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The role of ligand-independent activation of EGFR in E-cadherin-mediated squamous cell carcinoma cell survival

Author

Shen, Xiaodong,

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THE ROLE OF LIGAND-INDEPENDENT ACTIVATION OF EGFR IN E-CADHERIN-MEDIATED SQUAMOUS CELL CARCINOMA CELL SURVIVAL

by

XIAODONG SHEN

DISSERTATION

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ABSTRACT

THE ROLE OF LIGAND-INDEPENDENT ACTIVATION OF EGFR IN E-CADHERIN-MEDIATED SQUAMOUS CELL CARCINOMA CELL SURVIVAL

XIAODONG SHEN

The survival of squamous epithelial cells requires signals generated by integrin-matrix interactions. Following conversion to squamous cell carcinoma, the cells remain sensitive to detachment-induced anoikis, yet in tumor cell aggregates, which are matrix deficient, these cells are capable of suprabasal survival. Their survival is enhanced through a process we call *synoikis*, whereby junctional adhesions between neighboring cells generate specific downstream survival signals. Here we tested the hypothesis that activation of EGFR and downstream signaling pathway(s) are required for E-cadherin-mediated cell survival.

We found that in squamous cell carcinoma cells, both c-erbB family members EGFR and erbB2 were expressed. By using immunofluorescence staining and immunostaining, we have demonstrated the colocalization of EGFR and E-cadherin at cell-cell junctions in SCC cells grown as multicellular aggregates. E-cadherin-mediated cell-cell adhesion specifically induced activation of EGFR, but not erbB2 or another tyrosine kinase receptor c-Met.

Next we demonstrated that EGFR activation in turn triggered the ERK/MAPK signaling module, leading to elevation of anti-apoptotic Bcl-2. Using pharmaceutical inhibitors, we found that EGFR and the downstream ERK/MAPK activation were required for E-cadherin-mediated cell survival. Ectopic expression of E-cadherin in a receptor-negative cell line was sufficient to confer resistance to anoikis.

In additional studies, we investigated the mechanism of EGFR activation and found that after intercellular adhesion, formation of adherens junctions triggers the recruitment of E-cadherin–EGFR complexes to the sites of cell-cell contacts, correlating with EGFR transactivation. E-cadherin-mediated EGFR activation was also dependent on

cytoskeleton organization. Analysis of the process with a dominant-negative EGFR mutant indicates that activation of EGFR was ligand independent.

Our data implicate cell-cell adhesion–induced activation of EGFR as a cooperative mechanism that generates compensatory survival signaling, protecting cells from detachment-induced death.

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CHAPTER ONE: INTRODUCTION

Survival of normal epithelial cells depends on signals generated by their interaction with a thin extracellular matrix called the basement membrane. In the absence of these signals, the cells die, exhibiting molecular characteristics of apoptosis (Ruoslahti and Reed, 1994; Frisch and Ruoslahti, 1997). This form of apoptosis is also called anoikis (Frisch and Francis, 1994; Meredith et al., 1996). By contrast, many cancer cells of epithelial origin are anoikis resistant: they can survive in the absence of contact with the basement membrane inside three-dimensional tumor nests and in the absence of matrix attachment during metastasis (Frisch and Screaton, 2001). Therefore, acquisition of anoikis resistance plays an essential role in the progression of certain cancers.

Some of the mechanisms that confer anoikis resistance on epithelial cells during tumor progression have been described (Khwaja et al., 1997; Rosen et al., 2000; Jost et al., 2001; Tran et al., 2002). Our previous work using a three-dimensional spheroids model implicated E-cadherin–mediated cell-cell adhesion in protecting oral squamous cell carcinoma (SCC) cells from anoikis (Kantak and Kramer, 1998). However, the exact mechanism of E-cadherin–mediated anoikis resistance is unclear.

Because cadherins lack enzymatic activity, their ability to induce cell survival signals may depend on their association with other signaling systems. E-cadherin can physically associate with a number of signaling effectors, such as PI3K and PTP1B, in adherens junctions (Pece and Gutkind, 2000; Shinohara et al., 2001; Arregui et al., 2000; Kovacs et al., 2002). In this study we focused on the growth factor receptor tyrosine kinase, epidermal growth factor receptor (EGFR), since it is well known that in most oral SCCs and in cell lines established from these tumors EGFR is overexpressed (Todd and Wong, 1999) and may have a role in cell survival. We hypothesized that E-cadherin–

mediated cell-cell adhesion transactivates EGFR and that activation of EGFR and the downstream pathways promotes resistance to anoikis.

