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Targeting expression of keratinocyte growth factor to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice

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Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor (FGF) family. Synthesized by cells of the dermal component of skin, KGF's potent mitogenic activity is on the epidermal component, which harbors the receptors for this factor. To explore the possible role of KGF in mesenchymal-epithelial interactions in skin, we used a human keratin 14 promoter to target expression of human KGF cDNA to the stratified squamous epithelia of transgenic mice. Mice expressing KGF in their epidermis typically appeared frail and weak, and often had grossly wrinkled skin. These mice exhibited a gross increase in epidermal thickness accompanied by alterations in epidermal growth and differentiation. Most remarkably, animals displayed several striking and unexpected changes, including a marked suppression of hair follicle morphogenesis and suppression of adipogenesis. With age, some animals developed gross transformations in the tongue epithelium and in epidermis. In addition, they exhibited elevated salivation and their salivary glands showed signs of altered differentiation. Collectively, our findings provide new and important insights into the roles of KGF, implicating this potent growth factor in eliciting global effects not only on growth, but also on development and differentiation, of skin and other tissues. In particular, KGF seems to interfere with signalling of some mesenchymal-epithelial interactions.

Key words: epidermis/hyperproliferation/keratinocyte growth factor/terminal differentiation

Introduction

Keratinocyte growth factor (KGF) is made by stromal fibroblasts and belongs to the seven-member fibroblast growth factor (FGF) family (Finch *et al.*, 1989; Aaronson *et al.*, 1991). KGF is synthesized as a single peptide-containing precursor that is cleaved upon secretion to liberate mature KGF (Finch *et al.*, 1989). It differs from other FGFs in that its mitogenic activity appears to be specific for keratinocytes, and not fibroblasts or endothelial cells (Finch *et al.*, 1989). This paracrine pathway for stimulating keratinocyte growth is activated by an alternative splicing variant of the surface receptor FGFR2, whose tyrosine kinase is inducible by either KGF or aFGF (Rubin *et al.*, 1989; Bottaro *et al.*, 1990; Miki *et al.*, 1992; Peters *et al.*, 1992). *In vitro* studies have shown that the stimulating effects of KGF on DNA synthesis in keratinocytes is 2- to 10-fold stronger than that of autocrine keratinocyte growth factors such as TGFα and EGF (Rubin *et al.*, 1989). Together with the finding that the expression of KGF mRNA (and presumably protein) is dramatically elevated after skin injury (Werner *et al.*, 1992), KGF appears to be a major factor in promoting epidermal cell proliferation. Little is known about the possible effects of KGF on other cell types, organs or tissues, although the FGFR2 receptor is expressed in epithelial tissues at times in embryonic development when epidermal-mesenchymal interactions may play a role in organogenesis (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992).

The skin is a complex organ, composed of many different cell types. The major structural components of the skin are the superficial epidermis, its appendages, the underlying dermis and the cutaneous fat (Fitzpatrick *et al.*, 1987). To coordinate and maintain the appropriate proportions and differentiation states of cell types within the skin, there must be intricate and intertwined mechanisms of intercellular signalling. In this regard, it is well established that certain factors produced and secreted by mesenchymal cells are necessary for epidermal cells to grow, develop and differentiate (Wessels, 1977; Dhouailly *et al.*, 1978; Hardy *et al.*, 1983; Jahoda *et al.*, 1984). Mesenchymal factors that are likely to play a role in this process include extracellular matrix components (Bissell *et al.*, 1982), cell surface-associated proteins (Sariola *et al.*, 1988; Hirai *et al.*, 1992) and soluble growth factors such as KGF (Finch *et al.*, 1989; Orr-Urtreger, 1991; Peters *et al.*, 1992).

Little is known about the mechanisms by which autocrine and paracrine growth factors act in concert to orchestrate the balance between growth and differentiation within a specialized cell type of a tissue. Recently, we utilized the human keratin 14 (K14) promoter to over-express an autocrine growth factor, TGFα, in the epidermis of transgenic mice (Vassar and Fuchs, 1991). At birth, TGFα mice were frail, had prematurely open eyelids and exhibited wrinkled skin that developed flakiness within several days. Epidermal hyperproliferation and thickening also occurred, and animals were smaller than normal (Vassar and Fuchs, 1991). Interestingly, these phenotypic differences disappeared at ~5 weeks into postnatal development, concomitant with EGF receptor downregulation (Green *et al.*, 1983; Vassar and Fuchs, 1991).

In the present study, we focused on (i) exploring the role of KGF in mesenchymal-epithelial interactions and in keratinocyte growth and differentiation, and (ii) comparing the roles of paracrine and autocrine growth factors in epidermal growth and differentiation. To achieve these goals, we used the same human K14 promoter previously employed for our TGFα studies, this time engineering transgenic mice that artificially expressed human KGF in their keratinocytes. This converted the action of KGF from paracrine to autocrine. Our results have uncovered some interesting and

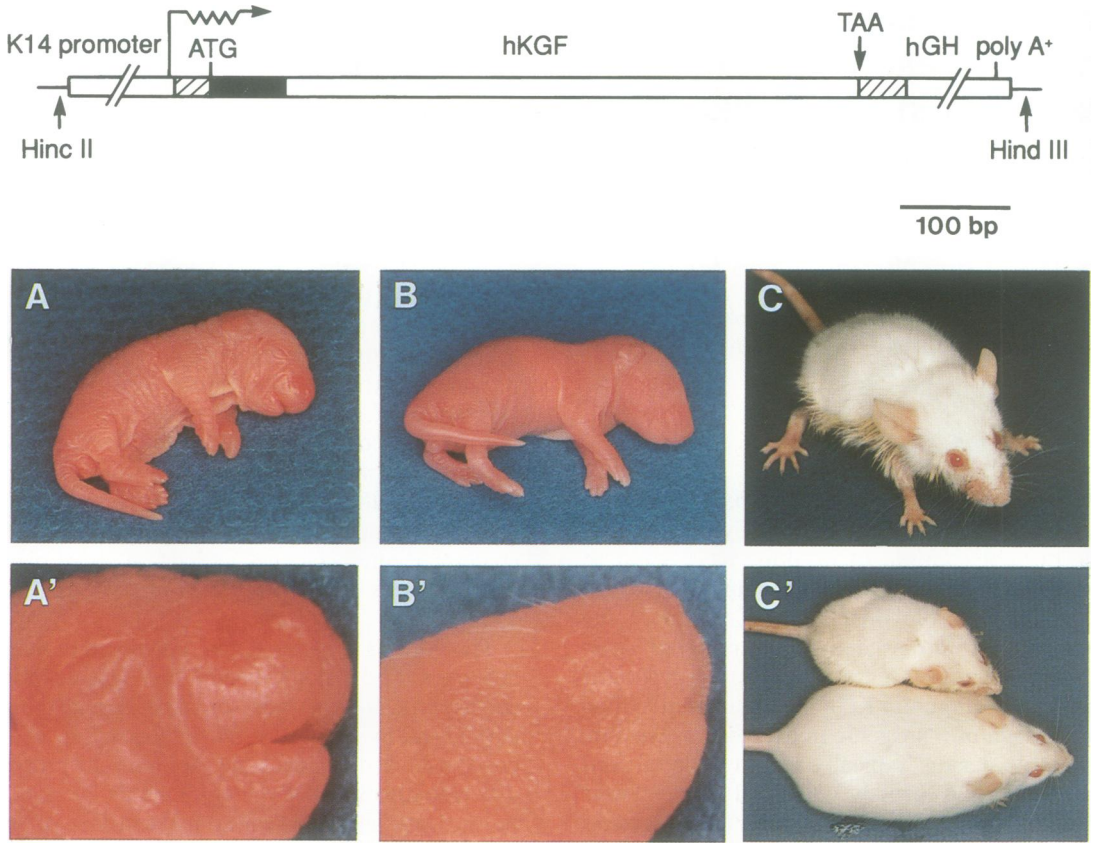

Fig. 1. Transgene construct and K14-KGF transgenic mice. (**Top**) Human KGF (hKGF) cDNA was cloned into the *Bam*HI site of an expression vector containing the human K14 promoter/enhancer (Vassar *et al.*, 1989). Human growth hormone (hGH) sequences were also inserted into the *Xba*I site of this vector and used as intron-containing, 3′ noncoding sequences and as a source of a polyadenylation signal. Abbreviations: ATG, translation start codon; TAA, termination codon; poly(A)$^+$, polyadenylation signal. (**A** and **A′**) Representative example of a newborn K14-KGF transgenic mouse (KGF-NB2) exhibiting a severe phenotype. Note grossly wrinkled skin and defined facial features relative to newborn control mouse in (**B**) and (**B′**). Note also that the transgenic mouse does not have whiskers (compare A′ with B′). (**C** and **C′**) A representative example of an ~4 month old K14-KGF transgenic mouse (KGF-m86), relative to control littermate. Transgenic animals that reached adulthood were smaller than normal and exhibited fewer hairs around the eyelids, nose, limbs and abdomen. Note also that these animals had a wet undercoat, due to excessive salivary secretions.

major differences between KGF- and TGFα-mediated regulation of keratinocyte growth and differentiation, which included the appearance of gross transformations in tongue and epidermis of KGF-expressing animals as they aged. Perhaps even more importantly, our studies have yielded some completely unexpected and dramatic effects of keratinocyte KGF expression on hair follicle morphogenesis, adipogenesis and salivary gland differentiation, providing new and intriguing insights into the mechanisms underlying cell fate choices during development.

Results

Newborn transgenic mice expressing keratin promoter-driven KGF exhibit grossly wrinkled skin and reduced hair follicle density

The construct used in generation of our transgenic mice is outlined in Figure 1. It has previously been shown that ~2100–2300 bp of 5′ upstream sequence of the human K14 gene was sufficient to drive expression of reporter genes in the basal cells of most stratified squamous epithelia, including epidermis (Vassar *et al.*, 1989; Vassar and Fuchs, 1991). This promoter was selected to generate transgenic mice that could express human KGF cDNA in a keratinocyte-specific fashion. Human KGF cDNA was cloned from WI-38 human fibroblast RNA using reverse transcription and polymerase chain reaction (PCR). The clone was sequenced in its entirety and corresponded exactly to the published sequence (Finch *et al.*, 1989). The strategy of introducing human growth hormone (hGH) sequences as an intron-containing 3′-non-coding segment and polyadenylation signal was employed previously as a necessary measure to generate appreciable levels of transgene messages (Sandgren *et al.*, 1990; Vassar and Fuchs, 1991; Cheng *et al.*, 1992). The K14/KGF/hGH fusion gene was then used to generate transgenic mice as described previously (Vassar *et al.*, 1989).

