An unexpected role for keratin 10 end domains in susceptibility to skin cancer

Jiangli Chen[1], Xing Cheng[1], Maria Merched-Sauvage[1], Carlos Caulin[1], Dennis R. Roop[1,2] and Peter J. Koch[1,2,*]

[1]Department of Dermatology and [2]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030, USA

*Author for correspondence (e-mail: pkoch@bcm.tmc.edu)

Accepted 9 October 2006
Journal of Cell Science 119, 5067-5076 Published by The Company of Biologists 2006
doi:10.1242/jcs.03298

Summary

Keratin 10 (K10) is a type I keratin that is expressed in post-mitotic suprabasal keratinocytes of the skin. Based on cell culture experiments and transgenic mouse studies, it has been proposed that K10 suppresses cell proliferation and tumor formation in the skin. Furthermore, the ability of K10 to suppress cell proliferation was mapped to its unique N- and C-terminal protein domains. In the present study, we modified the endogenous keratin 14 (K14) gene of mice using a knock-in approach to encode a chimeric keratin that consists of the K14 rod domain fused to the K10 head and tail domains (K1014chim). This transgene was expressed in the basal layer of the epidermis and the outer root sheath of hair follicles. Unexpectedly, we found that the K10 end domains had no effect on basal keratinocyte proliferation in vivo. Moreover, when subjected to a chemical skin carcinogenesis protocol, papilloma formation in mutant mice was accelerated instead of being inhibited. Our data suggest that the increased tumor susceptibility of K1014chim mice is in part due to a suppression of apoptosis in mutant keratinocytes. Our results support the notion that intermediate filaments, in addition to their function as cytoskeletal components, affect tumor susceptibility of epithelial cells.

Key words: Intermediate filaments, Keratin 10, Keratin 14, Apoptosis, Skin carcinogenesis, Keratinocytes

Introduction

Keratins are cytoskeletal proteins that are expressed in epithelial cells. More than 50 keratin genes have been identified in the mammalian genome [mainly mouse and human were analyzed (e.g. Hesse et al., 2004; Rogers et al., 2004; Rogers et al., 2005)]. The corresponding proteins are classified as type I or type II keratins based on biochemical characteristics such as molecular weight and isolelectric point (reviewed by Coulombe and Wong, 2004; Kirfel et al., 2003). Type I and II keratins form obligatory heteropolymers that assemble into higher order cytoskeletal structures, the intermediate filaments (IF).

The secondary structure of epidermal keratin proteins is conserved. A central α-helical rod domain is essential for assembly of the IF polymer (reviewed by Coulombe and Omary, 2002; Coulombe and Wong, 2004; Kirfel et al., 2003). The non-α-helical N-terminal head and C-terminal tail domains are thought to project laterally from the IF and are thus available for direct interactions with a variety of cytoplasmic proteins, such as desmosomal proteins (reviewed by Getsios et al., 2004) (see also Kim et al., 2006).

In contrast to the keratin rod domains, head and tail domains do not show a high degree of amino acid sequence conservation. For example, the rod domains of the epidermal type I keratins K14 and K10 show an overall sequence identity of ~70%, whereas the sequence identity in the head (~57%) and tail (~33%) domains is significantly lower, suggesting that the keratin end domains might be involved in keratin subtype-specific functions.

In most epithelial cells, IF extend from the periphery of the nucleus to the plasma membrane where they attach to desmosomes, a subclass of epithelial cell junctions (reviewed by Cheng et al., 2005; Cheng and Koch, 2004). The three dimensional network of IF and desmosomes plays a crucial role in providing tissue resilience against biomechanical stress. This conclusion is supported by the identification of skin fragility disorders linked to impaired IF or desmosome function (reviewed by Cheng and Koch, 2004; Kirfel et al., 2003; Kottke et al., 2006).

The observation that the mammalian genome encodes a large number of keratins that show highly specialized tissue-specific and cell-type-specific expression patterns, suggests that individual keratins are highly adapted for a specific function within a given epithelial cell type. The present work focuses on epidermal keratins. Two main pairs of keratins are expressed in the mammalian interfollicular epidermis; keratin 5 (K5) and K14 together form the IF network in the basal layer of the epidermis and the outer root sheath (ORS) of hair follicles, cell layers that contain the stem cell compartments of the skin (Bickenbach, 2005; Braun and Watt, 2004; Kaur, 2006; Morris et al., 2004). This skin compartment also contains proliferating keratinocytes, so called transient amplifying (TA) cells. These cells go through a few cell cycles before they stop proliferating and initiate a terminal differentiation program that leads to the formation of the various strata of the epidermis and the hair follicles. The last step in keratinocyte differentiation is the formation of the stratum corneum, a cell layer that consists of dead enucleated cells (corneocytes) that cover the body surface.

The second major keratin pair expressed in the epidermis is

K1/K10. These proteins are synthesized in post-mitotic suprabasal keratinocytes. Based on cell culture experiments and transgenic over-expression of K10 in mouse skin, it has been suggested that K10 expression inhibits cell cycle progression and thus directly contributes to terminal keratinocyte differentiation (Koch and Roop, 2004 and references therein). Cell culture experiments suggested that this effect can be attributed specifically to the K10 head and tail end domains (Paramio et al., 2001). Interestingly, loss of K10 in the suprabasal epidermis leads to increased proliferation of basal cells and accelerated migration of keratinocytes from the basal cell layer to the skin surface (Reichelt et al., 2004). The observations summarized above suggest that spatial-temporal controlled expression of epidermal keratins is a prerequisite for normal epidermal development and homeostasis.

The experiments summarized above suggested that ectopic expression of the K10 end domains in the basal layer of the epidermis might suppress keratinocyte proliferation. A potential caveat of the in vivo experiments published so far is that they relied on over-expression of the full length K10 protein in the basal cell layer. In the present paper we used a different approach: our experiments were designed to assess whether K10 end domains, when present in a 1:1 molar ratio with K5 end domains in basal keratinocytes, would affect epidermal development and homeostasis. To this end, we modified the endogenous K14 gene locus of mice so that it encoded a chimeric keratin protein consisting of the K14 rod domain fused to the K10 head and tail domains (K1014chim). Our data demonstrate that the presence of this fusion protein in the basal layer and ORS is compatible with normal skin development. Specifically, it does not suppress keratinocyte proliferation. Unexpectedly, we observed that K1014chim mice are more susceptible to benign tumor development when subjected to a chemical skin carcinogenesis protocol. We also found that mutant skin showed a reduction in the apoptotic index when exposed to genotoxic stress, such as UV radiation. It is thus likely that a reduced apoptotic rate contributes to the increased tumor burden and tumor kinetics in mutant mice.

Results

Generation of a gene targeting vector encoding a chimeric keratin consisting of the K14 rod domain fused to the K10 head and tail domains

The mouse K14 gene consists of eight exons (Fig. 1a). The N-terminal head domain of the protein is encoded by exon 1, while the C-terminal tail domain is encoded by exons 7 and 8. First we cloned the K14 gene, including 5′ and 3′ flanking regions, from a mouse 129SV genomic library (for details, see Materials and Methods). Next we replaced exon 1 sequences encoding the K14 head domain with those of the K10 gene. Exons 7 and 8 were deleted and a cDNA encoding the K10 tail domain was fused with K14 exon 6 (Fig. 1b).

Upon transfection of the chimeric K1014 construct into PtK2 cells, a kidney epithelial cell line, we found that the 2.3 kb promoter element in this vector was sufficient to drive transgene expression. As expected, the K1014chim protein could be detected in the transfected cells with an antibody against the K10 C-terminus. Furthermore, as observed for other ectopically expressed keratins, K1014chim co-assembled with the endogenous K8/K18 proteins into an intermediate filament (IF) network, indicating that the targeting vector construction was successful (data not shown).

The biochemical properties of the K1014chim protein (Fig. 1e) are almost identical to those of mouse K10, with an amino acid sequence of 567 residues (K10, 570 residues; SwissProt accession no. P02535), a predicted molecular weight of 57,624 (K10, 57,842), and a calculated isolelectric point of 4.91 (K10, 4.84). This similarity is due to the fact that the rod domains of both keratins show a high degree of sequence similarity (see Introduction). Unfortunately, this prevented us from separating K10 and the K1014chim protein in SDS-PAGE gels. Nevertheless, as described below, we could easily detect expression of the K1014chim transgene in mice by immunofluorescence microscopy based on the different tissue distribution of K10 (suprabasal layers of the epidermis) and K1014chim (basal layers of the epidermis).

