Keratin 15 Promoter Targets Putative Epithelial Stem Cells in the Hair Follicle Bulge

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Putative epithelial stem cells in the hair follicle bulge are thought to play pivotal roles in the homeostasis, aging, and carcinogenesis of the cutaneous epithelium. Elucidating the role of bulge cells in these processes has been hampered by the lack of gene promoters that target this area with specificity. Here we describe the isolation of the mouse keratin 15 (K15) promoter and demonstrate its utility for preferentially targeting hair follicle bulge cells in adult K15/lacZ transgenic mice. We found that patterns of K15 expression and promoter activity changed with age and correlated with levels of differentiation within the cutaneous epithelium; less differentiated keratinocytes in the epidermis of the neonatal mouse and in the bulge area of the adult mouse preferentially expressed K15. These findings demonstrate the utility of the K15 promoter for targeting epithelial stem cells in the hair follicle bulge and set the stage for elucidating the role of bulge cells in skin biology. ***Key words: transgenic/wound healing/alopecia/aging/carcinogenesis. J Invest Dermatol 121:963–968, 2003***

Epithelial stem cells in adult self-renewing tissues, such as the epidermis and hair follicle, are thought to maintain normal tissue homeostasis and to serve as a reservoir for tissue replacement after wounding or hyperproliferative stimulation (Cotsarelis *et al*, 1989, 1990; Wilson *et al*, 1994; Lehrer *et al*, 1998; Slack, 2000). As undifferentiated stem cells divide, they generate daughter cells that retain the stem cell phenotype and daughter cells (called transient- or transit-amplifying (TA) cells) that undergo a finite number of cell divisions before terminally differentiating (Watt and Hogan, 2000; Cairns, 2002; Potten and Booth, 2002). Epithelial stem cells persist for long periods and therefore can be detected as label-retaining cells (LRC; Bickenbach and Mackenzie, 1984; Cotsarelis *et al*, 1990; Morris and Potten, 1999). Although they rarely proliferate, they have a high proliferative potential and proliferate at times of tissue expansion such as during development, during wound healing, and at the onset of a new hair growth cycle (anagen; Lavker and Sun, 1982; Bickenbach and Mackenzie, 1984; Morris *et al*, 1986; Cotsarelis *et al*, 1989; Wilson *et al*, 1994; Lyle *et al*, 1998). Much evidence suggests that cutaneous tumors arise from epithelial stem cells (reviewed in Morris, 2000), thus the ability to target these cells is important for studying carcinogenesis.

We first localized putative epithelial stem cells as LRC to the hair follicle bulge of both mouse and human skin (Cotsarelis *et al*, 1990; Lyle *et al*, 1998). The bulge area of the hair follicle consists of a cluster of cells located at the lowermost portion of the "permanent" follicle (Cotsarelis *et al*, 1990; Lyle *et al*, 1998, 1999). Cells below this area degenerate during the catagen stage of the hair follicle cycle and regenerate at the onset of anagen (Cotsarelis *et al*, 1990; Cotsarelis and Millar, 2001). Bulge cells are strategically located to repopulate both the underlying follicle and the overlying epidermis (**Fig 1*F***), and the ability of bulge cell progeny to differentiate into both of these cell populations has been implied (Taylor *et al*, 2000; Oshima *et al*, 2001). Keratinocyte bulge cells, like stem cells in other epithelia such as the cornea (Schermer *et al*, 1986; Cotsarelis *et al*, 1989), possess other characteristics of epithelial stem cells: ultrastructurally they appear less-differentiated, and biochemically they possess a unique cytokeratin profile (Cotsarelis *et al*, 1990; Lyle *et al*, 1998). Recent evidence demonstrating that melanocyte precursors also localize to the bulge supports the notion that this area forms a niche for the maintenance of the stem cell phenotype (Nishimura *et al*, 2002).

Because cytokeratins generally are expressed in a differentiation-specific manner (Fuchs and Cleveland, 1998), keratin (K) promoter regions have proven invaluable for targeting transgene expression to specific compartments within epithelia. For example, the K5 and K14 promoters drive expression of transgenes to the relatively undifferentiated basal cell layer of the epidermis and hair follicle, whereas the K10 promoter targets the more differentiated suprabasal cells of these tissues (Vassar *et al*, 1989; Bailleul *et al*, 1990; Byrne and Fuchs, 1993; Ramirez *et al*, 1994). The use of these promoters in transgenic mice has resulted in major advances in understanding of epidermal and hair follicle development, proliferation, differentiation, and carcinogenesis (Oro *et al*, 1997; Brown *et al*, 1998; Gat *et al*, 1998; Grachtchouk *et al*, 2000; Waikel *et al*, 2001; Santos *et al*, 2002). Nonetheless, none of the existing promoters target epithelial stem cells with specificity. This major limitation impedes determining the contribution of stem cells to the phenotype of transgenic mice.

We previously demonstrated that hair follicle bulge cells in human scalp specifically express K15 (Lyle *et al*, 1998, 1999), and here we describe that K15 is preferentially expressed in adult mouse bulge cells as well. We hypothesized that the K15 promoter would target hair follicle bulge cells. Therefore, we cloned the up-stream region of the K15 gene and generated K15/lacZ transgenic reporter mice. We demonstrated that the K15 promoter

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Abbreviations: K, keratin; LRC, label-retaining cell; PBS, phosphate-buffered saline.

