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Formation of a Normal Epidermis Supported by Increased Stability of Keratins 5 and 14 in Keratin 10 Null Mice

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The expression of distinct keratin pairs during epidermal differentiation is assumed to fulfill specific and essential cytoskeletal functions. This is supported by a great variety of genodermatoses exhibiting tissue fragility because of keratin mutations. Here, we show that the loss of K10, the most prominent epidermal protein, allowed the formation of a normal epidermis in neonatal mice without signs of fragility or wound-healing response. However, there were profound changes in the composition of suprabasal keratin filaments. K5/14 persisted suprabasally at elevated protein levels, whereas their mRNAs remained restricted to the basal keratinocytes. This indicated a novel mechanism regulating keratin turnover. Moreover, the amount of K1 was reduced. In the absence of its natural partner we observed the formation of a minor amount of novel K1/14/15 filaments as revealed by immunogold electron microscopy. We suggest that these changes maintained epidermal integrity. Furthermore, suprabasal keratinocytes contained larger keratohyalin granules similar to our previous K10T mice. A comparison of profilaggrin processing in K10T and $K10^{-/-}$ mice revealed an accumulation of filaggrin precursors in the former but not in the latter, suggesting a requirement of intact keratin filaments for the processing. The mild phenotype of $K10^{-/-}$ mice suggests that there is a considerable redundancy in the keratin gene family.

INTRODUCTION

The epidermis has become a paradigm for the understanding of intermediate filament (IF) function. Its IF cytoskeleton is formed from several combinations of type I and II keratins. K5/14/15 are expressed in the basal layer, and they become sequentially replaced by K1/2e/10 in suprabasal keratinocytes during terminal differentiation (Moll *et al.*, 1982; Fuchs and Weber, 1994). This change in keratin pattern is accompanied by an altered arrangement and a massive increase in the amount of suprabasal keratin; although basal cells present individual IF, K1/2e/10 are organized in bundles and become oriented parallel to the cell surface. This process, although not understood in molecular terms, is being taken as one indicator of a cell type–specific function of individual keratin pairs.

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Abbreviations used: EM, electron microscopy; IF, intermediate filament.

Keratins have a major function in providing stability to epithelial cells under conditions of mechanical stress. This is exemplified by cell fragility after keratin point mutations in human inherited keratin disorders, including epidermolytic hyperkeratosis (Cheng *et al.*, 1992; Rothnagel *et al.*, 1992; Yang *et al.*, 1994), and in transgenic and knockout mice expressing dominant-negative keratin subunits (Fuchs *et al.*, 1992; Bickenbach *et al.*, 1996; Porter *et al.*, 1996). Moreover, the massive cytolysis accompanying the knockout of K14 (Lloyd *et al.*, 1995), K5 (Peters *et al.*, 2001), K8/19 (Tamai *et al.*, 2000), and K18/19 (Hesse *et al.*, 2000) has demonstrated that an intact IF cytoskeleton is essential to maintain tissue integrity in the basal layer of epidermis.

Tissue integrity is normally maintained through an interaction of keratins with constituent proteins of hemidesmosomes and desmosomes (Guo *et al.*, 1995). Biochemical and yeast 2-hybrid data have provided evidence that the latter associate predominantly with type II epidermal keratins (Kouklis *et al.*, 1994; Meng *et al.*, 1997), whereas the former interact with the type I keratin 14 (Geerts *et al.*, 1999). In the upper epidermis, K1/10 become covalently cross-linked to

cornified envelope proteins such as involucrin (Ming *et al.*, 1994; Steinert and Marekov, 1995, 1997; Candi *et al.*, 1998). Despite these well-known interactions, it is not yet known in mechanical terms how mutations in keratins or their loss lead to cytolysis. Most notably, the analysis of knockout mice for K8 and 18 demonstrated that their absence did not lead to increased tissue fragility in internal epithelia (Baribault *et al.*, 1994; Magin *et al.*, 1998). On the other hand, the expression of dominant-negative keratin 18 and the ablation of keratins 8/19 and 18/19 (Hesse *et al.*, 2000; Tamai *et al.*, 2000) were accompanied by tissue disease. Collectively, these data raise the issue of whether all keratins exert a purely structural function and how much keratin per cell is required to provide mechanical stability.

Previously, we have generated knockout mice expressing a deletion mutant of K10 (K10T), which represented a good model for epidermolytic hyperkeratosis (Porter *et al.*, 1996; Reichelt *et al.*, 1997). In those mice, the truncated K10 acted in a dominant-negative way and led to the accumulation of large keratin aggregates, followed by cytolysis of suprabasal keratinocytes, a strong induction of K6/16, and the perinatal death of homozygous mice. Now we have generated $K10^{-/-}$ mice to analyze the contribution of suprabasal keratins to the stability of the upper epidermis. We report that despite the absence of K10, which is the single most prominent protein in epidermis (Fuchs and Weber, 1994), $K10^{-/-}$ mice are viable and do not suffer from tissue fragility. Our data represent the first comparison of a dominant-negative keratin mutant with a complete knockout of the same keratin, generated by homologous recombination, revealing that both have completely different consequences for the stability of the concerned tissue.

MATERIALS AND METHODS

Generation of the Knockout

The 5′ flank was a 1.6-kb (–2015 to –414) fragment and the 3′ flank was a 4.3-kb *Eco*RI–*Bam*HI (1595 to ~5900) fragment of the mouse K10 gene. Both were derived from a 129 SVJ mouse genomic library (λ FIX II library; Stratagene, Heidelberg, Germany). The sequence of the K10 locus from –2578 to + 2524 was submitted to GenBank (accession no. AF245658). A lox P-flanked 2.7-kb phosphoglycerate kinase promoter-driven HPRT minigene (Porter *et al.*, 1996) replaced 414 bp of the promoter region and exons 1 and 2 of the K10 gene (–414 to –1595). The construct was cloned in Bpt SKII+ (Stratagene) and electroporated into HM-1 cells (Magin *et al.*, 1998). After PCR, positive clones were verified by Southern blotting, with the use of 5′ and 3′ probes as well as an HPRT probe. The targeting frequency was 8%. Correctly targeted embryonic stem cells were injected into blastocysts of BALB/c mice and returned to CBA recipients. Offspring of transgenic chimaeras were mated to BALB/c mice for further analysis. Additionally, some mice were backcrossed to yield a 129/Ola background.

Immunofluorescence and Electron Microscopy

For conventional electron microscopy (EM) of skin samples, see Bussow (1978) and Porter *et al.* (1996). For processing of cryosections, see Reichelt *et al.* (1999). Primary antibodies were anti-K6 (693-1), 1:1000; anti-K10 (LH2), undiluted; anti-K5 and anti-K1 (AF138 and AF109; Babco, Richmond, CA), 1:5000; anti-K15 serum, 1:200; and anti-K17 serum, 1:1000 (McGowan and Coulombe, 1998b). Secondary antibodies were Texas Red–coupled goat anti-mouse immunoglobulin G1 (Southern Biotechnology Associates, Birmingham, AL) and Alexa 594-coupled goat anti-rabbit (Molecular Probes, Eugene, OR). For immunogold EM, 4-μm sections on coverslips were fixed for 10 min with acetone at –20°C, permeabilized with 0.3% Triton-X 100, and after a short rinse with PBS, incubated for 2 h with antibodies against K1 (8.60; Sigma, Deisenhofen, Germany; 1:5000) and against K14 (guinea pig serum, 1:1000). After 3 washes with PBS, sections were incubated overnight with secondary antibodies coupled to 5- or 10-nm gold particles for double staining and with nanogold-coupled antibodies for single staining. Silver enhancement for the nanogold probes and fixation and embedding in Epon were carried out as described previously (Rose *et al.*, 1995).

