The expression of Keratin K10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis

Authors:
Mirentxu Santos\(^{(1,3)}\), Jesús M. Paramio\(^{(1,3,4)}\), Ana Bravo\(^{(2)}\), Angel Ramirez\(^{(1)}\) and José L. Jorcano\(^{(1)}\)

Affiliation:
1.- Project on Cell and Molecular Biology and Gene Therapy, CIEMAT Av. Complutense 22, E-28040 Madrid, (SPAIN).
2.- Dept. of Animal Pathology, Veterinary School, University of Santiago de Compostela, Lugo (SPAIN).
3.- These two authors contributed equally to this work.
4.- To whom correspondence should be addressed.
Phone: 34+ 91 3466598
Fax 34+913466484
e-mail: jesusm.paramio@ciemat.es

Running title: Keratin K10-induced inhibition of cell proliferation \textit{in vivo}

Key words: Keratin; cell cycle, signal transduction; skin tumorigenesis; epidermis; transgenic mice
ABSTRACT

Forced expression of K10, a keratin normally expressed in postmitotic, terminally differentiating epidermal keratinocytes, inhibits the progression of the cell cycle in cultured cells (Paramio et al., 1999 Mol. Cell Biol. 19: 3086-3094). This process requires a functional retinoblastoma (pRb) gene product, and is mediated by K10-induced inhibition of Akt and PKCζ, two signaling intermediates belonging to the PI-3 kinase signal transduction pathway (Paramio et al., 2001 Mol. Cell Biol. 21:7449-59). Extending earlier in vitro studies to the in vivo situation, this work analyzes the alterations found in transgenic mice that ectopically express K10 in the proliferative basal cells of the epidermis. Increased expression of K10 led to a hypoplastic and hyperkeratotic epidermis due to a dramatic decrease in skin keratinocyte proliferation, in association with the inhibition of Akt and PKCζ activities. The inhibition of cell proliferation and Akt and PKCζ activities was also observed, although to a minor extent in low hK10 expressing mice. These animals displayed no overt epidermal phenotype nor overexpression of K10. In these non-phenotypic mice, ectopic K10 expression also resulted in decreased skin tumorigenesis. Collectively, these data demonstrate that keratin K10 in vivo functions include the control of epithelial proliferation in skin epidermis.
INTRODUCTION

Keratins are the main components of the intermediate filament cytoskeleton in epithelial cells. They are a large family of proteins that includes ~30 different polypeptides expressed in a cell-type and differentiation-specific manner. Their functions have long been presumed to be predominantly structural. This role was clarified when human epithelial fragility syndromes became attributable to mutations within epidermal keratin genes (for reviews see 1-4). However, this shared function does not provide a clear explanation of the great diversity of these proteins, which suggests they may have additional specific functions.

Changes in keratin expression pattern are particularly important in epidermis. Keratins K5 and K14 are expressed in the mitotically active basal cells. As these cells enter the terminal differentiation program, becoming postmitotic and suprabasal, keratins K5 and K14 are substituted by keratins K1 and K10 (5). Under the influence of hyperproliferative stimuli, for example during wound healing and in certain disorders including cancer, epidermal expression of K1 and K10 is drastically reduced. Keratins K6 and K16, normally absent from interfollicular epidermis, are, however, induced (6). As a whole, these changes suggest that each keratin pair provides specific functional requirements to epidermal keratinocytes. This is also highlighted by recent findings in which K16 was expressed ectopically (7) to rescue the epidermal fragility promoted by inactivation of the keratin K14 gene (8). These rescued animals show neither epidermal fragility nor neonatal mortality, but they exhibit strong phenotypic alterations such as alopecia, chronic epidermal ulcers and alterations in other stratified epithelia (7). This demonstrates that these two proteins are not functionally equivalent.

In search of specific keratin functions, we have shown that the forced expression of particular keratin polypeptides may influence proliferation in cultured cells. It has been specifically demonstrated that the ectopic expression of keratin K10 inhibits cell proliferation (9). The modulation of cell growth exerted by keratin K10 is linked to the retinoblastoma (pRb) protein and the molecular machinery controlling cell cycle progression during G1, in particular...
cyclin D1 expression (9). This activity appears to involve the sequestration of Akt/PKB and atypical PKCζ to the keratin cytoskeleton, mediated by keratin K10 through its aminoterminus (10). Therefore, the presence of K10 leads to impaired phosphoinositide 3 kinase (PI3-K) signaling. Most of this information has been obtained in cultured cells; this paper reports work to confirm these results in vivo.

Transgenic mice were generated in which human keratin K10 (hK10) gene expression was targeted to the basal layer of the epidermis via the use of the bovine keratin K5 (bK5) promoter (11). These animals display severe alterations in their epidermis, including decreased proliferation which results in epidermal hypoplasia. Impaired activation of both Akt and PKCζ kinase activities in the skin of these animals was also observed. Finally, chemical skin carcinogenesis protocols demonstrated that ectopic K10 expression inhibits the formation of tumors in vivo.
MATERIALS AND METHODS

