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c-Jun Regulates Eyelid Closure and Skin Tumor Development through EGFR Signaling

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Summary

To investigate the function of c-Jun during skin development and skin tumor formation, we conditionally inactivated *c-jun* in the epidermis. Mice lacking *c-jun* in keratinocytes (*c-jun*$^{\Delta ep}$) develop normal skin but express reduced levels of EGFR in the eyelids, leading to open eyes at birth, as observed in EGFR null mice. Primary keratinocytes from *c-jun*$^{\Delta ep}$ mice proliferate poorly, show increased differentiation, and form prominent cortical actin bundles, most likely because of decreased expression of EGFR and its ligand HB-EGF. In the absence of c-Jun, tumor-prone K5-SOS-F transgenic mice develop smaller papillomas, with reduced expression of EGFR in basal keratinocytes. Thus, using three experimental systems, we show that EGFR and HB-EGF are regulated by c-Jun, which controls eyelid development, keratinocyte proliferation, and skin tumor formation.

Introduction

The skin consists of two major compartments, the epidermis and the dermis, which are separated by the basal lamina. The basal keratinocyte layer, which is directly attached to the basal lamina, contains the stem cell population. Continuous proliferation in the basal layer and a shift in the balance between proliferation and differentiation in cells of the adjacent layers are a prerequisite to replace the terminally differentiated keratinocytes from the surface of the skin (Angel et al., 2001; Kaufman and Fuchs, 2000; Watt, 2001).

A functional role for the transcription factor complex c-Jun/AP-1 in the skin has been suggested for keratinocyte differentiation, carcinogenesis, UV response, photoaging, and wound repair (for review see Angel et al., 2001). AP-1 consists of various dimers of the Fos (c-Fos, FosB, Fra-1, and Fra-2), Jun (c-Jun, JunB, and JunD), and CREB/ATF protein families, as well as other bZip proteins. In addition, associations have been observed between Fos or Jun and the p65 subunit of NFκB (Stein et al., 1993) and ATF-2 and p50-NFκB (Du et al., 1993).

Mice lacking c-Jun die at midgestation with heart defects and impaired hepatogenesis (Eferl et al., 1999), whereas fibroblasts derived from *c-jun*$^{-/-}$ mouse embryos exhibit a severe proliferation defect caused by upregulation of p53 and p21 (Schreiber et al., 1999). In the epidermis of newborn mice, c-Jun is expressed in the basal and spinous layer of the epidermis. Basal cells also express *c-fos*, *junB*, and *fra1*, whereas, in the granular layer, *junB* and *fra2* are the only AP-1 members expressed (Rutberg et al., 1996). Numerous genes controlling skin differentiation, e.g., Keratin 1, 5, 6, 8, 14, 18, and 19, filaggrin, loricrin, and involucrin, harbor AP-1 binding sites in their promoters (Angel et al., 2001). In addition, human keratinocytes cultured with mouse *c-jun*$^{-/-}$ fibroblasts in an organotypic coculture system proliferate slower and are less differentiated (Szabowski et al., 2000). A plethora of extracellular signals and stress stimuli lead to rapid and transient expression of AP-1 proteins, suggesting that AP-1 activity may be required under conditions where the balance of keratinocyte proliferation and differentiation has to be rapidly and temporally altered. The phenotypes of mice lacking c-Fos, FosB, or JunD in the epidermis do not provide evidence for a critical role of these proteins in normal skin (Jochum et al., 2001). In contrast, there is strong evidence that c-Jun/AP-1 might be involved in the process of wound healing. c-Jun, c-Fos, and ATF proteins are rapidly and transiently upregulated by wound edge cells (Martin and Nobes, 1992) and appear to be at the receiving end of signaling pathways initiated by cytokines and growth factors in appropriate target cells (Angel et al., 2001).

Another important regulator of keratinocyte proliferation and differentiation includes the epidermal growth factor receptor (EGFR) signaling pathway. Activation of the EGFR by several ligands, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), and heparin binding EGF (HB-EGF), is a central event in the regulation of epidermal development. Mice deficient for TGF-α develop a wavy coat and curly whiskers and show occasionally open eyes at birth (EOB) because of a defect in eyelid closure during embryonic development (Luetteke et al., 1993; Mann et al., 1993). A similar phenotype is observed in the hypomorphic EGFR mutant strain waved-2 (wa2), whereas mice deficient for EGFR are always born with open eyes and fail to develop a wavy coat (Fowler et al., 1995; Luetteke et al., 1994; Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995). Moreover, cell culture experiments have shown that EGFR signaling negatively affects keratinocyte differentiation (Hansen et al., 1997; Sibilia and Wagner, 1995; Wakita and Takigawa, 1999).

It is well established that members of the Jun and Fos protein families as well as their retroviral derivatives transform tissue culture cells and induce tumors in avian, rat, and mouse (Jochum et al., 2001). Furthermore,

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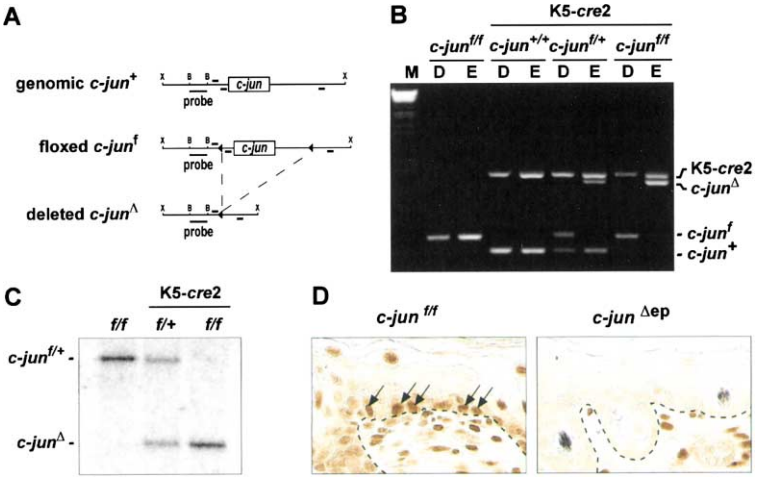

Figure 1. Epidermal-Specific Inactivation of *c-jun* by the K5-*cre*2 Transgene

(A) Schematic outline of the genomic wild-type (*c-jun*$^{+}$), floxed (*c-jun*^{f}), and deleted *c-jun* (*c-jun*$^{\Delta}$) locus. Location of loxP sites (black triangles), PCR primer binding sites, and the Southern probe are indicated. Genotyping by PCR (B), Southern blot (C), and immunohistochemistry for c-Jun in newborn mouse skin (D) demonstrating tissue-specific deletion of *c-jun*. The disruption of *c-jun* in the epidermis occurred very efficiently (>95%). Further indicated in (B) are the bands for the wild-type allele of *c-jun*$^{+}$ and the K5-*cre*2 transgene in epidermis (E) and dermis (D); mice without a transgene were used as controls.

(C) Southern blot with epidermal DNA showing the wild-type *c-jun*$^{+}$, the floxed *c-jun*^{f}, and the deleted *c-jun*$^{\Delta}$ allele in the epidermis.

(D) In contrast to *c-jun*$^{f/f}$ skin ([D], arrows, left side), no c-Jun-positive keratinocytes were detected in *c-jun*$^{\Delta ep}$ epidermis.

JunB and c-Jun are selectively upregulated and functionally implicated in fibrosarcoma development in vivo (Bossy-Wetzel et al., 1992). Skin-specific transgenic expression of a c-Jun transactivation mutant (TAM67) showed dramatic inhibition of papilloma induction in two-stage skin carcinogenesis experiments (Young et al., 1999). In addition, *c-fos*-deficient tumors failed to undergo malignant conversion, and v-H-ras-transformed *c-fos*-deficient keratinocytes grafted to nude mice are unable to form tumors (Saez et al., 1995). Skin papillomas develop with very high efficiency in K5-SOS-F transgenic mice and are strictly dependent on the presence of functional EGFR (Sibilia et al., 2000). Moreover, K5-SOS-F transgenic mice harboring an allele of *c-jun* (*junAA*) that cannot be phosphorylated by JNK show a reduction of tumor mass, suggesting that activation-dependent target genes of c-Jun are critically involved in the development of skin papillomas (Behrens et al., 2000).

To investigate the function of c-Jun during skin development and skin tumor formation, we conditionally inactivated *c-jun* in the epidermis. Here we show that mice lacking c-Jun in keratinocytes (*c-jun*$^{\Delta ep}$ mice) develop a normal skin but have open eyes at birth. In addition, primary keratinocytes lacking c-Jun proliferate poorly and display increased differentiation. Furthermore, in the absence of c-Jun, skin tumor development in K5-SOS-F transgenic mice is impaired, concomitant with reduced EGFR expression.