Thirteen newborn mice from females carrying K14-KGF injected embryos were extremely frail, and either died or were sacrificed within 12–24 h of birth. These mice exhibited pronounced phenotypic aberrations (Figures 1A and 1A′, KGF NB-2†; compare with 1B and 1B′, control). Most notably, their skin was significantly wrinkled. In addition, the eyelids of transgenic animals were more pronounced than those of control littermates, although neither transgenic nor control eyelids were open at birth (Figure 1A′ and 1B′). Finally, the whiskers of wrinkled skin transgenic mice were often sparser than those of control animals, and in two cases, they were absent (Figure 1A′; compare with

†Abbreviations used in transgenic mice designations are: NB, newborn; m, male of ≥12 days; f, female of ≥12 days.

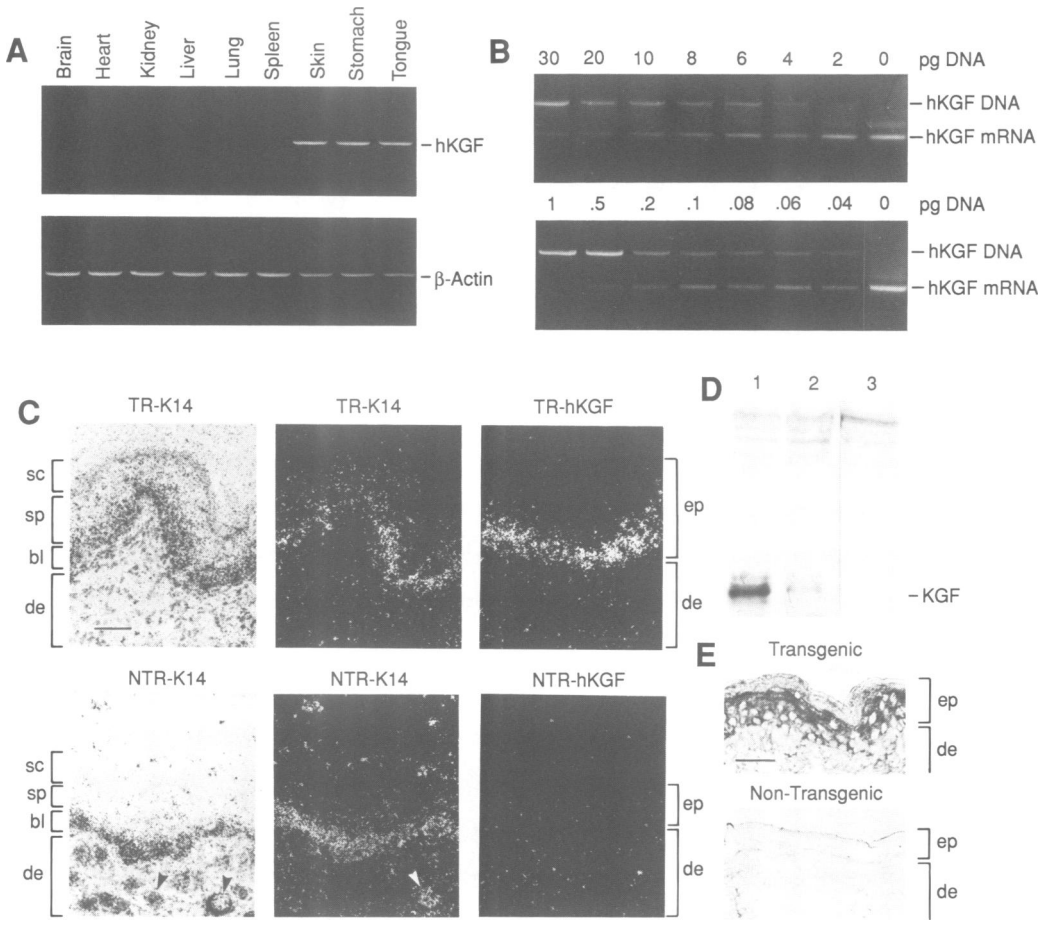

Fig. 2. Detection of hKGF mRNA and protein expression. (**A**) PCR analysis. Total RNAs were isolated from the organs of three transgenic and control mice (Chomczynski and Sacchi, 1987). PCR bands specific for hKGF and β-actin mRNAs were generated and resolved by agarose gel electrophoresis (Materials and methods). Bands were visualized by staining with ethidium bromide. Organ RNAs from which the PCR bands were generated are indicated above each lane. Ordering of samples in two gels was identical. Shown are data from KGF-NB7; data from KGF-NB8 were identical and those from KGF-m7 were similar with respect to tissue-specificity, but with reduced KGF signals. (**B**) Competitive PCR analysis of skin RNAs from different transgenic animals. Total skin RNAs were isolated from three frail newborn transgenic animals and three transgenic animals that reached adulthood. Competitive PCR analysis was carried out as described in Materials and methods. To compare relative levels of transgene RNAs, varying amounts of K14-hKGF-hGH DNA (in picograms, as indicated above each lane) were added to identical aliquots containing total cDNA made from a particular skin RNA, and oligonucleotide primers spanning an intron of hGH. In a PCR reaction, these primers generated a 1048 bp fragment from the transgene genomic DNA and a 492 bp fragment from the transgene cDNA. Shown are data from two of the six assays; top (KGF-NB13, a mouse with severely wrinkled skin); bottom (KGF-m86, mouse with milder phenotype). From these data, gels were scanned and matched molar amounts of gene and cDNA bands were used to estimate the pg hKGF mRNA/μg total skin RNA (see Table I). (**C**) *In situ* localization of transgene and K14 mRNAs. Skins from a newborn transgenic, KGF-NB2 (TR, top panels) and nontransgenic littermate (NTR, bottom panels) were fixed in 4% paraformaldehyde, sectioned (5 μm) and processed for *in situ* hybridization (Vassar and Fuchs, 1991). Sections were hybridized with [^{35}S]UTP-labelled cRNA probes complementary to (i) a 1.1 kb segment of human K14 mRNA (K14) that shares very high homology with mouse K14 mRNA (Vassar *et al.*, 1989) and (ii) the 1.9 kb hKGF/hGH transgene mRNA (hKGF). After hybridization, sections were washed, dipped in Kodak NTB2 liquid emulsion, and stored in the dark for 4 days at 4°C before developing. Localization of exposed silver grains was examined by brightfield (first panel of each set) and darkfield microscopy (last two panels of each set). Bar in upper left panel represents 12 μm for all panels. de, dermis; bl, basal epidermal layer; sp, spinous layers; sc, stratum corneum. Arrowheads denote hair follicles, which also express K14. (**D**) Immunoblot analysis. Sections (2 cm^2) from the skins of K14-KGF transgenic animals were processed for extraction of heparin–Sepharose-binding proteins as described in Materials and methods. Half of the sample was resolved by SDS–PAGE and subjected to immunoblot analysis, using an anti-KGF antiserum against a KGF peptide common to hKGF and mKGF. Samples are from: lane 1, KGF-NB11 wrinkled skin; lane 2, KGF-NB12 wrinkled skin; lane 3, a nontransgenic control littermate. Note that under the conditions used, endogenous mouse KGF was not detected (lane 3), indicating that the levels of transgene keratinocyte hKGF were substantially higher than endogenous levels. (**E**) Immunohistochemistry. Skin sections from three different newborn mice with wrinkled skin, KGF-NB9, KGF-NB11 and KBF-NG12 (shown at top is section from NB9), and a control littermate (bottom) were stained with anti-KGF antiserum. Bar represents 48 μm. ep, epidermis; de, dermis.

1B′). All of these mice tested positive for the transgene, as judged by PCR analysis of their tail DNAs.

Five additional animals appeared nearly normal at birth. These animals survived to adulthood. By ~5 days after birth, however, they began to lag in weight gain and produce excessive saliva, phenomena which persisted throughout life (Figure 1C, KGF-m86 at ~4 months of age—note wet abdominal fur due to salivation; Figure 1C′, the smaller KGF-m86 mouse and its non-transgenic littermate). As the animals grew, hairs appeared notably sparse around the eyes, limbs, nose and abdomen (Figure 1C). Curiously, some skin regions, such as the back, seemed thinner than normal, whereas other regions, such as the eyelids, seemed thicker than normal (see below). Only one of these mice was bred successfully, and this animal had one of the mildest phenotypes. These mice all tested positive for the transgene, and all eventually became frail.

