenotype results from Hedgehog-induced proliferation being too extensive to allow proper morphogenesis. Hedgehog antagonists may be useful in the treatment of epidermal tumors in which either Wnt or Hedgehog signaling is activated (Niemann et al., 2003; Taipale and Beachy, 2001).

Ectopic HF formation preceded any obvious change in proliferation or migration of bulge LRC. The effects of c-Myc and ΔNLef1 on lineage selection also precede

proliferation of LRC (Braun et al., 2003), and we have not observed migration of LRC upwards toward the SG and IFE in any of these models (cf. Oshima et al., 2001; Taylor et al., 2000). Even when LRC eventually divided in response to prolonged β-catenin activation, thereby losing the BrdU label, the location of the original bulge was still detectable by expression of K15 and CD34 (Morris et al., 2004; Trempus et al., 2003).

Even though new follicle formation preceded proliferation of LRC, we could not formally exclude the possibility that very small numbers of LRC, perhaps even a single cell, were required to found new follicles in the IFE. Lineage tracing experiments, however, showed that this was unlikely. Whenever a new CDP-positive epithelial outgrowth formed in the IFE, its β-galactosidase expression matched the surrounding IFE. Furthermore, when the new follicle formed at the boundary between β-galactosidase-positive and -negative regions, that follicle would be part β-galactosidase positive and part β-galactosidase negative, indicating that it was not clonal in origin. It has long been proposed that mouse IFE is organized into "epidermal proliferative units": at the base of each lies a single stem cell surrounded by committed progenitors (transit amplifying cells) (Potten and Morris, 1988). If this is indeed the case, the implication of the nonclonal origin of IFE hair follicles is that IFE-committed progenitors can be redirected to adopt a HF fate. Alternatively, IFE stem cells in mouse, as in human (Jones et al., 1995), may be clustered.

Remarkably, not only was new follicle formation initiated without any detectable involvement of existing bulge cells, but new follicles contained cells that expressed K15 and CD34 and were clonogenic in culture. Thus, cells with bulge stem cell characteristics can be formed from nonbulge epidermal cells. Ectopic follicles could undergo 4OHT-dependent cycles of growth and regression. In response to reapplication of 4OHT, there was no further increase in the number of HF, but the ectopic follicles enlarged. This implies that there is some mechanism to limit the number of new follicles: it might involve the same type of intercellular signaling events that control the spacing of new follicles during embryonic development (Millar, 2002).

The generation of new HF from IFE leads us to propose that given the appropriate signal, any proliferation-competent cell can form a new HF expressing both ORS and bulge markers. β-catenin activation in cultured human epidermal cells increases the number of stem cells (Zhu and Watt, 1999); whether this occurs through expansion of the preexisting stem cell pool, as we originally proposed, or through generation of new stem cells from committed progenitors remains to be explored (Pearton et al., 2004). The increased size of the colonies formed by K14ΔNβ-cateninER keratinocytes is interesting: since the cells were cultured in the absence of 4OHT, it must reflect an intrinsic increase in proliferative potential. A role of Wnt signaling in increasing stem cell number has now been reported in a wide range of tissues, including the blood, mammary gland, and nervous system (Liu et al., 2004; Megason and McMahon, 2002; Reya et al., 2003).

In addition to expressing bulge markers, β-catenin-induced follicles were populated by melanocytes and formed dermal papillae. The ability of ectopic follicles

to provide a home for melanocytes is of interest because melanocyte stem cells are dependent on interactions with the HF bulge (Nishimura et al., 2002). It is also intriguing that both melanocytes and dermal papilla cells are neural crest derived (Fernandes et al., 2004) and Wnt dependent. Wnt1 signaling is involved in the expansion and differentiation of melanocytes, and ablation of β-catenin in neural crest stem cells leads to lack of melanocytes (Le Douarin and Dupin, 2003).

Epidermal stem cells rarely feature in accounts of adult stem cell populations that exhibit plasticity (Niemann and Watt, 2002; Wagers and Weissman, 2004). This is probably because when epidermal cells are separated from their neighbors and deprived of extracellular matrix attachment, they withdraw irreversibly from the cell cycle and initiate terminal differentiation (Watt, 2002). Nevertheless, the finding that epidermal β-catenin activation induces dermal papillae opens up an unexpected way in which the epidermis could influence the homing, localization, and properties of a range of different cell types. Adult dermal cells have multilineage potential, including formation of neural precursors (Fernandes et al., 2004) and cells of the erythroid and myeloid lineages (Lako et al., 2002). Not only can epidermal β-catenin signaling control selection of the epidermal lineages, it may also stimulate the differentiation of other cell types by re-creating some of the inductive interactions that occur during normal epidermal development.

