

Manipulation of stem cell proliferation and lineage commitment: visualisation of label-retaining cells in wholemounts of mouse epidermis

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Accepted 2 July 2003

Development 130, 5241-5255
© 2003 The Company of Biologists Ltd
doi:10.1242/dev.00703

Summary

Mammalian epidermis is maintained by stem cells that have the ability to self-renew and generate daughter cells that differentiate along the lineages of the hair follicles, interfollicular epidermis and sebaceous gland. As stem cells divide infrequently in adult mouse epidermis, they can be visualised as DNA label-retaining cells (LRC). With whole-mount labelling, we can examine large areas of interfollicular epidermis and many hair follicles simultaneously, enabling us to evaluate stem cell markers and examine the effects of different stimuli on the LRC population. LRC are not confined to the hair follicle, but also lie in sebaceous glands and interfollicular epidermis. LRC reside throughout the permanent region of the hair follicle, where they express keratin 15 and lie in a region of high $\alpha\beta4$ integrin expression. LRC are not significantly depleted by successive hair growth cycles. They can,

nevertheless, be stimulated to divide by treatment with phorbol ester, resulting in near complete loss of LRC within 12 days. Activation of Myc stimulates epidermal proliferation without depleting LRC and induces differentiation of sebocytes within the interfollicular epidermis. Expression of N-terminally truncated Lef1 to block β -catenin signalling induces transdifferentiation of hair follicles into interfollicular epidermis and sebocytes and causes loss of LRC primarily through proliferation. We conclude that LRC are more sensitive to some proliferative stimuli than others and that changes in lineage can occur with or without recruitment of LRC into cycle.

Key words: Myc, Lef1, Hair follicle, Hair cycle, Stem cells, Epidermis, Sebocytes, Differentiation, Label-retaining cells, β -catenin

Introduction

Adult mammalian epidermis is maintained by stem cells that have the ability to self-renew and also to generate daughter cells that differentiate along the lineages of the hair follicle (HF), interfollicular epidermis (IFE) and sebaceous gland. Stem cells within the hair follicle can produce progeny that not only differentiate into hair, but into sebocytes and interfollicular epidermis as well (Taylor et al., 2000; Oshima et al., 2001). However, there is also evidence for the existence of distinct stem cell populations within the IFE and sebaceous gland (Ghazizadeh and Taichman, 2001). These observations can be reconciled by proposing that there are separate stem cell populations within the hair, sebaceous gland and IFE. Each of these is capable of generating daughters that differentiate along any of the epidermal lineages, but normally gives rise to a more restricted repertoire in response to signals from the local microenvironment (Ferraris et al., 1997; Niemann and Watt, 2002).

Epidermal stem cells can be distinguished from their non-stem progeny because they not only lack expression of markers of terminal differentiation, but also have distinct proliferative

characteristics. In particular, they divide infrequently in undamaged, steady-state epidermis (Potten and Morris, 1988; Cotsarelis et al., 1990), yet have the ability to undergo considerable proliferation in response to stimuli such as wounding. These properties form the basis of two different approaches to the identification of stem cells. The first is to give neonatal mice repeated injections of ³H-thymidine or 5-bromo-2'-deoxyuridine (BrdU) to label all the dividing cells in the epidermis at a time of rapid tissue expansion and then to identify those cells that do not divide subsequently and thus retain the label into adulthood (label-retaining cells, LRC) (Bickenbach, 1981; Morris et al., 1985; Bickenbach et al., 1986; Cotsarelis et al., 1990; Bickenbach and Chism, 1998; Morris and Potten, 1999). The second is to place epidermal cells in culture and perform clonal analysis: stem cells form large, self-renewing clones, whereas their non-stem daughters (known as transit amplifying cells or committed progenitors) (Potten and Morris, 1988; Watt, 1998) form abortive clones, because they divide a small number of times, then withdraw from the cell cycle and undergo terminal differentiation (Barrandon and Green, 1987; Jones and Watt, 1993; Morris and Potten, 1994). From experiments in which LRC and clonogenic

cells have been compared directly there is evidence that stem cells do indeed share label-retaining and clone-forming ability (Morris and Potten, 1994; Oshima et al., 2001).

Postnatal hair follicles undergo repeated rounds of growth (anagen), regression (catagen) and quiescence (telogen) (Hardy, 1992; Stenn and Paus, 2001). During catagen, the keratinocytes in the lower (cycling) region of the follicle are destroyed, leaving only the non-cycling upper one-third of the hair follicle intact, along with the mesenchymal cells of the dermal papilla. In mammalian hair follicles, stem cells are concentrated in the non-cycling region of the follicle in a specialised region of the outer root sheath (Cotsarelis et al., 1990; Lavker et al., 1993; Lyle et al., 1998; Morris and Potten, 1999; Akiyama et al., 2000; Taylor et al., 2000; Fuchs et al., 2001; Oshima et al., 2001). This region is defined by the point of insertion of the arrector pili muscle (Cotsarelis et al., 1990) and is known as the bulge.

The availability of promoters that target transgene expression to the epidermis has provided a wealth of information about the molecules that regulate epidermal self-renewal and differentiation (Fuchs and Raghavan, 2002; Niemann and Watt, 2002). However, the number of label-retaining cells is so small that it has proved hard to examine the effects of the transgenes on the epidermal stem cell compartment directly. One approach is to view LRC by preparing serial reconstructions of individual hair follicles (Morris and Potten, 1999); however, this is an extremely laborious technique that is not amenable to the screening of large numbers of follicles or large areas of IFE. In human epidermis, visualisation of the stem cell compartment has been facilitated by preparation of wholemounts, in which the epidermis is separated from the underlying dermis as an intact sheet, then labelled with antibodies to markers of proliferation or differentiation (Jensen et al., 1999). In the present report we describe the development of a similar whole-mount labelling technique for mouse epidermis.

We have used whole-mount labelling to determine the number and location of LRC and as a screen for molecular markers of LRC. We have also examined what happens to LRC during the normal hair growth cycle and when epidermal growth and differentiation are disturbed. By simultaneously visualising all the LRC within large numbers of HF and large areas of the IFE we have clarified some important aspects of their biology.

Materials and methods

Experimental mice

All mouse husbandry and experimental procedures were conducted in compliance with the protocols established by the Cancer Research UK animal ethics committee. K14MycER (founder line 2184c.1) (Arnold and Watt, 2001) and K14 Δ NLef1 (founder line L) (Niemann et al., 2002) transgenic mice have been described previously. These mice have been designated Tg(Krt14-dnLef1)LFmw and Tg(Krt14-MYCER)2184C.1Fmw, respectively, in the Mouse Genomic Informatics database of the Jackson Laboratory (<http://www.informatics.jax.org>). Transgenes were injected into fertilised (CBA \times C57BL/6) F1 embryos; founder mice were backcrossed to establish lines of mice. Wild-type littermates were used as control animals. The MycER transgene was activated in epidermis of adult K14MycER transgenic mice by daily topical application of 4-hydroxytamoxifen (OHT; Sigma) (1 mg OHT/0.2 ml acetone) (Arnold and Watt,

2001). To induce epidermal hyperplasia, tail skin was treated three times per week with 20 nmol of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Alexis Biochemicals, San Diego, CA) in 200 μ l acetone (Schweizer and Winter, 1982).

BrdU labelling

To generate label-retaining cells (LRC), the protocol described by Bickenbach and colleagues (Bickenbach et al., 1986; Bickenbach and Chism, 1998) was used. Ten-day-old mice were injected with 50 mg/kg bodyweight 5-bromo-2'-deoxyuridine (BrdU; 20 μ l of 12.5 mg/ml BrdU) every 12 hours for a total of four injections to label mitotic cells. By injecting neonatal mice we avoided the teratogenic effects of BrdU (Shah and MacKay, 1978; Bannigan et al., 1990). The mice were healthy and the only evidence that they were affected by BrdU was a transient loss of dorsal hair prior to the first postnatal hair cycle.

An extremely high percentage of keratinocytes incorporate the BrdU label because the epidermis is hyperproliferative in 10-day-old mice. Normally, mice were maintained for a minimum of 70 days after the final BrdU injection in order to detect LRC. In some experiments, the localisation of cells containing the BrdU label was assessed after a chase period of less than 70 days. To achieve short-term labelling of cells undergoing DNA synthesis, adult mice received an intraperitoneal injection of 100 mg/kg body weight 5-bromo-2'-deoxyuridine (BrdU; Sigma) 1 hour prior to sacrifice.

Preparation of wholemounts

The method described to prepare human epidermal wholemounts (Jensen et al., 1999) was modified for mouse tissue. To prepare wholemounts of mouse tail epidermis, a scalpel was used to slit the tail lengthways. Skin was peeled from the tail, cut into pieces (0.5 \times 0.5 cm²) and incubated in 5 mM EDTA in PBS at 37°C for four hours. Forceps were used to gently peel the intact sheet of epidermis away from the dermis and the epidermal tissue was fixed in 4% formal saline (Sigma) for 2 hours at room temperature. Fixed epidermal sheets were stored in PBS containing 0.2% sodium azide at 4°C for up to 8 weeks prior to labelling.

To prepare wholemounts from mouse back skin, the skin surface was shaved with electric clippers and treated topically with hair removal cream (ImmacTM) for 5 minutes. Remaining hair was scraped from the dorsal surface and the skin was removed from the mouse. Fat and most of the connective tissue were separated using scissors and the skin was cut into pieces (0.5 \times 0.5 cm²). Skin was incubated in 0.25% trypsin at 4°C for 24 to 48 hours; the epidermis was removed as soon as it could be separated from the dermis as an intact sheet. Fixation and storage conditions were the same as described above.

Antibodies

Rat monoclonal antibodies were used to detect BrdU (Oxford Biotechnology), α 6 integrin (Serotec) and β 1 integrin (MB 1.2, kindly provided by B. Chan) (Von Ballestrem et al., 1996). Polyclonal rabbit antiserum was used to detect keratin 6 (Babco), keratin 10 (Babco), keratin 14 (Babco), keratin 17 (kind gift of P. Coulombe) (McGowan and Coulombe, 1998), Ki67 (Novacastra), CCAAT displacement protein (CDP) (kindly provided by M. Busslinger) (Ellis et al., 2001) and NG2 chondroitin sulphate proteoglycan (Chemicon). Mouse monoclonal antibodies were used to detect BrdU (Becton Dickinson) and keratin 15 (kindly provided by I. Leigh) (Waseem et al., 1999). Secondary antibodies were conjugated to AlexaFluor 488 or AlexaFluor 594 (Molecular Probes).

Histological analysis and immunolabelling of tissue sections

Mice were killed with CO₂. Skin sections were harvested and either fixed overnight in 10% neutral-buffered formalin or frozen, unfixed, in OCT compound (Miles) on a frozen isopentane surface (cooled with liquid nitrogen). Formalin-fixed tissue was transferred to 70%

ethanol, embedded in paraffin wax and sectioned at 5 µm. Paraffin wax and frozen skin sections were stained with Haematoxylin and Eosin (H&E) and then examined microscopically. Lipid-containing cells, including sebocytes, were detected by performing Oil Red O staining on frozen sections essentially as described previously (Catalano and Lillie, 1975). To assess the hair cycle, skin sections were examined and the relative percent of follicles in anagen, catagen or telogen was estimated using criteria described elsewhere (Sundberg et al., 1996; Sundberg et al., 1997; Sundberg and King, 2000).

