Targeted Disruption of the Epidermal Growth Factor Receptor Impairs Growth of Squamous Papillomas Expressing the v-ras<sup>Ha</sup> Oncogene but Does Not Block in Vitro Keratinocyte Responses to Oncogenic ras

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ABSTRACT

We have assessed the role of epidermal growth factor receptor (EGFR) signaling in biological responses to the v-ras<sup>Ha</sup> oncogene using primary keratinocytes from Egfr <sup>-/-</sup> mice and wild-type littermates. On the basis of several criteria, Egfr <sup>-/-</sup> keratinocytes were unresponsive to either acute or chronic exposure to several EGFR ligands but were stimulated to proliferate in response to several other mitogens. Although conditioned medium from primary keratinocytes transduced with v-ras<sup>Ha</sup> retrovirus (v-ras<sup>Ha</sup> keratinocytes) was a potent mitogen for wild-type but not Egfr <sup>-/-</sup> keratinocytes, v-ras<sup>Ha</sup> transduction of primary keratinocytes of either genotype resulted in a strong mitogenic response, arguing against an obligatory role for EGFR activation in v-ras<sup>Ha</sup>-mediated stimulation of keratinocyte proliferation. Infection with high-titer v-ras<sup>Ha</sup> retrovirus altered the keratin expression pattern in keratinocytes of both genotypes, suppressing differentiation-specific keratins K1 and K10 while activating aberrant expression of K8 and K18. In wild-type but not Egfr <sup>-/-</sup> cultures, K1 and K10 were also suppressed following infection at lower retroviral titers, presumably as a result of paracrine EGFR activation on uninfected cells present in these cultures. Squamous papillomas produced by grafting Egfr <sup>-/-</sup> v-ras<sup>Ha</sup> keratinocytes onto nude mice were only 21% of the size of wild-type v-ras<sup>Ha</sup> tumors, and a striking redistribution of S-phase cells was detected by immunostaining for bromodeoxyuridine. In Egfr <sup>-/-</sup> v-ras<sup>Ha</sup> papillomas, the fraction of total labeled nuclei detected in suprabasal layers was increased from 19 to 39%. In contrast, the basal layer labeling index of Egfr <sup>-/-</sup> papillomas was reduced to 34%, compared to 43% in wild-type tumors. Our results indicate that, although autocrine EGFR signaling is not required for keratinocyte responses to oncogenic ras in culture or benign tumor formation in nude mouse grafts, disruption of this pathway impairs growth of v-ras<sup>Ha</sup> papillomas by a mechanism that may involve alterations in keratinocyte cell cycle progression and/or migration in vivo.

INTRODUCTION

Neoplastic transformation is frequently associated with alterations in the expression or function of growth factors and their receptors, suggesting that proliferation of tumor cells may be regulated in part by autocrine or paracrine growth factor stimulation (1). In a variety of epithelial tumors, the levels of TGF-α<sup>+</sup> and amphiregulin are commonly elevated, as is the expression of EGFR, implicating this particular signaling pathway in epithelial neoplasia (reviewed in Ref. 2). The study of chemically induced skin tumors in mice has helped elucidate some of the fundamental events occurring during the development and progression of epithelial neoplasms (reviewed in Ref. 3). Treatment of mouse skin with a low dose of carcinogen, typically dimethylbenz[a]anthracene, followed by repeated applications of tumor promoter, results in the formation of multiple benign squamous papillomas. The great majority of these tumors contain an A→T transversion in codon 61 of the c-ras<sup>Ha</sup> gene, which encodes a constitutively activated Ras protein (4). A critical role for Ras in epithelial tumor development was established by the demonstration that direct introduction of mutant viral ras genes into scarified mouse skin can substitute for the chemical carcinogen initiation step (5). Moreover, primary keratinocytes transduced with v-ras<sup>Ha</sup> retrovirus (v-ras<sup>Ha</sup> keratinocytes) produce squamous papillomas when grafted onto nude mice as a reconstituted skin (6), indicating that the presence of constitutively activated Ras is sufficient for transformation of normal keratinocytes into benign tumor cells. Because activating ras mutations occur in a large proportion of epithelial malignancies in humans (7, 8), studies aimed at gaining insight into how oncogenic Ras influences keratinocyte behavior may have important implications that are relevant to the prevention or treatment of human cancer.

