Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of β-catenin, and enhanced tumor cell invasion

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Summary

EGF receptor (EGFR) overexpression correlates with metastasis in a variety of carcinomas, but the underlying mechanisms are poorly understood. We demonstrated that EGF disrupted cell-cell adhesion and caused epithelial-to-mesenchymal transition (EMT) in human tumor cells overexpressing EGFR, and also induced caveolin-dependent endocytosis of E-cadherin, a cell-cell adhesion protein. Chronic EGF treatment resulted in transcriptional downregulation of caveolin-1 and induction of the transcriptional repressor Snail, correlating with downregulation of E-cadherin expression. Caveolin-1 downregulation enhanced β-catenin-TCF/LEF-1 transcriptional activity in a GSK-3β-independent manner. Antisense RNA-mediated reduction of caveolin-1 expression in EGFR-overexpressing tumor cells recapitulated these EGF-induced effects and enhanced invasion into collagen gels. We propose that EGF-induced negative regulation of caveolin-1 plays a central role in the complex cellular changes leading to metastasis.

Introduction

Epidermal growth factor (EGF) receptor overexpression has been reported in many human tumors, including lung, colon, breast, prostate, brain, head and neck, thyroid, ovarian, bladder, gliomas, and renal carcinoma (Berger et al., 1987; Gullick, 1991; Lemoine et al., 1991; Libermann et al., 1985; Salomon et al., 1995; Tillotson and Rose, 1991). EGF receptor (EGFR) overexpression correlates with poor clinical prognosis in tumors and is often accompanied by production of TGF-α or other EGF family ligands (Voldborg et al., 1997). Autocrine regulation through EGFR by such ligands contributes to tumor progression.

EGF promotes tumor cell motility and invasion (Price et al., 1996; Rosen and Goldberg, 1989; Shibata et al., 1996). Recently, we showed that EGF-induced dephosphorylation and inactivation of FAK leads to cell detachment, increased motility, and invasion, followed by integrin-dependent FAK reactivation during re-adhesion (Lu et al., 2001b). This dynamic regulation of FAK activity plays a role in EGF-associated tumor invasion and metastasis (Lu et al., 2001b). Tumor cell migration and metastasis is a highly coordinated process involving not only alteration of cell adhesion to extracellular matrix (ECM) proteins, but also the disruption of cell-cell junctions. The stimulation of epithelial cells with growth factors, such as hepatocyte growth factor/scatter factor (HGF/SF) (Muller et al., 2002; Savagner et al., 1997; Weidner et al., 1990), FGF (Shtutman et al., 1999; Valles et al., 1990), EGF (Blay and Brown, 1985; Geimer and Bade, 1991; Muller et al., 2002), and TGF-β (Miettinen et al., 1994), induces break-up of cell-cell junctions. The disruption of cell-cell junctions facilitates EMT and tumor cell migration (Birchmeier et al., 1995; Hay and Zuk, 1995). EMT can result from multiple extracellular stimuli; for instance, a synergistic effect on EMT has been observed with combined stimulation of EGF and TGF-β (Grande et al., 2002).

A major component of the epithelial adherens junction is E-cadherin. E-cadherin is a member of a family of functionally related transmembrane glycoproteins that mediate Ca^{2+}-dependent cell-cell adhesion via homophilic interaction with adjacent cells through their N-terminal ectodomains. The cytoplasmic C-terminal domain of E-cadherin directly interacts with β/γ-catenin (or plakoglobin). β-catenin binds α-catenin, which in turn binds to vinculin, α-actinin, ZO-1, and F-actin, and thus links E-cadherin to the actin cytoskeleton (Nagafuchi, 2001;

SIGNIFICANCE

EGF-induced signaling, downregulation of E-cadherin, and increased β-catenin have been reported separately to correlate with the invasive stage of tumor development. However, the exact relationship between them is not clear. We present data showing that endocytosis of E-cadherin and downregulation of caveolin-1 itself induced by EGF signaling result in depletion of E-cadherin protein, disruption of cell-cell contacts, and GSK-3β-independent β-catenin-TCF/LEF-1 transactivation. All these events contribute to invasive tumor development. This interplay between EGF and Wnt/Wingless signaling components provides an important mechanism for understanding tumor cell invasion and metastasis.

Rimm et al., 1995; Vasioukhin and Fuchs, 2001). Regulation of E-cadherin-mediated cell-cell adhesion by different mechanisms alters the dynamics of cell-cell junction formation and maintenance (Gumbiner, 2000). For instance, E-cadherin internalization in response to HGF is accompanied by the disruption of cell-cell adhesion and scattering of cells (Kamei et al., 1999). The critical role of E-cadherin-mediated cell-cell adhesion in metastasis is exemplified by the fact that the development of invasive tumors often correlates with the downregulation of E-cadherin expression (Berger et al., 1987). Moreover, inactivating mutations in the E-cadherin gene, consisting of splice site mutations and truncation mutations caused by insertions, deletions, and nonsense mutations, have been found in gastric, colon, and breast cancer (Berx et al., 1998; Efstathiou et al., 1999; Guilford et al., 1998; Salahshor et al., 2001). Loss of E-cadherin expression has been demonstrated as a causal factor for tumor progression in a transgenic mouse model of pancreatic β-cell carcinoma (Perl et al., 1998). Restored expression of E-cadherin enhances intercellular adhesion, inhibits tumorigenicity, and suppresses the invasiveness of epithelial tumor cells (Efstathiou et al., 1999; Frixen et al., 1991; Vleminckx et al., 1991).

Other components of the adherens junction, like α- and β-catenins, also play an important role in tumor progression. Loss of α-catenin compromises intercellular adhesion and results in hyperproliferation of epidermal cells (Vasioukhin et al., 2001). In addition to its role in cell-cell adherens junctions, β-catenin is also a key component of the Wnt/Wingless signal transduction cascade. Wnt signaling plays a central role in development, cell proliferation, and differentiation (Cox et al., 1996; Huelsken et al., 2000; Wodarz and Nusse, 1998). In the absence of a Wnt/Wingless signal, cytoplasmic β-catenin interacts with axin/conductin, glycogen synthase kinase-3β (GSK-3β), and the adenomatous polyposis coli protein (APC), which competes with E-cadherin for binding to the armadillo-like (ARM) repeats of β-catenin (Hulsken et al., 1994). β-catenin is phosphorylated in its N-terminal domain by GSK-3β, which leads to its degradation mediated by the SCF/ubiquitin/proteasome pathway (Aberle et al., 1997; Behrens et al., 1998; Ikeda et al., 1998; Orford et al., 1999; Rubinfeld et al., 1996; Yost et al., 1996). Activation of the Wnt/Wingless pathway inhibits GSK-3β-dependent phosphorylation of β-catenin. Stabilized, hypophosphorylated β-catenin translocates to the nucleus, where it interacts with transcription factors of the TCF/LEF-1 family, leading to increased expression of genes, such as c-*myc* and cyclin D1 (He et al., 1998; Shtutman et al., 1999; Tetsu and McCormick, 1999). HGF and EGF induce β-catenin signaling under conditions where they stimulate cell motility (Muller et al., 2002). Gain-of-function mutations in β-catenin, including N-terminal mutations that lead to stabilization of the protein, or loss-of-function mutations in proteins that participate in the regulated turnover of β-catenin, such as the tumor suppressor APC, all result in upregulation of β-catenin protein levels, thus increasing its transcriptional activity. Transgenic mice expressing an activated β-catenin under control of the keratin 14 promoter are predisposed to developing skin tumors resembling pilomatricomas (Gat et al., 1998). More than 75% of human pilomatricomas possess β-catenin-stabilizing mutations in *CTNNB1* (which encodes β-catenin) (Chan et al., 1999).

Increased β-catenin-TCF/LEF-1 transactivation, resulting from mutations in APC, axin, or β-catenin, has been also observed in several other types of cancers, including colon cancer, melanoma, and prostate cancer (Morin, 1999; Peifer and Polakis, 2000). However, the dynamic regulation of β-catenin protein levels by exchange of molecules between the spatially separated pools that function in cell-cell adhesion and in transactivation in the context of tumor cell invasion and metastasis is less well understood (Papkoff, 1997). Disruption of adherens junctions releases β-catenin from the adherens junction complex, thus making it available for nuclear import and increased transactivation of β-catenin-responsive genes. In colorectal tumors, dissociated cells at the invasive front that have lost E-cadherin expression show nuclearly localized β-catenin (Brabletz et al., 2001). c-Fos and IGF-2 induce nuclear translocation and transactivation of β-catenin during EMT (Eger et al., 2000; Morali et al., 2001), whereas Vitamin D_3 promotes differentiation of colon carcinoma cells by induction of E-cadherin expression and the translocation of β-catenin to the plasma membrane (Palmer et al., 2001). Paradoxically, the regulation of β-catenin transcriptional activity by E-cadherin appears to be independent of the cell-cell adhesion domain of E-cadherin (Gottardi et al., 2001) and may function in a cell adhesion-independent manner (Gottardi et al., 2001; Stockinger et al., 2001).

Although cadherins and catenins constitute the major structural components of cell adherens junctions, the ultrastructure of the plasma membrane itself is critical for the organization of adherens junctions. Discrete microdomains in the plasma membrane, such as caveolae and lipid rafts, serve to target, traffic, and/or anchor proteins to their specific subcellular sites of action. Caveolae, 50–100 nm protein-coated invaginations of the plasma membrane, play an important role in endocytosis and signal transduction (Anderson, 1998; Galbiati et al., 2001). Caveolin-1, a principal component of caveolar membrane coat, is expressed as α and β isoforms, which have overlapping but slightly different distributions in mammalian cells (Scherer et al., 1995). EGFR, platelet-derived growth factor receptor (PDGFR), Neu/ErbB2, Ha-Ras, c-Src, endothelial nitric oxide synthase (eNOS), and phosphatidylinositol 3′-kinase are known to localize in caveolae and are negatively regulated through their interaction with the caveolin-1 scaffolding domain (Garcia-Cardena et al., 1997; Okamoto et al., 1998). Caveolin-1 mRNA and protein expression levels are both downregulated during cell transformation by oncogenic Ha-Ras, v-Abl, Myc, Neu, and other oncoproteins (Koleske et al., 1995; Okamoto et al., 1998). Moreover, reduced caveolin-1 protein levels are also observed in a number of human cancers (Bagnoli et al., 2000; Bender et al., 2000; Racine et al., 1999; Razani et al., 2001b; Wiechen et al., 2001), suggesting a negatively regulatory role for caveolin-1 in tumor development. Consistent with these observations, the proliferation rate of caveolin-1-deficient cells is significantly increased (Drab et al., 2001; Razani et al., 2001a).