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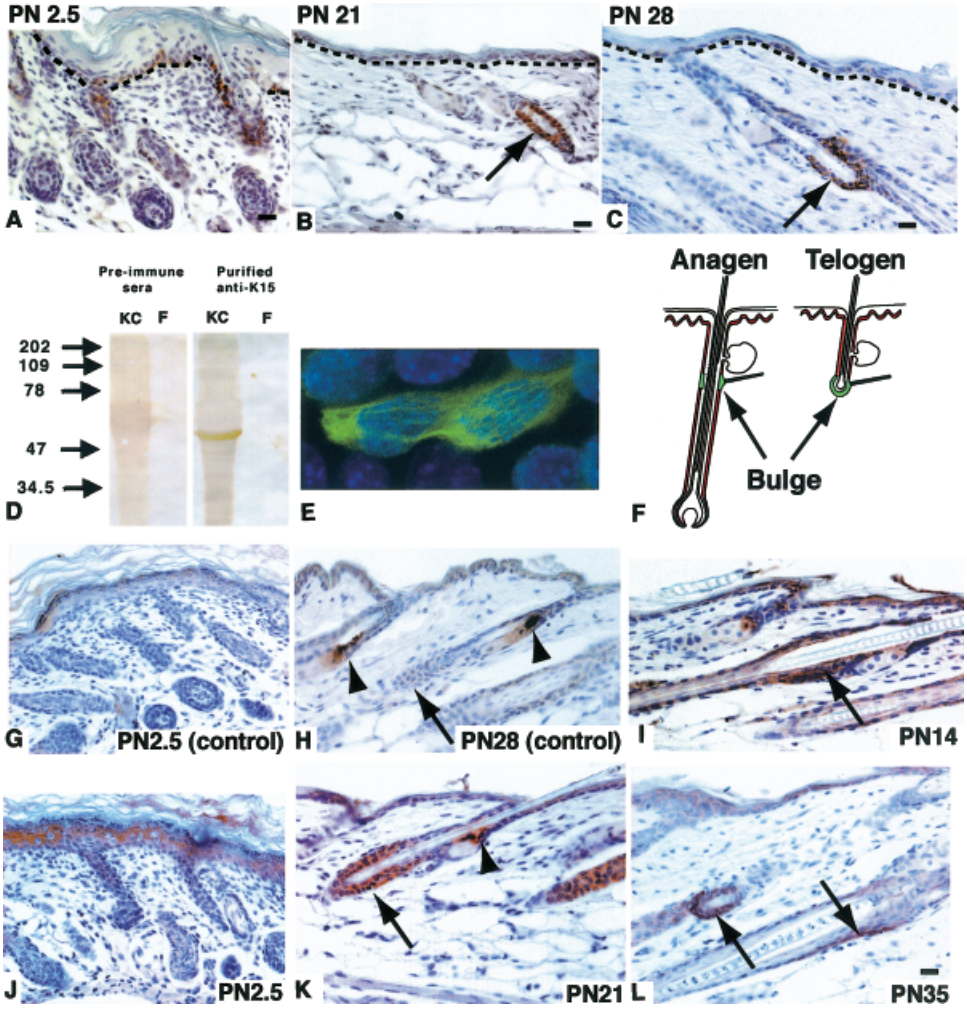

Figure 1. K15 gene and protein expression in mouse skin. (*A–C*) *In situ* hybridization with K15-specific riboprobe showed expression of K15 in basal cells of neonatal epidermis and in bulge cells in mature skin. The *dashed line* denotes the dermal/epidermal junction. *Arrows* point to the bulge. (*D*) Immunoblot using chicken anti-K15 antibody demonstrating detection of ~48-kDa band in protein from human keratinocytes (*KC*) and not fibroblasts (*F*). (*E*) Immunostaining of PAM212 cells (mouse keratinocyte cell line) with anti-K15 antibody demonstrated cytoplasmic staining in a filamentous pattern characteristic of intermediate filaments (K15, *green;* nuclear Hoechst stain, *blue*). (*F*) Diagram depicting hair follicles in anagen and telogen. K5 and K14 promoters are active throughout the epidermal and hair follicle basal layers (*red*), including bulge (*green*). (*G–L*) Immunostaining of mouse skin at different ages. Note positive staining for K15 in hair follicle bulge (*arrows*) and lack of staining for K15 in adult epidermis. Controls (*G,H*) performed with secondary (anti-chicken) antibody alone. *Arrowheads* denote sebaceous gland, which stains nonspecifically (*bars*, 25 μm).

specifically targets hair follicle bulge cells in adult transgenic mice and that the targeted cells are predominantly LRC. Thus, K15 expression correlates with a less differentiated and quiescent keratinocyte phenotype, and the K15 promoter serves as a valuable tool for targeting hair follicle bulge cells.

MATERIALS AND METHODS

In situ hybridization PCR-derived riboprobe templates were synthesized by introducing the T7 promoter into sense and antisense templates as described (Sitzmann and LeMotte, 1993) with some modification. One primer pair, mK15F (5′-GCAGTAGCAGCAGC AGCAATTTC-3′) and mK15R-T7 (5′-GTAATACGACTCACTATAG GGCCACTCAGAAGGAAGCCGAGAAAGC-3′), was used to generate the DNA template for the antisense riboprobe spanning 263BP of the 3′ end of the mouse K15 cDNA. Similarly, a primer pair consisting of mK15F-T7 (5′-GTAATACGACTCACTATAGGGCGCAGTAGCAGCAG CAGCAATTTC-3′) and mK15R (5′-CACTCAGAAGGAAGCCGA GAAAGC-3′) was used to amplify template for sense riboprobe. PCR procedures were carried out at 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min, for 30 cycles. The PCR products were separated on an agarose gel and purified with the QIAquick extraction kit (Qiagen, Chatsworth, CA). These templates were used to synthesize antisense and sense digoxigenin-labeled riboprobes with a DIG RNA labeling kit (Roche, Indianapolis, IN) according to the manufacturer's instructions.