In order to provide background information, several issues closely related to the current study will be introduced as follows.

Anoikis and tumor progression

Survival of normal epithelial cells is dependent on signals generated by the interaction of these cells with components of their basement membrane (Ruoslahti and Reed, 1994; Frisch and Ruoslahti 1997). The absence of such signals results in rapid activation of programmed cell death (apoptosis). This form of apoptosis is also termed anoikis or death of homelessness, as it is believed to affect cells outside of their proper tissue context (Frisch and Francis 1994; Meredith and Schwartz 1997). Anoikis is thought to play an important role in maintaining proper tissue architecture by precluding reattachment and growth of epithelial cells at ectopic locations (Frisch and Ruoslahti 1997).

Numerous kinase/phosphatase signaling molecules have been implicated in anoikis regulation. The best-characterized cell survival pathway that transmits the cell-matrix interaction-generated signals is the integrin-signaling pathway (Frisch and Ruoslahti, 1997). The key mechanism for the integrin-mediated signal transduction is the activation of intracellular tyrosine kinases such as FAK (Frisch and Ruoslahti, 1997; Hanks and Polte, 1997). Engagement of integrins induces phosphorylation and activation of tyrosine kinases. Activated tyrosine kinases recruit and phosphorylate intracellular

signaling adaptor proteins and trigger a number of signaling pathways that regulate cell survival and growth. The importance of the integrin/tyrosine kinase pathway in cell anchorage-dependent survival and growth has been demonstrated by introducing constitutively active form of FAK into the anchorage-dependent cells to render them anchorage-independent (Frisch et al, 1996). Two major cell survival signal and growth signaling pathways downstream of tyrosine kinases are the PI3K/Akt and MAPK pathways (Marte and Downward, 1997; Downward, 1998). The activation of both PI3K and MAPK are regulated by integrin-regulated tyrosine kinases. Transfection of cells with constitutively active forms of PI3K or Akt blocks loss-of-anchorage-induced apoptosis, whereas inhibition of PI3K induces apoptosis (Khwaja et al, 1997; Moro et al, 1998). The MAPK pathway has also been shown to be involved in regulating cell survival (Bonni et al, 1999; Ishikawa and Kitamura, 1999). Therefore, PI3K/Akt and MAPK pathways are crucial for mediating cell survival signals from cell-matrix interactions.

Clear evidence for the importance of anoikis in maintaining epithelia integrity comes from the contribution to neoplasia of the breakdown of anoikis. Several studies of anoikis from different aspects indicate that the acquisition of anoikis resistance plays a central role in the progression of tumors of epithelia origin. First, solid tumors grow *in vivo* as multicellular masses in which the cells are forced to survive in the absence of attachment to a properly formed basement membrane. Second, most cell lines derived from such solid tumors are capable of growing in an anchorage-independent manner as colonies in soft agar or suspension culture (Schwartz, 1997). Third, nonmalignant epithelial cells, selected for their ability to resist apoptosis in tissue culture,

simultaneously acquire a tumor-forming capacity (Rak et al., 1999). Fourth, suppression of the resistance to loss-of-anchorage-induced apoptosis in cultured transformed epithelial cells strongly inhibits their tumorigenicity (Rosen et al., 1998). Fifth, transfection of nonmalignant epithelial cells with various oncogenes commonly associated with epithelial malignancies, such as mutant H- or K-*ras*, induces resistance to loss-of-anchorage-induced apoptosis (Frisch and Francis, 1994; Rak et al., 1995).

E-cadherin

E-cadherin belongs to a family of classical adhesion molecules called cadherin. The term 'cadherin' was introduced by Takeichi and stems from "calcium-dependent cell-cell adhesion system" (Yoshida-Noro et al., 1984). As the name indicates, cadherins constitute a major class of single-pass transmembrane glycoproteins that mediate homophilic calcium-dependent cell-cell adhesion in all solid tissues of the body. Cadherins play a key role in cell-cell recognition by establishing tight cell-cell adhesion as well as defining adhesive specificities of cells. During development, segregation of cells into distinct tissues is correlated with regulated expression of different cadherin family members (Takeichi, 1991). In addition, cadherins have been implicated in a number of signaling pathways that regulate cellular behavior. Because of the importance of cadherins to cell recognition, adhesion, sorting, and signaling, disruption of cadherin function has significant implications in diseases, including cancer.