Previous studies from this laboratory and others have demonstrated that expression of oncogenic Ras has a profound effect on the EGFR signaling pathway in epidermal keratinocytes. TGF-α<sup>-</sup> levels are markedly increased in primary keratinocyte cultures transduced by a v-ras<sup>Ha</sup> retrovirus (v-ras<sup>Ha</sup> keratinocytes) as well as in squamous papillomas expressing oncogenic ras in vivo (9, 10). In addition, expression of EGFR mRNA and protein in papillomas is elevated when compared to normal skin (11, 12), and increased tyrosine-phosphorylation of a protein migrating in the vicinity of EGFR has been reported (13). In primary v-ras<sup>Ha</sup> keratinocytes, increased TGF-α expression is associated with stimulation of keratinocyte proliferation and distinctive changes in the terminal differentiation program, including suppression of the differentiation-specific keratins K1 and K10, coupled to aberrant induction of keratins K8 and K18 (14). Similar phenotypic changes are elicited in control keratinocytes treated with TGF-α or CM from v-ras<sup>Ha</sup> keratinocyte cultures (10), arguing that these responses to oncogenic Ras are mediated by autocrine stimulation of the EGFR. We recently reported that v-ras<sup>Ha</sup> induced similar phenotypic changes in TGF-α-null and wild-type keratinocytes studied both in vitro and in vivo (15), arguing against a critical function for TGF-α in ras-mediated keratinocyte neoplasia. In the course of these studies, however, we demonstrated that, in addition to inducing TGF-α, oncogenic Ras also induces expression of transcripts encoding the EGFR family members amphiregulin, betacellulin, and HB-EGF (15). The coordinate up-regulation of multiple EGFR protein kinases; Erk, extracellular signal-regulated kinase; BrdUrd, bromodeoxyuridine; TBS, Tris-buffered saline; PKC, protein kinase C.
ligands by v-rasH4 has led us to reexamine the role of this pathway in phenotypic responses to v-rasH4 using primary keratinocytes harboring a targeted disruption of the Egfr gene (16).

**MATERIALS AND METHODS**

**Materials.** Eagle’s minimal essential medium was obtained from Bio-Whittaker (Walkersville, MD), FCS was from Intergent (Parsippany, NY), and penicillin-streptomycin was from Life Technologies, Inc. (Gaithersburg, MD). KGF, acidic fibroblast growth factor, basic fibroblast growth factor, EGFr, TGF-α, betacellulin, amphiregulin (78-amino acid form, AR-s; similar results obtained with AR-I), and HB-EGF were from R&D Systems (Minneapolis, MN).

Cell Culture, CM Preparation, and Proliferation Assays. Primary keratinocytes were prepared as described previously (17). Standard growth medium contained FCS treated with 8% Chelex (Bio-Rad Laboratories, Hercules, CA) and 20 units/ml penicillin-20 μg/ml streptomycin in Ca2+- and Mg2+-free Eagle’s minimal essential medium, and the medium was adjusted to a final Ca2+ concentration of 0.05 mM using 300 mM CaCl2. Cells were plated in medium with the Ca2+ level adjusted to 0.25–0.3 mM to facilitate attachment, and on the following day (day 1), cultures were washed with Ca2+- and Mg2+-free PBS and replenished with standard growth medium with or without growth factors, as indicated in the figure legends. Cultures to be transduced with v-rasH4 retrovirus were treated with 1–10 ng/ml KGF beginning on day 1 to ensure a rapidly proliferating population of keratinocytes at the time of infection, which was performed using diluted supernatant from ψ-2 producer cells in the presence of 4 μg/ml polybrene. Undiluted viral supernatant had a tier of ~3 × 106 focus-forming units per ml. For experiments assessing keratinocyte responses to different titers of v-rasH4 retrovirus, ψ-2 supernatants were diluted between 1:1 and 1:24 with standard growth medium prior to infection. KGF treatment was continued until the day following infection, at which time the cells were washed with PBS and subsequently maintained in standard growth medium, which was replaced every 2–3 days. Terminal differentiation was induced by culturing keratinocytes in medium containing 0.12 mM Ca2+ for 2 days. CM was harvested from v-rasH4 keratinocyte cultures 4–6 days after infection, after a 24–48-h incubation. CM was centrifuged to remove debris and stored at −20°C until use. Thawed CM was diluted with standard growth medium prior to addition to wild-type or Egfr−− cultures.

For routine proliferation assays, cell counts were performed on trypsinized cells from replicate wells using a Coulter counter. For experiments measuring the mitogenic response to v-rasH4 transduction, a baseline cell count was performed on trypsinized keratinocytes at the time that replicate cultures were infected with retrovirus. Because a significant number of keratinocytes detach from the substrate and remain floating in the medium during the assessment of cumulative cell production by these cultures, the counting process was performed by counting cells in aliquots of culture medium obtained from wells at the time of each medium replacement. At the completion of the experiment, a final count was performed on cells present in the medium, and cells adherent to the substrate were harvested by trypsinization. Cumulative cell counts for each well are sums of all detached-cell counts and the final count performed on trypsinized cells.

**Antibodies.** The following antibodies were used: sheep anti-EGFr (Life Technologies); anti-phosphotyrosine clone 4G10 (Upstate Biotechnology, Inc., Lake Placid, NY); rabbit anti-Shc (Upstate Biotechnology, Inc.); rabbit anti-p44/p42 MAPK (New England Biolabs, Inc., Beverly, MA); anti-Ras mouse monoclonal (Transduction Labs) for immunoblotting; anti-Ras rat monoclonal antibody Y13-259 (Oncogene Science, Manhasset, NY) for immunofluorescence; mouse antitubulin clone B-5-1-2 (Sigma Chemical Co., St. Louis, MO); K1 and K10 (18), Ioricin and filagginin (19), and K13 (20) affinity-purified rabbit polyclonal antibodies; anti-K8 rat monoclonal TROMA-I (21) from Dr. Rolf Kemler (Max Planck Institute, Tubingen, Germany), provided by Dr. Ivan Damjanov (Thomas Jefferson University, Philadelphia, PA); and anti-K18 mouse monoclonal 24A3 (22), kindly provided by Dr. Janet Mitchell (University of Vermont, Burlington, VT). Activation of Erk-1/2 MAPKs was assessed using a rabbit polyclonal antibody (Promega, Madison, WI) that recognizes dually phosphorylated Erk-1/2, but not the unphosphorylated forms of these enzymes. Mouse monoclonal anti-BrdUrd was obtained from Becton Dickinson (San Jose, CA).