The phenotypic abnormalities observed in the K14-KGF

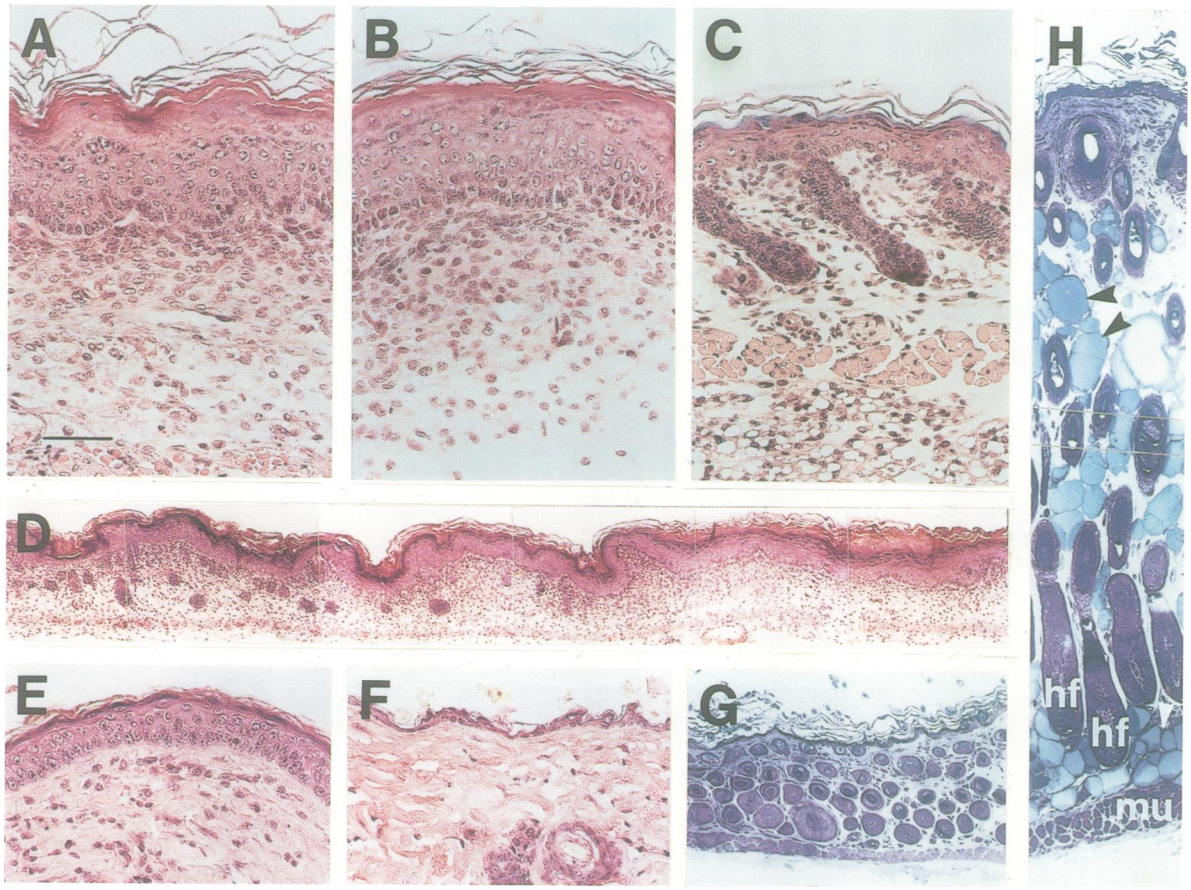

Fig. 3. Histology of transgenic and normal control skins. Skin samples were taken from representative examples of: the back of two different newborn full-transgenic KGF mice exhibiting a severe, wrinkled skin phenotype (panels **A** and **B**; KGF-NB4 and KBF-NB2, respectively); the back of a control newborn littermate (panel **C**); the back of a mosaic newborn KGF mouse exhibiting alternating patches of wrinkled and normal skin phenotypes (mouse KGF-NB12, panel **D**); the abdomen of an ~4 month old KGF mouse (KGF-m86) exhibiting a thin skin phenotype (panel **E**); the abdomen of an ~4 month old control littermate (panel **F**); the back of a 12 day old KGF mouse exhibiting a thin skin phenotype (F_1 offspring of KGF-m11, panel **G**); the back of a 12 day old control littermate (panel **H**). Skin samples were either placed in Bouin's fixative, for subsequent staining with hematoxylin and eosin (A–F), or embedded in epoxy resin for semi-thin (0.75 μm) sectioning followed by staining with toluidine blue (G and H). Notes: (i) for all KGF-expressing transgenic animals and in all body locations, a striking inverse correlation existed between epidermal thickening and hair follicle density; (ii) none of the KGF-expressing animals had fat pads, or cutaneous fat; this occurred even in the backskin of animals expressing low levels of KGF, which showed little evidence of epidermal or hair follicle changes (panel G). Abbreviations: hf, hair follicles; mu, muscle. Arrowheads in H denote adipocytes. Bar represents 48 μm in A–C, E and F; 200 μm in D, and 100 μm in G and H.

mice were markedly different from those seen in over-expressing K14-TGFα mice (Vassar and Fuchs, 1991). When newborn, TGFα animals had open eyelids; KGF mice did not. Whereas the TGFα mice exhibited flaky skin, the KGF mice did not. Wrinkling of skin occurred in both TGFα and KGF newborn animals, but was generally more severe in the K14-KGF mice. In addition, while both types of animal exhibited an apparent stunting of hair growth, this was manifested in strikingly different fashions: In TGFα mice, the only major aberration in hair was a delay in the first hair growth cycle, whereas in KGF mice, it was an age-independent sparsity of hair, which varied regionally in some mice, and in others seemed nearly absolute. Finally, while both TGFα and KGF mice were unusually small as juveniles, only adult KGF mice remained small as adults.

Phenotype correlates with transgene expression in K14-KGF mice

To verify that the atypical features of KGF mice were due to targeted expression of the human KGF (hKGF) transgene, total RNAs were isolated from nine different tissues of two newborn mice exhibiting wrinkled skin and one adult mouse exhibiting the milder phenotype. Following cDNA synthesis, PCRs were conducted using primers spanning the junction of hKGF and hGH sequences. As expected, relative to a control β-actin band, the diagnostic hKGF band was only produced from RNAs of transgenic organs with stratified squamous epithelial tissues, including skin, forestomach and tongue (Figure 2A). The specificity of the primers for transgene mRNA was verified by the absence of this band in all RNA reactions from non-transgenic tissues (not shown).

Based on the criteria that five surviving transgenic animals were less frail at birth and that the phenotypic effects on their skin were less, we anticipated that these animals expressed less KGF than the others. To determine the relative amounts of KGF mRNAs produced by the transgenic mice, we used competitive PCR analysis (Siebert and Larrick, 1992) on reverse transcribed cDNA from 1 μg total RNA isolated from skins of three of the most severely wrinkled mice and three mice with a range of milder phenotypes. By titrating in varying amounts of transgene genomic DNA into the cDNA mixtures, and using oligonucleotide primers spanning an intron within the hGH sequence, a PCR reaction

Table I. Competitive PCR determination of transgene mRNA levels in KGF mouse skin

	Molar ratios between amplified plasmid DNA and cDNA ([pDNA]:[cDNA])								Estimated range (pg) of hKG-hGH mRNA per μg of total RNA	
Plasmid DNA added (pg)	0.8	0.6	0.4	0.2	0.1	0.08	0.04	0	From	To
KGF-f29	–	7.3	8.9	8.0	3.8	1.4	0.9	0	0.005	0.01
KGF-m72	8.1	6.0	4.8	2.3	1.3	1.0	0.3	0	0.01	0.013
Plasmid DNA added (pg)	1	0.5	0.2	0.1	0.08	0.06	0.04	0		
KGF-m86	3.7	1.8	0.7	0.4	0.4	0.3	0.3	0	0.025	0.065
Plasmid DNA added (pg)	30	20	10	8	6	4	2	0		
KGF-NB4	0.7	0.3	0.3	0.3	0.2	0.1	0.1	0	>4.0	
KGF-NB9	1.1	0.9	0.6	0.5	0.4	0.3	0.2	0	2.6	4.0
KGF-NB13	1.6	1.1	0.8	0.7	0.5	0.5	0.3	0	1.3	2.6

The number of picograms of transgene mRNA per microgram of total skin RNA was calculated as described in Materials and methods (Siebert and Larrick, 1992). Values assume 100% efficiency of first strand cDNA synthesis of RNA. Molar ratios of [pDNA]:[cDNA] were determined by densitometer scanning of negative images of ethidium bromide-stained gels and adjusting for molarities. Phenotype severity was: KGF-NB4, KGF-NB13 > KGF-NB9 > KGF-m86 > KGF-m72, KGF-f29. KGN-NB13 was a mosaic.

could be generated where the molar amounts of bands generated from transgene DNA (1048 bp) and transgene cRNA (492 bp) were comparable, providing an estimate of the hKGF mRNA present in the mixture. Figure 2B illustrates two examples of such reactions (top set, reaction from wrinkled skin KGF mouse; bottom set, reaction from KGF mouse with milder phenotype). Table I provides the data from all six animals, after densitometry scanning and adjusting for molar rather than picogram ratios. From these data, it was clear that the relative levels of KGF transgene mRNA per microgram of total mRNA from the three severely wrinkled transgenic skins were substantially higher than those from the three phenotypically milder skins. Among the mice with milder phenotypes, a correlation existed between severity of phenotype and level of transgene expression.

To examine the location of transgene mRNA in skin, we conducted *in situ* hybridizations with radiolabelled cRNA specific for K14 mRNA and for hKGF-hGH mRNA (Figure 2C). As expected, both for transgenic and for control skin, K14 mRNAs were localized to the inner-most layers of the epidermis (upper left and middle frames, brightfield and darkfield, respectively, of a wrinkled skin KGF transgenic; lower left and middle frames, brightfield and darkfield, respectively, of a non-transgenic littermate). The K14 cRNA labelling seemed to extend higher in transgenic than in control skin, suggesting that the character of the inner suprabasal layers of transgenic epidermis might be more basal-like than those of control epidermis. Further studies were consistent with this notion.

In the wrinkled skin KGF transgenic, the pattern of transgene expression paralleled that of K14 mRNA expression (Figure 2C, upper right frame, hKGF probe, compare with upper middle frame, K14 probe). Labelling with the hKGF probe was not appreciable in control skin (lower right frame), a feature which most probably reflected a combination of (i) 9% sequence variation throughout the coding segment of hKGF and mKGF (Dr Clive Dickson, Imperial Cancer Research Fund, London, UK, personal communication); and (ii) substantially higher expression of transgene hKGF than endogenous mKGF mRNA levels. Finally, we were unable to generate appreciable hKGF mRNA hybridization signals in transgenic animals with milder phenotypes, consistent with our PCR quantification data indicating that the levels of hKGF mRNA in these animals were significantly lower than in the wrinkled skin animals.

To verify that the transgene mRNA was translated *in vivo*, we isolated heparin–sepharose-binding proteins, including KGF, from skins (~2 cm^2) of transgenic and control littermates (Rubin *et al.*, 1989). SDS–PAGE was then conducted on one half of these samples, followed by immunoblot analysis using a KGF antibody directed against a C-terminal peptide shared by both human and mouse KGFs (kindly provided by Dr Sabine Werner and Dr Lewis T.Williams). An immunoreactive band of 28 kDa was present in extracts of two wrinkled skin transgenic animals (Figure 2D, lanes 1 and 2). This band was of the expected size of mammalian KGF, which is known to be glycosylated (Rubin *et al.*, 1989). The band was not present in skin extracts of non-transgenic animals (lane 3), suggesting that it corresponded to human transgene KGF, rather then endogenous KGF. We were unable to detect appreciable hKGF in skin extracts from two of the mice expressing less KGF, consistent with RNA data indicating that transgene expression was markedly lower in these mice.

