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The EGF Receptor Provides an Essential Survival Signal for SOS-Dependent Skin Tumor Development

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Summary

The EGF receptor (EGFR) is required for skin development and is implicated in epithelial tumor formation. Transgenic mice expressing a dominant form of Son of Sevenless (SOS-F) in basal keratinocytes develop skin papillomas with 100% penetrance. However, tumor formation is inhibited in a hypomorphic (wa2) and null EGFR background. Similarly, EGFR-deficient fibroblasts are resistant to transformation by SOS-F and *ras*V12, however, tumorigenicity is restored by expression of the anti-apoptotic *bcl*-2 gene. The K5-SOS-F papillomas and primary keratinocytes from wa2 mice display increased apoptosis, reduced Akt phosphorylation and grafting experiments imply a cell-autonomous requirement for EGFR in keratinocytes. Therefore, EGFR functions as a survival factor in oncogenic transformation and provides a valuable target for therapeutic intervention in a broader range of tumors than anticipated.

Introduction

The epidermis is a stratified squamous epithelium composed mainly of keratinocytes, whose proliferation and differentiation must be tightly regulated and coordinated (Fuchs, 1990, 1992). Basal keratinocytes, which are attached to the basement membrane, are undifferentiated and have proliferative potential. Before entering the differentiation program, they withdraw from the cell cycle and migrate toward the surface of the epidermis, leading to the formation of the outermost layer of the epidermis composed of anucleated dead squames, which are continuously shed from the surface of the skin (Jones and Watt, 1993).

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Dedicated to our colleague Laura Stingl, who died from cancer on June 7, 2000.

Epidermal growth factor receptor (EGFR) activation is a central event in the regulation of epidermal development. The EGFR is activated by several ligands such as epidermal growth factor (EGF), transforming growth factor α (TGFα), amphiregulin, heparin binding EGF (HB-EGF), β-cellulin, and epiregulin (Prigent and Lemoine, 1992; Earp et al., 1995). Ligand binding to the EGFR induces receptor dimerization and activation of the intrinsic tyrosine kinase with subsequent autophosphorylation of key tyrosines located at the carboxyl terminal tail of the receptor (Prigent and Lemoine, 1992; Lemmon and Schlessinger, 1994; Earp et al., 1995). Phosphorylated tyrosine residues act as binding sites for proteins containing Src-homology 2 domains (SH2) such as Grb2, SHC, and PLCγ, which, in turn, activate complex downstream signaling cascades, thus transducing extracellular stimuli to the nucleus (Lemmon and Schlessinger, 1994; Weiss et al., 1997). The adaptor protein Grb2 seems to be critically involved in coupling signals from receptor tyrosine kinases to Ras through its association with Son of sevenless (SOS), a guanine nucleotide exchange factor that catalyzes the activation of Ras proteins by facilitating GDP–GTP exchange (Schlessinger, 1994; Weiss et al., 1997). Stimulation of cells with growth factors leads to the association of SOS–Grb2 complexes with activated receptors, and this is proposed to stimulate Ras through the juxtaposition of SOS and Ras at the membrane (Schlessinger, 1994; Weiss et al., 1997). Constitutive active SOS proteins can be obtained by targeting variants of SOS to the membrane via the addition of farnesylation signals (Aronheim et al., 1994). Moreover, deletion of the carboxyl terminal tail of SOS containing the Grb2 binding site also activates the SOS protein (Wang et al., 1995). These dominant forms of SOS have been shown to transform NIH3T3 fibroblasts in vitro and to constitutively activate the Ras/ERK pathway (Aronheim et al., 1994; Wang et al., 1995).

Numerous studies have documented alterations in growth factor signaling pathways in the development of human epithelial neoplasms (Derynck, 1992; Reichmann, 1994). Amplifications, rearrangements, and overexpression of the EGFR have been shown to occur at high frequency in human squamous cell carcinomas and glioblastomas (Libermann et al., 1985; Derynck, 1992) and activating mutations of the *ras* gene are observed in a variety of human neoplasms (Barbacid, 1990). Introduction of viral Ras into mouse epidermal cells in vivo and in vitro can initiate skin tumors (Brown et al., 1986; Roop et al., 1986) and expression of an activated form of Ha-Ras in the suprabasal layer of the epidermis of transgenic mice induces the development of benign papillomas at sites of promotional stimuli (Bailleul et al., 1990). Similarly, transgenic mice expressing the EGFR ligand TGFα in basal or suprabasal keratinocytes display thickening of the epidermis and develop papillomas prevalently at sites of mechanical irritations or wounding (Vassar and Fuchs, 1991; Dominey et al., 1993). In contrast, transgenic mice expressing an activated Ras in the outer root sheath of the hair follicles develop spontaneous papilloma-like skin tumors that frequently undergo conversion to squamous carcinomas (Brown et al., 1998).

EGFR signaling is also of physiological relevance dur-

ing normal epithelial development. Some of the EGFR ligands are synthesized by normal keratinocytes both in vitro and in vivo (Vardy et al., 1995). The EGFR is most strongly expressed in the basal layer of the epidermis and in the outer root sheath of hair follicles, in which the proliferating keratinocytes reside (King et al., 1990; Sibilia and Wagner, 1995). The number of receptors decreases as keratinocytes migrate to the suprabasal layers of the epidermis, entering the pathway of terminal differentiation (King et al., 1990). Recently, it was shown that EGFR signaling regulates keratinocyte survival, since antibody-mediated inhibition of the EGFR renders keratinocytes detached from the extracellular matrix susceptible to apoptosis (Rodeck et al., 1997a).

Mice deficient for the TGFα gene develop a wavy coat and curly whiskers (Luetteke et al., 1993; Mann et al., 1993). A similar phenotype is observed in the naturally occurring mouse mutant strain waved-2 (wa2), which is homozygous for a hypomorphic EGFR allele. Wa2 mice carry a point mutation in the kinase domain of the EGFR resulting in reduced kinase activity (Luetteke et al., 1994; Fowler et al., 1995). In contrast, mice harboring a null mutation in the EGFR gene exhibit strain-dependent phenotypes with defects in neural and epithelial tissues and die before weaning age (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995; Sibilia et al., 1998). These mutants show impaired epidermal as well as hair follicle differentiation and fail to develop a hairy coat, most likely because EGFR signaling is necessary for maintenance of hair follicle integrity (Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995; Hansen et al., 1997). Similar skin and hair phenotypes are observed in transgenic mice expressing a dominant negative EGFR (CD533) in the basal layer of the epidermis and outer root sheath of the hair follicles (Murillas et al., 1995). These results suggest that TGFα/EGFR signaling plays an essential role in epithelial cell proliferation and/or differentiation and is critical for the development of normal hair follicles and skin. However, it is unclear which signaling pathways downstream of the EGFR specifically control these processes.