In situ hybridization was performed using the digoxigenin-labeled riboprobes essentially as described previously (Hebert *et al*, 1994). Four percent paraformaldehyde-fixed and paraffin-embedded BALB/c mouse skin tissue sections were deparaffinized with xylene, rehydrated, treated with 20 μg per mL proteinase keratin, and postfixed with 4% paraformaldehyde for 10 min. The sections were then hybridized to digoxigenin-labeled antisense and sense K15 RNA probes (500 ng/mL) at 55°C overnight. The slides were washed at high stringency with 0.2X SSC at 68°C for 45 min, treated with anti-digoxigenin antibody (Roche) coupled to horseradish peroxidase, and amplified with the tyramide signal amplification kit (NEN Life Science, Boston, MA). The slides were then developed in diaminobenzidine/H_2O_2 substrate (Sigma Chemical Co., St. Louis, MO) and counterstained with hematoxylin Gill's Formulation No. 3 (Fisher Scientific, Hampton, NH).

Generation of K15-specific antisera A peptide corresponding to the carboxy-terminal 12 amino acids of human K15, CDGQVVSSHKREI (Lloyd *et al*, 1995), was synthesized and coupled through its N-terminal cysteine residue to keyhole limpet hemocyanin via MBS (Pierce 22310). The conjugated peptide was used to immunize chickens with standard protocols at Cocalico Biologicals, Inc. (Reamstown, PA). Briefly, for the initial inoculation, 100 μg of conjugated peptide was emulsified in complete Freund's adjuvant and injected into the breast muscle of chickens whose sera samples had been prescreened by immunohistochemistry to be certain that it contained no endogenous keratin antibodies. Three weeks later, 50 μg of conjugated peptide emulsified in incomplete Freund's adjuvant was injected to boost antibody production. After 10 d, the antibody titer of the first test serum sample was analyzed by immunohistochemistry. The chickens were further boosted with 50 μg of conjugate peptide and sera samples were collected 10 d after each boost. The sera samples were purified with an affinity column made from the peptide and cyanogen bromide-activated Sepharose 4B (Pharmacia) and then used for immunostaining and immunoblotting.

Immunoblotting Cell lysates from primary cultured human foreskin keratinocytes and fibroblasts were run on 10% SDS–PAGE and blotted onto polyvinylidene difluoride membranes. Blots were blocked in 5% nonfat dried milk in phosphate-buffered saline (PBS), 0.2% Tween 20 for 1 h at room temperature and then incubated with affinity-purified chicken

anti-K15 antibody (1:500) or preimmune serum for 2 h at room temperature, followed by horseradish peroxidase-conjugated anti-chicken immunoglobulins (1:2000, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for 1 h. After being washed, blots were developed with diaminobenzidine (Sigma Chemical Co.).

Anti-K15 Immunohistochemistry Immunostaining was performed similarly to previous descriptions (Lyle *et al*, 1998). Mouse skin was fixed in Bouin's fixative at room temperature overnight. Samples were then cleared with multiple 70% rinses and stored in 70% ethanol for 1 to 3 d before paraffin embedding and tissue sectioning. PAM212 cells were grown on glass coverslips and fixed with acetone. Endogenous peroxidases were quenched using 0.6% H_2O_2 in methanol. Samples were blocked for 20 min in 1X PBS, 0.1% Triton X-100, 0.1% bovine serum albumin (Sigma Chemical Co.), and 5% normal goat serum (Sigma Chemical Co.). Sections were incubated overnight at 4°C with chicken anti-K15 antibody (1:200). After being rinsed, samples were then incubated for 1 h in 1:200 biotinylated goat anti-chicken IgG (Kirkegaard & Perry Laboratories, Inc.) and then incubated in 1:500 streptavidin:horseradish peroxidase conjugate (Gibco BRL/Life Technologies, Inc., Gaithersburg, MD). Slides were washed and incubated for 7 min in Sigma Fast 3,3′-diaminobenzidine tablet sets diaminobenzidine substrate solution (Sigma Chemical Co.). The reaction was stopped using distilled water, and then samples were counterstained using a 1:10 dilution of hematoxylin Gill's Formulation No. 3 (Fisher Scientific), dehydrated through xylene, and coverslipped using Permount solution.

Cloning of K15 promoter The K15 (-5.0) promoter was cloned from C57 BL/SJ mouse genomic DNA (isolated from tail) by PCR using the Supermix High Fidelity Kit (GIBCO). The sequences of the forward and reverse primers were. CTGAGCTACCAGCGAGACTCC (K15F1) and TTCCTGTCCCTAGCAAGCAGGAGAG (K15R1), respectively. XhoI and EcoRI restriction sequences were added to the 5′ end of forward and reverse primers respectively for subsequent cloning of the PCR product into PBK/CMV vector (Stratagene). Similarly, the K15(-1.3) partial promoter sequence was cloned by using a forward primer K15F2 (AGCCTCAGTGCATTCCACG), 1342 bp upstream of K15 start codon and primer K15R1. K15(-3.7) was cloned by using primer K15F1 and primer K15R2 (GCTCTCCTTCGTGGAATGCAC) located 1315 to 1335 upstream of the K15 start codon.

