

The Functional Diversity of Epidermal Keratins Revealed by the Partial Rescue of the Keratin 14 Null Phenotype by Keratin 16

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Abstract. The type I epidermal keratins K14 and K16 are remarkably similar at the primary sequence level. While a structural function has been clearly defined for K14, we have proposed that a function of K16 may be to play a role in the process of keratinocyte activation that occurs after acute injury to stratified epithelia. To compare directly the functions of the two keratins we have targeted the expression of the human K16 cDNA to the progenitor basal layer of the epidermis of K14 null mice. Mice null for K14 blister extensively and die ~2 d after birth (Lloyd, C., Q.C. Yu, J. Cheng, K. Turksen, L. Degenstein, E. Hutton, and E. Fuchs. 1995. *J. Cell Biol.* 129:1329–1344). The skin of mice expressing K16 in the absence of K14 developed normally without evidence of blistering. However, as the mice aged they featured extensive alopecia, chronic epidermal ulcers in

areas of frequent physical contact, and alterations in other stratified epithelia. Mice expressing a control K16-C14 cDNA also rescue the blistering phenotype of the K14 null mice with only a small percentage exhibiting minor alopecia. While K16 is capable of rescuing the blistering, phenotypic complementation in the resulting skin is incomplete due to the multiple age dependent anomalies. Despite their high sequence similarity, K16 and K14 are not functionally equivalent in the epidermis and other stratified epithelia and it is primarily the carboxy-terminal ~105 amino acids of K16 that define these differences.

Key words: epidermis • hair • keratin • transgenic mice • gene replacement

THE complexity of skin epithelia is reflected by the large number of keratin genes expressed in these tissues. Of the >40 cloned keratin genes, at least 11 can be expressed in the epidermis alone including the type II keratins K1, K2e, K5, and K6, and the type I keratins K9, K10, K14, K15, K16, K17, and K19. The dividing basal cells of the epidermis synthesize K5, K14 (36), and low amounts of K15 (25). In addition, a small subset of basal cells express K19 (33, 44). As a basal cell stops dividing and begins the process of terminal differentiation that ultimately results in squame production, K5 and K14 gene expression is halted while K1 and K10 synthesis begins (12). These suprabasal keratinocytes also synthesize K2e as they proceed along the differentiation pathway (4). The genes for K6, K16, and K17 are not expressed in the normal interfollicular epidermis, but are expressed in a patchy fashion in palmar and plantar epidermis. In addition, these keratins are expressed in the outer root sheath of the hair

follicle and in several epithelia of the oral mucosa and upper digestive tract including the tongue, gingiva, esophagus, and forestomach (30, 35, 38).

Keratin function in the epidermis has been clearly elucidated by numerous transgenic mouse studies. Expression of dominant negative mutant keratin proteins (9, 13, 42, 47, 63) or the absence of a keratin (25) resulted in fragile epidermides that were susceptible to blistering. In addition, several human blistering genodermatoses including epidermolysis bullosa simplex (K5 and K14), epidermolytic hyperkeratosis (K1 and K10), ichthyosis bullosa of Siemens (K2e), and palmoplantar keratoderma (K9) have been found to be caused by dominant negative mutations affecting specific keratin genes (7, 19, 31). Mutations that result in tissue blistering have also been found in the keratins expressed in the oral mucosa (K4, K13) and the cornea (K3, K12) (17, 45, 50). Clearly, a general function of keratins is to provide mechanical support in the various complex epithelia. Whether keratins perform additional functions in complex epithelia is unknown at the present time. However, studies in simple epithelia, such as liver, strongly suggest a distinct role for simple epithelial keratins during metabolic stress (21).

Inherited mutations in the coding sequences of K6, K16,

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and K17 result in the diseases Pachyonychia Congenita (2, 32), nonepidermolytic palmoplantar keratoderma (K16), and steatocystoma multiplex (K17; references 53, 54). However, K16 mutations do not result in keratinocyte cytolysis, which is surprising since comparable mutations in the previously mentioned epidermal keratins do (7, 31). K6, K16, and K17 are noted for their rapid induction in suprabasal keratinocytes at the wound edge following injury to skin (28, 40, 61). This induction occurs as early as ~6 h after injury (28, 30, 40, 59). The expression of these keratins along with other major changes in gene expression that occur during a ~16–24-h period, termed the activation phase (6, 14), precedes migration into the wound site. The activated keratinocytes increase in size and their keratin filaments fragment and relocate adjacent to the nucleus. In addition, the intercellular space between them increases along with changes in the number and structure of desmosomes. K6, K16, and K17 are also expressed in stratified epithelia undergoing abnormal differentiation or hyperproliferation, including psoriasis and cancer (30, 55, 66). K6 and especially K17 are also prominently expressed during prenatal skin development (29). Expression of these keratins, therefore, is associated with very plastic states of a keratinocyte.

We have presented evidence that K16 may play a role in the process of keratinocyte activation. Initial experiments revealed that K16 formed relatively short filaments that were preferentially located adjacent to the nuclei of cells (40). The expression of K16 in cultured epithelial cells or in the suprabasal keratinocytes of transgenic mouse epidermis caused a relocalization of the endogenous keratins resulting in perinuclear aggregation (40, 58). An additional transgenic mouse study, in which K16 was expressed in the basal layer of the epidermis, also resulted in the bundling and relocalization of keratin filaments along with decreases in cell-cell adhesion (39). The morphologies observed in all of these keratinocytes are strikingly similar to that of the activated keratinocyte. Ectopic expression of K16 in the basal layer also resulted in the delayed maturation of mouse skin which was strikingly similar to what occurs when the EGF receptor is activated in skin (39). This potential involvement of K16 with signaling and cytoarchitectural modulation strongly argues that there are other functions for keratins in addition to providing mechanical support.

The inactivation of the mouse K14 gene resulted in basal keratinocytes that lacked a substantial keratin filament network and exhibited severe cytolysis due to their inability to withstand mechanical trauma (25). A clear structural function for K14 in the epidermis was established as this severe, EBS-like phenotype resulted in the death of the K14 null mice generally ~2 d after birth (25). This genetic alteration has provided a unique opportunity in which to test the functional significance of keratin sequence diversity. Our objective has been to express human K16 in the basal layer of these mice to determine if the lethal phenotype of these mice can be complemented by another epidermal keratin. Transgenic mice ectopically expressing K16 in the basal layer of mouse epidermis (39) were mated with the K14 null animals to generate the K16 replacement mice. In addition, as a control, replacement mice were generated that expressed a K16-C14 chimeric

protein that behaves functionally as K14 in various assays (39, 40, 65). While both types of replacement mice rescue the early blistering phenotype, we report here that there are dramatic differences between the two that further accentuate the unique nature of K16.

Materials and Methods

Generation of the Replacement Mice

All protocols involving mice were approved by the Johns Hopkins University Animal Care and Use Committee (Baltimore, MD). Transgenic mice expressing either the K16 cDNA or the K16-C14 cDNA under the control of the human K14 promoter have been previously described (39). In short, the cDNAs were subcloned into a modified version of the K14 cassette expression vector which contains 2 kb of human K14 promoter sequence, the rabbit β -globin intron, and 0.6 kb of human K14 poly A sequence (52). The purified constructs were micro-injected into fertilized C57B6/BalbC3 F₂ mouse embryos. K14 null mice were generated in the 129/SV background as previously described (25). Two K16-C14 and four K16 transgenic lines were generated with each having a single insertion site. The approximate transgene copy number for the K16 lines ranged from ~10 (no. 13 line), to ~15 (no. 6 and no. 10 line), to ~45 (no. 21 line). To generate the replacement mice, mice heterozygous at the insertion site for the transgene were mated with mice that were heterozygous for the K14-targeted (null) allele (25). Offspring were screened for the presence of the transgene and the K14-targeting event by Southern blotting as previously described (25, 39). The desired mice were then bred until the replacement offspring were obtained.

Northern Blot Analysis

To determine the levels of transgene mRNA in the various mice, total RNA was isolated from the skin of 7-d-old mice. Skin obtained from killed animals (~250 mg) was frozen in liquid nitrogen and crushed using a mortar and pestle kept cold with dry ice. The pulverized pieces were homogenized in 2 ml of Trizol reagent (GIBCO BRL) and the total RNA was isolated according to the manufacturer's protocol. Equivalent amounts of isolated RNA (30–40 μ g) were electrophoresed using a 1% agarose gel containing formaldehyde and transferred to a nylon membrane (NEN Life Science Products). Membranes were prehybridized for 30 min at 65°C in hybridization buffer (50 mM Tris, pH 7.4, 1 M NaCl, 1% SDS, and 10% PEG 8000). Hybridization was carried out overnight at 65°C. Blots were washed for 20 min at 65°C four times with 0.1 \times SSC, 0.5% SDS. A 500-bp HindIII-BamHI fragment from the K14 cassette (51) was used as a probe to specifically detect the human transgene mRNAs (K16 or K16-C14 mRNA). An ~140-bp FspI-HindIII fragment from a plasmid containing the mouse K14 cDNA (18) was used to detect mouse K14. Mouse actin was detected using a probe derived from DNA from Ambion.

Western Blot Analysis

Urea soluble proteins were isolated from dorsal skin of killed animals as previously described (39). Equivalent amounts of isolated proteins (~20 μ g, as determined by spectrophotometry and confirmed by Coomassie blue staining) were resolved via 8.5% SDS-PAGE and transferred to nitrocellulose. Western blotting was performed using the alkaline phosphatase method (Bio-Rad Laboratories). Human K16 was detected using the previously described rabbit polyclonal no. 1275 (58). Mouse K16 was specifically detected using RpmK16, a rabbit polyclonal antibody (43). Mouse K14 and human K16-C14 were detected with the mouse monoclonal antibody LL001 (44) that recognizes an identical sequence shared between the human and mouse K14 tail domains. Mouse K5 was detected using the rabbit polyclonal antibody no. 5054 (24) while K6 was detected using the rabbit polyclonal K6 general antibody (29). K17 was detected with the rabbit polyclonal α -K17 antibody (29) and K15 was detected with the rabbit polyclonal antibody UC54 (25).

For the solubility experiments, skin from killed animals was crushed as previously described and homogenized in a buffer containing 1% Triton X-100 in PBS and 5 mM EDTA. After centrifugation, the supernatant was isolated (soluble fraction) and the pellet was homogenized in HSE buffer (26). After another centrifugation, the insoluble pellet was homogenized

in buffer A (39). This urea soluble fraction is referred to as the insoluble fraction.