Results

Embryonic Eyelid Closure Depends on c-Jun

To investigate the role of c-Jun during skin development, we tissue-specifically inactivated *c-jun* in keratinocytes of mice carrying a floxed *c-jun* allele (*c-jun*$^{f/f}$) by employing the K5-*cre*2 transgenic line (Behrens et al., 2002; Tarutani et al., 1997; Figure 1A). Tissue-specific deletion of *c-jun* in newborn skin was assessed by PCR analysis, Southern blot, and immunohistochemistry, which showed that the disruption of *c-jun* in the epidermis occurred with almost 100% efficiency (Figures 1B–1D). As expected, some deletion of *c-jun* was also observed in other epithelia, e.g., esophagus, tongue, and stomach (data not shown), where the K5 promoter is known to be active (Byrne and Fuchs, 1993). As previously reported in wild-type skin, c-Jun was expressed in the stratum basale and the stratum spinosum (Rutberg et al., 1996) but was completely absent in *c-jun*$^{f/f}$ K5-*cre*-2 mice (*c-jun*$^{\Delta ep}$; Figure 1D).

c-jun$^{\Delta ep}$ mice were born with Mendelian frequencies and showed a fully penetrant eye open at birth (EOB) phenotype (Figure 2A). One hundred percent of mutant newborns and embryos at late stages of development (E16.5) could be readily identified by this phenotype (Figures 2A and 2C). Histological analyses of the eyes revealed that eyelids failed to fuse during ontogenesis (Figure 2C). This first became evident at E16.5, at which stage the developing eyelids of control embryos had already fused (Figure 2B). Other significant differences at this stage involved the developing corneal layers and the iris. In *c-jun*$^{\Delta ep}$ eyes the corneal stroma was less well organized and the iris, which was clearly separated from the cornea in control embryos, was attached to the posterior side of the cornea in the mutant embryos (Figures 2E–2G). In newborn mutants the eyelid fissure contained an inflammatory infiltrate. In some cases, most likely as a consequence of the open eyelid defect, we also saw microphtalmia and highly dysplastic retinal tissue with numerous folds and rosettes (data not shown). Adult *c-jun*$^{\Delta ep}$ mice showed corneal opacity (data not shown), and histological examination revealed corneal epithelial and stromal abnormalities. Extensive adherence of iridal tissue to the posterior surface of the cornea was observed, resulting in obliteration of the anterior chamber (Figure 2I, arrow).

As the eyelids in the wild-type embryo approach one another, they appear to zipper together at their nasal and lateral margins (Figure 3A). Transmission electron microscopy reveals that the leading epidermal cells at this stage are rich in filopodia and that confronting epidermal cells from the two eyelids exhibit filopodial interactions, including interdigitation (Figure 3C). In *c-jun*$^{\Delta ep}$

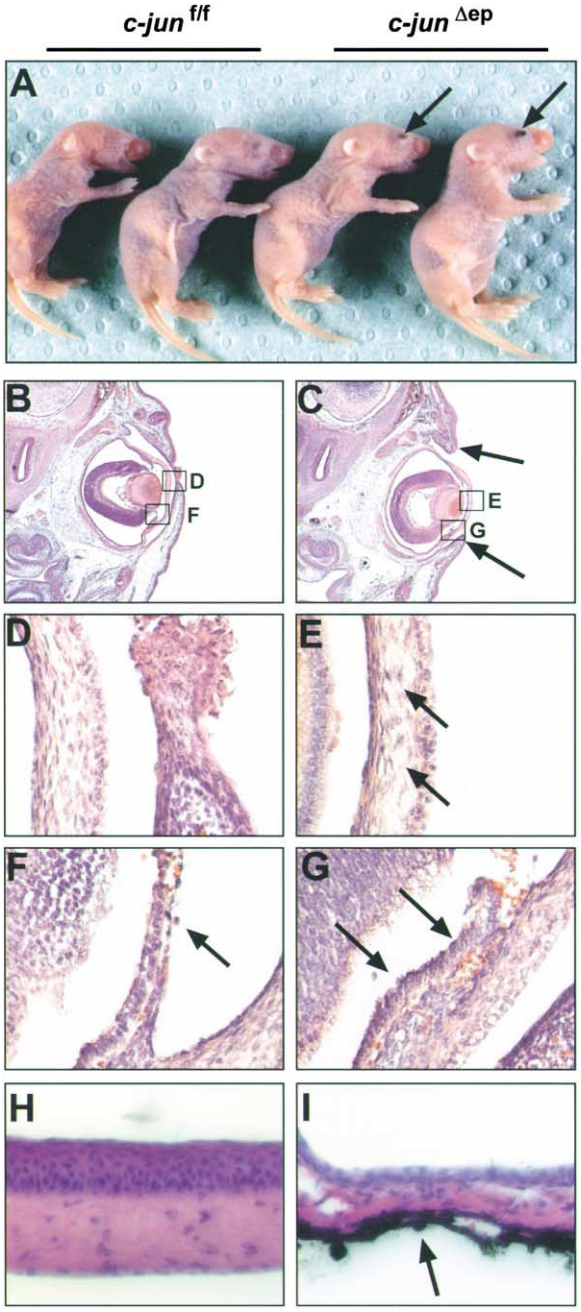

Figure 2. Mice without c-Jun in the Epidermis Show an EOB Phenotype

(A) *c-jun*$^{\Delta ep}$ mice are born with open eyes (arrows). In contrast to *c-jun*$^{f/f}$ embryos (B and D), late stage mutant fetuses (E16.5) have failed to close their eyelids (C). Histological analysis (H&E staining) of E16.5 embryos showed open eyelids, poorly differentiated corneal stroma ([E], arrows), and attachment of iridial tissue to the posterior side of the cornea ([G], arrows).

(H and I) H&E staining of adult cornea indicating severe cornea defects and extensive adherence of iridal tissue to the posterior surface of the cornea in *c-jun*$^{\Delta ep}$ mice ([I], arrow).

eyelids, the leading cells still formed filopodia, although they were slightly fewer in number and shorter (Figure 3D).

Since EGFR knockout mice show a similar phenotype,

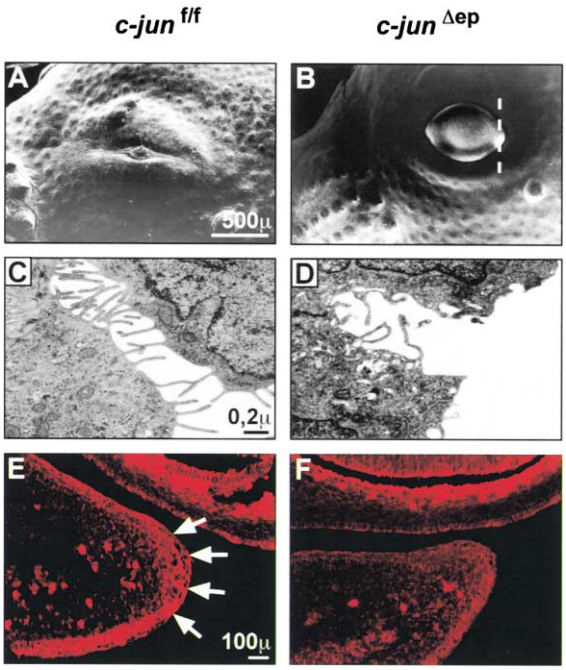

Figure 3. Filopodia that Do Not Express EGFR Are Formed in *c-jun*$^{\Delta ep}$ Eyelids

(A) Scanning electron microscopy (SEM) of an E16.0 wt embryo face with closed eyelid.

(B) A similar view of a *c-jun*$^{\Delta ep}$ sibling with a wide-open eye showing no apposition of eyelids at nasal or lateral margins.

(C and D) Transmission electron microscopy (TEM) detail of filopodial interdigitation, where leading edge epidermal cells confront one another as the eyelids zipper together in the wild-type embryo (C). (D) In the *c-jun*$^{\Delta ep}$ mutant leading edge, epidermal cells still express filopodia, although these appear to be fewer and shorter.

(E) Immunofluorescense studies of the E15.5–E16.0 eyelid just prior to eyelid fusion reveals expression of EGFR by the columnar epithelial cells at the leading edge. At the same stage, *c-jun*$^{\Delta ep}$ eyelids (F) express reduced levels of EGFR (red staining).

EGFR expression was analyzed in eyelids of E15.5 fetuses at the stage when keratinocyte migration starts. In contrast to wild-type embryos, reduced expression of EGFR was observed in keratinocytes at the leading edge of eyelids in *c-jun*$^{\Delta ep}$ embryos (Figures 3E and 3F). These results suggest that reduced EGFR expression in the leading edge keratinocytes of *c-jun*$^{\Delta ep}$ embryos might be responsible for the lack of eyelid closure.