Luciferase assays Mouse PAM212 cells (Roop *et al*, 1983) were grown to 30% to 50% confluence in six-well plates with 1:3 Ham's F-12 medium (Gibco) and Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were cotransfected with 1.5 nM K15/PGL3 construct and 0.4 μg of pSV-β- galactosidase control vector (Promega, Madison, WI) using 20 μL LipofectAMINE reagent (Gibco) according to the manufacturer's protocol. Cell extracts were prepared 48 h after transfection, and luciferase and β-galactosidase activities were determined using a Dual-Light kit (PE Biosystems) on a TD20e luminometer (Turner). β-Galactosidase activity was used to normalize transfection efficiency. Luciferase/β-galactosidase ratios of K15/pGL3 constructs were compared to pGL3-Basic Vector (baseline control) to determine the relative activity of various K15/PGL3 constructs. All transfections were performed in duplicate at least three separate times with reproducible results.

Generation of transgenic mice The lacZ reporter gene was cloned downstream of the K15 promoter (**Fig 2**). Transgenic mice were produced by the University of Pennsylvania Transgenic and Chimeric Mouse facility. The transgene was released by digestion with *Xho*I/*Sal*I (K15/lacZ) and microinjected into fertilized eggs from a B6SJLF1/J × B6SJLF1/J cross (Jackson Laboratories, Bar Harbor, ME). Transgenic mice were identified by southern blotting or PCR of tail biopsy DNA using transgene-specific probes or primers. All animal protocols were approved by the University of Pennsylvania IACUC.

Histochemical assay of β-galactosidase activity We initially evaluated several β-galactosidase staining techniques, including the use of frozen sections. We found that the formaldehyde/glutaraldehyde protocol described here provided the best morphology with equal intensity of signal as frozen sections. Mouse tissue samples were fixed in freshly prepared 2% formaldehyde/0.2% glutaraldehyde in PBS at 4°C for 1 to 2 h and then washed in three changes of PBS at room temperature for 1 h. Fixed tissue was incubated at 22°C overnight in 1 mg per mL X-Gal (5 mM K-ferrocyanide, 5 mM K-ferricyanide, 2 mM $MgCl_2$) in PBS. Then tissue was washed with PBS, fixed in formalin, and embedded in paraffin.

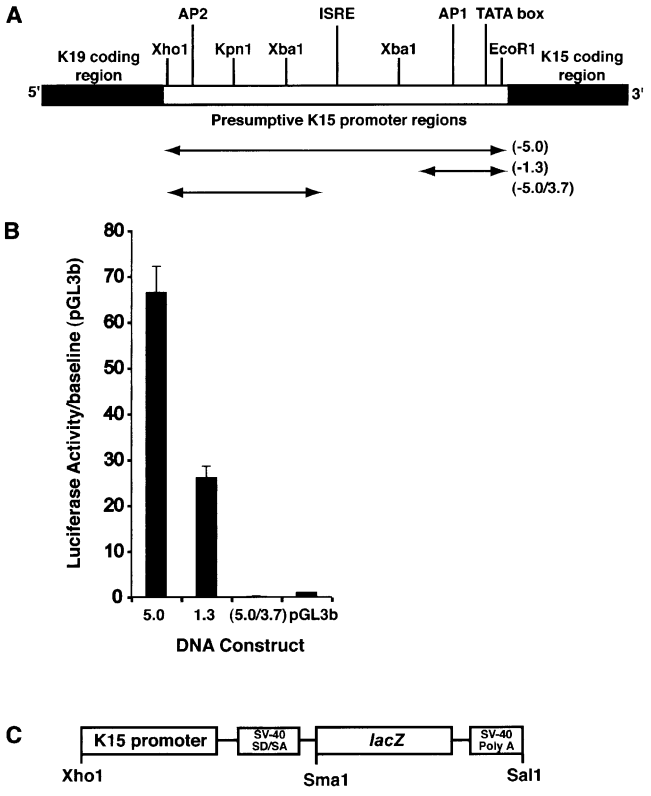

Figure 2. Isolation and characterization of K15 promoter. (*A*) Genomic organization of K15 promoter region. The K15 coding region lies ~5 kb downstream to the K19 coding region. We cloned three presumptive K15 promoter regions, indicated by the *double-headed arrows*. Restriction enzyme sites and consensus sequences for selected transcription factor binding sites are indicated. (*B*) Results of *in vitro* luciferase assays demonstrated that the full-length (5.0-kb) promoter has the highest activity. *PGL3b* refers to the luciferase construct lacking a promoter. (*C*) K15/lacZ construct. The 5.0-kb promoter region was used to generate transgenic mice. *SD/SA,* splice donor/splice acceptor.

Five-micrometer-thick tissue sections were counterstained with Fast Red nuclear stain.

Generation of LRC-containing K15/lacZ transgenic mice K15/lacZ neonatal mice were injected subcutaneously with tritiated thymidine (5 μCi per pup, twice per day, starting on postnatal day (PN) 3, for a total of 3 d) or bromodeoxyuridine (200 μg per pup per day, starting on PN3 for a total of 3 d), and then chased for 50 days, similar to previous descriptions to generate mice containing LRC (Cotsarelis *et al*, 1990; Morris and Potten, 1994). Autoradiography and immunohistochemistry were performed as described previously (Cotsarelis *et al*, 1989; Morris and Potten, 1994; Lyle *et al*, 1998).