Culture and Analysis of Primary Mouse Keratinocytes

Primary cultures of skin keratinocytes were established as previously described (39, 48). 1–4 d after plating, the primary keratinocytes were processed for immunofluorescence. The coverslips were washed three times in PBS and fixed for 15 min with 100% methanol at -20°C . After three washes with PBS, coverslips were blocked with 5% normal goat serum in PBS. Primary antibodies were diluted in blocking buffer and incubated for 45 min at room temperature. FITC-conjugated goat anti-mouse and rabbit, rhodamine-conjugated goat anti-mouse and rabbit, and FITC-conjugated goat anti-guinea pig secondary antibodies were used to detect bound primary antibodies. Coverslips were mounted onto slides and analyzed via fluorescence microscopy. Primary antibodies used were mouse monoclonals LL001, LL025 (anti-K16; reference 23), K8.60 (anti-K10; Sigma Chemical Co.), K8.12 (anti-K16; Sigma Chemical Co.), and rabbit polyclonals no. 1275, K6 general, α -K17, and no. 5054.

Morphological Analyses

Mouse tissues were fixed in Bouin's overnight at 4°C . The fixed tissues were embedded in paraffin and 5- μm sections were stained with hematoxylin and eosin or immunostained using the horseradish peroxidase procedure (HRP) by following the manufacturer's protocol (Kirkegaard and Perry Labs.). Primary antibodies used were mouse monoclonals LL001 and K8.60, and rabbit polyclonals no. 1275, K6 general, and α -K17. For electron microscopy, dorsal and ventral skin tissues were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate, post-fixed in aqueous 1% osmium tetroxide, and embedded in LX112 epoxy-resin (Ladd Research Industries Inc.). Ultrathin sections (50–70 nm) were placed on copper grids, counterstained with uranyl acetate and lead citrate, and visualized using a Zeiss EM10 transmission electron microscope operated at 60 kV.

Skin Challenges

Mice were anesthetized with avertin and their backs were depilated with Nair (Carter-Wallace). PMA treatment (phorbol-12-myristate-13-acetate; Sigma Chemical Co.) was performed by topically applying 200 μl (50 μM stock in acetone) on days 1, 3, 5, and 7 (56). The tissue was harvested for analysis on day 7. Two methods were employed to test the mechanical integrity of back skin. Scotch Guard tape (3M) was repeatedly applied (~ 20 times) to back skin and removed with a sudden motion. The stratum corneum could be seen attached to the tape. Alternatively, depilated back skin was vigorously rubbed using a thumb and forefinger for 30 s. This was repeated every other day (days 1, 3, 5, and 7) over the course of a week. It was also performed right before the tissue was harvested for analysis (day 7).

Results

Generation of the Replacement Mice

The four previously described K16 ectopic transgenic lines, no. 6, 10, 13, and 21 and the two K16-C14 ectopic transgenic lines, no. B1 and C1 in the C57B6/BalbC3 background (39) were bred with the 129/SV K14 null mice (25) to generate the K16 and K16-C14 replacement mice. Mice heterozygous for the K16 or K16-C14 transgene ($\text{Tg}^{+/-}$) were mated with mice that were hemizygous null at the K14 locus ($\text{K14}^{+/-}$). The desired F1 offspring ($\text{Tg}^{+/-}$, $\text{K14}^{+/-}$) were mated with each other to generate the replacement mice. While a total of nine different F2 genotypes are possible from this mating, the three of interest were: (a) K14 null mice ($\text{Tg}^{-/-}$, $\text{K14}^{-/-}$); (b) heterozygous replacement mice ($\text{Tg}^{+/-}$, $\text{K14}^{-/-}$); and (c) homozygous replacement mice ($\text{Tg}^{+/+}$, $\text{K14}^{-/-}$). In addition, wild-type mice ($\text{Tg}^{-/-}$, $\text{K14}^{+/-}$, or $\text{K14}^{+/+}$) were included as controls.

The K14 null mice develop gross blistering over their

body surface and die ~ 2 d after birth (25). Thus, this genetic background provides a clear readout for epidermal keratin function in the skin. All four of the K16 replacement lines and the two K16-C14 replacement lines were able to rescue the young mice from death. In addition, either replacement genotype ($\text{Tg}^{+/-}$ or $\text{Tg}^{+/+}$, $\text{K14}^{-/-}$) rescued the K14 null phenotype. No replacement mice displayed skin blistering at any body site at an early age.

Young Replacement Mice Have Normal Skin

The hyperproliferative phenotype of the K16 ectopic mice is most severe ~ 7 d after birth (39). To determine if there were any comparable morphological or molecular aberrations at the same age in the replacement mice, trunk skin from 7-d-old K16 and K16-C14 replacement mice was examined by light microscopy (Fig. 1, A and B). Skin tissue sections were analyzed by hematoxylin and eosin staining or by immunohistochemistry. The skin of both types of replacement mice appeared equivalent and normal compared with wild-type skin (Fig. 1, A and B, data not shown). In both cases, there were abundant hair follicles correctly oriented in the hypodermis and the epidermides were of normal thickness. No aberrations could be observed in any layers of the epidermis in either type of replacement mouse. In addition, there was no evidence of blistering in the basal layer.

The localization of the transgenic proteins was determined by performing immunohistochemical analysis with tail domain specific antibodies. Both transgenes were detected in the outer root sheath of hair follicles and in the basal layer of the epidermis (Fig. 1, C and F). There was no signal corresponding to K14 in the K16 replacement sample (Fig. 1 E) and there was no signal corresponding to K16 in the K16-C14 replacement sample (Fig. 1 D) as expected. Based on these data, the transgenes were correctly expressed in a K14-like fashion (39, 62).

K10 was used as a marker to determine if early terminal differentiation was normal in the replacement mice (Fig. 1, G and H). Both samples featured suprabasal staining of the epidermis using the K8.60 antibody, which was identical to a wild-type sample (data not shown). Filaggrin staining was also performed to determine if there were any differences in late terminal differentiation in the replacement mice. No differences were noted when compared with the wild-type sample (data not shown). Keratins K6 and K17, which are commonly associated with hyperproliferation or altered differentiation in skin (55, 57, 66) were also analyzed. The expression of both was restricted to the outer root sheath of the hair follicles in both cases (data not shown). In addition, BrdU labeling indicated no differences in the number of mitotic nuclei between the two replacement samples and a wild-type sample (data not shown). Based on these multiple criteria, the skin appears to develop and self-renew normally in both types of replacement mice at an early age. These results are in stark contrast to those observed in the K16 ectopic mice that featured multiple hyperproliferative abnormalities and aberrant keratin expression in the skin at ~ 7 d (39), suggesting that the keratin composition of a keratinocyte is crucial in determining the effects of K16 in the skin.

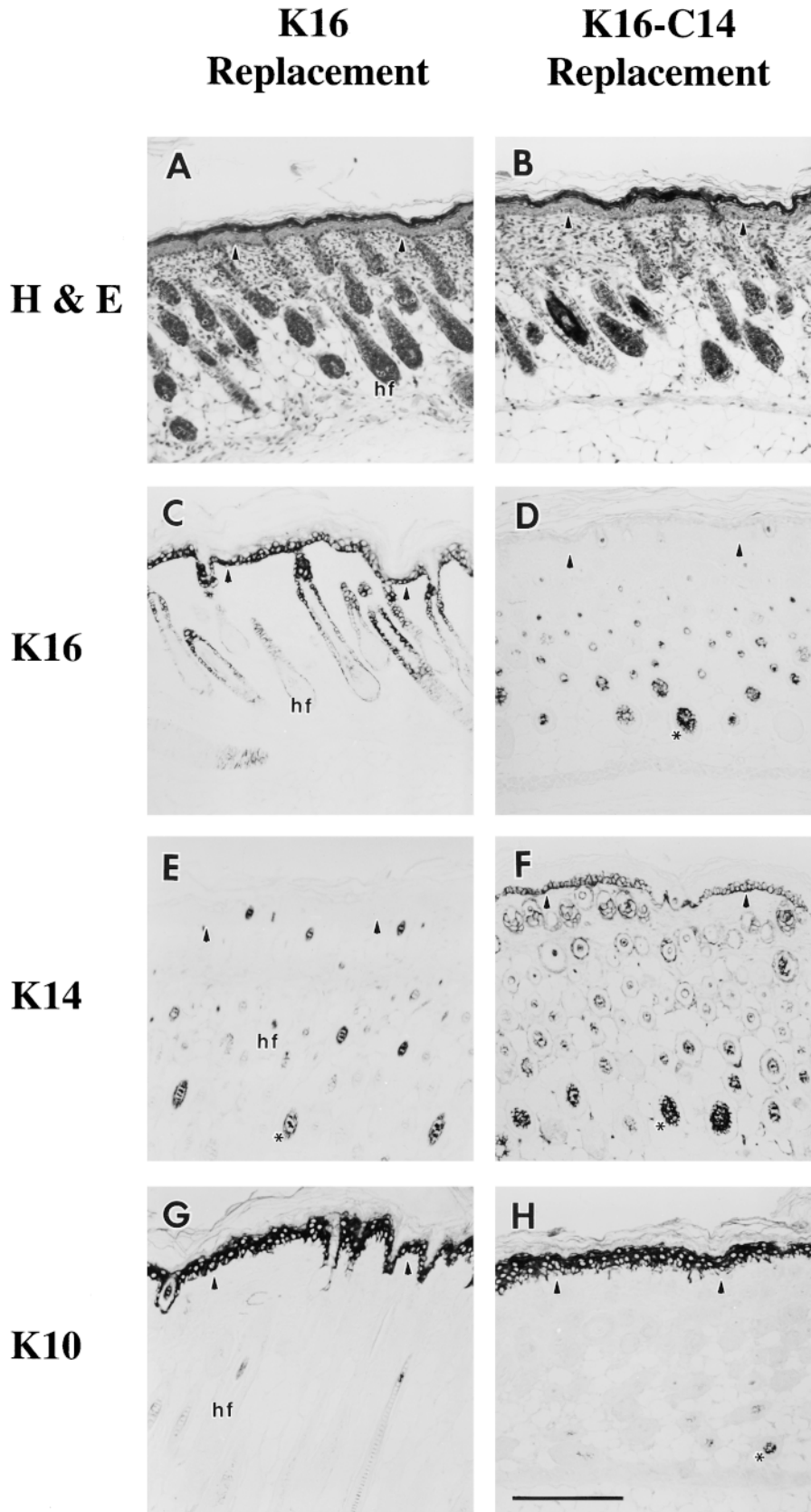


Figure 1. Light microscopy analysis and transgene expression in replacement skin. 7-d-old trunk skin was fixed, embedded in paraffin, and 5- μ m sections were stained with hematoxylin and eosin (H & E) or subjected to immunohistochemistry using the HRP procedure. H & E staining of K16 (A) and K16-C14 (B) replacement skin. Both epidermides featured a normal cellular morphology along with multiple anagen staged hair follicles. There were no detectable differences when compared with wild-type 7-d-old epidermis (data not shown). (C-F) Transgene detection. The K16 transgene protein was detected using the 1275 antibody (C) and was restricted to the basal layer of the epidermis and the outer root sheath of the hair follicle. The K16-C14 transgene protein was detected (F) using the LL001 antibody and showed the same distribution. There was no detection of K16 in the K16-C14 replacement sample (D) and there was no detection of K14 in the K16 replacement sample (E). (G and H) Both samples were also stained with an antibody against K10. K10 expression was detected throughout the suprabasal layers of the epidermis in both the K16 (G) and K16-C14 (H) samples indicating that there is no evidence of abnormal differentiation. Hf, hair follicle. Arrowheads, demarcate the dermal-epidermal junction. Asterisks, melanin granules in hair follicle profiles. Bar, 100 μ m.

