Hyperproliferation, induction of c-Myc and 14-3-3σ, but no cell fragility in keratin-10-null mice

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Summary

In the past, keratins have been established as structural proteins. Indeed, mutations in keratin 10 (K10) and other epidermal keratins lead to severe skin fragility syndromes. Here, we present adult K10$^{-/-}$ mice, which reveal a novel connection between the regulation of cell proliferation and K10. Unlike most keratin mutant mice, the epidermis of adult K10$^{-/-}$ mice showed no cytolysis but displayed hyperproliferation of basal keratinocytes and an increased cell size. BrdU labelling revealed a shortened transition time for keratinocytes migrating outwards and DAPI staining of epidermal sheets uncovered an impaired organization of epidermal proliferation units. These remarkable changes were accompanied by the induction of c-Myc, cyclin D1, 14-3-3σ and of wound healing keratins K6 and K16. The phosphorylation of Rb remained unaltered. In line with the downregulation of K10 in squamous cell carcinomas and its absence in proliferating cells in vivo, our data suggest that the tissue-restricted expression of some members of the keratin gene family not only serves structural functions. Our results imply that the altered composition of the suprabasal cytoskeleton is able to alter the proliferation state of basal cells through the induction of c-Myc. A previous model based on transfection of K10 in immortalized human keratinocytes suggested a direct involvement of K10 in cell cycle control. While those experiments were performed in human cultured keratinocytes, our data establish, that in vivo, K10 acts by an indirect control mechanism in trans.

Key words: Keratins, c-Myc, 14-3-3 proteins, Epidermis, Cell proliferation

Introduction

The epidermis is a multilayered epithelium consisting mainly of proliferating and differentiated, postmitotic keratinocytes (Watt and Hogan, 2000). The latter derive from transit amplifying cells originating from stem cells that represent a restricted number of basal keratinocytes, possibly residing in the bulge region of the hair follicle (Taylor et al., 2000; Oshima et al., 2001). The proliferation and differentiation of epidermal keratinocytes is regulated by a multitude of signalling cascades and transcription factors including Wnt/β-catenin (Huelsken et al., 2001), growth factors of the EGF and FGF family (Werner and Smola, 2001), TGF-β, members of the NFκB family (Kaufman and Fuchs, 2000), and c-Myc (Arnold and Watt, 2001).

c-Myc is an oncoprotein involved in both the regulation of cell cycle progression and growth control (Elend and Eilers, 1999). Expression of c-Myc in suprabasal cell layers of transgenic mice induced hyperproliferation and neoplastic changes (Waikel et al., 1999; Pelengaris et al., 1999). If restricted to the basal epidermis of transgenic mice, it was recently shown that c-Myc overexpression resulted in hyperproliferation but did not interfere with interfollicular terminal differentiation (Arnold and Watt, 2001). The hyperproliferation seemed to result from an increase in the number of transit amplifying cells, following depletion of the stem cell compartment (Arnold and Watt, 2001; Waikel et al., 2001). In agreement with this observation, the transgenic expression of cyclin D1, D2 or D3, which represent c-Myc target genes (Bouchard et al., 1999; Obaya et al., 1999), was also reported to stimulate epidermal proliferation upon overexpression in basal cells (Robles et al., 1996; Rodriguez-Puebla et al., 1999; Rodriguez-Puebla et al., 2000).

14-3-3σ is a member of the 14-3-3 protein family, which is predominantly expressed in epithelial cells (Leffers, 1993). Members of the 14-3-3 family of proteins bind to conserved phosphoserine-containing motifs of many proteins (Yaffe et al., 1997), including keratins (Ku et al., 1998). By sequestration of proteins involved in cell cycle progression 14-3-3σ is able to induce a G2 block (Hermeking et al., 1997). The depletion of 14-3-3σ in primary human keratinocytes using antisense technology led to the activation of telomerase and to the downregulation of p16, and forced these cells into continuous proliferation (Dellambra et al., 2000). It was argued that 14-3-3σ or one of its interaction partners could expand the proliferative potential of epidermal keratinocytes.

The differentiation state of epidermal keratinocytes is reflected by the intricate expression pattern of keratins (Fuchs and Green, 1980; Moll et al., 1982). Keratins are members of the large intermediate filament gene family (Hesse et al., 2001) and form the intermediate filament (IF) cytoskeleton in all epithelia including the epidermis. Basal keratinocytes express keratins (K) K5, K14 and K15, which are replaced by K1 and K10 once the cells lose contact with the basement membrane and move to the suprabasal compartment (Fuchs and Green, 1980). The functional significance of this change in keratin expression is not well understood. Given that keratins are

primarily regarded as cytoskeletal proteins, changes in their expression have been regarded as a result of differentiation but not as a means to influence the differentiation state of a cell or a tissue.

During the past decade it became evident that mutations in epidermal keratins interfere with the generation of a normal IF cytoskeleton, lead to epidermal fragility and cause blistering skin diseases including epidermolysis bullosa simplex and epidermolytic hyperkeratosis (Corden and McLean, 1996; Fuchs and Cleveland, 1998). Based on the patient data and from the knowledge that came from the study of transgenic mice, it is widely accepted that keratins are important structural proteins (Fuchs et al., 1992; Lloyd et al., 1995; Bickenbach et al., 1996; Porter et al., 1996; Hesse et al., 2000; Tamai et al., 2000; Arin et al., 2001; Cao et al., 2001; Peters et al., 2001). Recently, however, we demonstrated that $K10^{-/-}$ mice are fully viable and exhibit an intact skin that resists mechanical stress due to a compensatory suprabasal persistence of the basal keratins 5 and 14 (Reichelt et al., 2001).

In an attempt to understand the role of K10, we went on to analyse adult mice, given that in neonatal mice it was dispensable as a cytoskeletal protein. In adult mice, which still presented with an intact epidermis, we noted a considerable hyperproliferation of basal keratinocytes. This pointed to a specific suprabasal function of K10, or K1/K10 IF, that may not be fulfilled by other keratins. In fact, several studies in mice and man have shown that K10 is downregulated in most carcinomas (Ivanyi et al., 1989; Roop et al., 1988; Toftgard et al., 1985; Winter et al., 1983; Maddox et al., 1999). Moreover, the targeted expression of human K10 under the control of the K6 promoter showed a delay in tumor formation in mice (Santos at al., 1997). While the downregulation of K10 can be taken as a consequence of an altered differentiation known to occur in most tumors, another study pointed to an active involvement of K10 in cell cycle regulation (Paramio et al., 1999). Most recently, the same authors demonstrated that, in cultured human HaCaT keratinocytes, Akt kinase was sequestered by K10, which impaired its translocation to the cell membrane where it normally becomes activated by PI 3-kinase (Paramio et al., 2001a). The authors reasoned that K10 can directly inhibit cell proliferation via sequestration of Akt (Paramio et al., 2001a).

Here we show that the targeted deletion of K10 leads to epidermal hyperproliferation in adult mice. Our data imply that the altered composition of the suprabasal cytoskeleton is able to alter the proliferation state of basal cells through the induction of c-Myc. We discuss the possible links between K10, cell growth and proliferation.

Materials and Methods

Histological analysis and immunohistochemistry

Skin samples from 12-week-old mice were fixed over night with 4% formaldehyde in PBS at 4°C and then embedded in paraffin. Sections of 4 μm were placed on superfrost-plus slides (Menzel-Gläser, Braunschweig, Germany) and dried. After deparaffination, sections were either stained with hematoxylin and eosin or processed for 25 minutes in 10 mM sodium citrate buffer, pH 6 in the microwave oven for subsequent antigen detection. Sections were slowly cooled down to room temperature, rinsed in PBS and then incubated with primary antibodies diluted as follows: Ki-67 antiserum (Dianova, Hamburg, Germany), 1:50; 14-3-3σ antiserum N-14 (Santa Cruz Biotechnology, Heidelberg, Germany), 1:10; c-Myc monoclonal antibody Ab-3 (Oncogene Science, Cambridge, MA), 1:20; cyclin D1 monoclonal antibody DCS-6 (Progen, Heidelberg, Germany), 1:10. For detection with horseradish peroxidase-conjugated secondary antibodies the LSAB+ kit (Dako, Hamburg, Germany) was used with DAB as substrate. Sections were counterstained with hematoxylin, dehydrated and embedded in DPX (Sigma, Deisenhofen, Germany).