RESULTS

K15 expression marks the least differentiated cells of the neonatal epidermis and adult hair follicle bulge To determine K15 expression patterns in mouse skin during postnatal development and maturation, we used *in situ* hybridization with K15-specific riboprobes (**Fig 1*A*–*C***) and immunohistochemistry (**Fig 1*G*–*L***) with a chicken polyclonal antibody that we raised against a peptide corresponding to the 12 amino acids from the carboxy terminus of the human K15 protein (see Materials and Methods and Lloyd *et al*, 1995). The specificity of the antibody was confirmed by immunoblotting protein from human keratinocytes, which demonstrated a single band at ~48 kDa, the expected size for the K15 protein (**Fig 1*D***).

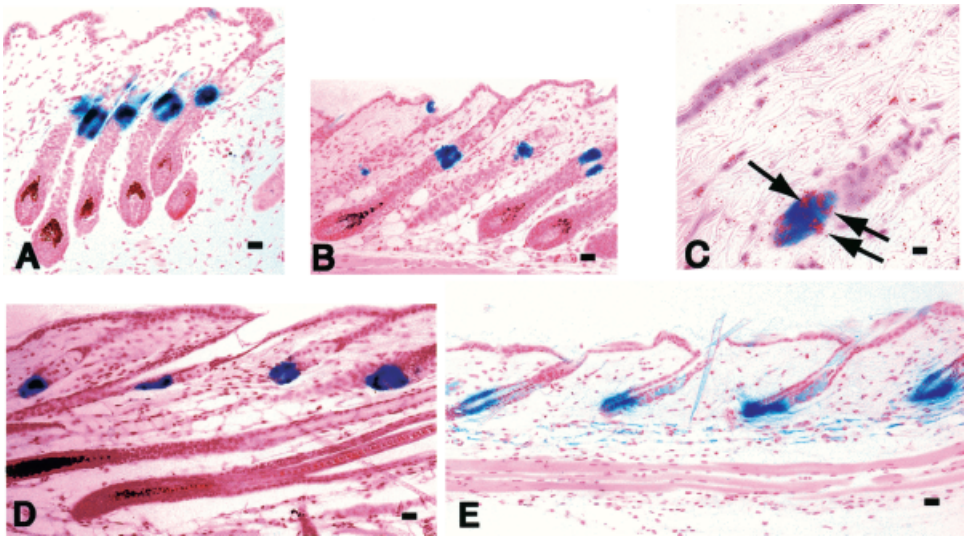

Figure 3. K15 promoter specifically targets bulge keratinocytes in adult K15/lacZ transgenic mice. Histology of X-Gal-treated K15/lacZ skin from different ages and body sites. *Blue stain* indicates transgene expression. (*A*) PN34, face; (*B*) PN28, back; (*C*) example of co-localization of K15/lacZ-positive cells and LRC (*arrows*). Silver grains are red-illuminated. (*D*) PN34, back; (*E*) PN58, back. Hair follicles are in anagen in *A,B,D* and telogen in *C,E* (*bars*, 10 μm). Sections are counterstained with Fast Red.

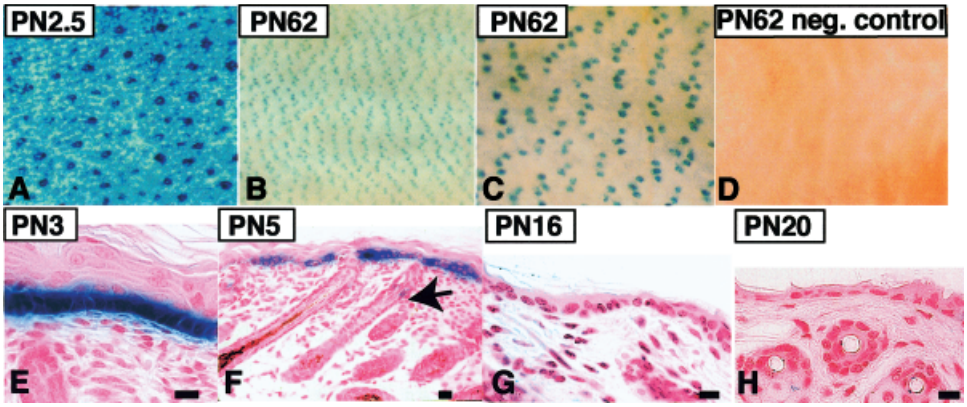

Figure 4. K15 promoter activity during postnatal development. Skin from K15/lacZ transgenic mice treated with X-Gal at indicated ages. Whole mounted (*A–D*) and sectioned (*E–H*) skin at indicated ages. Early time points (*A,E,F*) show epidermal K15 promoter activity. Later time points show specific K15 promoter activity in bulges and lack of promoter activity elsewhere (*B,C,G*). X-Gal-treated nontransgenic control skin (*D,H, bars*, 10 μm).

This antibody also stained a subset of PAM212 cells, a mouse keratinocyte cell line, in a pattern characteristic of intermediate filament proteins (**Fig 1*E***).

Strong expression of K15 mRNA and protein was uniformly concentrated in the hair follicle bulge of older mice (**Fig 1*B,C,K,L***), similar to the human scalp (Lyle *et al*, 1999). Epidermal expression of K15, at both the mRNA and the protein levels, was present in younger mice, but markedly decreased in mice older than approximately 2 wk of age (**Fig 1**). The preferential expression of K15 protein in the bulge cells was present in all four types of hair follicles found in the back skin of mice (**Fig 3** and data not shown). In approximately one-third of adult mice, variable weak expression of K15 in epidermal keratinocytes was detected by immunohistochemistry. Nevertheless, in all cases, K15 expression was markedly weaker in the epidermis compared with the bulge keratinocytes, which were uniformly positive. No difference in the distribution patterns of K15 protein was noted based on whether we used frozen or paraffin sections for staining with the antibody. In general, Bouin's-fixed tissue generated the strongest signal for this antibody.