In this report, we extend our studies on the mechanisms of EGF-induced tumor cell invasion and metastasis and examine the effect of EGFR activation on interactions between caveolin-1 and cell-cell adhesion components. We demonstrate that EGF treatment leads to negative regulation of caveolin-1, which results in downregulation of E-cadherin and disassembly of cell-cell contacts, increased transcriptional activity of β-catenin, and enhanced invasion by tumor cells.

Results

EGF treatment results in disruption of cell-cell contacts, EMT, and caveolae-dependent endocytosis of E-cadherin in tumor cells that overexpress EGFR

The development of invasive tumors often correlates with the downregulation of E-cadherin, and EGFR overexpression is frequently associated with poor clinical prognosis. Therefore, we investigated the relationship between E-cadherin-mediated cell-cell contacts and EGF-dependent signaling mechanisms in A431 human epidermoid carcinoma cells, which overexpress EGFR. Cells were treated with EGF (100 ng/ml) for different periods of time. After short periods of EGF treatment (30 min), these cells exhibited a rounded and refractile morphology accompanied by alteration of cell-cell contacts. One day after EGF treatment, the cells began to grow on top of each other and became elongated and spindle-shaped, resembling the morphology of mesenchymal cells (Figure 1A, left panel). To investigate if the EGF-induced disruption of cell-cell contacts and EMT is restricted to A431 cells or represents a more generalized response of EGFR-overexpressing tumor cell lines, A549 human non-small lung cancer cells and DU145 human prostate cancer cells, which also overexpress EGFR, were treated with EGF for 5 days. These cells also exhibited phenotypic changes upon EGF treatment (Figure 1A, middle panels), similar to those observed for the A431 cells. Consistent with EMT, we observed an increase in expression of vimentin, which is a marker of mesenchymal cells (Tsarfaty et al., 1994), along with a concomitant decrease in expression of an epithelial protein cytokeratin in DU145 cells after EGF treatment for 5 days (Figure 1A, right panel).

Since treatment of A431 cells with EGF resulted in disruption of cell-cell contacts, we investigated the effect of EGF treatment on cellular E-cadherin localization. E-cadherin was concentrated near the apical surface at cell-cell contacts, indicative of the existence of E-cadherin-mediated interactions in these cells, although these cells do not form true tight junctions, as demonstrated by the absence of ZO-1 in cell-cell contacts (data not shown). Immunofluorescence staining experiments revealed that 2 hr of EGF treatment resulted in internalization of E-cadherin protein from the cell-cell contacts in A431 cells (Figure 1B), suggesting that E-cadherin is endocytosed following EGF treatment. Similar results were obtained with human A549 lung cancer and rat NBT-II bladder carcinoma cells (Figure 1B). Furthermore, EGF-induced internalized E-cadherin colocalized with the Golgi marker β-COP, a subunit of the COP1 endoplasmic reticulum (ER)-Golgi apparatus transport system (Marsh and McMahon, 1999). This indicates that E-cadherin translocates to a compartment that partially overlaps with the Golgi upon EGF treatment.

To investigate the mechanism of EGF-induced E-cadherin internalization, GFP-tagged Eps15 Δ95-295, which lacks the second and third EH (Eps15 Homology) domains, was transiently transfected into A431 cells. Eps15 associates with components of clathrin-mediated endocytotic machinery, such as Epsin, through its EH domains, and plays a role in assembly of clathrin-coated pits (CCP) (Marsh and McMahon, 1999). The Eps15 Δ95-295 mutant lacking EH domains inhibits CCP assembly (Benmerah et al., 1999, 2000) and inhibited clathrin-dependent endocytosis of transferrin in A431 cells (Figure 1D). However, the expression of Eps15 Δ95-295 did not prevent EGF-induced internalization of E-cadherin (Figure 1D). Thus, EGF-induced loss of E-cadherin from cell-cell contacts is not mediated through a clathrin-dependent endocytotic mechanism.

Caveolar uptake provides an alternative internalization mechanism to clathrin-mediated endocytosis, and caveolae have been shown to be involved in the cellular transport of albumin (Razani et al., 2001a), pathogens (Pelkmans et al., 2001), cholesterol (Smart et al., 1996), endothelin receptor type 1 (Okamoto et al., 2000), and growth hormone (Lobie et al., 1999) into cells. In addition, EGF triggers caveolin-1 redistribution from the plasma membrane to the early/sorting endocytotic compartment of hepatocytes (Pol et al., 2000). To test whether there is an interaction between E-cadherin and caveolin-1, reciprocal immunoprecipitation experiments using anti-E-cadherin or anti-caveolin-1 antibodies were undertaken and revealed that the proteins associated with each other in the absence of EGF treatment (Figure 1E). The association was still detected at 1 hr of EGF treatment, but gradually dissipated after prolonged EGF treatment (Figure 1E). Consistent with this, costaining with anti-caveolin-1 and anti-E-cadherin antibodies displayed partial colocalization of both proteins at cell-cell contacts in untreated cells and in an intracellular compartment after 2 hr of EGF treatment (Figure 1F). A fraction of total caveolin-1 and E-cadherin was recycled and colocalized at the cell membrane after 24 hr of EGF stimulation. Treatment of cells with the cholesterol-sequestering reagent filipin, which is known to disrupt cholesterol-rich caveolae (Ros-Baro et al., 2001; Torgersen et al., 2001), blocked endocytosis of E-cadherin (Figure 1G) as well as albumin uptake but not internalization of transferrin (data not shown). Identical results were obtained with β-cyclodextrin, another cholesterol-sequestering reagent (data not shown). These data suggest that E-cadherin is endocytosed from the cell-cell contacts by a clathrin-independent pathway and that EGF-induced E-cadherin endocytosis may involve a caveolae-dependent mechanism.

Chronic EGF treatment downregulates protein expression of caveolin-1 and E-cadherin at the transcriptional level

Short-term EGF treatment disrupted cell-cell contacts, whereas prolonged treatment not only prevented cells from contacting each other but also caused EMT. To investigate the basis for these long-term effects of EGF, we tested the effect of chronic EGF treatment on expression levels of caveolin-1 and the cell adherens junction proteins E-cadherin, β-catenin, and α-catenin in A431 cells. Immunoblotting analyses revealed that the levels of caveolin-1 and E-cadherin proteins were dramatically reduced at 3 days of EGF treatment and became undetectable after 5 days of treatment (Figure 2A). In contrast to caveolin-1 and E-cadherin, there were only modest reductions in the levels of β-catenin and α-catenin (Figure 2A) and the focal adhesion protein FAK (data not shown), and the levels of the ERK1/2 MAP kinases (Figure 2A) were unaffected. EGF treatment of A549 and DU145 cells also caused downregulation of caveolin-1 and E-cadherin proteins (Figure 2A). The removal of EGF for 3 days resulted in the restoration of expression of both caveolin-1 and E-cadherin with similar kinetics (Figure 2B).

To determine whether EGF-induced downregulation of E-cadherin and caveolin-1 was regulated at the posttranslational level, a pulse-chase analysis was carried out following

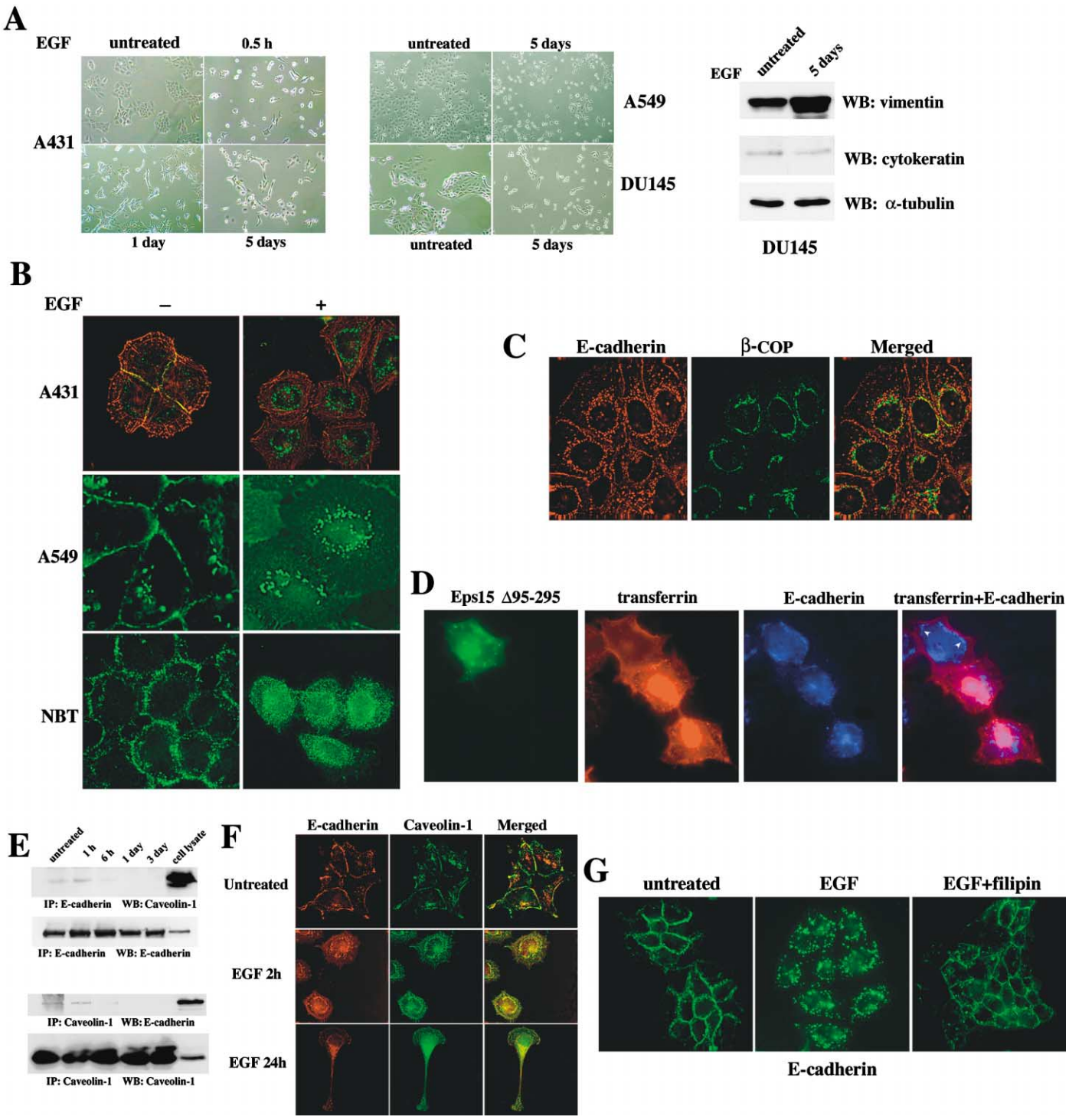

Figure 1. EGF treatment results in disruption of cell-cell junctions, EMT, and caveolae-dependent endocytosis of E-cadherin in tumor cells that overexpress EGFR

A: A431 (upper panel) and A549 and DU145 (middle panel) cells were treated with EGF (100 ng/ml) for the indicated times, and morphological changes were observed by phase-contrast microscopy. The expression of vimentin and cytokeratin in DU145 cells treated with or without EGF (100 ng/ml) for 5 days was assessed by immunoblotting analysis (right panel).