Experimental Procedures

Generation and Experimental Treatment of Mice

The D2 and D4 lines of K14ΔNβ-cateninER transgenic mice were generated as described (Lo Celso et al., 2004). For lineage marking studies, the D2 line was crossed with mice that were homozygous for the Rosa26 Cre reporter (R26R) (Soriano, 1999) and K14CreER (Vasioukhin et al., 1999; Hong et al., 2004). At the start of each experiment, the mice were 6 to 8 weeks old, and therefore in the resting phase of the hair cycle. Wild-type, nontransgenic littermates were used as controls.

The ΔNβ-cateninER transgene was activated by topical application of 4-hydroxytamoxifen (4OHT; Sigma) dissolved in acetone. Tail skin was treated by applying 4OHT with a paintbrush (0.5, 1.5, or 3 mg per mouse every second day). For the microarray experiment, 1 mg 4OHT was applied with a micropipette to a clipped area of dorsal skin either once or every day for 7 days. In some experiments, mice received daily topical applications of cyclopamine (50 μM in ethanol) and 4OHT every second day. LRC were generated as described previously (Braun et al., 2003).

Antibodies

The antibodies used have been described previously (Braun et al., 2003; Niemann et al., 2003; Lo Celso et al., 2004), except for the following: anti-CD34 (RAM34; BD Pharmingen), c-Kit (01902-D; Pharmingen), and nestin (nestin 130; gift from R. McKay) (Marvin et al., 1998).

Histochemistry, Immunolabeling of Sections, and Whole Mounts

Conventional frozen or fixed, paraffin-embedded sections were prepared and immunolabeled as described previously (Lo Celso et al., 2004). LacZ staining was performed with X-Gal as described previously (Soriano, 1999); when sections were double labeled, the antibody incubation was performed after the X-Gal incubation and the antibody was visualized with DAKO Visualization Systems (Lo Celso et al., 2004).

Whole mounts of tail epidermal sheets were prepared and immunolabeled as described previously (Braun et al., 2003; Niemann et al., 2003). To visualize melanocytes, epidermal sheets were incubated with 0.2% L-Dopa (Acros Organics, Netherlands) for 3–16 hr at 37°C in the dark. Images were obtained using a Zeiss 510 confocal microscope as described by Braun et al. (2003).

To quantify CDP expression in whole mounts, 30–50 units of epidermis (Figure 1L) per tail were scored in three separate mice for each experimental condition. The percentage of units with ectopic CDP expression and the number of ectopic CDP-positive patches per unit ± SEM were determined. Within each unit IFE, SG, and HF were scored separately.

Western Blotting and Luciferase Assay

Spontaneously immortalized keratinocyte lines were generated from adult mouse skin and cultured with a feeder layer, essentially as described previously (Romero et al., 1999), except that skin trypsinization was carried out at 4°C and cells were disaggregated without stirring. Cell lysis and Western blotting conditions are described in Supplemental Data.

Keratinocytes were transiently transfected with Promega luciferase reporter constructs pRL (Renilla luciferase control) and TOPFLASH or FOPFLASH (firefly luciferase) using Genejuice (Novagen). Luciferase activities were measured as described previously (Lo Celso et al., 2004).

FACS and Clonogenicity Assay

Keratinocytes were isolated and cultured from adult dorsal skin as described by Romero et al. (1999), with the minor modifications described above. Three further changes were made to increase colony-forming efficiency: keratinocytes were grown on collagen-coated dishes (BD Biocoat), the feeder layer was confluent, and the basal FAD culture medium was calcium free. 1000–5000 keratinocytes were plated per 35 mm dish. After 14 days, the cultures were fixed with 4% PFA and stained with 1% Rhodamine B. Colonies were defined as clusters of five or more keratinocytes.

For two-color FACS, cell suspensions were incubated with FITC or biotin-conjugated primary antibodies for 30 min on ice, washed twice for 10 min at 4°C, then incubated with streptavidin PE for 30 min on ice. Cell viability was assessed by 7AAD (BD Bioscience) staining. Cells were sorted with a FACSVantage sorter according to CD34 and α6 integrin expression (Morris et al., 2004) after gating out dead cells and cells with high forward and side scatter.