For the preparation of epidermal sheets, ears were excised immediately after the animals were killed. The ears were then torn with forceps to separate the outer epithelial and the inner side. Both halves were then immersed in 0.5 M ammonium thiocyanate (Sigma) solution in 0.1 M phosphate buffer, pH 6.8, for 20 minutes at 37°C. The epidermis was separated from the dermis with forceps and washed three times for 5 minutes with PBS. The epidermal sheets were subsequently immersed in PBS containing 0.5 μg/ml DAPI (Sigma) for 60 minutes followed by washing in PBS. After a brief rinse in double distilled water and ethanol, the sheets were mounted and embedded in Mowiol (Calbiochem, Bad Soden, Germany).

BrdU labelling

Mice were injected with 1 ml BrdU labelling reagent/100 g mouse weight i.p. (RPN 201, Amersham Pharmacia Biotech, Freiburg, Germany) 2 or 24 hours before they were sacrificed. Skin samples were fixed, embedded and sectioned as described above. After deparaffination, the sections were placed in 2 N HCl for 30 minutes at 37°C and subsequently rinsed thoroughly in PBS. This was followed by a 30 minute treatment with 0.1% (w/v) trypsin (Invitrogen, Karlsruhe, Germany) in PBS at 37°C. After rinsing in PBS, the sections were covered with the monoclonal anti-BrdU antibody BU 33 (Sigma), 1:1000. After 1 hour, the slides were washed three times for 5 minutes in PBS and incubated with an Alexa-488-conjugated anti-mouse secondary antiserum (Molecular Probes, Leiden, The Netherlands), 1:400, for 40 minutes. After washing as before, sections were briefly rinsed once with double-distilled water and once with ethanol and mounted in Mowiol (Calbiochem, Germany).

Northern blotting

Skin samples were immediately frozen in liquid nitrogen. RNA was extracted with Trizol (Invitrogen) according to the manufacturer's instructions. Thirty micrograms were loaded per lane, separated, transferred and hybridized as described before (Reichelt et al., 1999). Wnt-probes for Wnt-3, Wnt-4, Wnt-5a (a gift of Rolf Kemler, Freiburg, Germany), a 720 bp probe for 14-3-3σ and a K5 probe used for normalization were derived from cDNA clones and labelled with Decalabel (MBI, St Leon-Rot, Germany).

SDS-PAGE and western blotting

Total skin protein was extracted, blotted and stained as described before (Reichelt et al., 1999). Gels of 8, 10 and 18% polyacrylamide were used to separate proteins (see Fig. 5 legend). Primary antibodies were diluted as follows: K1 antiserum AF 109 (Babco, Richmond, CA), 1:400,000; K5 antiserum AF 138 (Babco), 1:100,000; K14 antiserum (a gift of Manfred Blessing, Mainz, Germany), 1: 50,000; K15 antiserum (a gift of Elaine Fuchs, Chicago, IL), 1:5000; K6 antiserum (a gift of Manfred Blessing), 1:10,000; K16 antiserum (a gift of Rebecca Porter, Dundee, UK), 1:25,000; 14-3-3σ antiserum N14 (Santa Cruz Biotechnology) 1:1000; p53 antiserum CM.5, 1:50,000; p21 antiserum C-19 (Santa Cruz Biotechnology), 1:500; p27 antiserum (Biosource, Nivelles, Belgium), 1:2000; β-catenin monoclonal antibody (Beckton Dickinson-Transduction Laboratories, Heidelberg, Germany), 1:10,000; Rb monoclonal antibody G3-245 (Beckton Dickinson-Pharmingen, Heidelberg, Germany), 1:4000; phospho-Rb (Ser780) antiserum (Cell Signaling Technology, Beverly,

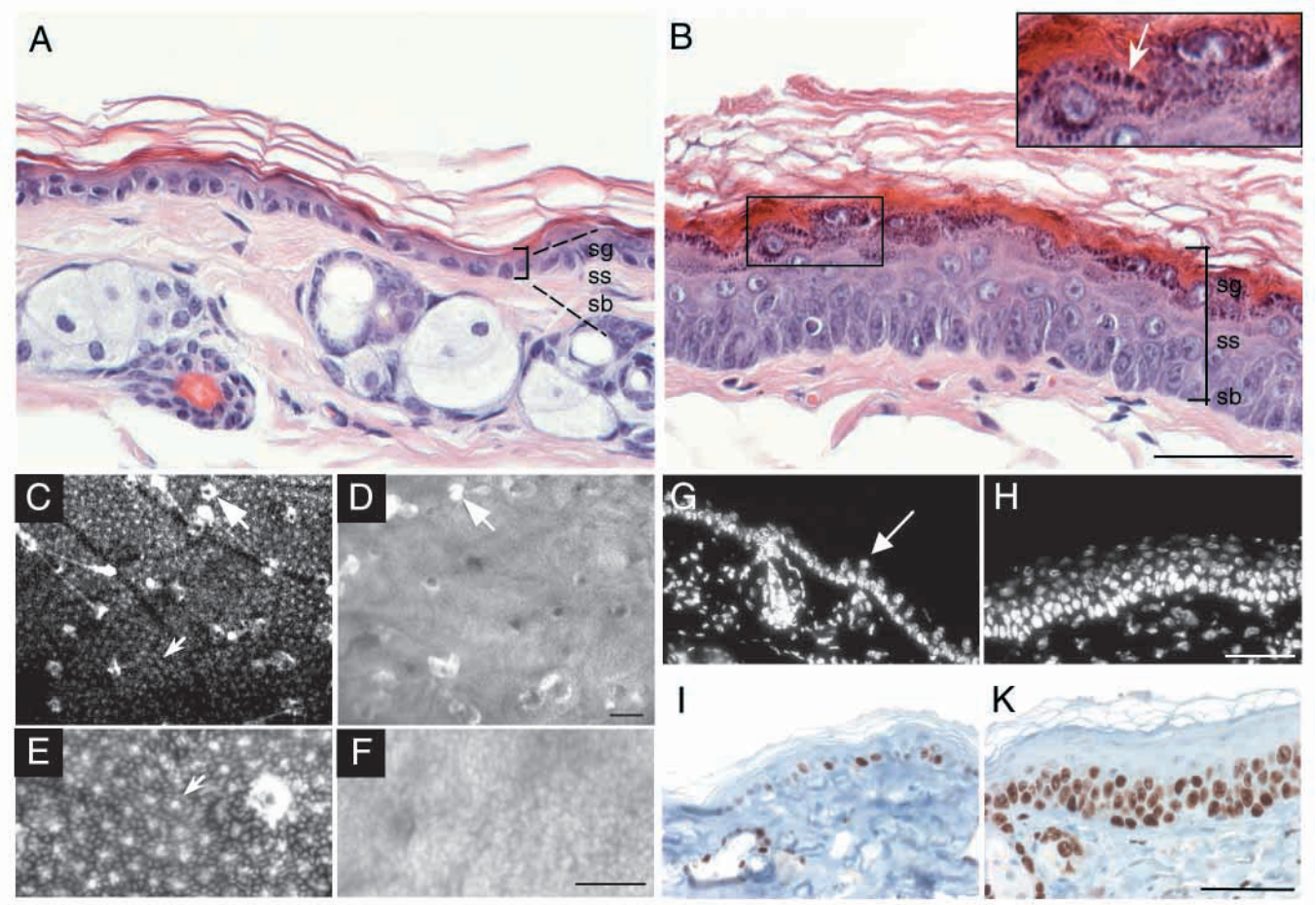

Fig. 1. The epidermis of adult K10$^{-/-}$ mice showed hyperkeratosis and acanthosis; cells and nuclei were enlarged (B). Keratohyalin was increased (B, inset, arrow) compared with that in the wild-type (A). A and B show H&E-stained paraffin sections of ear skin. DAPI staining of normal ear epidermis showed epidermal proliferative units (C, epidermal sheet, small arrow; E, higher magnification, arrow; G, section, arrow; large arrow in C and D, hair follicle), which were absent in knockout mice (D, epidermal sheet; F, higher magnification of D; H, section). In normal epidermis Ki-67 was restricted to basal cells (I), whereas it was expressed throughout the basal layer and in two to three suprabasal layers in K10$^{-/-}$ mice (K). sb, stratum basale; sg, stratum granulosum; ss, stratum spinosum. Bars, 40 μm. The respective wild-type and knockout photos are of the same magnification.