B: Deconvolution microscope images of E-cadherin (green) and actin stained with TRITC-labeled phalloidin (red) in A431 cells (upper panel) untreated or treated with EGF (100 ng/ml) for 2 hr; yellow indicates the staining overlap. E-cadherin staining (green) in A549 cells (middle panel) and NBT-II cells (lower panel) untreated or treated with EGF.

C: A431 cells treated with EGF for 2 hr were immunostained with anti-E-cadherin (red) and anti-β-COP (green) antibodies.

D: A431cells were transiently transfected with GFP-tagged Eps15 Δ95-295 followed by EGF (100 ng/ml) treatment for 2 hr and incubated with Alexa Fluor 594-conjugated transferrin (5 μg/ml) (red) for 30 min. E-cadherin, stained with mouse anti-E-cadherin antibody followed by Cy-5-conjugated anti-mouse antibody, is represented by pseudo-blue color.

E: Immunoprecipitation of E-cadherin from cells that were untreated or treated with EGF (100 ng/ml) for the indicated times was followed by immunoblotting

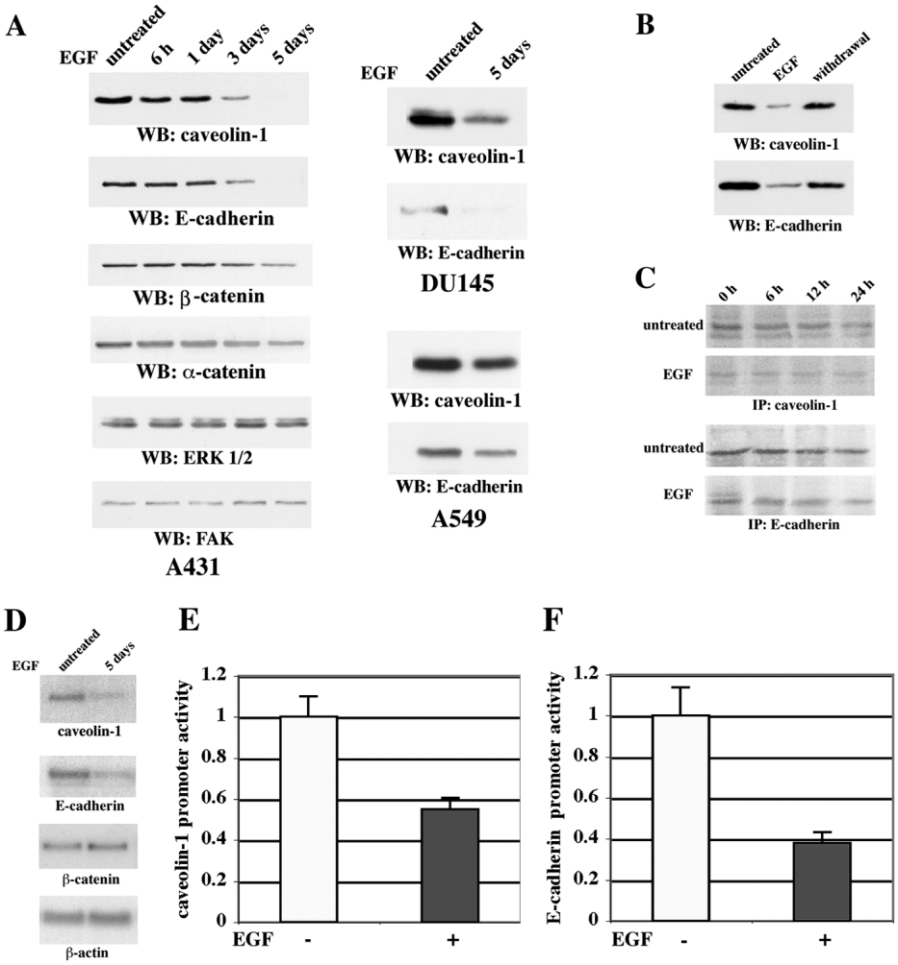

Figure 2. Chronic EGF treatment downregulates expression of caveolin-1 and E-cadherin proteins at the transcriptional level

A: The expression levels of caveolin-1, E-cadherin, β-catenin, α-catenin, ERK1/2, and FAK in A431 (left panel) or caveolin-1 and E-cadherin in DU145 cells and A549 cells (right panel) were assessed by immunoblotting with indicated antibodies.

B: A431 cells were either untreated or treated with EGF for 3 days or with removal of EGF for 3 days after EGF treatment for 3 days. The cell lysates were assessed by immunoblotting with the indicated antibodies.

C: A431 cells treated with EGF (100 ng/ml) for 3 days were labeled with [^{35}S]methionine for 1 hr and pulse-chased for the indicated time in the presence or absence of EGF treatment. Caveolin-1 or E-cadherin was immunoprecipitated, analyzed by SDS-PAGE, and transferred to nitrocellulose membranes for autoradiography.

D: Northern blot analyses for caveolin-1 (upper panel), E-cadherin (second panel), β-catenin (third panel), and β-actin (lower panel) for A431 cells treated with or without EGF (100 ng/ml) for 5 days.

E and F: 293T cells were transiently transfected with pEGFR expression vector and pA3Luc-caveolin-1 (**E**) or pGL 3-E-cad (−178/+92) vector containing the luciferase (Luc) gene under the control of the −178/+ 92 fragment of the human E-cadherin promoter (**F**). Cells were either left untreated or treated with EGF (100 ng/ml) for 12 hr before harvesting. Cell extracts were processed for measurement of luciferase activity. Data represent the mean ± standard deviation of 3 independent experiments.

chronic EGF treatment for 3 days and in untreated control cells; EGF treatment caused no major change in the half-life of either caveolin-1 or E-cadherin protein (Figure 2C). To determine whether the decrease in protein was due instead to a change in mRNA levels, Northern blotting analysis was carried out after 5 days of EGF treatment. The levels of both E-cadherin and caveolin-1 mRNAs were dramatically reduced, whereas β-catenin mRNA levels exhibited little change in these cells when compared to the β-actin mRNA control (Figure 2D). Additionally, EGF treatment suppressed the promoter activity of both caveolin-1 and E-cadherin in reporter gene assays in which 293T cells were transiently cotransfected with pEGFR expression vector and either a caveolin-1 promoter- (Engelman et al., 1999; Park et al., 2001) or E-cadherin promoter-luciferase reporter (Figures 2E and 2F; Poser et al., 2001). These data suggest that chronic EGF treatment of A431 cells causes downregulation of both caveolin-1 and E-cadherin expression at the transcriptional level.

EGF treatment induces β-catenin dissociation from E-cadherin, translocation to the nucleus, and increased transactivation by GSK-3β-independent mechanisms

β-catenin associates with E-cadherin at cell-cell junctions. Therefore, the internalization of E-cadherin and the disruption of cell-cell junctions in response to EGF treatment might result in the redistribution of membrane-localized β-catenin. To test this, E-cadherin was immunoprecipitated followed by immunoblotting with anti-β-catenin antibody. The level of E-cadherin-associated β-catenin was reduced after short periods of EGF treatment (1 hr) and showed ~70% reduction after 1 day (Figure 3A). Relatively similar amounts of E-cadherin protein were detected in the immunoprecipitates, in contrast to the reduced

with anti-caveolin-1 antibody (upper panel). The blot was reprobed with anti-E-cadherin antibody (second panel). Reverse immunoprecipitation with anti-caveolin-1 antibody was followed by immunoblotting with anti-E-cadherin antibody (third panel). The blot was reprobed with anti-caveolin-1 antibody (bottom panel).

F: Deconvolution microscope images for E-cadherin (red) and caveolin-1 (green) in A431 cells treated with or without EGF (100 ng/ml) for indicated time.

G: Deconvolution microscope images for E-cadherin (green) in A431 cells either untreated or treated with EGF (100 ng/ml) alone for 2 hr or followed by 30 min of filipin (5 μg/ml) or methyl-β-cyclodextrin (5 mM) treatment.