Double-immunolabelling was used to detect keratin 14 and BrdU in paraffin wax-embedded sections of mouse tail skin. Formalin-fixed sections were deparaffinised in xylene and rehydrated in graded alcohols. Tissue sections were microwaved in 10 mM sodium citrate (pH 6.0) for 3 minutes, incubated for another 15 minutes in the hot solution and rinsed in Automation Buffer (Biomedica, Foster City, CA). Sections were incubated in 2 M HCl at 37°C, washed in borate buffer, and digested in 0.01% trypsin in 0.05 M Tris for 3 minutes at 37°C. After blocking in 10% goat serum for 20 minutes, sections were incubated for 1 hour at room temperature with mouse BrdU antisera (Becton Dickinson; 1:25) and keratin 14 antisera (1:10000) in 1% bovine serum albumin.

Immunolabelling, staining and confocal microscopy of epidermal sheets

Epidermal sheets were blocked and permeabilised by incubation in PB buffer for 30 minutes (Jensen et al., 1999). PB buffer consists of 0.5% skim milk powder, 0.25% fish skin gelatin (Sigma) and 0.5% Triton X-100 in TBS (0.9% NaCl, 20 mM HEPES, pH 7.2). Primary antibodies were diluted in PB buffer and tissue was incubated overnight at room temperature with gentle agitation. Epidermal wholemounts were then washed for at least 4 hours in PBS containing 0.2% Tween 20, changing the buffer several times. Incubation with secondary antibodies was performed in the same way. Samples were rinsed in distilled water and mounted in Gelvatol (Monsanto, St Louis, MO) containing 0.5% 1,4-Diazabicyclo[2.2.2]octane (DABCO) (Sigma). To detect BrdU-labelled cells, after permeabilisation and prior to incubation with the anti-BrdU antibody, epidermal sheets were incubated for 20-30 minutes in 2M HCl at 37°C. Apoptotic cells were detected in epidermal sheets by using the DeadEnd™ Fluorometric TUNEL System (Promega). To stain lipids present in sebaceous glands, epidermal sheets were incubated in Nile Red (0.1 µg/ml in PBS; Sigma) for 30 minutes at room temperature, washed several times in PBS and mounted.

In some experiments, a dissecting microscope was used to isolate individual hair follicles from labelled epidermal sheets. The epidermis was placed in PBS with the basal layer facing upwards. Fine forceps were used to grasp a hair follicle at the infundibulum and the follicle was pulled gently until it separated from the epidermal sheet. Hair follicles were mounted on a slide in Gelvatol with 0.5% DABCO.

Images were acquired using a Zeiss 510 confocal microscope. Approximately 30 optical sections of each epidermal sheet were captured with a typical increment of 1-3 µm. Scans are presented as z-projections. Objectives used were Zeiss 10/NA 0.45, Zeiss 20/NA 0.75 and Zeiss 40/NA 1.2. The samples were scanned from the dermal side towards the epidermal surface to a total thickness of 40-80 µm, which encompassed the epidermis from the hair follicle bulb to the basal layer of interfollicular epidermis.

Results

Development of the whole-mount method to localise LRC

To assess the spatial organisation of stem cells, our objective was to prepare epidermal wholemounts from adult mice that contained LRC. Previous whole-mount preparations have

involved separation of the epidermis from the dermis with cold acetic acid and visualising actively cycling cells or LRC by ³H-thymidine incorporation (Hamilton and Potten, 1972; Schweizer and Marks, 1977a; Morris et al., 1986). The disadvantages of those methods include rupture of the hair follicles at the level of the sebaceous glands (Schweizer and Marks, 1977a) and lack of preservation of epitopes for antibody labelling. We improved the method by harvesting the epidermis with EDTA and labelling cells with BrdU rather than ³H-thymidine, thereby allowing dual-label immunofluorescence with markers of proliferation and differentiation.

In pilot studies, it proved difficult to produce consistently high-quality wholemounts from mouse dorsal skin because of the high density of hair follicles and thin interfollicular epidermis. By contrast, mouse tail skin was amenable to the production of epidermal wholemounts in which the pilosebaceous units remained intact (Fig. 1A). Mouse tail hair follicles are known to be arranged in parallel rows, the follicles being grouped in sets of three (triplets) (Schweizer and Marks, 1977a; Schweizer and Marks, 1977b), and this organisation was clearly visible in the wholemounts (Fig. 1A-D). Wholemounts were labelled with an antibody to keratin 14 (K14) in order to visualise all the cells in the basal layer of the IFE, the outer root sheath (ORS) of the hair follicle and the outer layer of the sebaceous gland (Fig. 1A-D).

To determine whether BrdU-containing cells could be detected in wholemounts, adult mice were administered a single injection of BrdU 1 hour prior to sacrifice to label cells undergoing DNA synthesis. BrdU-labelled cells were present throughout the IFE, sebaceous glands and the HF in tail wholemounts isolated from these mice (Fig. 1A). In the anagen stage of the hair cycle, the highest degree of BrdU labelling was within the bulb of follicles, as would be expected (Fig. 1A). In anagen follicles the bulge was visible as a distinct, asymmetric protuberance of the ORS, beneath the sebaceous gland (Fig. 1A). Keratinocytes within the bulge were rarely labelled with BrdU, which indicated that cells located within this region were not actively cycling.

LRC were generated using a previously reported protocol (Bickenbach et al., 1986; Bickenbach and Chism, 1998) in which 10-day-old mice received an injection of BrdU every 12 hours for a total of four injections. BrdU initially labels the majority of cells in the epidermis that are capable of proliferating, including the stem cells. During the chase period, the label is lost from dividing cells, so that only infrequently cycling cells, the putative stem cells, retain the label. To assess the efficiency of BrdU labelling, mice were killed 2 days after the final injection of BrdU: the majority of cells within the basal layer of the IFE, hair follicle outer root sheath and outer layer of the sebaceous glands contained the BrdU label (Fig. 1B). From 24 days post-labelling (anagen of the first post-natal hair cycle), the pattern of incorporation remained relatively constant. Some keratinocytes in both the HF and IFE still retained the BrdU label after a 140 day chase (Fig. 1C), which demonstrated that long-term labelling of infrequently cycling cells could be achieved using this protocol.

As previous studies of LRC have focused on mouse dorsal skin (Cotsarelis et al., 1990; Taylor et al., 2000) we compared the number and location of LRC in hair follicles on the dorsal and tail skin. We had some success in preparing dorsal

Fig. 1. Localisation of LRC in different compartments of wild-type mouse epidermis. Epidermal sheets (A-H) or tissue sections (I) were labelled for keratin 14 (red; A-E; H-I) or Ki67 (red; F,G) and BrdU (green) by double-label immunofluorescence. Skin was collected from tail (A-D,F-I) or back (E) of adult wild-type mice. (A) One hour BrdU label. (B-I) Mice received repeated injections of BrdU to generate LRC. Wholemounts prepared following a chase of 2 days (B) or 140 days (C). Asterisk in C indicates site of insertion of dermal papilla. (D,E) Epidermis from 42-day-old mouse (telogen; 31 days post-BrdU injection). (F-I) Epidermis collected following a 70 day chase period. (F) Arrows indicate LRC located in the interfollicular epidermis. (G) Hair follicle plucked from labelled wholemount. Arrowhead indicates a LRC in the infundibulum. Inset is higher power view of boxed area, showing two cells double-labelled for Ki67 and BrdU. Cells with intense (BrdU heavy) and weak (BrdU light) BrdU labelling are indicated. (H) High power confocal micrograph of sebaceous gland; arrows indicate the position of LRC. (I) Hair follicle in tissue section; arrows indicate keratin 14-positive LRC in the outer root sheath. HF, hair follicle; IFE, interfollicular epidermis; SG, sebaceous gland; BG, bulge. Scale bars: 100 μ m.

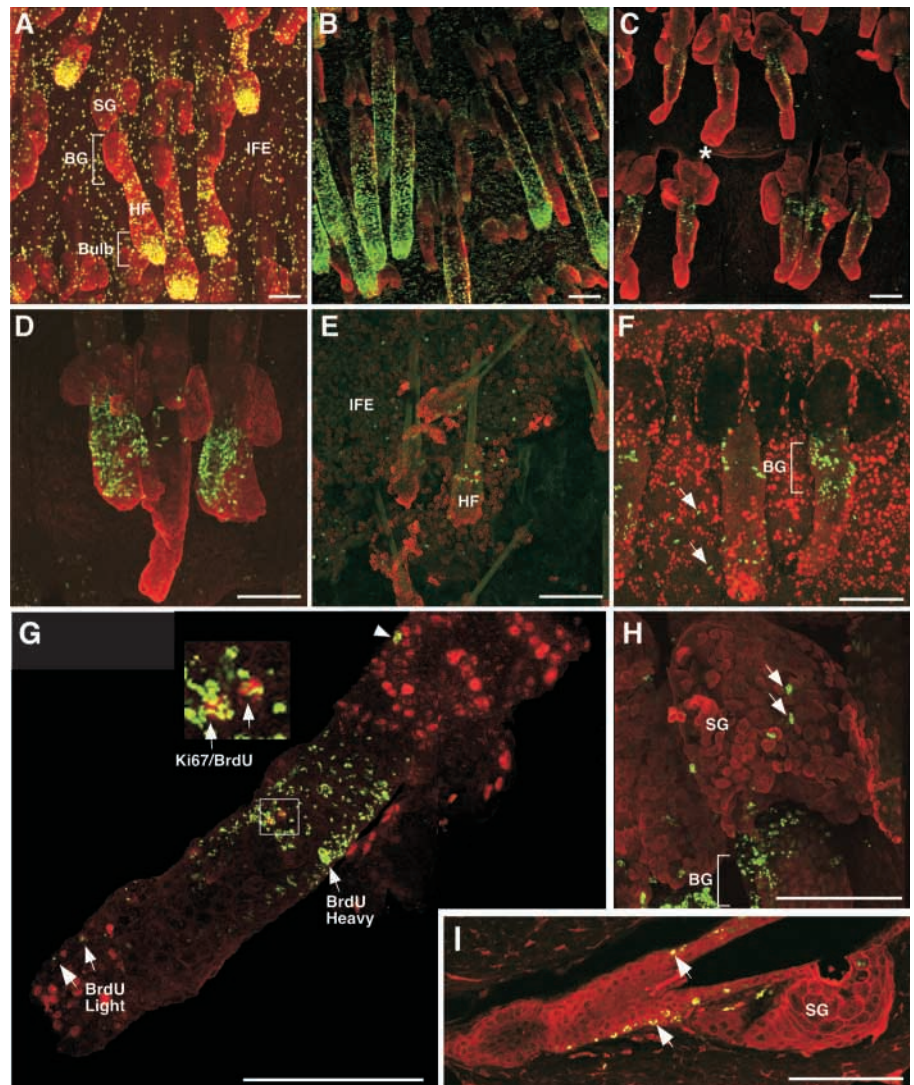


Table 1. Number and distribution of BrdU label-retaining cells in tail hair follicles (HF) of wild-type mice after a 70 day chase period