A mouse line expressing K1014chim in the epidermis

To construct a gene targeting vector, we next inserted a neomycin minigene and a HSV-TK selection cassette into the K1014chim vector (Fig. 1b). This construct was electroporated into ES cells and recombinant clones were isolated. Approximately 14% of the antibiotic-resistant ES-cell clones that were tested had undergone homologous recombination at the K14 gene locus (schematic representation of recombinant K10 locus in Fig. 1c). Several genetically independent K1014 mouse lines were established. Interestingly, the presence of the floxed neomycin minigene resulted in a mosaic expression pattern of the K1014chim transgene in the epidermis (data not shown). We therefore crossed K1014chim mice with CMV-Cre transgenic mice to delete the neomycin minigene from the mutant genome (the resulting K1014chim allele is shown in Fig. 1d). Next we back-crossed mice without neomycin cassette to C57Bl/6 mice. All experiments described below were conducted with mutant mice that were congenic with the C57Bl/6 background. Homozygous mutant mice were viable and fertile and did not show an overt skin phenotype as newborns or young adults. Standard histology did not reveal defects in skin development or homeostasis. Furthermore, based on immuno-staining with a panel of antibodies against epidermal differentiation markers (e.g. K1, K5, loricrin, filaggrin), we concluded that keratinocyte differentiation was not impaired in homozygous mutant mice (data not shown).

We also stained newborn mutant skin with antibodies against K6; in normal newborn mouse epidermis, K6 expression is restricted to the companion cell layer of hair follicles. Upon exposure to mechanical or biochemical stress, or in response to keratinocyte differentiation defects, K6 expression is induced in the interfollicular epidermis (e.g. Rothnagel et al., 1999; Wojcik et al., 1999; Wojcik et al., 2000; Wojcik et al., 2001). K6 expression in newborn K1014chim mice was restricted to the companion cell layer of the hair follicles (data not shown), further supporting our conclusion that development and function of the newborn epidermis was largely normal in mutant mice.

However, all homozygous mutants developed cornea lesions by 12 months of age, and animals older than 18 months developed chronic skin erosions (data not shown). The underlying pathology will be described elsewhere.

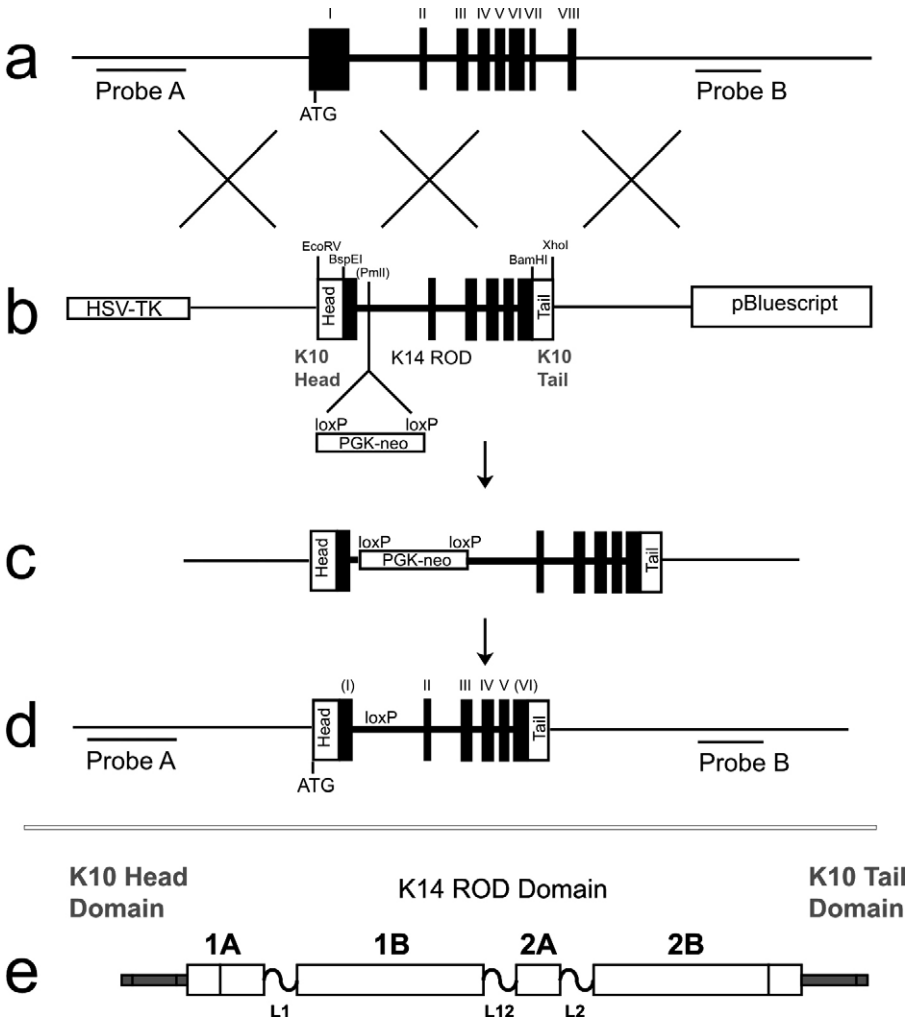

Fig. 1. Generation of K1014chim mice. (a) The mouse K14 gene consists of 8 exons (labeled I-VIII). (b) We generated a gene-targeting vector in which the sequence encoding the K14 N-terminal head domain in exon 1 was replaced with the sequence for the K10 head domain (*Eco*RV/*Bsp*EI fragment). Next, we deleted exons 7 and 8 and fused a cDNA encoding the K10 tail domain (*Bam*HI/*Xho*I fragment) with K14 exon 6. A neomycin selection cassette (PGK-neo), flanked by LoxP sites, was inserted into a unique *Pml*I site in intron 1. (c) The targeting construct was introduced into mouse ES cells and recombinant clones were identified by Southern blot analysis using an array of internal and external probes (data not shown). Recombinant ES cell clones were injected into blastocysts and several independent mouse lines were established. (d) Next, we crossed a mouse line carrying the recombinant K1014chim locus with CMV-Cre mice to remove the floxed neomycin selection cassette from K14 intron 1, thereby generating the founder for the K1014chim line used in the present study. Using RT-PCR and DNA sequence analysis, we demonstrated that the K1014chim locus generated an mRNA that encodes the predicted K1014chim fusion protein. (e) Schematic representation of the K1014chim protein and the keratin sub-domains (1A, 1B, 2A, 2B, L1, L12, L2) (Kirfel et al., 2003 and references therein).

K1014chim expression in the skin of mutant mice

The experiments described below were done with homozygous K1014chim mice. Unless indicated otherwise, we will refer to the homozygous state whenever we described mutant mice.

To ensure that the K1014chim transgene did not acquire any mutations during the many steps that were necessary to generate K1014chim mice, we isolated epidermal RNA from newborn K1014chim mice, amplified the K1014chim cDNA by RT-PCR, and analyzed the sequences encoding the head and tail domains, as well as the junctions of rod and end domains. No mutation was found, indicating correct processing and expression of the chimeric mRNA.

Next we stained newborn skin with an antibody raised against the C-terminus of K10. Only suprabasal keratinocytes were stained in wild-type sections (Fig. 2d), whereas the entire epithelium of K1014chim mice stained for the K10 epitope (Fig. 2b). This finding demonstrates that the K1014chim protein is synthesized in the correct cellular compartment, i.e. the basal layer of the interfollicular epidermis and the outer root sheath (ORS) of hair follicles. As expected, homozygous mutant skin did not stain with an antibody raised against the tail domain of K14, indicating that the wild-type keratin was not synthesized (data not shown). K5 staining was comparable in wild-type and mutant samples (Fig. 2a,c).

Given that ectopic over-expression of the K10 end domains has been associated with reduced cell proliferation (see Introduction), we determined the proliferation index of newborn mutant and control skin. No difference was found between mutants and wild-type controls (data not shown). Furthermore, BrdU-positive keratinocytes were clearly present in the K1014chim-expressing basal cell layer of the epidermis and the ORS of mutant skin (white arrows in Fig. 2b).

Characterization of mutant keratinocytes in vitro

To determine whether K1014chim can co-assemble with K5 into a well developed IF network, primary mutant keratinocyte cultures were established in low calcium medium to suppress differentiation, and stained with antibodies against K10 and K5 (Fig. 3a-c). Both keratin antibodies were raised against the tail domains of the proteins. Consequently, the K10 antibody recognizes endogenous K10 as well as the K1014chim protein. As predicted, under the culture conditions used in this experiment, K10 protein was not synthesized in wild-type cells (Fig. 4). Therefore any signal detected with the K10 antibody in mutant keratinocytes indicated K1014chim expression. K1014chim cells showed a well developed IF network that stained for both K5 and the mutant K1014chim protein (example in Fig. 3a-c).