Supplemental Data

Supplemental Data include two tables and Supplemental Experimental Procedures and can be found with this article online at http://www.developmentalcell.com/cgi/content/full/9/1/121/DC1/.

References

Arnold, I., and Watt, F.M. (2001). c-Myc activation in transgenic mouse epidermis results in mobilization of stem cells and differentiation of their progeny. Curr. Biol. 11, 558–568.

Braun, K.M., Niemann, C., Jensen, U.B., Sundberg, J.P., Silva-Vargas, V., and Watt, F.M. (2003). Manipulation of stem cell proliferation

and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis. Development *130*, 5241–5255.

Duman-Scheel, M., Weng, L., Xin, S., and Du, W. (2002). Hedgehog regulates cell growth and proliferation by inducing Cyclin D and Cyclin E. Nature *417*, 299–304.

Fernandes, K.J., McKenzie, I.A., Mill, P., Smith, K.M., Akhavan, M., Barnabe-Heider, F., Biernaskie, J., Junek, A., Kobayashi, N.R., Toma, J.G., et al. (2004). A dermal niche for multipotent adult skin-derived precursor cells. Nat. Cell Biol. *6*, 1082–1093.

Fuchs, E., Tumbar, T., and Guasch, G. (2004). Socializing with the neighbors: stem cells and their niche. Cell *116*, 769–778.

Gat, U., DasGupta, R., Degenstein, L., and Fuchs, E. (1998). De novo hair follicle morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin. Cell *95*, 605–614.

Ghazizadeh, S., and Taichman, L.B. (2001). Multiple classes of stem cells in cutaneous epithelium: a lineage analysis of adult mouse skin. EMBO J. *20*, 1215–1222.

Hong, K.U., Reynolds, S.D., Watkins, S., Fuchs, E., and Stripp, B.R. (2004). Basal cells are a multipotent progenitor capable of renewing the bronchial epithelium. Am. J. Pathol. *164*, 577–588.

Jones, P.H., Harper, S., and Watt, F.M. (1995). Stem cell patterning and fate in human epidermis. Cell *80*, 83–93.

Kielman, M.F., Rindapaa, M., Gaspar, C., van Poppel, N., Breukel, C., van Leeuwen, S., Taketo, M.M., Roberts, S., Smits, R., and Fodde, R. (2002). Apc modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. Nat. Genet. *32*, 594–605.

Lako, M., Armstrong, L., Cairns, P.M., Harris, S., Hole, N., and Jahoda, C.A. (2002). Hair follicle dermal cells repopulate the mouse haematopoietic system. J. Cell Sci. *115*, 3967–3974.

Le Douarin, N.M., and Dupin, E. (2003). Multipotentiality of the neural crest. Curr. Opin. Genet. Dev. *13*, 529–536.

Liu, B.Y., McDermott, S.P., Khwaja, S.S., and Alexander, C.M. (2004). The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc. Natl. Acad. Sci. USA *101*, 4158–4163.

Lo Celso, C., Prowse, D.M., and Watt, F.M. (2004). Transient activation of beta-catenin signalling in adult mouse epidermis is sufficient to induce new hair follicles but continuous activation is required to maintain hair follicle tumours. Development *131*, 1787–1799.

Marvin, M.J., Dahlstrand, J., Lendahl, U., and McKay, R.D. (1998). A rod end deletion in the intermediate filament protein nestin alters its subcellular localization in neuroepithelial cells of transgenic mice. J. Cell Sci. *111*, 1951–1961.

Megason, S.G., and McMahon, A.P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. Development *129*, 2087–2098.

Merrill, B.J., Gat, U., DasGupta, R., and Fuchs, E. (2001). Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. Genes Dev *15*, 1688–1705.

Millar, S.E. (2002). Molecular mechanisms regulating hair follicle development. J. Invest. Dermatol. *118*, 216–225.

Morris, R.J., Liu, Y., Marles, L., Yang, Z., Trempus, C., Li, S., Lin, J.S., Sawicki, J.A., and Cotsarelis, G. (2004). Capturing and profiling adult hair follicle stem cells. Nat. Biotechnol. *22*, 411–417.

Niemann, C., and Watt, F.M. (2002). Designer skin: lineage commitment in postnatal epidermis. Trends Cell Biol. *12*, 185–192.