Transgene construction and generation of transgenic mice. The plasmid bK5hK10 was generated by placing the 5.2 Kb of the bovine K5 regulatory sequences (Sal I-NruI fragment) (11) 5’ upstream of the human keratin K10 gene in plasmid HK10MC (12). This was performed using a SalI-HindIII digestion protocol. The plasmid pbK5Z, expressing the bacterial lacZ (β-Gal) gene under the bovine keratin K5 promoter, has previously been described (11). The constructs were released from the backbone, purified, and used for microinjection. Transgenic mice were generated by injection of bK5hK10 or coinjection of both bK5Z (13) and bK5hK10 constructs into a (C57 BL/10 x BALB/c) F2 or a (C57BL/10 x DBA/2) F2 genetic background (13). The presence of the transgene was analyzed by Southern blots of tail DNA and quantified using phosphorimager equipment (Bio-Rad). Primary keratinocyte cultures were established isolating keratinocytes from new-born mice, and cultured in Eagle's minimal medium containing 8% chelex-treated serum and 0.03 mM Ca2+ as described (14). Colony forming efficiency and BrdU labeling experiments were performed essentially as described (9, 10, 15-17).

Histological analysis. Dorsal skin samples and tumors were fixed in either formalin or ethanol and embedded in paraffin prior to sectioning. 4μm sections were stained with hematoxylin-eosin. Mice were injected with bromodeoxyuridine (BrdU) in PBS (100μg/g body weight) 1 hour prior to sacrifice. To analyze the epidermal-labeling index, paraffin sections were stained using an anti-BrdU monoclonal antibody (Boehringer Mannheim) following the manufacturer's instructions. Keratin K10 staining was performed in ethanol-fixed tissue sections using LH2 or K8.60 mAbs (15, 17)(reacting with both, human and mouse K10). Filaggrin, Loricrin, keratin K6, and keratin K5 expression was monitored as described (15, 17). For ultrastructural analysis, skin samples were fixed in 2.5 % gluteraldehyde in PBS and postfixed in 1 % osmium tetroxide prior to dehydration and embedding in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate. The expression of total and phosphorylated Akt was visualized.
by immunofluorescence using fresh cryosections of whole mouse skin essentially as previously described (10).

**Chemical carcinogenesis.** TG.AC mice (18) were bred with bK5Z+bK5hK10 transgenic mice (13) to obtain single- and double-transgenic mice. Sixteen bK5Z+bK5hK10/TG.AC transgenic mice and 16 TG.AC littermates were used in chemical carcinogenesis experiments. The backs of 7-week-old animals were shaved and treated with TPA (Sigma Chemical Co; 10 nmol in 200 µl acetone) three times a week for 8 weeks. At this time, the TPA treatment was arrested. The number and size of tumors arising on each mouse were recorded at regular intervals during 50 weeks. The histopathological analysis of the tumors was routinely performed on formalin-fixed, paraffin-embedded histology sections.

**Western blot and kinase assays.** Whole skin extracts from new-born mice or primary keratinocytes were obtained in buffer A (1% Triton X-100, 10% glycerol, 137 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 μg/ml aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM NaF, 1 mM disodium pyrophosphate, and 1 mM Na$_3$VO$_4$) or in Laemmli buffer for K10 detection. The Akt and PKCζ kinase activity assays were performed upon immunoprecipitation of the endogenous kinase proteins as previously reported (10). Western blot analysis using antibodies against K10, pRb, Akt, phosphorylated Akt and PKCζ were performed according to conventional techniques as previously described (9, 10, 15-17).
RESULTS

Epidermal abnormalities in bK5hK10 transgenic mice

To study keratin K10 functions in vivo, we have generated transgenic mice that ectopically express K10 in the basal layer of the epidermis using the bovine keratin K5 promoter (11). Five transgenic lines bearing the bK5hK10 construct and eight bearing a co-injection of the bK5Z (carrying the β-Gal gene) and bK5hK10 constructs (13) were analyzed. The ectopic expression of hK10 in the basal layer of the epidermis of newborn and adult mice was confirmed by immunohistochemistry (Fig. 1A' and 2C'). This expression was absent in non-transgenic littermates (Fig. 1A and 2C). All animals appeared healthy at birth, but by day 21 those (four founders) with high copy numbers of the bK5hK10 transgene (>10 copies) showed a clear phenotype characterized by small size, decreased weight, delayed growth and paralysis of the rear extremities. This finally led to death between days 34 and 79. In agreement with the reported copy number dependence of the transgene expression (13), animals (nine founders) with lower transgene copy numbers showed no obvious phenotypic alterations or reduction in life span. However, when the bK5hK10 transgene was brought to homozygosity in these non-phenotypic animals, all the phenotypic characteristics were again observed. Western blots demonstrated a stronger expression of hK10 in homozygous than in heterozygous transgenic mice (Fig. 1B).

Although no overt phenotype in the epidermis of newborn animals was detected (irrespective of genotype, i.e.: number of transgene copies), homozygous adult mice displayed severe histological epidermal abnormalities first detectable between days 12-21. The thickness of the epithelium was markedly reduced. In fact, the 2-3 layers of epidermal cells observed in non-transgenic littermates (Fig. 2A) was reduced to a single cell layer with flattened and elongated cells (Fig. 2A'). In addition, the stratum corneum was expanded, rendering a hyperkeratotic phenotype (Fig. 2A, A').