Normal Skin Differentiation and Stress Responses in *c-jun*$^{\Delta ep}$ Mice

Histological examinations revealed a normal architecture of newborn *c-jun*$^{\Delta ep}$ skin, and the expression of the differentiation markers Keratin 5, 10, and 14 was comparable to that in controls (Figures 4A–4F; data not shown). Occasionally, curly whiskers and a distinctly wavy appearance of the first coat was observed, reminiscent of the phenotype seen in mice defective for EGFR signaling (Luetteke et al., 1993, 1994; Mann et al., 1993). The skin of *c-jun*$^{\Delta ep}$ mice was analyzed following treatment with the phorbol ester TPA, which induces keratinocyte hyperproliferation and activates c-Jun/

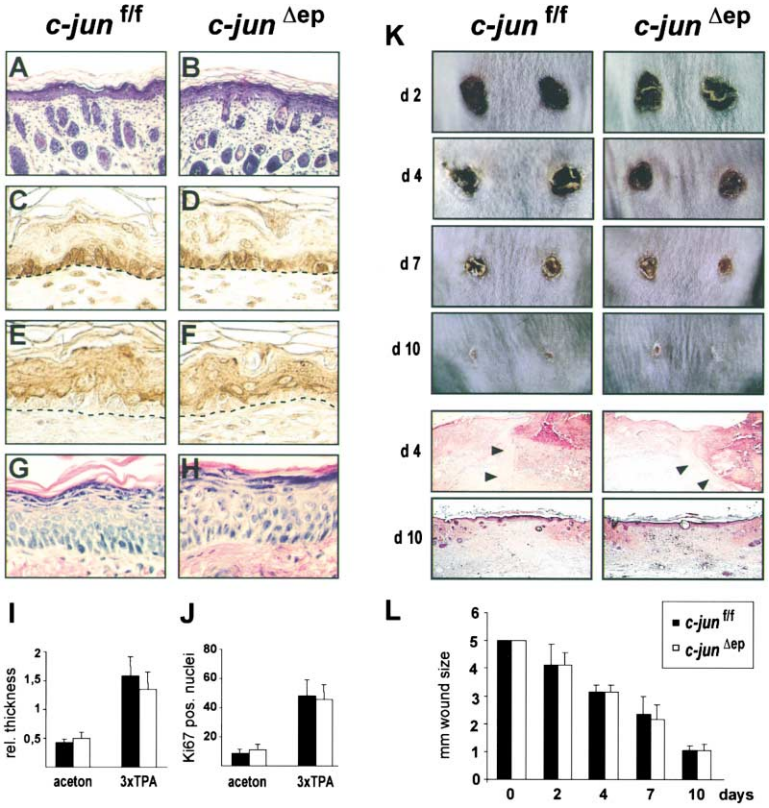

Figure 4. Normal Skin Histology and Stress Response in *c-jun*$^{\Delta ep}$ Mice

Normal development of skin and hair follicle in *c-jun*$^{\Delta ep}$ mice (B, D, and F) demonstrated by H&E staining (A and B) and immunohistochemistry for the skin differentiation markers Keratin 5 (C and D) and Keratin 10 (E and F) of P3 mouse skin. Treatment of adult epidermis with TPA revealed no difference between *c-jun*$^{\Delta ep}$ mice (H) and *c-jun*$^{f/f}$ littermates (G). Neither keratinocyte stratification (I) nor the number of proliferating keratinocytes stained for Ki67 was reduced (J).

(K) Wound healing upon excisional full-thickness wounding followed up to day 10 (d 10) postwounding did not reveal obvious healing defects in *c-jun*$^{\Delta ep}$ mice. Lower portion, histological analysis of day 4 and day 10 wounds (H&E staining) confirmed that keratinocyte migration (day 4, arrows), wound closure, and formation of a neoepidermis in *jun*$^{\Delta ep}$ mice are unchanged. Quantification of wound closure was comparable to that in *c-jun*$^{f/f}$ littermate mice (L).

AP-1 (Angel et al., 1988). Treatment of the epidermis with TPA revealed no differences between *c-jun*$^{\Delta ep}$ mice and controls (Figures 4G and 4H). Neither keratinocyte stratification nor the number of proliferating keratinocytes was reduced (Figures 4I and 4J).

During wound healing the adult skin has to reform a neoepidermis, and the wound edges must coordinate keratinocyte migration, proliferation, and differentiation (Martin, 1997). When full-thickness excisional wounds were analyzed in *c-jun*$^{\Delta ep}$ mice, no apparent repair defect was observed, and wound healing appeared comparable to littermate controls at all stages (Figures 4K and 4L). Histological analyses revealed no differences in keratinocyte proliferation and migration from the wound edges and adjacent hair follicles, and the wounds were completely closed within 10 days (Figure 4K). These results indicate that c-Jun is apparently not required under this condition for efficient wound healing.

Keratinocyte Proliferation In Vitro Depends on c-Jun

Primary keratinocytes were isolated from newborn *c-jun*$^{\Delta ep}$ mice to investigate whether the absence of c-Jun affects their proliferation and/or differentiation in vitro. Cultured keratinocytes lacking c-Jun grew very slowly and often had a very flat appearance (Figure 5B). The number of BrdU-positive cells was dramatically reduced, and the cells never reached confluency (Figures 5B and 5D). Staining for the keratinocyte differentiation marker Keratin 10 revealed a 6-fold increase in the number of differentiating keratinocytes (Figures 5E, 5F, and 5M), and TUNEL staining showed a significant increase of apoptotic cells in mutant cultures (Figures 5G, 5H, and 5N). In addition, actin microfilaments were organized primarily in cortical bundles in c-Jun-deficient keratinocytes, suggesting that c-Jun is involved in the organization of the actin cytoskeleton (Figure 5J).

In an attempt to rescue these defects, we supplemented the cultures with conditioned medium as well as with paracrine and autocrine growth factors. Interestingly, conditioned medium from wild-type keratinocytes completely rescued the proliferation defect of *c-jun*$^{-/-}$ keratinocytes (Figure 5O). Moreover, addition of autocrine factors, such as HB-EGF, EGF, and TGF-α, as well as the paracrine factors KGF and GM-CSF to the culture medium also rescued the proliferation defect, although high concentrations of EGF and TGF-α were required (Figures 5P and 5Q). High concentrations of EGF also reduced the number of differentiating and dying keratinocytes to wild-type levels and rescued the actin cytoskeleton defect, suggesting that EGFR signaling might be impaired in keratinocytes lacking c-Jun (Figures 5M, 5N, and 5L).

K5-SOS-F-Dependent Tumor Formation in the Absence of c-Jun

To determine the function of c-Jun in skin tumor development, we bred tumor-prone K5-SOS-F mice to *c-jun*$^{\Delta ep}$ mice. K5-SOS-F transgenic mice develop skin tumors in a strictly EGFR-dependent manner. The first hyperplastic abnormalities induced by K5-SOS-F are visible already 2 weeks after birth and then progress into highly