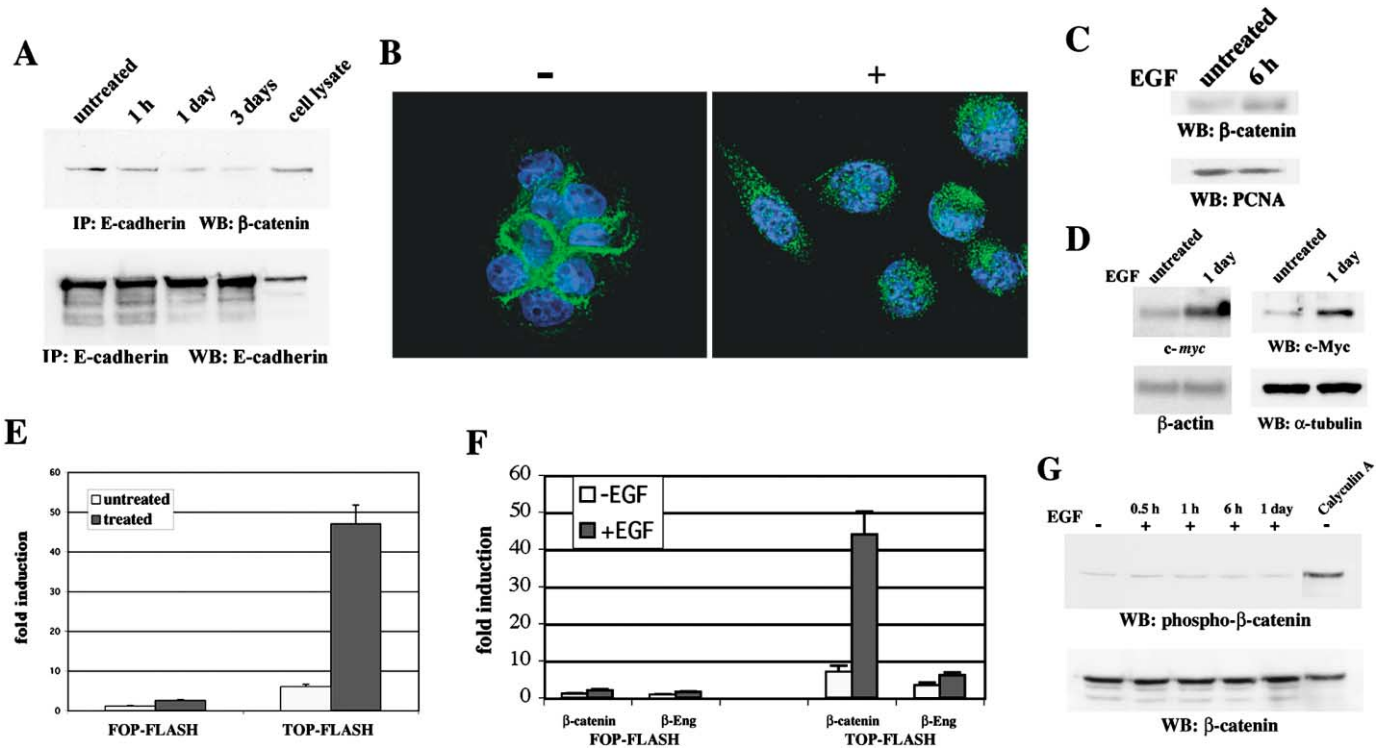

Figure 3. EGF treatment induces β-catenin dissociation from E-cadherin, translocation to the nucleus, and increased transactivation by GSK-3β-independent mechanisms

A: Immunoprecipitated E-cadherin from A431 cells untreated or treated with EGF (100 ng/ml) for the indicated times were analyzed by SDS-PAGE and immunoblotted with anti-β-catenin antibody (upper panel). The blot was reprobed with E-cadherin antibody (lower panel).

B: Deconvolution microscopy for β-catenin (green) and Hoechst 33342-stained nuclei (blue) in A431 cells either untreated or treated with EGF (100 ng/ml) for 1 hr.

C: Nuclear fractions of A431 cells were processed for immunoblotting with anti-β-catenin or PCNA antibody.

D: Northern blot analyses for c-Myc and β-actin (left panel) and immunoblotting for c-Myc and α-tubulin (right panel) in A431 cells treated with or without EGF (100 ng/ml) for 1 day were carried out.

E: FOP-FLASH or TOP-FLASH plasmids with pEGFR were transiently transfected into 293T cells. Cells were either untreated or treated with EGF (100 ng/ml) for 12 hr before harvesting. Cell extracts were measured for lucifererase activity. Data represent the mean ± standard deviation of 3 independent experiments.

F: FOP-FLASH or TOP-FLASH plasmids with pEGFR were transiently transfected with vectors expressing wild-type β-catenin or β-Eng into 293T cells. Luciferase assay was carried out on the lysates prepared from these cells.

G: The level of GSK-3β-phosphorylated β-catenin in A431 cells in the presence or absence of EGF (100 ng/ml) for the indicated time or calyculin A (50 nM) for 1 hr was examined by analyzing cell lysates by SDS-PAGE, immunoblotting with phospho-β-catenin (S33/S37/T41)-specific antibody, and then reprobing with anti-β-catenin antibody.

level of total E-cadherin observed by immunoblotting whole-cell protein (Figure 2A). This is because the amount of E-cadherin antibodies used for the immunoprecipitation was limiting and the antibodies were saturated with E-cadherin. Immunofluorescence staining analyses with β-catenin-specific antibodies also revealed reduced β-catenin staining at cell membrane following EGF treatment for 2 hr, in contrast to untreated controls in which β-catenin was primarily restricted to the cell-cell contacts (Figure 3B). Thus, β-catenin dissociates from E-cadherin and is internalized from cell-cell contacts in response to EGF treatment.

To test whether β-catenin released from E-cadherin accumulates in the nucleus following EGF treatment and interacts with transcription factors of the TCF/LEF-1 family, nuclei were isolated from A431 cells. Immunoblotting experiments detected an increase in nuclearly localized β-catenin following 6 hr of EGF treatment, whereas the amount of the nuclear protein PCNA remained unchanged (Figure 3C). Moreover, we did not detect any E-cadherin in the nuclear fractions, indicating that the nuclear preparations were free of cytosolic contaminants (data not shown). Consistently, we also observed that EGF treatment increased the levels of both c-*myc* mRNA and c-Myc protein, a downstream target gene for β-catenin transactivation (Figure 3D). To examine the effect of EGF on the transcriptional activity of TCF/LEF-1, which is activated after binding β-catenin, the TCF/LEF-1 luciferase reporter TOP-FLASH or a control vector FOP-FLASH was cotransfected with a vector expressing EGFR into 293T cells. Upon EGF treatment for 6 hr, there was a significant increase in TCF/LEF-1 transcriptional activity (Figure 3E). In contrast to wild-type β-catenin, β-Engrailed (β-Eng), in which the C-terminal transactivation domain of β-catenin is replaced with the transcriptional repression domain of *Drosophila* Engrailed (Montross et al., 2000), failed to enhance TCF/LEF-1 transcriptional activity after EGF treatment in this system (Figure 3F). This indicates that EGF induces β-catenin-dependent TCF/LEF-1 activation. Thus, in response to EGF treatment, β-catenin dissociates from cell-cell contacts, accumulates in the nucleus, and promotes TCF/LEF-1 transcriptional activity.

β-catenin protein and transactivation activity can be upregulated by inhibition of N-terminal phosphorylation by GSK-3β. However, in contrast to the significant increase of β-catenin phosphorylation induced by a phosphatase inhibitor, calyculin A, we did not detect any dramatic change in GSK-3β-dependent β-catenin phosphorylation in response to EGF stimulation using a phospho-β-catenin (S33/S37/T41)-specific antibody (Figure 3G). Moreover, there was no significant change in the level of phosphorylated GSK-3β in response to EGF as determined using a phospho-GSK-3β (S9)-specific antibody (data not shown). These data indicate that EGF-stimulated β-catenin transactivation is not mediated by GSK-3β inactivation.

Downregulation of caveolin-1 is sufficient for the upregulation of Snail and downregulation of E-cadherin

Caveolin-1 is downregulated in a number of cell types when transformed by different oncoproteins. Therefore, we next examined whether there is a direct causal relationship between the downregulation of caveolin-1 and E-cadherin. A431 cells stably transfected with a vector that expresses antisense caveolin-1 RNA (Nasu et al., 1998) exhibited reduced expression of endogenous caveolin-1 (Figure 4A). Moreover, the expression level of E-cadherin, but not ERK1/2, was also significantly reduced in these cells (Figure 4A). The residual levels of caveolin-1 and E-cadherin in A431 cells expressing antisense caveolin-1 RNA remained sensitive to long-term EGF treatment and showed a further decrease over 5 days (Figure 4A).

To determine whether E-cadherin downregulation induced by expression of antisense caveolin-1 RNA is regulated at the mRNA level as it is by EGF treatment, E-cadherin mRNA levels were analyzed by Northern blotting. Although β-catenin mRNA levels remained unchanged, as did β-actin mRNA, E-cadherin mRNA levels showed a dramatic reduction in the cells expressing antisense-caveolin-1 RNA (Figure 4B), compared to the parental cells. These data indicate that downregulation of caveolin-1 through expression of antisense caveolin-1 RNA recapitulates the effect of EGF and is sufficient for downregulation of both E-cadherin mRNA and protein expression.

To explore the mechanism of EGF-induced transcriptional downregulation of E-cadherin, we next examined the effect of EGF on Snail expression, since Snail, Slug, and SIP1 family transcription factors have been shown to bind to the E boxes in the E-cadherin promoter, repress transcription of E-cadherin, and induce EMT and invasion (Batlle et al., 2000; Cano et al., 2000; Comijn et al., 2001; Savagner et al., 1997). Snail mRNA levels measured by RT-PCR were increased significantly in response to EGF treatment (Figure 4C). Consistently, A431 cells expressing caveolin-1 antisense RNA contained an elevated level of Snail mRNA, similar to that in EGF-treated cells. Additionally, expression of Snail siRNA using the pRETRO-SUPER (pRS) vector, which reduced endogenous Snail mRNA levels in 293T cells (Figure 4D), increased the promoter activity of E-cadherin and inhibited EGF-induced suppression of its activity in an E-cadherin promoter-luciferase reporter gene assay (Figure 4E). These data indicate that EGF-induced transcriptional upregulation of Snail is necessary for downregulation of E-cadherin in 293T cells. Furthermore, luciferase assays with a Snail promoter-luciferase reporter gene (Peinado et al., 2003) showed that EGF-induced expression of Snail is due to increased Snail transcription (Figure 4F). Moreover, EGF-induced Snail transcription was blocked by expression of wild-type caveolin-1, whereas expression of antisense caveolin-1 RNA by itself enhanced Snail transcription, and a further increase was observed with EGF stimulation (Figure 4F). These data indicate that downregulation of caveolin-1 is necessary and sufficient for EGF-induced upregulation of Snail. EGF-induced Snail transcription, which is regulated by downregulation of caveolin-1, plays an important role in transcriptional downregulation of E-cadherin.