	Number of BrdU-labelled cells \pm s.d. (range)							
	Sebaceous gland (SG)		HF infundibulum		Non-cycling region of HF beneath SG		Cycling region of HF	
	Heavy BrdU	Light BrdU	Heavy BrdU	Light BrdU	Heavy BrdU	Light BrdU	Heavy BrdU	Light BrdU
Outer follicles ($n=20$)	0.3 \pm 0.6 (0-2)	0.6 \pm 0.8 (0-2)	0.2 \pm 0.4 (0-1)	1.1 \pm 1.0 (0-3)	46.9 \pm 19.1 (21-79)	35.3 \pm 12.7 (15-65)	0.7 \pm 1.0 (0-3)	14.3 \pm 11.7 (0-43)
Central follicles ($n=10$)	0.4 \pm 0.7 (0-2)	0.5 \pm 0.5 (0-1)	0.0 \pm 0.0 (0-0)	1.0 \pm 1.2 (0-4)	15.3 \pm 4.7 (10-23)	12.6 \pm 5.7 (4-21)	0.1 \pm 0.3 (0-1)	2.0 \pm 2.1 (0-7)

The number of BrdU-labelled cells was determined by microscopic examination of formaldehyde-fixed tail epidermal wholemounts prepared from three wild-type mice. Wholemounts were stained by double-label immunofluorescence to detect BrdU and keratin 14. Cells with the most intense BrdU labelling were counted as 'heavy BrdU' (Fig. 1G). BrdU-labelled cells that were estimated to have less than 50% of the maximum labelling observed for any cell in that follicle were classified as 'light BrdU' (Fig. 1G); these cells are likely to have undergone at least two cell divisions since incorporating the BrdU label.

epidermal wholemounts by harvesting skin at the telogen phase of the hair cycle (see also Sundberg et al., 1993; Zhang et al., 2001). Wholemounts of tail and dorsal epidermis were prepared from 42-day-old mice that were in telogen (31 days post-BrdU injection; Fig. 1D,E). In both types of epidermis, LRC were distributed as single cells within the IFE and clustered in the permanent portion of the hair follicle (Fig.

1D,E). The main difference between the two types of epidermis was that the number of LRC per hair follicle was greater in tail (range 10-79 LRC; see Table 1) than in dorsal (range 0-15 LRC; $n=10$) follicles (Fig. 1D,E). This difference reflects the fact that hair follicles in tail epidermis are much larger than dorsal follicles (compare Fig. 1D with 1E, same magnification).

Number and location of LRC within hair follicles, sebaceous glands and IFE

For a detailed assessment of the location of LRC within tail epidermis, wholemounts were prepared from wild-type mice killed after a 70-day chase period. The largest concentration of LRC within the tail epidermis was in the hair follicle just below the sebaceous glands (Fig. 1F,G). Immunolabelling of the proliferation marker Ki67 indicated that cells within this region of the hair follicle were relatively quiescent (Fig. 1F,G), confirming the results obtained with a 1 hour BrdU label (Fig. 1A).

The LRC were present in all follicles, irrespective of whether a distinct bulge was visible (Fig. 1C,F). This protuberance of the outer root sheath may be caused by the presence of the club hair (Morris and Potten, 1994; Narisawa and Kohda, 1996; Stenn and Paus, 2001). Whereas the visible bulge lies on one side of the follicle (Cotsarelis et al., 1990; Morris and Potten, 1994), the LRC were usually distributed symmetrically (Fig. 1C,D). The symmetrical location of LRC was also observed in dorsal follicles (Fig. 1E). Thus, in the context of LRC, 'bulge' refers to the permanent portion of the follicle below the sebaceous glands and not to a physical entity (e.g. Fig. 1F).

The number of LRC was counted in several anatomic locations within triplets of tail hair follicles (Table 1). While the outer two follicles in each triplet tended to go through the hair cycle concurrently, the central follicle frequently cycled asynchronously and generally had fewer LRC (Fig. 1D). Therefore the central follicles of each triplet were tabulated separately. Occasional LRC were Ki67 positive, indicating that they were capable of dividing (Fig. 1G, inset). The intensity of BrdU labelling varied in different cells, presumably as a result of dilution of the BrdU label during cell division (Fig. 1G). BrdU labelling is therefore described as 'heavy' or 'light' in Table 1.

In all hair follicles, the highest number of heavily BrdU-labelled cells (range 10-79) was clustered within the non-cycling region of the hair follicle beneath the sebaceous glands. This region of the follicle also contained many lightly labelled cells, which indicated that cells in this region were not completely quiescent (Table 1; Fig. 1G). By contrast, nearly all of the BrdU-containing cells located in the cycling region of the hair follicle, extending from the bulge toward the bulb, were lightly labelled and these cells tended to be located individually rather than clustered (Table 1; Fig. 1G). Lightly labelled cells rarely were present above the sebaceous gland (HF infundibulum; Table 1).

Although the greatest number of LRC was located in the non-cycling region of the hair follicle beneath the sebaceous glands, this compartment was not the exclusive location of LRC in tail epidermis. LRC were frequently observed as individual, non-clustered cells within interfollicular epidermis; these cells, like the LRC of the hair follicle, were usually negative for Ki67 (Fig. 1F). LRC occasionally were present in the sebaceous gland (Fig. 1H; Table 1) and, more rarely, in the infundibulum of the hair follicle, near the junction between the follicle and the IFE (Fig. 1G, arrowhead; Table 1). The fact that the LRC were keratin 14 positive was confirmed by double immunolabelling of histological sections of hair follicles (Fig. 1I, arrows). The double-labelled cells were confined to the outer root sheath, indicating that they had not undergone terminal differentiation along any of the hair lineages (Fig. 1I).

Expression of cell lineage markers and putative stem cell markers

In order to visualise different cellular compartments within the epidermis, we labelled the wholemounts with a range of antibodies. In contrast to keratin 14, which was uniformly expressed in the basal layer of IFE, the sebaceous gland and the outer root sheath of hair follicles (Fig. 2A), keratin 17 labelling was confined to the HF, extending from below the sebaceous glands to the bulb (Fig. 2B). Keratin 6 labelling was restricted to the permanent portion of the hair follicle below the sebaceous gland, including the bulge region, and thus coincided with the location of most of the LRC (Fig. 2C). The expression patterns of all three keratins was the same in tail as in dorsal hair follicles (e.g. McGowan and Coulombe, 1998).

Markers selective for sebocyte, IFE and hair follicle lineages also were examined in tail epidermal wholemounts. Sebocytes were readily identified with the hydrophobic dye Nile Red which is highly selective for lipids (Fig. 2D). K10 is expressed by suprabasal IFE keratinocytes and by those basal keratinocytes that are in the process of moving upwards from the basal layer (Schweizer et al., 1984; Regnier et al., 1986; Leigh et al., 1993). In mouse, as in human (Jensen et al., 1999), epidermal wholemounts, antibodies did not penetrate the suprabasal layers of the IFE and, thus, keratin 10 labelling was confined to basal cells that were committed to terminal differentiation (Fig. 2E). Keratin 10-positive basal cells were found scattered throughout the IFE and in the upper part of the HF, extending from the infundibulum to just below the sebaceous gland (Fig. 2E). The transcriptional repressor CCAAT displacement protein (CDP) (Ellis et al., 2001) regulates lineage choice within the hair follicle and is expressed in the hair bulb, in inner root sheath (IRS) progenitor cells of the matrix, the three differentiating layers of the IRS and the companion cell layer (Ellis et al., 2001; Niemann and Watt, 2002). In wholemounts, CDP labelling was confined to the bulb and companion cell layer (CCL) of anagen hair follicles (Fig. 2F).

We next examined putative surface markers of epidermal stem cells. β 1 integrins enrich for stem cells in human interfollicular epidermis (Jones and Watt, 1993; Jones et al., 1995; Jensen et al., 1999) and the proteoglycan NG2/MCSP also is upregulated in these cells (Legg et al., 2003). In both mouse and human epidermis, stem cells are included in the population of keratinocytes that express high levels of the α 6 β 4 integrin. Mouse epidermal stem cells are enriched by selection for keratinocytes that have high levels of α 6 integrin expression and low expression of the transferrin receptor (CD71) (Tani et al., 2000). In human epidermis, the combination of high expression of α 6 integrin and low expression of a proliferation-associated cell surface marker (10G7 ag) has been used to isolate epidermal stem cells (Li et al., 1998; Kaur and Li, 2000).

β 1 integrin expression was uniform in the IFE, sebaceous gland and most of the ORS, with more intense expression in the bulb of anagen follicles (Fig. 2G). By contrast, α 6 integrin labelling was most intense in the region, including the bulge, which contained LRC and was otherwise uniform in the IFE and sebaceous glands (Fig. 2H). An antiserum to NG2 chondroitin sulphate proteoglycan (NG2; rat homologue of MCSP) (Nishiyama et al., 1991), which crossreacts with the mouse homologue AN2, yielded uniformly weak labelling in