The homophilic cell-cell interaction is mediated by the extracellular domain of cadherin. Classical cadherins contain an extracellular domain consisting of five cadherin-

type repeats that mediate mainly, but not exclusively, homophilic interactions with cadherin molecules on neighboring cells. This means that cells adhere preferentially to other cells expressing the identical cadherin family members. This specificity of homophilic binding has been implicated in development as a mechanism by which various cells organize into tissues (Takeichi, 1991). Several lines of research suggest that the extracellular domain of cadherin exists as a lateral dimer and prompt the proposal that cadherins cluster into homophilic binding complexes via *cis* interactions between cadherins on the surface of the same cell (Shapiro et al., 1995; Nagar et al, 1996; Brieher et al, 1996).

The majority of classical cadherins are transmembrane components of adherens-type junctions and, as such, have a cytoplasmic domain that interacts with catenins, which in turn link the cadherin to the actin cytoskeleton and facilitate clustering into the junctional structure. Of all the proteins associated with the cadherin cytoplasmic domain, α- and β-catenin have been shown to participate in adhesion (Nagafuchi and Takeichi, 1988; Hirano et al, 1992; Watabe et al, 1994; Cox et al, 1996). β-catenin binds directly to the cadherin cytoplasmic tail via its armadillo repeats and serves as a linker to α-catenin, which as a whole called cadherin-catenin complex (Hulsken et al, 1994; Funayama et al, 1995). Other proteins that have been found to be associated with cadherin-catenin complex include kinases {Hoschuetzky, 1994; Pece et al, 1999; Arregui et al, 2000), phosphatases (Brady-Kalnay et al, 1995; Kypta et al, 1996), adaptor proteins (Xu et al, 1997; Shinohara et al, 2001) and the p120 src-kinase substrate (Staddon et al, 1995).

E-cadherin is arguably the prototype member of classical cadherin family and is typically found in the adherens junction, which is a cellular structure found near the

apical surface of polarized epithelial cells. The cadherin-catenin complex is characteristically present in the adherens junction in normal epithelium, which is critically important for the structural and functional integrity of epithelia. In contrast, loss of E-cadherin expression and/or disruption of cadherin-catenin complex has been demonstrated in carcinomas and been correlated with tumor dedifferentiation, metastasis, invasion, and poor prognosis (Bankfalvi et al, 2002).

Signal transduction and cadherins

The central role of cadherins in both stable cell-cell adhesion and dynamic tissue patterning, as well as the similarity between the homophilic ligation of cadherin cytoplasmic domain and other signaling systems such as growth factor receptors and integrins, raises a issue that whether cadherins participate in intercellular signaling. So far more and more evidence suggests that cadherins do exert some of their effects through signal transduction although direct cadherin-activated signaling has been hard to verify.

One attractive model of how cadherins may influence signal transduction is by organizing different signaling molecules into a signaling complex. In other words, cadherins and adherens junction may serve as a scaffold for individual signaling components. Many signaling molecules concentrate in adherens junction in epithelial cells, including src family members (Tsukita et al, 1991), PI3K (Pece et al., 1999; Tran et al., 2002), Gab1 (Shinohara et al., 2001) and PTP (Kypta et al, 1996). Although little direct evidence has been found for such a mechanism, there are studies showing that at

least some of the signaling molecules complexed with adherens junction transduce signals from cadherins to the cytoplasm (Pece et al., 1999; Tran et al., 2002).

Another indirect mechanism by which cadherins may affect signal transduction is by facilitating close cell-cell contact (Fagotto and Gumbiner, 1996). When cells are in close contact of each other, many juxtacrine-type membrane receptors are activated by their membrane-bound ligands, and transduce signals into the cells. Examples of this phenomenon include notch and delta in *Drosophila* nerve development (Artavanis-Tsakonas et al, 1995), sevenless and bride-of-sevenless in *Drosophila* eye development (Hafen et al, 1993) and the observation that transmembrane TGF-α precursors on one cell are able to activate EGF receptors expressed on an adjacent cell during intercellular adhesion (Bosenberg and Massague, 1993; Shi et al., 2000).

Although direct cadherin-activated signaling following homophilic ligation is difficult to identify, recent research has shed light into this area. Using recombinant cadherin-specific adhesive ligands to dissect the cellular consequences of homophilic adhesive binding from secondary effects, several groups have found that early-immediate Rho family GTPase signaling is regulated by cadherin homophilic ligation and in turn coordinate cadherin-actin integration at the cell membrane (Noren et al, 2001; Kovacs et al, 2002). State simply, the adhesive ligation of cadherin cytoplasmic domains stimulates the intracellular signaling, which suggests that cadherins function both as cell-cell adhesion mediators and adhesion-activated signaling receptors.