Electron Microscopy of Young Replacement Epidermis

To finely assess whether young replacement epidermis was truly similar to wild-type epidermis, ventral skin from

7-d-old wild-type and K16 replacement mice was examined by transmission electron microscopy. There were no discernible morphological differences between replacement and wild-type basal keratinocytes. K16 replacement

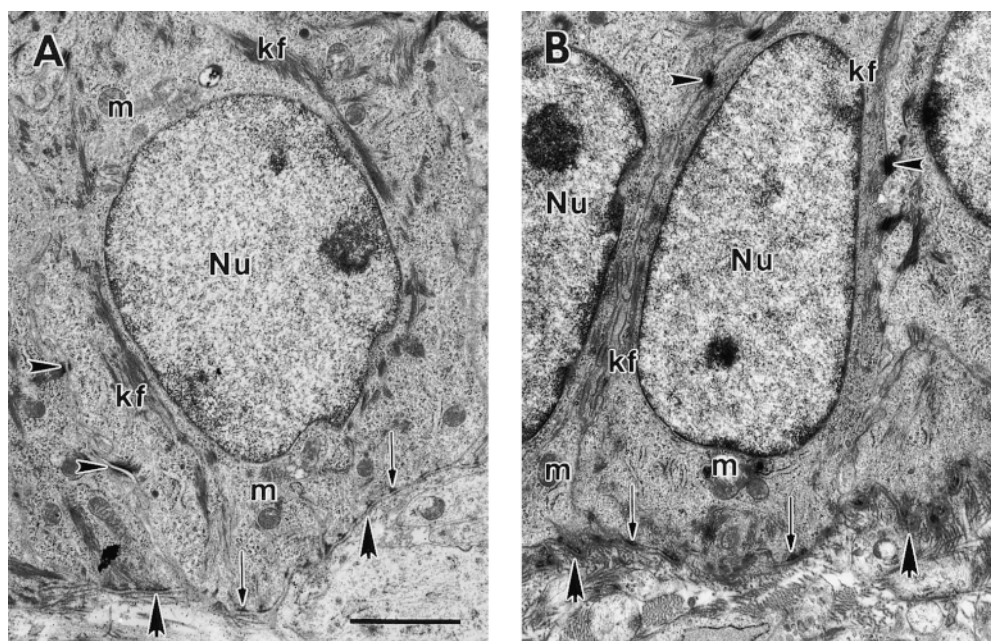


Figure 2. Transmission electron microscopy of wild-type and replacement mouse epidermis. Ventral skin was isolated from 7-d-old wild-type and K16 replacement mice and processed for electron microscopy. (A and B) Basal cells from wild-type (A) and K16 replacement epidermis (B). K16 replacement basal keratinocytes (B) are morphologically similar to wild-type keratinocytes (A). Both feature bundles of filaments (kf) distributed throughout the cytoplasm and multiple desmosomal (small arrowheads) and hemidesmosomal (arrows) attachments. There is no evidence of decreased cell-cell adhesion or blistering in any layers of the epidermis. Large arrowheads, the basal lamina. Nu, nucleus; m, mitochondria. Bar: (A and B) 2 μ m.

basal keratinocytes (Fig. 2 B) had a low-columnar, cuboidal shape and were tightly packed together. There were no obvious alterations in cell-cell or cell-matrix adhesion. Keratin filaments were loosely bundles and distributed throughout the cytoplasm. These same morphological characteristics were observed in both wild-type (Fig. 2 A) and K16-C14 replacement basal keratinocytes (data not shown). There was also no evidence of cell lysis or epidermal blistering. Spinous keratinocytes in the replacement epidermis (K16 or K16-C14) were also normal. Filament bundling occurred with the concomitant increase in the number of desmosomes (data not shown). Cells in the granular layer and the stratum corneum of replacement epidermis were also similar to wild-type (data not shown). By all morphological criteria, the replacement epidermides are equivalent to wild-type epidermis. This is in stark contrast to what was observed in the basal keratinocytes from ectopic K16 phenotypic epidermis in which the hypertrophic basal keratinocytes had large aggregations of keratin filaments and major decreases in cell-cell adhesion (39).

K16 Replacement Keratinocytes Have Aberrant Keratin Filament Networks in Culture

The electron microscopy results prompted the examination of the replacement keratinocytes in culture in order to analyze the global organization of the keratin filaments within the context of an intact keratinocyte. Primary cultures of newborn keratinocytes from K16 and K16-C14 replacement mice were established and analyzed by immunofluorescence to further determine if there were any possible abnormalities in the keratin networks of the K16 replacement mice. K16 replacement keratinocytes appeared normal after one day in culture (data not shown). All cells were positive for K5, K6, human K16, and K17,

and negative for K14 (data not shown). As the cells remained in culture, however, they began to display time-dependent changes in their keratin filament networks. In a subset of cells (<50%), the keratin filament networks began to appear fragmented and even absent in some areas of the cytoplasm (Fig. 3, B and C). There were also large bundles of filaments that were distributed throughout the cell (Fig. 3 A) rather than preferentially located adjacent to the nucleus as previously noted for the K16 ectopic keratinocytes (39). There was no evidence of keratin reorganization, fragmentation, or loss in any of the K16-C14 replacement keratinocytes regardless of the time spent in culture. These keratinocytes featured filament networks that were indistinguishable from wild-type keratinocytes (Fig. 3 D). These data suggest that in the absence of K14, K16 is not able to support a keratin filament network and that over time this fragile network is susceptible to fragmentation and loss. Furthermore, they provide further evidence that the carboxy-terminal \sim 105 amino acids of K16 are responsible for these differences in filament organization.

Transgene Expression in the Epidermis

We have previously shown that the expression of K16 protein at levels comparable to endogenous mouse K14 was able to cause the hyperproliferative skin phenotype of the K16 ectopic mice (39). The lack of a phenotype in the replacement mice at an early age raised the possibility that transgene expression was reduced in these mice. To test this idea, urea extractable proteins were isolated from the back skin of 7-d-old heterozygous and homozygous replacement mice from the no. 10 line and compared with wild-type, heterozygous, and homozygous ectopic mice from the same line (no. 10) by Western blot analysis (Fig. 4 A) using the no. 1275 antibody which specifically reacts

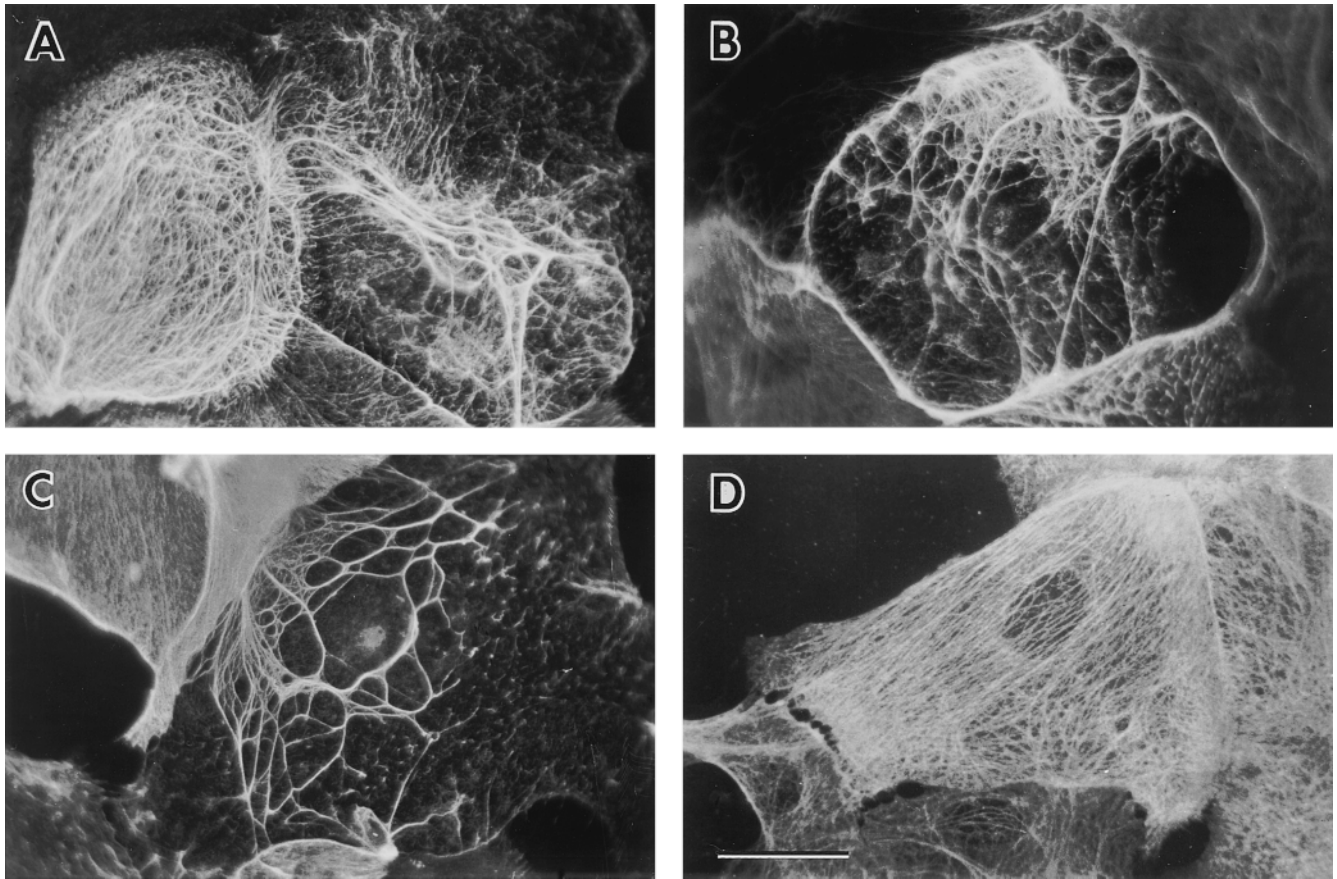


Figure 3. Immunofluorescence analysis of keratins in cultured primary keratinocytes from replacement mice. Keratinocytes were isolated from the epidermides of newborn K16 and K16-C14 replacement mice, cultured, and analyzed by indirect double immunofluorescence to examine the keratin filament networks. Keratinocytes from K16 replacement mice (A–C) were stained with the 1275 antibody to detect K16. Some K16 replacement keratinocytes had fragmented keratin networks and cytoplasmic areas devoid of filaments (B and C). Others exhibited large bundling of filaments randomly distributed throughout the cytoplasm (A). Keratinocytes from K16-C14 replacement mice (D) were stained with the LL001 antibody to detect K16-C14. These keratinocytes appeared identical to wild-type keratinocytes (data not shown) in all morphological aspects as there was no evidence of keratin reorganization, fragmentation, or loss. Bar, 25 μm .