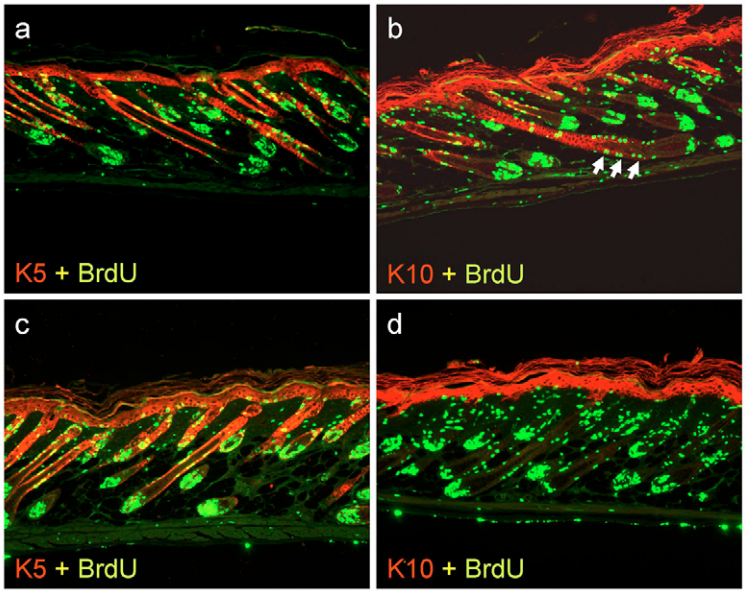

Fig. 2. K1014chim expression in the epidermis of newborn homozygous mutant mice. Sections of mutant (a,b) and wild-type (c,d) skin were stained with the antibodies indicated. (a,b) K5 and K1014chim are co-expressed in the basal layer of the epidermis and the ORS of hair follicles in mutant mice. Note that BrdU-positive nuclei (white arrows in b) are present in K1014chim-positive cell layers, demonstrating that the presence of the K10 end domains is compatible with keratinocyte proliferation. (c,d) In wild-type epidermis, the K10 antibody only stains suprabasal keratinocytes. Note the lack of K10 staining along the ORS of wild-type hair follicles.

Co-staining of mutant cells with antibodies against desmoplakin, a constitutive desmosomal protein, and K10 revealed that the K1014chim filaments extend to the plasma membrane where they terminate in desmosomal cell junctions (Fig. 3d-f). In essence, the K1014chim distribution in mutant keratinocytes appears to be identical to the K14 distribution in wild-type cells.

Next we conducted semi-quantitative western blotting to compare the steady state level of K14 and K1014chim in wild-type and mutant keratinocytes, respectively. It was important to demonstrate that our genetic manipulations of the K14 gene locus did not affect transgene expression and/or protein levels. Total cell extracts of keratinocytes from both genotypes were blotted with K5, K14 and K10 antibodies (Fig. 4). As expected, using antibodies specific for the end domain, K14 expression was not detected in mutant cells. More importantly, under the growth conditions used, K10 expression was not detectable in wild-type cells. The signal obtained with the K10 antibody in mutant cells therefore indicated K1014chim expression. As expected, the ratio of K5/K14 in wild-type cells was determined to be ~1:1. The ratio of K5/K1014chim in mutant cells was also ~1:1, demonstrating that the K10 end domains are expressed at physiological levels in mutant keratinocytes.

In order to determine whether the K1014chim mutation affected basic cellular behavior, we conducted migration (Fig.

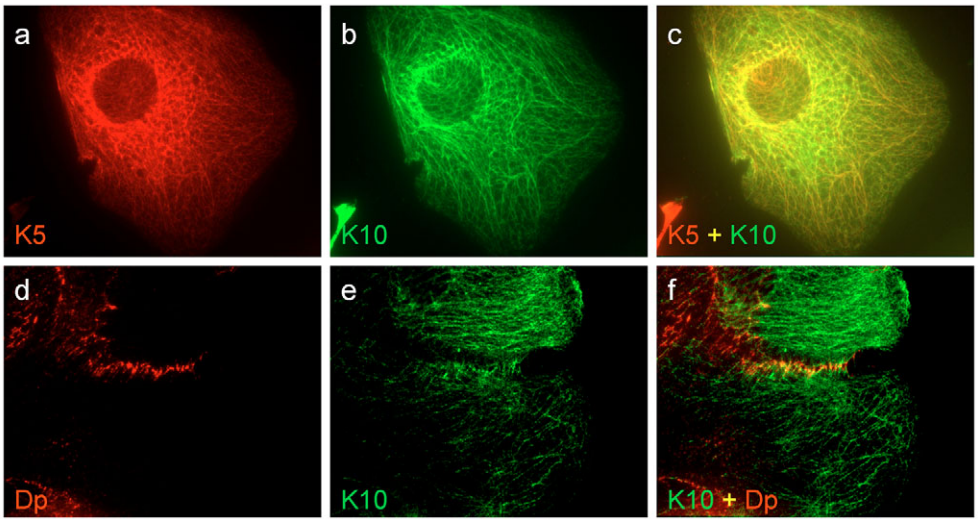

Fig. 3. K1014chim expression in cultured keratinocytes from newborn mutant mice. The antibodies used [K5, K10, Desmoplakin (Dp)] are indicated. Both keratin antibodies (K5, K10) used in this experiment were raised against the C-terminal tail domains of their targets. The K10 antibody therefore recognizes both K1014chim and K10. However, the keratinocytes shown were cultured for 8 days in low calcium medium to eliminate differentiating cells. Under these culture conditions, K10 is not expressed in wild-type control cells (see Fig. 4). The homogenous staining of mutant cultures with the K10 antibody therefore indicates expression of the K1014chim protein. (a-c) Staining of mutant cells (maintained in low calcium medium) with the keratin antibodies demonstrates co-distribution of K5 and K1014chim, suggesting that the chimeric keratin can form a functional intermediate filament cytoskeleton with its partner K5. (d-f) Keratinocytes cultured in high calcium medium for 1 hour to induce cell junction formation. Dp is a general marker for desmosomes (reviewed in Cheng et al., 2005; Cheng and Koch, 2004). Dp antibodies stain cell-cell borders in a punctuated fashion. Each dot represents a single desmosome. The double staining with Dp and K10 antibodies indicates that the K1014chim filaments terminate in desmosomes at the plasma membrane.

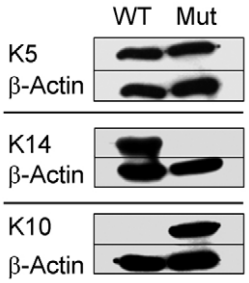

Fig. 4. Keratin expression in mutant (Mut) and wild-type (WT) keratinocytes as judged by western blotting. The antibodies used are indicated on the left (K5, K14, K10, β-actin). Total protein extracts were prepared from K1014chim and wild-type keratinocytes cultured in low calcium medium for 8 days. Expression of the differentiation marker K10 is not detectable in wild-type cells under these culture conditions. The signal obtained with the K10 antibody in K1014chim extracts therefore represents expression of the chimeric protein. Note that both the K10 and K14 antibody were raised against the C-termini of the keratins. Consequently, the K14 antibody does not detect its antigen in K1014chim extracts. β-actin was used as a loading control to normalize keratin expression. K5 expression was similar in mutant and wild-type cells. As expected, the molar ratio of K5/K14 in wild-type cells was ~1:1. The ratio of K5/K1014chim in mutant cells was also calculated as ~1:1, indicating that the steady state levels of K14 (wild-type) and K1014chim (mutant) are similar.

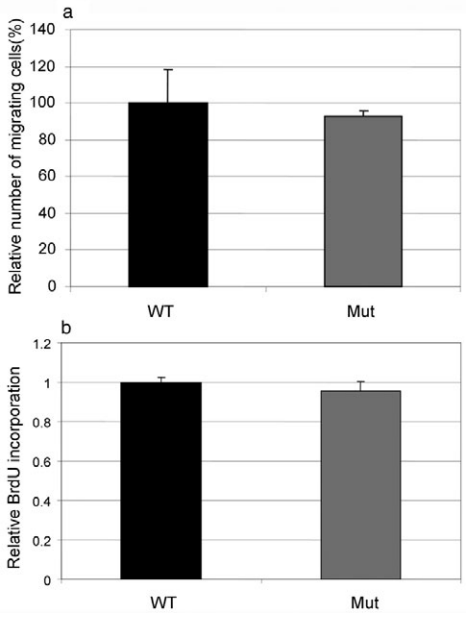

Fig. 5. Migration and proliferation of mutant cells. (a) Primary mouse keratinocytes from newborn K1014chim (Mut) and wild-type (WT) littermates were tested for their ability to migrate in vitro (see Materials and Methods for details). The number of migrating wild-type cells was set to 100%. Note that we did not observe a statistically significant difference in the migratory behavior of the two genotypes. The graphic summarizes the results obtained with three mutant and three wild-type isolates. (b) Keratinocyte proliferation as measured by BrdU incorporation by cells grown in low calcium medium at low cell density. Four mutant and four wild-type keratinocyte isolates were used in this experiment. BrdU incorporation of wild-type cells was defined as 1. Note that both genotypes showed essentially identical proliferation rates. Error bars show standard deviation.

5a) and proliferation (Fig. 5b) assays. No significant difference was found between wild-type and mutant cells with respect to these two parameters.