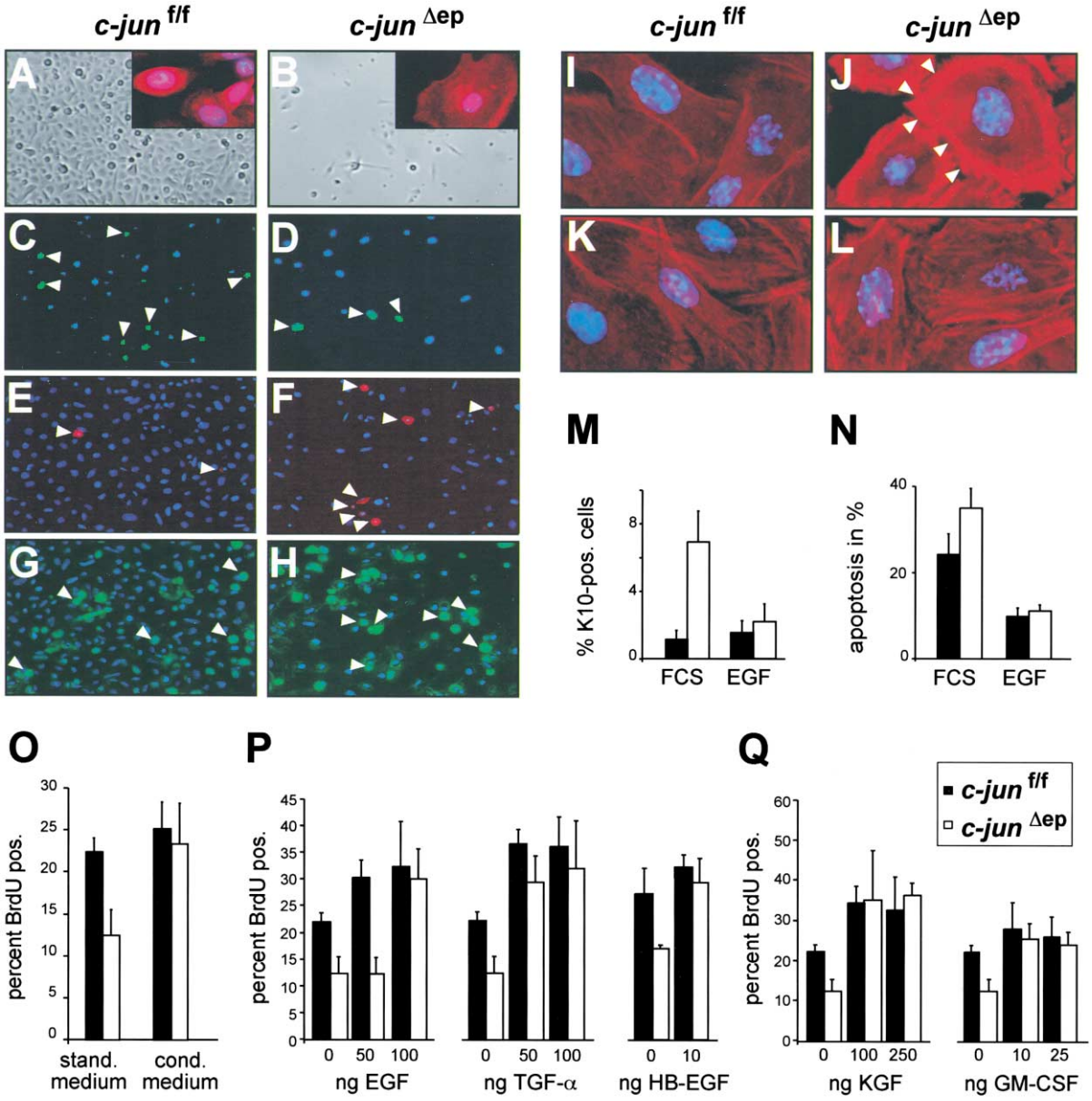

Figure 5. *c-jun*$^{\Delta ep}$ Keratinocytes Are More Differentiated and Have a Cytoskeleton Defect

Primary keratinocytes isolated from newborn *c-jun*$^{\Delta ep}$ mice (B and D) never reached confluency and showed a flat appearance, and the number of BrdU-positive cells was dramatically reduced compared to that in *c-jun*$^{f/f}$ keratinocytes (C and D).

(A and B) Phase contrast microscopy, Keratin 5 staining ([A and B], insets), and BrdU staining (C and D).

(E and F) Staining for Keratin 10 demonstrated a relative increase in differentiating keratinocytes in the absence of c-Jun (M).

(G and H) TUNEL staining showed a significant increase in the number of apoptotic keratinocytes when c-Jun was absent (N). Conditioned medium from *c-jun*$^{f/f}$ keratinocytes, as well as HB-EGF, EGF, TGF-α, KGF, and GM-CSF, could rescue the proliferation defect.

(O, P, and Q) Quantification of BrdU-positive keratinocytes in rescue experiments with conditioned medium (O), HB-EGF, EGF, and TGF-α (P), and KGF and GM-CSF (Q).

(M and N) High-dose EGF (100 ng) reduced the number of differentiating and dying keratinocytes to wild-type levels.

(I and J) Actin-phalloidin staining revealed a cytoskeleton defect in *c-jun*$^{\Delta ep}$ keratinocytes that is characterized by the formation of prominent cortical actin bundles ([J], arrows). This defect could be rescued by addition of high dose EGF (L).

disorganized papillomatous structures (Sibilia et al., 2000). In the absence of c-Jun (*c-jun*$^{\Delta ep}$ SOS^{+}), the tumor volume at the age of 5 weeks was decreased approximately 50%, whereas the average number of papillomas per mouse was unchanged (Figure 6A and data not shown). Despite a significant difference in tumor size, analysis of both mutant and control tumors revealed no striking differences in the histological appearance and cellular composition (Figures 6D and 6E). Staining with Keratin 5 and the proliferation marker Ki67 showed that the majority of basal cells were proliferating and that proliferating cells, which are normally confined to the basal layer, had expanded to the suprabasal compartments (Figures 6F and 6J, arrows). In contrast, Ki67- and

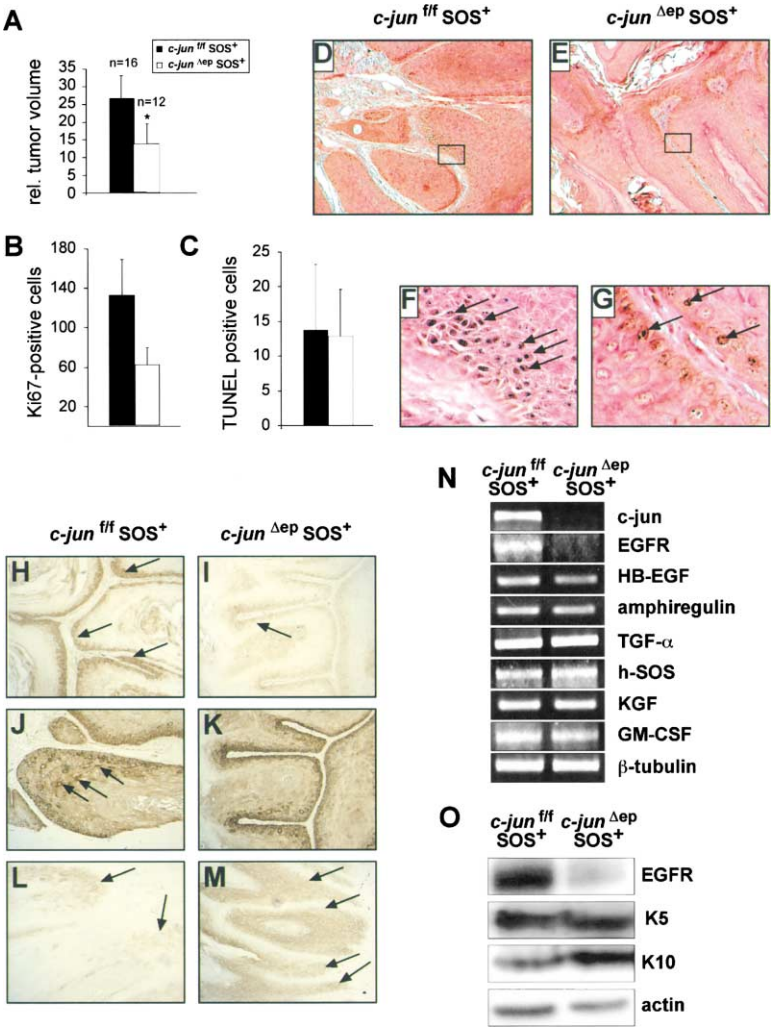

Figure 6. K5-SOS-F-Dependent Tumor Formation in the Absence of c-Jun

(A) Quantification of the relative tumor volume of K5-SOS-F *c-jun*$^{f/f}$ (*c-jun*$^{f/f}$ SOS^{+}) and K5-SOS-F *c-jun*$^{\Delta ep}$ (*c-jun*$^{\Delta ep}$ SOS^{+}) mice reveals an average decrease in tumor size of approximately 50%. Analysis of *c-jun*$^{f/f}$ SOS^{+} and *c-jun*$^{\Delta ep}$ SOS^{+} tumors showed no striking differences in the histological appearance (D and E) and cellular composition. In contrast to *c-jun*$^{f/f}$ SOS^{+} tumors (F), Ki67-positive keratinocytes in *c-jun*$^{\Delta ep}$ SOS^{+} tumors were mainly localized in the basal layer, and their number was significantly reduced (G and B), whereas TUNEL staining (data not shown) revealed no difference in the apoptotic index (C). EGFR was strongly expressed only in *c-jun*$^{f/f}$ SOS^{+} tumors (H), but not in tumors lacking c-Jun (I). Proliferating K5-positive keratinocytes had expanded to the suprabasal compartments in *c-jun*$^{f/f}$ SOS^{+} tumors ([J], arrows), whereas they were confined to the basal layer in *c-jun*$^{\Delta ep}$ SOS^{+} tumors (K). Keratinocytes in *c-jun*$^{\Delta ep}$ SOS^{+} tumors (M) exhibit increased differentiation, as shown by immunohistochemistry for Keratin 10 (L and M). RT-PCR (N) for EGFR, EGFR ligands, and paracrine factors from SOS-F-induced tumors and Western blot analysis (O) confirmed reduced expression of EGFR and increased expression of K10 in tumors lacking c-Jun.

K5-positive cells in *c-jun*$^{\Delta ep}$ SOS^{+} tumors were largely localized to the basal layer, and the number of proliferating cells was significantly reduced (Figures 6B, 6G, and 6K). No significant difference in the number of apoptotic cells was detected in the basal or suprabasal layers with TUNEL staining (Figure 6C).