To localize the 28 kDa anti-KGF-reactive protein, we stained frozen skin sections with anti-KGF (Figure 2E). As expected, staining was prominent in the epidermis, but not dermis, of mice that exhibited high, but not low, expression of hKGF mRNA (shown is an example from representative mouse KGF-NB9, top; control, bottom). Staining of skins from three different transgenic animals revealed similar staining patterns. The extension of staining in suprabasal layers indicated that at least some of the KGF protein made from basal KGF mRNA persisted in spinous cells. The surprisingly lower level of staining in basal cells may be reflective of the smaller cytoplasm:nucleus ratio in these

cells. It is also possible that antigenic masking of the peptide determinant occurred in a basal-specific fashion.

The failure to detect appreciable staining in the dermis was consistent with our immunoblot data. Given the lack of species specificity of the peptide used to generate the anti-KGF antiserum, the results further demonstrated that the level of epidermal human KGF was substantially higher than that of dermal mouse KGF in these animals. Finally, these findings were consistent with our RNA data indicating a correlation between the level of transgene expression and the severity of phenotype.

Epidermal KGF expression causes hyperthickening of the epidermis and suppression of both hair follicle morphogenesis and adipogenesis

Histopathology of the mice expressing high levels of KGF revealed gross abnormalities in the skin (Figure 3). The epidermis was markedly thickened (panels A and B, skin from KGF-NB4 and KGF-NB2, respectively; panel C, nontransgenic newborn control). One or two layers of basal-like cells were present, and the spinous and granular layers were increased greatly relative to control epidermis. The stratum corneum was moderately increased. Most strikingly, the density of cells within the epidermis was markedly elevated, with extremely tightly packed inner layers of highly columnar cells. Cells were atypically crowded, and appeared somewhat immature, with a larger nucleus to cytoplasm ratio than normal. This was in contrast to that of TGFα-overexpressing epidermis, where packing of basal cells appeared looser than normal and where the nuclear to cytoplasmic ratio was lower than normal (Vassar and Fuchs, 1991).

Skins of two KGF mice showing the most severe phenotype were nearly devoid of hair follicles (examples of KGF-NB4 and KGF-NB2, respectively, shown in panels 3A and B). This was even more apparent in skins of two seemingly mosaic mice, which exhibited alternating stretches of thickened epidermis and no hair follicles, followed by thinner epidermis and hair follicles (example of Figure 3D, KGF-NB12). In these animals, there was a gradient rather than a sharp demarcation in phenotype, suggesting that the local effects of KGF may have extended to neighboring epidermal cells. Examination of additional newborn transgenic animals revealed a good inverse correlation between the degree to which hair follicle density was reduced and the corresponding increase in epidermal thickness.

In transgenic animals expressing less KGF, this inverse correlation held, although the phenomenon seemed more sensitive to regional variations. Thus, for example, while backskin epidermis of these animals was comparable in thickness to control epidermis, abdominal epidermis was thicker and had fewer hair follicles than control samples (Figure 3E, KGF-m86; 3F, control). The regional variations at the histological level correlated well with those differences observed previously by visual inspection (see Figure 1C). Overall, analysis of these animals confirmed that the severity of aberrations in the epidermis and in hair follicle number were directly related to the level of transgene expression. The inverse correlation in epidermal thickening versus follicle density was interesting, given that the choice between hair follicle and epidermal morphogenesis is made in embryonic basal cells at a time during development when K14 is first expressed (see Kopan and Fuchs, 1989). The effects on hair follicle density seemed unique to K14-KGF mice and were not observed in K14-TGFα mice (Vassar and Fuchs, 1991).

Two pronounced and unexpected aberrations of KGF mice occurred in cells of fibroblast, rather than epithelial, origin. One was an increase in dermal cellularity seen directly beneath the hyperthickened epidermis of KGF mice (see e.g. Figure 3A and B). Extensive ultrastructural analyses of the dermis of these skins indicated that this increase in cellularity was primarily due to the presence of immature fibroblasts, with no appreciable changes in immune cells or mature fibrocytes. Another difference was a marked absence of fat. When dissected, adult KGF mice showed no intrascapular, perirenal or subcutaneous fat pads (data not shown). The skin of these animals was also notably thinner than control animals, and semi-thin sectioning confirmed that this difference was primarily attributed to the absence of fat (Figure 3G, F_1 offspring of KGF-m11, 12 days; panel H, control; arrowheads in H denote fat, which is missing from skin in G). Since these mice also began to show weight differences at $\sim 5-7$ days after birth, i.e. a time when fat begins to accumulate, it seems likely that adipogenesis was suppressed from the beginning in these animals. Paradoxically, in some mice expressing lower levels of KGF, the absence of fat caused the density of hair follicles to be greater in regions, e.g. backskin, where effects on epidermis and follicle number were not appreciable (Figure 3, compare panel G with H). These findings suggested that fat production must be especially sensitive to keratinocyte-mediated KGF expression, and that the effects on fat occurred even in regions of the body where there was no apparent effect on keratinocyte growth and differentiation.

The effects on adipogenesis and dermal cellularity were not seen in young K14-TGFα mice (Vassar and Fuchs, 1991). Moreover, they were unanticipated, since in contrast to TGFα or EGF receptors, high affinity KGF receptors have not been detected on fibroblasts *in vitro* (Bottaro *et al.*, 1990). These unusual results suggested that either dermal cells *in vivo* respond differently to KGF than NIH 3T3 cells *in vitro*, or the *in vivo* effects were an indirect, rather than direct, consequence of KGF expression in the overlying keratinocytes.

Electron microscopy studies: a closer look at KGF epidermis

Ultrastructural analysis enabled a more detailed investigation of terminal differentiation in the thickened skin regions of K14-hKGF-expressing transgenic mice (Figure 4). Skin samples from six different founder mice were analyzed ultrastructurally, and the findings were consistent. Panel A illustrates a section of backskin epidermis from a KGF mouse with wrinkled skin. Cells were very tightly packed with virtually no intercellular spaces. In addition, a number of transgenic suprabasal cells appeared similar to those in the basal layer.

The program of terminal differentiation was also aberrant. Transgenic cells that reached the skin surface often retained many of their organelles, including the nucleus (N, panel B) and mitochondria (arrows, panel C). This was in contrast to control cells, which were largely flattened and enucleated at this stage (panel D). In the upper transgenic layers, keratohyalin granules (KH) were atypically large (compare panels B and C with D). In addition, transgenic epidermal

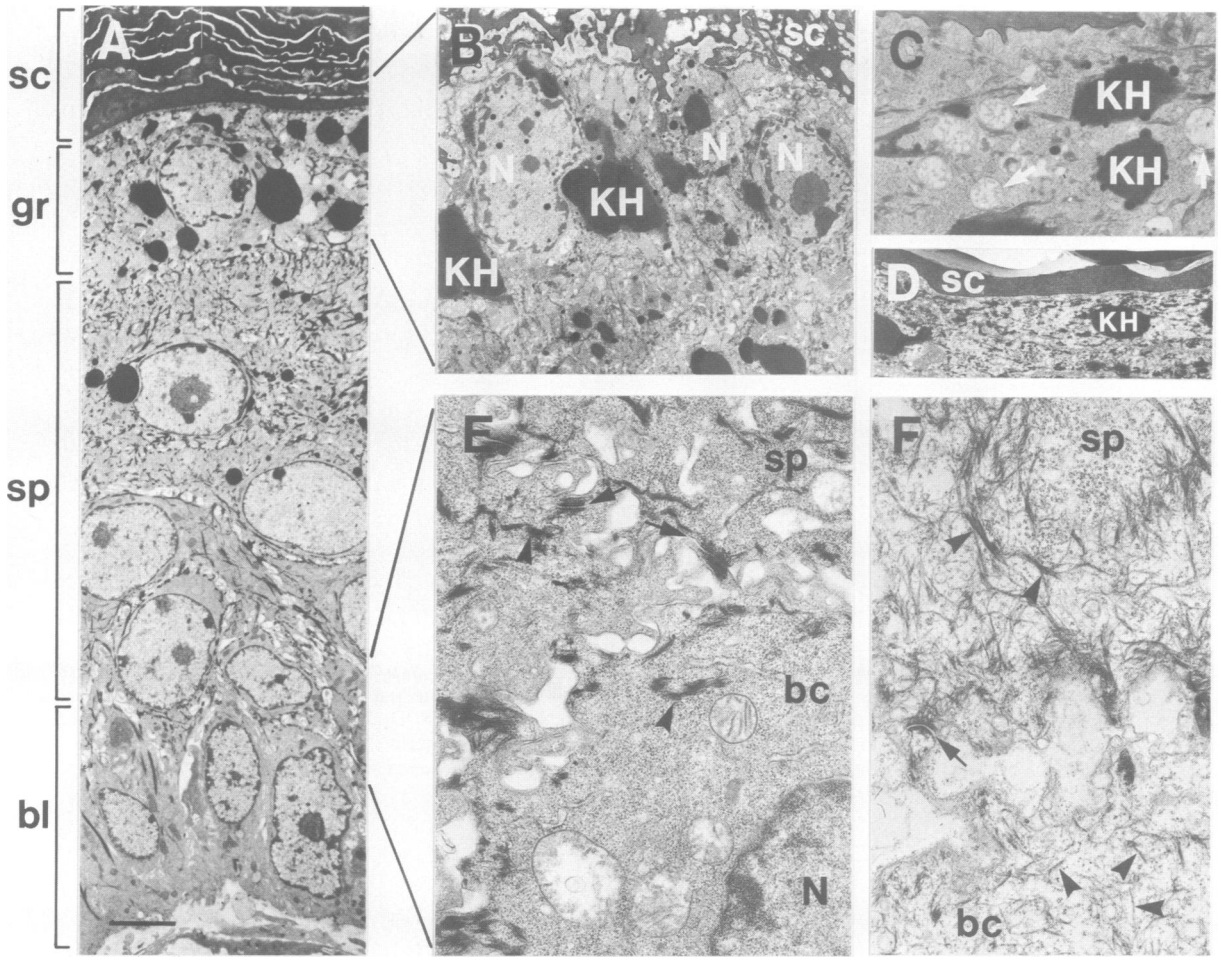

Fig. 4. Electron microscopy of skin sections from a newborn K14-KGF transgenic mouse expressing a severe, wrinkled skin phenotype. Skin samples (2 mm^2) from two newborn KGF mice (KGF-NB7 and KGF-NB2) expressing a severe, wrinkled skin phenotype and from a control newborn mouse were fixed and processed for electron microscopy (Coulombe *et al.*, 1989). Panel **A**, section of KGF-NB7 illustrating transgenic epidermis. bl, basal layer; sp, spinous layers; gr, granular layers; sc, stratum corneum. Panels **B** and **C**, higher magnifications of representative granular cells from KGF-NB7 and KGF-NB2, respectively, illustrating atypical presence of: panel B, nuclei (N) and enlarged keratohyalin granules (KH), and panel C, mitochondria (arrows) and enlarged KH. Panel **D**, granular layers of epidermis of newborn control for comparison. Panels **E** and **F**, higher magnifications of representative regions of the basal/spinous junction of KGF-NB7 epidermis (E) and control (F), illustrating presence of many fewer keratin filaments (arrowheads) in cytoplasm of transgenic suprabasal cells. Note also the typical increase in filament number and filament aggregation as cells leave the basal layer. sp, spinous cell; bc, basal cell. Arrows denote desmosomes; N, nuclei. Bars represent 5.5 μm in (A), 2.7 μm in (B and D), 1.2 μm in (C), and 0.7 μm in (E and F).

