

Aberrant Expression of Cortactin in Head and Neck Squamous Cell Carcinoma Cells Is Associated with Enhanced Cell Proliferation and Resistance to the Epidermal Growth Factor Receptor Inhibitor Gefitinib

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Abstract

The *CTTN* gene (formerly designated *EMSI*), encodes cortactin, a key regulator of dynamic actin networks. Both *CTTN* and *CCND1*, the latter encoding the cell cycle regulator cyclin D1, reside at chromosomal locus *11q13*, a region commonly amplified in breast cancers and head and neck squamous cell carcinoma (HNSCC). Previously, we identified a novel role for cortactin in cancer cells, whereby cortactin overexpression attenuated ligand-induced down-regulation of the epidermal growth factor (EGF) receptor (EGFR), leading to sustained signaling. However, how this affected growth factor-induced cellular responses was unclear. Here, by modulation of cortactin expression in a panel of HNSCC cell lines, we show that cortactin overexpression enhances serum- and EGF-stimulated proliferation under both anchorage-dependent and anchorage-independent conditions and also increases resistance to anoikis (detachment-induced apoptosis). These effects are associated with increased activation of extracellular signal-regulated kinase and/or AKT. Furthermore, we report that cortactin stabilizes the c-MET receptor tyrosine kinase and enhances hepatocyte growth factor-induced mitogenesis and cell scattering. Therefore, cortactin may modulate signaling by a broader range of receptors than originally proposed and thereby affect a variety of responses. Finally, we have determined that cortactin overexpression, either alone or in combination with cyclin D1 up-regulation, promotes resistance to the EGFR kinase inhibitor gefitinib. These findings indicate that cortactin may play multiple roles in progression of HNSCC and should be evaluated as a marker of prognosis, disease progression, and therapeutic responsiveness, particularly to EGFR-directed agents. [Cancer Res 2007;67(19):9304–14]

Introduction

Head and neck squamous cell carcinoma (HNSCC) represents a major worldwide health problem with patients exhibiting a 5-year survival rate of <50%, and despite intense efforts, this rate has not improved in the last 30 years (1, 2). Among the molecular alterations associated with development and progression of this disease are overexpression and/or mutation of particular receptor

tyrosine kinases (RTK). For example, increased expression of epidermal growth factor (EGF) receptor (EGFR) or its ligand, transforming growth factor- α , occurs in the majority of HNSCCs and elevated levels of these proteins are associated with reduced disease-free survival (3). This has led to ongoing clinical trials of two types of EGFR-selective therapeutic agent in this disease: small-molecule tyrosine kinase inhibitors (e.g., gefitinib and erlotinib) and monoclonal antibodies (mAb; e.g., cetuximab). Although these molecules exhibit modest activities as single agents, a combination of anti-EGFR strategies with cytotoxic chemotherapy or radiation therapy has seen improvements in response rates and patient survival (4, 5). Another RTK implicated in HNSCC progression is the receptor for hepatocyte growth factor (HGF), c-MET. Activation of this receptor, like the EGFR, promotes cancer cell proliferation and survival but also markedly enhances cell scattering, motility, and invasion (6). Consistent with the latter biological effects, overexpression or mutation of c-MET is implicated in the metastatic spread of HNSCC (7, 8).

A further characteristic of head and neck cancers, as well as those of the breast, esophagus, lung, and bladder, is amplification of chromosomal locus *11q13*. This occurs in ~40% of HNSCCs and is associated with the presence of lymph node metastasis and poor prognosis (ref. 9 and references therein). The *CCND1* gene, encoding the cell cycle regulatory protein cyclin D1, and the *CTTN* gene (formerly designated *EMSI*), encoding the actin-binding protein cortactin, localize to this region. In breast cancers, *CTTN* and *CCND1* are located on separate *11q13* amplicons that can be amplified independently (9). Studies by our group on a large breast cancer cohort revealed that *CTTN* was amplified independently of *CCND1* in a subset of samples and that there was no association between the degree of amplification of these two genes. Furthermore, amplification of *CTTN*, unlike that of *CCND1*, was associated with an increased risk of relapse and death in patients with estrogen receptor-negative disease (10). Subsequent work by Rodrigo et al. (11) extended these findings to HNSCC, where amplification of *CTTN*, but not *CCND1*, predicted early recurrence and reduced survival. More recently, cortactin, but not cyclin D1, overexpression was found to associate with lymph node metastasis of esophageal squamous cell carcinomas (12). Taken together, these data strongly suggest that cortactin plays a key, albeit incompletely understood, role in cancers with *11q13* amplification.

The function of cortactin is to promote the formation of dendritic actin networks critical to such processes as cell motility and receptor-mediated endocytosis (13). This stems from the ability of cortactin to bind the actin-related protein (Arp) 2/3 complex and F-actin via its NH₂-terminal acidic and repeat regions,

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respectively, and to stimulate the actin nucleation activity of Arp2/3 alone or in combination with N-WASP. In addition, cortactin stabilizes branched actin networks (13). Studies on a variety of cell types, including *11q13*-amplified HNSCC cells, have shown that cortactin overexpression enhances cell migration and invasion *in vitro* (13, 14). Supporting the hypothesis that cortactin promotes tumor invasion and systemic spread, overexpression of this protein in cancer cells increases metastasis in nude mouse models (12, 15). However, effects of cortactin on cell proliferation have not been reported.

Recently, we discovered that overexpression of cortactin in HNSCC cells attenuates ligand-induced down-regulation of the EGFR, leading to sustained receptor signaling to the mitogenic extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase pathway (16). This highlighted a potential oncogenic role for cortactin, although important questions that remained included the specificity of this effect and its biological consequences. To address these issues, we have used complementary strategies to alter cortactin expression in HNSCC cells and then characterized a variety of cellular responses relevant to the malignant phenotype and clinical treatment of this disease. The findings shed new light on the role of cortactin and *11q13* amplification in HNSCC.

Materials and Methods

Plasmids. The pRCMV/cortactin construct was as described previously (17). The mouse cortactin cDNA in pBabePuro was a kind gift from Dr. Danielle Lynch (Harvard Medical School, Boston, MA).

Tissue culture and generation of stable cell lines. The HNSCC cell lines Fadu, Detroit 562, Scc9, Scc15, and Scc25 were maintained as described previously (16, 18). Scc9 and Scc25 cells were transfected with pRCMV/cortactin or empty vector using PolyFect (Qiagen Pty Ltd.) following the manufacturer's protocol. Stable pools were generated in the presence of 500 $\mu\text{g}/\text{mL}$ geneticin (Life Technologies). Scc9 clones F5 and F7 overexpressing cyclin D1 (18) were transfected with pBabePuro/cortactin or the empty vector and stable pools were selected in the presence of 1 $\mu\text{g}/\text{mL}$ puromycin (Sigma).