To investigate EGFR responsive pathways in the skin, we have generated transgenic mice expressing a dominant form of hSOS (SOS-F) under the control of a full-length K5 promoter, which is active in EGFR-expressing cells. Here, we show that all K5-SOS-F mice develop spontaneous skin tumors and that tumor development is impaired in the absence of EGFR function, likely due to increased apoptosis, thereby demonstrating that the EGFR functions as a survival factor in oncogenic transformation.

Results

K5-SOS-F Transgenic Mice Exhibit Skin Hyperplasia and Develop Papillomas

The full-length regulatory region of the K5 gene was used to express an activated form of human Son of Sevenless (SOS-F) in the basal cell compartment of stratified epithelia and in the outer root sheath of the hair follicles (Aronheim et al., 1994; Ramírez et al., 1994) (Figure 1A). Four transgenic founder mice (892A-6*, 893A-1, 893B-1, and 893B-2) were obtained and SOS-F expression was found to be copy number dependent since the founders harboring 3–5 (893A-1) or more than 50 copies of the transgene (893B-1 and 893B-2) showed the highest levels of expression of the SOS-F protein in the skin (Figure 1B and data not shown). The latter displayed dramatic skin hyperplasias on the entire body (Figure 1D), open eyes (Figure 1D), runted limbs (Figure 1E) and died soon after birth probably due to the inability to breathe caused by a dramatic thickening of the tongue epithelium (data not shown). Founder 893A-1 carrying 3–5 copies of the transgene displayed no abnormalities at birth, but started to develop severe skin papillomas after 3 weeks and had to be sacrificed (data not shown). Founder 892A-6* carried only one or two copies of the transgene and showed low expression levels of the SOS-F protein similar to the endogenous SOS protein (Figure 1B). Moreover, the SOS-F protein was expressed in the stomach, thymus, and unaffected skin and papillomas, but was undetectable in other organs (data not shown). Founder 892A-6*, which was used to establish the transgenic line, started to develop papillomas after two weeks of age (Figure 1F). All transgenic offspring developed severe skin alterations on the body and tail and histological examination revealed a hyperplastic epidermis displaying more than the usual 3 to 4 suprabasal cell layers present in controls (data not shown). Within the next 2–3 weeks, these hyperplastic lesions developed into macroscopically visible papillomatous structures with no or only very few hair follicles present in the affected areas (Figures 1F, 1G, and 4A). RNA in situ hybridization using a transgene-specific probe revealed expression of K5-SOS-F in all layers of hyperplastic skins and papillomas. Keratin 14 (K14), which is normally coexpressed with K5 in the basal layer, was also detectable throughout the epidermis of the hyperplastic lesions and papillomas (data not shown). These results show that expression of the transgene in the skin results in a hyperproliferative skin disease leading to hyperplasia and papilloma formation characterized by an expanded basal compartment.

Tumor Development Is Impaired in a Hypomorphic EGFRwa2/wa2 Background

To explore a possible genetic interaction between SOS and EGFR, the K5-SOS-F transgene was bred into an EGFR null ($-/-$) or hypomorphic (wa2/wa2) background. Interestingly, skin tumor development was attenuated in EGFR$-/-$K5-SOS-F mice. The transgene partially rescued the hair growth defects in the few mutants obtained and prolonged the lifespan up to 6 months (M. S. and E. F. W., unpublished data). In contrast, in a wa2/wa2 or wa2/$-$ background, K5-SOS-F expression did not rescue the curly hair phenotype, suggesting either that SOS controls hair growth but not hair follicle orientation or that the levels of expression of K5-SOS-F are not appropriate to rescue this phenotype.

Skin tumor development was also severely impaired in a wa2/wa2 or wa2/$-$ transgenic background (Figure 2A). After 6 months, 85% of wa2/wa2 or wa2/$-$ K5-SOS-F mice were still tumor-free, whereas 100% of $+/+$, $+/-$ or wa2/$+$ K5-SOS-F mice had developed spontaneous tumors within the first two months (Figure 2A). The average tumor volume was dramatically different between the groups. Whereas more than 90% of the papillomas in an EGFR wild-type background were bigger than 1 cm^3 after 3 weeks of age, tumor volume was drastically reduced in the presence of mutant EGFR alleles (Figure 2B). In addition, the few lesions which developed in a mutant EGFR background only started

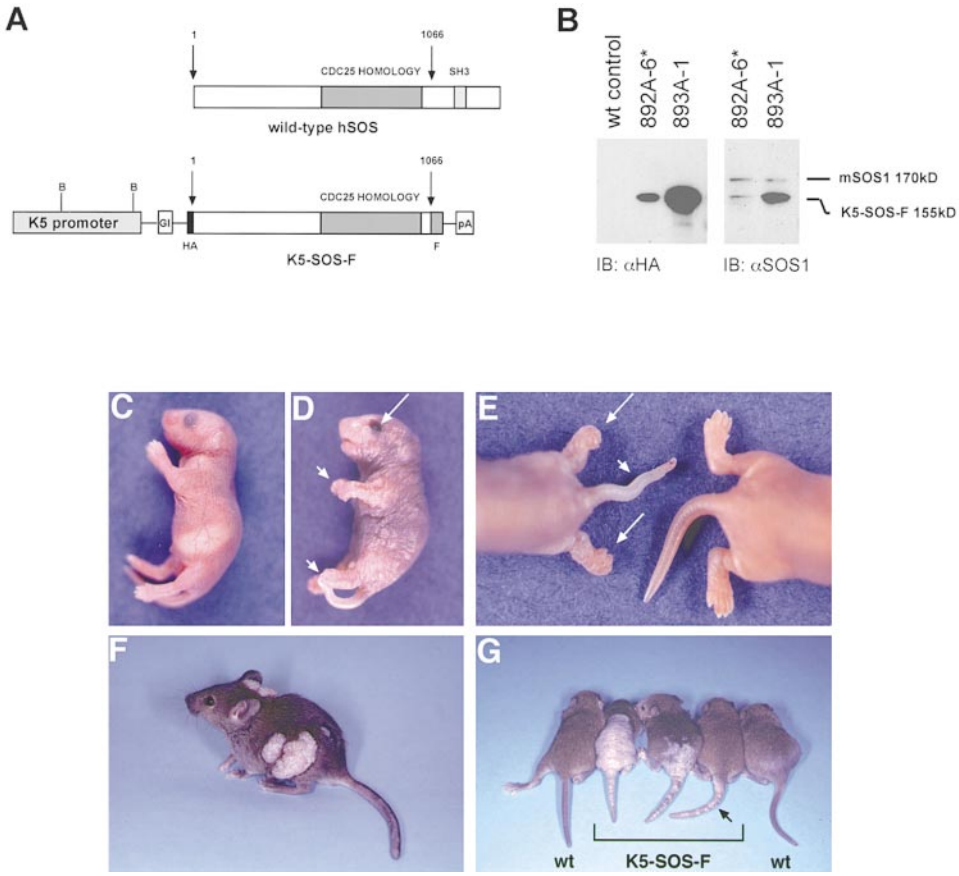


Figure 1. K5-SOS-F Transgenic Mice Exhibit Skin Hyperplasia and Develop Papillomas

(A) Schematic representation of the K5-SOS-F transgene construct. The upper line shows the structure of the wild-type human SOS (hSOS) cDNA with the position of the catalytic pocket denoted by the Cdc 25 homology domain and the proline-rich region binding the SH3 region of Grb2. The activated hSOS expressed from the keratin 5 promoter harbors an HA tag at the N terminus, lacks the C-terminal region containing the Grb2 binding site, and carries the c-Ha-ras farnesilation site (F) instead. B, Bam HI.