cells in all layers displayed fewer keratin filaments than their control counterparts (see e.g. panel E, transgenic epidermis at the basal, bc, and spinous, sp, junction; panel F, equivalent region of control). Collectively, these finding suggested that certain steps of the terminal differentiation program in transgenic epidermis may be retarded, allowing cells to reach the skin surface prematurely. Immunohistochemical studies (see below) were consistent with this notion.

One additional feature consistently noted from ultrastructural analyses was the presence of some swollen mitochondria and vacuoles specifically in the epidermal cells of those mice exhibiting high levels of transgene expression. These features were not present in the epidermis from control littermates that had been sacrificed and processed simultaneously. Coupled with the highly compacted nature of the cells within the epidermis of these animals, these findings suggest a possible hemostatic deprivation of nutrients caused by a KGF-mediated overpopulation of epidermal cells within the tissue. Further studies will be necessary to explore this possibility.

While some of the ultrastructural features of K14-KGF epidermis were similar to those of K14-TGFα epidermis, the marked crowding of cells appeared unique to KGF epidermis, and the epidermal hypertrophy seen in TGFα mice was notably absent in KGF mice (for TGFα data, see Vassar and Fuchs, 1991). Moreover, while the nucleus to cytoplasm ratio was reduced in the basal layer of TGFα skin, it was increased in KGF skin.

Aberrancies in terminal differentiation and proliferation in KGF-expressing epidermis

The expression of keratin and the IF-associated protein, filaggrin, are often used as biochemical indications of whether the program of epidermal differentiation is normal (Weiss *et al.*, 1984; Dale *et al.*, 1985). In normal epidermis, K14 and K5 are synthesized in the basal layer (Nelson and Sun, 1983; Lersch and Fuchs, 1988), K1 and K10 are expressed in suprabasal layers (Fuchs and Green, 1980) and filaggrin is expressed in the granular layer (Dale *et al.*, 1985). In a variety of hyperproliferative skin diseases, (i)

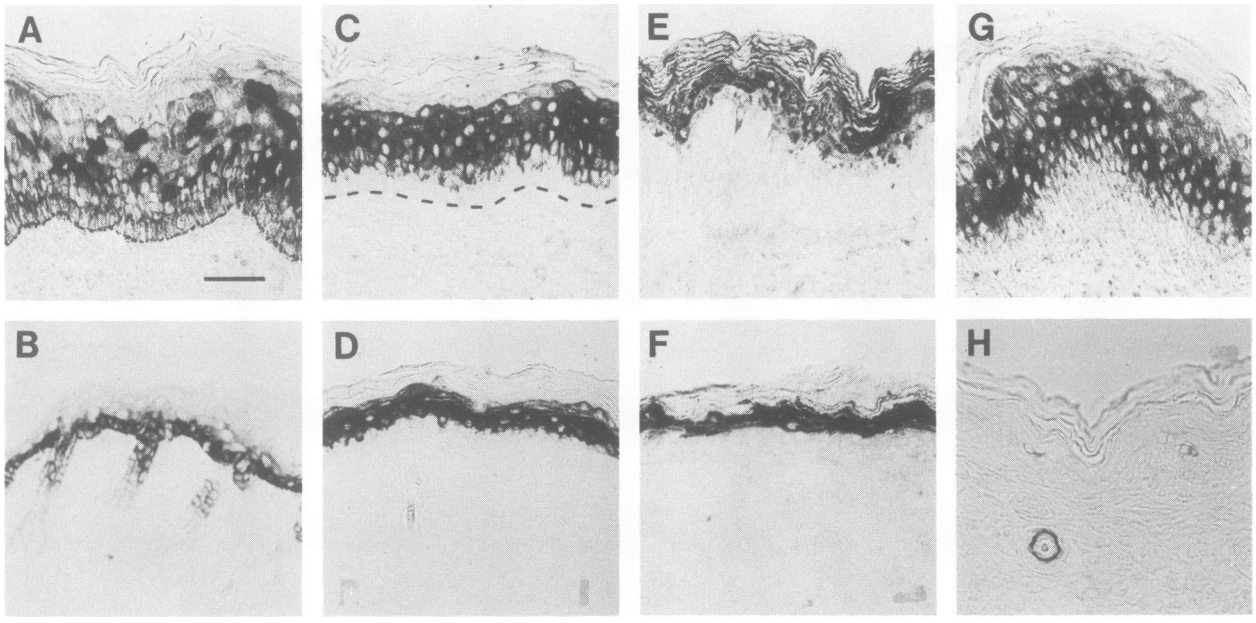

Fig. 5. Expression of keratins and filaggrin in transgenic and control newborn mice. Wrinkled skins from KGF-NB2, KGF-NB4 and KGF-NB6 and from a newborn nontransgenic control mouse were fixed with Bouin's solution, sectioned (5 μm) and then subjected to immunohistochemistry. The top sections (A, C, E and G) are from transgenic skin of KGF-NB2 and the bottom sections (B, D, F and H) are from control skin. Sections were stained with: (A,B), anti-K14 (1:250 dilution); (C,D), anti-K1 (1:300 dilution); (E,F), anti-filaggrin (1:300 dilution) and (G,H), anti-K6 (1:300 dilution). Details about the antisera and conditions and procedures used for staining can be obtained from Choi and Fuchs (1990). Bar represents 48 μm.

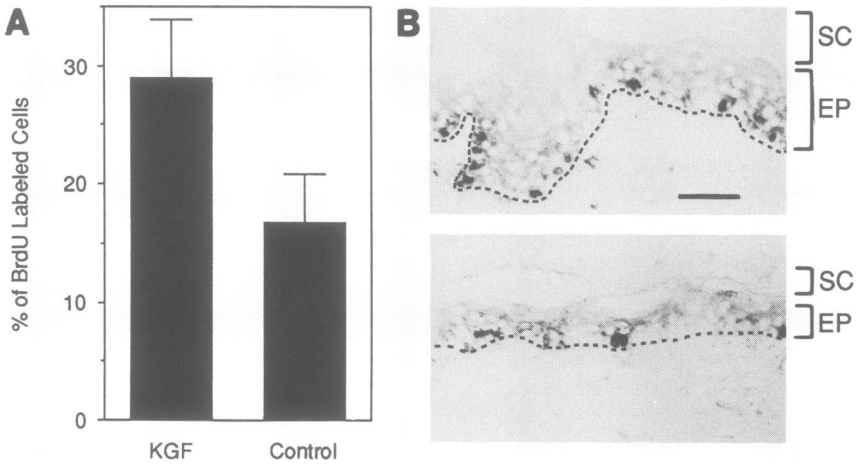

Fig. 6. BrdU labelling reveals an increase in proliferation within the epidermis of KGF transgenic mice. 1 × 1 mm skin samples were taken from two newborn transgenic mice with severely wrinkled skin and from their newborn non-transgenic littermates. Skins were placed in medium containing bromodeoxyuridine (BrdU) for 5 h as described by Sieweke *et al.* (1989). Following labelling, samples were washed, fixed in 2% paraformaldehyde, and sectioned (5 μm), followed by staining with an anti-BrdU antibody (Vassar and Fuchs, 1991). (**A**) Percentages of labelled basal epidermal cells were estimated by counting labelled and unlabelled cells in photographs from 10 random fields. Levels of significance for comparisons between samples were determined using the two-tailed Student's *t*-test. Statview software was used to calculate mean and standard deviations. Shown are average of two sets of data, which were highly similar. (**B**) Representative examples of BrdU labelling of skin from transgenic (top) and control (bottom) mice. Bar represents 48 μm.

K5 and K14 expression often extends into the suprabasal layers, (ii) a new pair of keratins, K6 and K16, are induced in the differentiating cells, and (iii) filaggrin expression changes in accordance with the enhancement or disappearance of granular cells (Weiss *et al.*, 1984; Fisher *et al.*, 1987; Stoler *et al.*, 1988).