Northern Blotting and In Situ Hybridization

For Northern blot analysis, trunk skin was obtained from neonatal mice and immediately frozen in liquid nitrogen. RNA was isolated with TRIzol (Life Technologies, Karlsruhe, Germany). Fifteen micrograms of RNA were loaded per lane. For processing of gels and hybridization, see Reichelt *et al.* (1999). Probes for mouse K1, 5, 10, and 14 were derived from the 3′-noncoding regions (K5, laboratory of T.M.M.; K1, 10, and 14, kind gifts from H. Winter, German Cancer Research Centre, Heidelberg, Germany). Quantitative analysis was performed with Image Master VDS software (Amersham Pharmacia Biotech, Freiburg, Germany). The ribosomal RNA from ethidium bromide–stained gels was compared with that of the mRNA from the respective autoradiographs.

In situ hybridization was performed with the use of RNA probes derived from 3′-noncoding sequences from K5 and 14. Probes were labeled with biotin-16-UTP (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions (RNA polymerases and ribonuclease inhibitor, Fermentas, St. Leon-Rot, Germany). Five-micrometer cryosections of neonatal back skin were placed on Superfrost slides (Menzel-Gläser, Braunschweig, Germany), air dried, and fixed with 4% paraformaldehyde (in PBS) for 20 min. Sections were washed 2 times for 5 min each with PBS and then blocked for 10 min with 0.1 M triethanolamine (Sigma; 2.7 ml triethanolamine, 200 ml double-distilled water, 0.33 ml HCl, and 533 μl acetic anhydride) followed by 2 washes with PBS for 5 min each. Prehybridization was performed with 50 μl of hybridization solution (0.3 M NaCl, 5 mM EDTA, 20 mM Na-phosphate, 20 mM Tris, pH 6.8, 50% deionized formamide [ultrapure, Merck, Darmstadt, Germany], 5% dextran sulfate, 1× Denhardt's, 10 mM DTT, 0.5 mg/ml yeast tRNA, and 100 μg/ml salmon sperm DNA) per section. After 1 h at 42°C, hybridization solution was replaced by 25 μl of fresh hybridization solution containing 250 ng biotin-labeled probe. A coverslip was placed on top, and the probes were heated for 5 min at 90°C before they were allowed to hybridize for 16 h at 42°C. The sections were then washed briefly with 2× SSC (prepared from a 20× stock: 3 M NaCl and 0.3 M Na citrate, pH 7.0) until the coverslips had come off, and then 30 min with 2× SSC, 50% formamide, and 20 mM DTT and another 30 min with 1× SSC, 50% formamide, and 20 mM DTT both at 50°C, followed by a 5-min wash with 1× SSC and 0.1% SDS at ambient temperature. Sections were incubated for 30 min at 37°C with 1× SSC containing 20 μg/ml RNase A (Roche Molecular Biochemicals) and afterward were washed for 30 min with 0.5× SSC, 50% formamide, and 20 mM DTT at 50°C. The slides were then washed 2 times with PBS for 5 min at ambient temperature and incubated for 30 min with streptavidine-alkaline phosphatase (Dako, Hamburg, Germany). After that, they were washed 2 times with PBS as before and incubated for 5 min in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$). Finally, 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium substrate solution (Dako) was added. After 30 min, the reaction was terminated by washing with double-distilled water, and sections were embedded in Mowiol (Calbiochem, Schwalbach, Germany).

Two-dimensional Gel Electrophoresis and Western Blotting

Trunk skin was prepared from neonatal mice and immediately frozen in liquid nitrogen. For SDS-PAGE, total protein extraction, separation and electrotransfer, see (Reichelt *et al.*, 1999). For 2-dimensional gel electrophoresis of keratin complexes, protein extracts enriched in cytoskeletal proteins were prepared from neonatal trunk skin (Hatzfeld and Franke, 1985). The resulting pellet was resuspended in isoelectric focusing sample buffer as before (Magin *et al.*, 1998), except that 5.5 M urea was used. This extract was dialyzed with the use of collodion bags (Sartorius, Göttingen, Germany) to urea concentrations up to 9.5 M. The samples were first separated by isoelectric focusing at the respective urea concentration and subsequently by SDS-PAGE. Western blotting was performed as described before (Reichelt *et al.*, 1999). The composition of ampholines was 0.8% of pH 4–6 and pH 5–7 and 0.4% of pH 3–10 (Amersham Pharmacia Biotech). Primary antibodies were diluted as follows: anti-K10 (LH 2), 1:1000; anti-K1 (AF109), 1:400,000; anti-K5 (AF138), 1:100,000; anti-K6, 1:10,000; anti-K15, 1:5000; anti-K17, 1:1000; anti-K 14, 1:50,000; and anti-filaggrin (AF111), 1:10,000 (AF109, AF138, and AF111, Babco). Before application of antibodies, polyvinylidene difluoride membranes were stained with Coomassie blue and photographed.

Dye Penetration Assay

The assay was used before by Hardman *et al.* (1998) on mouse embryos. Neonatal mice were killed by ether inhalation and then taken up and down a methanol series (25, 50, 75, and 100% methanol, 1 min each), equilibrated for 1 min in PBS, and subsequently stained with 0.2% toluidine blue (in water) for 5 min. Mice were rinsed 3 times for 1 min in 90% ethanol. After a brief wash in water, they were embedded in 0.4% agarose and immediately photographed.

RESULTS

Compensation by Basal Keratins 5, 14, and 15 Prevents Cytolysis and Permits Normal Epidermal Differentiation in K10$^{-/-}$ Mice

In accordance with their well-documented cytoskeletal function, keratins 10 and 1 are the most abundant proteins in epidermis, representing ~60% of total protein (Fuchs and Green, 1980). In accordance with previous K10 transgenic and knockout mice (Fuchs *et al.*, 1992; Bickenbach *et al.*, 1996), we expected neonates to display a fragile epidermis. Surprisingly, all K10$^{-/-}$ mice analyzed so far were born at the expected Mendelian ratio, were fully viable, and showed no obvious skin defect resulting from birth stress. Histological analysis of neonatal skin revealed normal stratification and an unaltered number of epidermal cell layers, although we noticed a slight increase in granular cell size and the size of keratohyalin granules and a loose appearance of the stratum corneum (Figure 1B). Most importantly, we detected no cytolysis in K10$^{-/-}$ epidermis, suggesting that filaments consisting of K10 and 1 are not essential for maintaining the integrity of suprabasal epidermis in these mice.

