Epidermal growth factor receptor (EGFR) expression 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 relocation 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. 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) 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-
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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 Hank's 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 trypsinized (Invitrogen). The reaction was stopped with soybean trypsin inhibitor (Sigma) and centrifuged. The pelleted was resuspended in keratinocyte-SFM medium (KSF) (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 gentamicyn, and 100 units/ml nystatin (Invitrogen). EPC cells were grown at 37 °C and 5% CO2 with KSF, 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 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 KSF 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 on 24-well plates (1.0×10^4 cells/well) and grown in KSF 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—pBsd-neo retroviral vectors (Stratagene) were used to infect EPC1 and EPC2. In addition, we subcloned into the pBsd-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 Millipore) media and grown in KSF 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.

In Vivo Experimental Techniques—To generate EGFR transgenic mice, the cDNA encoding a functional (i.e. nonphosphorylated) EGFR was isolated by RNA ligase-mediated polymerase chain reaction using primers specific for the transmembrane and intracellular domains of the human EGFR. Full-length cDNA was inserted into the pFB-neo retroviral vectors (Stratagene). pFB-neo-retroviral vectors were packaged by a three-plasmid system using Phoenix-Ampho cells. Cells were infected with filtered (0.45-μm Millipore) media and grown in KSF 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.

EGFR transgenic mice and cell lines of mouse esophageal keratinocytes. We employed the Epstein-Barr virus ED-L2 provirus to transduce the human EGFR cDNA to create a transgene which contains 0.1% unchelated newborn calf serum. Then cells were infected with filtered (0.45-μm Millipore) media and grown in KSF 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.

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 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 10 μM Na3VO4, 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% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM sodium orthovanadate, and 1 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 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 1 protease inhibitor mixture tablet (Roche Molecular Biochemicals)). Membranes were probed with primary antibody diluted 1:1000 in 5% bovine serum albumin and 1% sodium dodecyl sulfate buffer containing dithiothreitol for 1 h. Membranes were washed three times with 1 ml of TBST, washed with anti-mouse or anti-rabbit horseradish peroxidase antibody diluted 1:2000 in 5% bovine serum albumin and 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. Immunohistochemistry and immunofluorescence were performed on paraffin sections which were deparaffinized in xylene and rehydrated 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 exposed to Eastman Kodak Co. X-Omat LS film.

Objects were washed in PBS and blocked with 1% bovine serum albumin for 1 h. Incubated with primary antibody 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, Eugene, OR) or fluorescein isothiocyanate-conjugated secondary antibody (Roche Molecular Biochemicals) for 1 h.
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![Image](image.jpg)

**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.

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 TTA CAG GGA ATA; 3′-CTT CCA AAA GAG GAT CAT CCA), MMP-2 (5′-AGG CCA CAA TGT CCA CTG TT; 3′-TGG TGC CAG ACC ACA TCT TT), GAPDH (5′-CAC CCA TTO 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 Thermoscientific (QIAgen).

**Western blotting** and **SYBR green Real Time PCR—**Quantification of specific mRNA expression levels in EPC2, EPC2-GFP, and EPC2-EGFR cells. Phospho-EGFR levels are increased in EPC2-EGFR cells compared with parental cells 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).

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 EGFR 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. EGFR 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. 3A). 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. 3B). Proliferating cell nuclear anti-
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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 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. 6A). After the addition of AG1478 to the cell culture medium, the up-regulation of MMP-1 was reversed (Fig. 6B). 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. 6B).

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-GFP and EPC2-EGFR cells that was not present in EPC2-GFP cells (Fig. 5B). 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 (DECA-M1) to the cells in suspension during the 1-h incubation period. Samples that were incubated in the presence of DECA-M1 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-
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).

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**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).

**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.
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**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 in EGFR overexpressing cells (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 pre-malignant 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 mouse 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 EGFR signaling stimulates directionally persistent mammary epithelial cell migration (14).

**EGFR-mediated Cell Migration and MMP-1**—Many proteases 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 EGFR 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 E2C2-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 homoeostatic 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.

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