Suppression of cortactin expression by small interfering RNA. Three RNAs were chemically synthesized (Ambion, Inc.) based on the sequence 5'-GACUGGUUUUGGAGGCAAUUUU-3' (19), 5'-AAGCUGAGGGAGAAU-GUCUUGU-3' (19), and 5'-AAGACUGAGAAGCAUGCCUCC-3' [small interfering RNA (siRNA) 3; ref. 20]. Knockdowns used either siRNA 1 or a mixture of siRNA 2 and siRNA 3 (20), with similar results. A siRNA against lamin A/C or green fluorescent protein was used as a control. For annealing of siRNA, 20 μL of each single-stranded RNA (50 $\mu\text{mol}/\text{L}$) were incubated with 10 μL 5 \times annealing buffer [100 mmol/L potassium acetate, 2 mmol/L magnesium acetate, 30 mmol/L HEPES (pH 7.4)] for 1 min at 90°C and then 1 h at 37°C. The RNA duplexes were then stored at -20°C until use. One day before transfection, Fadu or Detroit 562 cells were plated at 1.4×10^5 cells per well in six-well plates. Each well contained 2 mL medium. For transfection of the cells in one well, 3 μL siRNA duplex (20 $\mu\text{mol}/\text{L}$) was diluted into 200 μL Opti-MEM (Invitrogen Corp.) in tube 1. In tube 2, 12 μL Oligofectamine (Invitrogen) was added to 48 μL Opti-MEM. Tubes 1 and 2 were incubated for 7 to 10 min at room temperature before combining the solutions. Following incubation for 20 to 25 min at room temperature, 152 μL Opti-MEM was added to the mixture, which was then added to the cells. Efficient knockdown of cortactin on siRNA treatment persisted for 5 to 6 days.

Growth factor treatment of cells. Cells were starved overnight in serum-free medium and then simulated for the indicated time period with 10 to 100 ng/mL HGF (R&D Systems) as indicated.

Cell lysis, immunoprecipitation, and immunoblotting. Cell lysates were prepared using radioimmunoprecipitation assay buffer (21) containing 10 $\mu\text{g}/\text{mL}$ aprotinin, 10 $\mu\text{g}/\text{mL}$ leupeptin, 1 mmol/L sodium orthovanadate,

and 1 mmol/L phenylmethylsulfonyl fluoride. Protein concentrations were determined using protein assay reagent (Bio-Rad). Samples were analyzed by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and subjected to Western blot analysis. Detection of bound antibodies was by enhanced chemiluminescence (Amersham Biosciences Pty Ltd.).

Antibodies. The cortactin monoclonal 4F11 antibody and c-MET mAb were purchased from Upstate Biotechnologies, Inc. Polyclonal antibodies specific for phosphorylated ERK (T202/Y204), AKT (S473), and Rb (S780), as well as antibodies against total ERK and AKT, were from Cell Signaling Technology. The mAbs against cyclin D1 (clone DCS6) and total Rb were from Novacastra Laboratories and BD PharMingen, respectively. The β -actin mAb (clone AC-15) was purchased from Sigma.

Flow cytometry. Flow cytometry was done on a FACSCalibur with CellQuest 2.0 software (Becton Dickinson Immunocytometry Systems) and analysis used ModFit LT analysis software (Verity Software House, Inc.).

Proliferation assays and determination of gefitinib resistance. Cells (500–2,000) were plated in 96-well microtiter plates (six replicates per treatment) according to the growth characteristics and optimal plating densities for the respective lines (18). Cells were incubated overnight to allow attachment before addition of growth factors or gefitinib (a gift from AstraZeneca Pharmaceuticals) at time 0. Assays to determine gefitinib sensitivity were done in the presence of 10% (v/v) fetal bovine serum (FBS) and vehicle control cells were treated with the equivalent concentration of DMSO. Cell proliferation was determined using the CellTiter 96 assay (Promega) in accordance with manufacturer's instructions at multiple time points as indicated or after 5 days for IC₅₀ determination.

Colony-forming assays. These were done as described previously (18). Cells (6,000–12,000) were plated in six-well plates, observed twice weekly, and fixed and stained with Diff-Quick stain (Lab Aids) in accordance with the manufacturer's directions. Images were captured using a Leica DFC280 camera with Leica Firecam software (version 1.9) and colonies containing greater than ~40 cells were quantified using the ImageJ software (version 1.34s) particle analysis module.

Anchorage-independent growth. This was assayed by determining the ability of cells to form colonies and grow in soft agar. DMEM/F12 containing 10% (v/v) FBS and 1% (w/v) DNA grade agarose (Quantum Scientific) was prepared at 37°C and 2 mL/well, added to six-well plates, and allowed to set at room temperature to form the bottom agarose layer. Cells (1×10^3) were resuspended in DMEM/F12 containing 10% (v/v) FBS and 0.3% (w/v) DNA grade agarose at 37°C and plated on top of the bottom layer. The upper layer was allowed to set at room temperature and then overlaid with 1 mL DMEM/F12. EGF or HGF (100 ng/mL) was added to the bottom and top layer as indicated (22). All assays were done in triplicate and were incubated for 21 days. Images of colonies were captured using a Leica DFC280 camera with Leica Firecam software (version 1.9) and colonies >100 μm in diameter were counted.

Anoikis. Anoikis was induced by preventing cell attachment using plates coated with poly-HEMA (Sigma). A stock solution of 120 mg/mL poly-HEMA in 100% ethanol was prepared and diluted to 5 mg/mL in 95% ethanol. This solution (0.1 mL/cm²) was pipetted into six-well plates and left to dry at room temperature in a sterile environment. Before use, wells were washed with PBS. Cells (5×10^4 per well) were suspended in DMEM/F12 containing 10% (v/v) FBS (2 mL/well) and incubated in poly-HEMA-coated wells for 36 h at 37°C. Anoikis was assayed by determination of the sub-G₁ (apoptotic) fraction of cells by flow cytometry as described previously (23). All experiments were repeated in triplicate and mean values were reported.

Colony dissociation assay. Evaluation of colony dispersion (cell scattering) was done as described previously (24). Briefly, cells were subcultured and maintained in growth medium in six-well plates until colonies were established. Cultures were deprived of growth factors and serum for 16 h before treatment with HGF (100 ng/mL) for 18 h. Colony dispersion was documented by photography using a Leica DFC280 camera with Leica Firecam software (version 1.9). Representative images of at least six independent experiments are shown.

Results

Overexpression of cortactin in HNSCC cells promotes cell proliferation and mitogenic signaling. We showed previously that high cortactin expression impairs EGFR down-regulation leading to sustained receptor signaling (16). We therefore explored the biological consequences of this effect using a panel of five HNSCC cell lines. Fadu, Detroit 562, and Scc15 cells feature *11q13* amplification and/or cortactin overexpression and express high levels of the EGFR. Scc9 and Scc25 cells also display EGFR overexpression; however, these cells have a normal *11q13* copy number and cortactin levels (Fig. 1A; refs. 18, 25). To determine the functional effects of altered cortactin expression, we isolated stable pools of Scc9 and Scc25 cells overexpressing cortactin via transfection or suppressed its expression in both Fadu and Detroit 562 cells to levels intermediate between Scc25 and Scc15 cells using cortactin selective-siRNAs (Fig. 1B).

In agreement with our previous work (16), stable pools of cortactin-overexpressing Scc9 cells exhibited impaired EGFR down-regulation and sustained ERK activation (data not shown). Therefore, we determined whether this was associated with enhanced cell proliferation. Under anchorage-dependent conditions and in 10% FBS, cortactin-transfected cells proliferated faster than vector control cells (Fig. 1C). To confirm that cortactin could enhance EGF-dependent proliferation, we repeated this assay in 1% FBS supplemented with EGF. Consistent with the effect of cortactin on EGFR down-regulation, proliferation under these conditions was also enhanced by cortactin overexpression (Fig. 1C). Similar effects were observed in two independent stable pools of Scc9 cells, and in stable pools of transfected Scc25 cells, as well as on transient transfection of a cortactin expression construct (data not shown).

To complement these data, we suppressed cortactin expression in *11q13*-amplified Fadu and Detroit cells using selective siRNAs. Reduction of cortactin levels in Fadu cells by RNA interference accelerates ligand-induced EGFR down-regulation and attenuates receptor signaling (16), and consistent with these data, cortactin knockdown impaired the proliferation rate of Fadu cells, both in 10% FBS and in 1% FBS + EGF (Fig. 1C). Similar results were observed in Detroit 562 cells (data not shown).