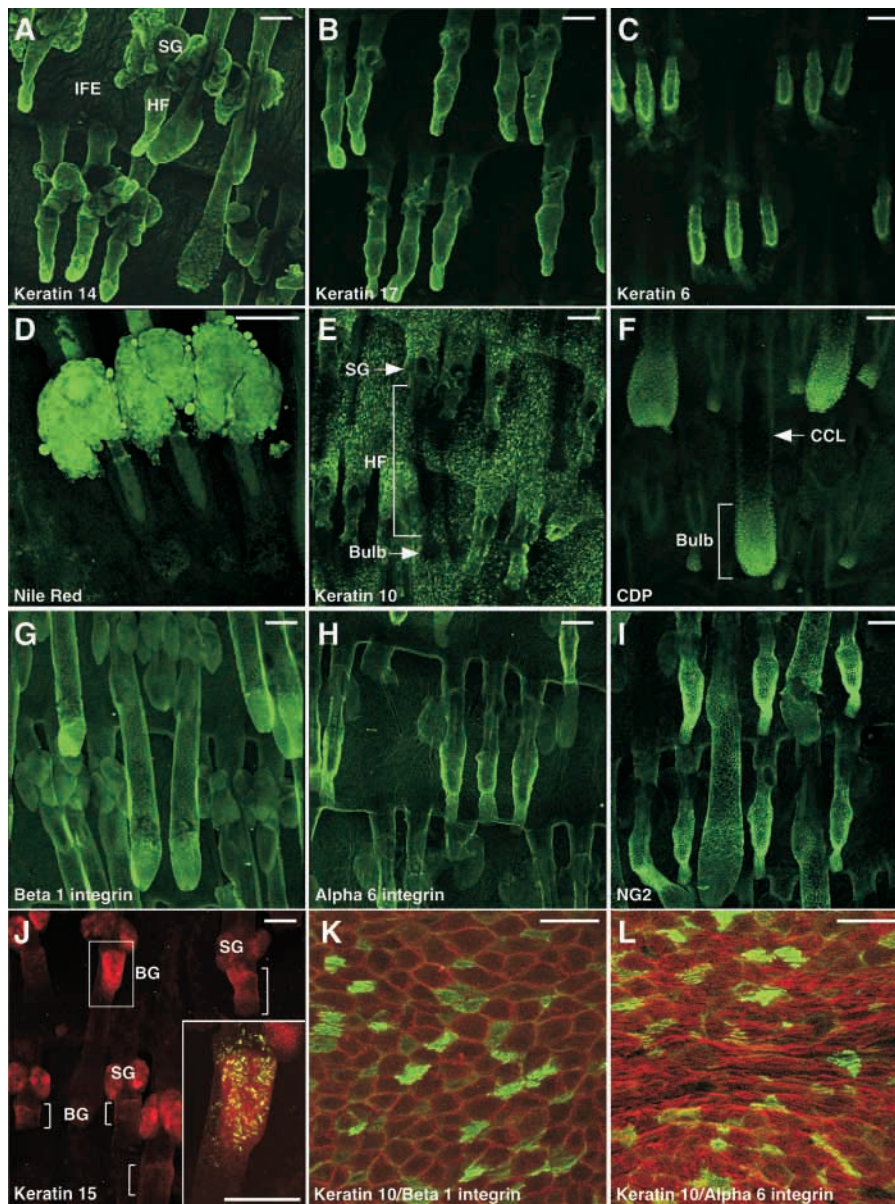


Fig. 2. Localisation of cell lineage markers and putative stem cell markers in tail wholemounts from adult mice. (A–J) Tail epidermal wholemounts from wild-type mice were immunolabelled to detect the proteins indicated or stained with Nile Red (D) to visualise sebocytes. (E) Bracket shows lack of labelling of HF outer root sheath from below the sebaceous gland to the bulb. (J) Brackets show that labelling for keratin 15 is present within the bulge region of hair follicles. Inset is a higher power view of boxed area, showing double-label immunofluorescence for keratin 15 (red) and BrdU (green) in a mouse labelled according to the LRC protocol followed by a chase of 32 days. Staining of sebaceous glands is non-specific because of the use of a mouse primary antibody. (K,L) High-power micrographs of IFE labelled by double-label immunofluorescence for keratin 10 (green) and either $\beta 1$ integrin (red; K) or $\alpha 6$ integrin (red; L). BG, bulge; HF, hair follicle; IFE, interfollicular epidermis; SG, sebaceous gland; CCL, companion cell layer. Scale bars: 100 μm in A–J; 20 μm in K–L.

10-positive cells is not random, but confined to regions of the basal layer surrounding the stem cell clusters (Jensen et al., 1999). The observations that LRC within mouse IFE were scattered rather than clustered (Fig. 1F) and that there was no obvious pattern to the location of the Ki67-positive cells (Fig. 1F) suggested that the keratin 10-positive basal cells would also be randomly distributed. This was examined by double labelling wholemounts for keratin 10 and $\beta 1$ or $\alpha 6$ integrin (Fig. 2K,L) and viewing the IFE at high magnification. As predicted, keratin 10-positive cells were distributed in an apparently random fashion in mouse IFE. As previously reported for human epidermis, the $\beta 1$ integrins were primarily

clustered at the periphery of the ventral plasma membrane of basal cells (Fig. 2K), while the $\alpha 6$ integrins were localised across the entire membrane surface (Fig. 2L). The distribution of $\alpha 6\beta 4$, which is a component of hemidesmosomes, was dense and somewhat filamentous, in contrast to the punctate distribution of $\alpha 6\beta 4$ in the ventral membrane of human IFE basal cells (Jensen et al., 1999).

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the IFE and sebaceous glands, but there was intense labelling in the HF extending from below the sebaceous glands to the bulb or just above the bulb (Fig. 2I). In contrast to $\beta 1$ integrin expression, which was highest in the bulb of anagen follicles, NG2 expression was higher in the region of the ORS that contained the LRC.

The marker that showed the best co-localisation with LRC was keratin 15 (Lyle et al., 1998; Waseem et al., 1999) (Fig. 2J, inset; note that the sebaceous gland labelling is non-specific). Keratin 15 staining was restricted to the permanent portion of the follicle directly beneath the sebaceous gland (Fig. 2J) and had a more restricted distribution than $\alpha 6\beta 4$ (Fig. 2H) or keratin 6 (Fig. 2C). There was variation in both the overall intensity of label (most intense during mid to late anagen) and location (symmetric in some follicles; asymmetric in others) (Fig. 2J and data not shown).

In human IFE, there is clustering of the putative stem cells and as a result the location of the actively cycling and keratin

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Assessing the fate of BrdU-labelled cells during the hair cycle

We were able to exploit the whole-mount labelling method to gain a better understanding of the fate of LRC during the hair growth cycle. Mice were sacrificed at the age of 28 days (early anagen), 35 days (anagen) or 42 days (telogen). Paraffin sections of dorsal and tail skin from the same mice were examined and the relative percent of follicles in anagen, catagen or telogen was estimated to confirm the stage of the hair cycle. Although the timing of entry and exit into the first

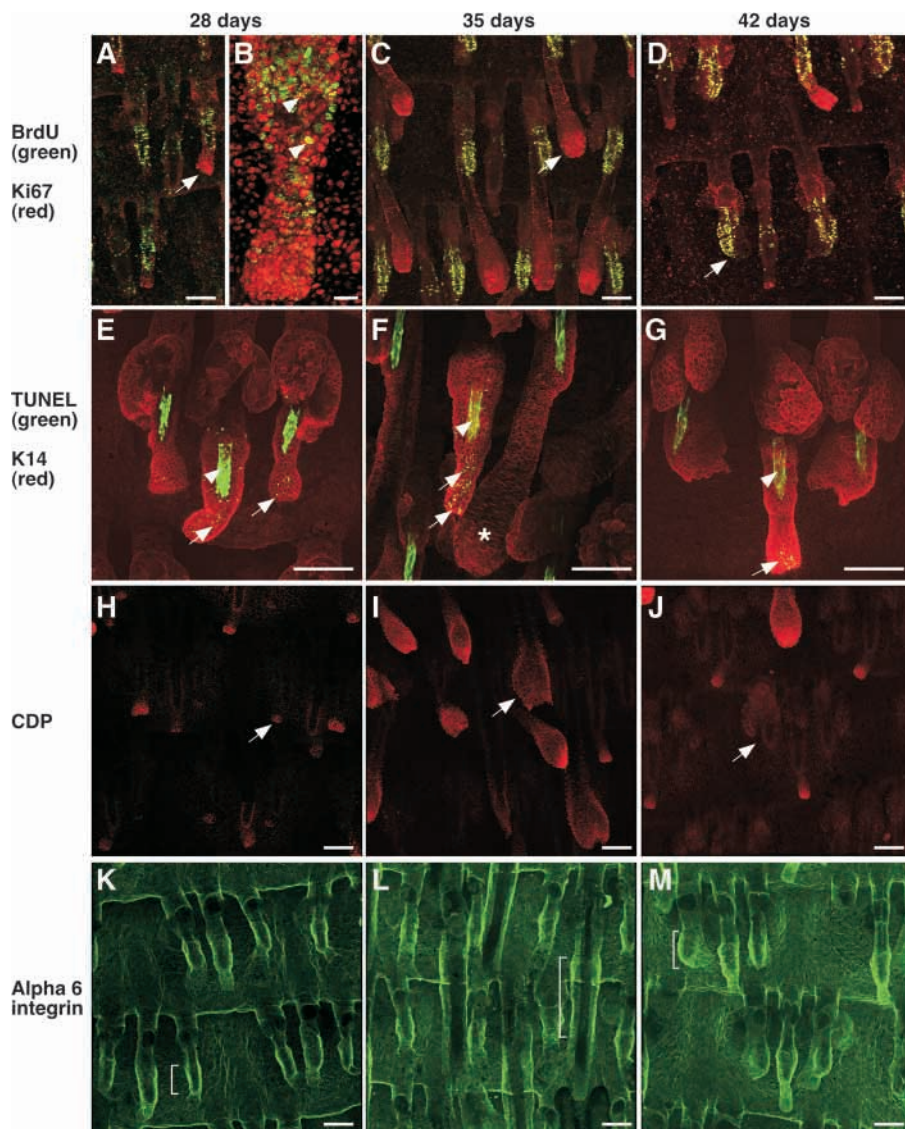


Fig. 3. Examination of tail wholemounts at various stages of the hair growth cycle. Wild-type mice were injected with BrdU according to the LRC protocol and wholemounts were prepared from mice of the following ages: 28 days (early anagen; A,B,E,H,K), 35 days (anagen; C,F,I,L) and 42 days (telogen; D,G,J,M). (A-D) Double labelling for BrdU (green) and Ki67 (red). Arrows indicate representative follicles in early anagen (A), full anagen (C) and telogen (D). Arrowheads in B indicate high-magnification examples of double-labelled cells in early anagen. (E-G) Double labelling for TUNEL positive cells (green) and keratin 14 (red). Arrows indicate TUNEL-positive cells. Arrowheads indicate non-specific staining of hair shafts. Asterisk indicates a follicle in full anagen. (H-J) Immunolabelling for CDP. Arrows indicate representative follicles in early anagen (H), full anagen (I) and telogen (J). (K-M) Immunolocalisation of $\alpha 6$ integrin. Brackets indicate regions of the hair follicle with strong $\alpha 6$ integrin labelling during early anagen (K), anagen (L) and telogen (M). Scale bars: 100 μm in A,C-M; 20 μm in B.

hair cycle was very tightly regulated in dorsal epidermis (data not shown), there was more variability in follicles in tail epidermis. In one study of tail skin, the proportion of follicles ranged from 60-95% anagen at 28 days ($n=6$ mice), 70-100% anagen and 0-30% telogen at day 35 ($n=3$), and 80-90% telogen at day 42 ($n=3$). Thus, at each of the selected time points a high percentage of the follicles in the tail epidermis were at the expected stage of the hair cycle. In addition, the status of any given follicle was generally obvious from its size and overall morphology (Fig. 3).