with the tail of human K16 and exhibits partial cross-reactivity with mouse K16 (8, 30). As previously documented, the amount of K16 transgene expressed in the homozygous ectopic mouse sample was approximately twice the amount expressed in the heterozygous ectopic sample (39). Interestingly, there was no difference in transgene expression between the heterozygous and homozygous replacement mice. Furthermore, the level of transgene expression was lower than that observed in the homozygous ectopic sample but higher than the heterozygous ectopic sample. This possible difference between the replacement and the homozygous ectopic samples may be accounted for by cross-reactivity of the 1275 antibody with mouse K16, which is induced in the homozygous ectopic sample (see below, Fig. 4 E).

The same type of Western blot analysis was performed with mice from the K16-C14 no. B1 ectopic and replacement lines using the LL001 antibody which detects the K14 moiety of the chimera (Fig. 4 B). As was the case with the K16 transgenics, the amount of K16-C14 approximately doubled from the heterozygous ectopic sample to

the homozygous ectopic sample (the upper band in these and the control sample is endogenous mouse K14). The level of transgene expression was similar between the heterozygous and homozygous samples also. However, in contrast to the results observed in the no. 10 line, the amount of transgene expressed in the replacement background was greater than in the homozygous ectopic sample.

Further Western blot analysis of urea extractable proteins from 7-d-old trunk skin from the four K16 replacement lines was performed to determine if there were differences in transgene expression between the various replacement lines or between the two types of replacement genotypes (Tg^{+/-} and Tg^{+/+}). Equivalent amounts of protein from both heterozygous and homozygous replacement mice from each of the four K16 lines were analyzed using rabbit polyclonal antibody no. 1275. As was the case with the no. 10 line (Fig. 4 A), the amount of transgene expression was the same within a given line regardless of the genotype (Fig. 4 C). However, the level of expression varied slightly between the lines. Compared with the ectopic

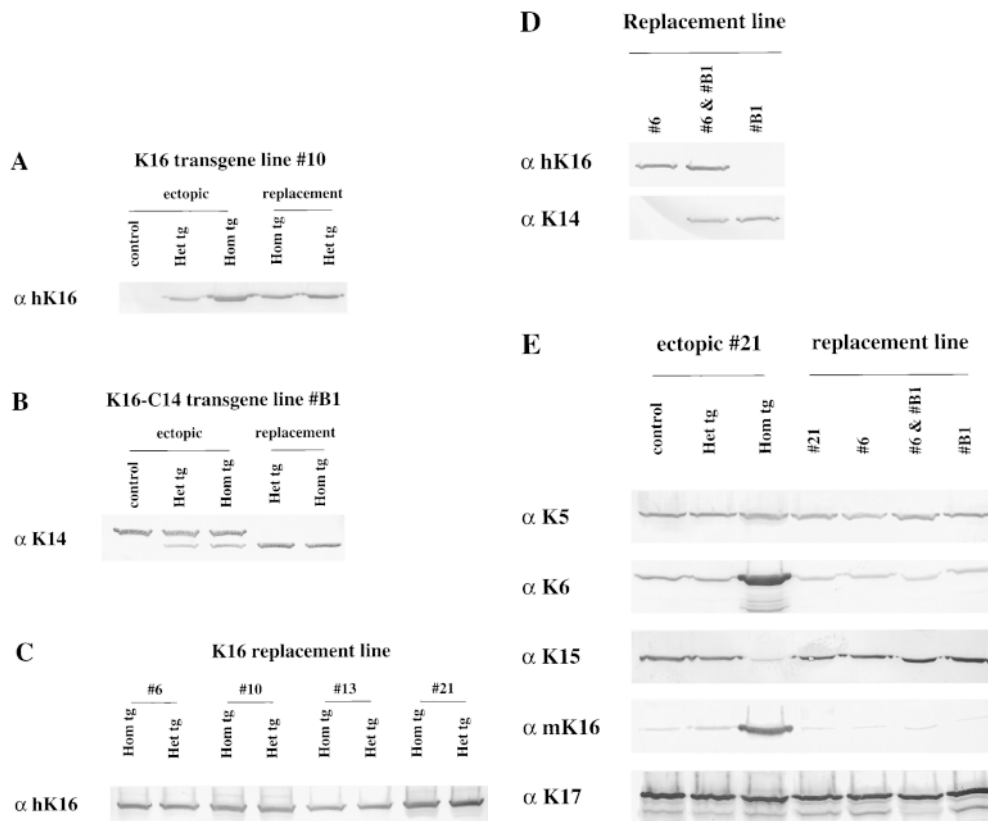


Figure 4. Analysis of transgene and keratin expression in the skin. Urea-soluble protein extracts were obtained from the skin of various 7-d-old replacement and ectopic mice from the K16 lines no. 6, no. 10, no. 13, and no. 21, and the K16-C14 line no. B1. Equivalent amounts of proteins ($\sim 20 \mu\text{g}$) were electrophoresed via SDS-PAGE and transferred to nitrocellulose for Western blot analysis. (A and B) Comparing K16 and K16-C14 expression in the replacement vs. ectopic background. A wild-type, heterozygous transgenic, homozygous transgenic, heterozygous replacement, and homozygous replacement samples from a K16 (no. 10) and a K16-C14 line (no. B1) were analyzed by Western blot analysis and probed with either the no. 1275 antibody to detect K16 (A) or with the LL001 antibody to detect the K14 moiety of the chimera (B). The amount of K16 transgene expressed in the heterozygous

and homozygous replacement samples were equivalent and slightly less than that expressed in the homozygous ectopic sample (A). In contrast, K16-C14 expression was increased in the replacement background compared with the ectopic background and the amount was equivalent independent of the replacement genotype. (C) Determination of the amount of K16 expression in the four replacement lines. Equivalent amounts of K16 were detected regardless of the genotype within each line. (D) Determination of transgene expression in the double replacement mice. The amount of K16 and K16-C14 expressed in the double replacement was similar to the amount made in either of the individual replacement mice. (E) Antibodies against K5, K6, K15, mouse K16, and K17 were used to determine keratin levels in the skin of various mice. K5 levels were slightly increased in the homozygous ectopic and double replacement samples. K6 and mouse K16 were greatly increased in the homozygous ectopic sample as expected due to the hyperproliferative epidermis of these mice (39). While K17 levels were equivalent in all mice, K15 was greatly reduced in the homozygous ectopic sample.

mice, the maximal K16 transgene levels are slightly reduced in the replacement background with the exception of the no. 13 line in which the levels are increased (data not shown). Thus, in the K14 null background there appears to be a tightly regulated range of transgene protein levels allowed in a keratinocyte which may be modulated by the levels of K5 and possibly K6 (59). Collectively these data strongly suggest that K14 has a dramatic impact on the regulation of K16 (or K16-C14) in a keratinocyte.

The fact that K14 has an impact on K16 levels prompted the mating of the two types of replacement mice to generate double replacement mice that express both transgenes. Urea extractable proteins from 7-d-old double replacement trunk skin was analyzed by Western blot analysis to determine if the two transgenes could influence the expression levels of each other. Proteins from K16, K16/K16-C14, and K16-C14 replacement mice were probed with the 1275 or the LL001 antibody to detect the transgenes. The levels of both K16 and K16-C14 in the double replacement sample were equivalent to the amount produced in the single replacement samples (Fig. 4 D). Although the chimeric transgene contains the last ~ 105 amino acids of K14, it

was not able to affect the levels of K16 transgene expression.

In addition to transgene expression, the levels of other epidermal keratins were examined by Western blot analysis to determine if there were any differences in keratin expression that may account for the phenotypic differences observed between the ectopic and replacement mice at 7 days of age. Equivalent amounts of urea proteins from trunk skin of various ectopic and replacement samples were probed with specific antibodies against K5, K6, K15, mouse K16, and K17 (Fig. 4 E). K5 levels were equivalent in all samples except in the homozygous ectopic and double replacement where the amount of K5 was slightly increased. K6 and mouse K16 levels were equal in all mice except for the homozygous ectopic sample where both were greatly increased. This is consistent with the fact that the epidermis of these mice is hyperproliferative and that K6 staining was observed in the suprabasal layers of these mice (39). While K17 levels were equivalent among all samples, K15 was dramatically reduced in the homozygous ectopic sample. K15 is expressed in a pattern analogous to K14 in the epidermis but at much lower levels (25). It is in-

interesting to note that a type I keratin that is such a minor component of basal cells is decreased to such levels while K14 levels remain unchanged (39). It has recently been reported that K15 mRNA levels in hyperproliferative human skin are decreased (64). These results suggest that the keratin profiles of the replacement samples are very similar to control and nonphenotypic, heterozygous ectopic samples for those keratins examined.