Next, we performed immunofluorescence (Figures 2 and 3), Northern blotting (Figure 4), and Western blotting (Figure 5), all confirming the absence of K10 in all epidermal strata of the knockout mice. The distribution of K1 was unaltered, although immunofluorescence (Figure 2) and Western blotting demonstrated a noticeable reduction in homozygotes (Figure 5A) and a milder reduction in het-

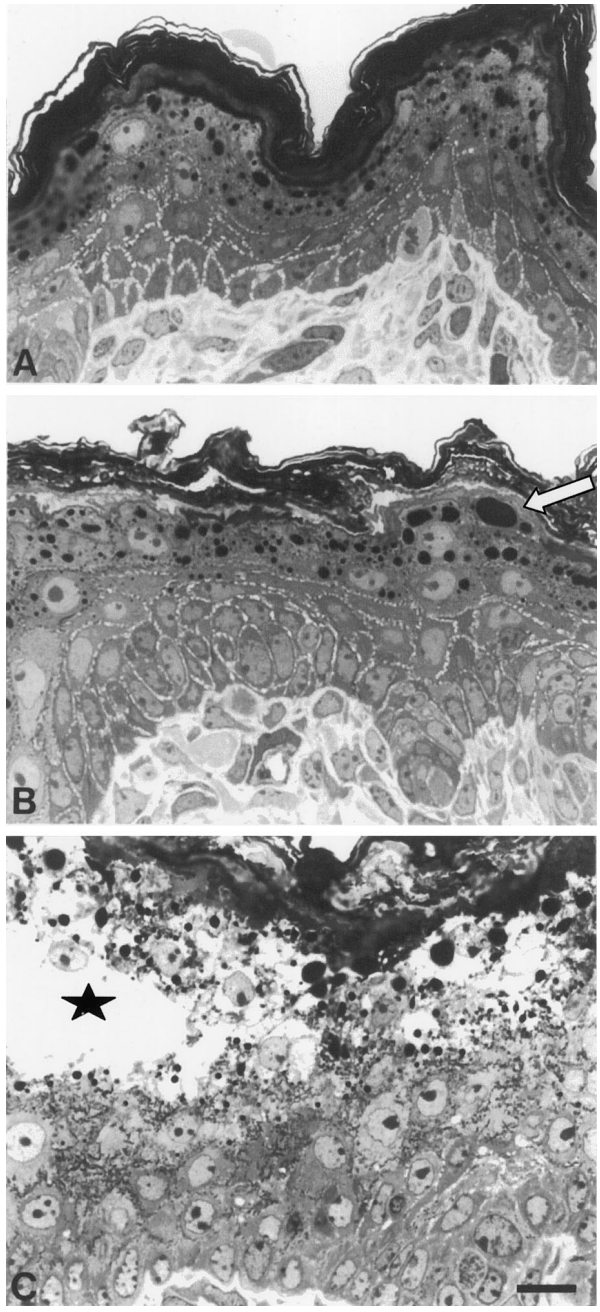

Figure 1. Semithin sections of mouse back skin. Comparison of K10$^{-/-}$ (B) with wild-type (A) mice revealed a normal differentiation in the K10$^{-/-}$ animal. In the granular layer of K10$^{-/-}$ mice, cell size was increased, but the number of cell layers was identical in both genotypes. We noted an increase in the size of keratohyalin granules (B, arrow) in K10$^{-/-}$ mice. The stratum corneum appeared irregular compared with that of the wild-type mice. In contrast to homozygous neonatal K10T mice (C, asterisk), which carry the dominant-negatively acting K10T, the K10$^{-/-}$ epidermis did not show any sign of cytolysis (B). In K10T skin the massive suprabasal accumulation of keratin aggregates was clearly visible (C, dark matter), whereas K10$^{-/-}$ keratinocytes exhibited a clear cytoplasm (B). Bar, 10 μm.

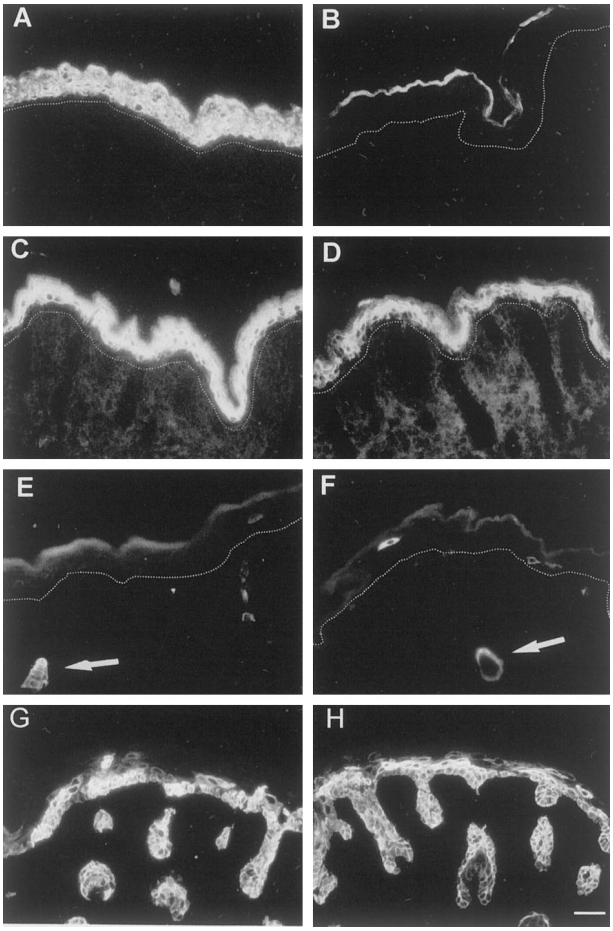

Figure 2. Immunofluorescence analysis of suprabasal keratins. The loss of K10 in the knockout mice (B) was confirmed (A, wild-type) and the suprabasal K1 expression was clearly reduced in in the $K10^{-/-}$ mice (D) compared with the wild-type mice (C). K6 expression was not induced in $K10^{-/-}$ (F) but showed the normal expression pattern of wild-type skin in hair follicles (E and F, arrow) as well as in rare single interfollicular cells. K17 expression was patchy in both wild-type (G) and $K10^{-/-}$ (H) epidermis. The dotted line in A–F marks the basal membrane, ignoring hair follicles. Bar, 64 μm.