To evaluate keratin and filaggrin expression, we subjected sections of newborn transgenic and control skin to immunohistochemistry (Figure 5). Similar to hyperproliferative diseases of the skin, anti-K14 staining was prevalent throughout the epidermis, extending up to the stratum corneum (Figure 5A). Basal cells stained less intensely than normal, consistent with our ultrastructural studies showing a reduced number of tonofilaments in these cells. Anti-K1 staining was restricted to spinous and granular layers of both transgenic and control cells (Figure 5C, transgenic; Figure 5D, control skin). Again, staining in transgenic epidermis appeared less intense, in agreement with the reduced levels of tonofibrils in these cells. The broad zone of anti-filaggrin staining reflected the thickened granular layer of KGF-expressing transgenic mice, and the atypical staining of the stratum corneum layers was suggestive that

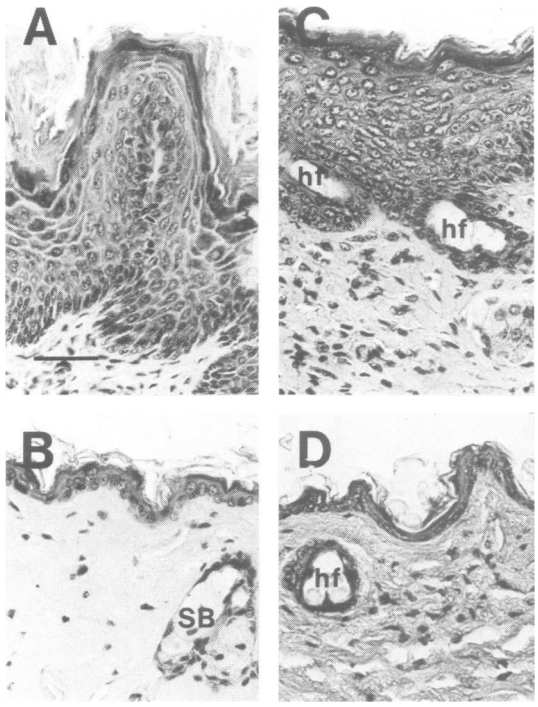

Fig. 7. Early signs of transformation in epidermis from adult KGF-expressing transgenic mice. Skin samples were taken from the eyelid and abdominal skin of an ~4 month old KGF mouse exhibiting a thin skin phenotype (KGF-m86) and its control littermate. Skin samples were placed in Bouin's fixative, for subsequent staining with hematoxylin and eosin. Panel **A**, eyelid skin of transgenic, showing invaginations into the dermis, epidermal thickening and gross irregularities in the organization of cells within the epidermis; panel **B**, eyelid skin from control littermate; panel **C**, abdominal skin of transgenic, showing irregularities in keratinocyte organization, particularly in regions surrounding hair follicles; panel **D**, abdominal skin of control littermate. Note: these aberrations are typical of the early signs of transformation seen in papilloma formation. Abbreviations: hf, hair follicle; SB, sebaceous gland. Bar in (A) represents 35 μm.

the cells entering this layer had not fully processed their filaggrin (compare Figure 5E, transgenic, with 5F, control). This was in agreement with our ultrastructural data, and provided additional evidence that the destructive phase of terminal differentiation in these mice was incomplete.

Thus far, our data suggested that the hyperthickened epidermis seen in some of our KGF mice was due to KGF-stimulated hyperproliferation. This notion was strengthened by the detection of anti-K6 staining within the suprabasal layers of these mice (Figure 5G). While suprabasal K6 expression was also a characteristic of TGFα mice (Vassar and Fuchs, 1991); control skin only exhibited anti-K6 staining in hair follicles (Figure 5H).

That the hyperthickened epidermis of KGF-expressing mice was truly hyperproliferative was demonstrated by comparing the number of epidermal cells synthesizing DNA in skin samples of two transgenics expressing high levels of KGF and two controls. As judged by anti-BrdU staining of skin sections of bromodeoxyuridine-labelled transgenic and control skins (Sieweke *et al.*, 1989), the level of proliferating cells in both KGF-expressing epidermises was nearly twice that of the control (Figure 6A; data shown is average of the two sets). Within the epidermis, labelling was localized to the basal layer of both transgenic and control skin (see Figure 6B for representative examples). The doubling in BrdU-labelled basal cells confirmed that KGF enhanced the proliferation of basal keratinocytes *in vivo*. Furthermore, the presence of only a single layer of BrdU-labelled cells indicated that, even though there were a number of basal-like cells in the suprabasal layers of the skin of KGF-expressing mice (see Figure 4A), the mechanisms that control cessation of DNA synthesis as cells leave the basal layer did not seem to be perturbed as a consequence of elevated KGF expression.

Effects of prolonged KGF expression: early signs of transformation

In newborn KGF-expressing transgenic animals, epidermal thickening was oftentimes dramatic, but no signs of disorganization or transformation were detected. However, in certain regions of hyperthickened skin of older KGF-expressing transgenic animals, there appeared to be early signs of transformation. Thus, for example, in the thickened eyelid skin of an ~4 month old transgenic mouse, a number of epidermal invaginations into the dermis were seen (Figure 7A). This was in striking contrast to control eyelid skin (panel B). Similarly, transgenic abdominal skin often exhibited irregularities in epidermal thickening and displayed numerous aberrations in epidermal organization. Aberrations were particularly prominent in regions surrounding hair follicles (panel C; compare with control in panel D). Collectively, these abnormalities were remarkably similar to those that were observed in the early stages of papilloma formation that occurred when K14-TGFα expressing transgenic animals were treated with tumor promoting agent (Vassar *et al.*, 1992). Curiously, in the TPA-treated K14-TGFα mice, transformations were also most prominent near intersections of epidermis and hair follicles, suggesting that these regions may be more prone to transformation than others. The remarkable difference between adult K14-TGFα and K14-KGF mice is that generation of epidermal aberrations in the K14-KGF mice did not require treatment with TPA.

KGF-induced abnormalities in other tissues

The human K14 promoter is active in a number of other stratified squamous epithelia, particularly the tongue (Vassar *et al.*, 1989). Indeed, histopathological analysis of the tongues of KGF-expressing transgenic mice revealed marked changes in both the dorsal and the ventral epithelia (Figure 8). In newborn mice, expressing higher levels of KGF, the dorsal epithelium was particularly affected, showing marked undulations of the basal layer, and reduced terminal differentiation of the upper layers (panel A, transgenic; compare with panel B, control). The ventral tongue epithelium of these animals was hyperthickened and showed signs of cell crowding in the basal layer, but was otherwise similar to control epithelium (not shown).

Interestingly, the effects of KGF on tongue were even more striking in our adult transgenic animals, even though these mice expressed lower levels of KGF. In particular, the dorsal epithelium exhibited gross signs of disorganization, with highly enlarged and irregular filliform papillae (fp; panels C and D, representative transgenic; compare with panel E, control). In some regions, invaginations of basal-like cells occurred, giving the appearance of islands of epithelial cells within the mesenchyme (panel D). As in

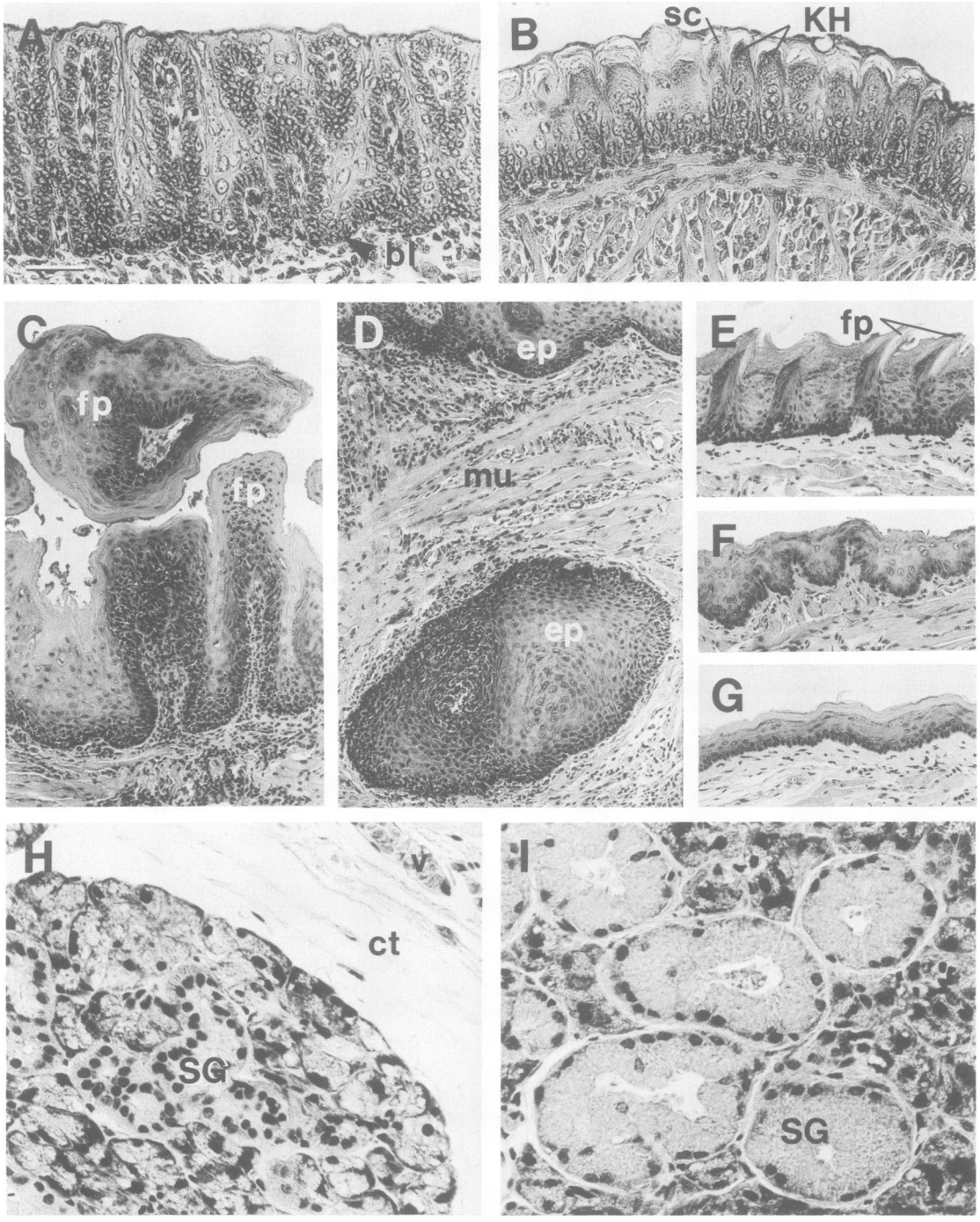

Fig. 8. KGF-mediated effects on tongue and salivary gland epithelium. Samples were taken from the tongues and salivary glands of two newborn KGF mice and two adult male KGF mice and their control littermates. Samples were placed in Bouin's fixative, for subsequent staining with hematoxylin and eosin. Data shown are from KGF-NB2 and KGF-m86, although data from KGF-NB4 and KGF-m73, respectively, were indistinguishable. Panel **A**, dorsal region of newborn transgenic tongue, showing invaginations of the basal layer (bl), crowding of filliform papilla, thickening of the stratified epithelium, and reduction in terminally differentiated cells at the tongue surface; panel **B**, dorsal region of newborn non-transgenic control, showing marked keratohyalin granules (KH) and stratum corneum (SC), missing in transgenic; panel **C**, dorsal region of ~4 month-old transgenic tongue, showing invaginations into the mesenchyme and gross distortions and protrusions of the filliform papilla (fp); panel **D**, another region of the transgenic tongue, illustrating region where the epithelium (ep) invaded the underlying mesenchyme and is shown here beneath the muscle layer (mu); panel **E**, dorsal segment of non-transgenic control tongue, illustrating uniform filliform papillae (fp); panels **F** and **G**, ventral regions of same tongues shown in panels C and E, respectively, depicting an increase in invaginations and thickening of the stratified squamous epithelium in the transgenic; panel **H**, region of submandibular gland of 4 months old KGF-m86, depicting numerous tubular cells with a paucity of secretory granules (SG). The high number of ring-like structures, their location near the edges of the lobes, and the presence of some secretory granules in these cells indicated that these were immature tubular cells, rather than ductal cells. Panel **I**, region of submandibular gland of control male littermate, illustrating columnar intercalated tubular cells filled with secretory granules (SG). Abbreviations: v, vein; ct, connective tissue. Bar in (A) represents 35 μm for A and B, 70 μm for C–G and 23 μm for H and I.