K1014chim mice are more susceptible to papilloma formation when subjected to a skin carcinogenesis protocol

It has been suggested that K10 might function as a tumor suppressor in the skin. To test whether the ectopic expression of the K10 end domains in the epidermal stem cell compartment can affect the susceptibility of K1014chim mutant mice to skin cancer, we subjected mutant and control mice to a chemical skin carcinogenesis protocol (DMBA/TPA protocol). We used mutant mice that were congenic on the C57Bl/6 background in this experiment. Gender- and age-matched C57Bl/6 mice served as wild-type controls. The back skin of adult mice (n=20 for each genotype; a total of 40 mice were used) were treated with a single dose of the carcinogen DMBA (for details see Materials and Methods). The animals were then treated with the tumor promoter TPA once a week for 25 weeks. Wild-type mice developed papillomas in response to this treatment, most of which regressed after the TPA treatment was stopped. However, a few papillomas progressed to squamous cell carcinomas (SCC). Mutant mice developed papillomas earlier than control mice (Fig. 6b). After 10 weeks of TPA treatment, for example, more than 50% of the mutants had developed at least one papilloma, whereas only 5% of the control animals carried a tumor (Fig. 6b). At the end of the TPA phase, 85% of the controls and all mutants had developed tumors. Most interestingly, mutants carried on average 2-3 times as many tumors as controls during the entire experiment (Fig. 6a). After termination of the TPA treatment, we monitored the animals for another 25 weeks. Most tumors regressed in both genotypes, with comparable regression rates (reduction of tumor burden over time; data not shown). Both mutants and wild-type mice developed SCC. Mutants developed 2-3 times as many SCC as wild-type controls after 50 weeks (data not shown). However, given that mutants also developed 2-3 times as many papillomas, we concluded that the papilloma/SCC conversion rate was not affected by the K1014chim transgene.

Primary SCC in the skin of mutants homogenously expressed the K1014chim transgene in all tumor cells (example in Fig. 7a). Furthermore, BrdU labeling experiments again demonstrated that the presence of the K10 end domains did not interfere with tumor cell proliferation. Interestingly, the only mouse that developed metastases was a mutant. Tumor cells that homogenously expressed the K1014chim transgene were identified in the lungs (Fig. 7b-d) and a lymph node (data not shown) of a mutant mouse. The lung tumor was classified as a squamous cell carcinoma. Lack of TTF-1 expression in tumor cells (a marker for lung epithelial cells; Fig. 7d) supported the conclusion that this SCC was a metastasis derived from a primary skin SCC.

Reduced epidermal apoptosis in the skin of K1014chim mice in response to UV radiation

Several recent publications have suggested that keratins can affect the apoptotic response of epithelial cells (Caulin et al., 1997; Caulin et al., 2000; Inada et al., 2001; Oshima, 2002; Tong and Coulombe, 2006; Yoneda et al., 2004; Zhuang et al.,

1999). A natural stimulus for apoptosis in the sun-exposed skin is UV-B radiation. We therefore determined the susceptibility of mutant mice to UV-induced apoptosis. Newborn mutant and control pups were exposed to UV-B radiation (100 mJ/cm^2). Twenty-four hours later, we determined the proliferation and apoptotic indices in these mice. The K1014chim transgene is expressed in the basal layer of the interfollicular epidermis. However, given that K5/K14 are known to persist in suprabasal keratinocytes, we decided to count BrdU- and TUNEL-positive cells in all cell layers of the interfollicular epidermis.

Non-irradiated pups served as a control. As expected, we observed only a few scattered TUNEL-positive cells in the epidermis of wild-type and mutant pups not exposed to UV-B (data not shown). A significant number of TUNEL-positive cells were observed in both mutants and wild-type mice that were treated with UV-B. Mutants, however, showed 27% fewer apoptotic keratinocytes in the interfollicular epidermis (Fig. 8a), which suggests that the K1014chim transgene suppresses the apoptotic response to environmental stress. The proliferative response, as measured by BrdU incorporation in the interfollicular epidermis, was similar in both genotypes (Fig. 8b).

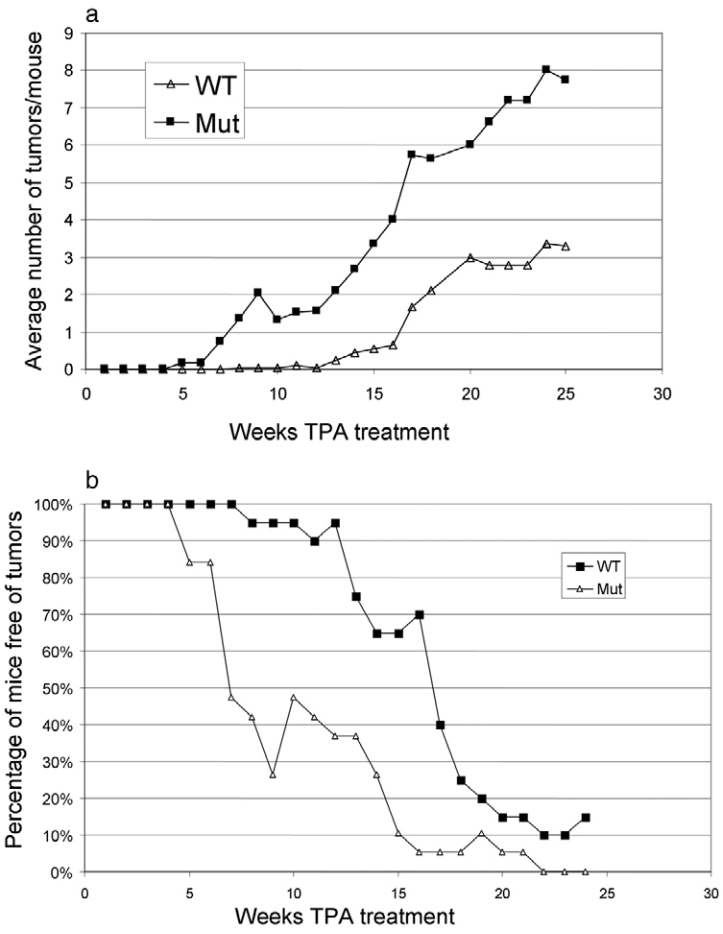

Fig. 6. Effects of K1014chim expression on tumor development in mice subjected to a skin carcinogenesis protocol (DMBA/TPA protocol). 20 mutants and 20 wild-type mice (gender and age matched) were subjected to the protocol. We used mutants that were congenic with the C57Bl/6 background for this experiment. All mice were treated once with DMBA (50 μg/mouse), followed by TPA treatment (20 μg/mouse) once a week for 25 weeks. (a) K1014 mutants developed papillomas earlier than control mice and on average carried 2-3 times as many tumors per mouse. (b) The percentage of tumor-free mutant mice declined more rapidly throughout the 25-week period than the percentage of tumor-free wild-type controls. For example, 10 weeks into the TPA treatment about 50% of the mutants were tumor free, whereas 95% of the wild-type controls had not developed tumors (P<0.05).

Mutant keratinocytes are less susceptible to TNFα-induced apoptosis in vitro

Both UV-B radiation (Brink et al., 2000; Niizeki et al., 2002; Strickland et al., 1997; Yarosh et al., 2000; Zhuang et al., 1999) and TPA treatment (Scott et al., 2003 and references therein) are known to induce the TNFα pathway, which in turn can trigger keratinocyte apoptosis. We therefore decided to test the hypothesis that an inhibition of TNFα-mediated apoptosis occurs in K1014chim keratinocytes.

We isolated primary keratinocytes from newborn mutant and wild-type littermates, cultured them in low calcium medium for 8 days to remove differentiated cells, and then exposed these cells to various concentrations of TNFα in the presence of cycloheximide. This protocol has been shown to induce apoptosis in epithelial cells (Caulin et al., 2000). As a quantitative marker for this assay, we analyzed caspase 3 activation in mutant and wild-type cells by western blotting (Fig. 9a). Caspase 3 is a downstream effector of the extrinsic apoptotic pathway (reviewed in Thorburn, 2004). As shown in Fig. 9b, mutant keratinocytes showed a significant reduction in their apoptotic response to TNFα at concentrations ⩾10 ng/ml.

Discussion

We have generated mice that express a chimeric keratin consisting of the K10 head and tail domains fused to the K14 rod domain in the basal layer of the epidermis and the ORS of hair follicles. Young mice were phenotypically normal. Specifically, they did not show any defects in skin development and maintenance as judged by conventional histology and immuno-histochemistry with antibodies against a panel of epidermal differentiation markers (data not shown). However, older mice (>12 months) developed blindness due to cornea lesions (100% penetrance) and most animals that reached 18 months of age developed skin lesions (data not shown). The underlying histo-pathology of these phenotypes is currently under investigation.

As judged by immuno-fluorescence microscopy of primary keratinocytes from newborn mice, the chimeric keratin appeared to co-assemble efficiently with its type II partner K5 into a well-developed IF cytoskeleton. Furthermore, based on the fact that we did not observe skin fragility phenotypes, we concluded that the mutant IF network is fully functional and effectively connected to desmosomal junctions at the plasma membrane of basal keratinocytes.