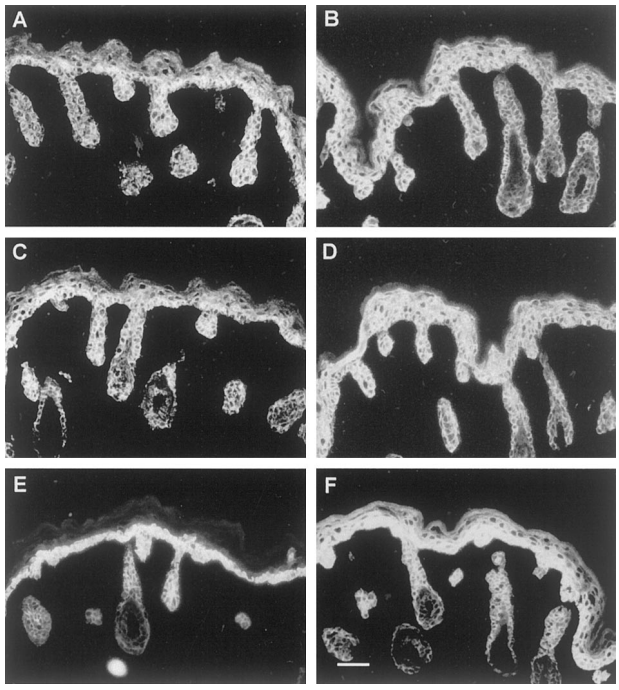

Figure 3. Immunofluorescence analysis of basal keratins. We noted a suprabasal increase in the basal cell keratins K5 (A and B), K14 (C and D), and K15 (E and F). In the wild-type mice, these keratins are predominantly found in the basal layer (A, C, and E), whereas the knockout had a significant suprabasal persistence of these keratins (B, D, and F). Bar, 64 μm.

erozygotes (Figure 5A), in agreement with immunofluorescence data (see Figure 2). This prompted us to investigate whether K1 remained as a single keratin or formed complexes with another partner in vivo. To investigate whether other keratins or IF proteins were expressed in $K10^{-/-}$ mice as a means to stabilize the epidermis, we examined the expression patterns of potential candidate skin keratins (K2e, 5, 6, 9, 14, 15, 16, and 17), of keratins specific for internal epithelia (K8, 18, and 19) and of vimentin by immunofluorescence staining. Most of these IF proteins were either not expressed or not up-regulated (our unpublished data). Surprisingly, the distribution of K5, 14, and 15 was altered in the epidermis of $K10^{-/-}$ mice. In comparison with normal epidermis, $K10^{-/-}$ mice showed an increased staining throughout the suprabasal epidermis up to the uppermost granular layer (Figure 3). As judged by immunofluorescence, all 3 keratins were expressed at similar levels. Western blotting, however, revealed an increase in K14 but not in K5 or 15 (Figure 5B). This result suggested that the synthesis of various epidermal keratins is regulated individually. To get an insight into at which level the expression of keratins was regulated, we performed Northern blotting. This demonstrated that the mRNAs of K1 and 5 remained unaltered, whereas that of K14 was increased (Figure 4A). Quantitative analysis of the mRNA levels showed that the increase in K14 was ~36%, whereas the amount of K1 and 5 remained unaltered. Remarkably, neither K14 nor K5 mRNA was expressed suprabasally but remained exclusively restricted to the basal epidermal layer, as indicated by in situ hybridization (Figure 4B). At present, our data do not allow us to discriminate whether the increase in K14 mRNA results from increased transcription or increased stability. The persistent suprabasal expression of the K14 protein suggests that the half-life of K14 is increased in $K10^{-/-}$ mice.

The induction of K6, 16, and 17 in interfollicular epidermis is taken as one of the most sensitive indicators of an altered epidermal differentiation program as in hyperproliferation or wound healing (Coulombe, 1997; McGowan and Coulombe, 1998a). Remarkably, antibody staining revealed no differences between wild-type and $K10^{-/-}$ littermates as shown for K6 and 17 (Figure 2). These findings imply that the presence of keratins 5 and 14 in suprabasal cells as well as K15 and 17 are able to compensate for the absence of K1/10 IF in neonatal $K10^{-/-}$ mice. We cannot exclude that

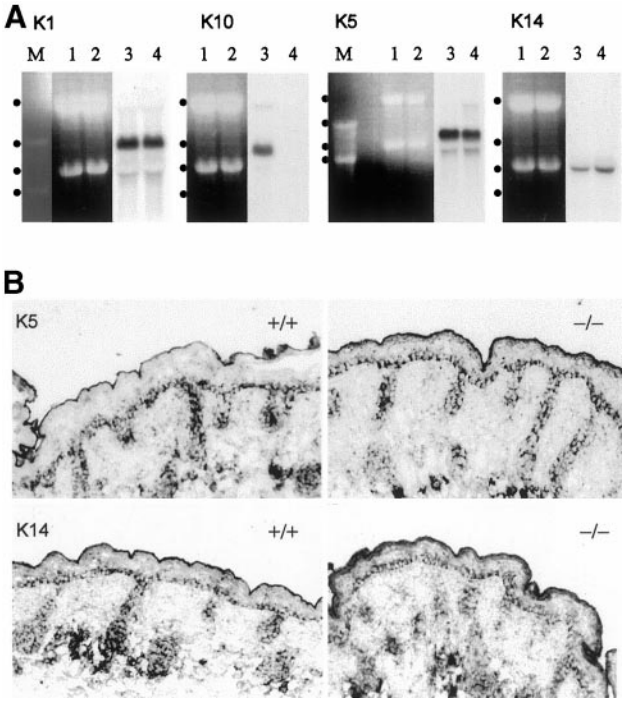

Figure 4. K14 mRNA is increased, but it remains restricted to basal cells. (A) Northern blot analysis revealed that K1 and 5 expression were unaltered in K10 knockout mice (−/−), whereas K14 mRNA was increased by 36%. In addition, the complete loss of K10 in the knockout mice was confirmed. Quantitation was performed by densitometric measurement. Lanes M, marker; lanes 1 and 2, ribosomal RNA; lanes 3 and 4, corresponding autoradiograph; dots from top to bottom, 4.4, 2.9, 1.9, and 1.5 kb. Northern blots for K1, 10, and 14 were derived from the same gel. (B) In situ hybridization showed that in $K10^{-/-}$ epidermis, both K5 and 14 mRNA remained restricted to basal keratinocytes.

other, yet unknown mechanisms contribute to the integrity of the epidermis in $K10^{-/-}$ mice.

Ultrastructural and Biochemical Analysis Reveal Unexpected Properties of Epidermal Keratins

Normally, the change in keratin expression from basal keratins 5 and 14 to those indicating terminal differentiation, i.e., K1 and 10, is accompanied by a reorganization from a loosely bundled array of individual filaments in basal cells to a bundling of IF resulting in the "keratin pattern" typical of epidermis (see Figure 7D). EM revealed the presence of IF in suprabasal epidermis of $K10^{-/-}$ mice (Figure 6C) and normal desmosomes (our unpublished data). The former were very similar to wild-type IF (Figure 6A) with regard to their distribution but also exhibited the bundling otherwise typical of K1/10, although they predominantly consisted of K5/14 IF (Figure 6, B and D). Apart from normal filaments, we detected small, isolated keratin aggregates in rare granular cells (Figure 6D, arrow), which resembled those large abundant aggregates typical of those previously described in K10T mice (Figure 6, E and F). We suspect that in $K10^{-/-}$ mice, these consisted of residual K1, which might either assemble into novel IF with the type I keratin 14 or form aggregates on its own. Immunogold EM with the use of antibodies directed against K1 and 14 confirmed the rare occurrence of suprabasal filament bundles, which contained both keratins (Figure 7, A–C). Additionally, we found that these filaments were able to attach to desmosomes (Figure 7B). K1/14 filaments were never detected in wild-type epidermis. They constituted only a small amount of the suprabasal IF network in $K10^{-/-}$ mice, indicating that the major part consisted of K5/14 filaments.