**Immunoprecipitation and Western Blotting.** To prepare immunoblotting of EGFr, keratins, Ras, and tubulin, cultures were washed with PBS and lysed in buffer containing 0.25 M Tris (pH 6.8), 5% SDS, and 20% β-mercaptoethanol. The same buffer was used to prepare lysates for analysis of phosphorylated and total Erk-1/2 MAPKs following TGF-α treatment, except that cultures were washed twice with cold PBS containing 1 mM Na2VO4 prior to harvest. Proteins were separated in polyacrylamide gels and transferred to reinforced nitrocellulose (BA-S 83; Schleicher & Schuell, Keene, NH) in buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20% methanol, for 1 h 15 min at 100 V. Nitrocellulose filters were stained for total protein with Ponceau S (Sigma) to assess uniformity of loading and transfer (23). Immunoblot analysis of multiple protein markers in a set of cell lysates was assessed either by reprobing filters repeatedly or by probing additional replicate filters. Nonspecific binding sites were blocked during a 30-min incubation with 5% milk in TBS (20 mM Tris (pH 7.4)-500 mM NaCl). Filters were exposed to primary antibodies diluted in 1% BSA in TBS for 1 h at room temperature, washed for 20–30 min using three changes of TBS containing 0.2% Tween 20 (Bio-Rad), and incubated with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) that were diluted 1:10,000 in 2% milk in TBS for 30 min. Filters were washed as described previously, and horseradish peroxidase was localized using enhanced chemiluminescence reagents purchased from Amersham (Arlington Heights, IL).

Phosphotyrosine immunoprecipitations were performed as described previously (24) with minor modifications. Cells were washed twice with cold PBS containing 1 mM Na2VO4 and harvested in cold buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM Na2VO4, and complete protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN), used according to the manufacturer’s instructions. After extraction on ice for 45 min, lysates were centrifuged for 15 min in a refrigerated microfuge at 12,000 rpm, and supernatants were used for immunoprecipitation, which was carried out overnight at 4°C in a 520-μl volume containing 750-1000 μg of protein in lysis buffer and 20 μl of phosphotyrosine antibody 4G10-agarose (Upstate Biotechnology, Inc.). Agarose beads were washed three or four times using radioimmunoprecipitation assay buffer and boiled in Laemmli sample buffer, and samples were loaded on a polyacrylamide gel and transferred to nitrocellulose as above.

**Immunofluorescence and Apoptosis Assays.** To examine K1 and Ras expression in vitro, cultures were washed with PBS and fixed using 1:1 methanol/acetone for 5 min. Dishes were air-dried and stored at −20°C until staining. Double-immunofluorescence was performed as described previously (19) using rat anti-Ras and rabbit anti-K1 antibodies. Ras antibody was detected using FITC-conjugated antirat IgG (Vector Laboratories, Inc., Burlingame, CA); K1 was detected using antirabbit IgG labeled with Texas Red (Vector Laboratories). Apoptosis was assessed in frozen tumor sections post-fixed in 10% methanol/acetone using the ApopTag kit (Intergen, Purchase, NY), according to the manufacturer’s instructions. Nuclei in viable cell layers were visualized by counterstaining with 0.1 μg/ml propidium iodide in PBS.

**Nude Mouse Skin Grafting and Tumor Analysis.** Reconstituted skin grafts were established on athymic mice essentially as described (17), using keratinocytes infected with a 1:6 dilution of v-rasH4 retrovirus. Briefly, mice were anesthetized, and a ~1-cm piece of dorsal skin was removed to allow positioning of a silicone dome with a 3-mm hole at its apex. A slurry containing 8 × 106 primary dermal fibroblasts and 5–6 × 106 keratinocytes was applied to the exposed dorsal fascia within the silicone chamber. The grafting chambers were removed 1 week later, and tumor dimensions were periodically measured using calipers, and approximate tumor volumes were determined by multiplying tumor height X length X width. Approximately 1 h prior to sacrifice, animals were injected with 300 μl of a 16.6 mg/ml solution of BrdUrd in sterile saline (~0.25 mg of BrdUrd per g of animal body weight). Tumor samples were fixed in 70% ethanol and embedded in paraffin for H&E analysis and immunohistochemistry. Secondary antibodies, obtained from Jackson ImmunoResearch, were used with Vectastain (Vector Laboratories) peroxidase reagents and 3,3′-diaminobenzidine (Sigma). Hematoxylin (Sigma) was used as a counterstain. Prior to immunostaining for BrdUrd, sections were treated with trypsin and NaOH, as recommended by the manufacturer. To assess the proliferative status of tumors, basal and suprabasal BrdUrd-labeled nuclei, as well as unlabeled basal nuclei, were quantitated in several randomly...
selected regions of each tumor. Between 200 and 1400 cells were counted in each of six EGFR-deficient and five wild-type papillomas.