Transgene mRNA Expression in the Ectopic and Replacement Mice

We performed Northern blot analysis on total RNA isolated from 7-d-old ectopic and replacement mouse skin to determine if the protein levels were an accurate reflection of the steady state levels of transgene mRNA. Equivalent amounts of total RNA were analyzed using a transgene specific probe that detects both the K16 and K16-C14 messages but does not cross react with nontransgenic control RNA (Fig. 5). The heterozygous ectopic no. 10 sample featured one band that reacted strongly with the transgene probe. The intensity of the K16 transgene in the no. 10 homozygous ectopic sample was increased at least fourfold compared with the heterozygous sample which is not consistent with the protein results. However, when the same samples were analyzed using a probe specific for mouse K14 the amount of this message was also increased in the homozygous sample (Fig. 5). These findings were reproducible as other comparable sets of samples from the no. 10 and no. 21 lines yielded similar results. This is consistent with a previous report that showed that the activity of the K14 promoter increases during hyperproliferation (42). In addition, the mRNA levels of K6 and mouse K16 are elevated only in the ectopic homozygous samples (data not shown). Thus, the human K14 promoter that drives transgene expression is stimulated in the homozygous ectopic sample because of the hyperproliferative conditions prevalent at 7 days of age.

The steady state level of K16 transgene mRNA in the homozygous replacement no. 10 sample was lower than that observed in the two no. 10 ectopic samples despite the fact that the amount of K16 protein in the no. 10 replacement line is intermediate between the two heterozygous ectopic samples (Fig. 4 A). The level of transgene mRNA from a homozygous replacement no. 21 sample was much greater compared with the no. 10 replacement sample. However, the amount of transgene protein expressed in skin is essentially equivalent for the two lines (Fig. 4 C) suggesting that despite the wide range in steady state transgene mRNA levels between the lines there is a limit to the amount of K16 protein that can be expressed in a replacement basal keratinocyte.

In the no. B1 chimera line, there was more transgene mRNA in the homozygous ectopic sample than in the heterozygous replacement sample (Fig. 5) despite the protein levels being higher in the heterozygous replacement sample (see Fig. 4 B). These results provide further evidence that the presence of K14 protein in the skin is acutely critical in determining the levels of transgene protein expression and that the mechanisms responsible may occur at both the transcriptional and post-transcriptional levels.

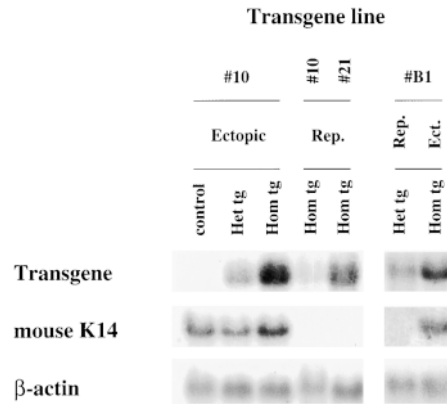


Figure 5. Transgene mRNA expression in ectopic and replacement mice. Total RNA was isolated from the skin of 7-d-old K16 and K16-C14 ectopic and replacement mice. ~40 μ g of each sample was electrophoresed in a formaldehyde containing gel and subsequently transferred to a nylon membrane. A transgene specific probe was used to detect both the K16 and K16-C14 transgene mRNAs. This probe did not react with any endogenous mRNA (see control lane, data not shown). β -actin was used as a probe to control for loading. Steady state levels of the K16 transgene mRNA were more than doubled in the homozygous ectopic sample compared with the heterozygous ectopic sample. This discrepancy between the steady state mRNA levels and protein levels may be partially accounted for by the increase in the activity of the K14 promoter under hyperproliferative conditions (42) as determined by the increase in mouse K14 mRNA in the homozygous ectopic sample. K16 transgene mRNA varied widely in the K16 replacement samples (no. 10 and no. 21 lines) despite the fact that comparable amounts of protein was expressed in each (see Fig. 4 C). K16-C14 mRNA levels were higher in the homozygous ectopic sample than in the replacement sample despite the fact that more protein is expressed in the replacement epidermis (see Fig. 4 B). The same blot was used for all three probes. Rep., replacement. Ect., ectopic.

Keratin Solubility in the Epidermis

We have previously shown that a single proline residue in the 1B rod domain of human K16 (residue 188) completely accounts for the reduced stability of K16-containing heterotetramers under denaturing buffer conditions *in vitro* (65). The reduced stability of these tetramers correlated with the diminished ability of K16 to form 10-nm filaments efficiently *in vitro*. This led to the possibility that the solubility and partitioning of K16 between the soluble and insoluble keratin pools in the epidermis may be an important factor in determining its effect on keratinocytes (65). To determine if the partitioning of K16 was different among the various types of mice, skin from 7-d-old mice was lysed and the soluble and insoluble (cytoskeletal) fractions were isolated and subjected to Western blot analysis using various keratin antibodies (Fig. 6).

There was very little detectable human K16 protein in the soluble fractions from any of the mice. The small amount detectable was proportional to the amount observed in the corresponding insoluble fractions. Thus, there were no obvious differences in K16 solubility among the samples. The same results were observed for mouse K16, which does not contain a destabilizing proline resi-

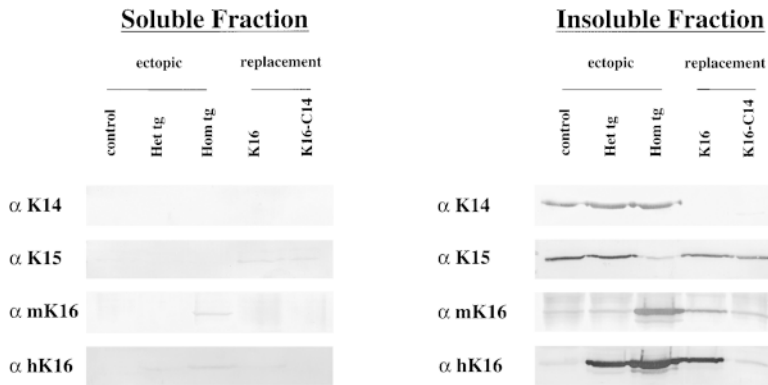


Figure 6. Keratin solubility in the skin. Skins from 7-d-old ectopic and replacement mice (no. 6 line) were homogenized in 1% Triton-X in PBS with 5 mM EDTA to obtain the soluble fraction of keratins. The remaining insoluble cytoskeletal fraction was solubilized in buffer A to obtain the insoluble fraction of keratins. 100 μ g of total protein from the soluble fractions and 15 μ g of total protein from the insoluble fractions were electrophoresed via SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blot analysis. Keratins analyzed included K14, K15, mouse K16, and human K16. No major differences were noted in the partitioning of any of the keratins between the soluble and insoluble fractions.

due in the 1B rod domain (McGowan, K., K. Hess, and P.A. Coulombe, unpublished data, also see reference 43). There were also no differences observed in the partitioning of K5, K6, and K17 (data not shown). The only difference observed was that more K15 appeared to be soluble in the two replacement samples compared with the ectopic samples. These results suggest that there are no major differences in keratin solubility among the types of mice analyzed that could account for the phenotypic differences observed and that the mechanism by which K16 functions probably does not involve a change in its *in vivo* solubility.

Age-dependent Changes in the Skin and Other Stratified Epithelia

Beginning as early as 4–5 wk after birth, the K16 replacement mice begin to lose hair (Fig. 7 A, middle mouse). This alopecia generally initiates at the crown of the head and proceeds in a head-to-tail fashion. The loss occurs primarily on the dorsal surface but also occurs to a lesser extent on the ventral surface. Once lost, the hair does not regrow. While \sim 80% of the K16 replacement mice exhibit alopecia, about one in three develop severe skin lesions (Fig. 7 A, right mouse) characterized by epidermal ulceration and scar contraction. These lesions are more prevalent in areas of hair loss and frequent physical contact (limbs, paws, eyes, nose, and mouth areas). Interestingly, the amount of K16 transgene expressed in skin does not change as a function of time. In fact, K16 transgene levels from hairless or lesional skin from the 8-mo-old mice were equivalent to the levels observed in 7-d-old skin (data not shown).

These phenotypes occur in mice of either the heterozygous or homozygous replacement genotype, which is expected given that transgene expression levels are equivalent. In addition, the phenotypes observed occur in all four of the K16 replacement lines. However, this phenotype is not fully penetrant as \sim 20% of the K16 replacement mice do not exhibit hair loss or skin lesions. This incomplete penetrance may partially be due to the mixed genetic background of the mice (see Materials and Methods). On the other hand, only \sim 33% of the K16-C14 replacement mice exhibit minor alopecia (Fig. 7 B, right mouse) and none have developed lesions.

As mentioned, a subset of the K16 replacement mice ex-

hibit hair loss and the development of skin ulcerations. To understand these further, tissue samples from 8-mo-old K16-C14 control and phenotypic K16 replacement mice were taken from skin and a variety of other stratified epithelia and examined by light microscopy. Nonphenotypic, hairy skin from a K16 replacement mouse (Fig. 8 A) was similar to wild-type and K16-C14 control skin (Fig. 8 E). The epidermis was thin and there were many hair follicles. However, the hair follicles were in the telogen stage as opposed to wild-type skin in which the follicles were in anagen (data not shown). K16 transgene expression was still restricted to the basal layer (data not shown). There was no evidence of hyperproliferation as the epidermis did not stain for K6 and K17 (data not shown).

In contrast, K16 replacement skin that exhibited hair loss in the absence of any visible lesions was abnormal in many respects (Fig. 8 B). The epidermis was significantly thickened due to acanthosis and an increase in the number of cell layers. While there were many anagen staged hair follicles that extended deep into the hypodermis, some of the follicles were misoriented and some were without hair shafts (large asterisk in Fig. 8 B). In addition, there appeared to be more sebaceous glands than in the wild-type. K6 expression was observed in the suprabasal layers indicative of hyperproliferation (data not shown).

Lesional K16 replacement skin also displayed many abnormalities (Fig. 8, C and D). The epidermis and the outer root sheaths of the hair follicles were hyperplastic and acanthotic. There was also strong K6 and K17 expression in the suprabasal layers of the epidermis (data not shown). Embedded in the dermis were many large cysts derived from pilosebaceous units. Migration in the K16 replacement epidermis was not inhibited as epithelial sheets appeared to be migrating into an epidermal ulcer (Fig. 8 D). The dermis also featured increased cellularity suggestive of an ongoing inflammatory response. The hair follicles in the lesional skin also extended very deeply into the hypodermis.