Previous data have shown that the stability of individual keratins depended on the expression of a partner keratin (Kulesh *et al.*, 1989; Lersch *et al.*, 1989). Given the persistence of K1 as demonstrated by IF and Western and Northern blotting, we examined whether K1 might become stabilized in the suprabasal epidermis by complex formation with the suprabasally increased K14. To that end, cytoskeletal keratin preparations were isolated from wild-type and $K10^{-/-}$ mice and dissolved in 4 M urea, which has been demonstrated to maintain oligomeric building blocks of keratins (Franke *et al.*, 1983; Hatzfeld and Franke, 1985). Dialysis of in vivo keratin complexes against increasing concentrations of urea leads to the dissociation of individual keratin complexes. At appropriate urea concentrations, keratin complexes can be visualized by isoelectric focusing followed by SDS-gel electrophoresis (Franke *et al.*, 1983; Hatzfeld and Franke, 1985). In accordance with previous data (Hatzfeld and Franke, 1985; Coulombe and Fuchs, 1990), K1, 5, 10, and 14 migrated in their authentic complex at 5.5 M urea in wild-type samples (Figure 8A). At higher urea concentrations, complexes dissolved, and keratins migrated to their individual isoelectric points (Figure 8B). In extracts from $K10^{-/-}$ mouse skin, most of K1 had already moved to its isoelectric point at 5.5 M urea, indicating the absence of its type II keratin partner K10 (Figure 8C). A minor portion, however, resided in a complex position together with K14 (white arrow). This indicates that in the absence of its natural partner, K1 can in part form novel heteromeric keratin complexes. These biochemical data are in agreement with immunogold EM findings and support the classical "promiscuity" concept of keratins (Hatzfeld and Franke, 1985).

Altered Processing of Profilaggrin in K10T but Not in $K10^{-/-}$ Mice

In contrast to the strong increase in keratohyalin, which we previously described in our K10T mice (Porter *et al.*, 1996), $K10^{-/-}$ mice showed only a mild increase in the size of granules in the stratum granulosum. Although Western blot analysis revealed a normal amount of profilaggrin and filaggrin, similar to that of wild-type mice (Figure 9B), K10T mice showed an impairment of profilaggrin processing and an accumulation of the major filaggrin precursors (Figure 9B). As judged by its size, profilaggrin is processed down to the penultimate stage, i.e., a dimer, but is not cleaved to the monomer stage. This is compatible with a model suggesting that the final proteolytic cleavage requires the presence of intact keratin filaments.

Barrier Formation in $K10^{-/-}$ Mice

Finally, we examined whether $K10^{-/-}$ mice formed a normal epidermal barrier with the use of an assay described

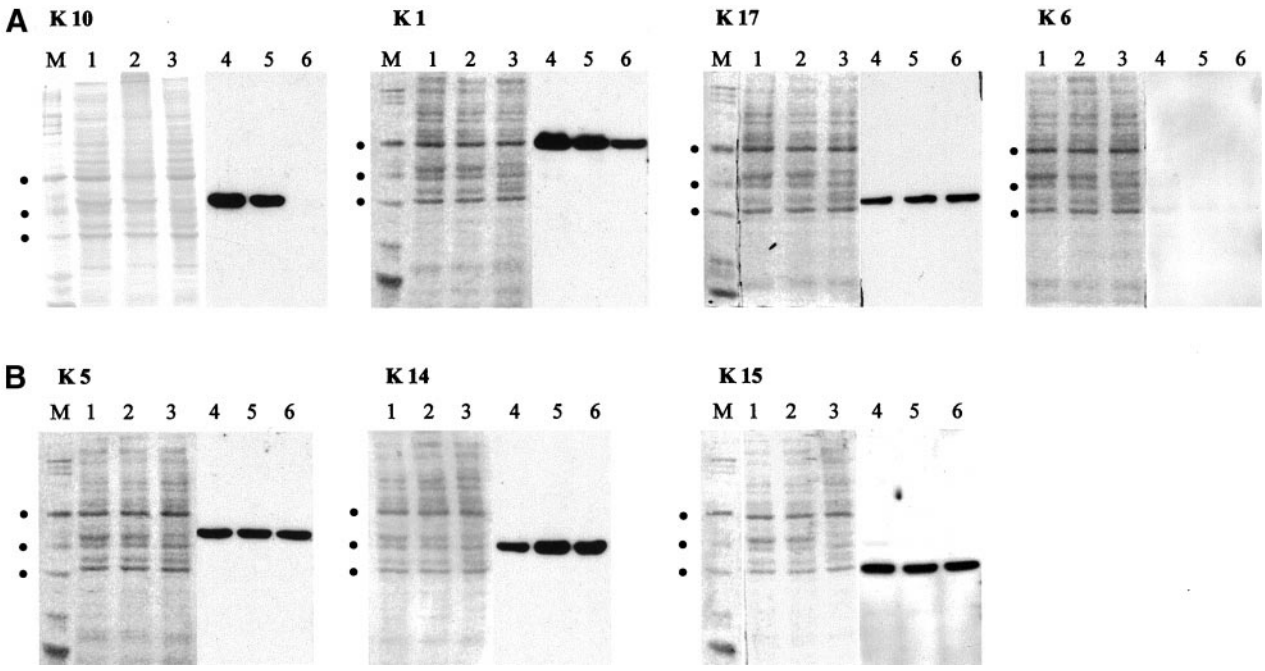

Figure 5. Western blot analysis of epidermal keratins. (A) The loss of K10 expression in $K10^{-/-}$ epidermis was confirmed by Western blotting, and its reduced expression in heterozygote skin (+/–) was shown. Note a remarkable decrease in K1 in $K10^{-/-}$ pups and a slight decrease in the skin of heterozygotes. (B) The amount of K5 remained unaltered, whereas its partner K14 was slightly increased. The amount of the third basal keratin, K15, was unaltered. Equal loading was verified by quantitative comparison of Coomassie blue staining. Lanes 1–3, Coomassie blue–stained polyvinylidene difluoride membrane; lanes 4–6, corresponding Western blot; M, marker; dots from top to bottom, 66.4, 55.6, and 42.7 kDa.

before (Hardman *et al.*, 1998). When homozygous K10T mice (Porter *et al.*, 1996) were analyzed, the previously reported barrier defect (Reichelt *et al.*, 1999) was confirmed here in a dye penetration assay (Figure 10C). The epidermis showed multiple lesions at different sites and was highly susceptible to mechanical trauma. In contrast, $K10^{-/-}$ mice displayed a well-developed epidermal barrier all over the body, with the exception of the forepaw sole (Figure 10B). Here, a transient barrier disruption became apparent. After 2–3 d, however, the barrier recovered and did not seem to retard the normal development of the pups.

DISCUSSION

How Little Keratin Is Enough to Maintain an Intact Epidermis?

In conjunction with previously established gene-targeted mice expressing a dominant-negative K10 mutation (K10T; Porter *et al.*, 1996), to our knowledge, the present study provides the first comparison with the use of gene targeting of partial and complete IF protein deletions in the same tissue. The observation that $K10^{-/-}$ mice survived after birth and displayed a normal epidermis with all the hallmarks of terminal differentiation challenges some established beliefs and may be of significance for the treatment of dominant skin disorders, including epidermolytic hyperkeratosis.