Downregulation of caveolin-1 is necessary and sufficient for EGF-induced β-catenin transcriptional activity

Caveolin-1-mediated endocytosis and downregulation of E-cadherin may lead to the disassembly of the E-cadherin/β-catenin complex and contribute to the release of β-catenin from its membrane anchor and thereby allow transactivation. To test this, 293T cells were transiently transfected with the TOP-FLASH reporter and an EGFR expression vector, and with antisense or wild-type caveolin-1 expression vector. The expression of antisense caveolin-1 RNA in the absence of EGF significantly enhanced β-catenin-TCF/LEF-1 transcriptional activity as quantified by the luciferase assays. Conversely, the expression of wild-type caveolin-1 dramatically inhibited both basal and EGF-induced LEF-1 transcriptional activity (Figure 5). These data indicate that the downregulation of caveolin-1 is necessary and sufficient for EGF-induced β-catenin transcriptional activity.

The effects of EGF autocrine regulation on cell morphology and the expression levels of caveolin-1 and E-cadherin

EGFR overexpression is often accompanied by tumor cell production of TGF-α or other EGF family ligands, and autocrine regulation via EGF family ligands has been implicated in tumor progression (Ethier et al., 1996; Kumar and Mendelsohn, 1990; Ma et al., 1998; Tillotson and Rose, 1991). To test the effect of autocrine regulation on caveolin-1 and E-cadherin cell-cell adherens proteins, A431 cells were grown for 7 days in the absence or presence of AG1478 (300 nM). AG1478, an EGFR inhibitor, interrupts the autocrine regulation loop by inhibiting EGFR activation. As shown in Figure 6A, A431 cells treated with AG1478 became flatter and larger, formed tight cell-cell adhesions, and grew in a monolayer, thus recapitulating the phenotypes of nontransformed cells. Consistent with the tight cell-cell contacts formed by cells treated with AG1478, the expression of caveolin-1 and E-cadherin, but not of FAK, was substantially increased in these cells (Figure 6B). These data imply that EGFR autocrine regulation in tumors plays a critical role in the downregulation of caveolin-1 and E-cadherin, which in turn contribute to disruption of cell-cell contacts, EMT, and tumor progression.

Downregulation of caveolin-1 is sufficient for EGF-induced cell invasion

The downregulation of E-cadherin and increased β-catenin-TCF/LEF-1 transcriptional activities contribute to the development of invasive tumors. To examine whether the downregulation of caveolin-1 induced by EGF plays a role in EGF-induced tumor cell invasion, an in vitro collagen gel analysis was carried out. In contrast to the parental cells, which are flat and form tight cell-cell contacts on the collagen gel surface, A431 cells expressing antisense caveolin-1 RNA exhibited typical mesen-

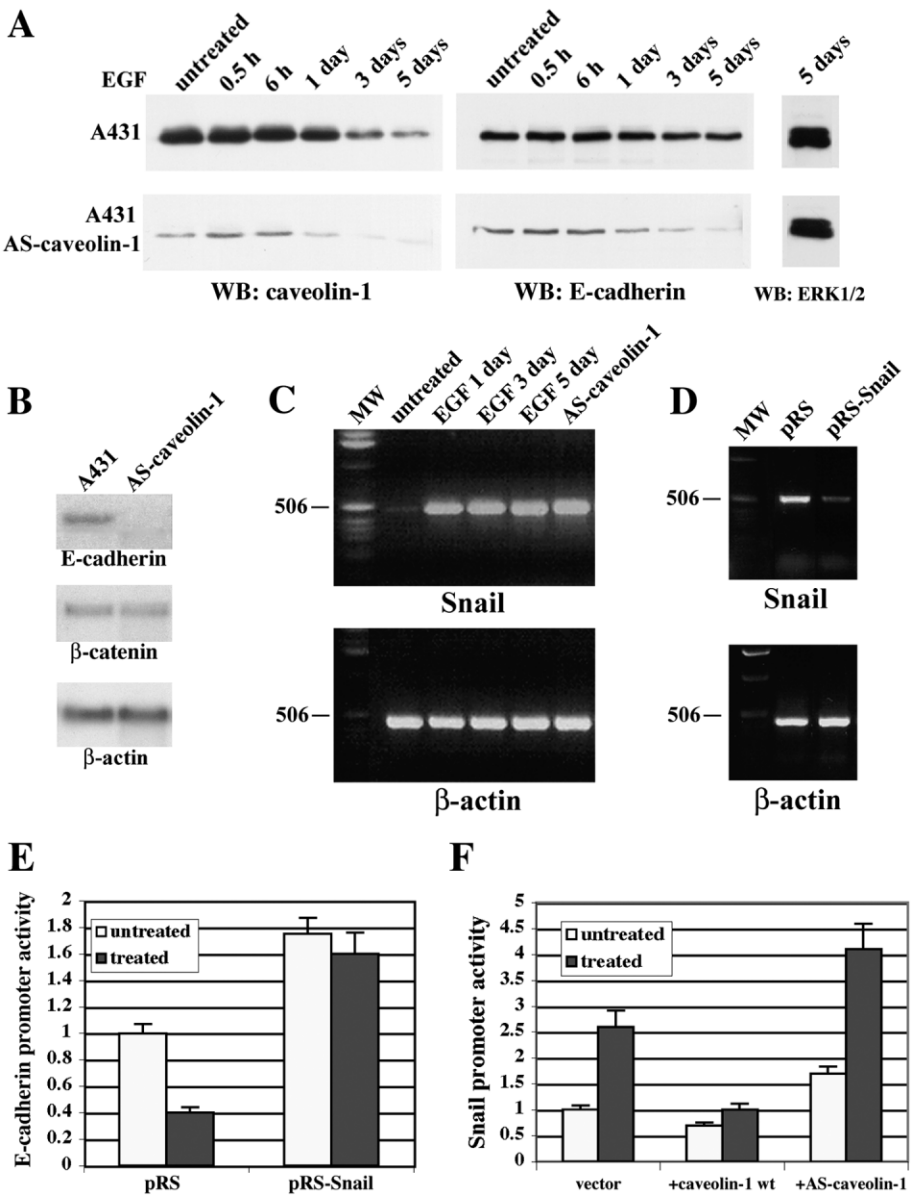

Figure 4. Downregulation of caveolin-1 is sufficient for the upregulation of Snail and the downregulation of E-cadherin

A: The expression of caveolin-1 (left panel), E-cadherin (middle panel), and ERK1/2 (right panel) in A431 cells (upper panel) and A431 cells expressing antisense caveolin-1 RNA (lower panel) treated with or without EGF (100 ng/ml) for the indicated times were analyzed by SDS-PAGE and immunoblotting with the indicated antibodies.

B: The mRNA levels of E-cadherin (upper panel), β-catenin (middle panel), and β-actin (lower panel) in A431 cells or A431 cells expressing antisense caveolin-1 RNA were analyzed by Northern blotting.

C: Snail mRNA expression was measured by RT-PCR analyses from A431 cells treated or untreated with EGF or expressing the antisense caveolin-1 construct. β-actin mRNAs from the same cDNA were amplified as a control.

D: RT-PCR analyses were carried out for mRNA expression of Snail and β-actin in 293T cells transfected with pRS or pRS-Snail.

E: pRS or pRS-Snail plasmids with pEGFR and pGL 3-E-cad were transiently transfected into 293T cells. Cells were either left untreated or treated with EGF (100 ng/ml) for 12 hr before harvesting. Cell extracts were processed for measuring luciferase activity. Data represent the mean ± standard deviation of 3 independent experiments.

F: pEGFR and pLuc-Snail were transfected into 293T cells with or without plasmids expressing wild-type caveolin-1 or antisense caveolin-1. Luciferase activities were determined after cells were treated with or without EGF (100 ng/ml) for 12 hr.

chymal morphology and loss of cell-cell adhesions (Figure 7A, left panels). Seven days after seeding, a larger number of A431 cells expressing antisense caveolin-1 RNA were able to penetrate into collagen gel in the absence of EGF treatment compared to the parental cells (Figure 7A, middle panels). EGF treatment (100 ng/ml), which caused further downregulation of caveolin-1 and E-cadherin in antisense caveolin-1-expressing cells, enhanced the invasiveness of A431 cells expressing antisense caveolin-1 RNA compared to parental cells treated with EGF (Figures 7A, right panels, and 7B). These data indicate that the downregulation of caveolin-1 plays an important role in EGF-induced tumor cell invasion.

Discussion

EGFR overexpression correlates with tumor progression, metastasis, and poor clinical prognosis. Metastasis is a complex process involving changes in cell-ECM and cell-cell junctions. Although E-cadherin downregulation also correlates with tumor development, whether there is a connection between EGFR overexpression and E-cadherin downregulation is not known, nor is the mechanism by which EGF affects cell-cell contacts. Here we demonstrate that EGF treatment of human tumor cells that overexpress EGFR results in the disruption of cell-cell contacts by two distinct mechanisms: internalization of both E-cadherin and β-catenin induced at early times of EGF treatment and depletion of E-cadherin upon prolonged EGF treatment (Figure 8). Importantly, EGF-dependent regulation of caveolin-1 plays a critical role in both processes. Our studies were restricted to examining the effect of EGF on tumor cell lines, and it will be interesting to ask whether these processes are restricted to metastasis and the invasive phase of tumor progression or whether this mechanism is also utilized during normal development and/or differentiation of epithelia. In this re-

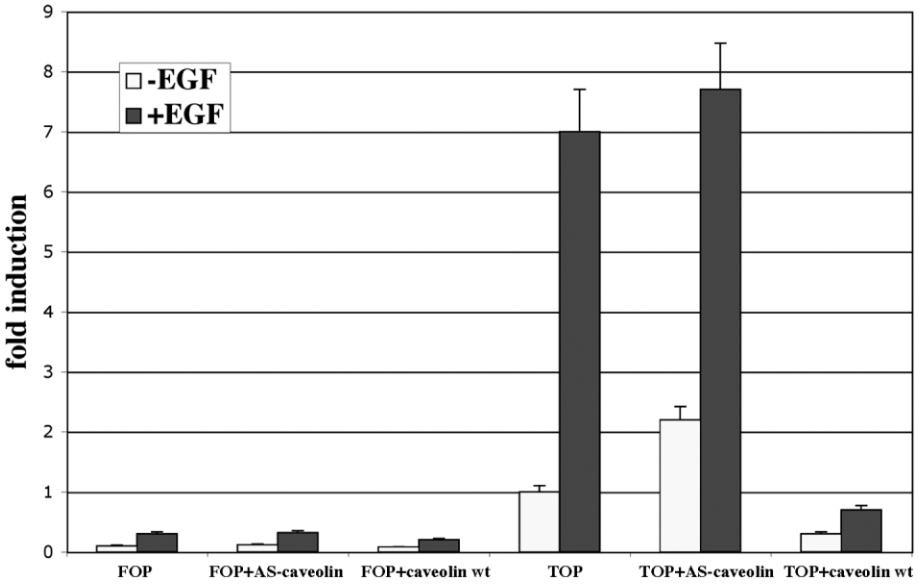

Figure 5. Downregulation of caveolin-1 is necessary and sufficient for EGF-induced β-catenin transcriptional activity

FOP-FLASH or TOP-FLASH plasmids with pEGFR were transiently transfected with vectors expressing antisense or wild-type caveolin-1 into 293T cells. Cells were either left untreated or treated with EGF (100 ng/ml) for 12 hr before harvesting. Cell extracts were processed for measurement of luciferase activity. Data represent the mean ± standard deviation of 3 independent experiments.

gard, a recent report indicates that EGF treatment of normal Madin-Darby canine kidney (MDCK) cells results in increased tight junction function through downregulation of claudin-2 and upregulation of claudins-1 and 3, without an effect on E-cadherin levels (Singh and Harris, 2003).