These phenotypic changes suggested possible alterations in the proliferation and/or differentiation program of the epidermal cells. The expression of several differentiation markers...
in the epidermis of homozygous mice were therefore studied and compared to those of non-transgenic littermates. A similar pattern of expression was found for keratin 5 (Fig. 2B, B'), Loricrin (Fig. 2D, D'), keratin K1 and Filaggrin (not shown). However, the expression of keratin K6, which is normally confined to the outer root sheath of the hair follicle (Fig. 6E), was also observed in patches in some interfollicular regions of the transgenic mice. Interestingly, these K6 positive patches also showed a more normal epidermal morphology and thickness (Fig. 2E').

To study the phenotype more closely, electron microscopic analyses were performed. In agreement with the histological studies, electron microscopy showed control animals to have a two-three cell deep epidermis with the nuclei of the basal layer regularly distributed (Fig. 2A). However, in transgenic mice, only a single cell layer was observed (Fig. 3A'). In addition, multiple abnormalities were observed in the epidermal cells of the transgenic mice. Abnormally flattened nuclei lying parallel to the basal membrane (Fig. 3B) as well as degenerative mitochondria (Fig. 3B) were frequently observed. Clear cytoplasmic areas depleted of organelles and suggestive of nuclear degeneration were observed around the nuclei (Fig. 3A' and C and data not shown). No differences were seen between control and transgenic cells in the number and structure of desmosomes and hemidesmosomes (arrows in Figs 3B and C, respectively). Finally, the keratin filaments appeared more densely packed in transgenic than in control cells (Fig. 3C), resembling the intermediate filament bundles observed in suprabasal cells expressing keratins K1 and K10 (19). However, no keratin aggregates were observed in these basal cells (Fig. 3B, C). This absence of keratin aggregates is of particular importance since they have been associated with keratin overexpression in cultured cells (20-22). In addition, aggregates have also been linked to skin disorders caused by mutant keratins, including mutations in keratin K10 that give rise to epidermolytic hyperkeratosis (23,24). In agreement with the absence of keratin aggregates, no blisters or any sign of epidermal fragility were observed in the bK5hK10 transgenic mice.
Decreased epidermal proliferation in bK5hK10 transgenic mice

Since K10 expression in cultured cells inhibits progression of the cell cycle (9,10), epidermal cell proliferation in bK5hK10 transgenic mice was analyzed. In newborn skin, significant inhibition of BrdU incorporation was seen. This was greater in homozygous than in heterozygous transgenic mice, which showed decreased BrdU incorporation compared to non-transgenic littermates (Fig. 4A, A', A'', B). A similar decrease in proliferation was also observed in the skin of adult transgenic mice, although the level of BrdU incorporation was reduced with respect to that of newborns due to the lesser proliferation of adult skin (Fig. 4C). It is important to remark that this decreased proliferation was observed even in the absence of overt phenotypic changes, as in the case of low K10-expressing heterozygous mice irrespective of their age, and in homozygous newborn epidermis (epidermal hypoplasia and hyperkeratosis appeared at day 21-30, see above).

To extend these in vivo data, the proliferative potential of primary keratinocytes derived from heterozygous, homozygous or non-transgenic newborn mice was studied. Two different parameters were considered: the capacity of these cells to incorporate BrdU, and colony forming efficiency (9,10, 15-17). Both types of experiment clearly demonstrated the diminished proliferative potential of primary keratinocytes derived from homozygous transgenic mice compared to their heterozygous counterparts. In turn, the latter showed less proliferative potential than non-transgenic littermates (Fig. 5A, B).

Since K10-induced cell cycle arrest in cultured cells is associated with reduced cyclin D1 expression and, concomitantly, decreased pRb phosphorylation (9,10), these features were analyzed by Western blotting. A marked decrease in phosphorylated pRb and cyclin D1 expression was observed alongside a parallel increase in non-phosphorylated pRb in the homozygous samples. The levels observed in heterozygous animals were intermediate between homozygous and non-transgenic mice (Fig. 5C). The mechanism underlying the inhibition of the cell cycle promoted by keratin K10 appears to be mediated, in human keratinocytes, by alterations in the PI3-K signal transduction pathway. In fact, K10 sequesters...
Akt and PKCζ to the intermediate filament cytoskeleton, and thus impairs translocation and subsequent activation (10). It was therefore investigated whether similar inhibition takes place in primary keratinocytes derived from bK5hK10 transgenic mouse epidermis. Whereas the levels of total Akt were similar in samples from the three different genotypes, those of phosphorylated, active Akt were severely reduced in heterozygous mice and barely detectable in homozygotes.

**Impaired Akt/PKB and PKCζ activities in the epidermis of bK5hK10 transgenic mice**

The reduced Akt activation in primary keratinocytes derived from bK5hK10 transgenic mice invited studies to determine whether a similar inhibition takes place in the epidermis in vivo. In agreement with previous results (10), immunofluorescence studies revealed that Akt is strongly expressed throughout all epidermal layers in the epidermis of non-transgenic and homozygous mice (Fig. 6A, A'). However, when an antibody that reacts with phosphorylated Akt was used, only the basal layer of the epidermis stained in non-transgenic animals (Fig. 6B; see also 10). This staining, indicative of activated Akt, was clearly reduced in the homozygous epidermis, suggesting a decreased Akt activation in these animals (Fig. 6B'). Confirming these data, Western blot analyses using whole skin extracts demonstrated that, although the levels of Akt were similar for all three genotypes, the amounts of phosphorylated Akt in homozygous were lower than in non-transgenic mice (Fig. 6D). Finally, the activities of endogenous Akt and PKCζ kinase were analyzed in total extracts from non-transgenic, heterozygous and homozygous mouse epidermis. Both activities were clearly diminished in the transgenic epidermis, particularly so in the homozygous extracts (Fig. 6E). These results demonstrate that the presence of K10 in skin in vivo impairs the activation of Akt and PKCζ, as in cultured human keratinocytes (10).