MA), 1:8000; Akt antiserum (Cell Signaling Technology), 1:2000; phospho-Akt (Thr308) antiserum (Cell Signaling Technology), 1:2000; phospho-Akt (Ser473) antiserum (Cell Signaling Technology), 1:2000. HRP-conjugated secondary antisera (Dianova, Hamburg, Germany) were diluted 1:30,000. For detection, Super Signal (Pierce, Rockford, Illinois) was used.

Immunofluorescence analysis

Immunofluorescence analysis was performed as described before (Reichelt et al., 1999). The K1 antiserum AF 109 (Babco), 1:5000; K5 antiserum AF 138 (Babco), 1:5000; K6 antiserum (a gift of Manfred Blessing), 1:1000; K14 antiserum (a gift of Manfred Blessing), 1:5000; K15 antiserum (a gift of Elaine Fuchs), 1:200; K16 antiserum (a gift of Rebecca Porter), 1:500; the Alexa 488-conjugated secondary antiserum (Molecular Probes, The Netherlands) was diluted 1:400.

Allele-specific PCRs

To detect allelic variants of p16 exon 1 we used a previously published protocol (Zhang et al., 1998) with minor modifications. Briefly, exon 1 was amplified from DNA preparations from BALB/c, 129/Ola (both inbred strains) and K10$^{-/-}$ mice as well as wild-type mice, which were used for our studies (the two latter are on a mixed BALB/c×129/Ola background) using the following primers: (forward) 5′-ACTGAATCTCCGCGAGGAAAGCGAACT-3′ and (reverse) 5′-GAATCGGGGTACGACCGAAAGAGT-3′. Cycling conditions were 94°C for 1 minute, followed by 35 cycles of 94°C for 1 minute, 62°C for 45 seconds, and 72°C for 2 minutes. The PCR products were digested with *Nla*III (New England Biolabs, Frankfurt, Germany). Exon 2 was amplified from the same DNA preparations using primers: (forward) 5′-GTGATGATGATGGGCAACGTTCA-3′ and (reverse) 5′-GGGCGTGCTTGAGCTGAAGCTA-3′. Cycling conditions were 94°C for 1 minute, followed by 35 cycles of 94°C for 1 minute, 68°C for 30 seconds, 72°C for 1 minute. The PCR products were digested with *Bsa*AI (New England Biolabs).

Results

Acanthosis and mild hyperkeratosis in adult K10$^{-/-}$ epidermis

Our previous analysis of neonatal K10$^{-/-}$ mice revealed that the loss of K10, which is the most prominent protein in the epidermis, was compensated by the suprabasal persistence of

K14 and K5, which together with K1 formed filaments (Reichelt et al., 2001). These filaments were fully functional in providing mechanical stability to the epidermis. Additionally, we showed that the altered composition of the suprabasal keratins in those mice did not lead to an induction of proliferation-associated keratins K6 or K16 (Reichelt et al., 2001). Here we demonstrate that adult mice maintained an intact epidermis, although histological analysis revealed acanthosis and a mild hyperkeratosis (Fig. 1A,B). In that setting, cells and nuclei of all strata were significantly enlarged but did not show any signs of cytolysis. Interestingly, the observed acanthosis was not homogeneous. About 10% of the K10$^{-/-}$ epidermis showed normal stratification, which was comparable with that of wild-type mice. We did not notice dyskeratosis, although the flattening of granular cells was impaired and the stratum corneum was less orderly stacked than in the wild-type. Additionally, we observed an increase in keratohyalin in the upper epidermis (Fig. 1B, inset). The hyperkeratotic skin of K10$^{-/-}$ mice was not covered with scales but appeared as smooth as wild-type skin. This was in apparent contrast to previously described K10T mice (Porter et al., 1996), which expressed a dominant-negative mutant of K10.

A columnar organization of epidermal keratinocytes has been documented for many species ranging from rodents to humans (for a review, see Potten, 1981). This ordered pattern of keratinocytes with normally regularly spaced epidermal proliferative units (EPUs) was impaired in K10$^{-/-}$ mice as demonstrated by DAPI staining of sections and epidermal sheets. Epidermal sheets viewed from the top as well as transversal sections showed that the spacing of nuclei in K10$^{-/-}$ epidermis appeared unordered (Fig. 1D,F,H), while wild-type epidermis (Fig. 1C,E,G) showed a regular spacing of nuclear columns. The observed alterations in the normal structural organization of K10$^{-/-}$ epidermis suggested a deregulation of cell proliferation.

The amounts of K1 (Fig. 2A), K5 (Fig. 2B) and K14 (Fig. 2C) protein were slightly increased while K15 (Fig. 2D) was unaltered, as revealed by western blotting. The distribution of K1 protein was unaltered in K10$^{-/-}$ epidermis (Fig. 2F) compared with that in the wild-type, while the basal keratins K5 and K14 were also present in suprabasal cells in K10$^{-/-}$ epidermis (Fig. 2H and K, respectively, G and I, wild-type) as described before for neonatal epidermis (Reichelt et al., 2001). In contrast to K5 and K14, another basal keratin, namely K15, remained restricted to basal keratinocytes (Fig. 2M, K10$^{-/-}$, L, wild-type).