Immunohistochemistry for EGFR showed that the receptor is efficiently expressed in *c-jun*$^{f/f}$ SOS^{+} tumors, whereas, in the absence of c-Jun, EGFR protein levels were strongly reduced (Figures 6H and 6I). Downregulation of EGFR is linked to the differentiation of keratinocytes in vivo and in vitro, and SOS^{+} tumors in an EGFR-deficient background are more differentiated (Peus et al., 1997; Sibilia and Wagner, 1995; Wakita and Takigawa, 1999; Sibilia et al, 2000). Similarly, *c-jun*$^{\Delta ep}$ SOS^{+} tumors were more differentiated and showed increased K10 expression (Figures 6L, 6M, and 6O). To analyze the regulation of EGFR by c-Jun, we performed semiquantitative RT-PCR and Western blot analysis. EGFR mRNA and protein were expressed at reduced levels, whereas the expression of hSOS as well as autocrine and paracrine factors was unchanged (Figures 6N and 6O). These results suggest that impaired EGFR expression is most likely responsible for reduced tumor growth because of induction of keratinocyte differentiation, rather than proliferation (Figure 7F).

c-Jun Regulates EGFR in Keratinocytes and Fibroblasts

c-Jun-deficient fibroblasts proliferate more slowly, likely because of increased p53 and p21 levels (Figure 7A; Schreiber et al., 1999). In contrast, in c-*jun*$^{\Delta ep}$ keratinocytes, the levels of p53 and p21 were comparable to those in controls, suggesting that the molecular mechanism responsible for the proliferation defect in the two cell types is different (Figure 7A). Semiquantitative RT-PCR analysis in primary keratinocytes revealed that EGFR and HB-EGF mRNA were downregulated, whereas amphiregulin and TGF-α expression were unchanged (Figure 7B). Furthermore, the reduced levels of EGFR expression in c-Jun-deficient keratinocytes were confirmed by Western blot analysis (Figure 7C). Interestingly, EGFR expression was also found to be regulated by c-Jun in 3T3 fibroblasts. Western blot analysis demonstrated reduced EGFR expression levels in c-Jun-deficient cells, whereas high levels of EGFR were observed when c-Jun was stably overexpressed in *c-jun*$^{-/-}$

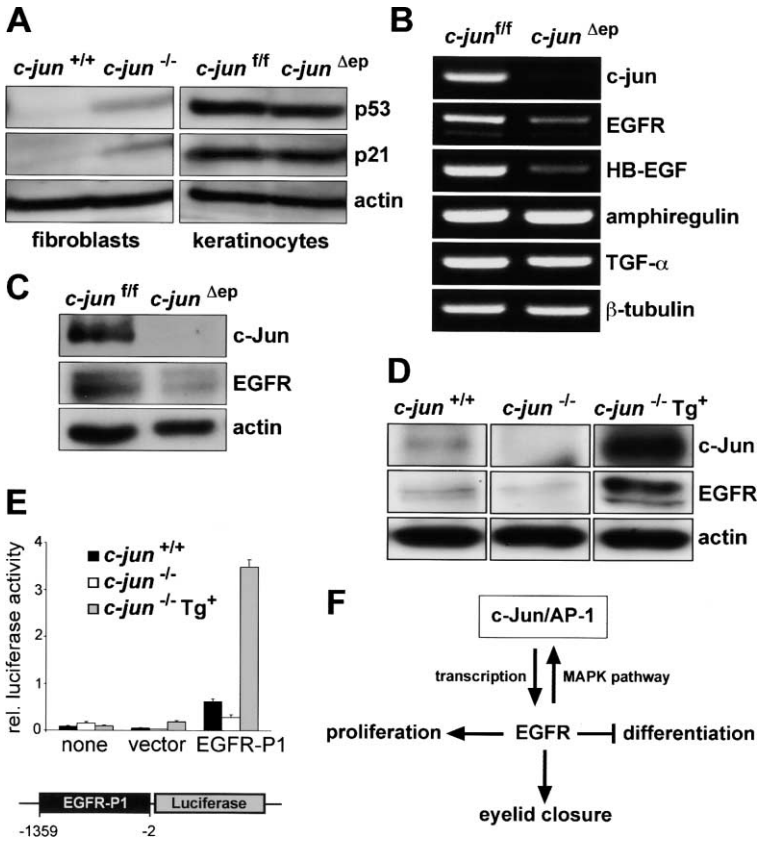

Figure 7. c-Jun Regulates EGFR Expression

(A) Western blot analysis for p53 and p21 in fibroblasts (left side) and in primary keratinocytes lacking c-Jun (right side). In contrast to fibroblasts, the levels of p53 and p21 were comparable in c-*jun*$^{\Delta ep}$ keratinocytes.

(B) RT-PCR for EGFR and EGFR ligands (HB-EGF, amphiregulin, and TGF-α) show reduced EGFR and HB-EGF expression in primary keratinocytes lacking c-Jun (*c-jun*$^{\Delta ep}$).

(C) Western blot of primary keratinocytes demonstrating reduced expression of EGFR in *c-jun*$^{\Delta ep}$ keratinocytes.

(D) EGFR expression is regulated by c-Jun in 3T3 fibroblasts. Western blot analysis demonstrated reduced EGFR expression in c-Jun-deficient cells, whereas high levels of EGFR were observed when c-Jun was stably overexpressed.

(E) Luciferase reporter assays performed in fibroblasts demonstrated reduced EGFR expression in the absence of c-Jun and strong upregulation in *c-jun*$^{-/-}$ fibroblasts overexpressing exogenous c-Jun.

(F) Schematic model summarizing the processes in keratinocytes, which require c-Jun to regulate the expression of EGFR. At the eyelid tips expression of EGFR is required for eyelid closure during development. Primary keratinocytes lacking c-Jun show a severe proliferation defect and increased differentiation and subsequently die by apoptosis, which is likely caused by reduced EGFR signaling. SOS-F-induced tumors lacking c-Jun fail to efficiently express EGFR, leading to an imbalance between proliferation and differentiation, thereby yielding reduced tumor sizes.

fibroblasts (Figure 7D). To analyze whether the regulation of EGFR by c-Jun occurs at the transcriptional level, we performed luciferase reporter assays by employing a mouse EGFR promoter fragment ranging from −1359 to −2. Compared to that in controls, basal EGFR expression was significantly reduced in *c-jun*$^{-/-}$ fibroblasts, but strongly upregulated in fibroblasts overexpressing exogenous c-Jun (Figure 7E). These data indicate that the regulation of EGFR by c-Jun occurs at the transcriptional level.

Discussion

To study the role of c-Jun in skin development and skin tumor formation, we tissue-specifically deleted *c-jun* in keratinocytes of mice. These *c-jun*$^{\Delta ep}$ mice displayed a normal epidermal architecture, and their skin responded normally to stress stimuli, like treatment with the phorbol ester TPA or excisional full-thickness wounding. However, *c-jun*$^{\Delta ep}$ mice were born with open eyes, likely because of insufficient expression of EGFR at the leading edges of developing eyelids during embryonic development. Primary keratinocytes lacking c-Jun exhibited a severe proliferation defect that could be rescued by conditioned medium, but also by autocrine and paracrine growth factors such as HB-EGF, EGF, TGF-α, KGF, and GM-CSF. In addition, skin tumor formation was impaired in K5-SOS-F transgenic mice lacking c-Jun in the epidermis. In both cultured keratinocytes and K5-SOS-induced tumors lacking c-Jun, reduced expression of EGFR was observed, suggesting a regulatory loop from EGFR signaling to c-Jun-dependent EGFR expression (Figure 7F).

c-Jun Is a Regulator of Embryonic Eyelid Closure

Although an increasingly large number of mutant mice are born with open eyes (EOB), the primary events involved in eyelid fusion are still not known (Carroll et al., 1998; He et al., 2002; Juriloff et al., 2000; Li et al., 2001). Deletion of *c-jun* in the epidermis leads to an EOB phenotype. Electron microscopy of leading edge cells revealed slightly fewer and shorter filopodia, suggesting that the zippering machinery may not be responsible for the failure to close the eyelids. Calcium-stimulated filopodia formation and directed actin polymerization have been shown to be the driving force for epithelial cell-cell adhesion in keratinocytes (Vasioukhin et al., 2000). Here we show that, in primary keratinocytes lacking c-Jun, the actin microfilaments are primarily organized in prominent cortical bundles. This cytoskeleton defect could be rescued by high doses of EGF, further suggesting that insufficient EGFR expression in the absence of c-Jun might be causal for this defect. In addition, the results presented by Li et al. (2003) in this issue of *Developmental Cell* and Y. Xia and M. Karin (personal

communication) showed cytoskeletal defects in keratinocytes deficient for the MAPK/JNK/c-Jun pathway. These leading edge cells fail to elongate centripetally, as they do in wild-type fetuses, and these defects may well be responsible for the failure of *c-jun*$^{\Delta ep}$ eyelid epidermis to spread forward over the developing cornea. Several mutations in mice that result in a failure of prenatal eyelid fusion cause a variety of ocular pathologies resulting from subsequent postnatal inflammation and corneal damage (Carroll et al., 1995; Fujii et al., 1995; Luetteke et al., 1993; Mann et al., 1993; Vassalli et al., 1994). Similar to the defects observed in *c-jun* mutant mice, disruption of the genes encoding EGFR, TGF-α, and MEKK1 or overexpression of *fra-2* and h-SOS resulted in an EOB phenotype (Luetteke et al., 1993; Mann et al., 1993; Miettinen et al., 1995; Sibilia and Wagner, 1995; McHenry et al., 1998; Sibilia et al., 2000). Since the failure of eyelid closure in *c-jun*$^{\Delta ep}$ fetuses was associated with reduced EGFR expression, these data indicate the existence of a signaling pathway from the EGFR via activation of MAPK to AP-1-dependent gene expression. EGFR signaling has been linked to the activation of c-Jun/AP-1 in keratinocytes (Malliri et al., 1998). In addition, AP-1 was found to be required for EGF-induced activation of Rho-like GTPases, cytoskeletal rearrangements, motility, and in vitro invasion of human squamous carcinoma-derived A431 cells. Expression of the dominant-negative mutant of c-Jun (TAM67) inhibited EGF-induced membrane ruffling, lamellipodia formation, cortical actin polymerization, and cell rounding in vitro (Malliri et al., 1998). These results indicate a prominent role for c-Jun in the regulation of cytoskeleton remodeling, which seems to be necessary for embryonic eyelid closure (see Li et al., 2003).

Skin Development and Stress Responses Are Not Affected in Mice Lacking c-Jun

Specific deletion of c-Jun in cells of the stratum basale did not yield an obvious skin phenotype, suggesting that c-Jun is not required for skin development under these conditions and that other members of the Jun protein family may functionally compensate for the loss of c-Jun. This idea is supported by the observation that *c-jun*$^{\Delta ep/+}$ *junB*$^{\Delta ep}$ double-mutant mice spontaneously develop papillomas (3 out of 12), suggesting that a balanced level of activators and inhibitors of keratinocyte proliferation is required for epidermal differentiation (R.Z., unpublished data).

Treatment of the epidermis with TPA known to activate c-Jun/AP-1 and to induce keratinocyte hyperproliferation revealed no differences between *c-jun*$^{\Delta ep}$ mice and controls. Neither keratinocyte proliferation nor their stratification was reduced. This is in agreement with results from Young et al. (1999), who showed that a transactivation mutant of c-Jun (TAM67) expressed under the Keratin 14 promoter did not prevent TPA-induced hyperproliferation. These results exclude a functional role for c-Jun in TPA-induced stress response.

Healing upon full-thickness wounding also did not exhibit an obvious defect in *c-jun*$^{\Delta ep}$ mice, and wound closure was comparable to that in control littermates. Histological analyses revealed no significant differences in the proliferation and migration of keratinocytes from the wound edges and adjacent hair follicle, suggesting that c-Jun is not absolutely required during wound healing. However, a difference at later stages of wound healing was observed in the study by Li et al. (2003), which may be explained by the use of a different promoter to express the *cre* transgene or by different genetic backgrounds of the mice under study.

Severe Proliferation Defect in Keratinocytes Lacking c-Jun

Primary keratinocytes isolated from newborn *c-jun*$^{\Delta ep}$ mice exhibited a severe proliferation defect, with increased numbers of differentiating and apoptotic cells. Cultured keratinocytes loose their attachment to the basal lamina, and paracrine factors are no longer provided by the adjacent dermal cells. Fibroblast-derived KGF and GM-CSF were identified as key regulators of paracrine signaling responsible for the balance of keratinocyte proliferation and differentiation in an organotypic coculture system of human keratinocytes and mouse fibroblasts (Szabowski et al., 2000). Indeed, the proliferation defect was rescued by exogenous addition of KGF and GM-CSF, suggesting that, in vivo, the cell-autonomous proliferation defect might be compensated by paracrine factors. The rescue by conditioned medium from wild-type keratinocytes indicates that the production or release of a soluble factor is impaired in *c-jun*$^{\Delta ep}$ keratinocytes. HB-EGF was strongly reduced in keratinocytes lacking c-Jun and is the likely candidate for the factor present in the conditioned medium. Addition of HB-EGF to cultured keratinocytes rescued the proliferation defect. Previous reports showed HB-EGF expression to be dependent on c-Jun and MAPK signaling (Fu et al., 1999). Furthermore, HB-EGF release from the membrane by matrix metalloproteases, which are often regulated by AP-1, have been described (Tokumaru et al., 2000). A defect in the release of HB-EGF from the membrane would impair the proliferation of keratinocytes in vitro. This idea is supported by the less severe proliferation defect observed when keratinocytes were plated at very high cell density, suggesting that membrane-bound HB-EGF might act via juxtacrine signaling (data not shown). EGF could also rescue the proliferation defect and reduced the number of differentiating and dying keratinocytes to wild-type levels. However, high concentrations were needed, suggesting that EGFR signaling is impaired in keratinocytes lacking c-Jun. Primary keratinocytes deficient for the EGFR do not show an obvious proliferation defect (M.S., unpublished data), suggesting that reduced EGFR and reduced HB-EGF levels together are responsible for the drastic phenotype seen in c-Jun null keratinocytes.

Differentiation of keratinocytes has been linked to the inhibition of EGFR (Sibilia and Wagner, 1995; Wakita and Takigawa, 1999), and it has been shown that, after EGFR inhibition, the differentiation markers Keratin 1 and 10 are induced in human keratinocytes (Peus et al., 1997). Interestingly, seven functional AP-1 sites were found in the human EGFR promoter, and c-Jun was shown to regulate EGFR promoter activity in a dose-dependent manner in transformed epithelial cell lines and mouse 3T3 fibroblasts (Johnson et al., 2000). Using *c-jun*$^{+/+}$, *c-jun*$^{-/-}$, and *c-jun*$^{-/-}$ Tg^{+} fibroblasts, we demonstrate, at the protein level and by luciferase reporter

assays, that c-Jun is indeed a transcriptional regulator of EGFR expression. Accordingly, we observe insufficient expression of EGFR under conditions where induced EGFR expression seems to be important—during eyelid closure, in cultured keratinocytes, and during tumor formation. Moreover, sequence comparison of the human and mouse EGFR genes revealed highly conserved sequences in the promoter and first intron, both containing AP-1 binding sites (data not shown). Reduced EGFR expression was also found in fibroblasts lacking c-Jun, which exhibit a cell cycle defect due to elevated p53 and p21 protein levels (Schreiber et al., 1999; Shaulian et al., 2000). However, p53 and p21 expression were not changed in mutant keratinocytes, suggesting that different molecular mechanisms control cell proliferation in fibroblasts and keratinocytes.

K5-SOS-F-Dependent Tumor Formation Requires c-Jun

Tumor formation by the K5-SOS-F transgene is impaired in the absence of c-Jun, although the histological appearance and cellular composition was comparable to control tumors. An increase in the number of differentiated keratinocytes was observed in *c-jun* mutant tumors. Again, similarly to data obtained with cultured keratinocytes, this was associated with reduced expression of the EGFR in these tumors. Interestingly, K5-SOS-F-induced tumor formation was strongly reduced in tumors lacking EGFR, where the EGFR provides an essential survival signal (Sibilia et al., 2000). In *c-jun*$^{\Delta ep}$ SOS^{+} tumors there was no increase in the number of apoptotic cells, suggesting that EGFR is most likely sufficiently expressed to provide this survival signal. In wild-type tumors EGFR signaling appears to delay the commitment to differentiate, suggesting that maintaining cells in the proliferative compartment may yet be another function of EGFR in rapidly proliferating tissues. Thus, we argue that, in tumors lacking c-Jun, the levels of EGFR expression are sufficient to signal survival but are too low to maintain keratinocytes in a proliferative state, and, therefore, increased differentiation is observed, which leads to reduced tumor growth.

Activation of the EGFR provides important signals essential to several aspects of keratinocyte biology, including cell cycle progression, differentiation, cell movement, and cellular survival. Here we have identified c-Jun as a transcriptional regulator of EGFR and HB-EGF in three experimental systems. Through modulation of HB-EGF and EGFR expression, c-Jun controls eyelid development, keratinocyte proliferation, and skin tumor formation and thereby strongly influences the fate of keratinocytes.

Experimental Procedures

Generation of *c-jun*$^{\Delta ep}$ and K5-SOS-F *c-jun*$^{\Delta ep}$ Mice

Mice carrying a floxed *c-jun* allele (*c-jun*$^{f/f}$; Behrens et al., 2002) were crossed to transgenic mice expressing the Cre recombinase under the control of the keratinocyte-specific Keratin 5 promoter (K5-*cre*2; Tarutani et al., 1997). To obtain K5-SOS-F *c-jun*$^{\Delta ep}$ transgenic and K5-SOS-F transgenic control littermates, we crossed offspring of the *c-jun*$^{f/f}$ EGFRwa2/wa2 intercross with K5-SOS-F EGFRwa2/wa2 transgenic mice (Sibilia et al., 2000) with the K5-*cre*2 line. The genetic background was C57Bl/6 × 129 for c-*jun*$^{f/f}$, B6C3F1 for K5-*cre*2, LVC for EGFRwa2/wa2, and a C57Bl/6 × CBA backcross to LVC for K5-SOS-F mice.

PCR

Tail epidermis was separated from dermis by dispase II-digest (Roche), and DNA was extracted according to standard protocols. The following primers were used for genotyping: Jun1, CTCATACCA GTTCGCACAGGCGGC; Jun2, CCGCTAGCACTCACGTTGGTAGGC; Jun3, CAGGGCGTTGTGTCAC-TGAGCT; Cre1, CGGTCGATGCAAC GAGTGATGAGG; Cre2; CCAG-AGACGGAAATCCATCGCTCG.

Histology, Immunohistochemistry, and TUNEL Assay

Tissues were fixed overnight with neutral buffered 4% PFA at 4°C and embedded in paraffin. Five-micrometer sections were stained either with hematoxylin and eosin (H&E) or processed further. Immunohistochemistry staining for Ki67 (Novocastra), c-Jun (Transduction Laboratories), Keratin 5, Keratin 10 (BabCO), and EGFR (Santa Cruz) was performed with the ABC staining kit (Vector Laboratories) according to the manufacturer's recommendations. TUNEL staining was performed with the in situ cell death detection kit (Roche). Immunfluorescence for Keratin 5 and 10 (BabCO) and F-actin (Alexa Fluor 568 phalloidin; Molecular Probes) was performed after fixation with 4% PFA, and nuclei were counterstained with the DAPI reagent.

Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), and Resin Histology

Specimens for SEM, TEM, or resin histology were fixed in half-strength Karnovsky's fixative (Karnovsky, 1965) at 4°C. After fixation embryonic heads were dissected sagitally in the midline (for SEM) or eyelids were dissected free and the lenses were removed (for resin histology and TEM), rinsed in 0.1 M sodium cacodylate buffer, postfixed in 1% osmium tetroxide in sodium cacodylate, and further rinsed in cacodylate buffer before being dehydrated through a graded ethanol series. Specimens for SEM were then critical point dried in the standard way and sputter coated with approximately 30 nm of gold before they were viewed on a Jeol 5410LV scanning electron microscope. Specimens for resin histology were embedded in Araldite, sectioned at 5 μm, and stained with Toluidine blue. For examination by TEM, ultrathin Araldite sections were cut, stained with uranyl acetate and lead citrate, and examined with a Jeol 1010 transmission electron microscope.

TPA-Induced Hyperproliferation

Eight-week-old littermates (n = 5) were shaved and, after 24 hr, treated three times every 48 hr with 5 μg TPA or solvent alone (aceton). Forty-eight hours after the last treatment, the back skin was isolated and fixed for histological analysis. Ten high-power fields per back skin were scored for epidermal thickness and Ki67-positive cells.

Wound-Healing Experiment

Eight-week-old littermates were anesthetized (Avertin) and shaved, and excisional full-thickness wounds were placed on their back skins by skin biopsy punches (5 mm; KAI). The healing of the wounds was followed, and, after 2, 4, 7, and 10 days postwounding (n = 5–6 for every time point), the wound size was measured and the wounds were isolated and fixed for histological analysis.

Quantification of Tumors

c-jun$^{\Delta ep}$ SOS^{+} mice (n = 12) and their control littermates (*c-jun*$^{f/f}$ SOS^{+}; n = 16) were killed at 5 weeks of age, and the number and weight of tumors were determined. For quantification the ratio of total tumor weight to body weight was calculated. Statistical significance was confirmed by Student's t test. Isolated tumors were frozen in liquid nitrogen or fixed in neutral-buffered 4% PFA at 4°C and processed further.

RNA Isolation and RT-PCR

Total RNA was isolated from cultured keratinocytes and tumors by the TRIZOL protocol (Sigma). cDNA synthesis was performed with the SuperScript Preamplification System (GibcoBRL) according to the manufacturer's instructions. RT-PCR analysis was performed with primers described by Sibilia et al. (2000) and Szabowski et al. (2000).

Isolation and Culture of Mouse Keratinocytes

Primary mouse keratinocytes were isolated as described by Hennings (1994) and cultured on vitrogen-fibronectin-coated dishes in low-calcium MEM (SIGMA M8167) medium containing 8% chelated FCS (Carroll et al., 1995). Conditioned medium was harvested and filtered (22 μm; Millipore) from primary mouse keratinocyte cultures at days 4, 7, and 10. Rescue experiments were performed with growth factors from Sigma (HB-EGF, EGF, TGF-α, and KGF) and R&D (GM-CSF) and quantified by BrdU staining (Becton Dickinson).

Southern and Western Blot Analysis

For Southern blots, 10 μg of epidermal DNA was digested with XbaI, yielding a 6.9 kb fragment for the floxed *c-jun* allele and a 3.3 kb fragment for the deleted *c-jun* allele. For detection of the bands, a 0.6 kb BamHI fragment from the *c-jun* promoter region was used as a probe (Behrens et al., 2002). Western blot analysis was performed according to standard procedures (Sibilia et al., 2000) with antibodies specific for c-Jun (Transduction Laboratories), actin, p53, p21 (Sigma), and EGFR (Santa Cruz).

Cloning of Reporter Construct, Transfection, and Luciferase Assay

The mouse EGFR gene promoter fragment ranging from −1359 to −2 (EGFR-P1) was amplified by PCR from a previously described EGFR genomic fragment of 129/Sv background (Sibilia and Wagner, 1995). The EGFR-P1 fragment was cloned into the BglII and HindIII sites upstream of the Firefly luciferase reporter gene of the pGL3-Basic vector (Promega, Madison, WI). The pGL3-EGFR-P1 reporter construct was transfected into *c-jun*$^{+/+}$, *c-jun*$^{-/-}$, and *c-jun*$^{-/-}$ *c-jun* Tg^{+} fibroblast cell lines (Schreiber et al., 1999) by employing the Lipofectamine Plus Reagent (Life Technologies, Gaithersburg, MD). Each transfection was performed in triplicates in 12-well plates, and the Renilla luciferase reporter pRL-SV40 (Promega, Madison, WI) was cotransfected as an internal control. Cells were harvested 24 hr after transfection, cell extracts were prepared, and the luciferase activity was measured according to the Dual-Luciferase Reporter Assay System protocol (Promega, Madison, WI). All Firefly luciferase reporter activities were normalized for transfection efficiency by determining the ratio between Firefly and Renilla luciferase activity.

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References

Angel, P., Hattori, K., Smeal, T., and Karin, M. (1988). The jun proto-oncogene is positively autoregulated by its product, Jun/AP-1. Cell *55*, 875–885.

Angel, P., Szabowski, A., and Schorpp-Kistner, M. (2001). Function and regulation of AP-1 subunits in skin physiology and pathology. Oncogene *20*, 2413–2423.

Behrens, A., Jochum, W., Sibilia, M., and Wagner, E.F. (2000). Oncogenic transformation by ras and fos is mediated by c-Jun N-terminal phosphorylation. Oncogene *19*, 2657–2663.

Behrens, A., Sibilia, M., David, J.P., Mohle-Steinlein, U., Tronche, F., Schutz, G., and Wagner, E.F. (2002). Impaired postnatal hepatocyte proliferation and liver regeneration in mice lacking c-jun in the liver. EMBO J. *21*, 1782–1790.

Bossy-Wetzel, E., Bravo, R., and Hanahan, D. (1992). Transcription factors junB and c-jun are selectively up-regulated and functionally implicated in fibrosarcoma development. Genes Dev. *6*, 2340–2351.

Byrne, C., and Fuchs, E. (1993). Probing keratinocyte and differentiation specificity of the human K5 promoter in vitro and in transgenic mice. Mol. Cell. Biol. *13*, 3176–3190.

Carroll, J.M., Romero, M.R., and Watt, F.M. (1995). Suprabasal integrin expression in the epidermis of transgenic mice results in developmental defects and a phenotype resembling psoriasis. Cell *83*, 957–968.

Carroll, J.M., Luetteke, N.C., Lee, D.C., and Watt, F.M. (1998). Role of integrins in mouse eyelid development: studies in normal embryos and embryos in which there is a failure of eyelid fusion. Mech. Dev. *78*, 37–45.

Du, W., Thanos, D., and Maniatis, T. (1993). Mechanisms of transcriptional synergism between distinct virus-inducible enhancer elements. Cell *74*, 887–898.

Eferl, R., Sibilia, M., Hilberg, F., Fuchsbichler, A., Kufferath, I., Guertl, B., Zenz, R., Wagner, E.F., and Zatloukal, K. (1999). Functions of c-Jun in liver and heart development. J. Cell Biol. *145*, 1049–1061.