These results are surprising given previous results suggesting that K10 expression is not compatible with cell cycle progression in epithelial cells. Several previous publications indicated that when expressed in fibroblasts (Kartasova et al., 1993) or epithelial cells (Kartasova et al., 1992; Paramio et al., 1999; Paramio et al., 2001; Paramio

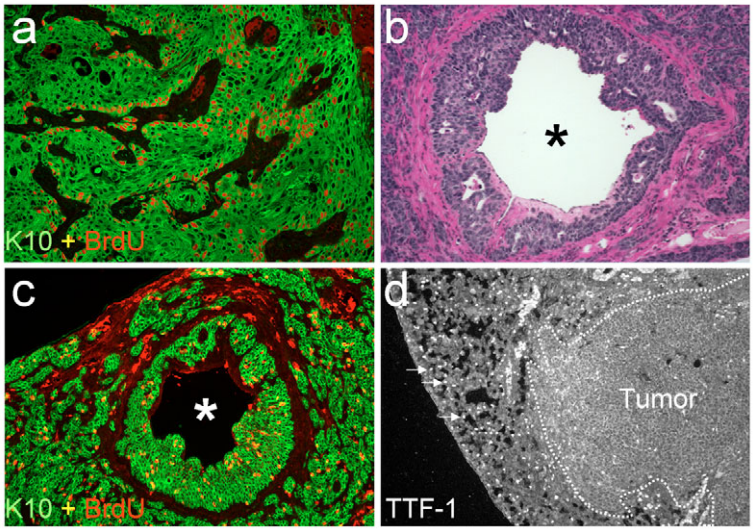

Fig. 7. K1014 transgene expression in an SCC of a K1014chim mouse subjected to the DMBA/TPA protocol. (a) SCC tumor cells from the skin stained with antibodies against K10 (green) and BrdU (red). Note that the K1014chim keratin is homogenously expressed throughout the tumor and apparently does not interfere with proliferation as indicated by the K10/BrdU double-positive cells. (b) Histological section of a lung SCC and (c) staining with K10 (green) and BrdU (red) antibodies. Note that the tumor tissue stains for the K1014chim protein. (d) As expected, the SCC was negative for TTF-1 (thyroid transcription factor-1), a marker for normal airway epithelial cells. Note the nuclear staining (white arrows) of normal lung tissue, suggesting that the SCC represents a metastasis of the primary tumor shown in panel a. The dashed line demarcates the border between the tumor and normal lung tissue. The asterisk indicates the bronchus.

and Jorcano, 2001), K10 suppresses cell proliferation. This inhibitory effect was mapped to the non-helical end domains of K10. It was shown that these domains can bind and thereby inhibit Akt and PKCζ in cell culture transfection experiments, and thus prevent cell cycle progression in a pRb (retinoblastoma protein)-dependent manner (Paramio et al., 2001).

Furthermore, when human K10 was over-expressed under the control of the bovine K5 promoter in the basal layer of the epidermis in transgenic mice, a hypoplastic and hyperkeratotic phenotype was observed (Santos et al., 2002; Santos et al., 2003). These mice also appeared to be somewhat resistant to papilloma development when subjected to a carcinogenesis protocol. Previously, the same investigators had reported that transgenic K10 expression under the control of the K6 promoter delays the onset of tumor development in mice subjected to a carcinogenesis protocol (Santos et al., 1997).

Since the K10 end domains inhibited cell cycle progression in vitro, one would have predicted that expression of the K10 end domains at physiological levels would lead to a hypoplastic epidermis and resistance to skin tumor development in K1014chim mice. Our results are clearly contrary to these expectations. It is therefore tempting to speculate that overexpression of K10 caused the previously observed phenotypes.

In this respect, it is interesting to note that transgenic mice expressing low levels of (full-length) K10 in the basal cell layer of the epidermis appeared to be phenotypically normal, whereas high levels lead to hypoplasia and hyperkeratosis (Santos et al., 2002).

In contrast to previous experiments, our K1014chim mice were designed to express the K10 end domains at levels similar to the K5 levels (1:1 molar ratio of type I and type II keratin end domains) to prevent toxic effects due to overexpression.

K1014chim mice showed increased susceptibility to benign tumor formation when subjected to a chemical carcinogenesis protocol. They developed papillomas earlier and in greater numbers than controls. However, conversions to malignant tumors or papilloma regression after TPA withdrawal were not affected by expression of the K1014chim protein. We identified one mutant mouse with K1014chim-expressing metastases in the lungs and lymph nodes. Given the previously published work suggesting suppression of tumor development by K10, it

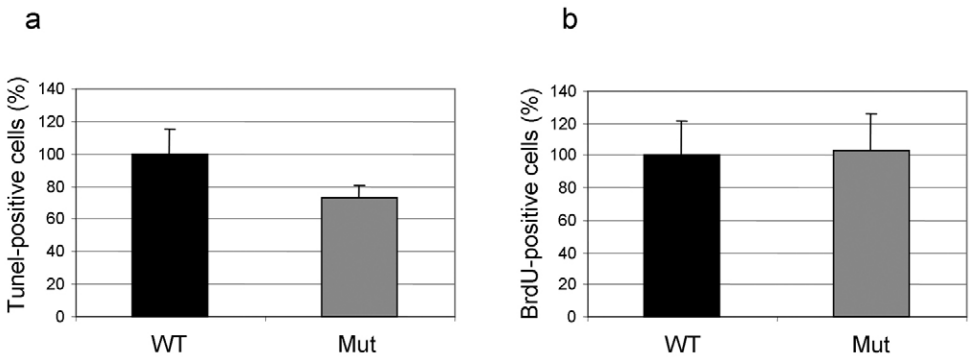

Fig. 8 Impaired apoptotic response of K1014chim skin in response to UV-B irradiation. The back skin of newborn K1014chim mutants (Mut; n=5) and wild-type littermates (WT; n=4) was irradiated with 100 mJ/cm^2 UV-B. 24 hours later, the skin was isolated and processed for (a) TUNEL and (b) BrdU staining. The number of TUNEL- and BrdU-positive keratinocytes in the interfollicular epidermis was determined (the average value for the wild-type samples was defined as 100%). Note that K1014 skin showed a 27% decrease in TUNEL-positive cells compared with wild-type control skin (Mut: 57±4, WT: 78±11; P<0.05). The proliferation indices of mutant and wild-type samples were essentially the same (Mut: 26±5; WT: 25±5). Error bars show standard deviation.

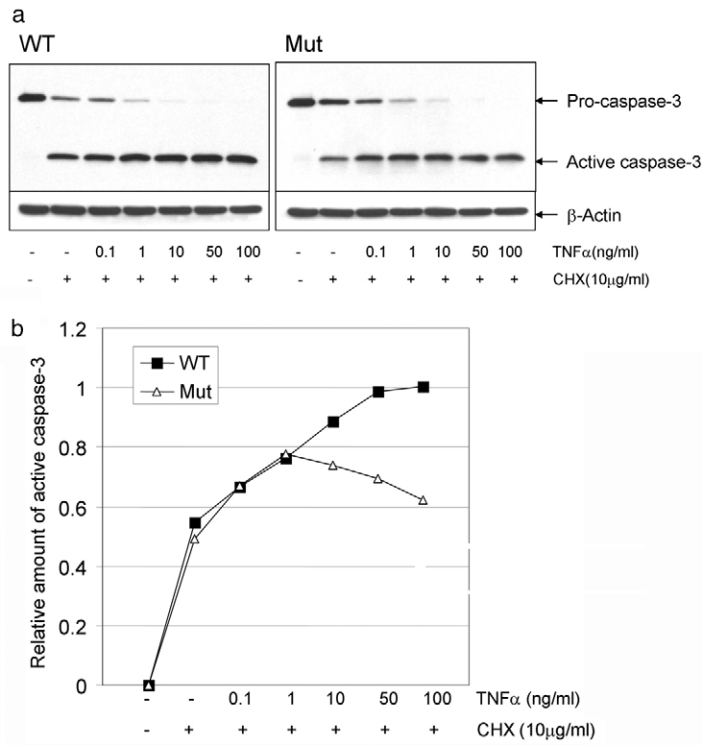

Fig. 9. Impaired apoptotic response of mutant keratinocytes exposed to TNFα. Primary keratinocytes from newborn mutants (Mut) and wild-type (WT) littermates were exposed to various concentrations of TNFα in the presence of cycloheximide (CHX), an inhibitor of protein translation (Caulin et al., 2000). (a) Western blot detection of pro-caspase 3 and active caspase 3. β-Actin was used as a loading control. (b) Quantitative evaluation of the western blot signals shown in panel a. The relative amount of active caspase 3 (normalized against β-actin) in the two genotypes is shown as a function of the TNFα concentration. Note the reduced apoptotic response of mutant cells compared with wild-type controls at a TNFα concentration ⩾10 ng/ml. This experiment was repeated twice with independent isolates of newborn keratinocytes from mutant and wild-type littermates. Similar results were obtained in these experiments (data not shown) (P=0.04).

is noteworthy that expression of the K1014chim transgene is compatible with invasive carcinoma growth.