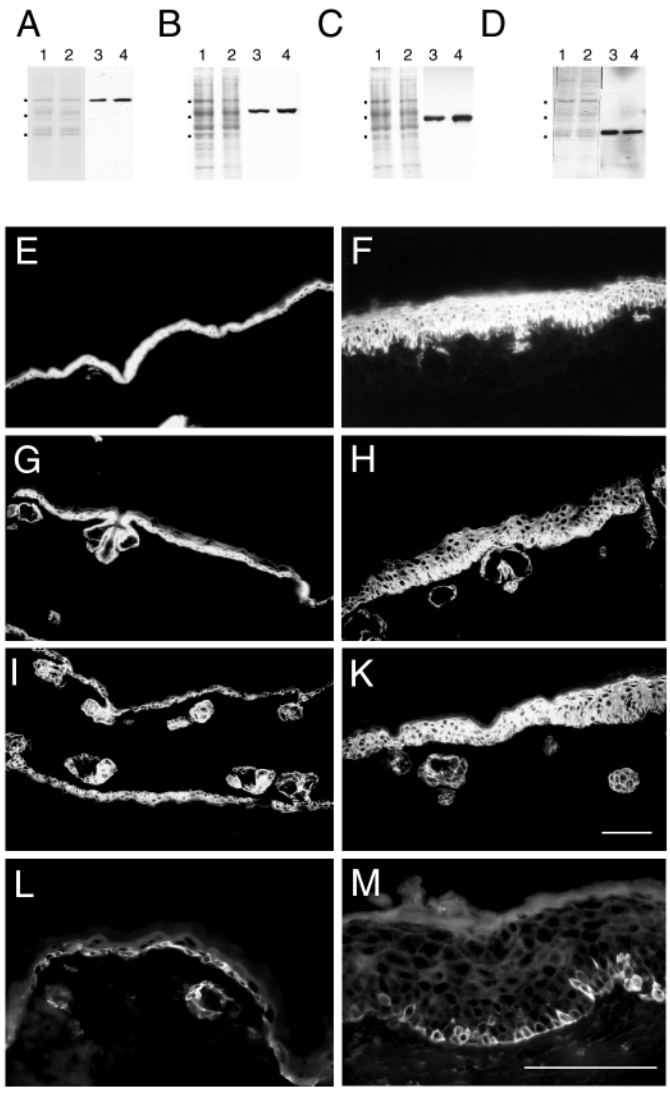

Fig. 2. Western blotting showed that the amount of K1 was slightly increased in K10$^{-/-}$ skin (A, lane 4) compared with that in wild-type skin (A, lane 3; lanes1,2, corresponding Coomassie-stained blot). K5 (B, lane 4, K10$^{-/-}$; lane 3, wild-type; lanes 1,2, corresponding blot) and K14 (C, lane 4, K10$^{-/-}$; lane 3, wild-type; lanes 1,2, corresponding blot) were also slightly increased, whereas K15 was unchanged (D, lane 4, K10$^{-/-}$; lane 3, wild-type; lanes 1,2, corresponding blot). Immunofluorescence analysis of ear epidermis showed unaltered suprabasal K1 localization (E, wild-type; F, K10$^{-/-}$), K5 and K14 were detected in basal and suprabasal layers (K10$^{-/-}$, H and K, respectively; wild-type, G and I). K15 remained restricted to the basal layer (K10$^{-/-}$, M; and wild-type, L). Bars, 50 μm.

Increased proliferation and induction of K6 and K16 in K10$^{-/-}$ epidermis

The reason for the thickening and the disordered architecture of the K10$^{-/-}$ epidermis became evident after Ki-67 labelling. In addition to an increased number of basal cells, a considerable number of suprabasal cells were Ki-67-positive (Fig. 1K). Scoring revealed that the knockout epidermis exhibited 32% stained cells (Fig. 1K), whereas in the wild-type, only 8% of all keratinocytes were Ki-67-positive (Fig. 1I). We reasoned that the increase in the labelling index could be directly or indirectly linked to the loss of K10. Of note, several groups reported the downregulation of K10 in mouse and human skin carcinomas (Nelson and Slaga, 1982; Roop et al., 1988; Toftgard et al., 1985; Winter et al., 1980; Winter et al., 1983), which led to the assumption that K10 might normally inhibit cell proliferation. In fact, it was recently reported that forced expression of K10 inhibited cell proliferation in the human epithelial cell line HaCaT, possibly involving Rb (Paramio et al., 1999). In activated keratinocytes, the loss or downregulation of K10 is accompanied by an increase in the so-called wound healing keratins K6 and K16 (McGowan and Coulombe, 1998; Freedberg et al., 2001). In contrast to neonatal K10$^{-/-}$ mice, adult animals displayed a

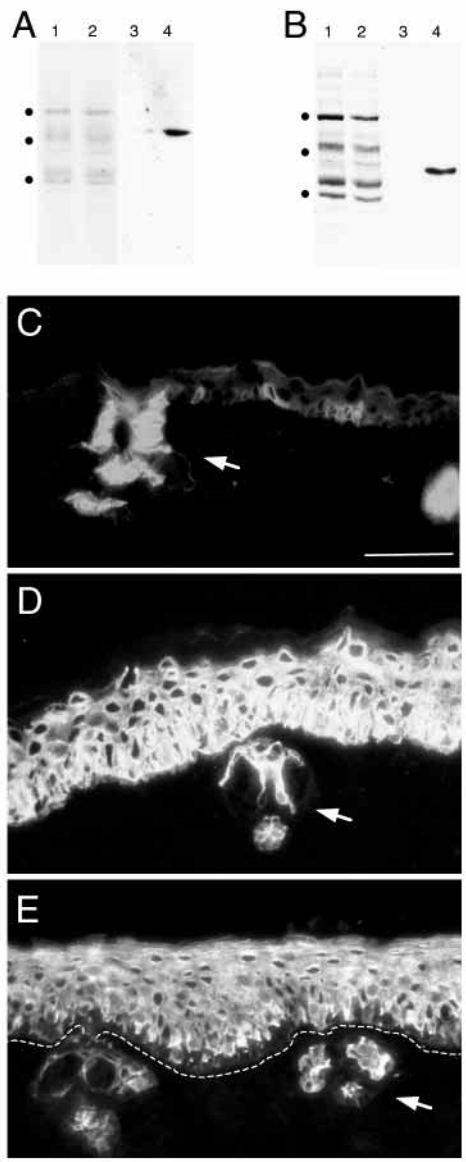

Fig. 3. K6 and K16, while hardly detectable in wild-type western blots (A, lane 3 and B, lane 3, respectively), were strongly increased in K10$^{-/-}$ skin (A, lane 4 and B, lane 4; A and B, lanes 1,2 are the corresponding coomassie-stained blot; dots indicate marker lanes at 66, 56 and 43 kDa). Immunofluorescence analysis of K6 showed that its expression in the wild-type was restricted to hair follicles (C, arrow) whereas K10$^{-/-}$ mice showed additional strong expression through all epidermal layers (D, arrow on hair follicle). In contrast to K6, K16 was induced only in the suprabasal layers of K10$^{-/-}$ epidermis (E, arrow on hair follicle; broken line indicates basement membrane). The sections show ear epidermis. Bar, 40 μm.

strong increase in K6 and K16 as indicated by western blotting (Fig. 3A,B). While it is often assumed that K6 and K16 are not only coexpressed but also colocalize, immunofluorescence analysis showed this not to be the case (see also Porter et al., 1998). Using an antiserum that recognizes both K6a and K6b, K6 was localized both in basal and in suprabasal keratinocytes (Fig. 3D). This is in agreement with data from knockout mice, according to which K6a is present both in basal and suprabasal keratinocytes, while K6b is restricted to the latter (Wojcik et al., 2000; Wojcik et al., 2001; Wong et al., 2000). By contrast, K16 was confined to the spinous and granular layer (Fig. 3E).

The alterations in the suprabasal keratin pattern may contribute to the increased suprabasal cell size observed in adult K10$^{-/-}$ mice. BrdU-labelling revealed that the increase in Ki-67-positive basal and suprabasal cells in K10$^{-/-}$ epidermis resulted from a decreased transition time of proliferating basal keratinocytes through the epidermal layers (Fig. 4). When skin samples were taken 2 hours after BrdU injection we found incorporation only in basal cells in both the wild-type and the knockout tissue, although the number of cells was markedly increased in the latter (Fig. 4A,B). Twenty-four hours after BrdU injection many basal cells in the wild-type had finished one round of division and entered the next cycle, which is shown by the frequent labelling of two adjacent cells (Fig. 4C). In contrast, in K10$^{-/-}$ mice, proliferating cells have cycled more than once and a large number of suprabasal cells were labelled (Fig. 4D), similar to the observed Ki-67 staining pattern (Fig. 1K). Most basal cells lost the BrdU label 24 hours after the injection, which underlines the increased transition time of proliferating cells in K10$^{-/-}$ mice (Fig. 4D). These results established that the loss of K10 stimulated primarily basal cells to proliferate.

c-Myc and 14-3-3σ induction in K10$^{-/-}$ epidermis

To investigate the molecular mechanism underlying the increase in basal cell proliferation, we investigated changes in a number of proliferation-associated proteins.

c-Myc and one of its targets, cyclin D1, are cell cycle-associated proteins that in epidermis are normally expressed in a subset of basal cells (Hurlin et al., 1995). Targeted transgenic overexpression of each of the proteins resulted in a strong increase in cell growth and epidermal proliferation (Arnold and Watt, 2001; Waikel et al., 2001; Robles et al., 1996; Rodriguez-Puebla et al., 1999). We show here that both proteins were upregulated in the epidermis of K10$^{-/-}$ mice, where they were detected in almost all basal cells and in some suprabasal cells (Fig. 5A-D). Given that c-Myc and cyclin D1 have been identified as targets of the Wnt signalling pathway (He et al., 1998; Shtutman et al., 1999), we monitored Wnt expression by RNA analysis. Using probes for Wnt-3, Wnt-4 and Wnt-5a, northern blotting did not provide evidence for an activation of Wnt signalling (Fig. 5E). In agreement with the drastic phenotype resulting from transgenic overexpression of β-catenin in mouse epidermis (Gat et al., 1998), we conclude that the increase in c-Myc and cyclin D1 was not mediated by Wnt signalling. In line with that notion, the distribution of β-catenin in the epidermis [i.e. at sites of cell to cell contact (Fig. 5E, wild-type; F, K10$^{-/-}$)] as well as its overall level as judged by western blotting (Fig. 7D) were unchanged.