The abundance and function of cell-surface molecules can be dynamically regulated by endocytosis. We demonstrated that EGF treatment of human tumor cells overexpressing EGFR caused a dramatic alteration in cell-cell contacts accompanied by internalization of E-cadherin. Moreover, caveolin-1, the principle constituent of caveolae, relocalized into endosomal compartments that partially overlapped the Golgi, consistent with the observations of Pol et al. (2000). Structural disruption of caveolae by cholesterol depletion with filipin or β-cyclodextrin, which inhibits caveolar function and blocks caveolae-mediated endocytosis (Ros-Baro et al., 2001; Torgersen et al., 2001), resulted in the retention of both caveolin-1 and E-cadherin at the cell surface. These results suggest that caveolin-1 might play an important role in the endocytosis of E-cadherin. However, we cannot rule out the possibility that E-cadherin internalization is mediated by a caveolin-independent endocytotic pathway that is directly or indirectly disrupted by cholesterol depletion. Caveolar vesicles transport many macromolecules to the endoplasmic reticulum, the Golgi apparatus, or across the cell (transcytosis) (Shin and Abraham, 2001), but this does not lead to lysosomal degradation of proteins. Consistent with this, there was little effect of EGF treatment on the half-life of E-cadherin, and this suggests that EGF-induced internalization of E-cadherin plays a role in the redistribution but not downregulation of E-cadherin. The internalized E-cadherin returns to cell surface and concomitantly cell-cell contacts are reestablished after removal of EGF during transient treatments (data not shown). Thus, clathrin-independent E-cadherin endocytosis and dissociation of E-cadherin/β-catenin complexes during the early stages of EGF treatment serve to disrupt cell-cell contacts by removal of major adherens components from the cell surface.

In contrast to the constitutive clathrin-mediated endocytic pathway, caveolae are normally immobile plasma membrane domains kept in place by the actin cytoskeleton under unstimulated conditions, and there is a very limited exchange of caveolin-1 with the intracellular pool (Lippincott-Schwartz et al., 2001; Thomsen et al., 2002). Much like caveolin-1, only a small fraction of E-cadherin, likely that present in the extrajunctional regions of the lateral surface rather than the adherens junctions, recycles in stable epithelial monolayers of MDCK cells, possibly through the clathrin-dependent pathway (Le et al., 1999). The pool of

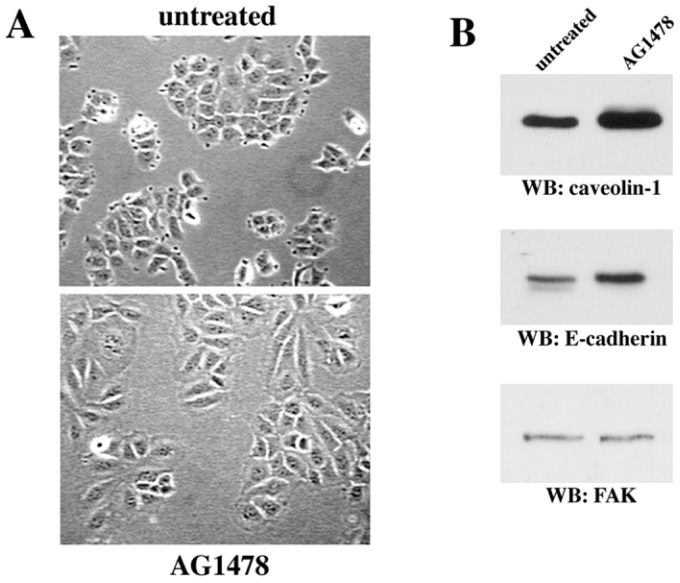

Figure 6. The effects of EGF autocrine regulation on cell morphology and the expression levels of caveolin-1 and E-cadherin

A431 cells were grown in the absence or presence of AG1478 (300 nM) for 7 days. The morphology of the cells (**A**) and the expression levels of caveolin-1, E-cadherin, and FAK (**B**) as assessed by immunoblotting were examined as described in Figure 1.

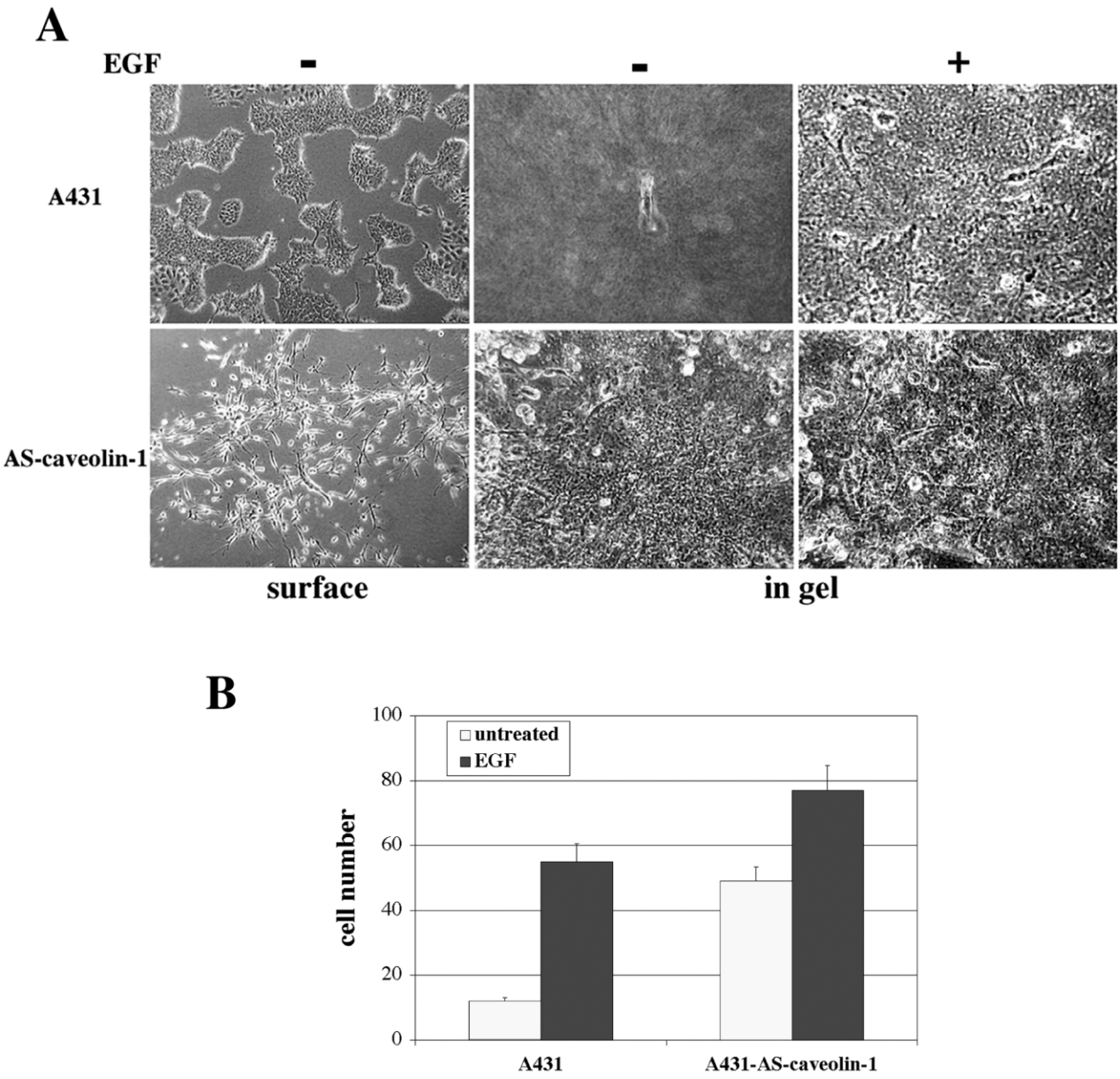

Figure 7. Downregulation of caveolin-1 is sufficient for EGF-induced cell invasion

A431 cells and A431 cells expressing antisense caveolin-1 RNA were plated on the top of collagen gel, and EGF (100 ng/ml) was added to the medium 1 day later.

A: Seven days after plating, cells were photographed either at the top surface of gel (left panels) or at a focal plane beneath the surface to visualize cells that have penetrated the gel (middle and right panels).

B: The numbers of invading cells in 10 photographic fields from 3 separate experiments were counted. Data represent the mean ± standard deviation of 3 independent experiments.