RESULTS

EGFR Function Is Blocked in Cultured Epidermal Keratinocytes Isolated from Newborn Egfr −/− Mice. The phenotype produced by targeted disruption of the Egfr gene in mice is dependent on genetic background (16, 25, 26). For the experiments presented in this study, primary keratinocyte cultures were established using newborn offspring produced by mating adult Egfr +/+ CD-1 mice. Immunoblot analysis for EGFR revealed a ∼170-kDa protein in Egfr +/+ and Egfr +/−, but not Egfr −/−, primary keratinocytes (Fig. 1A, arrow). Low levels of a protein with a molecular weight of ∼140 kDa were detected in Egfr −/− and Egfr +/+ lysates (Fig. 1A, large arrowhead), consistent with an in-frame exon 1→5 splice variant detected by reverse transcriptase-PCR using liver from these mice (16). A faint additional band migrating slightly slower than native EGFR was also detected in Egfr −/− keratinocyte lysates following prolonged film exposures (Fig. 1A, right, small arrowhead). Immunoblot analysis of phosphorytrosine immunoprecipitates was performed to assess EGFR-mediated signaling in wild-type and Egfr −/− keratinocytes. Treatment of wild-type cultures with TGF-α for 3 min resulted in increased tyrosine phosphorylation of EGFR and the adapter protein Shc, as well as activation of Erk-1/2 MAPKs, detected using a phosphorylation state-specific antibody (Fig. 1, B and C). These responses were not observed in Egfr −/− keratinocytes. Interestingly, phosphorytrosine immunoprecipitates of both Egfr −/− and wild-type keratinocytes contained a constitutively phosphorylated protein recognized by EGFR antibody; treatment with TGF-α did not increase tyrosine phosphorylation of this band (Fig. 1B).

EGFR function was examined further in a series of proliferation assays. Treatment of cultures for a period of several days with the EGFR ligands TGF-α, EGF, amphiregulin, betacellulin, or HB-EGF resulted in a severalfold increase in cell number in wild-type, but not Egfr −/− keratinocytes (Fig. 2A). In contrast, multiple other mitogens acting through pathways independent of EGFR elicited a comparable proliferative response in wild-type and Egfr −/− keratinocytes. Treatment of wild-type cultures with TGF-α for 3 min resulted in increased tyrosine phosphorylation of EGFR and the adapter protein Shc, as well as activation of Erk-1/2 MAPKs, detected using a phosphorylation state-specific antibody (Fig. 1, B and C). These responses were not observed in Egfr −/− keratinocytes. Interestingly, phosphorytrosine immunoprecipitates of both Egfr −/− and wild-type keratinocytes contained a constitutively phosphorylated protein recognized by EGFR antibody; treatment with TGF-α did not increase tyrosine phosphorylation of this band (Fig. 1B).

The Mitogenic Response of Normal Keratinocytes to v-rasH-Mediated Keratinocyte Neoplasia

EGFR dependence on v-rasH keratinocyte CM is EGFR-dependent, but increased proliferation in v-rasH keratinocyte cultures does not require autocrine EGFR signaling. We have previously shown that CM from v-rasH keratinocytes is mitogenic when added to cultures of naive primary keratinocytes and that this effect is EGFR-dependent (31). However, the mitogenic effect of CM from these tumors is not strictly autocrine, as CM from v-rasH keratinocytes cultured in the absence of CM from normal keratinocytes does not stimulate proliferation above control levels (16). v-rasH keratinocytes also show a marked increase in spontaneous proliferation when cultured in the absence of mitogens (Fig. 2B). Together, these results establish that primary keratinocytes isolated from Egfr −/− mice are unresponsive to either acute or chronic stimulation by recombinant EGFR ligands.

Fig. 1. Altered EGFR expression and signaling in primary epidermal keratinocytes from Egfr −/− mice. A, EGFR immunoblot analysis performed on keratinocyte lysates as described in "Materials and Methods," using a sheep polyclonal antibody recognizing the intracellular domain of EGFR. Native EGFR in Lane +/+ is detected as a broad band migrating at ∼160–170 kDa (arrow). Low levels of a ∼140-kDa species were detected in Egfr −/− and +/+ keratinocyte lysates (large arrowhead), and prolonged film exposures revealed a faint band at ∼165 kDa in Egfr −/− lysates (right, small arrowhead). B, Western blot analysis of phosphotyrosine immunoprecipitations. Lysates were prepared from control cultures and cultures treated with 10 ng/ml TGF-α for 3 min. Phosphotyrosine-containing proteins were immunoprecipitated, separated on an 8.5% polyacrylamide gel, and transferred to nitrocellulose as described in "Materials and Methods." TGF-α increases the phosphotyrosine content of EGFR and Shc in wild-type but not Egfr −/− keratinocytes. Lanes Contr., controls. C, TGF-α activates Erk-1/2 MAPKs in wild-type, but not Egfr −/−, keratinocytes. Lysates were prepared using cells that were either untreated or exposed to 1 μg/ml TGF-α for 3 min. Following polyacrylamide gel electrophoresis and transfer to nitrocellulose, activated Erk-1/2 was detected in lysates from TGF-α-treated wild-type, but not Egfr −/−, keratinocytes using an antibody that selectively recognizes the dually phosphorylated form of these enzymes. The total level of Erk-1/2 in wild-type and Egfr −/− keratinocytes was similar (bottom).
keratinocytes (10). To assess the role of secreted EGFR ligands in this response, proliferation assays were performed using CM from wild-type v-rasHa keratinocytes and either wild-type or Egfr —/— primary keratinocytes as target cells. Cell number in wild-type cultures was increased ~3-fold by v-rasHa CM diluted 1:1 with standard culture medium; this degree of growth stimulation is comparable to that achieved by treating wild-type cells with 1 ng/ml TGF-α (Fig. 3). At a 1:3 dilution of CM, a 40% increase in cell number was observed, whereas no growth stimulation was detected at a 1:9 dilution of CM.