Previous work established that 14-3-3σ is involved in cell cycle regulation (Hermeking et al., 1997; Chan, T. A. et al., 1999) and in the control of keratinocyte proliferation and differentiation (Dellambra et al., 2000). By immunohistochemistry, we found a prominent staining of 14-3-3σ in the cytoplasm of suprabasal cells of K10$^{-/-}$ mice compared with that of wild-type epidermis (Fig. 6A,B). The increase in protein (Fig. 6C) was accompanied by an equally strong increase in the mRNA as revealed by northern blotting (Fig. 6D). In basal keratinocytes, 14-3-3σ was unaltered in

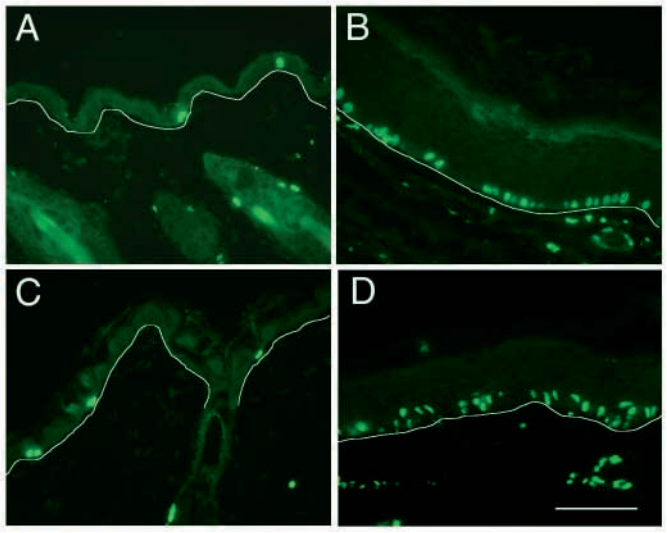

Fig. 4. Two hours after a BrdU pulse, wild-type mice showed ~8% labelled basal cells (A), while more than 50% of basal cells were labelled in K10$^{-/-}$ mice (B). 24 hours after the pulse the wild-type showed predominantly labelling of daughter cells in the basal layer (C). In contrast, K10$^{-/-}$ mice showed massive suprabasal labelling (D) indicating increased turnover of keratinocytes. The line depicts the dermo-epidermal junction. Bar, 40 μm.

comparison with wild-type mice, and appeared very weakly expressed in K10$^{-/-}$ mice (Fig. 6A,B).

Additional proteins have been linked to epidermal proliferation and differentiation. Here we analysed the expression and proliferation status of some candidate proteins by western blotting. Rb, one of the key regulators of the cell cycle, is controlled by phosphorylation (Kitagawa et al., 1996; Lundberg and Weinberg, 1998). Therefore, we included a phosphorylation-specific antibody against Rb in our studies. The experiment revealed that neither the overall amount of Rb (Fig. 7E) nor of Rb phosphorylated at serine 780 (Fig. 7F), was changed in total protein extracts from skin. Whether the subfraction of cells expressing c-Myc was also positive for phospho-Rb could not be resolved, as the antibody available was not suitable for immunocytochemistry. Akt kinase is a key component of the PI 3-K/Akt kinase cascade. One of its major tasks is to promote cell survival via phosphorylation of various target proteins (Datta et al., 1999). In this function, Akt might be linked to the hyperproliferation observed in K10$^{-/-}$ epidermis. The kinase is activated by phosphorylation on two domains, both of which are essential for maximal activation (Alessi et al., 1996). While multiple stimuli are known to trigger Akt phosphorylation (Vanhaesebroeck and Alessi, 2000), Dimmeler and co-workers reported an activation of Akt kinase in response to shear stress in endothelial cells (Dimmeler et al., 1998). Therefore, we presumed that the replacement of K1/K10 by K1/K5/14 in the K10$^{-/-}$ mice may render suprabasal keratinocytes more susceptible to mechanical strain, which could lead to an activation of Akt. Additionally, in cell culture transfection experiments it has been shown that unphosphorylated Akt colocalized with K10-containing IF (Paramio et al., 2001a). The authors concluded from their findings that Akt was sequestered by K10, which impaired its translocation to the cell membrane, where it is normally activated through phosphorylation, thereby causing cell cycle

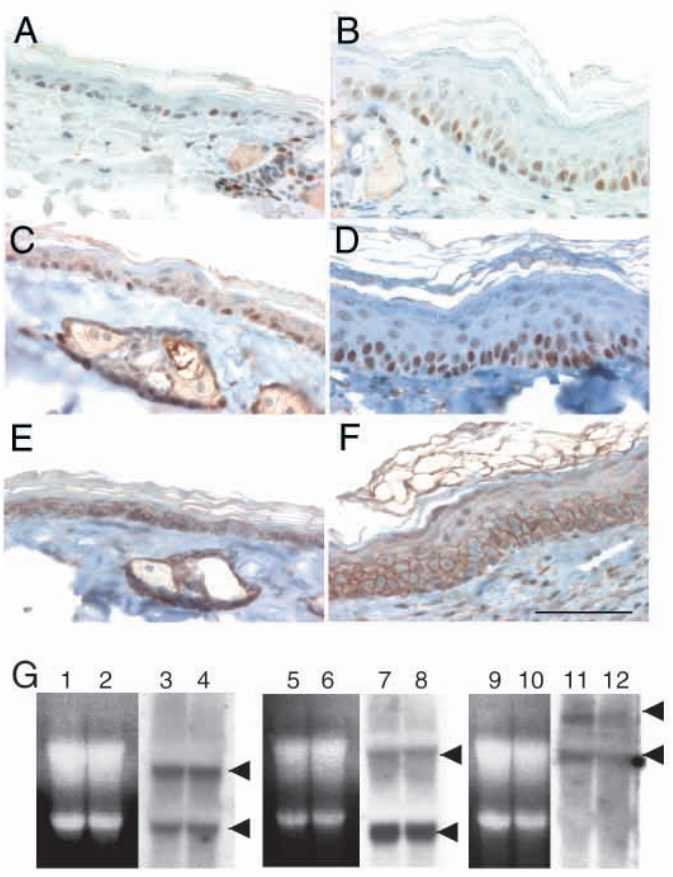

Fig. 5. Immunohistochemical analysis showed an increase in cyclin D1 (B) and c-Myc (D) in K10$^{-/-}$ epidermis compared with the wild-type, where both proteins are exclusively found in a restricted number of basal cells (A and C, respectively). The expression of β-catenin remained restricted to keratinocyte cell membranes (E, wild-type; F, K10$^{-/-}$). (G) Northern blotting revealed that the expression of Wnt-3 (lane 3, wt; lane 4, K10$^{-/-}$; loading control: lane 1, wt; lane 2, K10$^{-/-}$), Wnt-4 (lane 7, wt; lane 8, K10$^{-/-}$; loading control: lane 5, wt; lane 6, K10$^{-/-}$l) and Wnt-5a (lane 11, wt; lane 12, K10$^{-/-}$; loading control: lane 9, wt; lane 10, K10$^{-/-}$) were not altered in K10$^{-/-}$ skin. Arrowheads point to distinct RNA species. Bar, 40 μm.