Niemann, C., Unden, A.B., Lyle, S., Zouboulis, C.C., Toftgard, R., and Watt, F.M. (2003). Indian hedgehog and β-catenin signaling: role in the sebaceous lineage of normal and neoplastic mammalian epidermis. Proc. Natl. Acad. Sci. USA *100* Suppl. 1, 11873–11880.

Nishimura, E.K., Jordan, S.A., Oshima, H., Yoshida, H., Osawa, M., Moriyama, M., Jackson, I.J., Barrandon, Y., Miyachi, Y., and Nishikawa, S. (2002). Dominant role of the niche in melanocyte stem-cell fate determination. Nature *416*, 854–860.

Oliver, T.G., Grasfeder, L.L., Carroll, A.L., Kaiser, C., Gillingham, C.L., Lin, S.M., Wickramasinghe, R., Scott, M.P., and Wechsler-Reya, R.J. (2003). Transcriptional profiling of the Sonic hedgehog response: a critical role for N-myc in proliferation of neuronal precursors. Proc. Natl. Acad. Sci. USA *100*, 7331–7336.

Oro, A.E., and Higgins, K. (2003). Hair cycle regulation of Hedgehog signal reception. Dev. Biol. *255*, 238–248.

Oshima, H., Rochat, A., Kedzia, C., Kobayashi, K., and Barrandon, Y. (2001). Morphogenesis and renewal of hair follicles from adult multipotent stem cells. Cell *104*, 233–245.

Pearton, D.J., Ferraris, C., and Dhouailly, D. (2004). Transdifferentiation of corneal epithelium: evidence for a linkage between the segregation of epidermal stem cells and the induction of hair follicles during embryogenesis. Int. J. Dev. Biol. *48*, 197–201.

Potten, C.S., and Morris, R.J. (1988). Epithelial stem cells in vivo. J. Cell Sci. Suppl. *10*, 45–62.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409–414.

Romero, M.R., Carroll, J.M., and Watt, F.M. (1999). Analysis of cultured keratinocytes from a transgenic mouse model of psoriasis: effects of suprabasal integrin expression on keratinocyte adhesion, proliferation and terminal differentiation. Exp. Dermatol. *8*, 53–67.

Soriano, P. (1999). Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. *21*, 70–71.

Taipale, J., and Beachy, P.A. (2001). The Hedgehog and Wnt signalling pathways in cancer. Nature *411*, 349–354.

Taylor, G., Lehrer, M.S., Jensen, P.J., Sun, T.T., and Lavker, R.M. (2000). Involvement of follicular stem cells in forming not only the follicle but also the epidermis. Cell *102*, 451–461.

Trempus, C.S., Morris, R.J., Bortner, C.D., Cotsarelis, G., Faircloth, R.S., Reece, J.M., and Tennant, R.W. (2003). Enrichment for living murine keratinocytes from the hair follicle bulge with the cell surface marker CD34. J. Invest. Dermatol. *120*, 501–511.

Tumbar, T., Guasch, G., Greco, V., Blanpain, C., Lowry, W.E., Rendl, M., and Fuchs, E. (2004). Defining the epithelial stem cell niche in skin. Science *303*, 359–363.

van den Brink, G.R., Bleuming, S.A., Hardwick, J.C., Schepman, B.L., Offerhaus, G.J., Keller, J.J., Nielsen, C., Gaffield, W., van Deventer, S.J., Roberts, D.J., and Peppelenbosch, M.P. (2004). Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. Nat. Genet. *36*, 277–282.

Van Mater, D., Kolligs, F.T., Dlugosz, A.A., and Fearon, E.R. (2003). Transient activation of beta-catenin signaling in cutaneous keratinocytes is sufficient to trigger the active growth phase of the hair cycle in mice. Genes Dev. *17*, 1219–1224.

Vasioukhin, V., Degenstein, L., Wise, B., and Fuchs, E. (1999). The magical touch: genome targeting in epidermal stem cells induced by tamoxifen application to mouse skin. Proc. Natl. Acad. Sci. USA *96*, 8551–8556.

Wagers, A.J., and Weissman, I.L. (2004). Plasticity of adult stem cells. Cell *116*, 639–648.

Watt, F.M. (2002). Role of integrins in regulating epidermal adhesion, growth and differentiation. EMBO J. *21*, 3919–3926.

Zhu, A.J., and Watt, F.M. (1999). β-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. Development *126*, 2285–2298.