We then plated cells at low density and subjected them to colony formation assays (18). Under both serum-supplemented or EGF-dependent conditions, overexpression of cortactin in Scc9 and Scc25 cells increased both the number and size of colonies, whereas the converse effects were observed on cortactin knockdown in Fadu and Detroit cells (Fig. 1D). These results suggest that cortactin not only confers a proliferative advantage as shown by increased colony size but also may enhance cell survival, as indicated by increased colony number.

In the colony formation assays, Fadu cells with cortactin knockdown exhibited a significantly reduced percentage of cells in S phase (Fig. 2A), indicating that elevated cortactin levels promote G₁ phase progression. Furthermore, phosphorylation of the retinoblastoma protein (Rb), a critical regulator of the G₁-S phase transition, on Ser⁷⁸⁰ was reduced in siRNA-treated Fadu cells, and these cells predominantly exhibited the lower hypophosphorylated form of Rb (Fig. 2A). To characterize the mechanistic basis for this effect, we determined the activation status of growth factor receptor signaling pathways during the colony formation assays. In Fadu and Detroit 562 cells that had knockdown of cortactin, we detected significantly lower levels of activated ERK and also

decreased phosphorylation of AKT on Ser⁴⁷³, the latter indicative of reduced phosphatidylinositol 3-kinase (PI3K) activation (Fig. 2B). In the complementary experiment, Scc9 cells overexpressing cortactin exhibited enhanced activation of these pathways (Fig. 2C). Because both ERK and AKT regulate G₁-S transition by promoting the assembly and/or activation of cyclin D1/cyclin-dependent kinase (Cdk) 4 and cyclin E/Cdk2 complexes that target Rb (26), these data explain the increased proportion of cells in S phase observed on cortactin overexpression.

Taken together, these data show for the first time that cortactin provides a proliferative advantage to HNSCC cells that is commensurate with its ability to potentiate growth factor signaling pathways.

Cortactin overexpression attenuates c-MET down-regulation and enhances HGF-induced biological responses. Because we observed an increase in proliferation in cortactin-overexpressing cells maintained in 10% FBS, where cells are exposed to a variety of mitogens, we decided to explore whether cortactin overexpression affected the regulation of other growth factor receptors. The HGF receptor, c-MET, is strongly implicated in the development and progression of head and neck cancer (6–8). Therefore, we transiently transfected Scc9 cells with the empty vector or a cortactin expression construct and then assayed the extent of HGF-induced c-MET down-regulation at specific time points (Fig. 3A). This revealed that in vector-transfected cells, c-MET levels were reduced to 80% of starting levels at 60 min and to 30% at 120 min. However, in cortactin-overexpressing cells, a reduction in c-MET was not detected until 90 min of stimulation, when levels were 80% of their starting value, and no further decrease was observed at 120 min. Thus, c-MET down-regulation was both delayed and attenuated on cortactin overexpression. In addition, at 120 min, activation of p44 and p42 ERK was increased by 2.1- and 1.4-fold, respectively, in cortactin-overexpressing cells relative to controls. AKT activation was also enhanced at this time point, showing that cortactin overexpression leads to more sustained HGF-induced signaling. To complement this approach, we characterized c-MET down-regulation in Detroit cells, which express high endogenous levels of cortactin. In control cells, there was a very little HGF-induced receptor degradation over a 120-min time course (Fig. 3A). However, siRNA-mediated knockdown of cortactin markedly accelerated c-MET down-regulation in these cells (Fig. 3A). Therefore, the effect of cortactin on receptor down-regulation is not restricted to the EGFR and may affect multiple growth factor receptors.

To characterize the biological effects of impaired c-MET down-regulation, HNSCC cells exhibiting altered cortactin expression were subjected to proliferation or colony-forming assays in 1% FBS + HGF. Consistently, high cortactin expression was associated with an increased rate of proliferation and enhanced colony formation (Fig. 3B and C). Although a well-characterized response to c-MET activation is dispersal of epithelial colonies (6), this was not observed for Fadu and Detroit cells at the concentration of HGF used (10 ng/mL).

We then determined the effect of cortactin overexpression on cell scattering induced by a high concentration of HGF (100 ng/mL). This response was markedly enhanced in the cortactin-overexpressing Scc9 pool (Fig. 3D). Consequently, the ability of c-MET to promote both cell proliferation and dispersal is enhanced on cortactin overexpression. Interestingly, similar effects on c-MET-regulated responses were observed when receptor down-regulation was attenuated via mutation of the direct binding site for c-Cbl (27).