arrest. To identify activated Akt kinase, antibodies specific for each of the essential phosphorylated residues were used in addition to an antibody that recognizes total Akt protein. We found the amount of unphosphorylated Akt kinase unaltered (Fig. 7G, lanes 8,9, upper band), whereas phosphorylated Akt at threonine 308 (Fig. 7G, lanes 10,11) and serine 473 (Fig. 7G, lanes 12,13) was strongly reduced. This makes it unlikely that Akt kinase becomes activated following the loss of K10. The lower band in Fig. 7G, lane 8 represents phosphorylated Akt, as the antibody we used here detects total Akt (phosphorylation-state independent). As phosphorylated Akt was reduced in K10$^{-/-}$ mice the lower band was strongly reduced in the knockout (Fig. 7G, lanes 8,9). Finally, the amounts of the tumor suppressor p53 (Fig. 7A), and of the cell cycle inhibitors p21 (Fig. 7B) and p27 (Fig. 7D) remained unaltered. At present, the significance of p53 post-translational modifications on its activation and on its subcellular localization in vivo is not yet fully determined (Meek, 1999; Liang and Clarke, 2001) and has not yet been examined in our mice.

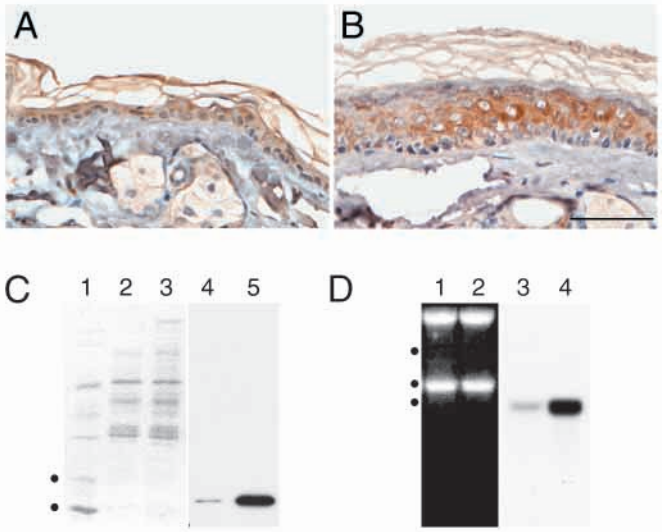

Fig. 6. 14-3-3σ was markedly increased in the cytoplasm of suprabasal $K10^{-/-}$ keratinocytes (B) compared with the wild-type (A). (C) Protein extracts of skin revealed a strong increase in 14-3-3σ expression in $K10^{-/-}$ mice (lane 5) compared with the wild-type (lane 4; lane 1, marker; lanes 2,3, Coomassie-stained blot of lanes 4,5; dots from top to bottom: 36 kDa and 26 kDa). (D) Northern analysis showed an equally strong increase in 14-3-3σ mRNA (lane 4) over the wild-type (lane 3; lanes 1,2, ethidium-stained agarose gel corresponding to lanes 3,4; dots from top to bottom: 2.8, 1.8 and 1.5 kb). Bar, 40 μm.

Another cell cycle regulator that has been shown to be involved in mouse epidermal differentiation and proliferation is $p16^{Arf4a}$ (Paramio et al., 2001b). This protein inhibits Cdk4 and Cdk6, thereby maintaining Rb in its active form, arresting cells in G1 phase. The $K10^{-/-}$ population as well as the wild-type animals presented here were on a mixed BALB/c×129/Ola mouse strain background. Based on an in vitro kinase assay, Zhang et al. concluded that BALB/c allelic variants in exons 1 and 2 of $p16^{Ink4a}$ are inefficient inhibitors of Rb phosphorylation (Zhang et al., 1998). Using PCR and subsequent specific restriction of the PCR products as previously described (Zhang et al., 1998), we found that $K10^{-/-}$ mice as well as the corresponding wild-type mice carry both allelic variants of exon 1 (Fig. 8A) and both allelic variants described for exon 2 (Fig. 8B). The fact that the $p16^{Ink4a}$ variants represent polymorphisms, as indicated by the presence of these variants in wild-derived strains of mice (Zhang et al., 1998), makes it unlikely that in vivo these alleles lead to the formation of an inefficient form of $p16^{Ink4a}$. In addition, the knockout of p16 did not show an epidermal phenotype (Krimpenfort et al., 2001; Sharpless et al., 2001). Moreover, our results demonstrated that the mice we used for this study carried both allelic variants and therefore could express $p16^{Ink4a}$ protein, which may efficiently inhibit Rb phosphorylation.

Collectively, the analysis of adult $K10^{-/-}$ mice suggested that K10 is indirectly involved in the regulation of cell proliferation in mice, possibly via c-Myc (Fig. 9).

Discussion

Within the past decade, it has been established that a major function of keratins is to confer mechanical stability to the epidermis. This property has been implicated first by the range of pathological phenotypes seen in patients bearing mutations in epidermal keratins and then by transgenic mouse models (for reviews, see Corden and McLean, 1996; Coulombe et al., 1991; Vassar et al., 1991; Porter et al., 1996; Peters et al., 2001; Arin et al., 2001). Nevertheless, the functional requirement for the complexity of keratins expressed in the epidermis is far from being understood, although the range of keratin mutant mice is beginning to deliver (Magin et al., 2000) (Herrmann et al., 2002). On the one hand, there is the severe tissue fragility resulting from the complete absence of the keratin cytoskeleton in $K5^{-/-}$ mice (Peters et al., 2001). More than any other, the knockout of this keratin underlined the importance of keratins as structural proteins. On the other hand, the targeted deletion of the suprabasal keratin K10 did not lead to tissue fragility in neonatal mice at all, despite the fact that K10 represents the most abundant epidermal protein (Reichelt et al., 2001). In an attempt to understand the role of K10, we went on to analyse its role in adult mice, where, unexpectedly, its loss resulted in an increase in basal cell proliferation. Based on our data, we argue that K10, in addition to being a cytoskeletal protein, has an additional function. Below, we discuss the evidence for a novel role of keratin K10, namely an indirect involvement in cell cycle regulation.

Is K10 excluded from proliferating cells?

Among keratins, K10 appears to be unique because its expression is restricted to postmitotic cells (Moll et al., 1982), and because it can form highly bundled IF typical of the upper epidermis. Given their abundance and the fact that their head and tail include glycine-rich domains, K10 and K1 have underscored the view that keratins represent the prototype cytoskeletal protein. The downregulation of K10 upon wound healing, where it becomes replaced by K6 and K16 in activated keratinocytes, has been taken as further evidence that the presence of K10 is not compatible with the state of a migratory or proliferating cell (McGowan and Coulombe, 1998). In line with this notion, a considerable number of publications has demonstrated the absence of K10 from carcinomas in mice (Roop et al., 1988; Toftgard et al., 1985; Winter et al., 1980; Nelson and Slaga, 1982) and humans (Winter et al., 1983). Although K10 has been found in rare cells throughout tumors (Moll et al., 1983; Moll, 1998), it was not resolved whether these were actively proliferating or rather resting cells. These in vivo situations, in which K10 is restricted to postmitotic keratinocytes and is absent in a number of malignant cancers, have prompted studies that intended to show an antiproliferative potential of K10 (Santos et al., 1997; Paramio et al., 1999). However, the expression of K10 under control of a K6 promoter, which is activated in hyperproliferative cells (Takahashi and Coulombe, 1997), could neither inhibit tumor formation nor influence the degree of malignancy in animals subjected to a skin carcinogenesis protocol but merely delayed tumor formation (Santos et al., 1997). Moreover, the forced expression of K10 in β-cells of the pancreas, using an insulin promoter, was compatible with the development of a normal organ (Blessing et al., 1993).