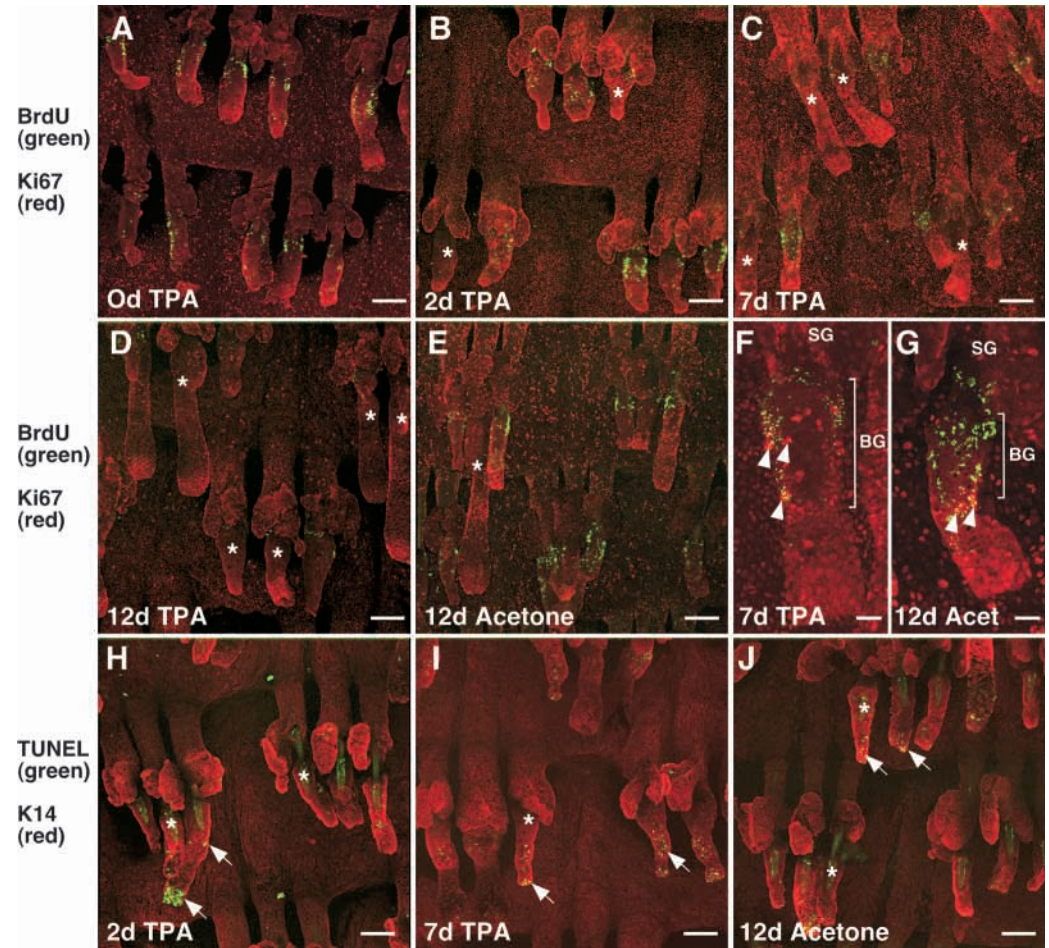
At all time points, the central follicle usually had a lower number of LRC than did the outer two follicles in each triplet (Fig. 3A,C,D). At 28 days (early anagen; 17 days post-BrdU injection) the LRC in the outer two follicles typically extended from the sebaceous gland nearly to the bulb (Fig. 3A). In 35-day-old mice (anagen; 24 days post-BrdU injection) the follicles were much longer and had more prominent bulges and bulbs than at 28 days (Fig. 3C). BrdU-labelled cells in 35-day-old mice were densely packed within the bulge region (Fig. 3C) and LRC were rarely seen within the elongating region of the

anagen hair follicle. At 42 days of age (telogen; 31 days post-BrdU injection), there were many follicles that had undergone regression; these follicles were very short and contained BrdU-labelled cells from beneath the sebaceous gland to the follicle base (Fig. 3D). The population of LRC was remarkably unchanged, both in terms of number and location, at the three time points examined and there was no evidence of significant depletion or migration of LRC during anagen. Nevertheless, the lightly BrdU labelled cells that we had observed previously (Fig. 1G; Table 1) were more abundant in the cycling region of anagen follicles (Fig. 3B).

We also examined the expression of markers of proliferation and lineage commitment at different phases of the hair growth cycle. During early anagen, a moderate number of cells in the hair matrix at the bulb of the hair follicle expressed the proliferative marker Ki67 (Fig. 3A,B). Ki67 labelling was normally low within the bulge; however, an increase in labelling was observed in anagen follicles and some Ki67-positive LRC were observed (Fig. 3B). Ki67 expression was greatly increased in the bulb during mid to late anagen and there was increased expression throughout the entire cycling region of the ORS (Fig. 3C). By contrast, Ki67 expression was completely absent at the bottom of telogen follicles (Fig. 3D).

The location of apoptotic keratinocytes was monitored by TUNEL labelling. In early anagen small numbers of scattered TUNEL-positive cells were present, primarily in the lower third of the follicle (Fig. 3E). In late anagen (Fig. 3F, asterisk) and telogen (Fig. 3G) there were very few TUNEL-positive cells. In regressing follicles TUNEL-positive cells were clustered at the bulb (Fig. 3G). These observations are in

Fig. 4. Loss of LRC following treatment with TPA. Wild-type mice were injected with BrdU according to the LRC protocol. After a chase period of 10 months, wholemounts of tail epidermis were collected from untreated mice (A) or after thrice-weekly treatment with TPA (B-D,F,H,I) or acetone (E,G,J) for the number of days indicated. Wholemounts were labelled to detect Ki67- (red, A-G), BrdU- (green, A-G), keratin 14- (red, H-J) or TUNEL-positive cells (green, H-J). (A-G) Asterisks indicate hair follicles that contain very few LRC. (F,G) Arrowheads in high magnification micrographs indicate double-labelled cells. (H-J) Arrows indicate TUNEL-positive cells. Asterisks indicate non-specific staining of hair shafts. BG, bulge; SG, sebaceous gland. Scale bars: 100 μ m in A-E,H-J; 20 μ m in F,G.



agreement with studies of the hair cycle in other body sites (reviewed by Stenn and Paus, 2001).

During early anagen, CDP was expressed by a few cells at the bottom of the follicles, indicating that differentiation along the IRS and companion layer lineages was being initiated (Fig. 3H). CDP expression in the hair bulb and companion cell layer was markedly increased as anagen progressed (Fig. 3I) and was absent from telogen follicles (Fig. 3J).

In early anagen and telogen follicles $\alpha 6$ integrin expression was greatest in the region of the follicles containing the LRC (Fig. 3K,M). As the hair follicle lengthened during anagen, strong $\alpha 6$ integrin labelling was maintained in this region but also extended nearly to the bulb (Fig. 3L); thus, in anagen follicles, $\alpha 6$ was not a specific marker of the LRC compartment.

Loss of LRC through phorbol ester induced proliferation

The presence of LRC in hair follicles that had undergone repeated cycles of growth and regression raised the possibility that LRC were incapable of proliferation. To investigate whether LRC in the tails of 10-month-old mice (Fig. 4A) could be stimulated to divide we treated the tails three times per week with the phorbol ester TPA (Schweizer and Winter, 1982). By 2 days after initiation of TPA treatment there was increased Ki67 labelling throughout the IFE, sebaceous glands and along the length of the hair follicles, indicating widespread

stimulation of cell proliferation (Fig. 4B). By 7 days, there was a decrease in the number of BrdU-positive cells (Fig. 4C) and those cells that did retain label were more lightly labelled (Fig. 4F) than controls (Fig. 4G). Individual cells were positive for BrdU and Ki67 (Fig. 4F, arrowheads). By 12 days, LRC were extremely rare (Fig. 4D) relative to acetone treated controls (Fig. 4E). In contrast to its strong effects on proliferation, TPA did not stimulate apoptosis, as evaluated by TUNEL labelling (Fig. 4H-J). These experiments establish that although LRC can be quiescent for many months, they nevertheless retain the ability to divide in response to an appropriate stimulus.

Characterising the changes that occur following activation of Myc in epidermis

We next applied the whole-mount labelling method to a transgenic mouse model in which activation of Myc by topical application of OHT stimulates proliferation and terminal differentiation of the IFE and promotes sebocyte differentiation at the expense of differentiation along the hair lineages (Arnold and Watt, 2001). Constitutive activation of Myc in the basal layer of the epidermis is reported to cause depletion of LRC in the IFE (Waikel et al., 2001), but this has not been examined in the inducible model.

K14MycER transgenic mice and nontransgenic littermates were administered BrdU to mark LRC. After a 70 day chase period, Myc was activated by daily, topical treatment of tail epidermis with OHT for up to 14 days (Arnold and Watt,