newborn KGF animals, the ventral portion was affected, but to a lesser degree than the dorsal region (panel F, transgenic; compare with panel G, control). Thus, in tongue as well as in skin, gross abnormalities typical of early signs of transformation were present in the adult KGF-expressing transgenic mice. A striking feature was that for tongues of

two different adult transgenic animals studied, the entire epithelium seemed affected, suggesting that this was not merely a clonal aberration, but rather an effect elicited within the entire population of cells.

Other stratified squamous epithelia, including forestomach, esophagus and cornea, did not seem to be as affected as the skin and tongue of newborn and adult K14-KGF-expressing transgenic mice (data not shown). That tongue and skin were most affected is probably reflective of the relative strength of the human K14 promoter in these two tissues, since these two tissues were also most affected in K14-TGFα-expressing transgenic mice (Vassar and Fuchs, 1991). Additionally, the levels of KGF receptors may be especially high in these tissues.

Since older KGF animals salivated excessively, we anticipated that there might be aberrations in these glands, where K14 is known to be expressed (Dardick *et al.*, 1988; S.Zinkel, L.Guo and E.Fuchs, unpublished). Indeed, upon dissection, the submandibular glands of two male transgenic animals [KGF-m86 (~4 months old) and KGF-m73 (~6 months old] appeared selectively and markedly smaller than those of their respective control male littermates. Histological analysis revealed marked differences between transgenic and control tubular cells within the gland. Transgenic tubular cells had central nuclei and cytoplasms with few or no secretory granules (SG; panel H). Their abundance, location and morphology were consistent with their being immature tubular cells, which are somewhat similar in appearance to ductal cells. In contrast, tubular cells from control male littermates were filled with secretory granules (SG), pushing the nuclei to the cell periphery (panel I). No differences in the alveoli were apparent. The appearance of the submandibular glands of these two transgenic males was similar to those of young males, suggesting an immaturity in differentiation. Whether excessive salivation was an indication that glandular differences extended beyond this, or whether it was due to aberrations in the releasing ducts, remains to be investigated.

Discussion

Deregulation of proliferation and incomplete differentiation in KGF-expressing epidermis: intriguing differences between KGF and TGFα

Prior to the present study, the known effects of KGF were limited to cell culture studies. We have now extended these studies to show that KGF has a profound hyperproliferative effect on epidermal growth *in vivo*. This increase in proliferation was reflected not only in patterns of keratin expression, which were typical of hyperproliferative epidermis, but also in BrdU labelling, which was higher in transgenic than control mice.

The major autocrine growth factor for epidermal cells is TGFα (Coffey *et al.*, 1987), which utilizes the EGF receptor that is abundant on the surface of basal epidermal cells (Massague *et al.*, 1983a,b; Green *et al.*, 1983). Unlike EGF, TGFα is made by keratinocytes (Coffey *et al.*, 1987), and it is more potent than EGF in stimulating keratinocyte growth and cell migration in culture (Barrandon and Green, 1987). It also has a proliferative effect on epidermal cells when overexpressed in the epidermis of transgenic mice (Vassar and Fuchs, 1991). *In vitro*, KGF is significantly more potent than either EGF or TGFα in eliciting a proliferative response in keratinocytes (Rubin *et al.*, 1989; Marchese *et al.*, 1990).

Our studies revealed that *in vivo*, K14-KGF differed from K14-TGFα in its ability to increase the total number of cells within the epidermal basal layer. In part, this overcrowding may have been due to a more potent effect of KGF than TGFα on epidermal proliferation *in vivo*. Thus, even though the highest increase in proliferation that we observed was an approximate doubling for both the KGF and the TGFα mice, the severe overcrowding in the basal layer of KGF mice seems to have led to some starvation and necrosis, and this could have obscured an otherwise higher rate of proliferation. While this notion is likely, it does not explain why epidermal cells were more loosely interconnected than normal in TGFα-overexpressing skin (Vassar and Fuchs, 1991) and more highly compacted than normal in KGF-expressing skin.

The opposing effects of these two growth factors on cell compaction within the epidermis suggest that there may be fundamental differences in the mechanisms underlying the action of KGF and TGFα. If, for instance, these factors differ in their influence on such processes as cell migration (Barrandon and Green, 1987) and/or basement membrane interactions (Turksen *et al.*, 1991 and references therein), then movement of basal cells into suprabasal layers might be quite different in K14-KGF and K14-TGFα transgenic animals. This could readily account for overcrowding of cells in response to one factor, and undercrowding of cells in response to the other. Further studies will be necessary to explore fully the nature of these differences.

In vivo, KGF and TGFα exhibited similar effects on epidermal thickening, and neither growth factor disrupted the maintenance of a single layer of mitotically active basal cells. This said, the functions of these two growth factors in controlling epidermal proliferation are likely to be quite different. In skin, TGFα is constitutively expressed by the epidermis (Coffey *et al.*, 1987), and its response appears to be autoregulated by EGF receptor levels (Green *et al.*, 1983; Vassar and Fuchs, 1991). Thus, TGFα may play a natural role in controlling epidermal thickening during development and in the adult (Vassar and Fuchs, 1991). In contrast, KGF is made by dermal fibroblasts, and its level is likely to increase upon skin injury (Werner *et al.*, 1992). This suggests a natural role for this factor in wound-healing and in mesenchymal–epidermal interactions (Finch *et al.*, 1989; Rubin *et al.*, 1989; Werner *et al.*, 1992).

While a major effect of these two growth factors in the epidermis was to enhance basal cell proliferation, both factors also elicited increases in the numbers of spinous, granular and stratum corneum layers, and both factors showed similar patterns of expression of biochemical markers of differentiation. This was surprising, given the opposing effects of EGF and KGF on K1 and filaggrin expression in cultured keratinocytes (Marchese *et al.*, 1990). Other features shared by mice expressing K14-TGFα and K14-KGF transgenes included the presence of organelles in the upper granular layer, and other signs generally attributed to incomplete terminal differentiation.

Despite certain similarities, KGF and TGFα elicited some differences on terminally differentiating keratinocytes. For example, while TGFα overexpression caused epidermal hypertrophy and a higher cytoplasm to nucleus ratio (Vassar and Fuchs, 1991), KGF expression produced keratinocytes with a lower cytoplasm to nucleus ratio. In addition, the

reduced number of keratin filaments in spinous cells of KGF mice was more notable than in TGFα mice. Collectively, while the *in vivo* effects of KGF on epidermal growth and differentiation shared some parallels with those of TGFα, there were also distinct differences. Irrespective of whether these differences are direct or indirect, KGF and TGFα must transmit different signals to the basal epidermal keratinocyte.

KGF and the choice between epidermal and hair follicle programs of differentiation: interfering with mesenchymal–epithelial signalling

Perhaps the most striking finding of our studies, and a major difference between K14-TGFα and K14-KGF mice, was the near complete suppression of hair follicle formation in those KGF transgenic animals exhibiting the thickest and most wrinkled skin. Moreover, among all of our K14-KGF transgenic animals, follicle number correlated inversely with relative levels of keratinocyte-derived KGF expression and with the relative thickness of the epidermis. From this correlation, it seems likely that the basis for regional sensitivity in follicle density in the animals expressing less KGF reflects regional variation either in the levels of transgene expression or the levels of KGF receptors on the surface of epidermal cells. Further experiments will be necessary to test this possibility.

We do not yet know how KGF elicits these unusual effects on hair follicle morphogenesis. However, it is unlikely that transgene expression in the follicle is involved, since this is likely to occur only after birth, when hair morphogenesis is complete (Kopan and Fuchs, 1989). Rather, the effects of KGF seem to be at an earlier stage, and in this regard it seems relevant that both hair follicles and epidermis arise from a common embryonic precursor. The commitment to hair follicle morphogenesis is thought to occur at a critical time during development when a specialized mesenchymal component, the dermal papillae, comes into contact with a pluripotent embryonic basal cell (Davidson and Hardy, 1952; Wessels and Roessner, 1965; Dhouailly *et al.*, 1978; Meyer and Gorgen, 1986; Hirai *et al.*, 1992). In the absence of this signal, an embryonic basal cell follows an epidermal cell fate.

Since the fewer follicles that formed in KGF-expressing animals seemed normal in morphology, it seems most likely that a threshold level of K14-KGF expression at a critical time during embryonic development may have interfered with receipt of the dermal papilla signal by a percentage of embryonic basal cells. This notion is intriguing given that at the time when the choice is made between hair follicle and epidermal fates of development, K14 (and presumably K14-KGF) is expressed by embryonic basal cells (Kopan and Fuchs, 1989), as is the KGF receptor (Orr-Urtreger *et al.*, 1991; Peters *et al.*, 1992). Moreover, at this time, there is also a restricted and transient loss of EGF receptors in embryonic basal cells directly above mesenchymal condensates (Green *et al.*, 1984), suggesting that down-regulation of growth is critical to morphogenesis. While further studies will be necessary to elucidate the molecular mechanisms underlying KGF-mediated suppression of this process, an unregulated, elevated growth response might block the mesenchymal–epithelial signalling necessary for hair follicle morphogenesis. This block was not elicited by TGFα (Vassar and Fuchs, 1991), perhaps due to natural downregulation of EGF receptors at this critical time.