In another attempt to study the correlation between K10 and cell proliferation, it was transfected into the human keratinocyte cell line HaCaT, where it was found to inhibit cell

proliferation. This block was shown to depend on the inactivation of Rb and seemed to require the non-helical end domains of K10 (Paramio et al., 1999). In this setting, the cell cycle block imposed by K10 could be released by K16. Most recently it has been shown by the same authors, that K10 can directly interact via its non-helical N-terminal domain with Akt kinase and PKCζ, thereby impairing their translocation and subsequent activation (Paramio et al., 2001a). Furthermore, they showed that this inhibition impeded Rb phosphorylation and reduced the expression of cyclins D1 and E (Paramio et al., 2001a).

While these findings suggested a direct and negative involvement of K10 in the regulation of human keratinocyte proliferation, the analysis of adult K10-deficient mice allows another conclusion. Most importantly, the in vivo ablation of K10 in suprabasal keratinocytes in mice did not induce proliferation of the same cells but rather acted on neighbouring cells residing in the basal layer. Therefore, the hyperproliferation is most likely an indirect consequence following the absence of K10. With respect to the role of Rb suggested by cell transfection studies (Paramio et al., 1999), we cannot completely rule out its contribution in vivo, given that we adressed its phosphorylation state in total skin extracts. To resolve this issue, a phospho-Rb antibody suitable for immunocytochemistry would be useful. Given the extent of proliferative changes in $K10^{-/-}$ epidermis, we would argue, however, that Rb does not appear to be a major candidate in mice. It is also possible that human and mouse cells use different mechanisms involving different pathways to regulate proliferation.

The changes in $K10^{-/-}$ epidermis are compatible with the increase in c-Myc

c-Myc expression is normally very low in the epidermis (Hurlin et al., 1995). We show here that the loss of K10 led to an increase in c-Myc predominantly in the basal epidermal layer. Collectively, the histological changes in the epidermis of adult $K10^{-/-}$ mice, namely hyperproliferation and an increase in cell size, were strikingly similar to those exhibited by transgenic mice overexpressing c-Myc in basal (Arnold and Watt, 2001; Waikel et al., 2001) or in suprabasal keratinocytes (Pelengaris et al., 1999; Waikel et al., 1999). Remarkably, all of these transgenic lines shared an impairment of the ordered arrangement of keratinocytes, while other aspects of terminal differentiation proceeded unimpaired.

In addition to the increase in basal cell proliferation, $K10^{-/-}$ mice displayed an increase in cell size and in the size of keratohyalin granules. This was similar to transgenic mice generated by Pelengaris et al., which also displayed a distortion of the normal epidermal architecture, an increase in granular cell size accompanied by the failure of these cells to flatten, and an increase in keratohyalin (Pelengaris et al., 1999). The change of cell size is compatible with the induction of c-Myc which, in addition to its established involvement in cell-cycle progression and oncogenesis (Pelengaris et al., 2000), is known to regulate cell growth (Elend and Eilers, 1999; Johnston et al., 1999). Given that basal cells show a different morphology than suprabasal cells, it is also possible that the replacement of the normal suprabasal K1/K10 cytoskeleton by one that contains the basal keratins K5/K14 in addition to K1 may influence suprabasal cell size and morphology in $K10^{-/-}$ epidermis.

$K10^{-/-}$ mice differ from c-Myc transgenic mice (Arnold and Watt, 2001; Waikel et al., 2001) in various aspects. Our mice did neither display ulcerated lesions nor an impairment of wound healing (Reichelt and Magin, unpublished). This is best explained by the higher level of c-Myc in transgenic mouse

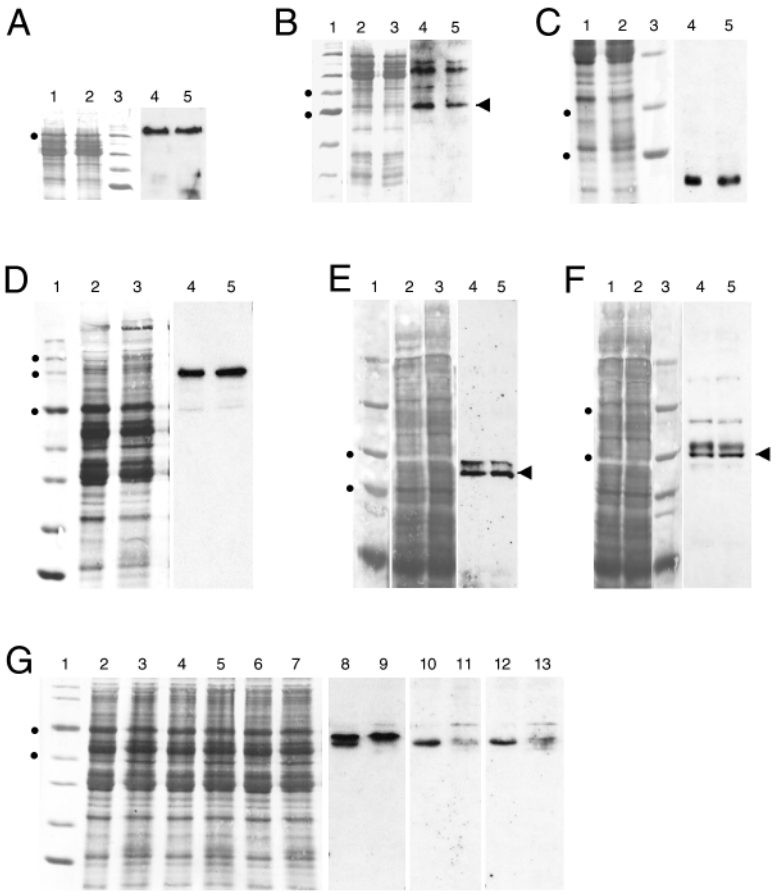

Fig. 7. Western blot analysis of proliferation-associated proteins showed no alterations in p53 (A, lane 4, wild-type; lane 5, $K10^{-/-}$; lanes 1,2, a corresponding coomassie-stained blot; lane 3, marker; dot, 56 kDa), a slight decrease in p21 (B, lane 4, wild-type; lane 5, $K10^{-/-}$; lanes 2,3, loading control; lane 1, marker; dots, 36 and 26 kDa) and p27 (C, lane 4, wild-type; lane 5, $K10^{-/-}$; lanes 1,2, loading control; lane 3, marker; dots, 36 and 26 kDa), no changes in β-catenin (D, lane 4, wild-type; lane 5, $K10^{-/-}$; lanes 2,3, loading control; lane 1, marker; dots from top to bottom, 116, 97 and 66 kDa), Rb (E, lane 4, arrowhead, wild-type; lane 5, $K10^{-/-}$; lanes 2,3, loading control; lane 1, marker; dots, 116 and 97 kDa) or phosphorylated Rb (Ser780) (F, lane 4, arrowhead, wild-type; lane 5, $K10^{-/-}$; lanes 1,2, loading control; lane 3, marker; dots, 158 and 116 kDa). (G) The level of unphosphorylated Akt kinase was unaltered (lane 8, wild-type; lane 9, $K10^{-/-}$, upper band; lanes 2,3, loading control; lane 1, marker; dots from top to bottom, 66 and 56 kDa) while phosphorylated Akt (Thr308-specific detection: lane 10, wild-type; lane 11, $K10^{-/-}$; Ser473-specific detection; lane 12, wild-type; lane 13, $K10^{-/-}$; lanes 4-7, loading control) was reduced. A and B show blots of 18%, E and F of 8% and C, D, G and H of 10% polyacrylamide gels.