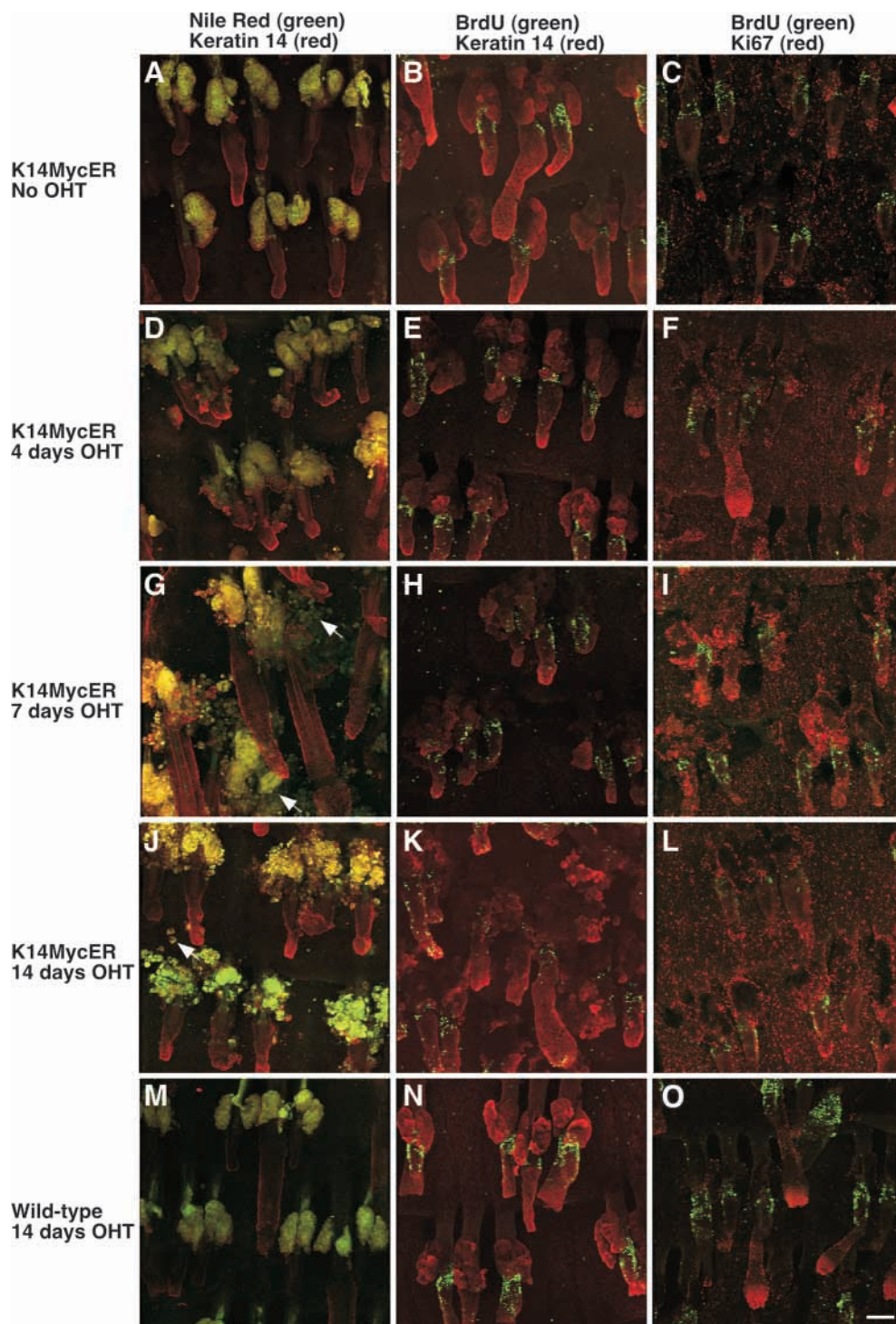


Fig. 5. Consequences of Myc activation in the epidermis of K14MycER transgenic mice. Transgenic mice and nontransgenic (wild-type) littermates were injected with BrdU according to the LRC protocol. After a 70 day chase period, wholemounts of tail epidermis were examined immediately (no OHT) or after daily treatment with 4-hydroxytamoxifen (OHT) for the number of days shown. Epidermis was labelled to detect keratin 14 (red) and Nile Red (green) (A,D,G,J,M), keratin 14 (red) and BrdU (green) (B,E,H,K,N), or Ki67 (red) and BrdU (green) (C,F,I,L,O). Arrows (G,J) indicate individual or clusters of sebocytes located in the interfollicular epidermis. Scale bar: 100 μ m.

2001). Mice were sacrificed at pre-determined time points and wholemounts of tail epidermis were prepared. Transgenic mice that were not treated with OHT had normal epidermis (Fig. 5A-C) (Arnold and Watt, 2001). Nontransgenic mice treated for 14 days with OHT also were completely normal (Fig. 5M-O). Activation of Myc did not lead to a decrease in the number of LRC in the interfollicular epidermis or hair follicles by 14 days (Fig. 5B,E,H,K). By contrast, expression of Ki67 was increased in the IFE and sebaceous glands as early as four days

after treatment with OHT (Fig. 5F) and was still elevated at 7 and 14 days (Fig. 5I,L).

High-power confocal microscopy was used to examine the increase in proliferation in the IFE in more detail (Fig. 6A,B). In untreated K14MycER transgenic mice, there was a moderate level of Ki67 labelling present in the IFE (Fig. 6A). However, both the number and intensity of Ki67-positive cells is increased significantly in IFE of K14MycER transgenic mice after 4 days of Myc activation (Fig. 6B). Despite the high

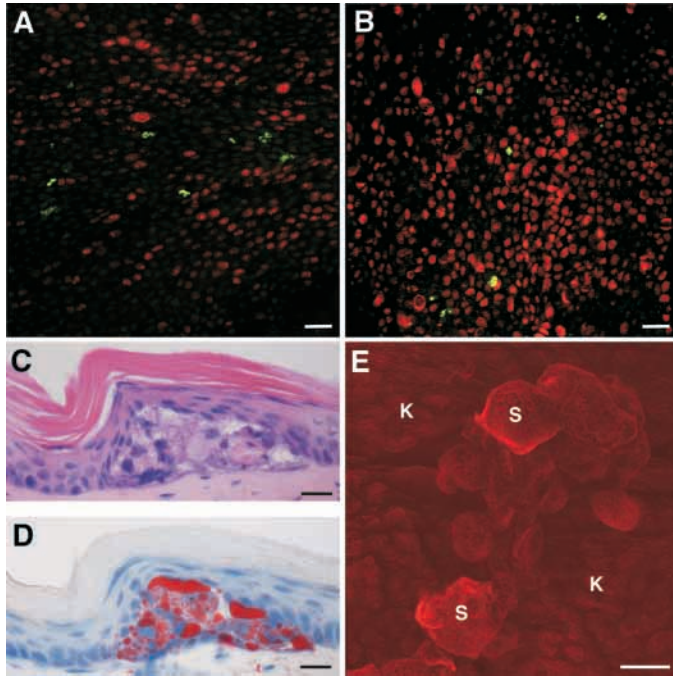


Fig. 6. Effects of Myc on interfollicular epidermis of K14MycER mice. (A,B) Wholemounts labelled by double-immunofluorescence for Ki67 (red) and LRC (green). (A) No OHT treatment; (B) 4 days of OHT treatment. (C,D) Serial sections of tail epidermis treated with OHT for 7 days, stained with Haematoxylin and Eosin (C) or Oil Red O with Haematoxylin counterstain (D). (E) Wholemount immunolabelled for keratin 14 (red) after 14 days of OHT treatment. K, basal keratinocytes; S, sebocytes. Scale bars: 20 μ m.

degree of proliferation both in normal and in Myc-activated IFE, heavily labelled LRC frequently were present throughout the epidermis (Fig. 6A,B).

After just 4 days of OHT treatment the sebaceous glands were enlarged and disorganised (Fig. 5D); this disorganisation became progressively more pronounced after longer treatment periods (Fig. 5G,J). By 7 days post-treatment, single sebocytes and clusters of sebocytes were present in the interfollicular epidermis (arrows in Fig. 5G,J; see also Fig. 6C-E). One potential explanation for the presence of sebocytes in the IFE following Myc activation would be that the cells had broken away from the disorganised sebaceous glands and come to rest on the basal layer of the IFE during the whole-mount preparation procedure. To exclude this possibility, it was necessary to establish the precise localisation of sebocytes within the IFE. Serial frozen sections of tail epidermis from a mouse that was treated with OHT for 7 days were stained with Haematoxylin and Eosin (Fig. 6C) or with Oil Red O (Fig. 6D) to detect the lipids present in sebocytes. These tissue sections demonstrated that at least some sebocytes present in the IFE were integrated among the basal keratinocytes. Furthermore, high power confocal microscopy of whole-mount IFE from a K14MycER transgenic mouse after 14 days of Myc activation showed sebocyte clusters interspersed with the keratinocytes of the IFE (Fig. 6E).

Effects of expressing Δ NLef1 in the epidermis

The second transgenic mouse model we examined is one in

which an N-terminally truncated form of the transcription factor Lef1 is expressed under the control of the K14 promoter (Niemann et al., 2002). The transgene blocks β -catenin signalling and there is conversion of hair follicles into cysts of interfollicular epidermis. Mice were examined up to 120 days after labelling (i.e. 132 days after birth). At later stages it was more difficult to prepare wholemounts because the cysts had become so large and complex.

Wholemounts revealed that the changes in the hair follicles were much more extensive and complex than anticipated from analysing Haematoxylin and Eosin stained skin sections (Fig. 7B,D). Each hair follicle did not give rise to a single cyst, but to multiple cysts that budded off from the outer root sheath anywhere from just beneath the sebaceous glands to the bulb of the follicle (Fig. 7F,H). The first changes in the mice previously had been observed 6 weeks after birth and were loss of hair and delayed onset of the first postnatal hair cycle (Niemann et al., 2002). However, wholemounts revealed changes in the follicles by 3-4 weeks of age, consisting of ectopic sebocyte differentiation (Fig. 7J) and bending of the base of the follicles associated with early cyst formation (Fig. 7E, arrows).

There was a clear depletion of LRC in the hair follicles of transgenic mice compared with wild-type animals from 60 days after birth (Fig. 7H, Fig. 8B). Mice that were greater than 2 years old lacked LRC but still had intact, histologically normal, interfollicular epidermis, as well as large epidermal cysts (data not shown) (Niemann et al., 2002). The loss of LRC correlated with increased proliferation, as judged by Ki67 labelling (Fig. 8A-D). Ki67-positive cells were found throughout the deformed hair follicles of transgenic mice but mainly at sites of epithelial cyst formation (Fig. 8B). There were also more Ki67-positive cells in the interfollicular epidermis of K14 Δ NLef1 mice than in wild-type mice (Fig. 8C,D). LRC in the IFE were reduced in number but not lost completely (data not shown).

Nile Red staining revealed that sebocyte differentiation was far more extensive in the K14 Δ NLef1 transgenic mice than we previously had thought (Fig. 7J,L). The degree of sebocyte differentiation was higher in transgenic tail epidermis than in back skin (Fig. 7B,D) (Niemann et al., 2002). Nile Red staining was observed throughout the deformed follicles, but not all cells of the follicle were positive (Fig. 7J,L). The increase in Nile Red-positive keratinocytes in the hair follicles from skin of K14 Δ NLef1 mice was seen very early during postnatal skin development, from about 3 weeks of age (Fig. 7J). There was no increase in sebocyte differentiation above the sebaceous gland or in the IFE.

Consistent with the change in differentiation from hair follicle lineages to IFE, CDP was completely absent from the deformed hair follicles of transgenic mice (Fig. 7P). However, those follicles that retained a normal morphology at 3.5 weeks continued to express CDP (Fig. 7N). In K14 Δ NLef1 mice, K10 expression was no longer restricted to the IFE and upper follicle but was also seen in the lower part of the deformed hair follicles (arrows in Fig. 8F). The K10-positive cells were not in the outer root sheath but were within the inner cell layers and were often seen in developing cysts (Fig. 8F, asterisks). In whole-mount samples from K14 Δ NLef1 mice, keratin 6 was expressed throughout the structures of the deformed follicles, albeit at low levels (Fig. 8H). In skin from transgenic animals,