**K10 expression in the basal layer of epidermis results in impaired skin tumorigenesis**

The expression of K10 is rapidly downregulated in epidermis under hyperproliferative stimuli, including epidermal tumors (25). This, together with results demonstrating K10
expression inhibits cell proliferation in vivo (Fig. 3) and in vitro (Fig. 4; see also 10,11), suggests that K10 expression may affect skin tumorigenesis. This hypothesis is reinforced by two recent observations. The partial reduction in tumor development observed in transgenic mice bearing K10 under the control of the bovine keratin K6β regulatory region (26), a keratin not expressed in interfollicular epidermis but strongly induced by hyperproliferative stimuli (27). Further, we have recently reported that Akt-dependent signaling, the process that results inhibited by K10 (10), plays a major role in mouse skin carcinogenesis (28), and the simple overexpression of Akt increases the tumorigenic potential of mouse keratinocytes (28).

To confirm this, bK5hK10 transgenic mice were crossed with the sensitive TG.AC strain (18) and skin carcinogenesis experiments performed. It is worth mentioning that these experiments had to be performed in the heterozygous mice due to the early lethality observed in homozygotes. As mentioned above, these heterozygotes displayed no overt epidermal alterations, but showed a partial inhibition of keratinocyte proliferation and Akt and PKCζ activation. A clear delay in tumor onset was found in the transgenic animals with respect to their non-transgenic littermates (Fig. 7A). In addition, the number of papillomas per mouse was strikingly decreased in the transgenic group (Fig. 7B), and an average five-fold fall was found in the size of the tumors (Fig. 7C, C'). Finally, the number of carcinomas was drastically reduced in the transgenic animals (Fig. 7D), indicating that malignant conversion was also dramatically impaired (malignant conversion rate of 0.001 compared to 0.024; p=0.01). Collectively, these data demonstrate that K10 expression leads to a severe decrease in tumorigenesis and provides clear evidence for a role for K10 in preventing tumor development in vivo.
DISCUSSION

The shared functional role of keratins in providing physical resilience to epithelial cells is well established (1-4). Nonetheless, the careful tissue- and differentiation-specific expression patterns displayed by these proteins have not been yet explained in terms of specific functions. This highly regulated, accurate expression suggests additional, precise roles for the members of this complex family of proteins. Using cultured cells, the present authors have recently demonstrated that the expression of keratin K10 inhibits cell cycle progression through its ability to bind and inhibit the activation of Akt and PKCζ (9,10). In this work, these findings were extended to the in vivo situation using transgenic mice. Similar approaches have been used previously to study the possible functions of keratins (7, 12, 20, 22, 29, 30), and in most cases the observed phenotype is due to the overexpression of the transgene (20, 22, 29, 30). In agreement, severe epidermal abnormalities were found in bK5hK10 transgenic mice in close association with K10 overexpression (Figs. 2 and 3). Nonetheless, a clear biochemical phenotype was also seen, this affected keratinocyte proliferation (Figs. 4, 5 and 6) in mice that displayed no overt histological phenotype or K10 overexpression. A similar correlation between the level of expression of a keratin in transgenic mice and the corresponding phenotype has been reported for K16 (20, 22, 31). In this case, however, a clear skin phenotype characterized by a hyperkeratotic, scaly and hyperproliferative interfollicular epidermis was also demonstrated in the absence of K16 overexpression in vivo (20).

Keratins as putative modulators of cell signaling

The possibility that keratins play roles in cell signaling has long been a matter of controversy. Initially based on indirect evidence, for example the interaction of keratins with molecules implicated in signal transduction such as PKCε (32) or 14-3-3 proteins (33, 34), this possibility has now been reinforced by direct experimental data. In fact, the evidence that K8 and K18 can modulate TNF-dependent signaling (35-37), and the transitory epidermal hyperproliferation associated with increased EGF receptor phosphorylation found upon ectopic
expression of k16 in basal epidermal keratinocytes (20), clearly implies that keratins are putative modulators of cell signaling processes. In this regard, we have previously shown that keratin K10 can inhibit cell cycle progression in a pRb-dependent manner (9) through the interaction and inhibition of Akt and PKCζ (10). The present data clearly confirm this. We show that the ectopic expression of K10 reduces keratinocyte proliferation and pRb phosphorylation, and impairs activation of Akt and PKCζ both in primary keratinocytes and in epidermis in vivo (Figs 4 and 5). This implicates that, in agreement with others (38, 39), pRb lies somehow downstream from the PI-3K signaling pathway. Interestingly, we have recently reported that the PTEN tumor suppressor, which acts in opposition to PI-3K, may also prevent cell cycle progression in a pRb-dependent manner in keratinocytes (16). This is in clear parallel with K10 (9, 10).