While nonphenotypic hairy skin from a K16-C14 mouse was normal in most morphological aspects (Fig. 8 E, see above), skin from a hairless region (Fig. 8 F) displayed many of the same abnormalities observed in the K16 replacement hairless skin (Fig. 8 B). The epidermis was thickened and the hair follicles were anagen staged. There appeared to be more sebaceous glands and there were also

K16 Replacement



K16-C14 Replacement



Figure 7. Age-dependent phenotypic changes in the replacement mice. (A) Three 8-mo-old K16 replacement littermates. The genotypes are from left to right: K16^{-/-}, K14^{+/+} (wild-type), K16^{+/+}, K14^{-/-}, and K16^{+/-}, K14^{-/-}. Alopecia in these mice can begin at ~4–5 weeks of age and usually proceeds in a head-to-tail direction. The mouse on the right has extensive epidermal ulcers. These ulcers usually occur in areas of hair loss and frequent physical contact. While ~80% of these mice exhibit alopecia, approximately one in three develop lesions. (B) Two 7-mo-old K16-C14 replacement littermates. The genotypes of both mice are K16-C14^{+/-}, K14^{-/-}. Alopecia occurs at a much lower frequency (~33%) in these mice and lesion formation has not been noted.

hair follicles that lacked hair shafts (large asterisk). There was, however, no evidence of cyst formation.

In addition to skin, other stratified epithelia that feature expression of the K16-C14 control and K16 transgenes in the basal layer, including forestomach and cornea, were

examined. Forestomach from a K16 replacement mouse (Fig. 8 H) exhibited extensive blistering and basal layer cytolysis along the length of the tissue. On the other hand, forestomach from a K16-C14 replacement mouse (Fig. 8 G) appeared completely normal when compared with

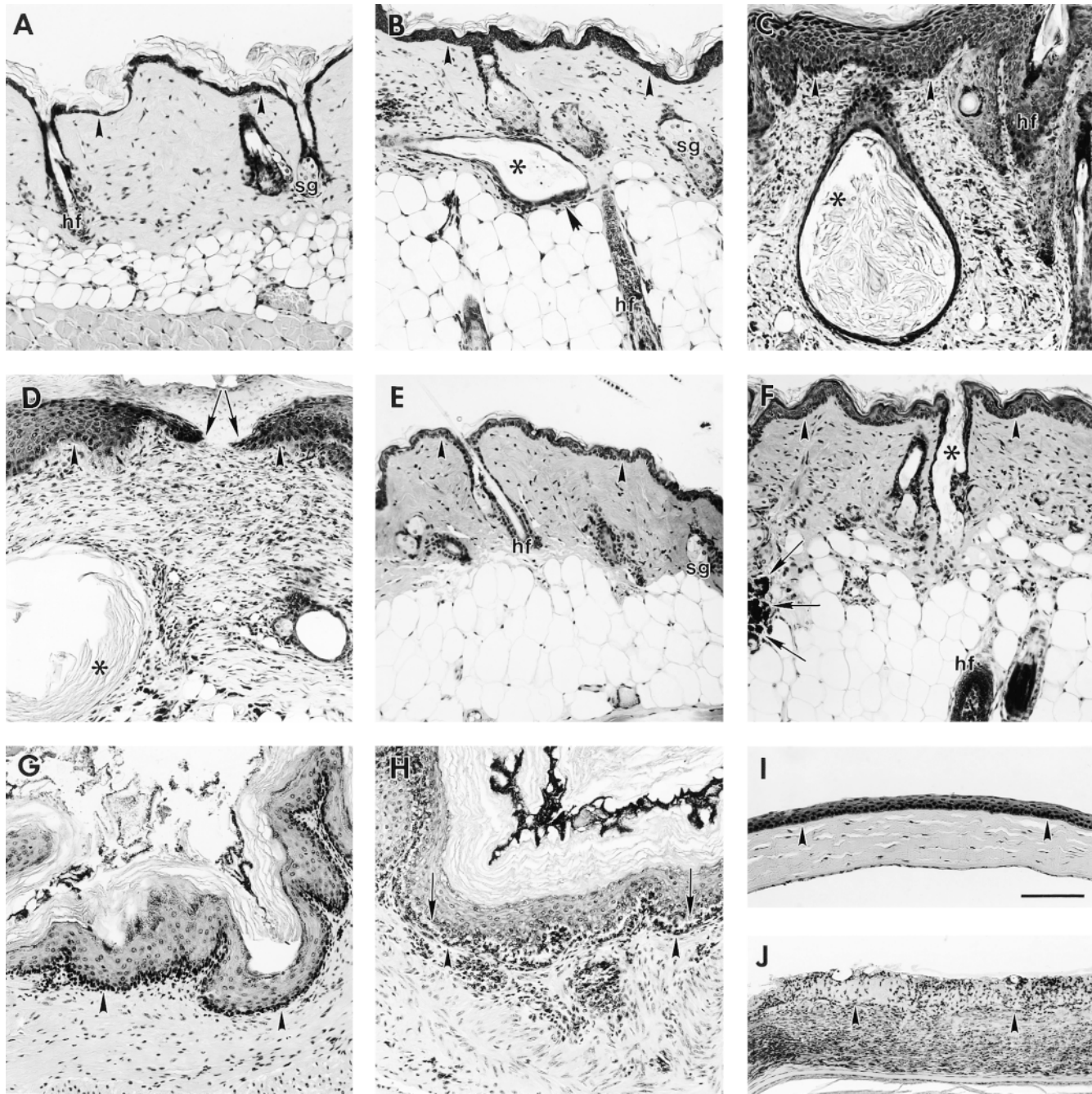


Figure 8. Light microscopy of various stratified epithelia from K16-C14 control and phenotypic K16 replacement mice. 5- μ m paraffin sections from 8-mo-old K16-C14 and K16 replacement mice were counterstained with hematoxylin and eosin (H & E). (A–D) Dorsal skin sections from a K16 replacement mouse. Hairy skin (A) has a thin epidermis and telogen staged hair follicles. Skin from a hairless region (B) features a thickened epidermis and an increase in the number of sebaceous glands. Some of the anagen staged hair follicles are improperly oriented (large arrowhead) and are missing their hair shafts (large asterisk). Skin from lesional areas (C and D) has a hyperplastic epidermis, an expansion of the outer root sheath of the hair follicles, and a large dermal infiltrate suggestive of an inflammatory response. There are also large cysts derived from pilosebaceous units (large asterisk). There are signs of migration of the epidermis into ulcerated areas of the skin (arrows in D). Dorsal skin sections from a K16-C14 replacement mouse. Hairy skin (E) was morphologically similar to K16 replacement hairy skin (A). Hairless K16-C14 skin (F) also exhibited many of the same aberrations observed in the K16 replacement hairless sample (B). There were also groups of melanocytes in the dermis not associated with hair follicles (arrows). This was also observed in K16 hairless and lesional skin (data not shown). Forestomach epithelium from a K16-C14 control (G) and a K16 replacement mouse (H). In the K16 replacement sample (H) there is extensive basal layer cytolysis along the length of the forestomach (arrows), while the K16-C14 sample features a normal morphology (G). Cornea from a K16-C14 control (I) and a K16 replacement mouse (J). The normal morphology and differentiation of the cornea is completely disrupted when compared with control (I) and there is a large dermal infiltrate in the underlying connective tissue (J). hf, hair follicle; sg, sebaceous gland. Arrowheads, indicate the junction between the stratified epithelium and the underlying connective tissue (A–D, the dermal-epidermal junction). Bar, 100 μ m.

wild-type (data not shown). Some of the older K16 replacement mice had obvious corneal opacities (data not shown). When corneal tissue from a K16 replacement mouse (Fig. 8 J) with opacities was observed under the light microscope, the normal morphology was completely absent (compare with K16-C14 control cornea in Fig. 8 I). The underlying connective tissue had a dramatic increase in the cellularity, probably due to an inflammatory response. It was also very difficult to discern basal, suprabasal, and differentiating keratinocytes in the cornea. These data clearly demonstrate that K16 expression in the basal layer of various stratified epithelia in the absence of K14 does not result in the normal differentiation and maintenance of these tissues.

Morphological Changes in K16 Replacement Hair Follicles

Hair follicles from K16 replacement and wild-type mice were examined using transmission electron microscopy to

determine if there were ultrastructural changes that might account for the hair loss observed. Outer root sheath keratinocytes from the isthmus (permanent) portion of a telogen staged wild-type hair follicle (Fig. 9 A) had a normal morphology and the hair fiber stained darkly with OsO₄, uranyl acetate, and lead citrate, as expected for wild-type hair (46). In contrast, anagen staged hair follicles from hairless K16 replacement skin (Fig. 9, B and C) exhibited severe vacuolization within the hair fiber which accounts for the improper formation and absence of hair. In addition, the outer root sheath of the follicle had been invaded by inflammatory cells (Fig. 9 B). There was also vacuolization observed in the K16 replacement keratinocytes of the outer root sheath. Hair follicles from hairless skin that did not exhibit as severe vacuolization in the hair fiber (Fig. 9 D) still featured extensive vacuolization in the keratinocytes of the outer root sheath. The vacuolization observed in the keratinocytes of the outer root sheath may be an event that precedes the improper formation of hair observed in the hairless skin from K16 replacement mice.