Isolation of K15 promoter We cloned the entire upstream region of the mouse K15 gene, which comprises approximately 5.0 kb between the K19 and K15 genes (Nozaki *et al*, 1994; **Fig 2*A***). To evaluate the presumptive K15 promoter regions, we generated three different K15/luciferase constructs utilizing the 5.0, 1.3, and 5.0/3.7-kb regions located upstream to the K15-coding region (**Fig 2*A***). We transfected PAM212 cells with these constructs and examined promoter activity with luciferase assays (**Fig 2*B***). Both the entire (5.0 kb) region and the truncated (1.3 kb) region resulted in high expression levels *in vitro* (**Fig 2*B***). Interestingly, the 5.0/3.7-kb region was not active and resulted in 100-fold lower luciferase activity compared with baseline control levels, suggesting that the 5′ region of the promoter may possess inhibitory *cis*-acting elements. This was supported by our sequencing of the promoter region (deposited in NCBI, accession numbers BankIt486710 and AF542050), which revealed several potential *cis*-regulatory elements, such as AP-1 and Sp1 sites (**Fig 2*A***), that are important for regulation of other keratin genes (Byrne *et al*, 1994; Ramirez *et al*, 1994; Whitbread and Powell, 1998; Maytin *et al*, 1999; Brembeck and Rustgi, 2000). These findings suggest that regulatory elements within the 5′ region of the promoter may inhibit K15 expression at some level and could be important for specificity of expression.

The K15 promoter targets hair follicle bulge cells in adult transgenic mice To determine whether the presumptive K15 promoter region functions *in vivo*, we generated transgenic mice carrying the full-length (5.0 kb) K15 promoter fused to the *Escherichia coli lacZ* reporter gene (K15/lacZ; **Fig 2*C***). Four K15/lacZ founder mice were generated. Three founder mice expressed β-galactosidase in their tail skin (by X-Gal staining), and one was negative. One positive founder mouse appeared runted. The other two positive founder mice had strong expression of β-galactosidase in skin and phenotypically appeared completely normal. We therefore used lines from these two founders for subsequent analyses.

In mice older than approximately 2 to 3 wk of age, K15/lacZ expression strikingly localized to the bulge cells of hair follicles from all areas of the body, including facial, back, and tail skin throughout all stages of the hair cycle (**Fig 3**). This expression pattern persisted in older mice (>50 days old), which also displayed strong β-galactosidase activity specifically in the bulge. This pattern was observed in two separate transgenic lines. Overall, the activity of the 5.0-kb K15 promoter appeared more specific than the expression pattern of endogenous K15 protein that was occasionally detected in the epidermis.

Majority of K15/lacZ-positive bulge cells are LRC To examine the association between K15 promoter activity and the stem cell phenotype in the K15/lacZ transgenic mice, we identified quiescent cells as LRC. LRC identify presumptive epithelial stem cells in both rodent skin and human skin grafted to immunodeficient mice (Bickenbach and Mackenzie, 1984; Cotsarelis *et al*, 1990; Lyle *et al*, 1998; Morris and Potten, 1999). We counted the number of lacZ-positive cells that were also LRC in six nonconsecutive tissue sections from the back skin of five mice that had received tritiated thymidine or bromodeoxyuridine over a 3-d period as neonates as previously described. We found that 92% ± 3% of the cells expressing lacZ were also LRC (**Fig 3*C***).

K15 promoter activity in neonatal mouse skin To further characterize the activity of the K15 promoter, we examined skin from K15/lacZ transgenic mice for β-galactosidase activity during the neonatal time period (**Fig 4**). Although significant transgene activity was present in the interfollicular epidermis of the neonate (**Fig 4*A*,*E*,*F***) by approximately PN16, epidermal promoter activity decreased, and epidermal expression generally was lost (**Fig 4*G***). Adult epidermis was devoid of K15/lacZ promoter activity (**Figs 3, 4**). K15 promoter activity was evident in the bulge during folliculogenesis at PN5 (**Fig 4*F***) and became more prominent in the bulge during the first telogen stage at approximately 3 wk of age.

DISCUSSION

Previously, using the K5 or K14 promoters, investigators simultaneously targeted transgenes to the epidermal and hair follicle basal layers, which are contiguous (**Fig 1*F***). The basal layer contains stem cells, transient amplifying cells, and even terminally differentiating cells. Thus, the use of these existing keratin promoters precludes determining whether a phenotype results from the targeting of stem or transient amplifying cell populations. The use of the K15 promoter, which selectively targets the hair follicle bulge, where stem cells are thought to reside, allows for determining the role of bulge cells in processes such as wound healing, aging, alopecia, and carcinogenesis. In contrast to a sheep promoter construct described previously (Whitbread and Powell, 1998), which likely included portions of both the K15 and the K19 genes and had a much wider distribution of activity, the mouse K15 promoter described here clearly targets hair follicle bulge cells with specificity in the adult. Interestingly, promoter activity was more specific for bulge cells than protein expression. Our findings of K15 expression in the epidermis, albeit at variable and markedly lower levels than in the bulge, are in line with previous reports of K15 protein in human epidermis (Porter *et al*, 2000). Whether this expression is due to persistence of K15 protein in cells that have migrated from the bulge or due to expression of K15 in pre-existing epidermal cells remains to be determined. The presence of K15 in the epidermis also suggests that using K15 protein expression as a sole criterion for defining an epithelial stem cell would be naive. Rather, we argue that K15 protein expression only correlates with keratinocytes that are less differentiated. K15's precise role in keratinocyte biology has yet to be defined.