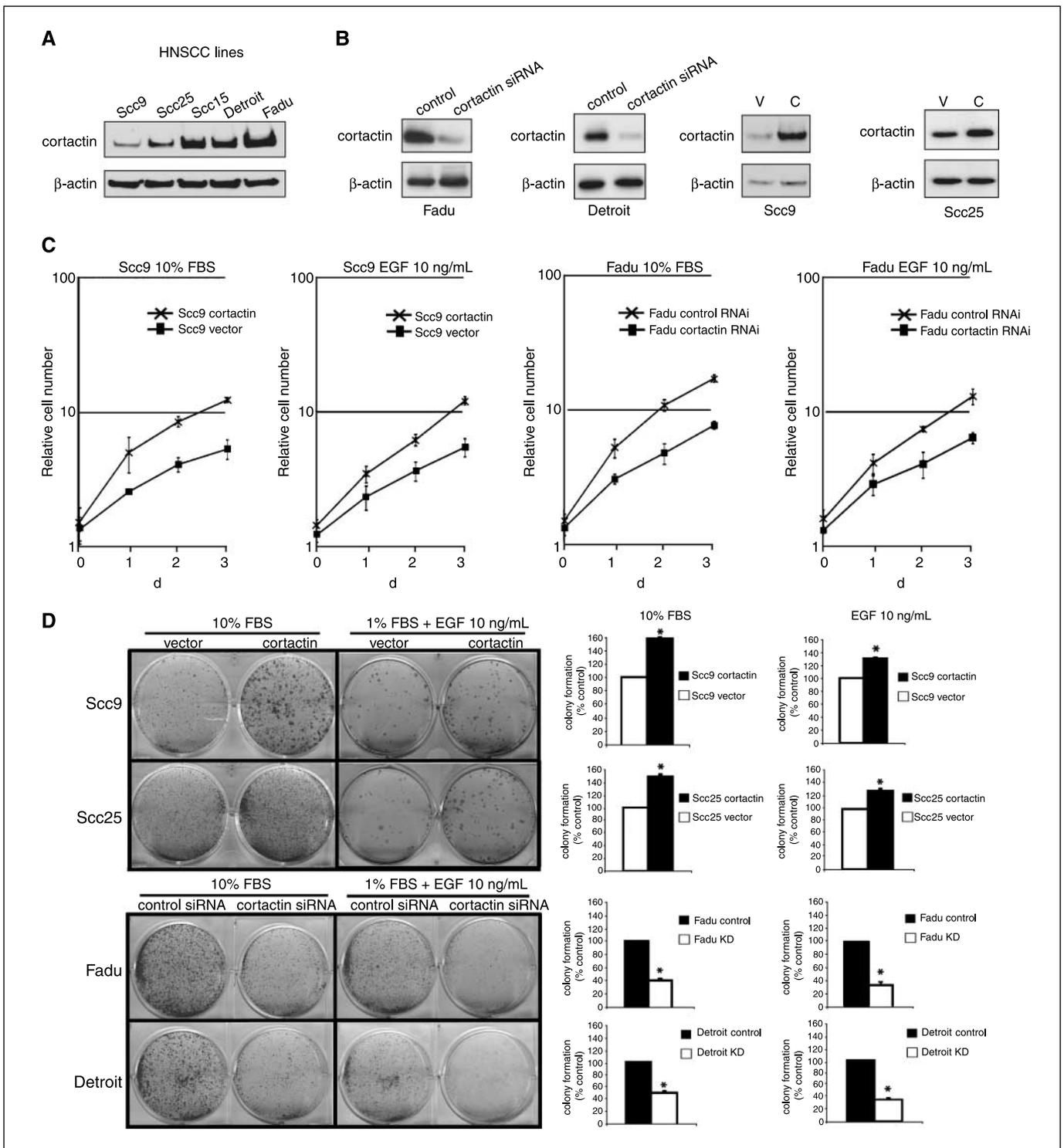


Figure 1. Overexpression of cortactin enhances anchorage-dependent proliferation of HNSCC cells. *A*, cortactin expression levels in a panel of HNSCC cell lines. Scc9 and Scc25 cells exhibit a normal *CTTN* gene copy number, whereas Fadu and Detroit cells are *11q13* amplified. Scc15 cells overexpress cortactin in the absence of *CTTN* amplification. *B*, alteration of cortactin expression in HNSCC cells. Cortactin expression was suppressed in Fadu and Detroit cells using cortactin-selective siRNAs (*left and middle left*) or increased in Scc9 and Scc25 cells by plasmid transfection (*middle right and right*). For the latter approach, cells were transfected with cortactin/pRcCMV (*C*) or with empty vector (*V*), and stable pools were isolated. Equivalent amounts of cell lysate were Western blotted for cortactin, with β-actin providing a loading control. *C*, proliferation of Scc9 and Fadu cells with altered cortactin expression. Cells were maintained in 10% FBS or 1% FBS + 10 ng/mL EGF, and cell numbers were indirectly assayed as described in Materials and Methods. Day 0 represents 24 h after plating. In each experiment, six replicates were obtained for each data point, and experiments were repeated at least thrice. *Points*, mean; *bars*, SE. *D*, effect of cortactin overexpression on colony formation. Colony-forming assays were done in six-well plates in 10% FBS or 1% FBS + 10 ng/mL EGF. Colonies were fixed and stained after 12 to 14 d (Scc9 and Scc25) or 7 to 10 d (Fadu and Detroit). In each experiment, treatments were done in triplicate. Representative images of at least six independent experiments. Colonies containing greater than ~40 cells were quantified using ImageJ software and represented as a percentage of the value for control cells (designated as 100%). *Columns*, mean; *bars*, SE. *, $P < 0.05$ by unpaired Student's *t* test, significant difference between cortactin-overexpressing and control cells. *KD*, cortactin knockdown.

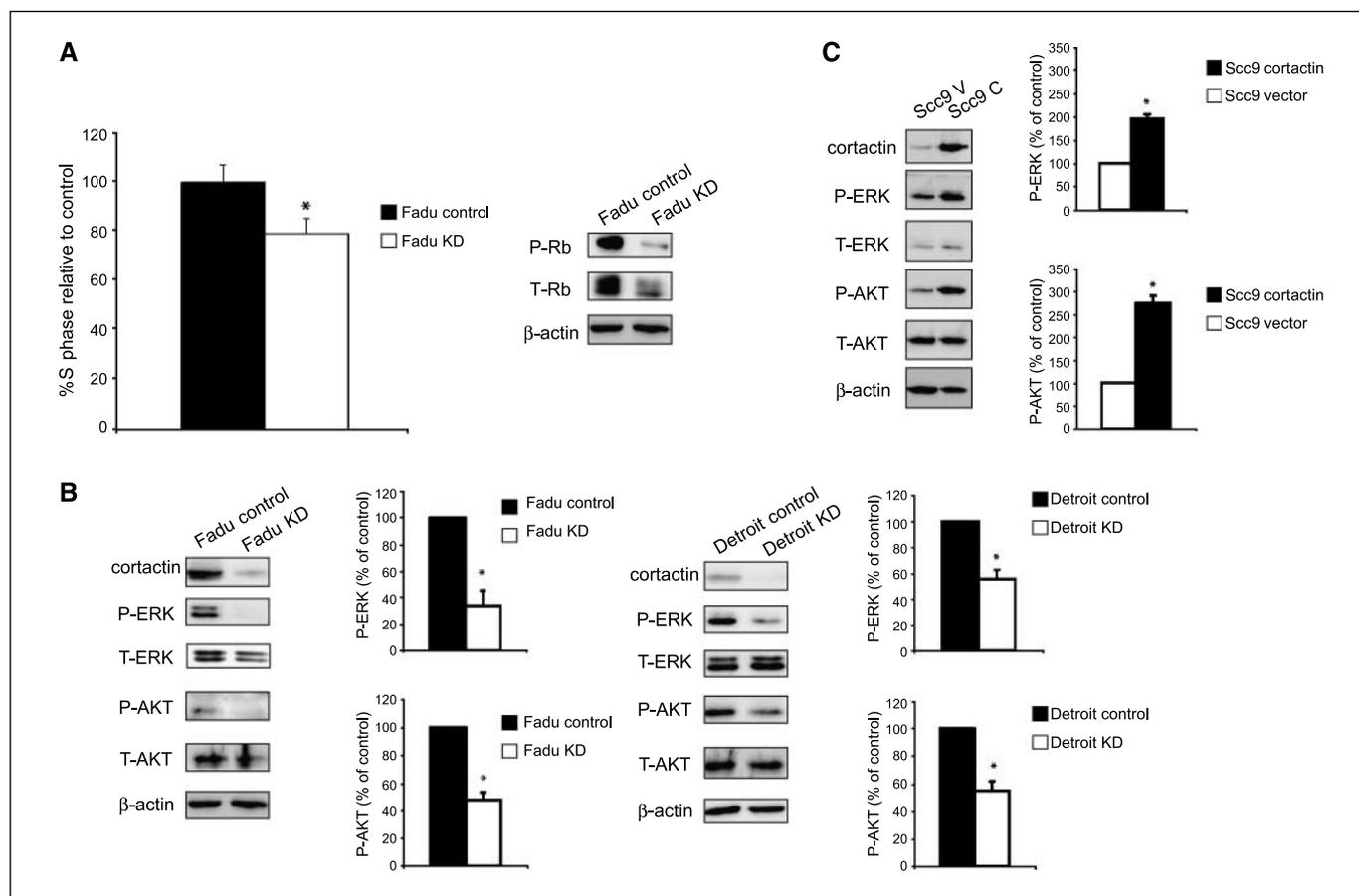


Figure 2. Elevated cortactin levels enhance specific growth factor signaling pathways and promote cell cycle progression. *A*, effect of cortactin knockdown in Fadu cells on cell cycle progression and Rb phosphorylation. Cells undergoing a colony formation assay in 10% FBS were analyzed by flow cytometry 5 d after plating. The histogram represents data from three independent experiments. In addition, cell lysates were prepared and Western blotted for phosphorylation of Rb (*P-Rb*) at Ser⁷⁸⁰ and for total Rb (*T-Rb*), with β -actin providing a loading control (*right*). *B*, effect of cortactin suppression in Fadu and Detroit cells on cellular signaling pathways. Cells were plated as for colony formation assays and harvested 5 d later. Lysates were Western blotted as indicated, with β -actin providing a loading control. The levels of phosphorylated ERK (*P-ERK*) and phosphorylated AKT (*P-AKT*) were normalized for the total amounts of each protein and expressed as a percentage of the value for control cells. The histograms represent data from three independent experiments. *C*, effect of cortactin overexpression in Scc9 cells on activation of cellular signaling pathways. Data were obtained as for (*B*), except cells were harvested on day 6. *Columns*, mean; *bars*, SE. *, $P < 0.05$, by unpaired Student's *t* test. *T-ERK*, total ERK; *T-AKT*, total AKT.