Most importantly, $K10^{-/-}$ mice exhibited an epidermis stable enough to withstand the mechanical demands of birth and the stress exerted by the mother's care. This was obviously due to the suprabasally extended expression of K5/14/15, although being less abundant than K1/K10 filaments normally would be. Of note, K6, 16 and 17, three of the most sensitive indicators of a disturbed epidermal homeostasis (Weiss *et al.*, 1984; McGowan and Coulombe, 1998a), were not induced in our mice. Because these keratins are otherwise detected in the interfollicular epidermis after wounding or in hyperproliferative conditions, their regular expression pattern in $K10^{-/-}$ neonates suggests a normal state of the epidermis. Although McGowan and Coulombe (1998a) described K17 expression as absent from the interfollicular epidermis, our mice showed patchy expression all over the epidermis. Because their mice had a genetic backgound different from those used in this study, we suggest that strain-specific differences account for the distinct K17 expression patterns.

Taking into consideration that no other keratins were induced and that epidermal thickness remained unaltered in $K10^{-/-}$ mice, we estimate that the amount of keratin per cell was reduced by more than half. Preliminary data indicate that this amount is also sufficient to sustain an intact, although slightly hyperkeratotic, epidermis in adult $K10^{-/-}$ animals. Our finding that the loss of K10 is not accompanied by extensive tissue damage is supported by the recent description of a K14 patient who represents the closest to a human knockout.

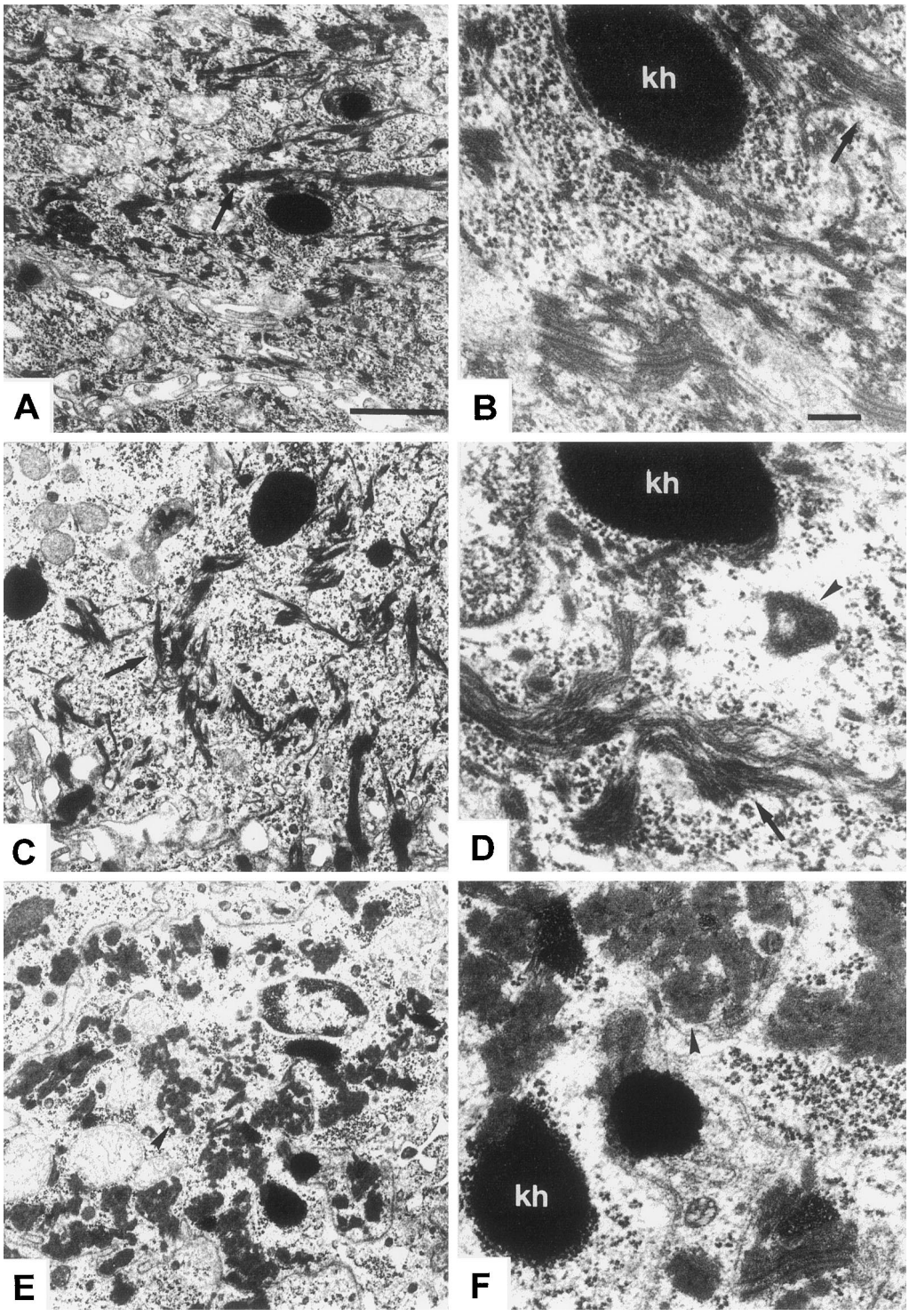

Figure 6. Ultrastructure of K10$^{-/-}$ epidermis. The granular layer of K10$^{-/-}$ epidermis maintained the typical content and distribution of IF bundles (C, arrow, survey EM). Higher magnification shows the regular shape of these filament bundles (D, arrow). For comparison, see survey micrograph of the wild type in A and a higher magnification in B (arrows on filament bundles) and the completely different setting in K10T mice, where the cytoplasm of granular layer cells was filled with a large amount of keratin aggregates (E, arrows, survey; F, details). The bundling otherwise typical of K1/10 was also noted for K5/14 in granular cells. Interestingly, in a few cells, we noted small keratin aggregates in the knockout epidermis (D, arrowhead), which were absent in wild-type littermates. These small aggregates closely resembled the aggregates observed in K10T mice (E and F, arrowheads). There were no signs of epidermal cytolysis in K10$^{-/-}$ neonates. Bars: A (also valid for C and E), 1 μm; B (also valid for D and F), 0.25 μm; kh, keratohyalin.