(B) Western blot analysis of transgenic skins. Protein extracts from control skin and tumor biopsies of the two surviving founders, 892A-6* and 893A-1, were immunoblotted with either anti-HA (αHA) or anti-SOS (αSOS1) antibodies as indicated. The positions of the 170 kDa endogenous mSOS1 and the 155 kDa transgenic SOS-F are indicated.

(C–D) Phenotype of K5-SOS-F transgenic founders and offspring. (C) Normal and (D) transgenic littermate founder 893B-1 at birth. Note open eyes (big arrow), abnormal limbs (little arrows), and abnormal skin wrinkling and thickening in the transgenic pup, which died shortly after birth.

(E) Runted limbs and kinky tail of the transgenic pup shown in (D) compared to the control littermate. Founder 893B-2 had very similar phenotypes and also died soon after birth (not shown).

(F) Adult founder transgenic mouse 892A-6* showing multiple papillomas at different body sites.

(G) Examples of 12-day-old offspring from founder shown in (F) exhibiting extensive and localized areas of abnormal epidermis on the body and tail compared to two control littermates.

to develop after an extended latency period (more than four months of age) and were frequently localized at the edges of ear-tag or tail-biopsy sites, suggesting that they were induced by wounding.

EGFR-Dependent Transformation of Immortalized Fibroblasts by Oncogenic SOS and Ras

To gain insight into the molecular mechanism of EGFR-dependent transformation and to examine whether EGFR signaling was a prerequisite for oncogenic transformation by components of the Ras signaling pathway in other cell types, immortalized 3T3 fibroblasts isolated from EGFR−/− fetuses were employed. Their proliferation potential compared to wild type was not affected (data not shown). Both −/− and +/+ immortalized fibroblasts infected with a control virus were not tumorigenic, whereas wild-type cells expressing SOS-F efficiently formed tumors in nude mice (Figure 3A). In contrast, fibroblasts lacking the EGFR failed to be transformed by SOS-F, indicating that EGFR signaling is required for oncogenic transformation by SOS-F (Figure 3A). Interestingly, expression of *bcl*-2 restored tumor formation of −/− fibroblasts expressing SOS-F, suggesting that the EGFR provides an antiapoptotic signal (Figure 3A).

To exclude the possibility that the resistance of −/− fibroblasts to SOS-F transformation was due to secondary genetic modifications, an independent fibroblast cell line expressing endogenous EGFR was analyzed. NIH3T3 fibroblasts were first infected with a retrovirus expressing a dominant-negative EGFR mutant (CD533), and then transfected with plasmids encoding SOS-F or *ras*V12 (Figure 3B). Both SOS-F and RasV12 efficiently

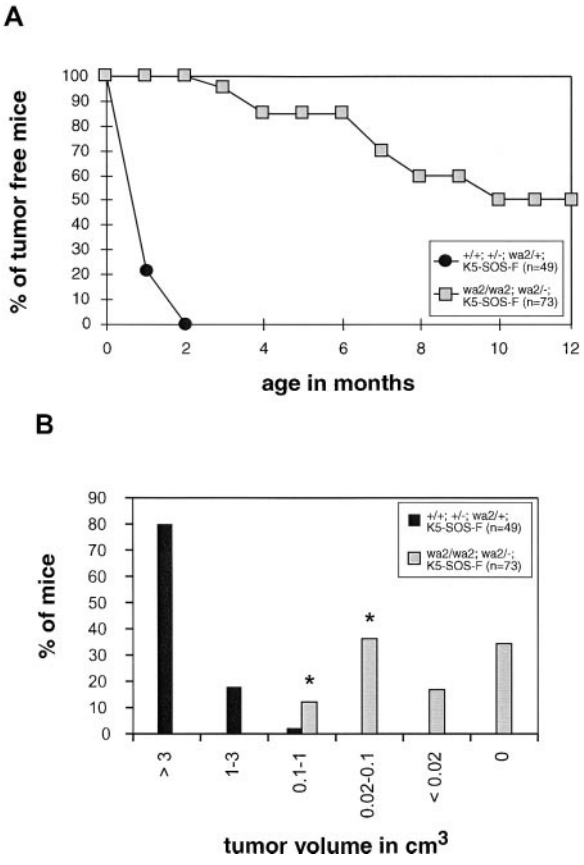
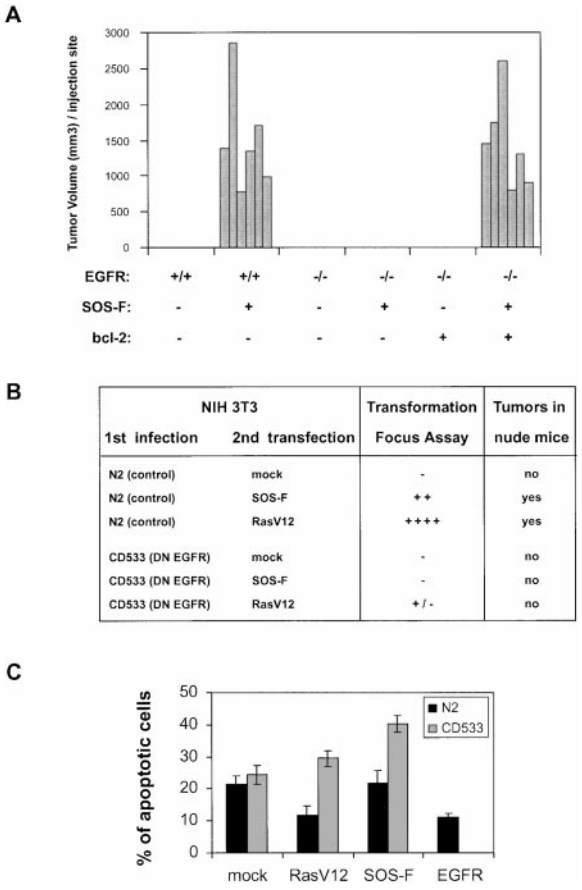


Figure 2. Tumor Development Is Impaired in a Hypomorphic EGFRwa2/wa2 Background

Tumor incidence (A) and volume (B) of K5-SOS-F mice in the presence of different EGFR alleles. One hundred percent of EGFR+/+, +/−, and wa2/+ mice carrying the K5-SOS-F transgene develop large skin papillomas (B, black bars) within 2 months of age (A, black circles), whereas 50% of EGFR wa2/wa2 and wa2/− remain tumor free for more than 12 months (A, gray squares) and never exceed the volume of 1 cm^3 (B, gray bars). Only skin lesions ≥ 0.02 cm^3 (marked by asterisks) were scored as tumors in (A). +/+, +/−, wa2/+, and wa2/wa2, wa2/− K5-SOS-F mice, respectively, were grouped since they showed similar tumor incidences and volumes.