Surprising effects of epidermal KGF expression on adipogenesis and salivation

Given that epidermal keratinocytes are the only cell type known to respond to KGF, we were surprised to see a near complete absence of cutaneous fat in our K14-KGF mice. In fact, fat production was blocked in all of our adult animals expressing lower levels of KGF, suggesting that adipogenesis was particularly sensitive to KGF expression.

We do not yet know how KGF mediated its effects on adipogenesis. KGF appears to bind specifically to a splice variant of FGFR2, and does not appear to bind appreciably to any of the other fibroblast growth factor receptors (Rubin *et al.*, 1989; Aaronson *et al.*, 1991). Consequently, it seems most likely that the effects of KGF observed in our transgenic animals were triggered by specific activation of its receptor. Although *in situ* hybridizations have convincingly demonstrated expression of FGFR2 in epidermal cells, expression of FGFR2 in adipocytes has not been ruled out (Peters *et al.*, 1992; Werner *et al.*, 1992). While an indirect mechanism may be involved, it is notable that adipocytes seemed to respond to levels of KGF not sufficient to generate a response in some keratinocytes (see Figure 3G and H). Future studies should resolve this issue.

While K14 is known to be expressed in the basal and myoepithelial cells of the salivary gland ducts (Dardick *et al.*, 1988), we were surprised by the unanticipated effects of KGF on the salivary glands of transgenic animals. Similar to hair follicle development, the salivary gland epithelium depends upon interaction with mesenchyme to develop its branched morphology and acquire glandular function. Thus, it was curious that the submandibular glands of adult mice expressing low levels of KGF were visibly smaller and histologically immature in appearance. The round, rather than convoluted, cross-sections of tubules, coupled with the marked paucity of secretory granules, suggested that developmental cues to the submandibular glands of these animals had been impaired. The excessive drooling of these animals may be related to the morphological aberrations in the submandibular gland, since this gland accounts for the bulk of saliva production. Alternatively, the excessive secretion of saliva could arise from abnormalities in the stratified squamous epithelium at the ductal opening. Clearly, more detailed studies on the salivary glands and their secretions will be a necessary prerequisite to a biological understanding of the alterations in the submandibular glands of these mice. However, it is intriguing that K14-KGF may have perturbed the mesenchymal–epithelial signalling in the salivary gland, as well as in the skin.

Finally, suppression of adipogenesis, impairment of submandibular gland development and excessive saliva secretion were not observed in juvenile or adult K14-TGFα animals (Vassar and Fuchs, 1991). These findings further emphasize the distinctly different effects of KGF and TGFα *in vivo*.

KGF and keratinocyte transformation

While skin from our adult K14-TGFα transgenic animals showed no epidermal invaginations or other early signs of transformation (Vassar and Fuchs, 1991), skin from our K14-KGF transgenic animals exhibited prominent irregularities in both the epidermis and tongue epithelium. The histopathological changes in the skin were reminiscent of those pre-papilloma changes that occurred within 3–4 weeks

of either (i) treating adult K14-TGFα transgenic mice with TPA, or (ii) simultaneously injecting control mice with TGFα and topically treating them with TPA (Vassar *et al.*, 1992). However, a major difference between KGF tongue aberrations and papilloma formation was that the effects on transformation appeared throughout the epithelium, rather than in a clonal fashion. In addition, the KGF mice showed no visible signs of skin papillomas.

It is interesting that the KGF-mediated, transformation-like alterations in the organization of keratinocytes within adult tissues were most prominent in eyelid, abdominal skin and tongue. A priori, it is possible that KGF is a more potent mitogen than TGFα, and that these tissues are especially sensitive to KGF, either as a consequence of higher KGF than EGF receptor levels or as a consequence of biochemical differences in receptor-mediated pathways. Alternatively, it could be that the secretions from eye and salivary glands provide some additional factor, which in combination with elevated KGF levels, causes morphological changes similar to benign transformation of a stratified squamous epithelium. As additional transgenic mouse studies are conducted, the mechanisms by which KGF elicits these unusual and striking effects should become apparent.

Summary

Our studies represent the first *in vivo* study of the effects of KGF on skin and other tissues. By artificially expressing KGF in basal keratinocytes, we have converted KGF from a paracrine to an autocrine growth factor. In the skin, this seemed to perturb mesenchymal–epidermal interactions, leading to a remarkable suppression of hair follicle morphogenesis, accompanied by gross epidermal proliferation. Keratinocyte-mediated KGF expression also had a profound inhibitory effect on adipogenesis. In addition, it inhibited development of the submandibular gland and caused marked proliferation and suppression of differentiation in the tongue. In K14-KGF expressing adult mice, early signs of transformation were seen throughout the tongue epithelium, and regionally in skin, including abdominal skin and eyelid epidermis. While additional studies are clearly needed to elucidate the precise molecular mechanisms involved in these KGF-initiated responses, these findings have provided new and important insights into how morphogenesis of epithelial–mesenchymal tissues is governed by paracrine and autocrine signals, and how this process can go awry when the normal autoregulatory mechanisms are overridden.

Materials and methods

PCR analyses

Total RNAs from different tissues of transgenic and control mice were isolated, and cDNAs were synthesized as described by Cheng *et al.* (1992). cDNAs were amplified by PCR. The PCR primers specific for transgene and β-actin are listed below, with predicted size of the amplified product given in parentheses. Primers were for (i) mouse β-actin (348 bp), 5′-TGGAATCCTGTGGCATCCATGAAAC-3′, 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′; and (ii) KGF/hGH (590 bp), 5′-GACATGGATCCTGCCAAC-3′ (in hKGF), 5′-ACAGGACCCTGAGTGGTT-3′ (in hGH).

Competitive PCR analyses were performed to quantify hKGF mRNA levels (Siebert and Larrick, 1992). For each mRNA sample, cDNA was made using 40 μl 5× reverse transcription (RT) buffer (Bethesda Research Laboratories), 20 μl 0.1 M dithiothreitol, 8 μl 25 mM dNTPs (Pharmacia), 10 μl of RNasin (Promega Biochemicals), 10 μl of 50 μM random hexamers (Pharmacia), 10 μl of murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and 10 μg of heat denatured total skin RNA. Diethyl pyrocarbonate-treated water was added to bring the final reaction volume to 200 μl, and the reaction was incubated at room temperature for 10 min, 42°C for 90 min, 100°C for 1 min and 0°C for 5 min.

Aliquots (20 μl each) of the above RT reactions were then used for competitive PCR analysis. A stock sample of competing plasmid DNA containing the K14-KGF-hGH gene sequence was serially diluted in water, and aliquots (10 μl) of these dilutions were added to each aliquot of RT reaction. To each reaction was added 70 μl of a master mix containing the following: eight parts 10× PCR buffer (Perkin Elmer–Cetus recipe), one part 20 μM primers (hGH538F and hGH1550R; see below), 0.5 parts AmpliTaq polymerase (Perkin Elmer–Cetus), and 59.5 parts dH_2O. The PCR reaction was carried out for 35 cycles of: 94°C, 1 min, 55°C, 1 min, and 72°C, 2 min. An extension of 72°C for 7 min was added at the end of the run. Primers were: hGH538F: 5′-AACCACTCAGGGTCCTGT-3′; hGH1550R: 5′-CAGCGTTTGGATGCCTTC-3′. On plasmid genomic DNA, these primers yield a 1048 bp fragment; on cDNA, the primers yield a 492 bp fragment.

In situ hybridizations

In situ hybridizations were performed essentially as described by Vassar and Fuchs (1991). Full-length antisense cRNA was produced from Bluescript-hKGF using T3 polymerase (Stratagene).

Light and electron microscopy studies

Light and electron microscopy methods are as described by Cheng *et al.* (1992).

Immunoblot analysis and immunohistochemistry

Mouse skin (~1 cm^2) was frozen in liquid nitrogen and subsequently stored at −70°C. Tissues were ground with a mortar and pestle, in the presence of liquid nitrogen. At this point, KGF extractions were carried out as recommended by Dr Sabine Werner (University of California, San Francisco). Tissue powders were scraped into 0.5 ml of cold 2× lysis buffer containing 2% Triton X-100, 40 mM Tris pH 8, 274 mM NaCl, 20% glycerol, 4 mM EDTA, 5 mM phenyl methyl sulfonyl fluoride, 10 μg/ml pepstatin, 15 μg/ml leupeptin, 0.5 U/ml aprotinin (Sigma Chemicals), and then homogenized at 4°C using a polytron. Homogenized solutions were centrifuged for 10 min at 4°C, and the supernatants were diluted (1:1 v/v) with ice-cold water. Centrifugation was repeated, and this time, 50 μl of 1:1 (v/v) slurry of heparin–Sepharose (Pharmacia) in water was added to the supernatant. The solution was then placed on a rotating apparatus and incubated for 6 h at 4°C. At the end of incubation, the solution was centrifuged briefly, and after aspirating the supernatant, the beads were washed with 1 ml buffer containing 0.3 M NaCl, 20 mM Tris pH 7.5, 1 mM PMSF and 0.25 U/ml aprotinin. Washings were repeated three times, and then heparin–Sepharose-bound material was eluted with 30 μl of gel sample buffer and subjected to electrophoresis through SDS–polyacrylamide gels (10%). Immunoblot analysis was performed as described by Choi and Fuchs (1990) using a rabbit anti-KGF antiserum (1:1000) and a Bio-Rad alkaline phosphatase-conjugated goat anti-rabbit antibody (1:3000). Color was developed according to the manufacturer's recommendations.

For immunohistochemistry, frozen sections (6 μm) were fixed mildly with 1% paraformaldehyde in PBS for 10 min at 4°C. After washing briefly in PBS, sections were permeabilized with methanol for 5 min. Washed slides were then air dried briefly and used for immunostaining as described by Vassar and Fuchs (1991). Rabbit anti-KGF was used at a 1:100 dilution as primary antibody, and gold-conjugated goat anti-rabbit antiserum was used at a 1:40 dilution as secondary antibody.

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