**Functional divergence of keratins in epidermis**

Changes in keratin expression in epidermis, either under normal or pathological conditions such as tumors (25), have suggested that epidermal keratins are not functionally redundant. This was confirmed by rescuing the K14 gene ablation (8) by ectopic expression of K16 in transgenic mice (7). Rescued animals do not display the epithelial fragility of K14-deficient mice (8), but show phenotypic alterations such as alopecia, chronic epidermal ulcers and alterations in other stratified epithelia (7). This demonstrates that K14 and K16 are not functionally equivalent. In this regard, we have reported that the overexpression of keratin K14 in human keratinocytes does not affect cell proliferation, while K16 accelerates and K10 inhibits S phase entry in these cells (9). However, these data seem to be at variance with those of Wawersik and Coulombe (31), who showed alteration in keratinocyte differentiation and migration but not increased proliferation through K16 expression. This apparent controversy can be explained by the activation of specific signaling pathways and the different experimental approaches employed. In this respect, the changes in cell cycle progression due to K16 expression have only been detected under inhibitory conditions, for example with specific.
inhibitors (10) or reduced amounts of serum (9), conditions not studied in the K16-expressing primary keratinocytes. We have shown that K16 expression rescues the inhibition of cell proliferation caused by wortmannin or LY294002, two well-known inhibitors of PI-3K, but not PD98059, an inhibitor of MEK (10). This suggests that K16 might activate PI-3K-dependent signaling pathways. This activation could be due to K16-induced phosphorylation of the EGF receptor (20), which is an activator of Akt in keratinocytes (40-42). The activation of Akt could be responsible for the observed alterations in cell differentiation (43-47) and migration (48-51) reported in primary keratinocytes derived from K16 transgenic mice.

**Keratin K10 as a component of a skin tumor suppressor network.**

The present data showing impaired tumor development in mice expressing K10 ectopically in the basal layer of epidermis are important in several respects. They suggest that under certain circumstances, K10 might act as a tumor suppressor, and they emphasize the relevance of PI-3K signaling in skin tumor development. With respect to the latter, the importance of Akt signaling in mouse skin tumors has already been suggested. In fact, in transgenic mice, the expression of insulin-like growth factor, whose signaling proceeds through PI3-K and Akt (52), increases susceptibility to chemical skin carcinogenesis protocols (53). In addition, the EGFR function, as a survival factor in keratinocyte oncogenic transformation, is associated with the activation of Akt (41). More recently, we have observed that Akt activation is one of the most relevant events during mouse skin tumorigenesis (28). It was found that Akt activity increases during the promotion and progression stages of this process, preceding the increase in cyclin D1 expression. In addition, Akt overexpression increases the tumorigenic potential of mouse keratinocytes (28). The action of Akt might be related, in this context, to the phosphorylation and inactivation of GSK3β, precluding the phosphorylation and degradation of cyclinD1 (54, 55). On the other hand, the expression of cyclin D1 has been proved essential for the development of mouse skin tumors (56). In the present work it is shown that the expression of K10 in bK5hK10 transgenic mice, even in the absence of histological abnormalities, reduces
the activity of Akt (Fig. 6) and consequently the expression of cyclin D1 (Fig. 5, see also 9, 10).
This mechanism is similar to that described for PTEN in keratinocytes (16), and suggests that
K10 acts as a tumor suppressor under certain circumstances.

One of the characteristics of tumor suppressors is their loss in tumors, usually by
mutational inactivation and loss of heterozygosity. However, in some cases, the inactivation of
their expression takes place through methylation of the promoter, and thus transcriptional
silencing. A clear example of this is the ink4a locus (reviewed in 57, 58). The expression of
keratin K10 gene is drastically reduced in mouse skin tumors (25), although the mechanism
underlying this silencing is presently unknown. However, the expression of K10 appears to be
modulated by well known tumor suppressors. In particular, pRb, through cooperation with its
relative p107, induces the expression of K10 in human keratinocytes (15). More recently, we
have observed defective expression of K10 gene in ink4aΔ2,3/p21-doubly-deficient mouse
epidermis (17). Although our data showing impaired tumor development in low K10-expressing
mice (Fig. 7), and the delayed tumor formation in K6βhK10 transgenic mice (26) strongly
support the notion that K10 may function as a tumor suppressor, further studies are required. In
particular, the use of the recently developed K10-/- mouse (59) in skin carcinogenesis
experiments will help to determine this, and will clarify the roles of K10 as modulator of cell
proliferation in epidermis.

Santos et al., 15
REFERENCES

1 Fuchs, E.V. (1997). *Mol. Biol. Cell* **69**: 899-902.

2 Fuchs, E.V. and Weber, K. (1994). *Annu. Rev. Biochem.* **63**: 345-382.

3 Irvine, A.D. and McLean, W.H.I. (1999). *Br. J. Dermatol.* **140**: 815-828.

4 McLean, W.H.I. and E.B. Lane. (1995). *Curr Op. Cell Biol.* **7**: 118-125.

5 Fuchs, E.V. and H. Green. (1980). *Cell* **19**: 1033-1042.

6 Weiss, R.A.R., Eichner, R. and Sun, T.T. (1984). *J. Cell Biol.* **98**: 1397-1406.

7 Paladini R.D. and Coulombe, P.A. (1999). *J Cell Biol.* **146**: 1185-1201.

8 Lloyd, C., Yu, Q.C., Cheng, J., Turksen, K., Degenstein, L., Hutton, E. and Fuchs, E. (1995). *J Cell Biol.* **129**: 1329-1344.