E-cadherin undergoing endocytosis and recycling is dramatically increased in cells without stable cell-cell contacts (Le et al., 1999). Various stimuli can lead to internalization of E-cadherin. Expression of constitutively active Rac1 results in disruption of cell-cell contacts and endocytosis of E-cadherin via a clathrin-independent but possibly a caveolae-mediated pathway (Akhtar and Hotchin, 2001). However, other processes that may not involve caveolae-dependent internalization also regulate endocytosis of E-cadherin. For instance, when E-cadherin is free on the plasma membrane of isolated MCF-7 cells, it is predominantly endocytosed by clathrin-independent and dynamin-dependent endocytosis in which ARF6 activity is involved (Paterson et al., 2003). In HGF-treated MDCK cells, ARF6 is required for clathrin/dynamin-dependent endocytosis of E-cadherin (Palacios et al., 2002). Moreover, an ARF6-dependent initial decrease in the level of Rac1 GTP appears to be necessary for v-Src-induced cell-cell dissociation (Palacios and D'Souza-Schorey, 2003). These data suggests that E-cadherin can be internalized by multiple pathways in different cellular contexts.

We demonstrated that caveolin-1 and E-cadherin interact

and colocalize at cell-cell contacts. Upon EGF treatment, cell-cell contacts were disrupted, accompanied by a dramatic morphological change. While the exact role of caveolin-1 in E-cadherin endocytosis is not clear, it was recently shown that when E-cadherin is tyrosine phosphorylated by Src, it recruits Hakai, a c-Cbl-like ubiquitin E3 ligase, which in turn ubiquitylates E-cadherin (Fujita et al., 2002). This may induce the endocytosis of E-cadherin. Moreover, a role for small G proteins in HGF-induced endocytosis of E-cadherin in MDCK cells has also been suggested (Kamei et al., 1999; Palacios et al., 2001). In response to EGF treatment, we found that caveolin-1 was tyrosine phosphorylated and monoubiquitylated, although we did not detect any change in the phosphorylation of E-cadherin (data not shown). We speculate that tyrosine-phosphorylated caveolin-1, or EGFR, which also localizes at caveolae, recruits an SH2 domain containing E3 ligase, such as c-Cbl or Hakai, which in turn ubiquitylates caveolin-1. Monoubiquitylation has attracted considerable attention as a mechanism for targeting receptor molecules for endocytosis (Hicke, 2001). Additionally, some of the components of clathrin-dependent endocytosis machinery, such as Eps15 and Eps15R (which are themselves monoubiquitylated), epsins, and Vps27p, have a ubiquitin binding domain (Polo et al., 2002; Shih et al., 2002). Therefore, it is tempting to speculate that either ubiquitylation of caveolin-1 itself or association of monoubiquitylated adaptor proteins may play an analogous role in the clathrin-independent endocytosis of caveolin-1 and possibly of caveolin-1-associated E-cadherin.

In contrast to the effects of transient stimulation with EGF, chronic treatment resulted in reduced expression of caveolin-1 and E-cadherin proteins due to downregulation of their mRNA levels. Interestingly, ERK1/2 MAP kinase signaling, an important target of EGFR activation, downregulates caveolin-1 transcription (Engelman et al., 1999). It has also been reported that treatment of human lung cancer cell lines with a ligand-blocking monoclonal antibody against EGFR results in increased expression of E-cadherin (Al Moustafa et al., 1999, 2002). We found that interruption of EGF autocrine signaling in A431 epidermoid carcinoma cells with the EGFR inhibitor AG1478 increased expression of E-cadherin and caveolin-1, accompanied by normal epithelial-like phenotypic change. The suppression of caveolin-1 and E-cadherin promoter activity by EGF indicates that their transcription is downregulated. Depletion of caveolin-1 by antisense RNA expression recapitulated the effect of EGF and downregulated E-cadherin expression, indicating that downregulation of caveolin-1 is sufficient for downregulation of E-cadherin. Consistent with this, antisense RNA-mediated depletion of caveolin-1, which mimics the effects of EGF treatment, resulted in the increased expression of Snail, a transcriptional regulator that directly represses E-cadherin promoter activity and induces EMT (Batlle et al., 2000; Cano et al., 2000). Moreover, EGF-induced upregulation of Snail transcription is dependent on downregulation of caveolin-1. Thus, while E-cadherin is functionally inactivated by a caveolin-1-dependent internalization mechanism during transient EGF treatment, long-term EGF treatment causes downregulation of caveolin-1, leading to transcriptional repression of E-cadherin through induction of Snail, and thus accounts for loss of E-cadherin during long-term EGF treatment. Therefore, caveolin-1 may play two distinct roles in EGF regulation of E-cadherin function and loss of cell-cell contacts: caveolin-1 may directly or indirectly participate in removal of E-cadherin from the plasma membrane during short-term treatment with EGF, and loss of caveolin-1 results in transcriptional downregulation of E-cadherin during long-term EGF treatment (Figure 8).

EGF-induced downregulation of E-cadherin transcription appears to involve upregulation of Snail, but the mechanism through which Snail is upregulated by EGF is unclear. Our data indicate that E-cadherin gene expression is dependent on the presence of caveolin-1 and possibly intact caveolae. Many signaling molecules such as EGFR, PDGFR, Neu/ErbB2, Ha-Ras, c-Src, and PI-3′-kinase localize in caveolae and are negatively regulated through their interaction with caveolin-1 (Okamoto et al., 1998). Thus, downregulation of caveolin-1 from caveolae through internalization at early times of EGF treatment and reduced expression through prolonged EGF treatment or antisense regulation of its mRNA could in principle result in the release of signaling proteins from caveolae that then upregulate transcription of the Snail gene and thereby indirectly reduce E-cadherin gene transcription. The resultant depletion of E-cadherin could prevent cells from reforming cell-cell contacts and facilitate EMT, possibly in combination with increased signaling due to loss of caveolin-1 and with enhanced β-catenin-TCF/LEF-1-mediated transcription due to release and nuclear import of β-catenin.

Caveolin-1 expression is reduced in lung cancer, colon cancer, ovarian cancer, and sarcoma (Bagnoli et al., 2000; Bender et al., 2000; Racine et al., 1999; Razani et al., 2001b; Wiechen et al., 2001). Moreover, a common sporadic caveolin-1 mutation has been found in human breast cancer (Hayashi et al., 2001; Lee et al., 2002). Reduced expression of caveolin-1 correlates with poor tumor prognosis. In addition, overexpressed caveolin-1 reduced transforming phenotypes of lung and breast cancer cells, supporting an inhibitory role of caveolin-1 in tumor progression (Lee et al., 1998; Racine et al., 1999). Paradoxically, caveolin-1 expression is increased in prostate cancer and esophageal cancer, suggesting a positive role in tumor development (Kato et al., 2002; Li et al., 2001; Mouraviev et al., 2002). These data imply that caveolin-1 has multifunctional activities in cancer dependent on interacting signal molecules and the specific cell type/tissue context. Caveolin-1 is reported to interact with the androgen and estrogen receptors and potentiate their transcriptional activity (Lu et al., 2001a; Razandi et al., 2003; Schlegel et al., 1999), and this may contribute to the growth of sex hormone-related tumors.

Upregulation of β-catenin transcriptional activity has been observed in several types of cancers, such as colon cancer, melanoma, and prostate cancer (Morin, 1999; Peifer and Polakis, 2000). We found that EGF treatment resulted in dissociation of β-catenin from E-cadherin, internalization from cell-cell contacts, increased nuclear localization, and stimulation of β-catenin-TCF/LEF-1 transcriptional activity. The internalization and depletion of both caveolin-1 and E-cadherin destabilizes the E-cadherin and β-catenin complex and allows translocation of β-catenin from the cell membrane to the nucleus. Consistent with this, overexpression of caveolin-1, which recruits β-catenin to caveolar membranes (Galbiati et al., 2000), blocks EGF-induced β-catenin-mediated transactivation, which is in line with transcriptional repression of cyclin D1 gene by caveolin-1, which is dependent on the TCF/LEF-1 binding site in the cyclin D1 promoter (Hulit et al., 2000). These results indicate that caveolin-1 plays an essential role in regulation of β-catenin transcriptional activity. β-catenin-TCF/LEF-1-dependent tran-

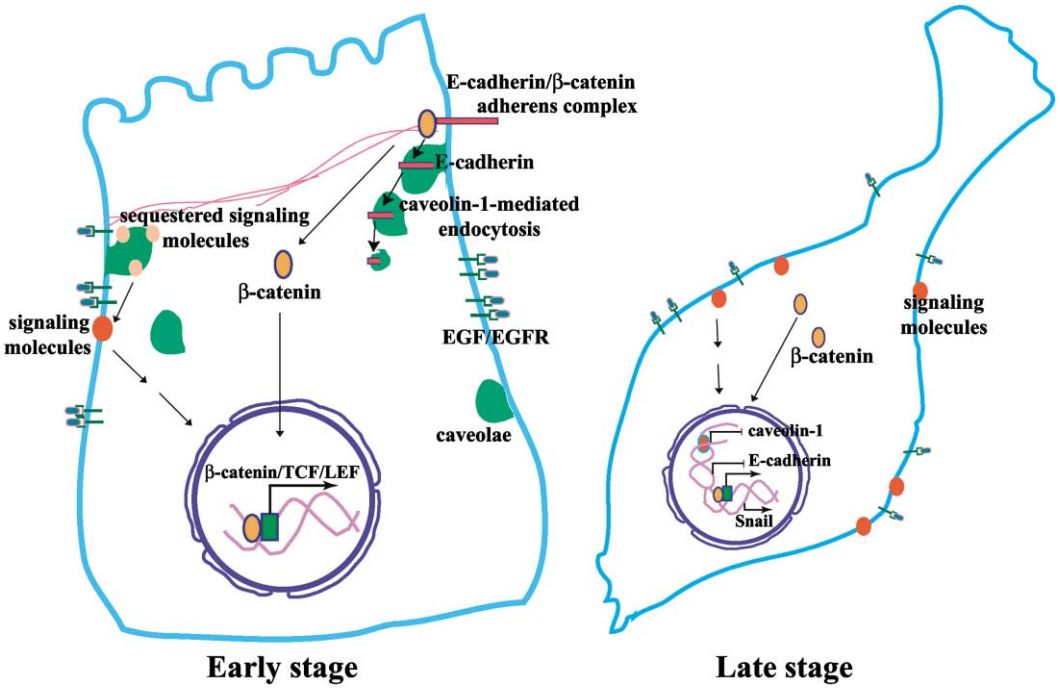

Figure 8. Disruption of cell-cell adhesion junction and β-catenin/TCF/LEF-1 transactivation

Left: Early phase of EGF treatment: caveolae-mediated E-cadherin endocytosis and dissociation of E-cadherin/β-catenin complexes during the early stages of EGF treatment serve to disrupt junctions by removal of these adherens components from cell surface. Release of signaling molecules sequestered by caveolin-1, which may also contribute to the activation of β-catenin, turn on the transcriptional machinery and suppress the transcription of caveolin-1 and E-cadherin.