Cortactin overexpression enhances anchorage-independent growth and protects against anoikis. An important characteristic of the transformed phenotype is the ability to grow under anchorage-independent conditions. Therefore, we determined whether cortactin could enhance the growth of HNSCC cells in soft agar and modulate cellular responsiveness to specific growth factors under these conditions. When cultured in 10% FBS, cortactin-transfected Scc9 cells formed significantly more colonies in soft agar than the corresponding vector controls (Fig. 4A). Interestingly, although the addition of either EGF or HGF enhanced colony formation for both the cortactin-transfected and control cells, the increase was greater for the former pool. These data are consistent with the ability of cortactin to enhance signaling by both the EGFR (16) and c-MET (Fig. 3).

The effect of cortactin on anchorage-independent growth suggested that it may also provide protection from detachment-induced apoptosis (anoikis). When plated as a semiconfluent monolayer, cortactin-overexpressing Scc9 cells exhibited a low level of apoptosis that was not significantly different from vector controls. Culture on poly-HEMA to prevent cell attachment resulted in a marked increase in apoptotic cell death for both

cell populations, but cortactin-overexpressing cells exhibited a significant survival advantage compared with vector controls (Fig. 4B). This was associated with increased activation of AKT (Fig. 4B), but not ERK (data not shown), in detached cells relative to controls. Consistent with these data, knockdown of cortactin in Detroit cells enhanced anoikis (data not shown). Therefore, depending on the conditions, cortactin can enhance either the proliferation or survival of HNSCC cells, and both effects may contribute to increased colony formation in soft agar.

Cortactin promotes resistance to the EGFR kinase inhibitor gefitinib. A major requirement for the effective clinical use of EGFR-targeted agents is the identification of markers of therapeutic responsiveness. Our observation that cortactin attenuates down-regulation of the EGFR (16) and enhances mitogenic signaling induced by serum and specific growth factors (Figs. 1–4) raised the important question of how cortactin overexpression might affect cellular sensitivity to gefitinib. Furthermore, deregulated expression of cyclin D1, which is also encoded at chromosome 11q13, leads to increased resistance to gefitinib *in vitro* (18). Because overexpression of both cortactin and cyclin D1 occurs in HNSCC (28), cells with aberrant levels of both proteins might

exhibit altered sensitivity to this drug compared with those overexpressing either protein alone.

Earlier work examining the differential sensitivity of cancer cells to gefitinib classified sensitive cells as having a IC_{50} of 0.07 to 1.4 $\mu\text{mol/L}$ and resistant cell lines as having a IC_{50} of 3 to 16 $\mu\text{mol/L}$ (29). Consistent with previous studies, Scc9 cells (that have low cortactin and cyclin D1 expression) fell within the sensitive range, and this was not significantly affected by vector transfection (Fig. 5A; ref. 18). In contrast, Fadu and Detroit cells (that have *11q13* amplification and high expression of both proteins) exhibited a IC_{50} of ~ 20 $\mu\text{mol/L}$, placing them in the gefitinib-resistant category. Overexpression of cortactin in Scc9 cells led to a shift in the dose-response curve to the right, indicating decreased

sensitivity to the drug (Fig. 5A). However, as with cyclin D1 overexpression (18), the level of resistance observed was not as marked as with Fadu or Detroit cells.

To determine the effect of combined overexpression of cortactin and cyclin D1, two clonal Scc9 lines engineered to overexpress cyclin D1 (F5.cyclin D1 and F7.cyclin D1; ref. 18) were transfected with the cortactin expression vector. The resulting doubly transfected pools clearly overexpressed cortactin, but not to the same level as the Fadu or Detroit lines (Fig. 5B). Determination of the gefitinib sensitivity of these lines and their respective controls revealed that although cyclin D1 overexpression reduced the effect of this drug, cortactin conferred further resistance (Fig. 5C). Indeed, the IC_{50} values for the Scc9 pools overexpressing both