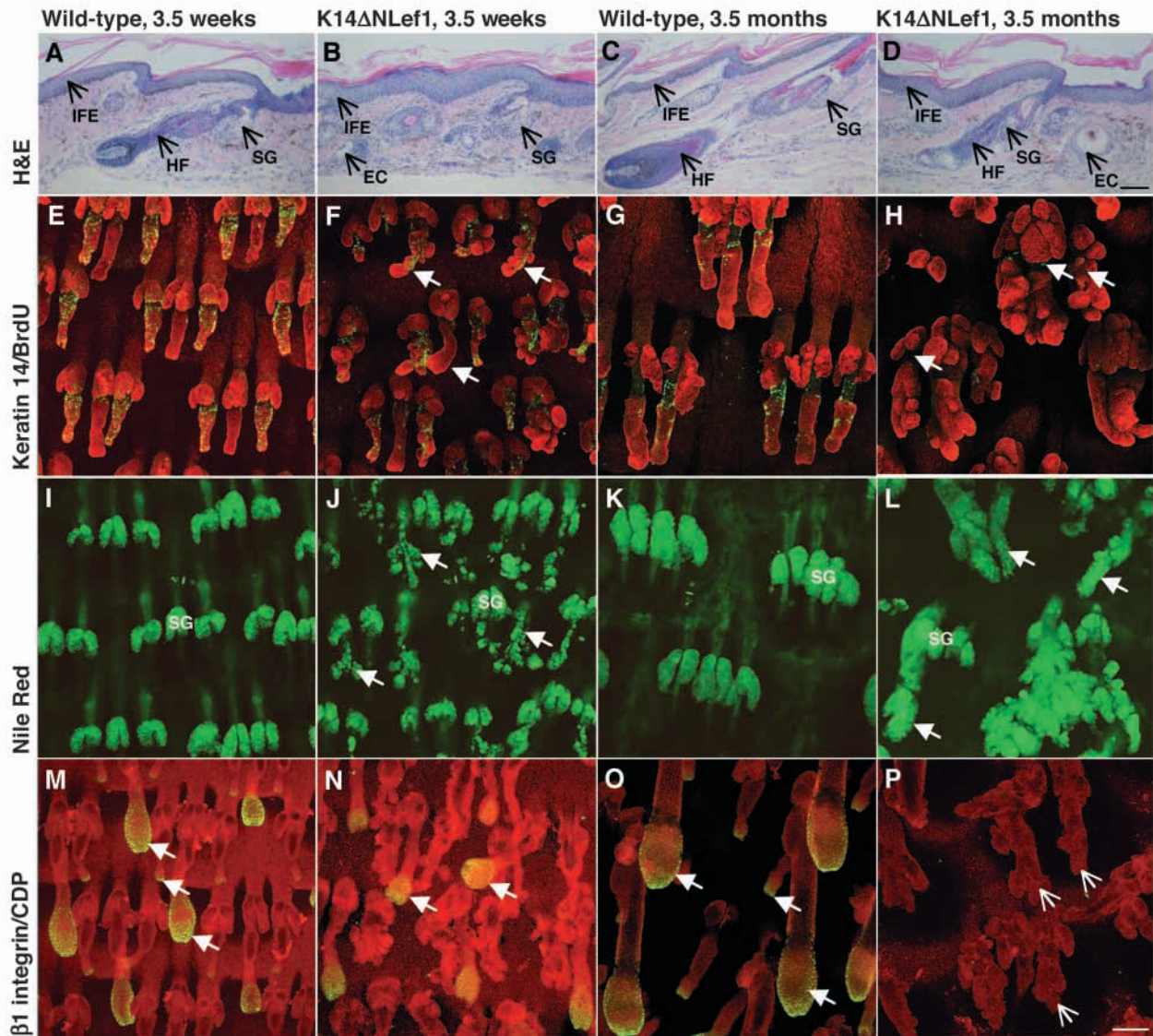


Fig. 7. Effects of Δ NLeF1 transgene on tail epidermis. Tissue sections (A–D) and wholemounts (E–P) of wild-type (A, C, E, G, I, K, M, O) and K14 Δ NLeF1 transgenic mice (B, D, F, H, J, L, N, P) at 3.5 weeks (A, B, E, F, I, J, M, N) and 3.5 months (C, D, G, H, K, L, O, P) of age. (A–D) Hematoxylin and Eosin staining. Note epithelial cysts (EC) at the base of hair follicles (HF) in transgenic skin. IFE, interfollicular epidermis; SG, sebaceous glands. (E–H) Wholemounts immunolabelled for keratin 14 (red) and LRC (green). Arrows in F indicate bending of base of hair follicles associated with early cyst formation. Arrows in H show loss of LRC in deformed hair follicles of transgenic mice. (I–L) Staining with Nile Red. (I, K) Staining restricted to sebaceous glands (SG). (J, L) Arrows indicate increased sebocyte differentiation along the length of hair follicles. (M–P) Immunolabelling for β 1 integrins (red) and CDP (green). Immunolabelling for CDP is observed in wild-type anagen follicles (arrows in M, O) and some follicles of 3.5-week-old transgenic mice (arrows in N), but is completely lost from the abnormal follicles of older animals (arrowheads in P). Scale bar: 100 μ m.

expression of the α 6 β 4 integrin was no longer elevated in the upper third of the follicles (Fig. 8E), but was uniformly expressed in the hair follicles, sebaceous glands and IFE (Fig. 8F). No changes in β 1 integrin expression were observed (Fig. 8G, H).

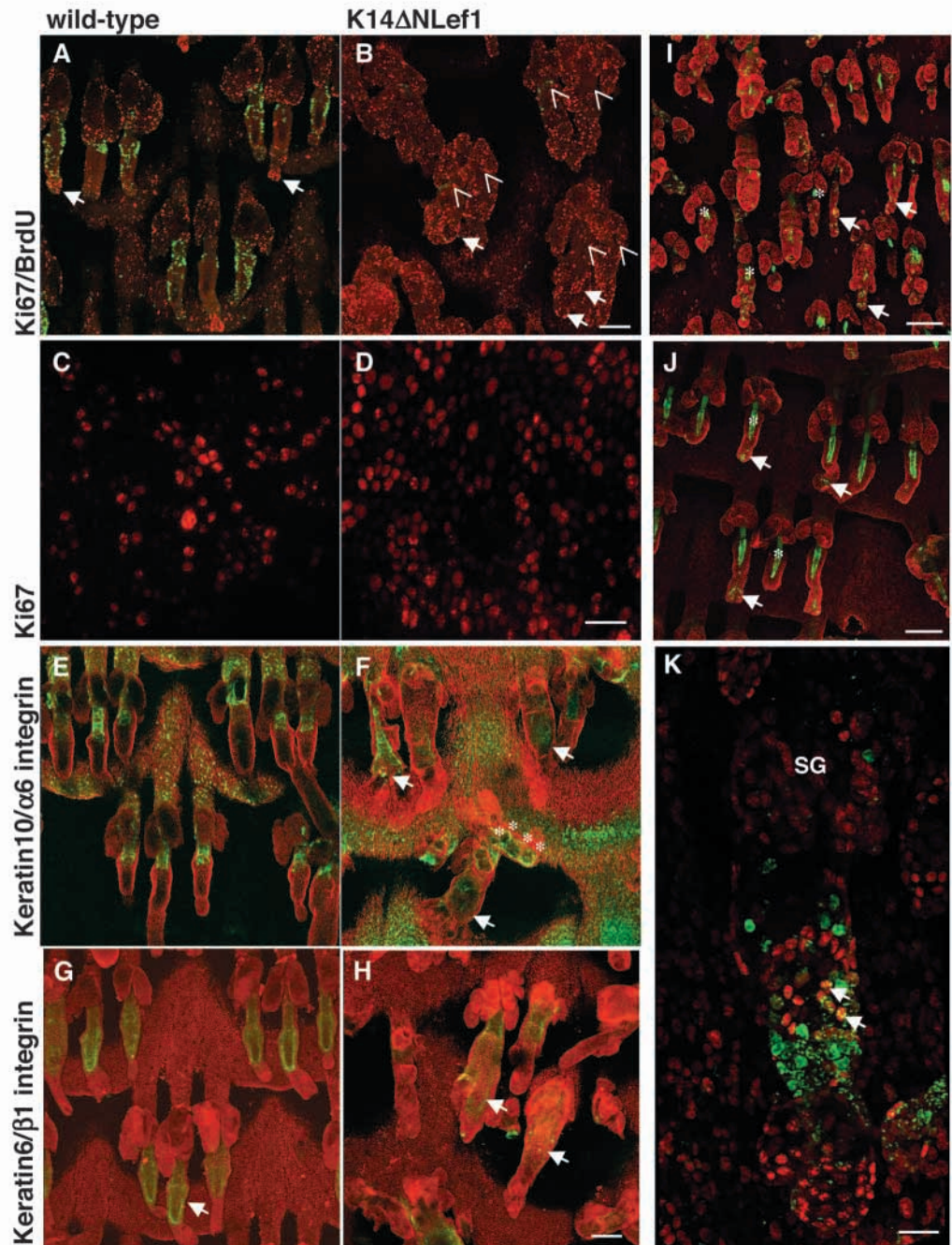
Although the increase in number of Ki67-positive cells (Fig. 8B, D) in K14 Δ NLeF1 transgenic epidermis relative to controls (Fig. 8A, C) suggested that LRC were being lost through proliferation, an alternative possibility was that they were undergoing apoptosis. In 3.5-week-old transgenics, there was an increase in TUNEL labelling (Fig. 8I) compared with wild type (Fig. 8J). However, the increase was modest and mainly

confined to the lower third of the follicle (Fig. 8I, arrows). Ki67-positive LRC and cells that were lightly labelled with BrdU were frequently observed in the transgenic follicles (Fig. 3K). Thus, although some LRC may be lost through apoptosis, we believe that in K14 Δ NLeF1 transgenics, as in TPA treated wild-type mice (Fig. 4), most cells lose BrdU label by undergoing cell division.

Discussion

The organisation of mammalian epidermis as a simple sheet penetrated by hair follicles and their associated sebaceous glands

Fig. 8. Effects of Δ NLef1 transgene on markers of proliferation and differentiation. Wholemounts of tail epidermis from 3.5-month-old wild-type animals (A,C,E,G), 3.5-month-old K14 Δ NLef1 transgenic mice (B,D,F,H), and 3.5-week-old K14 Δ NLef1 transgenic mice (I-K) were stained to detect TUNEL-positive cells (green in I,J) or labelled with antibodies against Ki67 (red in A-D,I,J), BrdU (to detect LRC; A,B,K), keratins 10 (green in E,F) and 6 (green in G,H), α 6 (red in E,F) and β 1 integrins (red in G,H). (A,B) Note reduction in number of LRC in deformed hair follicles of transgenic mice (arrowheads in B). In wild-type anagen hair follicles, Ki67 labelling is concentrated at the base of the hair follicles (arrows in A), whereas in aberrant transgenic follicles, Ki67-positive cells are found throughout the deformed hair follicles (arrows in B). (C,D) High power views of interfollicular epidermis. (E,F) In transgenic animals note labelling for keratin 10 extends to the middle and lower part of deformed follicles (arrows in F) and to developing cysts (asterisks in F). (G,H) Keratin 6 labelling is less restricted in transgenic than wild-type follicles (arrows in G,H). (I,J) TUNEL-positive cells are increased in transgenic (I) versus nontransgenic (J) hair follicles. Arrows indicate that TUNEL-positive cells are primarily located in the lower one-third of the hair follicles. Asterisks indicate non-specific staining of hair shafts. (K) High-power confocal micrograph of a hair follicle; arrows indicate examples of double-labelled cells. Scale bar: 100 μ m in A,B,E-H,I-J; 20 μ m in C,D,K.



makes the tissue amenable to whole-mount analysis. Detection of LRC in epidermal wholemounts permitted direct characterisation of the putative stem cell compartment and provided an effective screen for potential surface markers of stem cells. We also used this approach to characterise changes in LRC and cell lineage commitment in genetically modified mice.