NIH 3T3 1st infection	NIH 3T3 2nd transfection	Transformation Focus Assay	Tumors in nude mice
N2 (control)	mock	-	no
N2 (control)	SOS-F	++	yes
N2 (control)	RasV12	++++	yes
CD533 (DN EGFR)	mock	-	no
CD533 (DN EGFR)	SOS-F	-	no
CD533 (DN EGFR)	RasV12	+/-	no

Figure 3. EGFR-Dependent Transformation of Immortalized Fibroblasts by Oncogenic SOS and Ras

(A) Tumor formation in nude mice by EGFR+/+ and −/− mouse embryonic fibroblast cell lines infected or transfected with various constructs. Fibroblasts were infected with a retrovirus encoding SOS-F or an empty virus. Successively, −/− SOS-F fibroblasts were transfected with *bcl*-2. Tumorigenicity was assayed after subcutaneous injection of 1×10^6 cells into nude mice. There was no difference in the infection and transfection frequency between wild-type and −/− cells.

(B) Transforming activity of NIH 3T3 cells first stably infected with an empty (N2) retrovirus or with a retrovirus harboring a dominant negative EGFR (CD533) and successively with an empty plasmid (mock) or constructs encoding SOS-F or *ras*V12. Transformation was measured by focus assay or by the ability to form tumors in nude mice.

(C) Increased apoptosis in SOS-F or *ras*V12 transformed NIH 3T3 cells in the presence of a dominant-negative EGFR (CD533) as measured by flow-cytometry after annexinV-FITC/propidium iodide (PI) staining. The bar diagram shows the percetage of apoptotic cells as the sum of annexinV single and annexinV/PI double positive cells. Results of one experiment are shown and the data represent the mean ± SD of the analysis of three independent cultures of the respective genotypes. A second independent experiment gave very similar results (data not shown). Similar differences in apoptosis among the various cell lines were observed when fibroblasts were grown in 10% serum (data not shown). NIH 3T3 fibroblasts infected with a retrovirus expressing the wild-type human EGFR (EGFR) were taken as control. The expression of the respective transfected or infected proteins was verified by Western blot analysis (data not shown).

transformed NIH3T3 cells as judged by focus formation in vitro and tumor formation in nude mice (Figure 3B). Inhibition of EGFR function by expression of CD533 completely abolished transformation by SOS-F (Figure 3B). Moreover, CD533 expression almost completely abolished transformation by RasV12, suggesting that EGFR is not only required for oncogenic transformation by SOS-F, but also for transformation by other components of the Ras signaling pathway (Figure 3B).

When apoptosis was measured in these transfected fibroblasts, no difference in the percentage of apoptotic cells was detectable between controls and cells expressing a dominant-negative EGFR (Figure 3C). Strikingly, in SOS-F- or *ras*V12-transfected NIH3T3 fibroblasts, inhibition of EGFR function by CD533 resulted in a marked increase in the number of apoptotic cells (Figure 3C). These results suggest that EGFR plays an important role in antiapoptotic signaling in oncogenic transformation by components of the Ras signaling pathway.

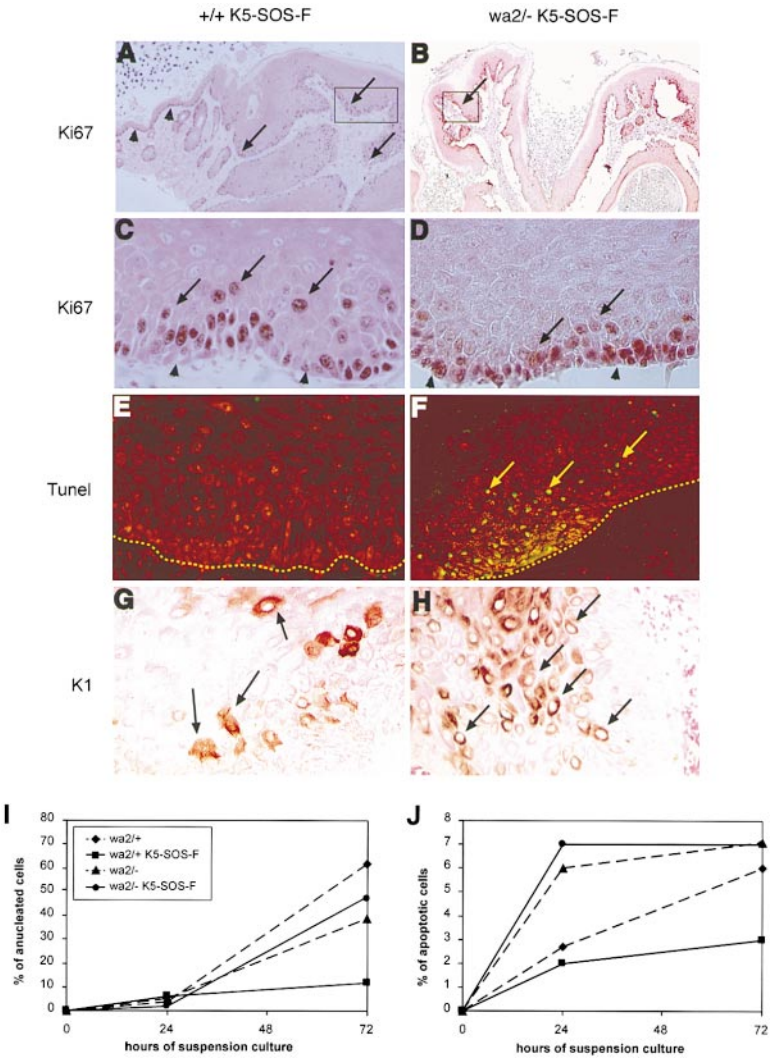


Figure 4. Increased Apoptosis and Impaired Differentiation in EGFRwa2/- K5-SOS-F Papillomas and Primary Keratinocytes

(A–D) Anti-Ki67 immunostaining of papillomas isolated from a 3-week-old +/+ (A and C) and a 10-month-old wa2/− (B and D) transgenic mouse. The number of proliferating cells which have extended to the suprabasal layers is increased to a similar extent in +/+ (arrows in A and C) and wa2/− (arrows in B and D) transgenic papillomas. Note that the majority of basal cells are also proliferating in the nonaffected transgenic skin (arrowhead in A). Hair follicles are absent in the affected areas. (C and D) Higher magnifications of the boxed areas shown in (A) and (B). Arrowheads point to basal cells.

(E and F) TUNEL staining (green) of adjacent sections to (A) and (B) showing a significantly higher number of apoptotic cells in the basal and suprabasal compartments of transgenic papillomas in the absence of a wild-type EGFR (arrows in F). Propidium iodide was used as a nuclear counterstain. The dotted lines in (E) and (F) delineate the basal membrane.