During the first 2 postnatal wk in the mouse, the epidermis undergoes a drastic alteration in its morphology, which changes from multiple cell layers with a thick stratum corneum in the neonate to its more mature phenotype of only three cell layers with a thin stratum corneum in the adult (Hanson, 1947). K15 expression in the epidermis correlated with the immature epidermal phenotype. Interestingly, K15 promoter activity in the follicle was limited to a small number of cells in the newly developing anagen follicle until after epidermal maturation when K15 promoter activity prominently appeared in the hair follicle bulge of the telogen follicle. Our results suggest that the loss of K15 expression in the basal epidermis and the persistence of K14 expression (Byrne *et al*, 1994), during the neonatal time period, indicate a switch to a more mature phenotype. Similar age-related changes in keratin expression patterns occur in the basal layer of the esophageal epithelium, in which K15 expression predominates in young mice, but is then replaced by K14, the other major type I keratin in the esophagus (Lloyd *et al*, 1995).

The correlation of K15 expression and K15 promoter activity with the least differentiated cell populations in the epidermis and hair follicle supports the notion that K15 promoter activity is a putative epithelial stem cell marker. To further test the correlation between K15 promoter activity and the epithelial stem cell phenotype, we examined the K15/lacZ-positive cells for their ability to retain label. A cell's ability to retain label after a long chase period indicates that it has a quiescent phenotype, a hallmark of putative epithelial stem cells (Waikel *et al*, 2001). It has been shown repeatedly that the bulge contains the most quiescent cells throughout the epidermis and hair follicle, in that these cells retain label for many months (Cotsarelis *et al*, 1990; Lyle *et al*, 1998; Morris and Potten, 1999; Taylor *et al*, 2000). We found that the large majority of K15/lacZ-positive cells are LRC. The small percentage of K15/lacZ cells that are not LRC likely represent tissue sections in which the tritiated thymidine-labeled nucleus was not sectioned or stem cells that had divided and diluted out their label. These results are in line with cell kinetic analysis of bulge cells isolated from the skin using a CD34 antibody (Trempus *et al*, 2003).

The isolation of a K15 promoter that targets bulge cells allows for the development of more advanced transgenic systems for studying the bulge. In combination with promoter/enhancer elements responsive to tetracycline or hormonal agents. for example (Diamond *et al*, 2000; Arnold and Watt, 2001; Liu *et al*, 2001), inducible systems that activate oncogenes exclusively in bulge stem cells in adult mice can now readily be developed. These are likely to yield insights into the role of bulge cells in carcinogenesis through lineage analysis and by specific targeting of oncogenes to the bulge cells. Similarly, an inducible K15 promoter driving Cre recombinase can be designed for deleting genes selectively in bulge cells of adult mice (see Cao *et al*, 2001). Transgenic mice expressing green fluorescent protein can be developed for isolation and characterization of bulge cells. Such approaches should allow for the identification of specific genes involved in hair follicle cycling, wound healing, and other processes.

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REFERENCES

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

Bailleul B, Surani MA, White S, *et al*: Skin hyperkeratosis and papilloma formation in transgenic mice expressing a Ras oncogene from a suprabasal keratin promoter. *Cell* 62:697–708, 1990

Bickenbach J, Mackenzie I: Identification and localisation of label retaining cells in hamster epithelium. *J Invest Dermatol* 82:618–622, 1984

Brembeck FH, Rustgi AK: The tissue-dependent keratin 19 gene transcription is regulated by Gklf/Klf4 and Sp1. *J Biol Chem* 275:28230–28239, 2000

Brown K, Strathdee D, Bryson S, Lambie W, Balmain A: The malignant capacity of skin tumours induced by expression of a mutant H-Ras transgene depends on the cell type targeted. *Curr Biol* 8:516–524, 1998

Byrne C, Fuchs E: Probing keratinocyte and differentiation specificity of the human K5 promoter *in vitro* and in transgenic mice. *Mol Cell Biol* 13:3176–3190, 1993

Byrne C, Tainsky M, Fuchs E: Programming gene expression in developing epidermis. *Development* 120:2369–2383, 1994

Cairns J: Somatic stem cells and the kinetics of mutagenesis and carcinogenesis. *Proc Natl Acad Sci USA* 99:10567–10570, 2002

Cao T, Longley MA, Wang XJ, Roop DR: An inducible mouse model for epidermolysis bullosa simplex: Implications for gene therapy. *J Cell Biol* 152:651–656, 2001

Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM: Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: Implications on epithelial stem cells. *Cell* 57:201–209, 1989

Cotsarelis G, Millar SE: Towards a molecular understanding of hair loss and its treatment. *Mol Med Today* 7:293–301, 2001

Cotsarelis G, Sun TT, Lavker RM: Label-retaining cells reside in the bulge area of pilosebaceous unit: Implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell* 61:1329–1337, 1990

Diamond I, Owolabi T, Marco M, Lam C, Glick A: Conditional gene expression in the epidermis of transgenic mice using the tetracycline-regulated transactivators Tta and Rta linked to the keratin 5 promoter. *J Invest Dermatol* 115:788–794, 2000

Fuchs E, Cleveland DW: A structural scaffolding of intermediate filaments in health and disease. *Science* 279:514–519, 1998

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

Grachtchouk M, Mo R, Yu S, Zhang X, Sasaki H, Hui CC, Dlugosz AA: Basal cell carcinomas in mice overexpressing Gli2 in skin. *Nat Genet* 24:216–217, 2000