Several mechanisms could explain the observed increase in tumor burden of mutants, for example an increased proliferative response to TPA treatment or a reduction in the apoptotic response. In the latter case, one would expect that fewer initiated tumor cells are eliminated from mutant skin after DMBA treatment, thus leading to a higher rate of papilloma formation upon TPA treatment.

A comparison of the proliferation indices of newborn skin and TPA-treated adult skin did not reveal a significant difference between mutants and wild-type controls. However, we observed a decrease in the apoptotic response of mutant epidermis in vivo (UV-B radiation) and in vitro (TNFα-induced apoptosis) when compared to wild-type (littermate) controls. Interestingly, Tamiji and colleagues recently provided evidence that K10-expressing HaCat cells appear to be partially protected from chemically induced apoptosis in vitro (Tamiji et al., 2005), further supporting the notion that K10 could be involved in suppressing apoptosis.

It has previously been demonstrated that keratins can affect the apoptotic response of epithelial cells in vivo. Most investigators have focused on the role of simple epithelial keratins [keratin 8 (K8)/keratin 18 (K18)] in this process (reviewed by Coulombe and Wong, 2004; Oshima, 2002). Nevertheless the epidermal keratins K14 and keratin 17 (K17) have been identified as components/regulators of epidermal apoptosis in vivo as well (e.g. Caulin et al., 1997; Caulin et al., 2000; Coulombe and Wong, 2004; Inada et al., 2001; Tong and Coulombe, 2006; Yoneda et al., 2004). It has been proposed, for example, that cytoplasmic sequestration of TRADD (TNFα receptor-associated death domain protein) by K14 and K17 suppresses TNFα-induced apoptosis. TRADD is a component of the 'death inducing signaling complex' (DISC) that consists of TNFα-family related death receptors and a cytoplasmic complex containing death domain proteins such as TRADD and FADD (Fas-associated death domain protein) (reviewed by Thorburn, 2004). Interestingly, TRADD has also been found in the cell nucleus where it appears to induce apoptosis through a mechanism that is different from the cytoplasmic apoptotic cascade (Bender et al., 2005; Morgan et al., 2002). TRADD binds to the 1A domain in the K14 rod (Inada et al., 2001). Since this domain is present in both wild-type K14 and the K1014chim protein, it is not obvious that differences in TRADD binding to the rod domain could explain the reduced apoptotic index of UV-B irradiated or TNFα-treated mutant keratinocytes. Furthermore, keratin end domains can bind a large number of cytoplasmic proteins (e.g. Coulombe and Wong, 2004; Kim et al., 2006). It remains to be seen which apoptotic effector molecules are involved in K1014chim-mediated resistance to TNFα-induced apoptosis. Nevertheless, the concept that the K10 end domains potentially bind mediators of epidermal apoptosis and thereby interfere with their activity is fascinating and warrants further investigation

It is important to note that K1014chim expression is not likely to act as a general suppressor of TNFα signaling. The pro-inflammatory effects of this signaling pathway are required for skin tumor formation, i.e. a general block of this signaling cascade would lead to decreased tumor susceptibility in mice exposed to a DMBA/TPA protocol (e.g. Arnott et al., 2004; Moore et al., 1999; Scott et al., 2003). Consequently, it is unlikely that the pro-inflammatory effects of TNFα are affected by K1014chim expression.

The role of IF as cytoskeletal components that confer resilience to mechanical stress is well documented (reviewed by Coulombe and Wong, 2004; Kirfel et al., 2003). Mutations in keratin genes have been associated with several skin fragility disorders. K1 or K10 mutations, for example, are the underlying cause for the human skin disorder bullous congenital ichthyosiform erythroderma (reviewed by Kirfel et al., 2003). The IF scaffold also affects basic cellular processes such as apoptosis, either by binding cell surface receptors that trigger apoptosis (in the case of K8/K18) or by binding (and controlling the activity) of cytoplasmic cell death mediators (K14, K17; see above). K10 is an intermediate marker of keratinocyte differentiation, which is expressed in spinous cells. K10 expression ceases in the granular layer of the epidermis. It is

tempting to speculate that one function of K1/K10 expression is a temporal suppression of cell death, thereby allowing completion of the keratinocyte differentiation program.

Materials and Methods

Animal husbandry

Animal experiments were conducted in accordance with local and federal guidelines and were approved by the 'Institutional Animal Care and Use Committee' (IACUC) of Baylor College of Medicine.

Generation of K1014chim mice

In order to generate the K1014chim gene targeting construct, we used two plasmids (E1, F8), provided by Tongyu Cao (Baylor College of Medicine), that contain K14 genomic DNA isolated from a 129SV genomic library. Furthermore, expression plasmid MK10 (provided by Tonja Kartasova, NIH, Washington, D.C.), which contains mouse keratin 10 cDNA, was used to generate the K10 head and tail cDNA. Using a PCR approach, we generated five DNA fragments that were fused to generate the final targeting vector shown in Fig. 1b: (1) the 5′UTR fragment (2.3 kb) was amplified with primers T7 and K1402 (TTTGAGAGAGGTGAGCAAG) using plasmid E1, (2) the K10 head (0.43 kb) was amplified with primers K1001 (GCCATGTCTGTTCTATACAGC) and K1002 (GGAGAGAAGGCTGCCACC; template was plasmid MK10), (3) the K14 rod (3 kb) was amplified with primers K1403 (GAGAAAGTGACCATGCAGA) and K1404 (CTCTCCCTCCAGCAG-ACGG; template was plasmid E1), (4) the K10 tail (0.35 kb) was amplified with primers K1003 (TCCAGCGGTGGCGGCGGCGGA) and K1004 (GTATCTTGG-TCCTTTAG; template was plasmid MK10), and (5) the K14 3′UTR (2.3 kb) was amplified with primers T3 and K1405 (AGCTGCTACATGCTGCTCAG) using plasmid F8 as a template. All DNA fragments were cloned and sequenced to ensure that no mutation was introduced during the PCR amplification and cloning steps. Next, we fused all five fragments using conventional restriction enzyme cloning. The restriction sites were chosen so that no missense mutation would be introduced into the head-rod and rod-tail junction, respectively, of the chimeric construct. Transfection experiments demonstrated that the construct encoded a protein that can co-assemble into a pre-existing intermediate filament network of simple epithelial cells (data not shown). Finally, we inserted a neomycin selection cassette (PGK-neo, provided by Allan Bradley, Baylor College of Medicine; Fig. 1) into intron 1 of the construct. A HSV-TK cassette (provided by John Lydon, Baylor College of Medicine) was also added to allow a positive/negative selection strategy in gene targeting experiments (Mansour et al., 1988). The targeting vector was then transfected into RW4 ES cells (Genome Systems, St Louis). The isolation and identification of recombinant ES clones was done essentially as described (Koch et al., 1997). Recombinant clones that had undergone homologous recombination at the K14 locus were analyzed by Southern blots using five different hybridization probes (5′ and 3′ probe, probe A and B in Fig. 1d; K10 head probe; K10 tail probe; neomycin minigene probe; data not shown). Twelve genetically independent recombinant ES cell clones were identified (data not shown). ES cell injections into C57BL/6 blastocysts were carried out at the 'Genetically Engineered Mouse Core' at Baylor College of Medicine (Francesco DeMayo, Director). Several genetically independent K1014chim lines were established, all of which showed the same phenotype. We then crossed one of the original mouse lines with a CMV-Cre transgenic line (provided by Allan Bradley) to delete the floxed PGK-neo cassette from the K1014chim genome. A mouse line that had lost the PGK-neo cassette (K1014chim allele in Fig. 1d) and the CMV-Cre transgene was established and then back crossed to the C57Bl/6 inbred strain. The experiments described in this manuscript were done with mice that were congenic with the C57Bl/6 background (10 back crosses of heterozygous mutant mice to wild-type C57Bl/6 mice).

Keratinocyte cell cultures

Keratinocytes were cultured in CnT-07 medium (Chemicon) following the manufacturer's recommendations. In certain experiments, the calcium concentration in the medium was raised to 1.5 mM to induce cell junction formation and keratinocyte differentiation. All experiments described below were done with keratinocytes isolated from newborn mutant and wild-type littermates. In order to prepare cells for migration, proliferation and in vitro apoptosis assays, the cultures were maintained for 8 days in Cn-T-07 medium to remove differentiated cells. The keratinocytes were then harvested and used for the assays described below.

Migration assays

In vitro keratinocyte migration assays were carried out essentially as described (Nieman et al., 1999) with the following modifications: newborn mouse keratinocytes were mitotically inactivated by incubation with 8 μg/ml mitomycin C (Sigma) for 2 hours. The next day, the cells were transferred into collagen IV-coated BioCoat invasion chambers (8 μm pore size; BD Biosciences; 1.7×10^5 cells/well). After 48 hours, the cells on the upper surface of the chamber were removed using a cotton tip and cells that migrated to the lower membrane surface were fixed by methanol, stained with hematoxylin and counted. All experiments were done in triplicate using mutant and wild-type keratinocytes derived from littermates.