(G and H) Immunostaining for keratin 1. Arrows point to differentiated cells which are significantly increased in number in wa2/− tumors (H). Impaired differentiation (I) and reduced apoptosis (J) in primary keratinocytes of wa2/+ K5-SOS-F mice. In vitro differentiation of primary keratinocytes was induced by suspension culture for the indicated times. Cells suspended in methyl-cellulose were harvested and air-dried on coverslips and scored for the following parameters: (I) Differentiation by the number of anucleated cells, (J) Apoptosis by the number of small cells with condensed, fragmented nuclei as judged by DAPI/TUNEL double staining or hematoxylin and eosin staining. Symbols in (I) and (J) correspond to the same genotypes. Data in (I) and (J) represent the respective percentages after counting 300 cells from randomly chosen fields. A second independent experiment gave similar results (data not shown). (Original magnifications: A, B 10×; C–H 40×)

Increased Apoptosis in wa2/− K5-SOS-F Transgenic Papillomas and Primary Keratinocytes

Although wa2/− or wa2/wa2 transgenic papillomas were much smaller in size and developed much later (Figures 2A and 2B), histologically they appeared very similar to the ones observed in a +/+ or wa2/+ background (Figures 4A and 4B). Staining with the proliferation marker Ki67 showed that the majority of basal cells were proliferating and that the number of proliferating cells was increased to a similar extent in +/+ and wa2/− transgenic papillomas (Figures 4A–4D). In both backgrounds, the proliferating cells, which are normally confined to the basal layer, had expanded to the suprabasal compartments (arrows in Figures 4C and 4D). In contrast, in adjacent nonaffected transgenic skin, Ki67-positive cells were present in the basal layer (Figure 4A, arrowheads). When skin tumor sections were labeled with the TUNEL technique, a significant increase in the number of apoptotic cells was detected in the basal and suprabasal layers of wa2/− K5-SOS-F papillomas (Figure 4F, arrows). Quantification of apoptosis on multiple sections of different tumors revealed a 3-fold increase in the number of apoptotic cells in wa2/− papillomas (145.3 ± 13.6) compared to wild-type tumors (50.6 ± 9.6). To assess the effect of K5-SOS-F expression on terminal differentiation, tumors of both backgrounds were stained for Keratin 1 (which is normally limited to the suprabasal layer of the epidermis). Whereas the expression of K1 was almost absent in +/+ papillomas, a significantly increased number of differentiated cells were still present in wa2/− tumors, suggesting that signaling by the wild-type EGFR might negatively affect terminal differentiation tumors (Figures 4G and 4H).

The survival capacity of primary transgenic keratinocytes was followed during differentiation in vitro, which was induced by deprivation of cell anchorage by means of suspension culture. Consistent with a differentiation defect, the number of anucleated cells was considerably lower in wa2/+ K5-SOS-F keratinocytes after 72 hr of suspension culture compared to cells of all three other

genotypes (Figure 4I). After 72 hr of suspension culture, a similar number of apoptotic cells was observed in nontransgenic wa2/+ and wa2/− control keratinocytes (Figure 4J). In contrast, K5-SOS-F keratinocytes in the presence of a wild-type EGFR displayed only half the number of apoptotic cells compared to transgenic keratinocytes of a hypomorphic EGFR background (Figure 4J). These results indicate that expression of K5-SOS-F renders basal keratinocytes prone to hyperproliferation, but that a functional EGFR is required to negatively regulate keratinocyte differentiation and to provide a survival signal for tumor development.

Impaired Akt but Normal ERK Activation in wa2/− K5-SOS-F Keratinocytes

To exclude the possibility that lack of tumor formation in a hypomorphic EGFR background was due to reduced transgene expression or impaired EGFR activation, primary basal keratinocyte cultures established from wa2/+ and wa2/− transgenic mice and nontransgenic littermates were analyzed for protein expression. Western blot analysis using antibodies against SOS1 confirmed that the levels of SOS-F protein were similar to the levels of the endogenous SOS protein and comparable in the wa2/+ and wa2/− background (Figure 5A). Likewise, the levels of EGFR protein were not different between the various genotypes, and EGF stimulation led to phosphorylation of the EGFR, although this was slightly reduced in a wa2/wa2 background (Figure 5A) (Luetteke et al., 1994). Activation of Akt-kinase, which positively regulates a pathway leading to cell survival in epithelial cells, was significantly reduced in wa2/− K5-SOS-F keratinocytes (Figure 5A). In contrast, ERK1/2 phosphorylation after EGF treatment was comparable and not influenced by the presence of the hypomorphic wa2 EGFR allele (Figure 5A). These results demonstrate that whereas ERK activation is not affected, Akt phosphorylation is impaired in wa2/− transgenic keratinocytes, thus providing a molecular explanation for the survival function of the EGFR.

To investigate if the presence of K5-SOS-F was leading to increased expression of EGFR ligands, thereby activating an autocrine loop, total RNA was extracted from different keratinocyte cultures. Quantitative RT-PCR analysis revealed that EGF stimulation resulted in an elevation of HB-EGF mRNA transcription and that HB-EGF induction was significantly enhanced by the expression of K5-SOS-F (Figure 5B). The transcription of the EGFR itself was not significantly altered (Figure 5B). These data suggest that an autocrine loop might be activated, but since the induction of HB-EGF expression is increased in both wa2/+ and wa2/− K5-SOS-F keratinocytes, this alone can not account for the differences in tumor formation observed in vivo.

We next investigated whether the lack of skin tumor formation in a wa2/− background could be attributed to differences between wa2/+ versus wa2/− dermis. For this purpose, primary dermal fibroblasts were isolated from mice of all four genotypes. Quantitative RT-PCR analysis did not reveal significant differences in expression levels of HB-EGF and EGFR in all fibroblast groups, which were not affected by the presence of K5-SOS-F, since the latter is not expressed in the dermis (Figure 5B).