In contrast to wild-type keratinocytes, Egfr —/— cultures exhibited minimal growth stimulation by v-rasHa CM, and this stimulation was demonstrated only at the highest concentration (1:1) tested (Fig. 3). CM harvested from EGFR-deficient v-rasHa cultures had a similar effect as wild-type v-rasHa CM in this assay; growth of wild-type target cells was stimulated severalfold with a negligible mitogenic response in Egfr —/— cultures (data not shown), indicating that disruption of EGFR function does not impair the ability of v-rasHa to up-regulate EGFR ligand expression in cultured keratinocytes.

Additional experiments were performed to assess whether EGFR signaling is required for the increased proliferation observed in v-rasHa cultures. Primary keratinocytes were transduced with v-rasHa retrovirus at several dilutions to assess the involvement of autocrine versus paracrine EGFR signaling in phenotypic responses to oncogenic ras (see below), and the cumulative increase in cell number was determined as described in “Materials and Methods.” Surprisingly, v-rasHa caused at least a 4-fold stimulation of cell growth in wild-type as well as Egfr —/— keratinocyte cultures (Table 1). In one experiment, Egfr +/+ cultures transduced using retrovirus dilutions ranging from 1:3 to 1:24 exhibited a 4.4- to 4.6-fold increase in cell number, whereas the growth of Egfr —/— v-rasHa cultures was stimulated between 4.3- and 5-fold (Table 1). In a second experiment, the fold increase in cell number for Egfr +/+ v-rasHa cultures ranged from 5.5 to 6.5, whereas that of Egfr —/— v-rasHa cultures was modestly reduced, ranging from 4.3 to 5.5 (Table 1). We did not detect consistent differences in the growth rate of wild-type and Egfr —/— keratinocytes grown under control culture conditions (data not shown). Thus, although v-rasHa CM contains substantial mitogenic activity that requires a functional EGFR (Fig. 3), the brisk mitogenic response observed in v-rasHa keratinocyte cultures is largely independent of EGFR function. Moreover, EGFR function does not appear to be required for sustaining basal growth of murine keratinocytes under the conditions used in these assays.

EGFR Signaling Is Required for Paracrine, but not Autocrine, Suppression of Differentiation-specific Keratins by v-rasHa. In addition to stimulating cell growth, introduction of v-rasHa into primary keratinocytes results in suppression of the differentiation-specific keratins K1 and K10, coupled to anomalous expression of K8 and K18 (14, 27). Similar changes are induced in normal keratinocytes by TGF-α, EGF, or CM from v-rasHa cultures (10), suggesting that this response to v-rasHa is mediated by autocrine-activated EGFR. Expression of K1 and K10 was blocked, whereas K8 and K18 were induced in both wild-type and Egfr —/— keratinocyte cultures. 

Table 1 Mitogenic response of Egfr +/+ and Egfr —/— primary keratinocytes to v-rasHa transduction (cumulative cell number per well × 10^6)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Baseline</th>
<th>Control</th>
<th>1:1</th>
<th>1:3</th>
<th>1:6</th>
<th>1:12</th>
<th>1:24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egfr +/+</td>
<td>5.42</td>
<td>12 (2.2)</td>
<td>24.6 (4.5)</td>
<td>24.2 (4.5)</td>
<td>23.6 (4.4)</td>
<td>24.7 (4.6)</td>
<td></td>
</tr>
<tr>
<td>Egfr —/—</td>
<td>4.45</td>
<td>9.51 (2.1)</td>
<td>21.6 (4.9)</td>
<td>22.1 (5.0)</td>
<td>21.8 (4.9)</td>
<td>19.0 (4.3)</td>
<td></td>
</tr>
<tr>
<td>Egfr +/+</td>
<td>4.31</td>
<td>ND†</td>
<td>28.1 (6.5)</td>
<td>24.7 (5.7)</td>
<td>23.5 (5.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egfr —/—</td>
<td>4.15</td>
<td>ND†</td>
<td>22.9 (5.5)</td>
<td>21.9 (5.3)</td>
<td>18.0 (4.3)</td>
<td></td>
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a Baseline indicates the number of keratinocytes per well at the time cells were transduced with v-rasHa retrovirus. Cumulative cell number was determined as described in “Materials and Methods,” with final counts performed 9 days after infection for experiment 1; 14 days after infection for experiment 2.

b Numbers in parentheses indicate fold increase in cumulative cell number relative to baseline, calculated as described in “Materials and Methods.”