Proliferation assays

Proliferation of cultured newborn mouse keratinocytes was measured in vitro using the 'BrdU cell proliferation assay kit' from Calbiochem. Briefly, 4×10^4 keratinocytes were plated in each well of a collagen IV-coated 48 well plate. For each keratinocyte isolate, 4 wells were used. After overnight culture, BrdU was added according to Calbiochem's recommendation, and the cells were maintained in the presence of BrdU for another 24 hours. BrdU incorporation was determined following the manufacturer's protocol.

In vitro apoptosis induction

Newborn mouse keratinocytes were cultured in the presence of 10 μg/ml cycloheximide (CHX, Sigma) and different concentrations of TNFα for 16 hours to induce apoptosis (Caulin et al., 2000). Adherent cells and cells floating in the culture medium were pooled and then lysed in Laemmli buffer. The amount of active caspase 3 was determined by western blotting. Caspase 3 blots were stripped and then re-probed with a β-actin antibody. The β-actin signal was used to normalize caspase expression. Western blot signals on Kodak films were scanned using a conventional flat bed scanner. Relative signal intensities were determined with the QuantiScan software package from Biosoft.

Chemical skin carcinogenesis protocol

We used 20 homozygous mutant and 20 wild-type mice for this experiment. The K1014 mutants were congenic to the C57Bl/6 background. Mutant and control mice were matched with respect to age and gender (10 male and 10 females were used for each genotype). The backs of 12-week-old mice were shaved and two days later a single sub-carcinogenic dose of the carcinogen DMBA (dimethylbenz[a]anthracene; Sigma) was applied (50 μg/mouse in acetone). One week later, we began treatment with the tumor promoter TPA (12-O-tetradecanoylphorbol-13-acetate; Sigma), which was applied to the back skin once a week for 25 weeks (20 μg in acetone/mouse). After the last TPA treatment, we observed the mice for an additional 25 weeks to assess squamous cell carcinoma (SCC) formation and tumor recession, respectively. Tumor development (onset, number of tumors per mouse, number of mice with tumors) was assessed weekly by visual inspection. The tumor type was determined by histology following standard procedures (Klein-Szanto, 1989).

UV treatment of mice and TUNEL assays

The back skin of newborn pups from K1014chim+/– intercrosses was irradiated with 100 mJ/cm^2 of UV-B using FS40T12 bulbs (Westinghouse, Philadelphia). The UV dosage was measured with a photometer from International Light (IL1400A Radiometer/Photometer with SCS280 filter). 24 hours after UV-B treatment, we injected the mice i.p. with 0.25 mg/g body weight BrdU (Sigma) in 0.9% NaCl solution. Two hours later, the animals were sacrificed and the skin was fixed in IHC zinc fixation buffer (BD Pharmigen), paraffin-embedded, sectioned and then processed for immuno-histochemistry with BrdU antibodies (proliferation index) or TUNEL assays (apoptotic index; DeadEnd fluorometric TUNEL system, Promega). The TUNEL assays were carried out essentially as recommended by the manufacturer of the kit. To determine the apoptotic and proliferative index, 14 photographs of skin sections from each mouse were analyzed (4 wild-type and 5 mutant mice were used). The average number of TUNEL- and BrdU-positive cells per millimeter of interfollicular epidermis was determined, and the Student's *t* test was used for statistical analysis of the experiment.

Antibodies, immunofluorescence microscopy, BrdU labeling, and histology

The following primary antibodies were used in this study: K5, K10, K14, K6, loricrin, filaggrin (Koch et al., 2000; Roop et al., 1984; Rothnagel et al., 1999; Wojcik et al., 1999; Wojcik et al., 2000; Wojcik et al., 2001; Yuspa et al., 1989), TTF-1 (provided by Francesco DeMayo, Baylor College of Medicine), BrdU (BioGenex), caspase 3 (BD Biosiences), β-actin (Abcam), and desmoplakin 1 and 2 (Dp, Research Diagnostics). Fluorochrome-labeled secondary antibodies (Alexa Fluor 488, Alexa Fluor 594) were purchased from Invitrogen. Immunofluorescence microscopy, immuno-histochemistry and western blots were carried out essentially as described previously (Cheng et al., 2004).

A Nikon Eclipse E600 microscope was used in conjunction with the MetaVue v6.1r5 imaging software (Universal Imaging Corp.) to photograph slides of conventional histology and tissue sections stained with fluorochrome-labeled antibodies. Deconvolution microscopy was carried out using an Applied Precision SoftWoRx Image Restoration Microscope (provided by Baylor's Integrated Microscopy Core).

We thank Mitsi Blount, Kusal Mihidukulasuria and Mary Ann Longley for technical assistance. Many thanks to Tongyu Cao, Tonja Kartasova, John Lydon, Allan Bradley, Francesco DeMayo and Andrew Thorburn for providing valuable reagents. We are also grateful to Neil Box, Andrew Thorburn and Francesco DeMayo for helpful discussions and Maranke Koster for critical reading of this

manuscript. This work has been supported in part by grants from the National Institutes of Health (NIH/NIAMS) to P.J.K. (AR050439) and D.R.R. (AR052263).

References

Arnott, C. H., Scott, K. A., Moore, R. J., Robinson, S. C., Thompson, R. G. and Balkwill, F. R. (2004). Expression of both TNF-alpha receptor subtypes is essential for optimal skin tumour development. *Oncogene* **23**, 1902-1910.

Bender, L. M., Morgan, M. J., Thomas, L. R., Liu, Z. G. and Thorburn, A. (2005). The adaptor protein TRADD activates distinct mechanisms of apoptosis from the nucleus and the cytoplasm. *Cell Death. Differ.* **12**, 473-481.

Bickenbach, J. R. (2005). Isolation, characterization, and culture of epithelial stem cells. *Methods Mol. Biol.* **289**, 97-102.

Braun, K. M. and Watt, F. M. (2004). Epidermal label-retaining cells: background and recent applications. *J. Investig. Dermatol. Symp. Proc.* **9**, 196-201.

Brink, N., Szamel, M., Young, A. R., Wittern, K. P. and Bergemann, J. (2000). Comparative quantification of IL-1beta, IL-10, IL-10r, TNFalpha and IL-7 mRNA levels in UV-irradiated human skin in vivo. *Inflamm. Res.* **49**, 290-296.

Caulin, C., Salvesen, G. S. and Oshima, R. G. (1997). Caspase cleavage of keratin 18 and reorganization of intermediate filaments during epithelial cell apoptosis. *J. Cell Biol.* **138**, 1379-1394.

Caulin, C., Ware, C. F., Magin, T. M. and Oshima, R. G. (2000). Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J. Cell Biol.* **149**, 17-22.

Cheng, X. and Koch, P. J. (2004). In vivo function of desmosomes. *J. Dermatol.* **31**, 171-187.

Cheng, X., Mihindukulasuriya, K., Den, Z., Kowalczyk, A. P., Calkins, C. C., Ishiko, A., Shimizu, A. and Koch, P. J. (2004). Assessment of splice variant-specific functions of desmocollin 1 in the skin. *Mol. Cell Biol.* **24**, 154-163.

Cheng, X., Den, Z. and Koch, P. J. (2005). Desmosomal cell adhesion in mammalian development. *Eur. J. Cell Biol.* **84**, 215-223.

Coulombe, P. A. and Omary, M. B. (2002). 'Hard' and 'soft' principles defining the structure, function and regulation of keratin intermediate filaments. *Curr. Opin. Cell Biol.* **14**, 110-122.

Coulombe, P. A. and Wong, P. (2004). Cytoplasmic intermediate filaments revealed as dynamic and multipurpose scaffolds. *Nat. Cell Biol.* **6**, 699-706.

Getsios, S., Huen, A. C. and Green, K. J. (2004). Working out the strength and flexibility of desmosomes. *Nat. Rev. Mol. Cell Biol.* **5**, 271-281.

Hesse, M., Zimek, A., Weber, K. and Magin, T. M. (2004). Comprehensive analysis of keratin gene clusters in humans and rodents. *Eur. J. Cell Biol.* **83**, 19-26.

Inada, H., Izawa, I., Nishizawa, M., Fujita, E., Kiyono, T., Takahashi, T., Momoi, T. and Inagaki, M. (2001). Keratin attenuates tumor necrosis factor-induced cytotoxicity through association with TRADD. *J. Cell Biol.* **155**, 415-426.

Kartasova, T., Roop, D. R. and Yuspa, S. H. (1992). Relationship between the expression of differentiation-specific keratins 1 and 10 and cell proliferation in epidermal tumors. *Mol. Carcinog.* **6**, 18-25.

Kartasova, T., Roop, D. R., Holbrook, K. A. and Yuspa, S. H. (1993). Mouse differentiation-specific keratins 1 and 10 require a preexisting keratin scaffold to form a filament network. *J. Cell Biol.* **120**, 1251-1261.

Kaur, P. (2006). Interfollicular epidermal stem cells: identification, challenges, potential. *J. Invest. Dermatol.* **126**, 1450-1458.