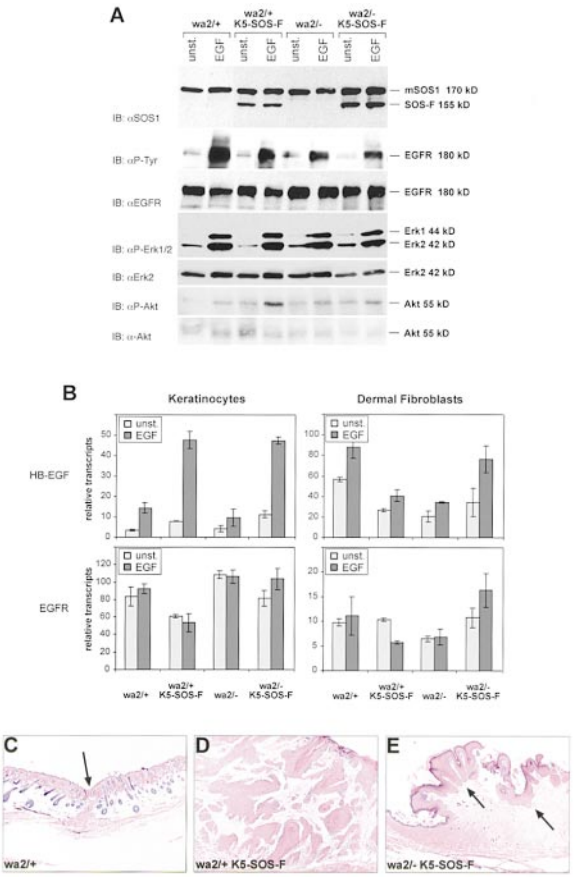


Figure 5. Signaling in Primary Keratinocytes and a Cell-Autonomous Function for EGFR

(A) SOS and EGFR expression and Akt and ERK activation in primary keratinocytes. Keratinocytes of various genotypes were starved for 48 hr in 0.5% serum and stimulated for 5 min with 20 ng/ml EGF. Protein extracts were separated on an 8% SDS-polyacrylamide gel, transfererred to a membrane and immunoblotted with the indicated antibodies.

(B) Quantitative RT-PCR measuring HB-EGF and EGFR transcripts in primary keratinocytes and dermal fibroblasts. Cells of the respective genotypes were starved for 48 hr in 0.5% serum and stimulated for 4 hr with 20 ng/ml EGF.

(C–E) Histological sections of skin lesions developing from wa2/+ (C), wa2/+ K5-SOS-F (D), and wa2/− K5-SOS-F (E) keratinocytes grafted in combination with wa2/− dermal fibroblasts onto the back of nude mice. Note the big papilloma-like structure of the tumor derived from wa2/+ K5-SOS-F keratinocytes (D) versus the small lesion observed in wa2/− K5-SOS-F keratinocytes (E). Arrows in (E) point to hyperplastic papilloma-like structures. No hyperplasias were detected in the control (C). Arrow in (C) points to the site of wound closure in the grafted skin. All the lesions were isolated 7 weeks after grafting. (Original magnifications: C–E, 5×)

A Cell-Autonomous Requirement for the EGFR in Keratinocytes

To analyze whether the EGFR was required cell-autonomously in transgenic keratinocytes and to demonstrate that mutant dermis was able to support tumor growth, grafting experiments were performed with transgenic keratinocytes in combination with wa2/− dermal fibroblasts. In grafts from wa2/+ K5-SOS-F keratinocytes, tumors became apparent after 4 weeks while no tumors could be detected in grafts established with wa2/− K5-SOS-F keratinocytes. Within the next 3 weeks, the tumors

derived from wa2/+ K5-SOS-F keratinocytes increased in size and appeared macroscopically and histologically as typical papillomas (Figure 5D). After 7 weeks, one of the grafts from wa2/− K5-SOS-F keratinocytes appeared as a small papillomatous lesion which was >20 times smaller than the papillomas derived from wa2/+ transgenic keratinocytes. Histological examination of the lesion derived from wa2/− K5-SOS-F keratinocytes revealed a hyperplastic epidermis (Figure 5E, arrows), whereas no signs of hyperplasia could be detected in control grafts established with wa2/+ keratinocytes (Figure 5C). These results indicate that a functional EGFR is needed in keratinocytes to promote tumor development in K5-SOS-F mice and that papilloma formation is most likely not influenced by the presence or absence of a functional EGFR in the dermis.

Discussion

K5-SOS-F transgenic mice develop spontaneous skin tumors, which resemble the lesions observed in transgenic mice expressing an activated Ras in hair follicles (Brown et al., 1998), suggesting that hyperkeratosis may result from activation of the Ras signaling pathway by SOS. Epidermal thickening and papilloma formation were also observed in K14-TGFα transgenic mice. However, the epidermal hyperplasia of K14-TGFα mice regressed with time, and terminal differentiation was not perturbed (Vassar and Fuchs, 1991). As EGFR expression is unaffected in K14-TGFα mice, it seems that ligand-induced EGFR stimulation alone is not capable of inducing epidermal hyperproliferation and that constitutive activation of the EGFR or SOS/Ras are required to trigger tumor formation (Vassar and Fuchs, 1991).

Ras activation has been shown to directly affect terminal differentiation of keratinocytes. Retroviral infection of v-*ras* in wild-type keratinocytes results in suppression of K1 and K10 expression and protein kinase C (PKC) activity, which is thought to promote keratinocyte differentiation (Denning et al., 1993; Dlugosz et al., 1994). In contrast, v-*ras* infected EGFR−/− keratinocytes do not downregulate K1 and K10 and maintain high PKC activity, indicating that EGFR might negatively regulate keratinocyte terminal differentiation (Denning et al., 1996; Dlugosz et al., 1997). A higher number of K1/10 positive keratinocytes was detected in wa2/− K5-SOS-F keratinocytes and papillomas, supporting the hypothesis that impaired EGFR signaling promotes keratinocyte differentiation. The premature differentiation might in part explain why papilloma development is severely impaired in an EGFR null or wa2/wa2 background. In K5-SOS-F papillomas as well as in primary keratinocytes, the number of proliferating cells was increased to the same extent in wild-type and wa2/wa2 background. Similarly, the EGFR does not seem to be required in v-*ras* mediated keratinocyte proliferation, since the proliferation capacity of v-*ras* infected EGFR−/− or +/+ keratinocytes was comparable (Dlugosz et al., 1997). These results suggest that a functional EGFR is not required for proliferation of SOS-F or Ras transformed cells, but that EGFR signaling might negatively affect terminal differentiation of basal keratinocytes.

In the absence of a wild-type EGFR, K5-SOS-F papillomas and keratinocytes show increased apoptosis, suggesting that the EGFR also provides a survival signal for tumor cells. It was shown that squamous cell papillomas produced by grafting v-*ras* infected EGFR−/− keratinocytes onto nude mice were smaller than EGFR wild-type Ras tumors, although no significant increase in apoptosis was detected (Dlugosz et al., 1997). It is possible that the high expression levels of constitutively active viral Ras results in maximal stimulation of direct downstream targets such as PI3-kinase, which mediates cell survival via Akt, so that an additional survival input from the EGFR is functionally insignificant. In K5-SOS-F keratinocytes, Ras, which is the direct target of SOS, would still be a limiting factor in the signaling cascade, thus avoiding activation of multiple effector pathways. Therefore, an additional SOS/Ras-independent survival signal originating from the EGFR becomes essential for tumor development.