9 Paramio, J.M., Casanova, M.Ll., Segrelles, C., Mittnacht, S., Lane, E.B. and Jorcano, J.L. (1999). *Mol. Cell Biol.* **19**: 3086-3094.

10 Paramio, J.M., Segrelles,C., Ruiz, S., and Jorcano, J.L. (2001a) *Mol. Cell Biol.* **21**: 7449-7459.

11 Ramírez, A., Bravo, A., Jorcano, J.L. and Vidal, M. (1994). *Differentiation* **58**: 53-64.

12 Blessing, M., Rüther U., and Franke, W.W. (1993). *J Cell Biol* **120**, 743-755.

13 Ramírez, A., Milot, E., Ponsa, I., Marcos-Gutierrez, C., Page, A., Santos, M., Jorcano, J., and Vidal, M. (2001). *Genetics* **158**, 341-350.

14 Hennings H., Michael D., Cheng C., Steinert P., Holbrook K., and Yuspa, S. H. (1980). *Cell* **19**, 245-254.

15 Paramio, J.M., Laín, S., Segrelles, C., Lane, E.B., and Jorcano, J.L. (1998) *Oncogene* **17**, 949-957.

16 Paramio, J.M., Navarro, M., Segrelles, C., Gómez-Casero, E. and Jorcano, J.L. (1999b). *Oncogene* **18**, 7462-7468.

17 Paramio, J.M., Segrelles, C., Ruiz, S., Martín-Caballero, J., Page, A., Martínez, J., Serrano, M. and Jorcano, J.L. (2001b). *J. Biol. Chem.*, **276**, 44203-44211.

Santos et al., 16
18 Leder A., Kuo A., Cardiff, R.D., Sinn E. and Leder, P. (1990). *Proc Natl Acad Sci U S A* **87**, 9178-9182.

19 Eichner, R., Sun, T.T. and Aebl, U. (1986). *J Cell Biol.* **102**, 1767-1777.

20 Paladini, R.D. and Coulombe, P.A. (1998). *J. Cell Biol.* **142**, 1035-1051.

21 Paramio, J.M. and Jorcano, J.L. (1994). *Exp. Cell Res.* **215**, 319-331.

22 Takahashi, K., Folmer, J. and Coulombe, P.A. (1994). *J. Cell Biol.* **127**, 505-520.

23 Cheng, J., Syder, A.J., Yu, Q.C., Letai, A., Paller, A.S., and Fuchs, E. (1992). *Cell.* **70**, 811-819.

24 Fuchs, E., Esteves, R.A. and Coulombe, P.A. (1992). *Proc Natl Acad Sci U S A.* **89**, 6906-6910.

25 Roop, D.R., Krieg, T.M., Mehrel, T., Cheng, C.K., and Yuspa, S.H. (1988). *Cancer Res.* **48**, 3245-3252.

26 Santos, M., Ballestín, C., García-Martín, R. and Jorcano, J.L. (1997). Mol. Carcinogenesis **20**, 3-9.

27 Ramírez, A., Vidal, M., Bravo, A., Larcher, F. and Jorcano, J.L. (1995). *Proc Natl Acad Sci U S A.* **92**, 4783-4787.

28 Segrelles, C., Ruiz, S., Perez, P., Murga, C., Santos, M., Budunova, I.V., Martinez, J., Larcher, F., Slaga, T.J., Gutkind, J.S., Jorcano, J.L., and Paramio, J.M. (2002) *Oncogene* **21**:53-64.

29 Albers, K.M., Davis, F.E., Perrone, T.N., Lee, E.Y., Liu, Y. and Vore, M. (1995). *J Cell Biol.* **128**: 157-169.

30 Casanova, M Ll., Bravo, A., Ramírez, A., Morreale de Escobar, G., Were, F., Merlino, G., Vidal, M., and Jorcano, J.L. (1999). *J. Clin. Invest.* **103**, 1587-1595.

31 Wawersik, M. and Coulombe, P.A. (2000). *Mol Biol Cell.* **11**, 3315-3327.

32 Omary, M.B., Baxter, G.T., Chou, C.F., Riopel, C.L., Lin, W.Y. and Strulovici, B. (1992). *J Cell Biol.* **117**, 583-593.

33 Ku, N.O., Liao, J. and Omary, M.B. (1998). *EMBO J.* **17**, 1892-1906.
34 Liao, J. and Omary, M.B. (1996). *J Cell Biol.* **133**, 345-357.

35 Caulin, C., Ware, C.F., Magin, T.M. and Oshima, R.G. (2000). *J Cell Biol.* **149**, 17-22.

36 Inada, H., Izawa, I., Nishizawa, M., Fujita, E., Kiyono, T., Takahashi, T., Momoi, T., and Inagaki, M. (2001) *J Cell Biol.* **155**: 415-426.

37 Gilbert, S., Loranger, A., Daigle, N., and Marceau, N. (2001) *J Cell Biol.* **154**: 763-73.

38 Brennan, P., Babbage, J.W., Burgering, B.M.T., Groner, B., Reif, K. and Cantrell, D. A. (1997). *Immunity* **7**, 679-689.

39 Klippel A., Escobedo, M.A., Wachowicz, M.S., Apell, G., Brown T.W., Giedlin, M. A., Kavanaugh, W.M. and Williams, L.T. (1998). *Mol. Cell Biol.* **18**, 5699-5711.