Kim, S., Wong, P. and Coulombe, P. A. (2006). A keratin cytoskeletal protein regulates protein synthesis and epithelial cell growth. *Nature* **441**, 362-365.

Kirfel, J., Magin, T. M. and Reichelt, J. (2003). Keratins: a structural scaffold with emerging functions. *Cell Mol. Life Sci.* **60**, 56-71.

Klein-Szanto, A. J. (1989). Pathology of human and experimental skin tumors. *Carcinog. Compr. Surv.* **11**, 19-53.

Koch, P. J. and Roop, D. R. (2004). The role of keratins in epidermal development and homeostasis – going beyond the obvious. *J. Invest Dermatol.* **123**, x-xi.

Koch, P. J., Mahoney, M. G., Ishikawa, H., Pulkkinen, L., Uitto, J., Shultz, L., Murphy, G. F., Whitaker-Menezes, D. and Stanley, J. R. (1997). Targeted disruption of the pemphigus vulgaris antigen (desmoglein 3) gene in mice causes loss of keratinocyte cell adhesion with a phenotype similar to pemphigus vulgaris. *J. Cell Biol.* **137**, 1091-1102.

Koch, P. J., de Viragh, P. A., Scharer, E., Bundman, D., Longley, M. A., Bickenbach, J., Kawachi, Y., Suga, Y., Zhou, Z., Huber, M. et al. (2000). Lessons from loricrin-deficient mice: compensatory mechanisms maintaining skin barrier function in the absence of a major cornified envelope protein. *J. Cell Biol.* **151**, 389-400.

Kottke, M. D., Delva, E. and Kowalczyk, A. P. (2006). The desmosome: cell science lessons from human diseases. *J. Cell Sci.* **119**, 797-806.

Mansour, S. L., Thomas, K. R. and Capecchi, M. R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature* **336**, 348-352.

Moore, R. J., Owens, D. M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, B., Pasparakis, M. et al. (1999). Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat. Med.* **5**, 828-831.

Morgan, M., Thorburn, J., Pandolfi, P. P. and Thorburn, A. (2002). Nuclear and cytoplasmic shuttling of TRADD induces apoptosis via different mechanisms. *J. Cell Biol.* **157**, 975-984.

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

Nieman, M. T., Prudoff, R. S., Johnson, K. R. and Wheelock, M. J. (1999). N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J. Cell Biol.* **147**, 631-644.

Niizeki, H., Inoko, H. and Wayne, S. J. (2002). Polymorphisms in the TNF region confer susceptibility to UVB-induced impairment of contact hypersensitivity induction in mice and humans. *Methods* **28**, 46-54.

Oshima, R. G. (2002). Apoptosis and keratin intermediate filaments. *Cell Death. Differ.* **9**, 486-492.

Paramio, J. M. and Jorcano, J. L. (2001). Transient transfections and heterokaryons as tools for the analysis of keratin IF dynamics. *Methods Mol. Biol.* **161**, 189-197.

Paramio, J. M., Casanova, M. L., Segrelles, C., Mittnacht, S., Lane, E. B. and Jorcano, J. L. (1999). Modulation of cell proliferation by cytokeratins K10 and K16. *Mol. Cell Biol.* **19**, 3086-3094.

Paramio, J. M., Segrelles, C., Ruiz, S. and Jorcano, J. L. (2001). Inhibition of protein kinase B (PKB) and PKCzeta mediates keratin K10-induced cell cycle arrest. *Mol. Cell Biol.* **21**, 7449-7459.

Reichelt, J., Furstenberger, G. and Magin, T. M. (2004). Loss of keratin 10 leads to mitogen-activated protein kinase (MAPK) activation, increased keratinocyte turnover, and decreased tumor formation in mice. *J. Invest Dermatol.* **123**, 973-981.

Rogers, M. A., Winter, H., Langbein, L., Bleiler, R. and Schweizer, J. (2004). The human type I keratin gene family: characterization of new hair follicle specific members and evaluation of the chromosome 17q21.2 gene domain. *Differentiation* **72**, 527-540.

Rogers, M. A., Edler, L., Winter, H., Langbein, L., Beckmann, I. and Schweizer, J. (2005). Characterization of new members of the human type II keratin gene family and a general evaluation of the keratin gene domain on chromosome 12q13.13. *J. Invest Dermatol.* **124**, 536-544.

Roop, D. R., Cheng, C. K., Titterington, L., Meyers, C. A., Stanley, J. R., Steinert, P. M. and Yuspa, S. H. (1984). Synthetic peptides corresponding to keratin subunits elicit highly specific antibodies. *J. Biol. Chem.* **259**, 8037-8040.

Rothnagel, J. A., Seki, T., Ogo, M., Longley, M. A., Wojcik, S. M., Bundman, D. S., Bickenbach, J. R. and Roop, D. R. (1999). The mouse keratin 6 isoforms are differentially expressed in the hair follicle, footpad, tongue and activated epidermis. *Differentiation* **65**, 119-130.

Santos, M., Ballestin, C., Garcia-Martin, R. and Jorcano, J. L. (1997). Delays in malignant tumor development in transgenic mice by forced epidermal keratin 10 expression in mouse skin carcinomas. *Mol. Carcinog.* **20**, 3-9.

Santos, M., Paramio, J. M., Bravo, A., Ramirez, A. and Jorcano, J. L. (2002). 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.

Santos, M., Perez, P., Segrelles, C., Ruiz, S., Jorcano, J. L. and Paramio, J. M. (2003). Impaired NF-kappa B activation and increased production of tumor necrosis factor alpha in transgenic mice expressing keratin K10 in the basal layer of the epidermis. *J. Biol. Chem.* **278**, 13422-13430.

Scott, K. A., Moore, R. J., Arnott, C. H., East, N., Thompson, R. G., Scallon, B. J., Shealy, D. J. and Balkwill, F. R. (2003). An anti-tumor necrosis factor-alpha antibody inhibits the development of experimental skin tumors. *Mol. Cancer Ther.* **2**, 445-451.

Strickland, I., Rhodes, L. E., Flanagan, B. F. and Friedmann, P. S. (1997). TNF-alpha and IL-8 are upregulated in the epidermis of normal human skin after UVB exposure: correlation with neutrophil accumulation and E-selectin expression. *J. Invest Dermatol.* **108**, 763-768.

Tamiji, S., Beauvillain, J. C., Mortier, L., Jouy, N., Tual, M., Delaporte, E., Formstecher, P., Marchetti, P. and Polakowska, R. (2005). Induction of apoptosis-like mitochondrial impairment triggers antioxidant and Bcl-2-dependent keratinocyte differentiation. *J. Invest Dermatol.* **125**, 647-658.

Thorburn, A. (2004). Death receptor-induced cell killing. *Cell Signal.* **16**, 139-144.

Tong, X. and Coulombe, P. A. (2006). Keratin 17 modulates hair follicle cycling in a TNF{alpha}-dependent fashion. *Genes Dev.* **20**, 1353-1364.

Wojcik, S. M., Imakado, S., Seki, T., Longley, M. A., Petherbridge, L., Bundman, D. S., Bickenbach, J. R., Rothnagel, J. A. and Roop, D. R. (1999). Expression of MK6a dominant-negative and C-terminal mutant transgenes in mice has distinct phenotypic consequences in the epidermis and hair follicle. *Differentiation* **65**, 97-112.

Wojcik, S. M., Bundman, D. S. and Roop, D. R. (2000). Delayed wound healing in keratin 6a knockout mice. *Mol. Cell Biol.* **20**, 5248-5255.

Wojcik, S. M., Longley, M. A. and Roop, D. R. (2001). Discovery of a novel murine keratin 6 (K6) isoform explains the absence of hair and nail defects in mice deficient for K6a and K6b. *J. Cell Biol.* **154**, 619-630.

Yarosh, D., Both, D., Kibitel, J., Anderson, C., Elmets, C., Brash, D. and Brown, D. (2000). Regulation of TNFalpha production and release in human and mouse keratinocytes and mouse skin after UV-B irradiation. *Photodermatol. Photoimmunol. Photomed.* **16**, 263-270.

Yoneda, K., Furukawa, T., Zheng, Y. J., Momoi, T., Izawa, I., Inagaki, M., Manabe, M. and Inagaki, N. (2004). An autocrine/paracrine loop linking keratin 14 aggregates to tumor necrosis factor alpha-mediated cytotoxicity in a keratinocyte model of epidermolysis bullosa simplex. *J. Biol. Chem.* **279**, 7296-7303.

Yuspa, S. H., Kilkenny, A. E., Steinert, P. M. and Roop, D. R. (1989). Expression of murine epidermal differentiation markers is tightly regulated by restricted extracellular calcium concentrations in vitro. *J. Cell Biol.* **109**, 1207-1217.

Zhuang, L., Wang, B., Shinder, G. A., Shivji, G. M., Mak, T. W. and Sauder, D. N. (1999). TNF receptor p55 plays a pivotal role in murine keratinocyte apoptosis induced by ultraviolet B irradiation. *J. Immunol.* **162**, 1440-1447.