The EGFR has been implicated in the regulation of cell survival in epithelial cells. EGFR activation in keratinocytes can lead to transcription of bcl-X_L, a member of the bcl-2 family of proteins, and human keratinocytes show increased apoptosis when treated in vitro with blocking EGFR antibodies or EGFR-specific tyrosine kinase inhibitors (Rodeck et al., 1997a, 1997b; Stoll et al., 1998). However, little is known about the signal transduction pathways linking the EGFR to regulation of cell survival. Oncogenic Ras has been shown to regulate both pro- and antiapoptotic pathways (Downward, 1998). Cell survival is mediated by PI3-kinase and Akt whereas the Raf pathway is required for Ras-induced apoptosis (Downward, 1998). In wa2/− keratinocytes, ERK1/2 activation, which is directly regulated by Raf, is not markedly altered even in the presence of K5-SOS-F. In contrast, Akt phosphorylation is reduced in wa2/− K5-SOS-F keratinocytes, indicating that an EGFR-dependent signal is necessary for maximal Akt activation. Although it is tempting to speculate that Akt is a mediator of the survival function of the EGFR, it can not be excluded that there are additional molecular targets of EGFR-dependent survival signaling. Recently, it was shown in the fruit fly *Drosophila melanogaster* that EGFR signaling via the Ras/ERK pathway promotes cell survival by downregulating the expression of the proapoptotic gene *hid* (Bergmann et al., 1998; Kurada and White, 1998), suggesting that two independent pathways, one by Ras/ERK and one by the EGFR, have to converge for efficient repression of such proapoptotic signals. The survival function of the EGFR is not only restricted to SOS-F-transformed keratinocytes, but can also be demonstrated in mesenchymal cells, since EGFR−/− fibroblasts and NIH 3T3 cells expressing a dominant-negative EGFR are also resistant to transformation by oncogenic forms of hSOS and Ras. Interestingly, expression of the antiapoptotic gene *bcl*-2 in SOS-F-transfected EGFR−/− fibroblasts restores transformation and the capability to form tumors in nude mice.

Overexpression of members of the Ras signaling pathway have been shown to induce the secretion of EGFR ligands and therefore activate an autocrine loop contributing to cellular transformation (McCarthy et al., 1995; Gangarosa et al., 1997). An increase in the expression of the EGFR ligand HB-EGF was observed in transgenic keratinocytes in both wa2/+ and wa2/− backgrounds. Therefore, the increased HB-EGF expression in the presence of K5-SOS-F might establish an autocrine stimulation of the EGFR that contributes to tumor development. However, since the HB-EGF gets activated in both wa2/+ and wa2/− transgenic keratinocytes, this alone is unlikely to account for the differences in tumor formation.

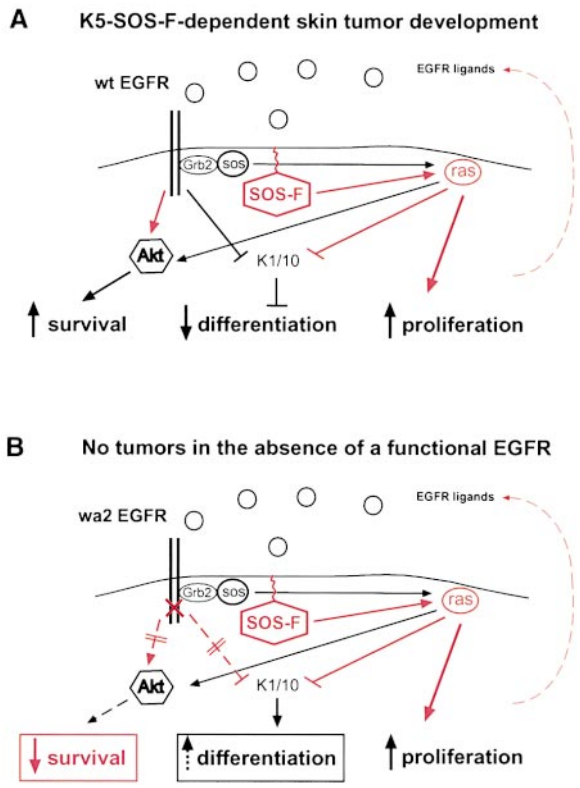


Figure 6. EGFR—A Potent Survival Signal for Tumor Cells?

Consequence of SOS-F expression in EGFR wild-type (wt) and mutant (wa2) epidermis. Keratinocyte survival, proliferation, and differentiation are tightly regulated processes during skin development. Whereas Ras induction is essential for keratinocyte proliferation, EGFR signaling in basal keratinocytes activates an antiapoptotic pathway, possibly via Akt, and inhibits keratinocyte differentiation in a SOS/Ras-dependent and/or -independent manner.

(A) Expression of a dominant SOS-F activates Ras and therefore leads to increased proliferation and reduced differentiation of keratinocytes. In addition, the wild-type EGFR acts as a potent survival signal leading to skin tumor development.

(B) In the presence of a hypomorphic EGFR, the survival pathway downstream of the EGFR is not sufficiently activated and, as a consequence, tumors can not develop.

Reduced tumor development in an EGFR hypomorphic background is most likely not caused by the lack of a wild-type receptor in the dermis, since wa2/+ K5-SOS-F keratinocytes grafted in combination with wa2/− dermal fibroblasts onto nude mice form papillomas that are similar to the ones developing in a wild-type EGFR background.

EGFR—A Potent Survival Signal for Tumor Cells

EGFR signaling appears to have two functions during skin development: it provides a survival signal by activating an antiapoptotic pathway and inhibits keratinocyte differentiation, thereby keeping basal cells in the proliferative compartment attached to the basal membrane. K5-SOS-F keratinocytes are prone to hyperproliferation independent of whether a wild-type or mutant EGFR is present. In a wild-type EGFR background, hyperproliferation induced by SOS-F together with the survival signal provided by the EGFR will lead to skin tumor development (Figure 6A). In a wa2/− transgenic background, SOS-F expression will still lead to hyperproliferation; however, keratinocytes will be induced to leave the basal compartment prematurely, since the absence of the EGFR favors terminal differentiation. Moreover, the survival signal activated by the EGFR (possibly by Akt), is missing, and due to these conflicting signals, keratinocytes would be committed to apoptose (Kauffmann-Zeh et al., 1997). As a consequence, no tumors develop in the absence of a functional EGFR (Figure 6B). Since in vitro keratinocytes are grown on plastic substrates, this might also explain why undifferentiated SOS transgenic keratinocytes in a wa2/wa2 background do not show increased apoptosis unless they are induced to differentiate by placing them in suspension.

A similar scenario could be true for other human tumors, which carry activating mutations in components of the Ras signaling pathway. The tumor cells would be prone to hyperproliferation, and the EGFR (or other growth factor receptors) may additionally provide survival signals. The EGFR itself is frequently amplified, overexpressed, or rearranged in human squamous cell carcinomas and glioblastomas (Libermann et al., 1985; Derynck, 1992). Thus far, it has never been clarified whether the EGFR simply provides a proliferation advantage to tumor cells or if other cellular processes are altered. Since EGFR amplifications and rearrangements often occur at later stages of tumor development, one would exclude that the only function of the EGFR is to provide a proliferation advantage. It is likely that the EGFR triggers multiple downstream survival pathways, rendering tumor cells more aggressive and resistant to chemotherapeutic drugs, which usually induce apoptosis by activating only one of the apoptotic programs (Nagane et al., 1998). Therefore, the conventional chemotherapy combined with EGFR-specific inhibitors might prove to be more successful in antitumor therapies.