Right: Late phase of EGF treatment: reduced expression of E-cadherin protein due to suppression of E-cadherin promoter activity and downregulation of its mRNA level through EGF-induced upregulation of Snail prevents cells from reforming cell-cell junctions and facilitates EMT.

scription can be positively regulated by both PKB/AKT-mediated phosphorylation and inactivation of GSK-3β (Weston and Davis, 2001). We did not detect reduced β-catenin phosphorylation at the GSK-3β sites, increased phosphorylation of GSK-3β itself at the site that inhibits its kinase activity, or any apparent change of half-life of β-catenin in response to EGF treatment (data not shown). Thus, it is unlikely that EGF-induced β-catenin-TCF/LEF-1 transcriptional activity is due to negative regulation of GSK-3β, which leads to stabilization of β-catenin complexed with APC-axin. EGF-induced β-catenin transactivation may be potentiated by posttranslational modification of β-catenin itself or of a β-catenin binding coactivator. Although the nature of this activation is not clear, our results suggest that the internalization and depletion of caveolin-1 leads to release of inhibited signaling molecules normally sequestered in caveolae, which in turn contribute to β-catenin transactivation. We conclude that β-catenin-TCF/LEF-1 transcriptional activity can be regulated through at least two distinct mechanisms: GSK-3β-dependent regulation mediated by Wnt/Wingless signaling and PKB/AKT- and GSK-3β-independent regulation mediated though EGF signaling by the regulation of caveolin-1.

The downregulation of E-cadherin, increased β-catenin-TCF/LEF-1 transcriptional activity, and EGFR overexpression and EGF-induced signaling have all been separately reported to correlate with an invasive stage of tumor development. Here we provide a mechanistic model for tumor cell invasion and metastasis that integrates these different components. EGF family members secreted during autocrine regulation of EGFR-overexpressing tumor cells function as signals to direct the disruption of cell adhesion to ECM mediated through dephosphorylation and inactivation of FAK (Lu et al., 2001b). Moreover, it also directs the disruption of cell-cell contacts and causes EMT through caveolin-1-mediated internalization and transcriptional downregulation of E-cadherin. Induction of protooncogenes, such as c-*myc* and cyclin D1, through a GSK-3β-independent increase in β-catenin-TCF/LEF-1 transcriptional activity provides a further drive for progression to an invasive stage of tumor development. Our studies reveal that the phenotypic effects of EGF, internalization and transcriptional downregulation of E-cadherin, and increased transactivation by β-catenin are all mediated through downregulation of caveolin-1, which represents a novel and important mechanism underlying the effects of EGF during tumor development. The demonstration of an interplay between EGF and Wnt/Wingless signaling components provides an important insight for further understanding tumor cell invasion and metastasis.

Experimental procedures

Cells and cell culture conditions

A431 human epidermoid carcinoma cells, DU145 prostate cells, A549 human non-small cell lung cancer cells, NBT-II rat bladder carcinoma cells, and 293T cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone). Cell cultures were made quiescent by growing them to confluence and then replacing the medium with fresh medium containing 0.5% serum for 1 day.

Materials

Polyclonal antibodies for ERK1 (K-23), FAK, PCNA, c-Myc, caveolin-1 (N20), vimentin, E-cadherin (H108), and monoclonal antibody for β-catenin (E-5) and cytokeratin were from Santa Cruz Biotechnology. Anti-caveolin-1 monoclonal antibody (C37120) was obtained from Transduction Laboratories, and the 4G10 anti-phosphotyrosine monoclonal antibody from Upstate Biotechnology. Polyclonal phospho-β-catenin (Ser33/Ser37/Thr41)-specific antibodies were from Cell Signaling Technology. Monoclonal antibody for ZO-1 was from Zymed laboratories. Polyclonal antibody against β-COP was from Affinity Bioreagents. Human EGF, filipin, methyl-β-cyclodextrin, TRITC-labeled phalloidin, monoclonal α-tubulin antibody, and FITC-conjugated albumin were purchased from Sigma. Hygromycin was from GIBCO. AG1478 and calyculin A were obtained from Calbiochem. FITC-conjugated antimouse antibody and Texas red-conjugated anti-rabbit antibody were from Southern Biotechnology Associates. Cy-5-conjugated anti-mouse antibody, Hoechst 33342, and Alexa Fluor 594 conjugated transferrin were from Molecular Probes. To generate the pRETRO-SUPER-Snail (pRS-Snail), the pRETRO-SUPER vector (pRS) (Brummelkamp et al., 2002) was digested with BglII and HindIII, and the annealed oligonucleotides GATCCCC**GATGCACATCCGAAGCCAC**TTCAAGAGA**GTGGCTTCGGATGTGCATC**TTTTTGGAAA and AGCTTTTCCAAAAA**GATGCACATCCGAAGCCAC**TCTCTTGAA**GTGGCTTCGGATGTGCATC**GGG were ligated into the vector.

Transfection

Cells were plated at a density of 4×10^4 per 60 mm diameter dish 18 hr prior to transfection. Transfection was performed using either calcium phosphate or LipofectAmine reagent (GIBCO) according to the vendor's instructions. Transfected cultures were selected with hygromycin (200 μg/ml) for 10 to 14 days at 37°C. At that time antibiotic-resistant colonies were picked, pooled, and expanded for further analysis under selective conditions.

Immunoprecipitation and immunoblotting analysis

Extraction of proteins from cultured cells was performed with a lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin (12 mg/ml), aprotinin (20 mg/ml), 100 mM sodium vanadate, 100 mM sodium pyrophosphate, and 1 mM sodium fluoride. Cell extracts were clarified by centrifugation at 12,000 rpm, and the supernatants (1.5 mg protein/ml) were subjected to immunoprecipitation with the indicated antibodies. After overnight incubation at 4°C, protein A or G agarose beads were added and left for an additional 3 hr. Immunocomplexes were washed with lysis buffer 3 times and then subjected to immunoblotting analysis as described previously (Lu et al., 1998).

Immunofluorescence and deconvolution microscopy

Cells were grown on poly-L-lysine-coated glass coverslips, fixed with 4% paraformaldehyde, permeabilized in PBS containing 0.2% Triton-X100, and blocked with 1% BSA. Cells were incubated with corresponding antibodies for 1 hr at room temperature, washed, and incubated with FITC-conjugated antimouse antibody, Texas red-conjugated anti-rabbit antibody, and Hoechst 33342. After the final washes and mounting, cells were examined using a laser scanning Olympus microscope with a 60× oil immersion objective. DeltaVision (Applied Precision Software) was used to deconvolve z series images. Transferrin and albumin endocytosis assays were carried out as described previously (Razani et al., 2001a).

In vitro invasion assay

Polymerized collagen gels (1.0 mg/ml final concentration) were prepared by neutralization of the collagen solution (Vitrogen 100, Collagen Corp.) with 1/6 volume of 7× DMEM concentrate. The mixed solution was diluted to give a 1× DMEM solution containing 10% serum. EGF (100 ng/ml) (chemokinesis) was added to the medium 1 day after seeding the cells. The cells were examined 7 days later for invasion beneath the surface using a digital camera mounted on a microscope with 100× magnification.

Isolation of nuclei

Nuclei were isolated by hypotonic lysis and isopycnic centrifugation on OptiPrep (Accurate Chemicals, Westbury, NY) iodixanol density gradient following the manufacturer's protocol.

Luciferase reporter gene assay

In order to measure the transcriptional activity of TCF/LEF-1, 293T cells were seeded in 12-well plates at 3×10^3 cells/well. 24 hr after seeding, cells were transiently transfected with 0.5 μg of pEGFR, 0.5 μg TCF/LEF-1 reporter (pTOP-FLASH), or control vector (pFOP-FLASH) with or without 0.5 μg wild-type caveolin-1 or antisense-caveolin-1 expression vector. Twelve hours after transfection, the medium was replaced with 0.1% serum for another 24–36 hr, and EGF (100 ng/ml) was added 6 hr before harvesting. Ten microliters out of the 100 μl cell extract were used for measuring luciferase activity. pA3Luc-caveolin-1, pGL 3-E-cad (−178/+92) vector, or pLuc-Snail was transfected as described above to measure the promoter activity of caveolin-1, E-cadherin, or Snail, respectively.

Pulse-chase analysis

A431 cells treated or untreated with EGF (100 ng/ml) for 3 days were incubated in the absence of methionine for 20 min, pulse-labeled for 1 hr with 250 μCi/ml [^{35}S] methionine, washed twice, and incubated in medium containing excess unlabeled methionine for 0, 2, 4, or 24 hr in the presence or absence of EGF (100 ng/ml). Proteins were immunoprecipitated with the indicated antibodies. Samples were then separated on 6% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for exposure to X-ray film.

Northern blot analysis

Total RNA was isolated from A431 cells using the RNeasy MiniKit (Qiagen, Santa Clarita, CA) according to the manufacturer's instructions. Aliquots (20 μg) of total RNA were separated electrophoretically on 1.2% (w/v) agarose/formaldehyde gels (equal loading was monitored by ethidium bromide staining of the gel), blotted on Hybond-N+ (Amersham) membranes, and hybridized with ^{32}P-labeled cDNA probes, which were prepared by using Prime-It II Random Prime labeling Kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Subsequent washes were performed at 65°C, using standard procedures from Current Protocols in Molecular Biology.

RT-PCR

First-strand cDNA from 2 μg of total RNA was synthesized using ProStar First Strand RT-PCR Kit (Stratagene, Cedar Creek, TX). For Snail RNA amplification, the first round of PCR used 2 μl of the cDNA and primers ATGCCG CGCTCCTTCCTGGTCAGG and TCAGCGAGGGCCTCCGGAGCAGCC. A nested PCR was performed as described previously (Poser et al., 2001). β-actin RNA amplification was carried out for 25 cycles using primers GTGGTGGTGAAGCTGTAGCC and GACGAGGCCCAGAGCAAGAGAGG.

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