40 Okano, J., Gaslightwala, I., Birnbaum, M.J., Rustgi, A.K. and Nakagawa, H. (2000). *J Biol Chem.* **275**, 30934-30942.

41 Sibilia, M., Fleischmann, A., Behrens, A., Stingl, L., Carroll, J., Watt, F.M., Schlessinger, J. and Wagner, E.F. (2000). *Cell* **102**, 211-220.

42 Wan, Y.S., Wang, Z.Q., Shao, Y., Voorhees, J.J. and Fisher, G.F. (2001). *Int J Oncol.* **18**, 461-466.

43 Bang, O.S., Park, E.K., Yang, S.I., Lee, S.R., Franke, T.F. and Kang, S.S. (2001). *J Cell Sci.* **114**, 81-88.

44 Fukumoto, S., Hsieh, C.M., Maemura, K., Layne, M.D., Yet, S.F., Lee, K.H., Matsui, T., Rosenzweig, A., Taylor, W.G., Rubin, J.S., Perrella, M.A. and Lee, M.E. (2001) *J Biol Chem.* **276**, 17479-17483.

45 Jiang, B.H., Aoki, M., Zheng, J.Z., Li, J. and Vogt, P.K. (1999). *Proc Natl Acad Sci U S A.* **96**: 2077-2081.

46 Rommel, C., Clarke, B.A., Zimmermann, S., Nunez, L., Rossman, R., Reid, K., Moelling, K., Yancopoulos, G.D. and Glass, D.J. (1999). *Science.* **286**, 1738-1741.

47 Vandromme, M., Rochat, A., Meier, R., Carnac, G., Besser, D., Hemmings, B.A., Fernandez, A. and Lamb, N.J. (2001) *J Biol Chem.* **276**, 8173-8179.
48 Bakin, A.V., Tomlinson, A.K., Bhowmick, N.A., Moses, H.L. and Arteaga, C.L. (2000). J Biol Chem. 275, 36803-36810.

49 Sasaki, T., Irie-Sasaki, J., Jones, R.G., Oliveira-dos-Santos, A.J., Stanford, W.L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., Joza, N., Mak, T.W., Ohashi, P.S., Suzuki, A. and Penninger, J.M. (2000). Science. 287, 1040-1046.

50 Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R., and Yamada, K.M. (1998). Science. 280, 1614-1617.

51 Tamura, M., Gu, J., Takino, T. and Yamada, K.M. (1999). Cancer Res. 59, 442-449.

52 Kulik G., and Weber, M.J. (1998). Mol Cell Biol. 18, 6711-6718

53 DiGiovanni J., Bol D.K., Wilker E., Beltran L., Carbajal S., Moats S., Ramirez A., Jorcano J.L., and Kiguchi, K. (2000). Cancer Res. 60, 1561-1570.

54 Alt, J.R., Cleveland, J.L., Hannink, M. and Diehl, J.A. (2000) Genes Dev. 14, 3102-3114.

55 Diehl J.A., Cheng, M., Roussel, M.F., and Sherr, C.J. (1998). Genes Dev. 12, 3499-3511.

56 Robles, A.I., Rodriguez-Puebla, M.L., Glick, A.B., Trempus, C., Hansen, L., Sicinski, P., Tennant, R.W., Weinberg, R.A., Yuspa, S.H. and Conti, C.J. (1998). Genes Dev. 12, 2469-2474

57 Roussel, M.F. (1999). Oncogene. 18, 5311-5317.

58 Sharpless, N.E. and DePinho, R.A. (1999). Curr Opin Genet Dev. 9, 22-30.

59 Reichelt, J., Bussow, H., Grund, C. and Magin, T.M. (2001). Mol Biol Cell. 12, 1557-1568.
FIGURE LEGENDS

Fig. 1. Expression of human keratin K10 in the epidermis of transgenic mice. Skin sections from newborn non-transgenic (A) and transgenic (A') littermates were stained using antibody LH2 for endogenous and transgenic keratin K10 (15). Note that in the transgenic skin there is a clear positive staining of the basal layer of the epidermis which is absent in non-transgenic skin (the dashed line in A and A' denotes dermal-epidermal junction). B) Western blot analysis of whole skin extracts demonstrating the expression of human K10 in the transgenic mice and that such expression is stronger in homozygous mice than in heterozygous littermates. K5 was used to normalize the loading. Bar in A = 50µm.

Fig. 2. Epidermal abnormalities in homozygous bK5hK10 transgenic mice. (A, A') Hematoxylin-eosin stained skin sections from 21 day old non-transgenic (A) and homozygous transgenic mice (A'), demonstrating that K10 expression in basal cells leads to epidermal hypoplasia and hyperkeratosis. Insets in A: A' shows higher magnifications of the interfollicular epidermis. (B, B') Peroxidase staining showing that in non-transgenic (B) and homozygous transgenic mice (B') K5 expression is confined to basal cells. (C,C') Immunohistochemical detection of keratin K10 in non-transgenic (C) and homozygous transgenic mice (C'). (D,D') Peroxidase staining showing that in non-transgenic (D) and homozygous transgenic mice (D') Loricrin is similarly expressed in most differentiated cells. (E,E') Immunodetection of K6 in non-transgenic (E) and homozygous transgenic mice (E'). Note that K6 in non-transgenic is confined to the hair follicles, whereas in homozygous mice it is also present in interfollicular areas in association with regions showing no hypoplasia. Bars = 100µm. sc in A' denotes the thickened hyperkeratotic stratum corneum in transgenic mice.