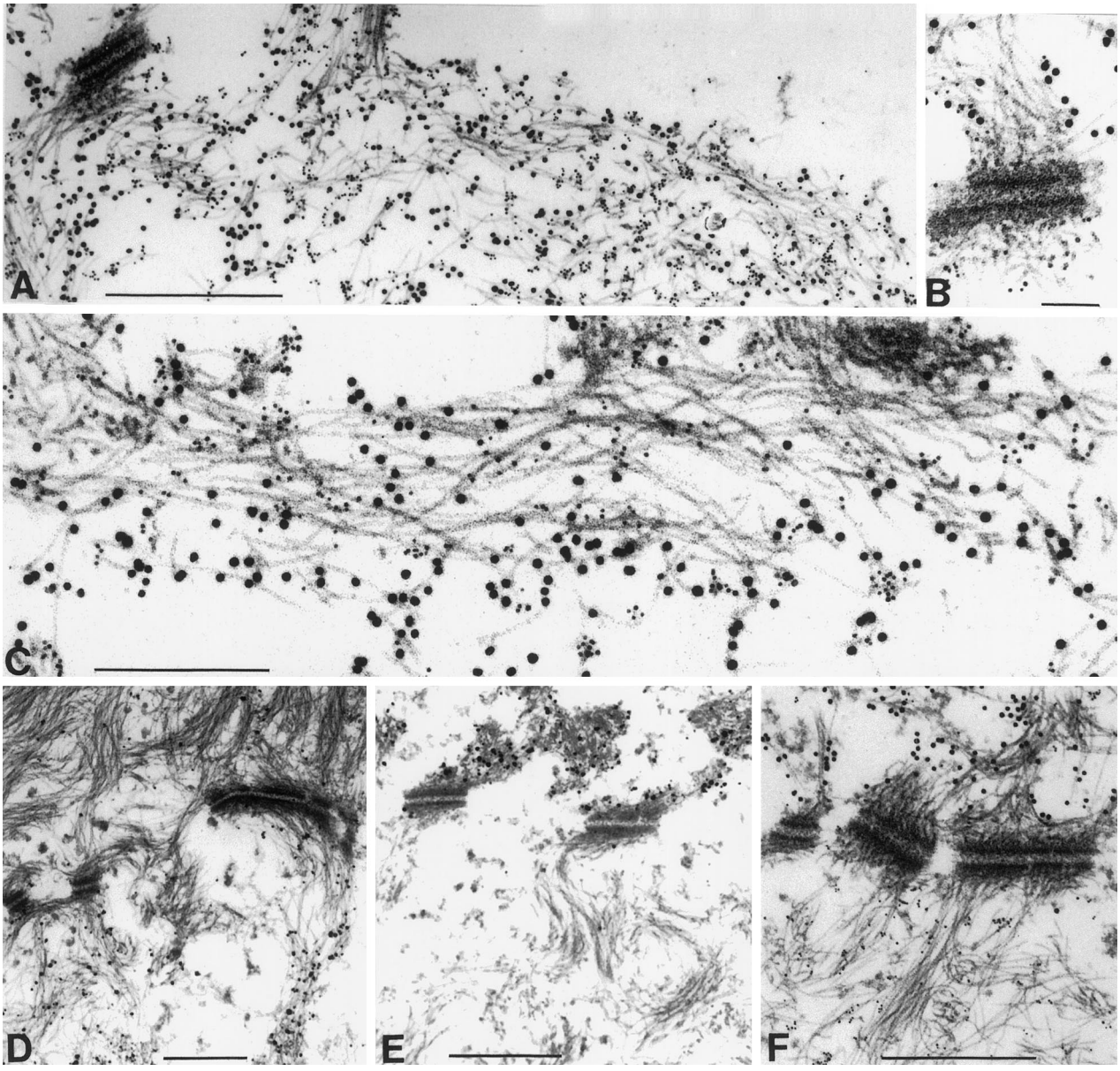

Figure 7. Immunogold EM revealed the formation of K1/K14 filaments in $K10^{-/-}$ mice. (A–C) IFs in the granular layer of $K10^{-/-}$ epidermis. (D–F) Basal–suprabasal transition zone of wild-type epidermis, with the basal cell at the bottom. Desmosomes mark the level of the cell membranes, which have been lost upon fixation. (A) Survey of keratin bundles, which were labeled with both K1 and 14 antibodies. Filaments that were composed of K1 and 14 were also found attached to desmosomes (B). (C) Higher magnification of a filament bundle showing that K1 and 14 were in close proximity in the filaments. In single-antibody–labeling experiments in wild-type skin, K14 was detected in the basal epidermal layer (D), whereas K1 was exclusively found in subrabasal cells (E). This was confirmed in a double-labeling experiment with both antibodies (F). K1, 10-nm gold particles; K14, 5-nm gold particles. Bars: A and D–F, 0.5 μm; B, 0.1 μm; C, 0.24 μm.

This patient had a mild recessive form of epidermolysis bullosa simplex, probably caused by compensation of K14 by K15 (Batta *et al.*, 2000). Collectively, results from K10 (this study), K18 (Magin *et al.*, 1998), and K19 (Tamai *et al.*, 2000) knockout mice and from the above-described patient demonstrate that the loss of a given keratin is far less detrimental than the expression of a mutant one, provided that compensation by another keratin of the same subfamily can take place. If this fails, loss of the keratin cytoskeleton prevails, leading to extensive tissue damage (Hesse *et al.*, 2000; Peters *et al.*, 2001).

How Specific Are Keratins in their Constituent Compartments?

Although our replacement experiment is one of nature, arguing that the K5/14 pair can complement K1/10 in supra-

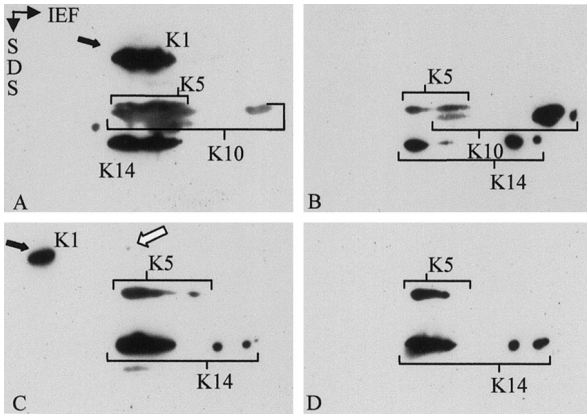

Figure 8. Two-dimensional gel electrophoresis of keratin complexes and subsequent Western blotting revealed that only a minor portion of the residual K1 formed filaments with K14. Keratin complexes were extracted from neonatal epidermis and resuspended in 5.5 M urea (A and C). To visualize keratins, the blots shown were incubated with a mixture of corresponding antibodies. At 5.5 M urea, the keratins of wild-type extracts still formed complexes with their partners and migrated at the complex-specific isoelectric point in the first dimension (A; IEF). Note that all keratins migrated at the same isoelectric position. Although K5 and 14 behaved in $K10^{-/-}$ null extracts (C), as in the control (A), and were exclusively migrating as a complex, most of K1 focused at its own basic IP (C, black arrow). Only a small amount of K1 (white arrow) was observed at the complex-specific IP. Dialysis to 6.5 M urea (B and D) resulted in almost complete dissociation of K1/K10 in the wild-type epidermis (exemplified by K10) and partial dissociation of K5/K14 complexes in both wild-type (B) and $K10^{-/-}$ (D) epidermis.

basal epidermis, experimental substitutions of a single keratin have been performed in the basal epidermis. In $K14^{-/-}$ mice, the highly related K16, a K16/14 hybrid, or the simple epithelial K18 did not fully rescue the phenotype of the knockout mice (Hutton *et al.*, 1998; Paladini and Coulombe, 1999). Although the ectopically expressed proteins formed IF with the endogenous K5, the resulting filaments were possibly too weak to withstand mechanical stress, leading to a skin phenotype in those mice. To resolve the question of whether keratins have cell-type–specific functions or can complement each other, as suggested in certain internal epithelia (Magin *et al.*, 1998), more refined experiments need to be performed. Our data suggest that even epithelia exposed to considerable stress, such as epidermis, do function

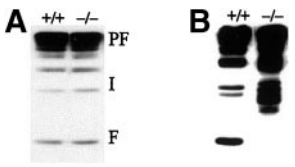

Figure 9. Normal profilaggrin processing in neonatal $K10^{-/-}$ epidermis. In contrast to the impairment of profilaggrin processing in K10T neonates (B), neither the amount of profilaggrin (PF) nor that of its processing intermediates (I) or mature filaggrin (F) was altered in neonatal $K10^{-/-}$ mice (A).