† ND, not determined.
ROLE OF EGFR IN v-ras-H-MEDIATED KERATINOCYTE NEOPLASIA

Fig. 5. Expression of K1 in Egfr−/− v-ras-H keratinocyte cultures is inversely correlated with Ras expression. Primary cultures transduced with low-titer v-ras-H were grown in medium containing 0.12 mM Ca²⁺ for 2 days to induce terminal differentiation. Cells were fixed and processed for double-immunofluorescence to detect Ras (FITC-green) and K1 (Texas Red) as described in "Materials and Methods." Note that cells exhibiting intense staining for Ras protein (arrowheads) do not contain appreciable levels of K1; conversely, K1-positive cells (arrows) are largely negative for Ras staining.

transduced with high-titer v-ras-H retrovirus (Fig. 4), arguing against a requirement for autocrine EGFR signaling in this response to v-ras-H. In wild-type cultures, K1 and K10 were suppressed even at reduced retroviral titers (Fig. 4), suggesting that differentiation-specific keratins are blocked not only in those cells expressing oncogenic ras, but also in "v-ras-H-negative" keratinocytes that are being stimulated by EGFR ligands in a paracrine manner. In support of this hypothesis, suppression of K1 and K10 was observed in Egfr−/− cultures that were infected using reduced titers of v-ras-H retrovirus (Fig. 4). Moreover, double-immunofluorescence analysis of Egfr−/− v-ras-H cultures revealed that cells expressing K1 contain low or undetectable levels of what is presumably endogenous Ras protein (Fig. 5, arrows), whereas cells that stain intensely for Ras protein, presumably viral, do not contain significant amounts of K1. Taken together, these data indicate that EGFR signaling is required for paracrine-mediated suppression of K1 and K10 expression in low-titer v-ras-H cultures, but they argue against an autocrine role for EGFR in this response in individual cells expressing oncogenic Ras. In contrast to the suppression of K1 and K10, the abundance of K8 and K18 was roughly proportional to the expression level of viral Ras protein in cells of both genotypes (Fig. 4), suggesting that EGFR signaling does not play a major role in the induction of these keratins by v-ras-H.

Disruption of EGFR Function Impairs Growth of v-ras-H-induced Epidermal Tumors and Alters the Distribution of S-Phase Nuclei in Vivo. The role of EGFR function in epidermal tumor growth was assessed by grafting v-ras-H-transduced wild-type or Egfr−/− keratinocytes onto nude mice as a reconstituted skin. Cells of both genotypes produced tumors; however, the Egfr−/− v-ras-H papillomas reached only 21% of the size of wild-type v-ras-H tumors at 26 days after grafting (Fig. 6). Histological examination confirmed that both sets of tumors were squamous papillomas; moreover, no remarkable differences were detected between tumor genotypes for expression or distribution of the keratinocyte differentiation markers K1, K10, loricrin, or filaggrin when the tumors were assayed by immunostaining (data not shown). Furthermore, epidermal transglutaminase activity, responsible for cross-linked cornified envelope formation during the terminal stage of epidermal differentiation, was similar among the tumor genotypes when determined by an in situ assay using the fluorescent transglutaminase substrate dansylcadaverine (28), and immunostaining for keratins K8 and K13, markers for neoplastic progression in epidermal tumors (29, 30), was also similar in all tumors (data not shown).

Additional analyses focused on exploring the potential basis for differences in tumor growth of wild-type and Egfr−/− v-ras-H papillomas. Terminal deoxynucleotidyl transferase-mediated nick end labeling staining did not reveal consistent differences between tumor groups (Fig. 7A), suggesting that persistent alterations in the rate of cell death due to disruption of the EGFR pathway were not playing a

Fig. 6. Targeted disruption of EGFR impairs growth of v-ras-H-induced squamous papillomas. Fibroblasts (8 × 10⁶), combined with 6 × 10⁶ Egfr+/+ or Egfr−/− primary v-ras-H keratinocytes, were grafted onto nude mice as a reconstituted skin as described in "Materials and Methods." Average tumor volumes were calculated as described in "Materials and Methods." Error bars, SE. Each group contained five animals. In a replicate experiment, in which 5 × 10⁶ primary v-ras-H keratinocytes were grafted per animal, with nine animals per group, the size of Egfr−/− papillomas was 23% of that produced by Egfr+/+ keratinocytes, measured 30 days after grafting.

DAYS AFTER GRAFTING

TUMOR VOLUME (mm³)

EGFR +/+  EGFR −/−

0 2 4 6 8 10 12 14 16 18 20 22 24 26
Fig. 7. A, similar incidence of apoptotic nuclei in Egfr −/− and Egfr +/+ v-rasH4 papillomas. Apoptotic cells were identified using the ApopTag kit (Oncor), which stains nuclei containing nicked DNA green (arrowheads). Propidium iodide was used as a nuclear counterstain. B, altered distribution of S-phase nuclei in Egfr −/− v-rasH5 CM, transduction of wild-type and Egfr −/− keratinocytes of both genotypes in v-rasH5 CM, transduction of wild-type and Egfr −/− keratinocytes of both genotypes produced papillomas when grafted onto nude mice, although EGFR-deficient tumors were smaller and the distribution of S-phase nuclei was altered when compared to wild-type papillomas. Our findings indicate that, although autocrine EGFR signaling is not required for keratinocyte neoplasia using cells in which the Egfr gene has been genetically disrupted. Despite the presence of abundant EGFR-dependent mitogens in v-rasH4 CM, transduction of wild-type and Egfr −/− keratinocytes with high-titer v-rasH4 retrovirus resulted in comparable growth stimulus and reprogramming of keratin expression in vitro. Moreover, v-rasH4 keratinocytes of both genotypes produced papillomas when grafted onto nude mice, although EGFR-deficient tumors were smaller and the distribution of S-phase nuclei was altered when compared to wild-type papillomas. Our findings indicate that, although autocrine EGFR signaling is not required for keratinocyte responses to v-rasH4 in vitro or benign tumor formation in vivo, maximal tumor growth appears to be dependent on a functional EGFR signaling pathway.