Fig. 3. Ultrastructural analysis of epidermal abnormalities. Electron microscopy analysis of non-transgenic (A) and homozygous (A') transgenic mouse skin demonstrated the presence of a single cell layer in the transgenic epidermis. Flattened elongated nuclei lying parallel to the
basal membrane were also observed (B). Higher magnification of a transgenic keratinocyte (C) revealed the presence of degenerative mitochondria (m in B), abundant densely packed keratin filaments (kfb in C) and a clear perinuclear area devoid of cytoplasmic organelles (h in C). Also of note is the presence of normal desmosomes (arrowsheads in B) and hemidesmosomes (arrows in C) in transgenic keratinocytes bm in B and C denotes the basal membrane. Bar in A = 10µm, in B and C = 1µm.

Fig. 4. Decreased epidermal proliferation in bK5hK10 transgenic epidermis.

(A, A', A'') Examples of newborn skin sections immunostained against BrdU, showing strongly decreased labeling of basal keratinocytes in homozygous transgenic skin (A'') compared to heterozygous (A') or control (A) littermates. B) Summary of the in vivo BrdU labeling experiments demonstrating the decreased proliferation in transgenic epidermis. Note also that the effect is much more evident in homozygous than in heterozygous skin. C) Similar BrdU labeling experiments to those in (B) but using skins from 15 days old mice. Note that, although the labeling index is lower than that of newborn samples, the antiproliferative effect of K10 is evident.

Fig. 5. Impaired proliferation in primary keratinocytes.

A) Summary of five independent BrdU labeling experiments using cultured primary keratinocytes derived from non-transgenic, heterozygous and homozygous transgenic mice, showing decreased proliferation induced by K10 expression in vitro (p=0.0012 and 0.016). B) Reduced colony formation efficiency displayed by transgenic compared to non-transgenic primary keratinocytes. C) Reduced pRb phosphorylation and Cyclin D1 expression in primary keratinocytes derived from bK5hK10 transgenic mice. Total protein extracts were analyzed by Western blotting against total pRb (slow migrating bands represent differently phosphorylated pRb species), hypophosphorylated pRb and cyclin D1 as previously described (9). D) Impaired Akt activation in primary keratinocytes derived from bK5hK10 transgenic mice. The same blots
shown in C were re-blotted using antibodies against Akt and Ser473 phosphorylated Akt (active form). Data in A and B come from three to five independent experiments (in A at least 500 total cells were counted per experiment), and are shown as mean ± S.D.

**Fig. 6. Decreased Akt and PKCζ activities in bK5hK10 transgenic mouse epidermis.**

Immunofluorescence analysis of the Akt expression in non-transgenic (A) and homozygous transgenic (A') new-born mouse epidermis, showing similar staining throughout the entire epidermis in both cases. B, B') The same field as in A, A') showing that the expression of phosphorylated (active) Akt is constrained to the basal layer in non-transgenic mice (B) and that staining is severely reduced in homozygous transgenic epidermis (B'). C, C') DAPI counterstaining of the sections. D) Western blot of whole skin extracts from the quoted genotypes demonstrating the decrease in phosphorylated Akt content in homozygous mice epidermis. E) Kinase assays of the immunoprecipitated endogenous Akt (upper panel) and PKCζ (middle panel) demonstrating the inhibition of both kinase activities in homozygous, and to a lesser extent in heterozygous, transgenic bK5hK10 epidermis. The lower panel shows the Western blot of the immunoprecipitated PKCζ, demonstrating that this enzyme is expressed to a similar level in all the genotypes. Bar in C = 10µm. Dashed line in A, A' denotes the dermal-epidermal junction.

**Fig. 7. Kinetics of tumor development in double (TG.AC/bK5hK10) or single (TG.AC) transgenic mice.**

Sixteen animals corresponding to each genotype were used in chemical carcinogenesis experiments. TPA was applied topically for eight weeks and the number of tumors arising in each mouse recorded at regular intervals. A) Kinetics of tumor appearance in TG.AC single or TG.AC/nK5hK10 double transgenic animals with a significant delay in the development of tumors in the presence of K10. B) The number of papillomas per mouse is also significantly reduced in TG.AC/nK5hK10 double transgenic animals. C, C') Distribution of total papillomas.
according to size and weeks after starting TPA treatment in TG.AC (C) and TG.AC/nK5hK10 double transgenic animals (C'). D) The number of mice bearing malignant squamous cell carcinomas (SCCs) demonstrates that the rate of malignant conversion is also severely impaired by hK10 expression.
ACKNOWLEDGEMENTS

We are greatly indebted to J. Martínez for excellent animal care, I. de los Santos for assistance in histology preparations, S. Moreno for help in photography and C. Murga for her help with the kinase assays. Special thanks to J.S. Gutkind and C.J. Conti for their helpful comments. This work was partially funded by grant PB94-1230 from the Spanish DGICYT (Dirección General de Investigación Científica y Tecnológica)
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6
Figure 7
The expression of Keratin K10 in the basal layer of the epidermis inhibits cell proliferation and prevents skin tumorigenesis

Mirentxu Santos, Jesus M. Paramio, Ana Bravo, Angel Ramirez and José L. Jorcano

J. Biol. Chem. published online March 11, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201001200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts