THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
Vol. 278, No. 3, Issue of January 17, pp. 1824–1830, 2003
Printed in U.S.A.

Epidermal Growth Factor Receptor Mediates Increased Cell Proliferation, Migration, and Aggregation in Esophageal Keratinocytes *in Vitro* and *in Vivo**

Received for publication, September 6, 2002, and in revised form, November 11, 2002
Published, JBC Papers in Press, November 14, 2002, DOI 10.1074/jbc.M209148200

Claudia D. Andl‡§¶, Takaaki Mizushima‡§¶, Hiroshi Nakagawa‡§¶, Kenji Oyama‡§, Hideki Harada‖, Katerina Chruma‖, Meenhard Herlyn‖, Anil K. Rustgi‡§‡‡**

*From the ‡Gastroenterology Division, **Department of Genetics, §Abramson Cancer Center and Family Cancer Research Institute, ‖Wistar Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Epidermal growth factor receptor (EGFR) overexpression is observed in a number of malignancies, especially those of esophageal squamous cell origin. However, little is known about the biological functions of EGFR in primary esophageal squamous epithelial cells. Using newly established primary human esophageal squamous epithelial cells as a platform, we overexpressed EGFR through retroviral transduction and established novel three-dimensional organotypic cultures. Additionally, EGFR was targeted in a cell type- and tissue-specific fashion to the esophageal epithelium in transgenic mice. EGFR overexpression in primary esophageal keratinocytes resulted in the biochemical activation of Akt and STAT pathways and induced enhanced cell migration and cell aggregation. When established in organotypic culture, EGFR-overexpressing cells had evidence of epithelial cell hyperproliferation and hyperplasia. These effects were also observed in EGFR-overexpressing transgenic mice and the esophageal cell lines established thereof. In particular, EGFR-induced effects upon aggregation appear to be mediated through the relocalization of p120 from the cytoplasm to the membrane and increased interaction with E-cadherin. EGFR modulates cell migration through the up-regulation of matrix metalloproteinase 1. Taken together, the functional effects of EGFR overexpression help to explain its role in the initiating steps of esophageal squamous carcinogenesis.

Epidermal growth factor receptor (EGFR)[1] is a transmembrane protein receptor with tyrosine kinase activity that triggers numerous signaling pathways (1–3). Activation of the EGFR tyrosine kinase results in the generation of a number of intracellular signals, which culminate in not only cell proliferation but also other processes that are crucial to cancer progression, including angiogenesis, metastatic spread, and the inhibition of apoptosis. These events are mediated through various downstream targets of EGFR (*e.g.* the serine/threonine kinase Raf and mitogen-activated protein/extracellular signal-regulated kinase 1/2). In addition, Ras activation by EGFR is required for a vast array of cellular functions, foremost of which is the regulation of cellular proliferation. Activation of EGFR also results in the activation of the lipid kinase phosphatidylinositol 3-kinase, generating the second messenger phosphatidylinositol 3,4,5-trisphosphate, which in turn activates Akt. We have previously demonstrated that there is differential activation of the Akt isoforms by EGFR in esophageal cancer cells (4). Apart from the mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways, EGFR also activates other pathways such as phospholipase-C and its downstream protein kinase cascades, small GTPases such as Rho, and multiple signal transducer and activator of transcription (STAT) isoforms.

EGFR activation is not only important in normal cellular processes, but it is frequently altered or overexpressed in many malignancies, especially those of squamous cell origin. Mechanisms that mediate EGFR overexpression include gene amplification, truncation of the carboxyl terminus, transcriptional activation, and posttranslational modifications. EGFR overexpression is a frequent genetic alteration in premalignant esophageal squamous dysplastic lesions and the early stages of esophageal squamous cell cancer (5–8). This has led us to focus upon the role of EGFR-mediated signaling in esophageal epithelial cell biology and examine its role in models of esophageal squamous cell carcinogenesis.

We describe herein novel organotypic esophageal cell culture and esophageal specific transgenic mouse models as the basis for examination of EGFR-mediated biological effects in physiologic environments that are often not possible in cancer-derived cell lines. We observe that EGFR overexpression results in hyperproliferation both *in vitro* and *in vivo* but is not sufficient to induce cancer, explaining its frequent association with premalignant stages. Furthermore, EGFR activation results in increased cell migration. Mechanistically, the increase in cell migration induced by EGFR is mediated through up-regulation of matrix metalloproteinase-1 (MMP-1). Whereas EGFR overexpression did not influence the assembly of adherens junctions and desmosomes, it did induce a translocation of p120 catenin from the cytosol to the cell membrane that mediates the increased cell aggregation. We believe that these effects help to explain how EGFR modulates the persistence of prolif-

* This work was supported by National Institutes of Health (NIH) Grants P01 DE12467 and P01 CA098101 (to A. K. R.), a grant from the Leonard and Madlyn Abramson Family Cancer Research Institute at the University of Pennsylvania Cancer Center (to A. K. R.), the NIH/NIDDK Center for Molecular Studies in Digestive and Liver Diseases (Grant P30 DK50306), and its Morphology, Molecular Biology, Mouse, and Cell Culture Core Facilities, and NIH Grants CA 80999 and CA 25874 (both to M. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ These three authors contributed equally to this work.
‡‡ To whom correspondence should be addressed: 600 CRB, University of Pennsylvania, 415 Curie Blvd., Philadelphia, PA 19104. Tel.: 215-898-0154; Fax: 215-573-5412; E-mail: anil2@mail.med.upenn.edu.

[1] The abbreviations used are: EGFR, epidermal growth factor receptor; STAT, signal transducers and activators of transcription; MMP, matrix metalloproteinase; EGF, epidermal growth factor; GFP, green fluorescent protein; PBS, phosphate-buffered saline; ECM, extracellular matrix; KSFM, keratinocyte-SFM medium; RT, reverse transcription.

This paper is available on line at http://www.jbc.org

erative basal cells into the suprabasal compartment of the squamous epithelium and that EGFR is critical for the initiating events in squamous carcinogenesis.

EXPERIMENTAL PROCEDURES

Cell Lines—Primary esophageal keratinocytes, designated as EPC1 and EPC2, from normal human esophagus were established. Surgical specimens from normal esophagi were promptly removed and transported aseptically in Hanks' solution (Invitrogen) with 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and 5 μg/ml gentamicin (Invitrogen). The tissue specimen was incubated with 1.5 units/ml dispase (Roche Molecular Biochemicals) at 4 °C overnight, and the epithelium was dissected away and incubated with trypsin (Invitrogen). The reaction was stopped with soybean trypsin inhibitor (Sigma) and centrifuged. The pellet was resuspended in keratinocyte-SFM medium (KSFM) (Invitrogen) supplemented with 40 μg/ml bovine pituitary extract (Invitrogen), 1.0 ng/ml EGF (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 5 μg/ml gentamycin, and 100 units/ml nyastatin (Invitrogen). EPC cells were grown at 37 °C and 5% CO_2 with KSFM, with 40 μg/ml bovine pituitary extract, 1.0 ng/ml EGF, 100 units/ml penicillin, and 100 μg/ml streptomycin.

When cells were starved, the medium was KBM supplemented with 0.5 μg/ml hydrocortisone (Bio Whittaker Inc., Walkersville, MD) and 0.09 mM calcium chloride (Bio Whittaker). Cells were starved for 48 h without EGF and then stimulated with 10 or 100 ng/ml EGF for the indicated periods at 37 °C, washed three times with ice-cold phosphate-buffered saline, lysed in buffer, and centrifuged for 15 min at 4 °C. A431 cells conditioned in KSFM served as a positive control.

The growth curves of EPC cells and those transduced with green fluorescent protein (GFP) or EGFR (see below) were generated by plating cells in six-well plates (1.0×10^4 cells/plate) and grown in KSFM supplemented with 40 μg/ml bovine pituitary extract. Cells were harvested at indicated periods and counted with a spectrophotometric quantitation method (9). All experiments were carried out at least three times in triplicate with generation of S.D. values.

Retroviral Vectors and Infection—pFB-neo retroviral vectors (Stratagene, La Jolla, CA) were used to infect EPC1 and EPC2. In addition, we subcloned into the pFB-neo vector the entire coding sequence for the human EGFR or green fluorescent protein (GFP). The inserted region in the resulting construct was sequenced, and the plasmid was transfected into Phoenix-Ampho cells by the calcium-phosphate precipitation method (Calphos; Clontech, Palo Alto, CA) according to the manufacturer's instructions. In brief, culture supernatants from individual Phoenix-Ampho cells were used to infect EPC1 and EPC2 cells. These cells were infected with filtered (0.45-μm pore size) supernatant from an overnight culture of Phoenix-Ampho cells, producing the pFB-neo retroviruses encoding EGFR or GFP. Cells were passaged 48 h after infection and selected in 300 μg/ml G418 for 14 days.

Organotypic Cell Culture—To grow human esophageal epithelial cells (keratinocytes), 5×10^5 cells were seeded on to the collagen matrix, containing 1× minimal essential medium with Earle's salts (Bio Whittaker), 1.68 mM L-glutamine (Cellgro, Herndon, VA), 10% fetal bovine serum (Hyclone, Logan, UT), 0.15% sodium bicarbonate (Bio Whittaker), 76.7% bovine tendon acid-extracted collagen (Organogenesis, Canton, MA), and 7.5×10^4 human skin fibroblast cells. Cells were fed with Epidermalization I medium for 2 days, which is Dulbecco's modified Eagle's medium (JRH Biosciences, Lenexa, KS)/Ham's F-12 (Invitrogen) (3:1) supplemented with 4 mM L-glutamine, 0.5 μg/ml hydrocortisone, 0.1 mM *O*-phosphorylethanolamine, 20 pM triiodothyronine, 0.18 mM adenine, 1.88 mM $CaCl_2$, 4 pM progesterone (Sigma); 10 μg/ml insulin, 10 μg/ml transferrin, 10 mM ethanolamine, 10 ng/ml selenium (ITES) (Bio Whittaker); and 0.1% chelated newborn calf serum (Hyclone). For the following 2 days, cells were fed with Epidermalization II medium, which is identical to Epidermalization I except that it contains 0.1% unchelated newborn calf serum. Then cells were raised to the air-liquid interface and cultured in Epidermalization III medium for 6 days containing the same growth supplements as Epidermalization II except 2% newborn calf serum. Cells were fixed with 10% formaldehyde and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Generation of EGFR Transgenic Mice and Cell Lines of Mouse Esophageal Keratinocyte Origin—We employed the Epstein-Barr virus ED-L2 promoter and fused it to the human EGFR cDNA to create a transgene from which founder lines were generated and maintained in the B6SJL-F1 gene background. The EGFR transgene is expressed in a tissue-specific fashion with targeting to the tongue, esophagus, and forestomach (10). The EGFR transgenic mice and age-matched wild-type mice were sacrificed at age 6 months for histology and immunohistochemistry. The cell lines were established in the same fashion same as described for EPC1 and EPC2.

Antibodies—Antibodies against EGFR were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against anti-phospho-EGFR (Y1173) and Akt1 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Antibodies specific for focal adhesion kinase were from BD Biosciences (San Diego, CA), Antibodies specific for Akt phosphorylated at Ser-473, phosphofocal adhesion kinase, phospho-STAT1, phosphoextracellular signal-regulated kinase 1/2 and total extracellular signal-regulated kinase 1/2 were obtained from Cell Signaling (Beverly, MA). Antibodies specific against desmoglein 1 and 2 (clone DG3.10) were purchased from Biodesign (Kennebunk, ME), and antibodies against Desmoplakin were from Serotec. E-cadherin-neutralizing antibody was purchased from Sigma. Antibodies against E-cadherin, p120, and β-catenin were obtained from Transduction Laboratories (Lexington, KY), and antibodies against MMP-1 were purchased from Neomarkers (clone X2A; Fremont, CA). Secondary anti-mouse and anti-rabbit horseradish peroxidase antibodies were purchased from Amersham Biosciences.

Immunoprecipitation—Preconfluent cells starved in KBM medium and stimulated with 10 ng/ml EGF for 15 min as well as unstimulated cells were washed with phosphate-buffered saline without calcium and magnesium (PBS) and incubated with 700 μl of lysis buffer (1% Triton X-100, 1% Nonidet P-40, 50 mM Tris, pH 8, and proteinase inhibitors 2 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 2 mM Na_3VO_4, 5 mM sodium pyrophosphate; less stringent buffer does not contain Nonidet P-40) for 30 min on ice. 70 μl of 4% bovine serum albumin and 140 μl of 1.5 M NaCl were added to the extracts, which were then preabsorbed with 10 μl of recombinant Protein G-agarose (Invitrogen) for 1 h at 4 °C. Preabsorbed extracts were incubated with antibodies against EGFR, plakoglobin, p120, and β-catenin. After a 1-h incubation at 4 °C, the antigen-antibody complex was incubated with 10 μl of recombinant Protein G-agarose for 1 h at 4 °C. The precipitates were washed three times with 1 ml of wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS; less stringent wash in PBS only) and boiled with 100 μl of lithium dodecyl sulfate buffer containing dithiothreitol for 10 min. Supernatants were used for Western blotting as described above.

Western Blotting—Subconfluent cells were lysed in lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM sodium orthovanadate, and a protease inhibitor mixture tablet (Roche Molecular Biochemicals)). Protein concentration was determined by the BCA protein assay (Pierce). The solution was subsequently solubilized in NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen, Carlsbad, CA) containing 50 mM dithiothreitol. Total protein samples (10 μg) were separated on a 4–12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA). The membrane was blocked in 5% nonfat milk (Bio-Rad) in TBST (10 mM Tris, 150 mM NaCl, pH 8.0, and 0.1% Tween 20) for 1 h at room temperature. Membranes were probed with primary antibody diluted 1:1000 in 5% TBST milk overnight at 4 °C, washed three times in TBST, incubated with anti-mouse or anti-rabbit horseradish peroxidase antibody diluted 1:3000 in TBST for 1 h at room temperature and then washed three times in TBST. The signal was visualized by an enhanced chemiluminescence solution (ECL Plus; Amersham Pharmacia Biotech) and was exposed to Eastman Kodak Co. X-Omat LS film.

Immunohistochemistry and Immunofluorescence—Immunohistochemistry for Ki67 was performed with the Vecta Elite kit (Vector Laboratories, Burlingame, CA) following the manufacturer's protocol. In brief, paraffin sections were pretreated with xylene and then placed in a microwave in 10 mM citric acid buffer. Endogenous peroxidases were quenched using hydrogen peroxide before sections were blocked in avidin D blocking reagent and biotin blocking reagent. Sections were incubated with primary and secondary antibody, and then signal was developed using the DAB substrate kit for peroxidase.

Reconstructs were embedded in ornithine transcarbamylase and frozen at −80 °C. Sections were cut and fixed in acetone for 10 min at −20 °C. Cells in culture were seeded into chamber slides (Nalge Nunc, Naperville, IL) and fixed in 1:1 methanol/acetone for 8 min. After fixation, objects were treated with 0.1% Triton X-100 in PBS for 5 min. Objects were washed in PBS and blocked with 1% bovine serum albumin (Sigma) for 1 h. Incubation of primary antibodies was for 1 h at room temperature or overnight at 4 °C. After washing with PBS, objects were incubated with Texas Red- conjugated secondary antibody (Molecular Probes, Inc., Eugene, OR) or fluorescein isothiocyanate-conjugated secondary antibody (Roche Molecular Biochemicals) for 1 h.

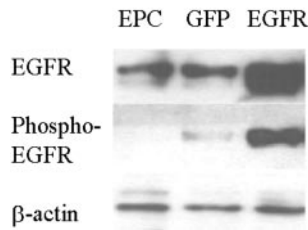


FIG. 1. **Retroviral transduction of EGFR results in overexpression and tyrosine kinase activation.** Western blotting shows EGFR expression levels in EPC2, EPC2-GFP, and EPC2-EGFR cells. Phospho-EGFR levels are increased in EPC2-EGFR cells after stimulation with EGF. β-Actin serves as a loading control.

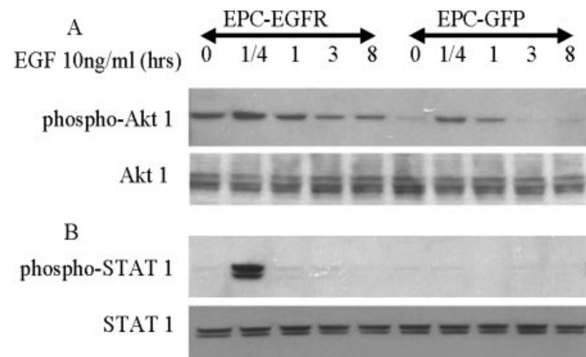


FIG. 2. **Western blotting of EGFR and its downstream target molecules demonstrates EGFR-mediated activation of intracellular signaling pathways.** *A*, Akt1 phosphorylation is increased in EPC2-EGFR cells compared with EPC2-GFP cells before and after stimulation with EGF. *B*, STAT1 phosphorylation peaks after 15 min of EGF stimulation in EPC2-EGFR cells, whereas STAT1 is not phosphorylated in EPC2-GFP cells. β-Actin serves as a loading control (data not shown).

Stained objects were examined with a Nikon Microphot microscope and imaged with a digital camera at magnifications as indicated. Confocal microscopy was performed using the Radiance2100 confocal and multiphoton imaging systems and documented with Laser Sharp software (Bio-Rad).

RT-PCR and SYBR Green Real Time PCR—RNA was isolated from EPC2-GFP and EPC2-EGFR cells using Trizol reagent (Invitrogen), and cDNA was synthesized using the Superscript first strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. Primer for PCR and SYBR green real time PCR were designed using the TaqMan probe software and synthesized by Invitrogen (primer sequences: MMP-1 (5′-TCC GGT TTT TCA AAG GGA ATA; 3′-CCT CAG AAA GAG CAG CAT CGA), MMP-2 (5′-AGA CCG CCA TGT CCA CTG TT; 3′-TGG TCG CAC ACC ACA TCT TT), GAPDH (5′-CAC CCA CTC CTC CAC CTT T; 3′-TCC ACC ACC CTG TTG CTG TAG)). SYBR green real time PCR was performed and analyzed using the ABI 6000 (Applied Biosystems, Foster City, CA) with reagents from the SYBR green PCR kit (QIAgen).

Cell Migration and Invasion Assay—Haptotactic cell migration assays were performed using 24-well inserts (Falcon cell culture inserts (8-μm pore size); BD Biosciences) with or without matrigel Biocoat (BD Biosciences) according to the manufacturer's instructions. In brief, the lower chamber was filled with 0.6 ml of KBM containing 0.5 μg/ml hydrocortisone with 10 ng/ml EGF, and 0.5-ml cell suspension in KBM under serum-starving conditions were plated in the upper chamber in duplicate or triplicate wells and incubated at 37 °C for 12 h. Then cells attached to the upper side of the membrane were removed gently with a cotton swab and rinsed. Cells that migrated through the membrane and attached to the bottom of the membrane were fixed and stained with reagents from the Diff Quik staining set (Dade Behring, Newark, DE). Membranes were cut out and photographed, so migrated cells could be counted on the pictures taken. All experiments were performed at least three times in triplicate. The specific inhibitor of EGFR tyrosine kinase, AG1478, was purchased from Calbiochem.

Cell Aggregation Assay—Cell aggregation assays were based upon previously described methods (11). Single cell suspensions, starved for 48 h in KBM and stimulated with 10 ng/ml EGF, and those that were unstimulated were obtained with 1 mM EDTA in 10 mM Hepes-buffered calcium- and magnesium-free Hanks' solution. Cells were then washed twice in Hepes-buffered Hanks' solution containing 10 ng/ml DNase I. Cells were resuspended, and 3×10^5 cells were incubated on a rotary shaker for 1 h in six-well plates coated with 2% bovine serum albumin. AG1478 was added to the cell suspension during the 1-h incubation period on the shaker. Plates were incubated for another 1 h without shaking, so cell aggregates settled for analysis using the NIH Image software.

RESULTS

EGFR Overexpression and Activation by Retroviral Transduction in Normal Human Esophageal Epithelial Cells—Human primary esophageal cells, designated as EPC1 and EPC2, were established and had morphologic, cytogenetic, and biochemical properties of normal cells as illustrated by normal diploid status, expression of cytokeratins 5 and 14 found in basal cells, absence of p53 mutation, and the ability to be differentiated in a postconfluent state or with high calcium concentration (1.0–1.2 mM) (data not shown). These cells also reach senesence after 40–44 population doublings.

The cells were transduced with retroviral vectors containing either wild-type human EGFR or GFP. Western blotting indicated EGFR overexpression in EPC1 and EPC2 compared with parental EPC or cells transduced with GFP (Fig. 1). In fact, EGFR expression was comparable with EGFR expression found in A431 cells, a vulvar squamous cancer cell line, and a prototypic cancer cell line for EGFR investigation. ^{125}I-EGF binding experiments and Scatchard analysis were performed to determine EGFR number: EPC2-GFP, K_d = 0.9 nM, $r = 0.9 \times 10^6$/cell; EPC2-EGFR: K_d = 1.2 nM, $r = 3.4 \times 10^6$/cell. All subsequent experiments are depicted for EPC2 cells but were identical in EPC1 cells as well.

Functionally, phosphorylation levels of EGFR were also increased in EPC2-EGFR cells compared with parental cells and EPC2-GFP cells (Fig. 1). These results indicated that EGFR can be overexpressed in primary human esophageal epithelial cells with functional activation of the EGFR by EGF ligand.

EGFR Overexpression Results in the Activation of Key Downstream Targets—Extracts of cells serum-starved overnight and then stimulated with EGF ligand were analyzed by Western blot for the activation of downstream targets of EGFR. EGF stimulation induced increased phosphorylation of Akt-1 and STAT1 in EPC2-EGFR cells (Fig. 2) compared with control cells. Akt1 phosphorylation was induced at early time periods but sustained for a longer phase in EPC2-EGFR cells. STAT1 phosphorylation was dramatically increased in EPC2-EGFR cells 15 min after stimulation compared with EPC2-GFP cells. In addition, EGFR activation induced phosphorylation of extracellular signal-regulated kinase 1/2 (data not shown).

EGFR Induces Esophageal Epithelial Cell Hyperproliferation in Organotypic Cell Culture and Transgenic Mice—To analyze the behavior of GFP- and EGFR-transfected cells in a more physiologic environment, we established and examined organotypic cultures of these cells. Parental cells as well as GFP and EGFR-EPC2 cells were grown on a collagen gel containing human skin fibroblasts submerged in the tissue culture medium for 4 days followed by cultivation at the air-liquid interface for 7 days to induce terminal differentiation. These epithelial reconstructs were evaluated by histology, immunohistochemistry for EGFR and proliferating cell nuclear antigen, and the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling method for apoptosis. Compared with normal and GFP-transduced primary human esophageal epithelial cells, EGFR overexpression resulted in a thicker epithelium and basal cell hyperplasia (Fig. 3*A*). EGFR was expressed in the basal and suprabasal cell layers and showed not only membrane staining but also diffuse cytoplasmic staining, whereas reconstructs of parental cells showed only faint staining in the basal cells (Fig. 3*B*). Proliferating cell nuclear anti-

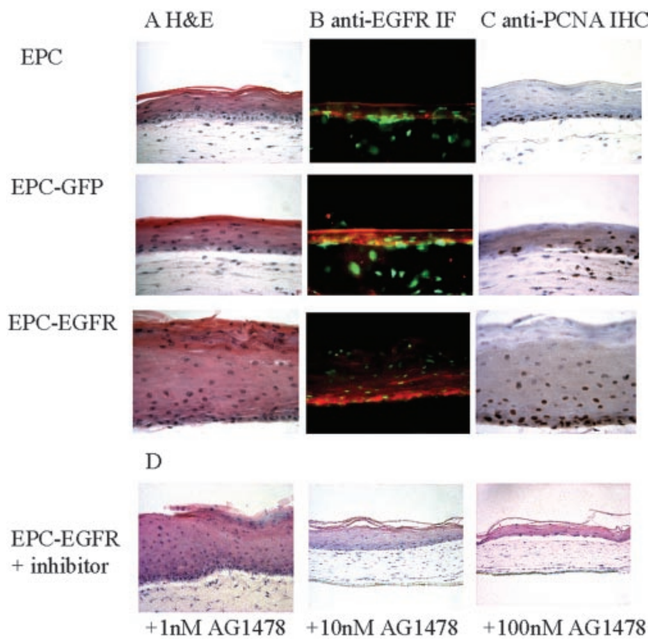


FIG. 3. **Organotypic cell culture demonstrates hyperplasia and hyperproliferation due to EGFR overexpression.** *A*, Hematoxylin and Eosin (*H&E*) staining of EPC2-, EPC2-GFP-, and EPC2-EGFR-overexpressing cells reveals hyperplasia as a function of EGFR overexpression (magnification, ×400). *B*, immunofluorescence staining (*IF*) reveals expression of EGFR in basal and suprabasal layers (EGFR-positive cells) in EPC2-EGFR cells, whereas EPC2 and EPC2-GFP cells only express EGFR in the basal layer (magnification, ×400). Staining in EPC2 and EPC2-GFP in the keratinized layer is nonspecific. *C*, the hyperplasia correlates with proliferating cell nuclear antigen (*PCNA*)-positive cells in the basal and suprabasal layers of EPC2-EGFR cells compared with only proliferating cell nuclear antigen-positive basal cells in EPC2 and EPC2-GFP as revealed by immunohistochemistry (*IHC*) (magnification, ×400). *D*, the addition of AG1478, an EGFR-specific tyrosine kinase inhibitor, leads to a dose-dependent decrease in the thickness of the epithelium in EPC2-EGFR cells.

gen staining revealed that EGFR-overexpressing cells were found in the basal and suprabasal cell layers and not restricted to the basal cell layer in parental and GFP-derived cells (Fig. 3*C*). Furthermore, there was no change in the scant apoptosis that was confined to the basal cell compartment between these control cells and EGFR-overexpressing cells (data not shown). By adding the EGFR tyrosine kinase inhibitor AG1478 to the medium of the organotypic culture, the hyperplasia induced by EGFR was dramatically diminished such that the epithelium was significantly thinner in a dose-dependent fashion (Fig. 3*D*).

In order to address the effects of EGFR overexpression *in vivo*, we generated transgenic mice in which the Epstein-Barr virus ED-L2 promoter was fused to the human EGFR cDNA. This promoter has been previously demonstrated to direct transgene expression specifically to the oral and esophageal squamous epithelia (10). Transgene expression in two different founder lines was confirmed by RT-PCR, Northern blotting, Western blotting, and immunohistochemistry (data not shown). Furthermore, there was increased tyrosine phosphorylation of the EGFR transgene product in transgenic mice when compared with age-matched littermates (data not shown). Compared with age-matched (6 months) littermate wild-type mice, EGFR transgenic mice revealed evidence of increased proliferation in the basal and suprabasal cells (Fig. 4), consistent with what was observed in organotypic culture.

EGFR Overexpression Increases Cell Migration and MMP-1 Expression Level—Activation of EGFR is associated with cell migration and invasion. To determine whether overexpression of EGFR in primary esophageal keratinocytes induces cell migration, we performed cell migration assays. Migration of

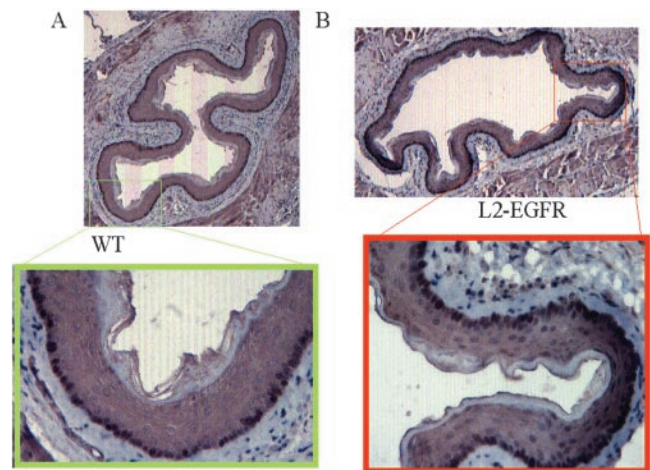


FIG. 4. **The esophageal epithelium of EGFR-overexpressing mice shows increased proliferation by Ki-67 immunohistochemistry.** *A*, Ki-67-positive cells are confined to the esophageal basal cell layer from wild-type mice (*WT*) (magnification, ×400). *B*, transgenic mice overexpressing EGFR under the control of the Epstein-Barr virus ED-L2 promoter (L2-EGFR) show increased proliferation in the esophageal basal and suprabasal layers based upon Ki-67 staining (magnification, ×400).

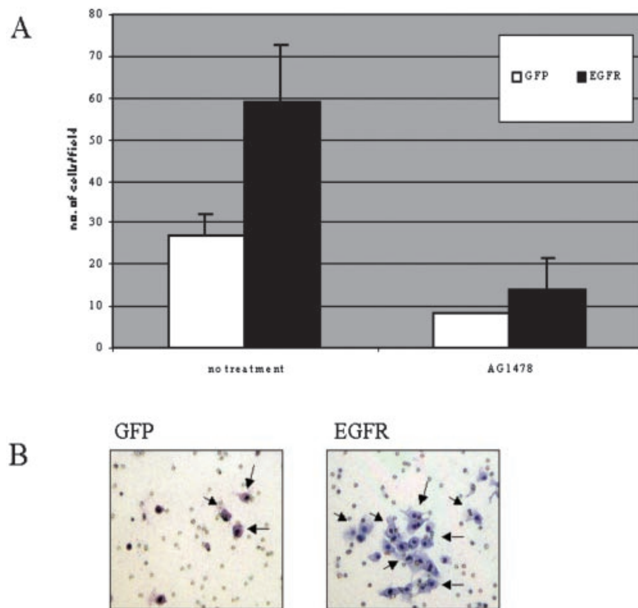
EPC2-EGFR cells was increased compared with control cells (Fig. 5). We could demonstrate that the observed enhanced migration is EGFR-dependent by use of the EGFR-specific inhibitor, AG1478 (Fig. 5).

In order to assess how EGFR mediates the increased cell migration, we determined whether matrix metalloproteinases may be up-regulated. Indeed, based upon RT-PCR data, we could show an up-regulation of MMP-1 in EPC2-EGFR cells. Levels for MMP-2 and MMP-9 were the same in both EPC2-GFP and EPC2-EGFR cells (Fig. 6*A*). After the addition of AG1478 to the cell culture medium, the up-regulation of MMP-1 was reversed (Fig. 6*B*). We confirmed the up-regulation of MMP-1 with SYBR Green real time PCR in EPC2-EGFR cells compared with EPC2-GFP cells, whereas MMP-2 and MMP-9 levels remained unchanged (data not shown).

Western blot analysis revealed increased MMP-1 in conditioned media of EPC2-EGFR cells compared with EPC2-GFP control cells. Furthermore, the AG1478 inhibitor abolished MMP-1 secretion (Fig. 6*B*).

EGFR Overexpression Increases E-cadherin-dependent Cell Aggregation—After staining of EPC2-GFP and EPC2-EGFR cells in migration assays, we observed clustering of EPC2-EGFR cells that was not present in EPC2-GFP cells (Fig. 5*B*). We investigated whether EGFR overexpression and stimulation with EGF have effects on cell-cell adhesion using a cell aggregation assay. EPC2-EGFR cells demonstrated stronger aggregation than EPC2-GFP cells without EGF stimulation (Fig. 7). After stimulation with EGF, cell aggregation is further enhanced, and the differences in aggregation between EPC2-GFP and EPC2-EGFR cells are accentuated. The addition of AG1478 nearly abolishes cell-cell aggregation, suggesting that aggregation is EGFR-dependent (Fig. 7). To prove that the observed aggregation is E-cadherin-dependent, we performed aggregation assays adding neutralizing E-cadherin antibody (DECMA-1) to the cells in suspension during the 1-h incubation period. Samples that were incubated in the presence of DECMA-1 were unable to aggregate (Fig. 7).

To examine whether EGFR overexpression induces changes in the assembly of adherens junctions and desmosomes, we performed immunoprecipitations and Western blot on cell extracts before and after EGF stimulation. Expression levels of components of adherens junctions and desmosomes (E-cadherin, β-cate-



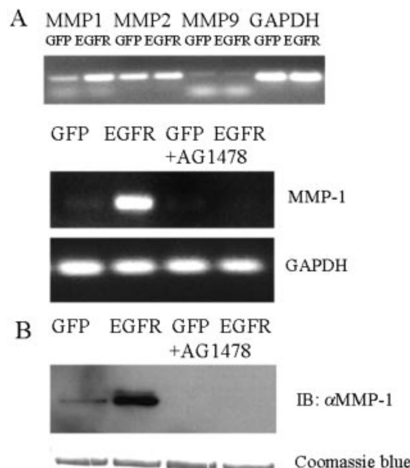


FIG. 5. **EGFR overexpression results in increased cell migration.** *A*, cell migration was measured in 8-μm pore size cell culture inserts and shows increased migration in EPC2-EGFR cells (*black bar*) compared with EPC2-GFP cells (*white bar*). Migration was inhibited by the addition of an EGFR-specific tyrosine kinase inhibitor, AG1478. *B*, staining of the cells migrated through the PET membrane shows single EPC2-GFP cells (*arrows*, *left panel*) compared with clusters of migrated EPC2-EGFR cells (*arrows*, *right panel*).

A MMP1 MMP2 MMP9 GAPDH
GFP EGFR GFP EGFR GFP EGFR GFP EGFR
GFP EGFR GFP EGFR +AG1478
MMP-1
GAPDH
B GFP EGFR GFP EGFR +AG1478
IB: αMMP-1
Coomassie blue

FIG. 6. **EGFR overexpression up-regulates MMP-1 mRNA and secreted protein levels.** *A*, RT-PCR reveals increased amplification of MMP-1 levels in EPC2-EGFR cells compared with EPC2-GFP cells, whereas levels for MMP-2 and MMP-9 were unchanged. The addition of EGFR inhibitor AG1478 reversed the up-regulation of MMP-1. *B*, Western blot of conditioned media revealed an increase of MMP-1 secretion in EPC2-EGFR cells compared with EPC2-GFP cells, which was inhibited by the addition of EGFR inhibitor AG1478 (Coomassie Blue stain of the gel serves as loading control).

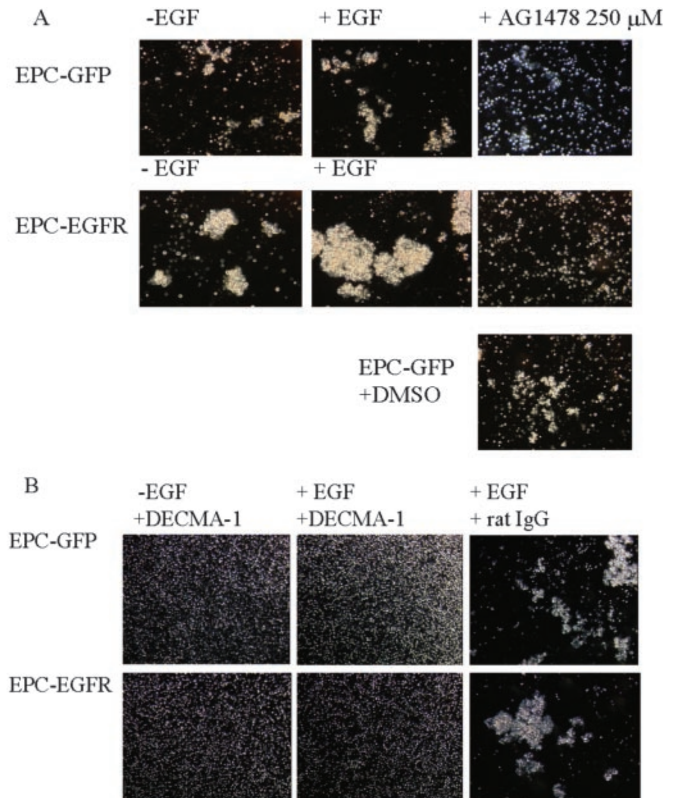


FIG. 7. **Stimulation of EGFR leads to increased cell-cell aggregation that is abolished by EGFR inhibitor AG1478.** *A*, cell aggregation assays show enhanced aggregation in EPC2-EGFR cells that is abolished by EGFR inhibitor AG1478. Cell aggregation assays were performed in six-well plates with capture and analysis after cells settled with the NIH image software. *B*, the addition of neutralizing anti-E-cadherin antibody abolishes aggregation, thereby demonstrating that aggregation is E-cadherin-dependent. Rat IgG serves as a control.

nin, α-catenin, plakoglobin, desmoplakin, and desmoglein) remained unchanged (data not shown). Furthermore, immunoprecipitations and Western blots of E-cadherin did not reveal changes in interaction with its cytoplasmic partners that link it to the cytoskeleton (data not shown). However (and importantly), analysis of its interaction with another member of the catenin family, p120, demonstrated that p120 had stronger interaction with E-cadherin in EPC2-EGFR cells, whereas p120 was predominantly in the cytosol in EPC2-GFP cells (Fig. 8).

Sequential detergent extraction followed by immunoprecipitation with an antibody against E-cadherin confirmed the strong interaction between E-cadherin and p120 in the Triton X-100 soluble pool (membrane fraction), whereas p120 but not E-cadherin could be immunoprecipitated from the saponin pool (cytoplasmic fraction) (data not shown).

p120 Translocation from Cytoplasm to the Membrane Correlates with Strong Adhesion—To determine the localization of p120 in organotypic culture (Fig. 8*C*) and monolayer cell culture (Fig. 8*D*), we performed immunofluorescence staining in EPC2-GFP and EPC2-EGFR cells. Confocal microscopy of organotypic culture shows that EGFR overexpression in the basal and suprabasal layers correlates with strong p120 staining. Merged scans demonstrate partial co-localization of EGFR with p120 at the cell membrane of EGFR-positive cells and co-localization of p120 with E-cadherin (Fig. 8*C*), whereas controls stained with antibody against desmoplakin, a component of the desmosome, did not show co-localization with p120. In order to analyze the localization of p120 in more detail, EPC2-GFP and EPC2-EGFR cells were stained with an antibody against p120, which demonstrates there is a large cytoplasmic pool of p120 in EPC2-GFP cells. In EPC2-EGFR cells, the distribution of p120 is shifted toward the cell membrane with little cytoplasmic staining (Fig. 8*D*). Furthermore, EPC2-EGFR cells display more sites of cell-cell contact in culture that stain positive for p120 than EPC2-GFP cells. In summary, EGFR induces a shift in the distribution of p120 from the cytoplasm to the cell membrane that coincides with a co-localization with E-cadherin and co-distribution of EGFR and p120 in organotypic culture. Interaction of p120 with E-cadherin is believed to promote adhesion and helps to explain the stronger aggregation we observe in EPC2-EGFR cells.

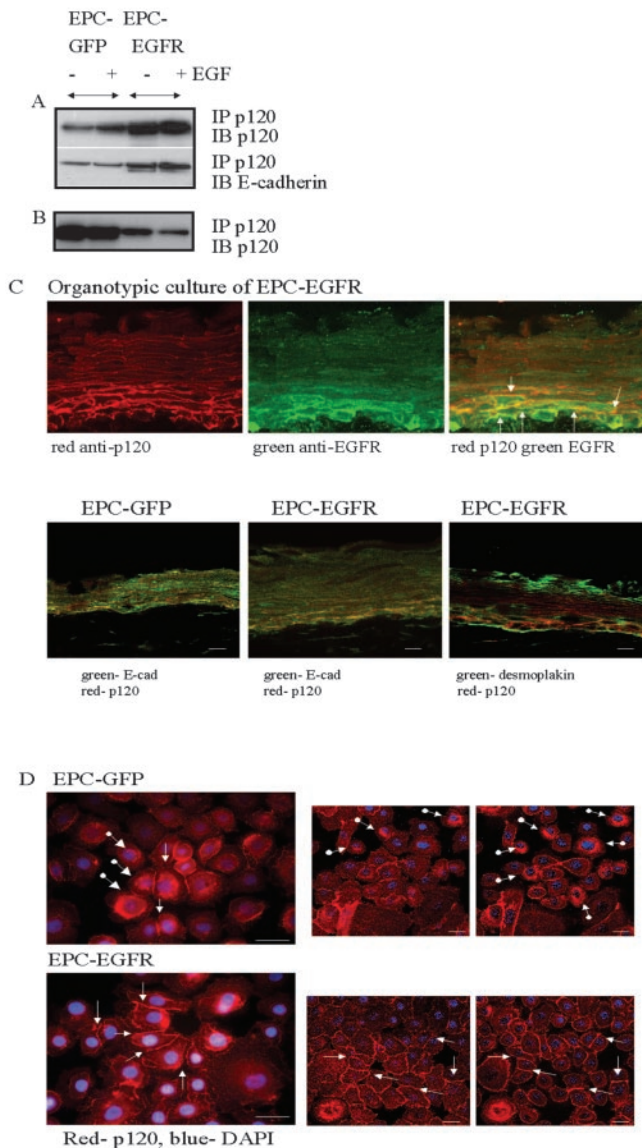


FIG. 8. **EGFR overexpression induces a shift in p120 from the cytoplasm to the membrane.** *A*, immunoprecipitation of p120 shows increased p120 levels in EPC2-EGFR and interaction of p120 with membrane-bound E-cadherin. *IB*, immunoblotting; *IP*, immunoprecipitation. *B*, immunoprecipitation using anti-p120 antibody after cell lysis under less stringent conditions shows less cytoplasmic p120 in EGFR-overexpressing cells than in GFP-expressing cells. *C*, confocal microscopy of organotypic culture of EGFR-overexpressing EPC2 cells demonstrates strong membranous staining of p120 (*red*) in cells that overexpress EGFR (*green*). The merged picture shows partial co-localization of p120 and EGFR (*yellow-white arrows*, *far right upper panel*). p120 localizes to the adherens junction as demonstrated in double staining with E-cadherin (*green*) and p120 (*red*), whereas the control experiment with desmoplakin reveals that localization of p120 to adherens junction can be distinguished from the desmosomal junction (*far right lower panel*) (magnification, ×60). *D*, immunofluorescence staining using p120 antibody shows a shift in the localization of p120 from cytoplasm (*round-stemmed arrows*) to membrane (*arrows*) in EGFR-overexpressing cells compared with GFP-expressing cells. EGFR-overexpressing cells adhere closer and provide more cell-cell contact (*arrows*) sites than GFP-expressing cells. Confocal microscopy (*four smaller pictures* to the *right*) allows one to follow localization of p120 from top to the bottom of the cells. Cytoplasmic localization in EPC2-GFP is marked by *round-stemmed arrows*, and localization in adherens junctions is marked with *arrows*.

DISCUSSION

EGFR-mediated activation of diverse signal transduction pathways results in protean cellular manifestations (1–3). Furthermore, EGFR overexpression is important in the premalignant stages of carcinogenesis. In order to address the biological roles of EGFR-mediated effects in physiological settings, we have developed and characterized primary human esophageal squamous epithelial cells that can recapitulate the stratified squamous epithelium in organotypic cell culture. In addition, we generated and characterized transgenic mice where EGFR is specifically targeted to the esophageal squamous epithelium. Notably, EGFR overexpression induces a hyperproliferative state, whereby proliferating basal cells persist into the normally differentiated suprabasal compartment, perhaps reflecting a combination of enhanced cell migration and cell aggregation. Furthermore, EGFR transgenic mouse-derived esophageal epithelial cells also reveal evidence of increased cell proliferation in the basal and suprabasal compartments.

Cell migration is a highly coordinated process involving the precise regulation of cell adhesion to and dissociation from extracellular matrix (ECM) proteins (12). In our experimental systems, EGFR overexpression and activation result in increased migration of human and mouse esophageal epithelial cells. This is reversed by use of an inhibitor of EGFR tyrosine kinase, indicating a direct effect of EGFR upon cell migration. Our results are consistent with the importance of EGFR-mediated cell migration in developmental and spatial paradigms. EGFR can substitute for fibroblast growth factor in modulating cell migration in *Drosophila* (13). It has also been observed that autocrine EGF signaling stimulates directionally persistent mammary epithelial cell migration (14).

EGFR-mediated Cell Migration and MMP-1—Many proteinases are capable of degrading ECM components, but one family of enzymes that appears to be particularly important for matrix degradation is the MMPs. Currently, the MMPs comprise a large family of over 20 secreted or transmembrane proteins that together can degrade all known components of the ECM and basement membrane (15, 16). MMP family members share functional and structural characteristics and can be categorized into the collagenase, gelatinase, stromelysin, and membrane-type MMP subfamilies. We find that EGFR-mediated cell migration appears to be coordinated specifically with increased secretion of MMP-1. Egfr−/− mice show low MMP expression in early lung development (17). Stimulation with EGF could up-regulate MMP in wild-type mice but not so in Egfr−/− mice, which suggests a direct link between EGFR and MMPs. MMP-9 has been shown to be up-regulated in ovarian cancer cell lines that harbor EGFR overexpression (18), and this may be mediated through phosphatidylinositol 3-kinase activity (19). Additionally, MMP-9 is up-regulated in head and neck squamous carcinoma cell lines with EGFR amplification (20).

Originally, MMPs were considered to be most important almost exclusively in invasion and metastasis. However, recent studies document that MMPs are involved in several steps of cancer development. MMPs can regulate cell growth in different ways (*e.g.* the release of membrane-bound growth factors like tumor growth factor-α) (21). ECM-bound growth factors also become more easily available after ECM degradation (22, 23). During metastasis, cancer cells must cross several ECM barriers. They cross the epithelial basement membrane, invade the surrounding stroma, and enter blood vessels or lymphatics. One of the first steps in invasion is migration.

Up-regulation of MMP-1 in malignant tumors compared with normal tissue has been described for cancer cells, stromal cells, and fibroblast in squamous carcinogenesis, but these observations have been only correlative in nature (24–28). Our findings support a direct and novel role of EGFR signaling and MMP-1 transcriptional regulation.

E-cadherin and p120 Modulate EGFR Effects upon Cell Adhesion—Through the promotion of increased migration, EGFR is thought to decrease cell adhesion by way of its interaction with β-catenin, which after tyrosine phosphorylation can no longer mediate the connection of the cadherin-catenin complex with the actin-cytoskeleton (29). Whereas we confirmed binding of EGFR to β-catenin, it did not result in changes of the phosphorylation status of β-catenin, since β-catenin is phosphorylated even in EPC2-GFP cells. It also has been reported that EGFR-mediated phosphorylation of plakoglobin after EGF stimulation leads to a loss of adhesion (30). We could not confirm this in our experimental conditions. By contrast, our results show that EGFR overexpression and activation do not induce changes in the assembly of adherens junctions and desmosomes but that EGFR mediates increased cell aggregation through relocalization of p120, a component of adherens junctions.

E-cadherin associates with p120, but this is not necessary for its link to the cytoskeleton (31), although it is important for cell adhesion and may serve as a scaffold for recruitment of other small RhoGTPases (32, 33). Furthermore, p120 appears to be an important regulator of adhesion through its interactions with RhoGTPase family members that modulate transitions between migration and adhesion (34–36). We find that the p120 cytosolic pool is decreased in EGFR-overexpressing esophageal epithelial cells, which may contribute to the increased cell aggregation by virtue of the fact that more p120 binds E-cadherin. Functionally, the cell aggregation assay is supportive of this notion. Furthermore, our confocal microscopy data show that p120 has predominantly a cytoplasmic distribution in EPC2-GFP cells, which shifts to a membranous localization in EPC2-EGFR cells. Furthermore, the localization of p120 in organotypic culture correlates with EGFR overexpression.

The clustering potential of E-cadherin is attributable to the juxtamembrane domain, which seems to be activated by the homophilic interaction between cadherin extracellular domains (37). Mutational analysis of the juxtamembrane domain has demonstrated the importance of this region for the regulation of adhesion mediated by p120 (38) as uncoupling of E-cadherin from p120 results in weaker adhesion.

In summary, EGFR overexpression leads to a hyperproliferative state with induction of enhanced cell migration. This permits cells in the basal compartment to migrate to the suprabasal compartment. The migration may be related to an increased secretion of MMP-1. We believe that there is activation of cell-cell aggregation once cells migrate to the suprabasal compartment. This appears to be modulated by the availability of p120 to bind E-cadherin, thus permitting a homeostatic balance between proliferation in the basal compartment and differentiation in the suprabasal compartment. Our studies allow for novel insights into EGFR-mediated effects on cellular processes in the normal esophageal epithelium and may help to explain the initiating events in squamous carcinogenesis.

REFERENCES

1. Blume-Jensen, P., and Hunter, T. (2001) *Nature* **411,** 355–365
2. Wells, A. (1999) *Int. J. Biol. Cell Biol.* **31,** 637–643
3. Schlessinger, J. (2000) *Cell* **103,** 193–200
4. Okano, J., Gaslightwala, I., Birnbaum, M. J., Rustgi, A. K., and Nakagawa, H. (2000) *J. Biol. Chem.* **275,** 30934–30942
5. Itakura, Y., Sasano, H., Shiga, C., Furukawa, Y., Shiga, K., Mori, S., and Nagura, H. (1994) *Cancer* **74,** 795–804
6. Kitagawa, Y., Ueda, M., Ando, N., Ozawa, S., Shimizu, N., and Kitajima, M. (1996) *Clin. Cancer Res.* **2,** 909–914
7. Mandard, A. M., Hainaut, P., and Hollstein, M. (2000) *Mutat. Res.* **62,** 343–353
8. Shimada, Y., Imamura, M., Watanabe, G., Uchida, S., Harada, H., Makino, T., and Kano, M. (1999) *Br. J. Cancer* **80,** 1281–1288
9. Mohler, W. A., Charlton, C. A., and Blau, H. M. (1996) *BioTechniques* **21,** 260–262, 264–266
10. Nakagawa, H., Wang, T. C., Odze, R., Zukerberg, L., Togawa, K., May, G. H., Wilson, J., and Rustgi, A. K. (1997) *Oncogene* **14,** 1185–1190
11. Hanakawa, Y., Amagai, M., Shirakata, Y., Sayama, K., and Hashimoto, K. (2000) *J. Cell Sci.* **113,** 1803–1811
12. Lauffenburger, D. A., and Horwitz, A. F. (1996) *Cell* **84,** 359–369
13. Dossenbach, C., Rock, S., and Affolter, M. (2001) *Development* **128,** 4563–4572
14. Maheshwari, G., Wiley, H. S., and Lauffenburger, D. A. (2001) *J. Cell Biol.* **155,** 1123–1128
15. Massova, I., Kotra, L. P., Fridman, R., and Mobashery, S. (1998) *FASEB J.* **12,** 1075–1095
16. Stetler-Stevenson, W. G., Hewitt, R., and Corcoran, M. (1996) *Semin. Cancer Biol.* **7,** 147–154
17. Kheradmand, F., Rishi, K., and Werb, Z. (2002) *J. Cell Sci.* **115,** 839–848
18. Alper, O., De Santis, M. L., Stromberg, K., Hacker, N. F., Cho-Chung, Y. S., and Salomon, D. S. (2000) *Int. J. Cancer* **88,** 566–574
19. Ellerbroek, S. M., Halbleib, J. M., Benavidez, M., Warmka, K., Wattenberg, E. V., Stack, M. S., and Hudson, L. G. (2001) *Cancer Res.* **61,** 1855–1861
20. O-charoenrat, P., Modjtahedi, H., Rhys-Evans, P., Court, W. J., Box, G. M., and Eccles, S. A. (2000) *Cancer Res.* **60,** 1121–1128
21. Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Castner, B. J., Johnson, R. S., Fitzner, J. N., Boyce, R. W., Nelson, N., Kozlosky, C. J., Wolfson, M. F., Rauch, C. T., Cerretti, D. P., Paxton, R. J., March, C. J., and Black, R. A. (1998) *Science* **282,** 1281–1284
22. Manes, S., Mira, E., Barbacid, M. M., Cipres, A., Fernandez-Resa, P., Buesa, J. M., Merida, I., Aracil, M., Marquez, G., and Martinez-A, C. (1997) *J. Biol. Chem.* **272,** 25706–25712
23. Manes, S., Llorente, M., Lacalle, R. A., Gomez-Mouton, C., Kremer, L., Mira, E., and Martinez-A, C. (1999) *J. Biol. Chem.* **274,** 6935–6945
24. Charous, S. J., Stricklin, G. P., Nanney, L. B., Netterville, J. L., and Burkey, B. B. (1997) *Ann. Otol. Rhino. Laryngol.* **106,** 271–278
25. Polette, M., Clavel, C., Cockett, M., Girod de Bentzmann, S., Murphy, G., and Birembaut, P. (1991) *Invasion Metastasis* **11,** 76–83
26. Muller, D., Wolf, C., Abecassis, J., Millon, R., Engelmann, A., Bronner, G., Rouyer, N., Rio, M. C., Eber, M., and Methlin, G., Chambon, P., and Basset, P. (1993) *Cancer Res.* **53,** 165–169
27. Gray, S. T., Wilkins, R. J., and Yun, K. (1992) *Am. J. Pathol.* **141,** 301–306
28. Pickett, K. L., Harber, G. J., DeCarlo, A. A., Louis, P., Shaneyfelt, S., Windsor, L. J., and Bodden, M. K. (1999) *J. Dent. Res.* **78,** 1354–1361
29. Hoschuetzky, H., Aberle, H., and Kemler, R. (1994) *J. Cell Biol.* **127,** 1375–1380
30. Gaudry, C. A., Palka, H. L., Dusek, R. L., Huen, A. C, Khandekar, M. J., Hudson, L. G., and Green, K. J. (2001) *J. Biol. Chem.* **276,** 24871–24880
31. Daniel, J. M., and Reynolds, A. B. (1995) *Mol. Cell. Biol.* **15,** 4819–4824
32. Fukata, M., and Kaibuchi, K. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 887–897
33. Nakagawa, M., Fukata, M., Yamaga, M., Itoh, N., and Kaibuchi, K. (2001) *J. Cell Sci.* **114,** 1829–1838
34. Anastasiadis, P. Z., and Reynolds, A. B. (2001) *Curr. Opin. Cell Biol.* **13,** 604–610
35. Braga, V. M., Machesky, L. M., Hall, A., and Hotchin, N. A. (1997) *J. Cell Biol.* **137,** 1421–1431
36. Evers, E. E., Zondag, G. C., Malliri, A., Price, L. S., ten Klooster, J. P., van der Kammen, R. A., and Collard, J. G. (2000) *Eur J. Cancer* **36,** 1269–1274
37. Yap, A. S., Niessen, C. M., and Gumbiner, B. M. (1998) *J. Cell Biol.* **141,** 779–789
38. Thoreson, M. A., Anastasiadis, P. Z., Daniel, J. M., Ireton, R. C., Wheelock, M. J., Johnson, K. R., Hummingbird, D. K., and Reynolds, A. B. (2000) *J. Cell Biol.* **148,** 189–202