Fowler, K.J., Walker, F., Alexander, W., Hibbs, M.L., Nice, E.C., Bohmer, R.M., Mann, G.B., Thumwood, C., Maglitto, R., Danks, J.A., et al. (1995). A mutation in the epidermal growth factor receptor in waved-2 mice has a profound effect on receptor biochemistry that results in impaired lactation. Proc. Natl. Acad. Sci. USA *92*, 1465–1469.

Fu, S., Bottoli, I., Goller, M., and Vogt, P.K. (1999). Heparin-binding epidermal growth factor-like growth factor, a v-Jun target gene, induces oncogenic transformation. Proc. Natl. Acad. Sci. USA *96*, 5716–5721.

Fujii, S., Hatakenaka, N., Kaneda, M., and Teramoto, S. (1995). Morphogenetic study of the eyelids in NC-eob mice fetuses with an open-eyelid malformation at birth. Lab. Anim. Sci. *45*, 176–180.

Hansen, L.A., Alexander, N., Hogan, M.E., Sundberg, J.P., Dlugosz, A., Threadgill, D.W., Magnuson, T., and Yuspa, S.H. (1997). Genetically null mice reveal a central role for epidermal growth factor receptor in the differentiation of the hair follicle and normal hair development. Am. J. Pathol. *150*, 1959–1975.

He, W., Li, A.G., Wang, D., Han, S., Zheng, B., Goumnans, M.J., ten Dijke, P., and Wang, X.J. (2002). Overexpression of Smad7 results in severe pathological alterations in multiple epithelial tissues. EMBO J. *21*, 2580–2590.

Hennings, H. (1994). Primary culture of keratinocytes from newborn mouse epidermis in medium with lowered levels of Ca^{2+}. In Keratinocyte Methods, I. Leigh and F. Watt, eds. (Cambridge: Cambridge University Press) pp. 21–23.

Jochum, W., Passegue, E., and Wagner, E.F. (2001). AP-1 in mouse development and tumorigenesis. Oncogene *20*, 2401–2412.

Johnson, A.C., Murphy, B.A., Matelis, C.M., Rubinstein, Y., Piebenga, E.C., Akers, L.M., Neta, G., Vinson, C., and Birrer, M. (2000). Activator protein-1 mediates induced but not basal epidermal growth factor receptor gene expression. Mol. Med. *6*, 17–27.

Juriloff, D.M., Harris, M.J., Banks, K.G., and Mah, D.G. (2000). Gaping lids, gp, a mutation on centromeric chromosome 11 that causes defective eyelid development in mice. Mamm. Genome *11*, 440–447.

Karnovsky, M.J. (1965). A formaldehyde-gluteraldehyde fix of high osmolarity for use in electron microscopy. J. Cell Biol. *27*, 137–138.

Kaufman, C.K., and Fuchs, E. (2000). It's got you covered. NF-kappaB in the epidermis. J. Cell Biol. *149*, 999–1004.

Li, C., Guo, H., Xu, X., Weinberg, W., and Deng, C.X. (2001). Fibroblast growth factor receptor 2 (Fgfr2) plays an important role in eyelid and skin formation and patterning. Dev. Dyn. *222*, 471–483.

Li, G., Gustafson-Brown, C., Hanks, S.K., Nason, K., Arbeit, J.M., Pogliano, K., Wisdom, R.M., and Johnson, R.S. (2003). c-Jun is essential for organization of the epidermal leading edge. Dev. Cell *4*, this issue, 865–877.

Luetteke, N.C., Phillips, H.K., Qiu, T.H., Copeland, N.G., Earp, H.S., Jenkins, N.A., and Lee, D.C. (1994). The mouse waved-2 phenotype results from a point mutation in the EGF receptor tyrosine kinase. Genes Dev. *8*, 399–413.

Luetteke, N.C., Qiu, T.H., Peiffer, R.L., Oliver, P., Smithies, O., and Lee, D.C. (1993). TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell *73*, 263–278.

Malliri, A., Symons, M., Hennigan, R.F., Hurlstone, A.F., Lamb, R.F., Wheeler, T., and Ozanne, B.W. (1998). The transcription factor AP-1 is required for EGF-induced activation of rho-like GTPases, cytoskeletal rearrangements, motility, and in vitro invasion of A431 cells. J. Cell Biol. *143*, 1087–1099.

Mann, G.B., Fowler, K.J., Gabriel, A., Nice, E.C., Williams, R.L., and Dunn, A.R. (1993). Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. Cell *73*, 249–261.

Martin, P. (1997). Wound healing—aiming for perfect skin regeneration. Science *276*, 75–81.

Martin, P., and Nobes, C.D. (1992). An early molecular component of the wound healing response in rat embryos—induction of c-fos protein in cells at the epidermal wound margin. Mech. Dev. *38*, 209–215.

McHenry, J.Z., Leon, A., Matthaei, K.I., and Cohen, D.R. (1998). Overexpression of fra-2 in transgenic mice perturbs normal eye development. Oncogene *17*, 1131–1140.

Miettinen, P.J., Berger, J.E., Meneses, J., Phung, Y., Pedersen, R.A., Werb, Z., and Derynck, R. (1995). Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. Nature *376*, 337–341.

Peus, D., Hamacher, L., and Pittelkow, M.R. (1997). EGF-receptor tyrosine kinase inhibition induces keratinocyte growth arrest and terminal differentiation. J. Invest. Dermatol. *109*, 751–756.

Rutberg, S.E., Saez, E., Glick, A., Dlugosz, A.A., Spiegelman, B.M., and Yuspa, S.H. (1996). Differentiation of mouse keratinocytes is accompanied by PKC-dependent changes in AP-1 proteins. Oncogene *13*, 167–176.

Saez, E., Rutberg, S.E., Mueller, E., Oppenheim, H., Smoluk, J., Yuspa, S.H., and Spiegelman, B.M. (1995). c-fos is required for malignant progression of skin tumors. Cell *82*, 721–732.

Schreiber, M., Kolbus, A., Piu, F., Szabowski, A., Mohle-Steinlein, U., Tian, J., Karin, M., Angel, P., and Wagner, E.F. (1999). Control of cell cycle progression by c-Jun is p53 dependent. Genes Dev. *13*, 607–619.

Shaulian, E., Schreiber, M., Piu, F., Beeche, M., Wagner, E.F., and Karin, M. (2000). The mammalian UV response: c-Jun induction is required for exit from p53-imposed growth arrest. Cell *103*, 897–907.

Sibilia, M., and Wagner, E.F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor. Science *269*, 234–238.

Sibilia, M., Fleischmann, A., Behrens, A., Stingl, L., Carroll, J., Watt, F.M., Schlessinger, J., and Wagner, E.F. (2000). The EGF receptor provides an essential survival signal for SOS-dependent skin tumor development. Cell *102*, 211–220.

Stein, B., Baldwin, A.S., Jr., Ballard, D.W., Greene, W.C., Angel, P., and Herrlich, P. (1993). Cross-coupling of the NF-kappa B p65 and Fos/Jun transcription factors produces potentiated biological function. EMBO J. *12*, 3879–3891.

Szabowski, A., Maas-Szabowski, N., Andrecht, S., Kolbus, A., Schorpp-Kistner, M., Fusenig, N.E., and Angel, P. (2000). c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. Cell *103*, 745–755.

Tarutani, M., Itami, S., Okabe, M., Ikawa, M., Tezuka, T., Yoshikawa, K., Kinoshita, T., and Takeda, J. (1997). Tissue-specific knockout of the mouse Pig-a gene reveals important roles for GPI-anchored proteins in skin development. Proc. Natl. Acad. Sci. USA *94*, 7400–7405.

Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., et al. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science *269*, 230–234.

Tokumaru, S., Higashiyama, S., Endo, T., Nakagawa, T., Miyagawa, J.I., Yamamori, K., Hanakawa, Y., Ohmoto, H., Yoshino, K., Shirakata, Y., et al. (2000). Ectodomain shedding of epidermal growth factor receptor ligands is required for keratinocyte migration in cutaneous wound healing. J. Cell Biol. *151*, 209–220.

Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. Cell *100*, 209–219.

Vassalli, A., Matzuk, M.M., Gardner, H.A., Lee, K.F., and Jaenisch, R. (1994). Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. Genes Dev. *8*, 414–427.

Wakita, H., and Takigawa, M. (1999). Activation of epidermal growth factor receptor promotes late terminal differentiation of cell-matrix interaction-disrupted keratinocytes. J. Biol. Chem. *274*, 37285–37291.

Watt, F.M. (2001). Stem cell fate and patterning in mammalian epidermis. Curr. Opin. Genet. Dev. *11*, 410–417.

Young, M.R., Li, J.J., Rincon, M., Flavell, R.A., Sathyanarayana, B.K., Hunziker, R., and Colburn, N. (1999). Transgenic mice demonstrate AP-1 (activator protein-1) transactivation is required for tumor promotion. Proc. Natl. Acad. Sci. USA *96*, 9827–9832.