Hanson J: The histogenesis of the epidermis in the rat and mouse. *J Anat* 81:174–197, 1947

Hebert JM, Rosenquist T, Gotz J, Martin GR: Fgf5 as a regulator of the hair growth cycle: Evidence from targeted and spontaneous mutations. *Cell* 78:1017–1025, 1994

Lavker R, Sun TT: Heterogeneity in epidermal basal keratinocytes: Morphological and functional correlations. *Science* 1982:1239–41, 1982

Lehrer MS, Sun TT, Lavker RM: Strategies of epithelial repair: Modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci* 111:2867–2875, 1998

Liu X, Alexander V, Vijayachandra K, Bhogte E, Diamond I, Glick A: Conditional epidermal expression of TGFbeta 1 blocks neonatal lethality but causes a reversible hyperplasia and alopecia. *Proc Natl Acad Sci USA* 98: 9139–9144, 2001

Lloyd C, Yu QC, Cheng J, Turksen K, Degenstein L, Hutton E, Fuchs E: The basal keratin network of stratified squamous epithelia: Defining K15 function in the absence of K14. *J Cell Biol* 129:1329–1344, 1995

Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, Cotsarelis G: The C8/144b monoclonal antibody recognizes cytokeratin 15 and defines the location of human hair follicle stem cells. *J Cell Sci* 111:3179–3188, 1998

Lyle S, Christofidou-Solomidou M, Liu Y, Elder DE, Albelda S, Cotsarelis G: Human hair follicle bulge cells are biochemically distinct and possess an epithelial stem cell phenotype. *J Investig Dermatol Symp Proc* 4:296–301, 1999

Maytin EV, Lin JC, Krishnamurthy R, Batchvarova N, Ron D, Mitchell PJ, Habener JF: Keratin 10 gene expression during differentiation of mouse epidermis requires transcription factors C/Ebp and Ap-2. *Dev Biol* 216:164–181, 1999

Morris RJ: Keratinocyte stem cells: Targets for cutaneous carcinogens. *J Clin Invest* 106:3–8, 2000

Morris R, Fischer S, Slaga T: Evidence That a Slowly Cycling Subpopulation of Adult Murine Epidermal Cells Retains Carcinogen. *Cancer Res* 46:3061–3066, 1986

Morris RJ, Potten CS: Highly persistent label-retaining cells in the hair follicles of mice and their fate following induction of anagen. *J Invest Dermatol* 112: 470–475, 1999

Morris RJ, Potten CS: Slowly cycling (label-retaining) epidermal cells behave like clonogenic stem cells *in vitro*. *Cell Prolif* 27:279–289, 1994

Nishimura EK, Jordan SA, Oshima H, *et al*: Dominant role of the niche in melanocyte stem-cell fate determination. *Nature* 416:854–860, 2002

Nozaki M, Mori M, Matsushiro A: The complete sequence of the gene encoding mouse cytokeratin 15. *Gene* 138:197–200, 1994

Oro AE, Higgins KM, Hu Z, Bonifas JM, Epstein EH Jr, Scott MP: Basal cell carcinomas in mice overexpressing sonic hedgehog. *Science* 276:817–821, 1997

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

Porter RM, Lunny DP, Ogden PH, *et al*: K15 expression implies lateral differentiation within stratified epithelial basal cells. *Lab Invest* 80:1701–10, 2000

Potten CS, Booth C: Keratinocyte stem cells: A commentary. *J Invest Dermatol* 119:888–899, 2002

Ramirez A, Bravo A, Jorcano JL, Vidal M: Sequences 5′ of the bovine keratin 5 gene direct tissue- and cell-type-specific expression of a lacz gene in the adult and during development. *Differentiation* 58:53–64, 1994

Roop DR, Hawley-Nelson P, Cheng CK, Yuspa SH: Expression of keratin genes in mouse epidermis and normal and malignantly transformed epidermal cells in culture. *J Invest Dermatol* 81:144s–149s, 1983

Santos M, Paramio JM, Bravo A, Ramirez A, Jorcano JL: The expression of keratin K10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis. *J Biol Chem* 277:19122–19130, 2002

Schermer A, Galvin S, Sun TT: Differentiation-related expression of a major 64K corneal keratin *in vivo* and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 103:49–62, 1986

Sitzmann JH, LeMotte PK: Rapid and efficient generation of PCR-derived riboprobe templates for in situ hybridization histochemistry. *J Histochem Cytochem* 41:773–776, 1993

Slack JM: Stem cells in epithelial tissues. *Science* 287:1431–1433, 2000

Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM: Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell* 102:451–461, 2000

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

Vassar R, Rosenberg M, Ross S, Tyner A, Fuchs E: Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. *Proc Natl Acad Sci USA* 86:1563–1567, 1989

Waikel RL, Kawachi Y, Waikel PA, Wang XJ, Roop DR: Deregulated expression of c-Myc depletes epidermal stem cells. *Nat Genet* 28:165–168, 2001

Watt FM, Hogan BL: Out of Eden: Stem cells and their niches. *Science* 287:1427–1430, 2000

Whitbread LA, Powell BC: Expression of the intermediate filament keratin gene, K15, in the basal cell layers of epithelia and the hair follicle. *Exp Cell Res* 244:448–459, 1998

Wilson C, Cotsarelis G, Wei ZG, *et al*: Cells within the bulge region of mouse hair follicle transiently proliferate during early anagen: Heterogeneity and functional differences of various hair cycles. *Differentiation* 55:127–136, 1994