A striking phenotypic difference between wild-type and Egfr −/− v-rasH4 cultures was detected following infection at reduced titers of retrovirus, resulting in transduction of only a limited number of keratinocytes. Under these conditions, K1 and K10 expression was suppressed in wild-type but not Egfr −/− cultures, indicating that EGFR-dependent signaling can block aspects of normal keratinocyte differentiation in a paracrine manner. The results of these experiments differ remarkably from those examining the proliferative response to v-rasH4 transduction, which was elevated in cells of either genotype at all dilutions of retrovirus tested and was thus largely independent of EGFR function. Our inability to demonstrate that autocrine EGFR signaling contributes to the phenotype of v-rasH4 keratinocytes in papillomas, it plays an important role in regulating the growth of these lesions once established. Moreover, the redistribution of BrdUrd-labeled cells to suprabasal compartments in Egfr −/− papillomas suggests that the EGFR influences tumor biology in part as a result of alterations in keratinocyte cell cycling or premature migration into the differentiating compartments.

DISCUSSION

Increased expression of mitogenic growth factors and their receptors in neoplastic epithelia suggests that autocrine and/or paracrine signaling contributes to the growth of these tumors. In this report, we have examined the role of EGFR in v-rasH4-mediated epidermal neoplasia using cells in which the Egfr gene has been genetically disrupted. Despite the presence of abundant EGFR-dependent mitogens in v-rasH4 CM, transduction of wild-type and Egfr −/− keratinocytes with high-titer v-rasH4 retrovirus resulted in comparable growth stimulus and reprogramming of keratin expression in vitro. Moreover, v-rasH4 keratinocytes of both genotypes produced papillomas when grafted onto nude mice, although EGFR-deficient tumors were smaller and the distribution of S-phase nuclei was altered when compared to wild-type papillomas. Our findings indicate that, although autocrine EGFR signaling is not required for keratinocyte responses to v-rasH4 in vitro or benign tumor formation in vivo, maximal tumor growth appears to be dependent on a functional EGFR signaling pathway.

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Table 2 Altered distribution of S-phase cells in EGFR-deficient v-rasH4 squamous papillomas

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Basal</th>
<th>Suprabasal</th>
<th>Total</th>
<th>N⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egfr −/−</td>
<td>34.3 ± 1.1% (61%)</td>
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<tr>
<td>Egfr +/+</td>
<td>42.7 ± 1.7% (81%)</td>
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</tbody>
</table>

⁴ The number of BrdUrd-labeled nuclei present in a field of 100 basal cells. The labeling index (% of BrdUrd-labeled cells in the Egfr +/+ basal cell compartment was 42.7%, compared with 34.3% for the Egfr −/− basal cell compartment. 

N, number of tumors analyzed.

Mean is significantly different from that of the corresponding wild type (P < 0.05, Student’s t test).

Numbers in parentheses indicate the relative proportion of labeled cells in basal versus suprabasal compartments for tumors of each genotype.
vitro was not anticipated given the evidence supporting this concept in our model system (10, 31), as well as results of studies implicating the EGFR pathway in ras-mediated transformation of other epithelial cell types (32–35). Thus, these findings raise several issues regarding interactions between oncogenic Ras and EGFR signaling in keratinocytes. Assuming that a major pathway coupling EGFR to nuclear events involves the Ras→Raf→MAP/Erk kinase→Erk cascade, it is conceivable that the high expression level of constitutively active viral Ras protein in v-rasH5 keratinocytes results in maximal stimulation of these downstream targets, so that any additional input due to autocrine-activated EGFR is functionally insignificant. This concept rests on the assumption that EGFR-mediated activation of endogenous Ras elicits qualitatively similar responses as does the constitutively active viral Ras protein; moreover, it implies that Ras-independent effector pathways operating downstream of activated EGFR (36) do not contribute to the phenotypic responses examined in this report.

An additional consideration is raised by the observation that oncogenic ras results in decreased responsiveness to exogenous EGF and/or down-modulation of 125I-EGF-binding in murine keratinocytes (37), as well as several other cell types (38–41). Although attributed to chronic EGFR stimulation by autocrine-acting ligands, the similar in vitro growth response of wild-type and Egrf −/− keratinocytes to v-rasH5 argues against this concept, suggesting that other mechanisms may play a role in altering EGFR function. We previously reported that PKCa activity measured in v-rasH5 keratinocyte lysates is increased relative to levels in control cells (42) and that selective inhibition of PKCa function reverses certain keratinocyte responses to v-rasH5 in vitro (42, 43). Because activation of the PKC pathway blocks both 125I-labeled EGF binding and EGFR tyrosine kinase activity (reviewed in Ref. 44), the reported down-modulation of EGFR function in v-rasH5 keratinocytes may be mediated by endogenously activated PKCa. Thus, rather than being a consequence of EGFR activation and down-regulation by autocrine-acting ligands, inhibition of EGFR function in ras-transformed cells could provide a mechanism for blocking the response to mitogenic stimuli in cells in which the Ras pathway is already constitutively activated. Additional experiments will be required to address this issue.