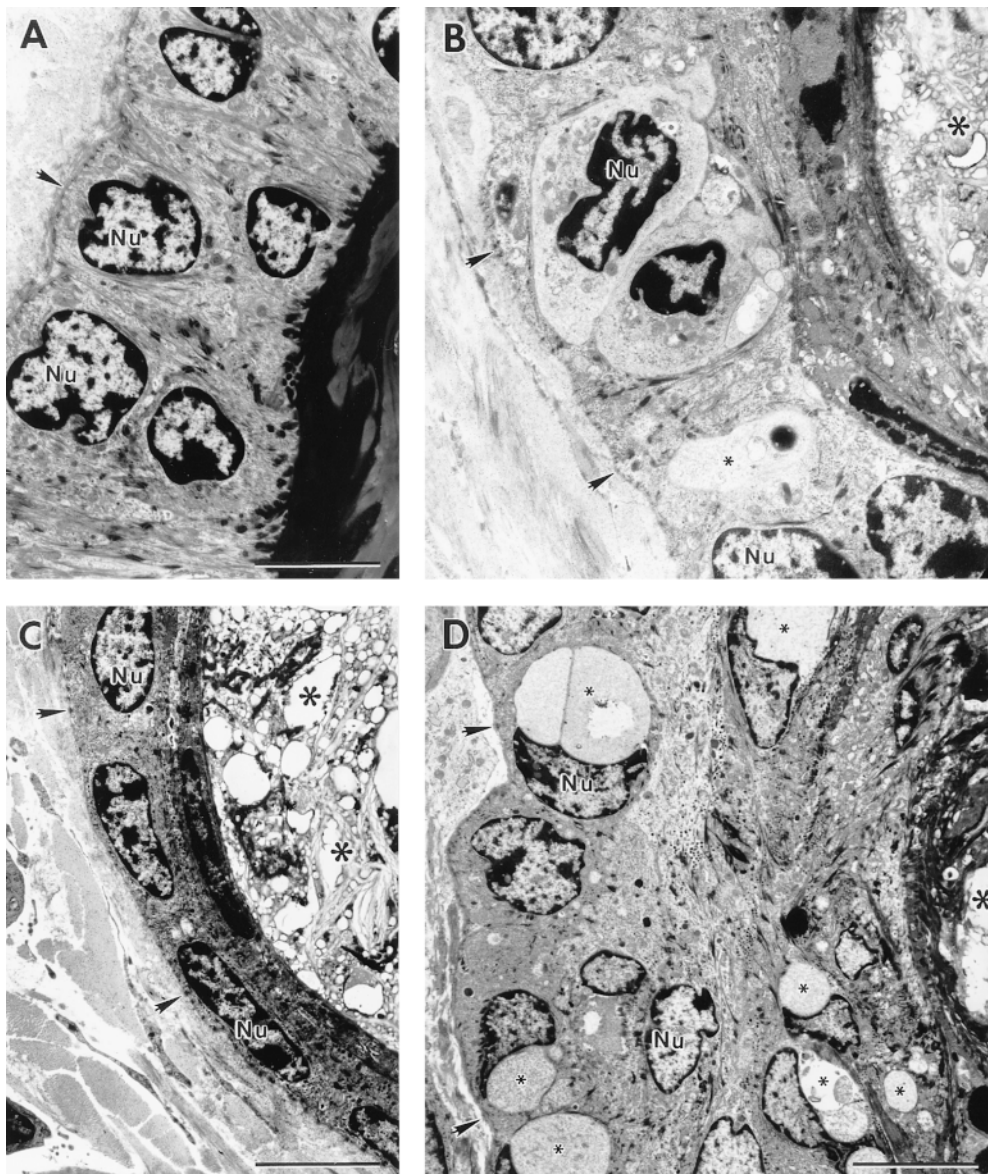


Figure 9. Transmission electron microscopy of wild-type and K16 replacement mouse hair follicles. The isthmus portion of hair follicles from wild-type control skin (A) and hairless K16 replacement skin (B–D) was analyzed. The outer root sheath of control follicles (A) has a normal morphology and the cortex stains darkly with OsO₄, uranyl acetate, and lead citrate, as expected for wild-type hair (46). In contrast to wild-type, the cortex from a K16 replacement follicle (B) is highly vacuolated (large asterisks). Vacuolization also occurs in some cells of the outer root sheath (small asterisks). Inflammatory cells have also invaded the outer root sheath (the two adjacent cells in the center). Another example (C) of the extreme vacuolization observed in the cortex of K16 replacement follicles. Preceding the major vacuolization observed in the cortex is the appearance of many keratinocytes in the outer root sheath (D) that are also vacuolated (small asterisks). Arrowheads, dermal-epidermal junction. Nu, nucleus. Bars: (A and B) 5 μ m; (C) 6.4 μ m; (D) 8 μ m.

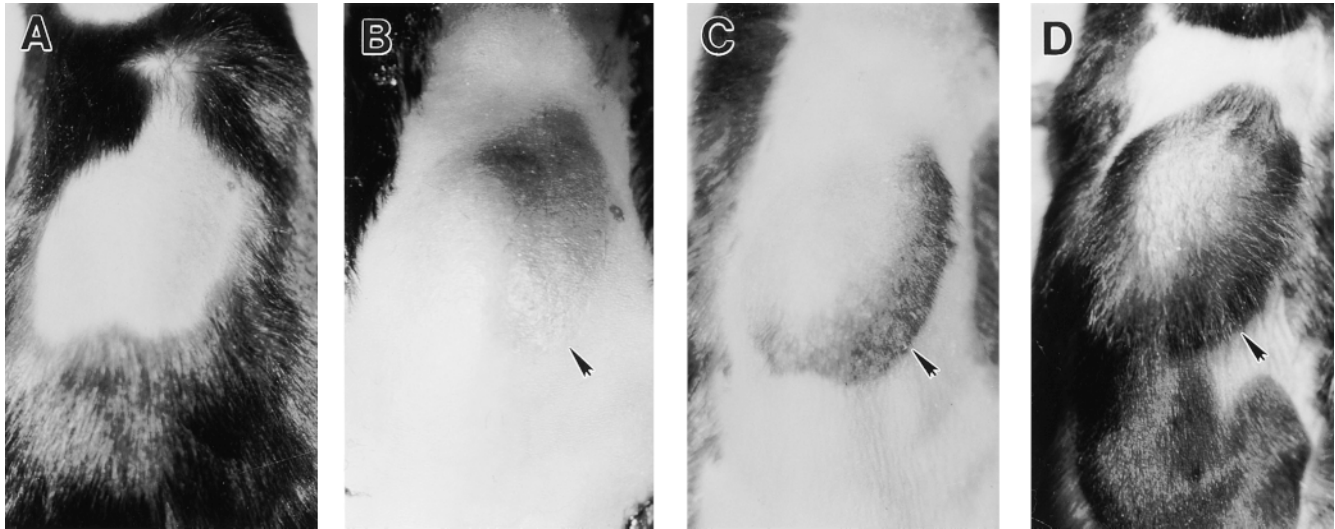


Figure 10. Hair regrowth in the K16 replacement mice. A K16 replacement mouse that featured dorsal alopecia was treated with a depilatory agent to determine if hair regrowth could occur (A). Immediately after depilation, the hairless area had a darker color than the surrounding skin (B). 5 d after treatment (C) hair regrowth in the previously hairless area was apparent while growth in the previously hairy areas had not yet occurred. 2 wk after treatment (D) hair regrowth in both areas is obvious. Arrowhead, indicates the boundary between the hairless and hairy skin areas.

Challenging the Replacement Epidermis

The electron microscopy data from hairless K16 replacement skin suggested that there were major morphological alterations in the outer root sheath of the hair follicle, the hair shaft, and in the correct cycling of the follicles. Hairless and hairy regions of K16 replacement skin were treated with a depilatory agent to remove existing hairs and to stimulate the anagen phase of the hair cycle (Fig. 10 A). Immediately after treatment the regions of skin that were previously hairless had a much darker color compared with the areas that were hairy (Fig. 10 B). As early as 2 d after treatment there were signs of hair regrowth in the region that was previously devoid of hair (data not shown). Five days after treatment with the depilatory agent hair regrowth was obvious in the previously hairless region (Fig. 10 C) while hair regrowth had not occurred in the previously hairy regions. After 2 wk (Fig. 10 D) the regrown hairs were quite long and hair growth in some of the previously hairy regions had also occurred. Hair regrowth in these regions was slower and more sporadic as some of these areas remained hairless for many weeks after the treatment (data not shown). These results clearly indicate that hair cycle progression and hair growth is aberrant in the K16 replacement mice.

Replacement skin was also subjected to mechanical trauma to determine if the epidermis was weakening with age. Skin from the three and 5-mo-old wild-type, K16, and K16-C14 replacement mice was subjected to acute mechanical trauma by tape stripping of the epidermis or to extensive mechanical trauma by vigorously rubbing the skin between a thumb and forefinger over the course of a week. Regardless of the treatment, there was no evidence of blistering in any sample as analyzed by light microscopy (data not shown). Despite the fact that alopecia and epi-

dermal ulcers occur, the K16 replacement skin is still resistant to vigorous acute and prolonged mechanical trauma.

Replacement skin was treated with PMA, a phorbol ester which stimulates hyperproliferation (56, and references therein), to determine if there were changes in the proliferative capacity of replacement basal keratinocytes as a function of age. 3-mo-old wild-type, K16, and K16-C14 mice were treated with 50 μ m PMA over the course of one week (56). The epidermides of all three samples were dramatically thickened to a comparable extent after PMA treatment and some spinous keratinocytes exhibited vacuolization (data not shown). However, there were no differences observed among the three samples. These data suggest that the ability of replacement skin to respond to the PMA treatment regimen applied is not compromised.

Discussion

K16 Does Not Fully Complement the K14 Null Phenotype: Implications for Keratin Sequence Diversity

The absence of K14 protein in the basal layer of mouse epidermis results in a lethal phenotype that resembles epidermolysis bullosa simplex (25). Neonatal mice exhibit severe blistering over their body surface and generally die early after birth. In the absence of K14, the residual keratin filament network (K5/K15) is not able to provide sufficient mechanical support to basal cells, which renders them susceptible to trauma induced rupturing (3, 25, 49). Thus, the integrity of basal cells in the epidermis is dependent on the formation of a keratin filament network containing K5 and K14. These results raise the question as to whether basal cells specifically require K14 to achieve a functional keratin filament network.

Among the type I epidermal keratins, K14, K16, and K17 share the highest amino acid identity. The identity between human K14 (472 amino acids) and K16 (473 amino acids) is 91%-86%-38% along their tripartite head-rod-tail domain structure (30, 60). Despite this high identity, they are quite distinct in both their assembly properties (40) and in their expression patterns (35, 66). Based on these contrasting facts, we wanted to determine if K16 could provide an equivalent function in the basal layer of mouse epidermis and substitute for K14 by rescuing the lethal blistering phenotype.

Expression of K16 in the basal layer of K14 null mice resulted in the rescue of the lethal skin blistering phenotype. The skin developed normally and there was no evidence of blistering at an early age. As the mice aged they exhibited extensive alopecia, developed chronic skin ulcerations in areas of repeated physical contact, and displayed morphological alterations in other stratified epithelia. Comparatively, a small percentage of replacement mice that expressed the K16-C14 chimeric protein exhibited only minor alopecia. While no obvious abnormalities were observed in the filament networks of replacement basal keratinocytes *in situ*, transmission electron microscopy of ultra-thin sections does not provide the optimal context in which to evaluate their three dimensional organization. In fact, when newborn K16 replacement keratinocytes were placed in primary culture, multiple abnormalities in the organization of their keratin filament networks were observed. Despite the fact that the K16 replacement skin is susceptible to hair loss and epidermal ulcers in areas of frequent trauma, and that an internal stratified epithelia such as the forestomach featured blistering and cytolysis of basal cells, we were unable to induce blistering in the epidermis using a variety of methods. To provide mechanical support to the tissue, keratinocytes from a particular stratified epithelium may have different mechanisms of organizing keratin filaments. These results indicate that K16 can functionally substitute for K14 to a significant, yet incomplete fashion, and that the carboxy-terminal ~105 amino acids are responsible for a significant fraction of the functional differences between these two keratins. These features are likely to be maintained in mouse K16 as the sequence identity between the orthologs is high (McGowan, K., K. Hess, and P.A. Coulombe, unpublished data, also see reference 43).