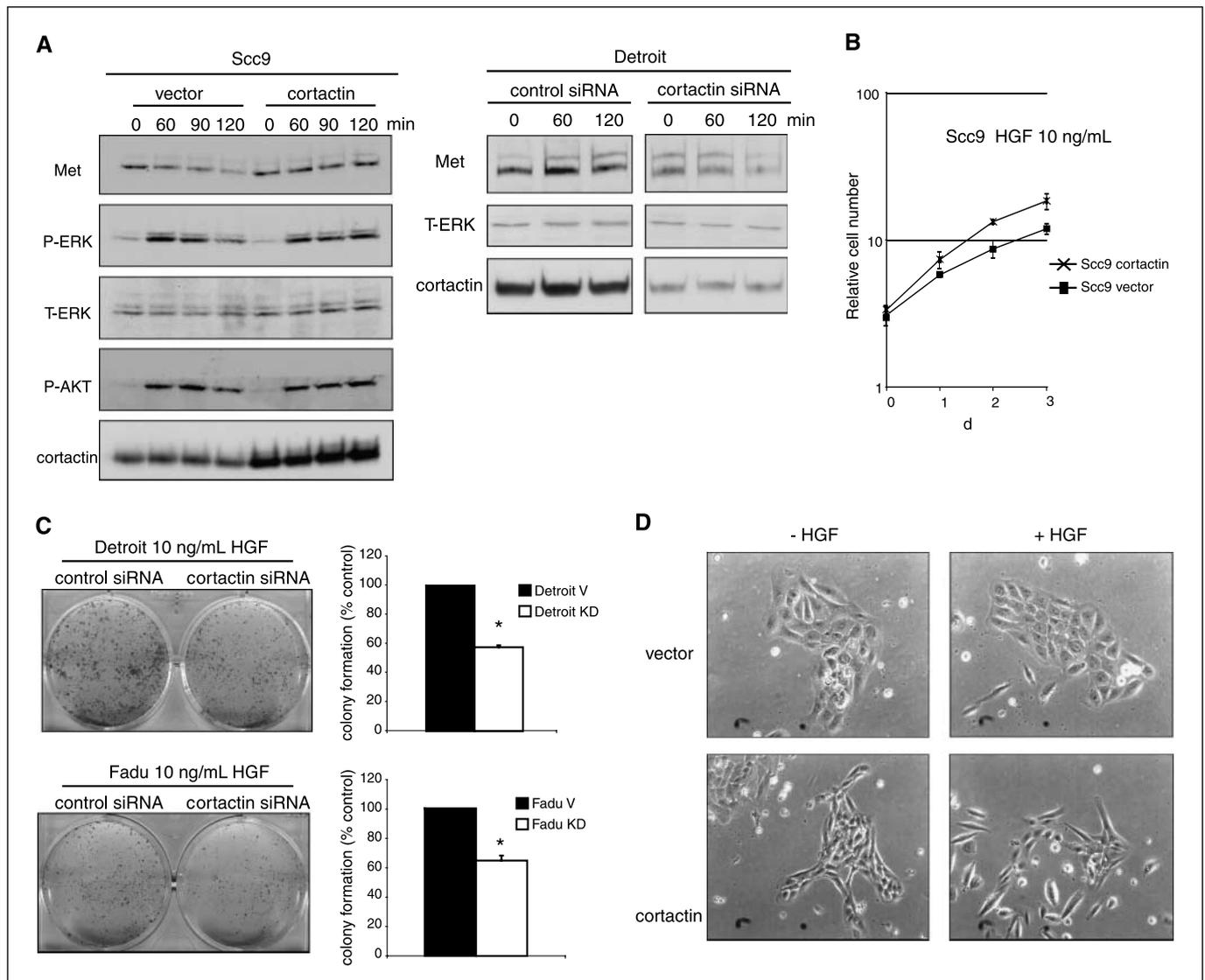


Figure 3. Overexpression of cortactin attenuates c-MET down-regulation and enhances HGF-induced biological responses in HNSCC cells. *A*, effect of cortactin on c-MET down-regulation. *Left*, control or cortactin-overexpressing Scc9 cells were stimulated with HGF (10 ng/mL) for the indicated periods and lysates were Western blotted as indicated; *right*, Detroit cells were transfected with a cortactin-selective siRNA or a control siRNA and then stimulated with HGF. Lysates were Western blotted as indicated. *B*, effect of cortactin overexpression on HGF-stimulated proliferation. Cells were maintained in 1% FBS + 10 ng/mL HGF and cell number was indirectly assayed as described in Materials and Methods. In each experiment, six replicates were obtained for each data point, and experiments were repeated at least thrice. *Points*, mean; *bars*, SE. *C*, effect on colony formation. Assays were done in six-well plates in 1% FBS + 10 ng/mL HGF. Colonies were fixed and stained after 7 to 10 d. In each experiment, treatments were done in triplicate. Representative images of at least six independent experiments. Images were quantified by densitometry and data are expressed as a percentage of the value for control cells (designated as 100%). *Columns*, mean; *bars*, SE. *, $P < 0.05$, by unpaired Student's *t* test. *D*, cortactin overexpression promotes HGF-induced cell dispersion. Cell colonies were serum starved and then stimulated with HGF (100 ng/mL) for 18 h. Representative images of at least six independent experiments.

cortactin and cyclin D1 (both ~10 μmol/L) were of similar magnitude to those for Detroit and Fadu cells.

To characterize the mechanistic basis for this effect, we assayed the effect of gefitinib on specific signaling pathways. Although gefitinib treatment of cells with low cortactin levels resulted in a marked inhibition of ERK and AKT activation, this effect was completely lost or significantly attenuated in cells with high cortactin expression (Fig. 6A). This also occurred in the independently generated lines exhibiting combined overexpression of cortactin and cyclin D1 (Fig. 6B; data not shown). Therefore, cortactin promotes gefitinib resistance by buffering specific growth factor signaling pathways against the action of this drug.

Discussion

Oncogenic subversion of RTK signaling commonly involves evasion of ligand-induced down-regulation by endocytosis (30). Previously, we reported that overexpression of cortactin represents a novel mechanism whereby cancer cells attenuate EGFR down-regulation, leading to sustained signaling (16). In this study, we have addressed the specificity and biological consequences of

this effect, providing novel insights into the role of cortactin in HNSCC.

Although effects of cortactin on cell proliferation have not been reported previously, our data clearly indicate that cortactin overexpression enhances proliferation of HNSCC cells in response to both EGF and HGF. This was evident in monolayer proliferation and colony formation assays and also under anchorage-independent conditions, where the differential in colony size between control and cortactin-overexpressing cells was significantly enhanced by the presence of these growth factors. These findings are consistent with the ability of cortactin to promote sustained signaling by the EGFR (16) and c-MET. However, serum-induced mitogenic signaling and proliferation were also increased. Under these conditions, cortactin may amplify signaling by additional RTKs and possibly other receptor types. For the EGFR, cortactin-mediated attenuation of down-regulation is associated with decreased coupling of the EGFR to the E3 ubiquitin ligase c-Cbl (16), and because c-MET is also subject to this negative control mechanism (27), this probably underlies the effect of cortactin on c-MET degradation. However, the platelet-derived growth factor receptor (PDGFR) and c-kit are also regulated by

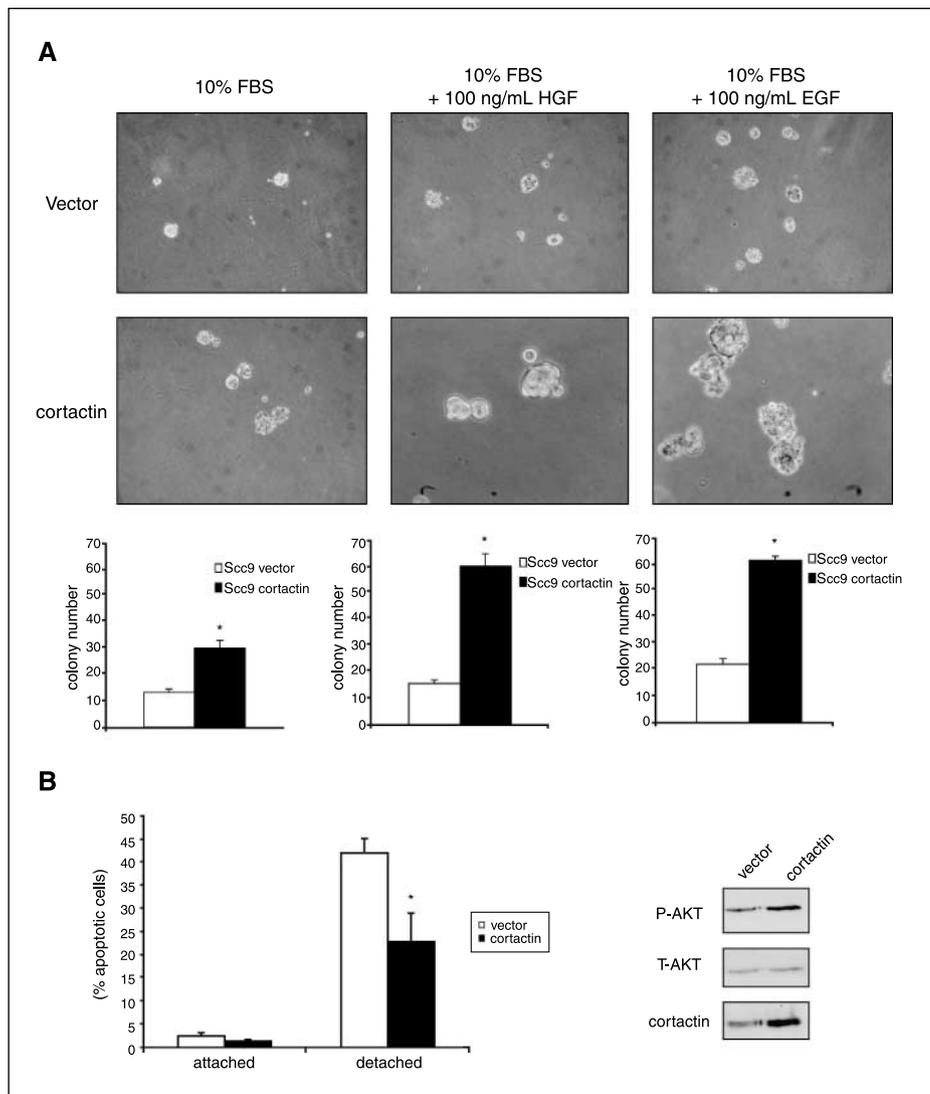
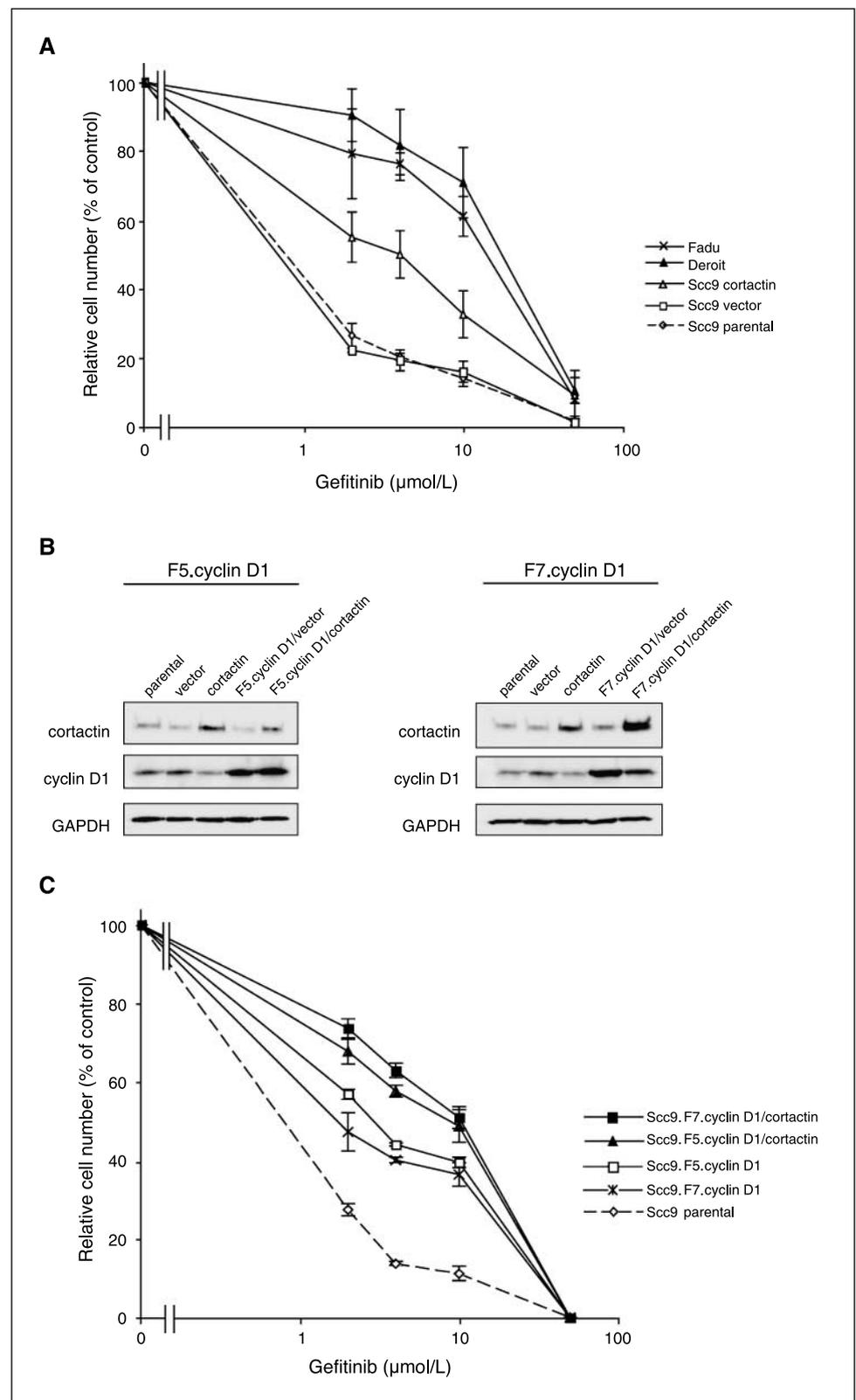