Finally, in recent studies it has been shown that betacellulin is capable of activating ErbB-4 independently of EGFR (45, 46), suggesting that this EGFR ligand may function as an autocrine growth factor even in EGFR-deficient cells. Although we found no evidence for growth stimulation of Egrf −/− keratinocytes using recombinant betacellulin (Fig. 2), v-rasH5 could modulate responsiveness to this growth factor if produced endogenously (15), as a result of changes involving either ligand or receptor.

Although it is conceivable that the low level of truncated EGFR present in EGFR-deficient keratinocytes is biologically active, our inability to detect ligand-mediated receptor activation or downstream signaling argue against this possibility. Furthermore, we have not detected constitutive phosphorylation of this mutant protein in primary cultures of Egrf −/− control or v-rasH5 keratinocytes (data not shown), suggesting that it is not involved in autocrine signaling under these conditions. Our results are in contrast with those reported for a spontaneously occurring EGFR mutant detected in gliomas (47) and several other tumors (48) that is produced by a transcript lacking exons 2–6. Ligand-mediated receptor activation is also not detected, but this truncated EGFR is constitutively phosphorylated (49), accelerates growth of a glioblastoma cell line in nude mice (50), and transforms NR6 fibroblasts (51).

Surprisingly, EGFR immunoblot analysis of phosphotyrosine immunoprecipitates revealed that Egrf −/− keratinocyte lysates contained a constitutively phosphorylated protein with an apparent molecular weight similar to that of EGFR from wild-type cultures (Fig. 2B). The phosphotyrosine content of this protein was comparable to that of EGFR from unstimulated wild-type keratinocytes, but it was not detectably increased by TGF-α treatment (Fig. 2B), arguing against an active role in signaling following exposure to EGFR ligand. Additional studies will be required to determine the identity and biological significance of this protein, as well as its possible relationship to the low-abundance band seen in Egrf −/− keratinocyte immunoblots probed with the same EGFR antibody (Fig. 1A, right).

Although grafting of either Egrf −/− or wild-type v-rasH5 keratinocytes onto nude mice resulted in the formation of squamous papillomas, the smaller size of Egrf −/− lesions indicates that growth of these tumors is in part regulated by the EGFR pathway. Immunohistochemical analysis for multiple keratinocyte differentiation markers argues that accelerated terminal differentiation is not contributing to the smaller size of Egrf −/− tumors. In addition, we found no evidence for changes in apoptosis between the two tumor groups using terminal deoxynucleotidyl transferase-mediated nick end labeling staining, although a modest, yet biologically significant, difference cannot be excluded. Despite these basically negative results in established tumors, it is conceivable that keratinocytes are most susceptible to undergoing programmed cell death at the initiation of grafting, with a substantially smaller fraction of Egrf −/− than that of wild-type v-rasH5 keratinocytes surviving to form a tumor.

We did detect a striking alteration in the distribution of cycling cells in Egrf −/− tumors, which contained an increased proportion of BrdUrd-labeled nuclei in suprabasal compartments and modest reduction in basal cell labeling index, compared to controls. These findings could explain the observed difference in tumor growth rate, assuming that only basal cells are capable of completing mitosis and thus providing additional cell mass to the developing tumor. The increased suprabasal labeling in Egrf −/− papillomas could be due to an alteration in keratinocyte cell cycling and/or premature migration of S-phase cells from basal into suprabasal compartments. Further in vivo and in vitro analyses will be required to explore these possibilities.

In addition to the apparent in vivo role for EGFR in regulating proliferation and/or migration of v-rasH5-keratinocytes, other explanations for the divergent tumor growth rates of wild-type and Egrf −/− papillomas need to be considered. For example, disruption of EGFR function may alter the ability of v-rasH5 keratinocytes to influence stromal elements involved in tumor growth. Because expression of vascular endothelial growth factor is up-regulated in murine skin tumors (52), it is conceivable that optimal expression of this or other angiogenic factors by ras-transformed keratinocytes requires an intact EGFR signaling pathway. Consistent with this concept, EGFR activation up-regulates levels of vascular endothelial growth factor mRNA or protein in cultured keratinocytes (53), as well as in glioma cell lines (54). EGFR function may also play a role in ras-mediated up-regulation of urokinase-type plasminogen activator (55, 56) or in metalloprotease expression (57), although these changes are more likely to be involved in malignant progression than benign papilloma growth. Although our data argue against an obligatory role for EGFR in the formation of papillomas by oncogenic ras, additional studies using this system are required to clarify the function of EGFR in late stages of tumor progression. In addition, it will be of interest to assess whether autocrine EGFR activation plays a role in epithelial neoplasia mediated by oncogenes other than ras, the products of which do not themselves lie in a signaling pathway downstream of activated EGFR.
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REFERENCES


Targeted Disruption of the Epidermal Growth Factor Receptor Impairs Growth of Squamous Papillomas Expressing the v-\textit{ras} Oncogene but Does Not Block \textit{in Vitro} Keratinocyte Responses to Oncogenic \textit{ras}

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