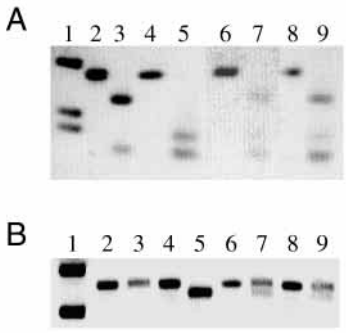

Fig. 8. The restriction patterns for *Nla*III in exon 1 (A) and *Bsa*AI in exon 2 (B) of the $p16^{Ink4a}$ gene revealed the presence of polymorphisms in $K10^{-/-}$ mice that were on a mixed BALB/c×129/Ola background. Lanes 2,4,6,8 (A,B) show undigested PCR products of the respective exons. p16 exon 1 of BALB/c mice (A, lane 3) showed a different restriction pattern from the 129/Ola p16 gene (A, lane 5). $K10^{-/-}$ mice as well as wild-type control animals of a mixed strain background showed an intermediate pattern (A, lane 7 and lane 9, respectively). Exon 2 of the BALB/c p16 gene (B, lane 3) lacked a restriction site that was present in the 129/Ola p16 gene (B, lane 5). $K10^{-/-}$ mice, as well as the wild-type mice of the same mixed strain background, also showed an intermediate pattern (B, lane 7 and lane 9, respectively). Size markers from top to bottom: (A) 200 bp, 100 bp and 80 bp; (B) 400 bp and 300 bp.

lines where it is present in all basal cells including stem cells. As previously argued (Waikel et al., 2001; Arnold and Watt, 2001), the expression of c-Myc resulted in a depletion of the stem cell compartment and stimulated the proliferation of transit amplifying cells. In due course, this could lead to the alterations seen in c-Myc transgenic mice. In contrast to the situation in c-Myc transgenic mice, c-Myc need not necessarily be induced in epidermal stem cells of $K10^{-/-}$ mice. Indeed, not all basal cells were labelled in the latter (data not shown); this would explain the morphological differences.

The induction of cyclin D1 in $K10^{-/-}$ mice may be caused by c-Myc (Daksis et al., 1994). Although cyclin D1 expression is present in multiple malign tumors, transgenic expression of cyclin D1 in the basal layer of the epidermis did not result in tumor formation but only in hyperproliferation (Robles et al., 1996), which is in line with our observations that $K10^{-/-}$ mice do not develop spontaneous skin tumors. Skin carcinogenesis experiments on $K10^{-/-}$ mice will reveal whether the susceptibility of these mice to skin tumors is increased. Given that both c-Myc and cyclin D1 are among the few known target genes of the Wnt/β-catenin-signalling pathway (He et al., 1998; Shtutman et al., 1999), we investigated the activity of different Wnt genes known to be active in skin (Millar et al., 1999; Saitoh et al., 1998). In agreement with the normal pattern of epidermal differentiation, no alterations in the transcription of Wnt genes were detectable by northern blotting. Additionally, we did not find alterations in β-catenin cellular distribution or protein levels. Consistent with this, hair follicle morphogenesis seemed completely normal in our mice (data not shown). It has been described recently that Wnt-signalling has a major impact on hair follicle development (Gat et al., 1998) and that the stabilization of β-catenin may lead to de novo hair follicle morphogenesis and malignant transformation (Chan et al., 1999a). The turnover of cyclin D1 can be upregulated by phosphorylation through GSK-3β, which in turn is negatively regulated by activated Akt kinase (Diehl et al., 1998). Although we found a decrease in phosphorylated Akt in skin extracts, we observed an increase in cyclin D1-positive nuclei in the basal cell layer and in some suprabasal cells in $K10^{-/-}$ epidermis. An explanation for this may be that the increase in cyclin D1 is restricted to a minor number of keratinocytes, while the decrease in activated Akt may concern the major cell population. Moreover, the relative contribution of c-Myc and Akt to cyclin D1 regulation has not been examined in an in vivo setting.

The increase in 14-3-3σ may explain the lack of malignant changes in $K10^{-/-}$ epidermis

Despite a strong increase in basal cell proliferation, combined with the disturbance of epidermal proliferative units, we have failed to observe any signs of malignant transformation in adult $K10^{-/-}$ epidermis, even in mice older than 18 months. 14-3-3σ is a p53-inducible gene which is involved in G2 arrest (Fu et al., 2000). In tumors including breast cancer, downregulation of 14-3-3σ has been reported to involve methylation of CpG islands and may represent an epigenetic event critical for the accumulation of mutations (Ferguson et al., 2000). Interestingly, the experimental treatment of breast cancer cell lines with 5-aza-2′-deoxycytidine, which demethylates DNA, leads to the transcriptional activation of the 14-3-3σ gene (Ferguson et al., 2000). Remarkably, we detected a significant increase of 14-3-3σ mRNA and protein in the epidermis of adult $K10^{-/-}$ mice. While at present we have no data to support whether the upregulation of 14-3-3σ involves demethylation of its gene, our histochemical localization of 14-3-3σ in suprabasal, postmitotic epidermal keratinocytes is consistent with the absence of malignant changes.

This localization is also in agreement with a recent report suggesting that 14-3-3σ is a key regulator of keratinocyte stem cells (Dellambra et al., 2000). Based on gene inactivation studies in a human colon carcinoma cell line it became evident that 14-3-3σ is required to sequester cdc2-cyclin B1 complexes in the cytoplasm to initiate a G2/M block (Hermeking et al., 1997; Chan et al., 1999b). Such a feature is consistent with the increased size of suprabasal keratinocytes that we observed in $K10^{-/-}$ mice, which might point to an arrest at this cell cycle stage. Moreover, c-Myc, when transfected into cultured keratinocytes, can also cause a G2/M block and initiate cell growth (Gandarillas et al., 2000).

In addition to their obvious role in cell cycle regulation (for a review, see Fu et al., 2000), 14-3-3 proteins can associate with K8/K18 tetramers, mainly through binding of phosphorylated K18 during the S/G2/M phase of the cell cycle. By keratin

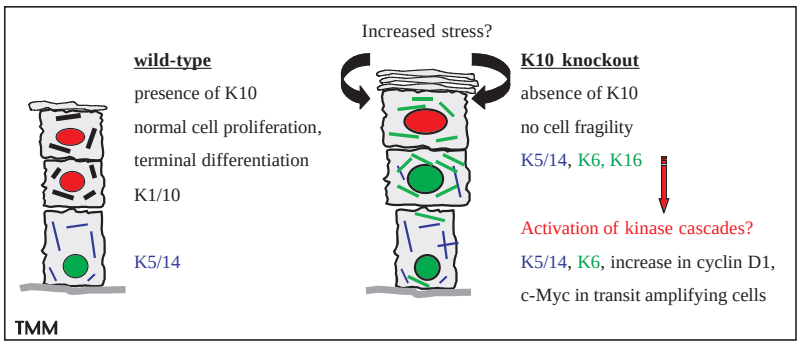

Fig. 9. Model for K10-mediated regulation of cell proliferation.

sequestration, they may be albe to influence keratin distribution and intermediate filament organization (Liao and Omary, 1996; Ku et al., 1998; Toivola et al., 2001). Whether 14-3-3σ can interact with epidermal keratins in an analogous way and whether such an interaction is of significance in the epidermis has yet to be explored.

In our work, we have demonstrated for the first time that the deletion of a keratin in one cell type leads to the stimulation of cell proliferation in neighbouring cells, involving an increase in c-Myc. The stimulation of basal cell proliferation upon the loss of K10 must involve transmission of a signal from the suprabasal to the basal compartment of the epidermis. This could involve paracrine-acting cytokines such as KGF, GM-CSF, IL-1 and/or members of the TGF-β superfamily (for a review, see Werner and Smola, 2001). Alternatively, the altered composition of the suprabasal IF cytoskeleton in $K10^{-/-}$ epidermis could render suprabasal cells more susceptible to mechanical stress, providing an appropriate signal (Takei et al., 1997; Kippenberger et al., 2000). With respect to the large number of keratins, it will be interesting to see whether K10 is unique.

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