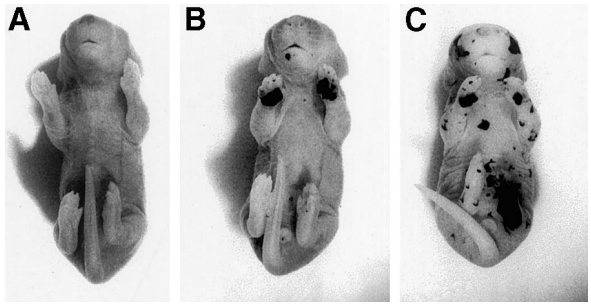

Figure 10. Transient barrier defect of forepaw sole epidermis in $K10^{-/-}$ neonates. During the dye perfusion assay, the skin of control neonates did not take up the color (A), whereas the forepaw sole skin of K10 null neonates was stained (B). K10T neonates were much more affected than K10 null mice, showing dye penetration at multiple body sites (C).

with another set of keratins. Despite the fact that overall sequence identities between the type II keratins 1 and 5 and the type I keratins 10 and 14 were only 66 and 58%, respectively, both keratin sets contain sequence motifs typical of epidermal keratins in their head and tail domains. Therefore, we propose that, in functional terms, both the keratin type and the amount of keratin per cell do matter. This is supported by the phenotype of patients carrying functional K14 null alleles (Chan *et al.*, 1994; Rugg *et al.*, 1994; Jonkman *et al.*, 1996; Batta *et al.*, 2000), which is far less severe than that of patients with point mutations (Corden and McLean, 1996). Increasing the level of endogenous keratins by pharmaceutical intervention in the appropriate compartment of the epidermis might be a useful therapeutic approach for keratin disorders.

Fundamental Differences between Knockout Mice of Type I and II IF Proteins

In contrast to a former hypothesis established from cell transfection studies, which stated that individual keratins were unstable (Kulesh *et al.*, 1989), a considerable amount of K1 persisted without its partner in $K10^{-/-}$ mice. Our analysis of keratin complexes has supported the view that most of keratin 1 remained stable without any other keratin and that a small part was able to form filaments with K14. This corresponded well with the presence of type I keratins in $K5^{-/-}$ mice (Peters *et al.*, 2001) and K8 in $K18^{-/-}$ mice without a partner keratin (Magin *et al.*, 1998), although the converse was not reported in $K8^{-/-}$ animals (Baribault *et al.*, 1994). On the other hand, K13 was still detectable in esophagus extracts from $K4^{-/-}$ mice (Ness *et al.*, 1998). The relative instability of some type I IFs against proteases is in agreement with the caspase-mediated cleavage of K18 at the linker L1/2 sequence VEVD/A (Caulin *et al.*, 1997; Ku *et al.*, 1997). It will be interesting to see whether individual keratins are stable per se, or whether other proteins (for candidates, see Nicholl and Quinlan, 1994) are involved. Moreover, the fact that neonatal $K10^{-/-}$ mice did not have cytolysis and were basically free of IF aggregates, whereas K10T knockout mice (Porter *et al.*, 1996) and keratin transgenic mice (Fuchs *et al.*, 1992) had extensive tissue damage raises again the issue of whether IF aggregates in general are

involved in tissue disease or represent a byproduct (Eyer *et al.*, 1998).

In view of other keratin knockout mice (for review, see Magin *et al.*, 2000) and patients (Corden and McLean, 1996), the normal appearance of $K10^{-/-}$ mice reported here supports the hypothesis that type I and II IF proteins are functionally different and that the deletion of type I is less damaging than that of type II keratins. In the epidermis, this could be due to the interaction of desmoplakin and cornified envelope proteins, which preferentially includes type II keratin interactions (Kouklis *et al.*, 1994; Steinert and Marekov, 1995; Meng *et al.*, 1997). Formal proof of this hypothesis has to await additional knockout mouse studies.

Altered Profilaggrin Processing in K10T but Not in $K10^{-/-}$ Mice

Filaggrin is a keratin-associated protein typical of mammalian epidermis (Harding and Scott, 1983). After its initial synthesis in granular keratinocytes, where its high molecular weight precursor profilaggrin is aggregated in insoluble keratohyalin granules, it becomes subsequently processed to functional filaggrin (Resing *et al.*, 1984). Monomeric filaggrin is supposed to bundle keratin filaments via interaction between keratin rods and β-turn repeat motifs present in all mammalian filaggrins (Mack *et al.*, 1993). Although previous studies have characterized the proteases and phosphatases involved in profilaggrin processing (Resing *et al.*, 1984, 1989; Haugen-Scofield *et al.*, 1988; Kam *et al.*, 1993), the comparison of $K10^{-/-}$ and K10T mice has drawn attention to the subcellular topology of this process. We suggest that the penultimate cleavage leading to the release of the filaggrin monomer depends on the presence of intact keratin filaments. In keeping with in vitro–binding studies (Dale *et al.*, 1978, 1989; Steinert *et al.*, 1981), these can be formed not only by K1/10 but also by K5/14. The presence of keratin aggregates per se does not prevent profilaggrin processing, at least in humans (Ishida-Yamamoto *et al.*, 1994). In patients with epidermolytic hyperkeratosis, an increase in profilaggrin, the processing of intermediates, and accumulation of filaggrin have been described (Ishida-Yamamoto *et al.*, 1994). K10T mice, although they display keratin aggregates similar to patients, differ significantly with respect to keratin mutations in humans. Although the latter have keratin point mutations, K10T mice only express a head–coil 1A fragment (Porter *et al.*, 1996), which is unable to form higher-order structures. Therefore, our results suggest that the last stage of profilaggrin processing requires interaction with keratin hetero-oligomers. This would be in agreement with recent data from transfection studies with filaggrin deletion constructs. These revealed that significant binding of filaggrin to keratin only occurred after disappearance of the granular profilaggrin morphology (Kuechle *et al.*, 1999). It cannot be excluded, however, that the extent of cytolysis in K10T mice has an impact on the final stages of profilaggrin processing.

In conclusion, we have demonstrated that the deletion of K10, which is the most abundant epidermal protein, does not lead to epidermal fragility or to the up-regulation of the hyperproliferative keratins 6 and 17. The increased size of granular cells might be a sign that K5/14 do not fully replace K1/10 in suprabasal keratinocytes. Given that a lower amount of keratin seems sufficient to maintain an epidermis, these findings suggest that K1/10 might have additional functions. In the future, it will be possible to address additional issues, including wound healing, in those mice.

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