Figure 4. Cortactin overexpression enhances anchorage-independent growth and protects against anoikis. **A**, anchorage-independent growth. Control or cortactin-overexpressing Scc9 cells were grown in soft agar in the presence of 10% FBS alone, 10% FBS + HGF (100 ng/mL), or 10% FBS + EGF (100 ng/mL). Cell colonies were scored after 21 d. Data are derived from four independent experiments. **B**, anoikis and survival signaling. Cells were analyzed by flow cytometry for the hypodiploid (apoptotic) fraction after culture on poly-HEMA for 36 h. Cell lysates were obtained from cells 5 h after detachment and Western blotted with the indicated antibodies (right). Columns, mean; bars, SE. *, *P* < 0.05, by unpaired Student's *t* test (A and B).

Figure 5. Overexpression of cortactin alone or in combination with cyclin D1 enhances gefitinib resistance. **A**, effect of cortactin overexpression in Scc9 cells on sensitivity to gefitinib. For each cell line, cell number was indirectly assayed after 5 d of treatment with different concentrations of gefitinib and expressed relative to the vehicle control value, arbitrarily set at 100%. In each experiment, six replicates were obtained for each data point, and experiments were repeated at least thrice. **B**, generation of cell pools overexpressing both cortactin and cyclin D1. Scc9.F5.cyclin D1 and Scc9.F7.cyclin D1 cells (parental controls) were transfected with a cortactin expression vector or the corresponding empty plasmid (vector controls) and stable pools were isolated. Scc9 cells overexpressing cortactin alone (cortactin) are included for comparison. Cell lysates were Western blotted as indicated. **C**, gefitinib sensitivity of cells expressing high levels of both cortactin and cyclin D1. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.



c-Cbl (30) and have been detected in HNSCC cells (31). Consequently, cortactin may also modulate down-regulation and signaling by these receptors. In addition, cortactin overexpression has been shown recently to enhance recycling and ERK activation

mediated by a different class of cell surface receptor, the G protein-coupled seven transmembrane receptor CXCR4 (32). This finding indicates that aberrant cortactin expression may alter receptor endocytic trafficking via Cbl-independent mechanisms,

for example by perturbing the normally transient association of actin networks with the endocytic machinery (19, 33), and supports the hypothesis that the effects of cortactin on mitogenic receptor signaling may not be solely mediated via the tyrosine kinase class of growth factor receptors.

In addition to enhancing cell proliferation, cortactin overexpression affected two other biological responses relevant to cancer progression. First, it increased HGF-induced scattering of Scc9 cells. This is consistent with the sustained activation of ERK and PI3K in response to HGF treatment, as both pathways

contribute to disassembly of adherens junctions on c-MET stimulation (34). Second, it significantly reduced anoikis, which likely reflects the increased levels of activated AKT detected in cortactin-overexpressing, detached cells. A prosurvival role for AKT under these conditions has been documented (35) and may be mediated via suppression of Bim, phosphorylation of Bad, or up-regulation of prosurvival Bcl2 family proteins (36). Lending support to this hypothesis, knockdown of cortactin in *11q13*-amplified esophageal cancer cells enhances anoikis and decreases AKT activation (12). Overall, our findings and those of others