Number and location of LRC within the hair follicle

In studies of the hair follicles of mouse back skin, Cotsarelis et al. (Cotsarelis et al., 1990) identified LRC as being concentrated in a permanent region of the hair follicle known as the bulge. As many hair follicles lack a physical bulge in the outer root sheath, the term 'bulge' has come to refer loosely

to the permanent region of the follicle below the sebaceous gland (Morris and Potten, 1994; Narisawa and Kohda, 1996; Stenn and Paus, 2001). In wholemounts of tail and dorsal follicles, LRC typically were clustered symmetrically along the entire length of the permanent section of the follicle below the sebaceous glands. Our observations on the location of hair follicle LRC are thus in broad agreement with those of others.

The number of LRC per follicle in wholemounts was also in good agreement with previous estimates. We observed a range of 0 to 15 LRC in dorsal follicles, consistent with the six to ten reported previously (Taylor et al., 2000) and the one to four found in 3D reconstructions of pelage follicles after a prolonged chase period of 14 months (Morris and Potten, 1999). In wholemounts of tail follicles, the number of LRC was

greater (range 10-79 LRC) than in dorsal follicles, which probably reflects the larger size of the tail follicles. This observation is similar to the results of Morris and Potten (Morris and Potten, 1999), who found 17-45 LRC in the large follicles of guard hairs.

There are several hypotheses regarding the role that stem cells play during the hair cycle. According to the bulge activation hypothesis of Cotsarelis et al. (Cotsarelis et al., 1990), during late telogen or early anagen, the LRC of the bulge area are activated to proliferate by signals from the dermal papilla and their progeny migrate to give rise to the lower follicle. In support of this model, Taylor et al. (Taylor et al., 2000) describe lightly labelled BrdU-positive cells in the lower part of anagen follicles. By contrast, Oshima et al. (Oshima et al., 2001) observed that in vibrissae follicles, which have such a long anagen that they appear to grow continuously rather than cyclically (J.P.S., unpublished), the stem cells themselves migrate to the base of the follicle before undergoing proliferation. Finally, Panteleyev et al. (Panteleyev et al., 2001) hypothesise that a second reservoir of stem cells, which has migrated from the bulge during anagen, exists at the periphery of the bulb; these cells survive catagen and give rise to the hair shaft and inner root sheath cell lineages.

Our whole-mount data are most consistent with the bulge activation hypothesis. Although there was normally little proliferative activity in the zone of LRC in the permanent region of the follicle, some anagen follicles showed Ki67 labelling in that region and we occasionally observed individual LRC that were Ki67 positive (e.g. Fig. 3B). Furthermore, cells lightly labelled with BrdU were often observed in the ORS of the cycling region of anagen follicles and these cells are most likely to be LRC daughters migrating downwards (e.g. Fig. 1G). By contrast, heavily labelled cells rarely were observed in the cycling region of the hair follicle, suggesting that LRC themselves neither migrate downwards in appreciable numbers (Oshima et al., 2001) nor constitute a permanent clustered population within the bulb (Panteleyev et al., 2001). We cannot, of course, rule out the possibility that unlabelled stem cells migrate to, or are residents of, the bulb.

We saw no obvious decrease in the number of LRC during the course of one hair growth cycle (Fig. 3) (Morris and Potten, 1999) and in mice sacrificed 10 months after labelling LRC were still present, albeit in smaller numbers and with less intense labelling than in younger animals (Fig. 4A). These observations indicate that very few LRC divide during each round of the hair cycle. The concept that an entire follicle is formed by the progeny of even a single stem cell would be quite consistent with the known regenerative potential of stem cells in the haematopoietic system (Lemischka et al., 1986). Equally plausible is the possibility that LRC are only a subset of the stem cell population within the hair follicle and that the unlabelled stem cells also contribute daughters to anagen follicles.

The persistence of LRC during successive hair cycles also raised the possibility that LRC were incapable of division. We were able to rule this out by applying a strong proliferative stimulus to the epidermis in the form of repeated applications of TPA (Schweizer and Winter, 1982). Within 7 days this treatment resulted in a major reduction in LRC without any corresponding increase in apoptosis. The loss of LRC correlated with the appearance of lightly labelled BrdU-

positive cells, some of which were Ki67 positive, demonstrating that LRC had been stimulated to divide.

LRC are present in IFE and sebaceous glands

The existence of LRC within the sebaceous glands and IFE has been contentious. Cotsarelis et al. (Cotsarelis et al., 1990) and Taylor et al. (Taylor et al., 2000) found no LRC in the IFE and presented evidence that bulge LRC migrate up the follicle to populate the sebaceous gland and IFE, implying that there are no stem cells in the IFE of hair bearing skin. However, this is not consistent with reports that LRC lie within the IFE and have a patterned distribution corresponding to the location of epidermal proliferative units, columns of terminally differentiated cells produced through the proliferation of a central stem cell surrounded by a ring of transit amplifying cells (Mackenzie, 1970; Christophers, 1971; Potten, 1974; Morris et al., 1986).

With whole-mount labelling, it is possible to examine large areas of IFE and many hair follicles simultaneously. Our observations leave no doubt that there are scattered LRC in the IFE of tail and dorsal skin. We also found LRC in the sebaceous glands. There was no evidence that lightly labelled cells were migrating upwards from the permanent region of the hair follicles, although such cells were readily detected in the lower part of the follicle. We did not see any pattern in the distribution of the IFE LRC that would be suggestive of epidermal proliferative units. In keeping with this, the K10-positive basal cells also appeared to have a random distribution, in contrast to their arrangement in human IFE (Jensen et al., 1999). The presence of numerous LRC within both tail and dorsal skin IFE suggests that mouse, like human, IFE is normally maintained by its own stem cell compartment (Ghazizadeh and Taichman, 2001; Niemann and Watt, 2002).

Evaluation of putative stem cell markers

There are two major limitations of label retention as a marker of the stem cell compartment. First, it cannot be used to isolate viable cell populations; and, second, it is only a marker of cells that are not dividing. There is therefore a pressing need for stem cell-specific surface markers. Whollemounts provide a rapid screen for candidate markers.

The best surface marker of those evaluated was $\alpha 6\beta 4$ integrin, which was specifically upregulated in the LRC zone of early anagen and telogen follicles. Keratin 15 (Lyle et al., 1998) showed even better colocalisation with LRC, yet neither $\alpha 6\beta 4$ nor keratin 15 was exclusively upregulated on individual LRC. More work is required to determine whether or not the keratin 15-positive, high $\alpha 6\beta 4$ -expressing keratinocytes in the bulge that are not LRC are also stem cells. One aspect of $\alpha 6\beta 4$ expression that had not been observed previously is that the zone of high expression extends to the bulb during anagen. This demonstrates that expression of putative stem cell markers can be highly dynamic and it is intriguing to speculate that the change in $\alpha 6\beta 4$ expression reflects movement of committed progenitor cells downwards from the LRC zone.

Manipulation of the LRC compartment and lineage commitment in transgenic mice

We used the whole-mount method to visualise the changes induced in the epidermis by activating Myc or expressing ΔN Lef1. In cultured human keratinocytes, activation of Myc results in depletion of the stem cell compartment, as evaluated

by an increase in abortive clones of terminally differentiated cells (Gandarillas and Watt, 1997). In transgenic mice in which Myc is constitutively expressed in the epidermal basal layer, there is a depletion of LRC in the IFE (Waikel et al., 2001). However, after 14 days of Myc activation in the tails of adult mice, we did not see any loss of LRC, demonstrating that LRC are not required to drive the marked hyperproliferation of IFE that is a feature of this transgenic model (Arnold and Watt, 2001). Instead, recruitment of LRC into cycle must occur after the initial wave of proliferation induced by Myc (Waikel et al., 2001).

What was most striking in the wholemounts of K14MycER epidermis was the increased sebocyte differentiation and disorganisation, which were far more extensive than appreciated from histological sections (Arnold and Watt, 2001). Nile Red staining revealed that the increased sebaceous differentiation was not confined to the hair follicles, but also occurred in the IFE. This suggests that Myc not only stimulates sebocyte differentiation at the expense of the hair lineages, but may also reprogram IFE keratinocytes to differentiate along the sebocyte lineage. In cultured human keratinocytes, Myc activation is not sufficient to induce conversion to sebocytes (Gandarillas and Watt, 1997). However, activation of Myc in keratinocytes in vivo causes changes in gene expression in the underlying dermis (Frye et al., 2003), which may facilitate reprogramming. What is clear is that Myc induced sebaceous differentiation occurs independently of LRC proliferation or terminal differentiation.

Expression of Δ NLef1 blocks β -catenin signalling and thereby converts hair follicles into cysts of IFE (Huelsen et al., 2001; Merrill et al., 2001; DasGupta et al., 2002; Niemann et al., 2002). Consistent with the change in differentiation programme from hair lineages to IFE (Niemann et al., 2002), CDP was absent from the aberrant follicles and keratin 10 labelling was observed in the cysts of K14 Δ NLef1 mice. Wholemounts revealed that changes in the Δ NLef1 transgenics took place much earlier and were more extensive than we had thought previously. By 3.5 weeks after birth, there were numerous cysts developing along the length of the ORS, demonstrating that reprogramming was neither confined to the LRC zone nor to cells in contact with the dermal papilla.

In K14 Δ NLef1 transgenics, as in the K14MycER mice, IFE hyperproliferation occurred without a corresponding loss of LRC. However, LRC were almost completely absent from the hair follicles and IFE in 3.5-month-old mice. Although a small increase in apoptosis was observed in K14 Δ NLef1 mice compared with nontransgenic mice, the apoptotic cells were mainly located in the lower follicle. By contrast, Ki67-positive LRC were present in the upper follicle, suggesting that most of the LRC are likely to be lost through proliferation. In spite of the loss of LRC there was no evidence of stem cell depletion as the IFE remained healthy and differentiated normally, even in mice older than 2 years (Niemann et al., 2002).

In conclusion, the whole-mount labelling method has allowed us to clarify a number of issues in epidermal stem cell biology. It will be of great use in the search for stem cell markers and in characterising the skin of genetically modified mice. Our data support the bulge activation hypothesis of cyclical hair growth, but demonstrate that there are distinct LRC populations in the IFE and sebaceous glands. LRC can readily be stimulated to divide in response to TPA, yet Myc

induced hyperproliferation and sebocyte differentiation can occur without changes to the LRC compartment. The entire ORS is responsive to Δ NLef1 induced IFE and sebocyte differentiation and loss of LRC does not compromise epidermal integrity. Finally, our results show that LRC are not synonymous with epidermal stem cells, but rather represent a subset of the stem cell population.

We are grateful to everyone who generously provided us with reagents; to the staff of the Cancer Research UK Histopathology Unit and of the Confocal Microscopy Laboratory; and to Angela Mowbray, Gillian Hutchinson, Julie Bee, Clare Millum and Carla Croucher for expert technical assistance. We thank T. T. Sun for an enlightening conversation regarding these data. This work was supported by Cancer Research UK, by an American Cancer Society Fellowship (PF-01-131-01-MGO) to K.B., by a European Union Marie Curie Fellowship to C.N., and by a grant from the National Institutes of Health (RR173) to J.P.S.

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