Experimental Procedures

Generation of K5-SOS-F Transgenic Mice and Establishment of Transgenic Lines in Different EGFR Backgrounds

A constitutively active, HA-tagged form of hSOS (SOS-F) (Aronheim et al., 1994) was excised from the plasmid vector as a HindIII fragment, blunt ended, and inserted into a Sna BI site downstream of the K5 regulatory region (Murillas et al., 1995). The K5-SOS-F fragment was excised from the plasmid vector after digestion with the restriction enzymes SalI and NotI. Transgenic mice were generated by pronuclear injection of the purified K5-SOS-F DNA fragment into fertilized oocytes from (C57BL/6 × CBA) F1 mice. Founders were identified by Southern blot analysis (data not shown)

Founder 892A6* was first backcrossed to EGFR+/− mice of C57BL/6 background and the resulting EGFR+/− K5-SOS-F transgenics were further backcrossed to EGFRwa2/wa2 mice of LVC background (MRC-Chilton) to obtain wa2/+ and wa2/− mice harboring the transgene. To monitor tumor incidence in the various EGFR backgrounds, offspring born from intercrosses between wa2/+ K5-SOS-F × wa2/wa2 and wa2/− K5-SOS-F × wa2/wa2 were kept and monitored over a year for the appearance of papillomas.

Histology, Immunohistochemistry, and TUNEL Staining

Mouse tissues were fixed overnight in 4% paraformaldehyde, dehydrated, and embedded in paraffin. 5 μm sections were stained either with hematoxylin and eosin or processed further. Immunohistochemical staining for Ki67 (Novocastra, NCL-Ki67p, 1:1000) was performed using the ABC Staining Kit (Vector Laboratories) according to the manufacturer's recommendations. Immunostainings of mouse skins with keratins were performed as described (Carroll et al., 1995).

TUNEL staining was performed using the in situ cell death detection kit II (Boehringer Mannheim).

Isolation and Culture of Mouse Keratinocytes and Dermal Fibroblasts

Mouse keratinocytes were isolated as previously described and cultured onto vitrogen-fibronectin coated dishes in low calcium MEM (Sigma M8167) medium containing 8% chelated FCS (Carroll et al., 1995). Keratinocytes were induced to undergo terminal differentiation by suspension culture and recovered at the indicated time points as previously described (Gandarillas et al., 1999).

For the isolation of dermal fibroblasts, mouse ears ears were split into dorsal and ventral side and placed in 1% trypsin for 45 min at 37°C. The epidermis was separated from the dermis and the latter cut into small pieces and incubated at 37°C for 60 min in 1 mg/ml collagenase/dispase (Boehringer) with gentle stirring. Cells were filtered through a 70 μm Teflon mesh, centrifuged, and resuspended in DMEM medium containing 10% FCS.

Immunoblotting Analysis

Keratinocytes and minced tissues were homogenized in lysis buffer as previously described (Redemann et al., 1992). The lysates were cleared by centrifugation and processed for Western blot analysis as previously described (Sibilia and Wagner, 1995). The following antibodies were used: anti SOS1 (Transduction Laboratories), anti EGFR #1001 (Santa Cruz), anti ERK2 (Santa Cruz), anti phosphorylated ERK1/2 (Biolabs), anti Phosphotyrosine 4G10 (Upstate), anti phosphorylated Akt (Biolabs), and anti Akt (Transduction Laboratories).

RNA Isolation and RT-PCR

Total RNA was isolated from keratinocytes using the RNeasy Mini Kit (Quiagen). cDNA synthesis was performed with the SuperScript Preamplification System (GibcoBRL) according to the manufacturer's instructions. The following primers were used for RT-PCR analysis: EGFR1, GGAGGAAAAGAAAGTCTGCC; EGFR2, ATCGCAC AGCACCAATCAGG; HB-EGF1 GCTGCCGTCGGTGATGCTGAAGC; HBEGF2, GATGACAAGAAGACAGACG; Tubulin1, CAACGTCAAGA CGGCCGTGTG; and Tubulin2, GACAGAGGCAAACTGAGCACC. Transcripts were quantified with Light Cycler (Roche Diagnostics) using SYBR Green I and TaqStart Antibody (Clontech). Purified PCR amplicons were used to obtain absolute standard curves.

Nude Mice Skin Grafting

Skin grafts were established on athymic mice as previously described (Dlugosz et al., 1997) using keratinocytes isolated from wa2/+ and wa2/− transgenic and nontransgenic controls and dermal fibroblasts isolated from wa2/− mice. Mice were anesthetized and a silicone dome with a 2 mm hole at its apex was positioned under the back skin. A slurry containing 4×10^6 primary dermal fibroblasts and 2×10^6 keratinocytes was applied to the silicone chamber. The grafting chambers were removed after 8 days and tumor growth was periodically monitored. Two out of three grafts from wa2/+ K5-SOS-F keratinocytes were attached and both started to form papillomas after 4 weeks. Only in one out of three wa2/− K5-SOS-F grafts could a tiny papillomatous lesion be detected after 7 weeks.

Fibroblast Cell Lines, Retroviral Infections, and Transfections

Primary mouse embryonic fibroblasts were isolated from wild-type and EGFR−/− E12.5 fetuses and immortalized according to the 3T3 protocol (Todaro et al., 1965). All fibroblasts were cultured in DMEM medium containing 10% FCS. The following retroviruses were employed for stable infection: pBabe-rasV12 (kindly provided by S. Lowe), pBabe-SOS-F (kindly provided by K. Matsuo), and pBabe (puromycin resistance) to infect EGFR+/+ and −/− 3T3 fibroblasts; and pNTK-HERCD533 and pNTK (neomycin resistance) to infect NIH 3T3 cells (Redemann et al., 1992). Stable transfections were performed with the following plasmids: CMV-bcl-2, RSV-rasV12, and RSV-SOS-F cotransfected with RSV-hygro.

Apoptosis in NIH 3T3 fibroblasts was measured with the flow-cytometer after labeling with AnnexinV-FITC/Propidium Iodide (PI) (Clontech) according to the manufacturer's recommendations.

Tumorigenicity Assays

Fibroblasts were plated at 3×10^5 cells per 10 cm dish and infected or transfected with the above-described retroviruses and plasmid vectors 24 hr later. For the focus assay, cells were cultured in the same dishes without selection for 2 weeks. Cells were fixed and stained with 0.2% methylene blue (v/v in methanol). The number of macroscopically visible foci was estimated by visual examination.

For the tumorigenicity assay, the transfected or infected cells were selected with G418, puromycin, or hygromycin for 1 week until all noninfected/transfected cells had died. 1×10^6 resistant cells were subcutaneously injected into 3- to 5-week old anaesthetized nude mice and the appearance of tumors was monitored.

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