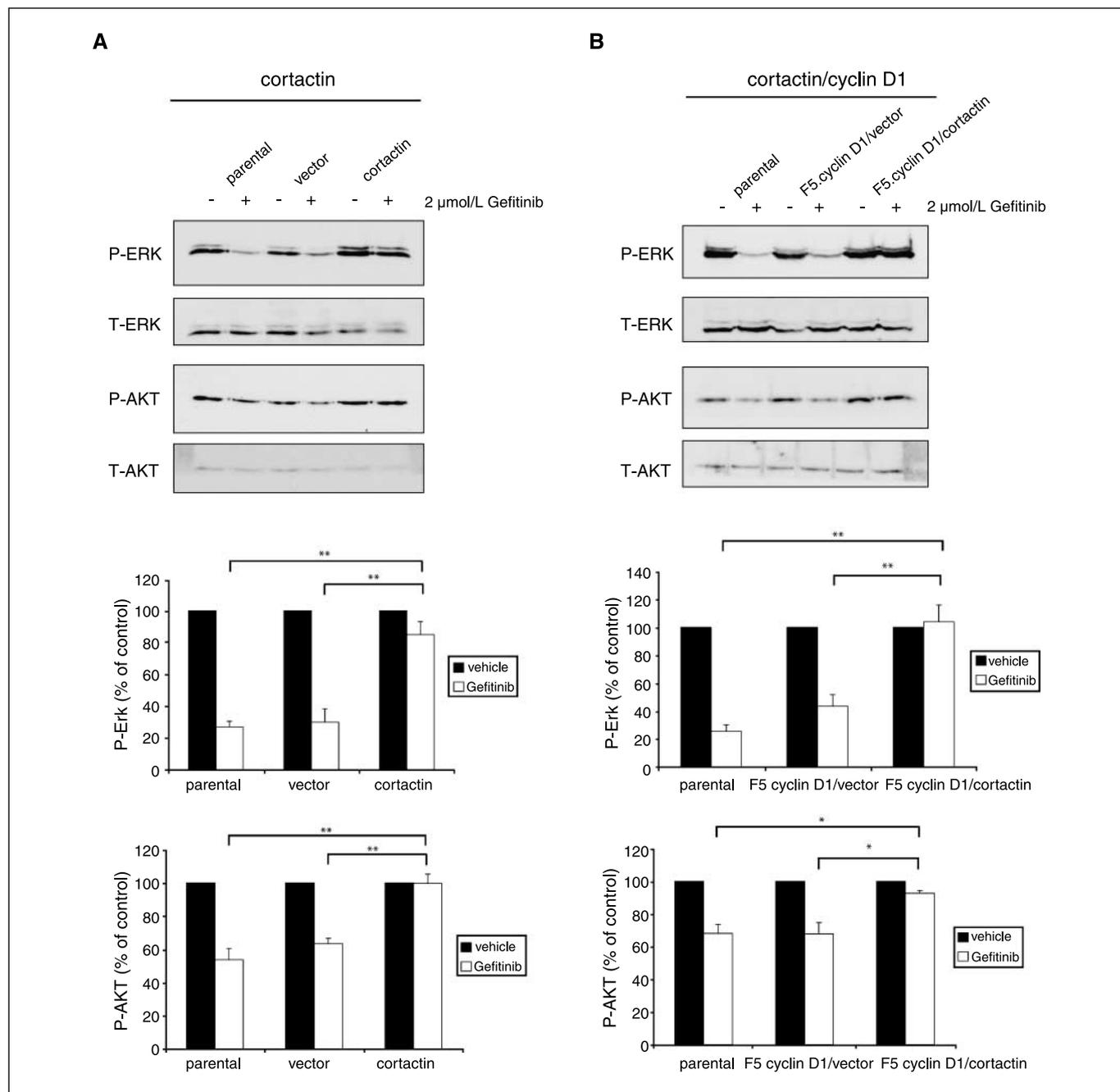


Figure 6. Effect of cortactin overexpression on inhibition of growth factor receptor signaling pathways by gefitinib. Scc9 cells overexpressing cortactin alone (A) or in combination with cyclin D1 (B) or their corresponding controls were treated with gefitinib (2 μ mol/L) for 24 h and lysates were subjected to Western blotting as indicated. The levels of phosphorylated ERK and phosphorylated AKT were normalized for the total amounts of each protein and expressed as a percentage of the control value without gefitinib treatment. The histograms represent data from three independent experiments. *, $P < 0.05$; **, $P < 0.01$, by unpaired Student's t test.

indicate that cortactin may contribute, albeit via different mechanisms, to multiple stages of tumor progression. These include proliferation of cells within the primary tumor, dissolution of cell-cell contacts, cell invasion (13), and systemic spread (12, 15). Importantly, the ability of cortactin to promote proliferative and survival signaling provides a more satisfactory explanation for *CTTN* amplification in primary tumors than its mitogenic effects.

Cortactin plays a direct role in regulating actin reorganization during cell migration, binding and activating the Arp2/3 complex, and stabilizing branched actin networks (13). Indeed, *in vivo*, cortactin binds preferentially to dynamic actin filaments, and cortactin-knockdown cells exhibit a lower production of uncapped actin filament barbed ends at the leading edge and a reduced persistence of lamellipodial protrusions (37). However, in light of the data presented in this article, an important consideration about interpretation of the effects of cortactin overexpression on cell motility is that enhanced mitogenic responses could be due to both increased activation of specific signaling effectors (due to effects of cortactin on receptor down-regulation) and direct effects of cortactin on actin dynamics. For example, the increased ERK signaling we observe in cortactin-overexpressing cells could promote motility by increasing phosphorylation of paxillin, thereby regulating focal adhesion turnover (38), or myosin light chain kinase, hence promoting contraction at the rear of the cell (39). The need to consider the potential effects of cortactin on cell signaling pathways is further highlighted by the recent work of Luo et al. (32). Here, although expression of a cortactin mutant defective in Src-mediated phosphorylation inhibited CXCL12-induced chemotaxis, consistent with other functional studies examining the role of cortactin tyrosine phosphorylation (13), this mutant also inhibited ERK phosphorylation induced by this chemokine (32). Consequently, future analyses of cortactin-regulated migration will need to consider both the direct and indirect mechanisms whereby cortactin can regulate this process.

Interestingly, cells exhibiting high levels of cortactin were less sensitive to the EGFR-selective inhibitor gefitinib. This might occur due to altered endosomal trafficking in cortactin-overexpressing cells, leading to increased dissociation of gefitinib-EGFR complexes in low pH endosomes. Alternatively, in light of our observation that the effects of cortactin on receptor down-regulation extend to c-MET and the recent report that cortactin can also modulate G protein-coupled receptor signaling (32), cortactin may amplify

proliferative signals from other receptors and thereby reduce cellular dependency on the EGFR. This is consistent with reports that depending on cell type, c-MET, the insulin-like growth factor-I receptor or PDGFR β can bypass the EGFR for mitogenic stimuli and promote gefitinib resistance (40–42). Also of note, cortactin further enhanced gefitinib resistance conferred by overexpression of cyclin D1, a cell cycle regulator also encoded at the *11q13* locus. Because gefitinib resistance due to *CCND1* amplification and/or cyclin D1 overexpression is associated with maintenance of cyclin D1 protein levels after drug treatment (18), the enhanced growth factor signaling that occurs in cortactin-overexpressing cells may block drug-induced modulation of other key regulators of the G₁-S phase transition, such as cyclin E and/or p27 (26, 43).

In summary, the data presented in this article and our earlier study (16) provide strong evidence that cortactin overexpression potentiates growth factor-induced signaling and biological responses. Because cortactin overexpression in both breast cancers (44) and HNSCCs (14) is tightly linked to *CTTN* amplification, this provides a novel explanation for the selective pressure underlying amplification of this *11q13*-localized gene. In some breast cancers and HNSCCs, *CTTN* can be amplified in the absence of *CCND1* amplification (10, 11), indicating that it can contribute to tumor progression independently of the latter oncogene, presumably by promoting proliferative and/or survival signaling. In others, coamplification occurs (10, 11), which may result in functional cooperation between the cell cycle regulatory role of cyclin D1 and the biological effects of cortactin. The ability of these two proteins to exert cooperative effects on cancer cell phenotype is highlighted by the further modulation of gefitinib sensitivity that occurs on their combined overexpression. Therefore, cyclin D1 and cortactin should be evaluated further as markers of response to EGFR-directed therapies.

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