The multiple anomalies observed in the hair of the K16 replacement mice may indicate a role for keratins in hair growth and follicle cycling. Hair follicles from K16 replacement mouse skin were consistently out of phase compared with those from control mice (data not shown). K16 replacement hairless skin featured large cysts derived from pilosebaceous units and anomalies in outer root sheath keratinocytes that likely preceded the loss of hair. The treatment of hairless K16 replacement skin with a depilating agent stimulated the regrowth of hair. These observations suggest that progression through the hair cycle is partially impaired in the K16, and to a lesser extent, in the K16-C14 replacement mice. A possible explanation for these changes may reside in the outer root sheath where the transgene is expressed. The transition from telogen to anagen is initiated when a subpopulation of K14-expressing keratinocytes in the

bulge region of the outer root sheath is induced to migrate down into the dermis when stimulated by a specialized group of fibroblasts, the dermal papillae (5). It may be that in this context K16 affects the ability of these cells to promote a timely progression through the stages of the hair cycle.

Recently, a similar experiment was performed in which the human simple epithelial type I keratin K18 was introduced into the epidermis of K14 null mice (16). While K5 and K18 were able to form keratin filaments both *in vitro* and *in vivo*, the resulting network was not strong enough to withstand mechanical trauma and only partially complemented the lethal phenotype (16). Paw skin, a body site of constant mechanical friction, exhibited extensive skin blistering in the K18 replacement mice. The cytoplasm of the basal cells were devoid of keratin filaments and featured the presence of densely packed keratin aggregates, which is reminiscent of dominant negative keratin mutants in mice and in Dowling-Meara EBS patients (1, 63). In contrast, no blistering was observed in back skin where the majority of basal cells featured seemingly normal keratin filament networks that were well distributed throughout the cytoplasm and attached at desmosomes and hemidesmosomes. However, when back skin was subjected to acute mechanical trauma, the clumping and aggregation of keratin filaments along with basal cell lysis and blistering occurred (16). These data suggest that a K5/K18 filament network can form in basal cells but that it is unable to withstand normal mechanical stress and is susceptible to blistering. These results provided evidence that epidermal and simple epithelial type I keratins are not equivalent *in vivo*. While this may have been predicted considering the comparatively low sequence identity between K14 and K18 (48%) (16), it contrasts sharply with the K16 replacement mice results. These observations along with the K16 and K18 replacement mice will provide a context for examining the relationship between keratin filament organization and the susceptibility of keratinocytes to mechanical trauma.

It has been proposed that the multiplicity of keratin sequences arose from successive gene duplication events (10). Given their remarkably high sequence identity and their proximity within the type I keratin gene cluster (11, 34, 52), it is highly probable that the genes for human K14, K16, and K17 arose from a common ancestor. Our evidence strongly argues that in addition to possessing distinct mechanisms of transcriptional regulation, the genes for K14 and K16 have also evolved to provide a combination of shared and distinct functions at the protein level. These conclusions may apply to the entire family of keratin genes. Another large group of evolutionarily conserved genes, the Hox family of transcription factors, are also organized in a tandem fashion with adjacent genes sharing common functions (15, 20), suggesting that this may be a general feature of large multigene families.

Context Dependent and Independent Effects of Human K16 in Mouse Skin

To date we have produced three different types of transgenic mice that express human K16 in skin. The tissue specific overexpression of human K16 (under the control of

its own promoter) led to widespread follicular keratosis in the skin beginning one week after birth (8, 58). Aberrant keratinization in the outer root sheath of hair follicles resulted in the hyperproliferation and aberrant differentiation of the adjacent inter-follicular epidermis. The appearance of keratin filament aggregates near the nucleus and cytoplasmic areas devoid of filaments in suprabasal keratinocytes preceded the phenotypic changes observed in the skin (40). In addition, cells were hypertrophic and there were decreases in the number of desmosomes at the cell surface that correlated with blister formation.

The ectopic expression of K16 directed to the basal layer of the epidermis (using the K14 promoter) led to a phenotype consisting of scaly, wrinkled skin that lacked fur (39). The epidermis was severely thickened and hair follicle morphogenesis and hair production was delayed. Basal cells were hypertrophic, hyperproliferative, and featured keratin filament aggregation. Cell-cell adhesion was also drastically altered in the basal and suprabasal layers of the epidermis. The phenotype improved beginning ~5 wk after birth. These effects were very reminiscent of what occurs when the EGF receptor signaling pathway is activated in skin.

In contrast to the other two mouse studies, K16 expression in basal keratinocytes lacking K14 (this study) did not result in phenotypic epidermis at an early age. There was no evidence of hyperproliferation or developmental and morphological anomalies. It was not until after ~5 weeks of age that the K16 replacement mice began to exhibit alopecia and lesion formation. What becomes apparent by comparing and contrasting these three different transgenic mouse phenotypes is that the effect that K16 can have on a keratinocyte depends on many factors. These include the location within the epidermis, the differentiation status, and the keratin composition of the keratinocyte. The level to which it is expressed is also critical. The properties of K16 can be classified into context dependent and independent effects. Independent of the expression context, K16 has the ability to reorganize keratin filaments and is able to perturb the normal development and cycling of hair follicles when it is expressed in the outer root sheath. Depending on the expression context, K16 can affect cell size and cell-cell adhesion. It can also affect proliferation, development, and differentiation within the epidermis.

An exquisite example of the dramatic effects that the expression context can have on K16 function is proliferation. A recent report has stated that K16 has the ability to promote proliferation when transfected into a variety of cultured epithelial cell lines (41). It further stated that K16 can antagonize the inhibitory effect that K10 has on proliferation. Our results have provided a direct *in vivo* test of the relevance of these claims. The presence of K16 in progenitor basal cells of the epidermis (including keratinocytes of the outer root sheath) can either have a positive, negative, or neutral effect on keratinocyte proliferation (39, this study). From these data, it appears that the relationship between K16 expression and the control of keratinocyte proliferation is complex and likely indirect. This interpretation is consistent with the regulation of K16 expression at the edges of skin wounds where the induction of K16 protein does not correlate spatially or tem-

porally with enhanced proliferation in the epidermis (30, 40).

Regulation of K16 Protein Expression

We generated four different transgenic mouse lines covering a wide range of copy numbers (see Materials and Methods) that expressed the human K16 cDNA in the basal layer of the epidermis (39). K16 protein levels were different in the four lines with the highest expressing lines having three times as much K16 as the lowest expressing line. The amount of K16 expressed in the highest expressing lines was approximately equivalent to the amount of endogenous mouse K14. Within each line, mice homozygous for the transgene expressed approximately twice as much transgene as mice heterozygous for the transgene. These data suggested that basal keratinocytes have the ability to accept a wide range of K16 protein. However, there appeared to be an upper limit as two of the lines (with different transgene copy numbers) expressed to approximately equivalent levels (no. 6 and no. 21). It is likely that the levels of the type II basal keratin K5, and possibly K6 (59), determine the limit of transgene expression at the protein level.

The amount of K16 expressed in the four lines varied when bred into the K14 null background. However, there was no variation within a replacement line as either the heterozygous or homozygous genotype expressed equivalent amounts of K16 protein. In fact, the amount of K16 transgene expressed in a heterozygous replacement mouse was greater than the amount expressed in a heterozygous ectopic mouse (within the same line). These facts clearly indicate that the presence of K14 in basal cells strongly influences the amount of K16 protein that is present. Similar results were also observed with the K16-C14 replacement mice. One simple possibility that may explain these results is that K16 does not effectively compete with endogenous K14 and may turn over faster in basal cells of the epidermis and the outer root sheath. In fact, in the double replacement there was no evidence of competition or of one keratin influencing the levels of the other. Considering the structure of the two transgenes and the protein data, the region responsible for this behavior lies somewhere between the head domain and the end of the rod domain of K16. One potential biochemical determinant of this behavior may be the proline residue at position 188 (65). While this proline does not appear to influence the solubility of human K16 *in vivo*, it may still affect its stability.

Despite the huge variation in K16 protein levels in the ectopic mice, the amount of K14 protein remained constant regardless of the transgene line and the genotype (39). Even when K14 mRNA levels are increased due to hyperproliferation (this study, reference 42) or decreased twofold due to the targeted inactivation of the K14 allele (K14 hemizygous null epidermis, reference 25), K14 protein levels remained unchanged. These data imply that despite various genetic changes and biological contexts, the level of K14 protein is tightly regulated in mouse epidermis and is not affected by the presence of K16. In addition, in the absence of K14 protein, there is no mechanism that increases the amount of K15 or other type I keratin proteins in the epidermis to compensate for the absence of

K14 (25). This renders the K14 knockout distinct from the targeted disruption of the genes for K4, K10, and K18 in which there were major changes in other keratins at the mRNA or protein level (27, 37, 42).

While K14 levels are tightly regulated, the amount of K15 protein was dramatically reduced in the phenotypic ectopic mice. This is consistent with a recent report stating that the levels of human K15 mRNA are greatly reduced in hyperproliferative epidermis (64). Surprisingly, in the soluble pool of the replacement epidermis there was an increase in the levels of K15 suggesting that there may be competitive inhibition of its partitioning into the insoluble pool by K16. Therefore, there may be multiple mechanisms that limit K15 protein levels in the epidermis. Interestingly, K5 protein levels in the K14 null mice were not as dramatically reduced as might have been expected based on the decreased amounts of type I proteins and the fact that in cell culture studies, K5 protein is degraded in the absence of K14 (3, 22, 24). In fact, in the phenotypic ectopic and the double replacement mice, the amount of K5 increased correlating with an increased amount of type I keratins. These observations serve to underscore the complexity of keratin regulation in the basal layer of the epidermis.

In addition to the regulation of K16 at the protein level, there also appear to be mechanisms that regulate it at the level of the mRNA. While the amount of transgene protein doubled from the heterozygous ectopic to the homozygous ectopic mouse, the amount of mRNA did not. In fact, the steady state mRNA levels in the homozygote were much higher (greater than fourfold) than expected compared with the heterozygote based on the protein data. This may be partially accounted for by the increase in the activity of the K14 promoter. Steady state levels of K16 transgene mRNA levels also varied widely among the different replacement lines despite the fact that the protein levels were similar. Cumulatively, these data suggest that there is not a straightforward relationship between the regulation of the K16 mRNA and the expression of the protein. The various transgenic mice that we have generated may provide significant insight into the mechanisms that regulate keratin expression at the level of both the mRNA and the protein.

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