EGFR and Squamous Cell Carcinoma

EGF receptor is the prototypal member of the tyrosine kinase receptor superfamily (reviewed in Wells, 1999). In mammals, the EGFR family consists of four members: EGFR/c-erbB1/HER1, c-erbB2/HER2, c-erbB3/HER3 and c-erbB4/HER4. The ability of one c-erbB family member to influence or function synergistically with another is a general feature of these receptors. The c-erbB ligands consist of more than 30 members including EGF, transforming growth factor alpha (TGF-α), amphiregulin, betacellulin, epiregulin, and heparin-binding-EGF-like growth factor. Some of these ligands like EGF, TGF-α and amphiregulin directly bind to the ectodomain of EGFR and provoke its activation through a mechanism that involves dimerization, activation of the cytoplasmic tyrosine kinase domain, and autophosphorylation of the receptor (Downward et al., 1984). This process leads to the recruitment of phosphotyrosine-binding effector proteins and initiates activation of subsequent multiple signaling pathways. Upon ligand binding and activation, EGFR undergo internalization via endocytosis (Wells, 1999). The fate of EGFR depends on the type of ligand as EGF mediates receptor degradation and activation by TGF-α leads to receptor recycling. In consequence, ligand-induced internalization and degradation results in signal attenuation.

Evidence suggests that EGFR is also part of signaling networks activated by stimuli that unrelated to EGFR ligands. These stimuli including other types of membrane receptors such as G protein-coupled receptor (GPCR), membrane depolarization, physical and chemical stressors like UV radiation and engagement of adhesion receptors of the integrin family (Hackel et al, 1999; Thomas and Brugge, 1997; Rosette and Karin, 1996;

Moro et al., 1998). The mechanisms of EGFR transactivation by these stimuli can be divided into two categories: ligand dependent and ligand independent (Carpenter, 2000). It has been shown that GPCR agonists (Prenzel et al, 1999) or ionizing radiation (Dent et al, 1999) transactivate EGFR through stimulation of a protease activity that results in the cleavage of EGF-like precursors and the production of soluble ligands. In contrast integrin-mediated EGFR transactivation has been proposed as ligand-independent (Moro et al., 1998; Liu et al, 2002). Activation of EGFR by such stimuli enables the receptor to be involved in more complex signaling events and cellular responses initiated by these seemingly unrelated and indirect means.

EGFR is widely expressed in non-hematopoietic tissues and thus is linked to a wide range of biological responses. After activation, EGFR kinase induces multiple downstream signaling pathways including Src, Ras/MAPK, PI3K, STATs, and PLCγ in different cell types (Schlessinger and Ullrich, 1992), which then lead to pleiotropic biological responses including mitogenesis, apoptosis or survival, enhanced cell motility, protein secretion, and differentiation or dedifferentiation (Schlessinger and Ullrich, 1992). In addition to being implicated in organ morphogenesis, maintenance and repair, upregulated EGFR signaling has been correlated in a wide variety of tumors (Partanen, 1990; Puddicombe et al., 2000; Mendelsohn and Baselga, 2000).

Comprehensive data over the past 20 years strongly support the role of EGFR and its ligand in tumor progression, providing one of the first links between an activated oncogene and tumor formation (Downward et al, 1984). A variety of studies have demonstrated that EGFR is overexpressed in a number of human solid tumors including

Squamous Cell Carcinoma (SCC). For example, overexpression of EGFR and its ligand TGF-α has been reported in 80% to 90% of head and neck SCC tumors (Grandis et al, 1993). EGFR mRNA and protein expression are also increased in dysplastic lesions and histologically normal mucosa from SCC patients (Bergler et al, 1989; Grandis et al, 1998), suggesting that EGFR upregulation represents an early event in SCC carcinogenesis. Besides its growth-regulatory effect, EGFR involves in SCC progression by promoting motility, invasion, survival and angiogenesis (Ozawa et al, 1987; Chen et al, 1994; P et al, 2001; Lee et al, 1990; Petit et al, 1997).

Spheroids model

While most cells lines are grown traditionally *in vitro* as flatted monolayers, certain methods can be employed to grow cells as three-dimensional aggregates, or spheroids. These methods generally require conditions that will not allow the cells to adhere to the substrate on which they are plated. For example, coating the tissue culture dishes with a thin layer of polyHEMA (2-hydroxylethyl-methacrylate) will prevent the deposition of matrixes for cells to attach. Under these conditions, many cell types, especially tumor-derived cell lines, will spontaneously form aggregates.

Three-dimensional spheroid cell culture has been used for over 50 years mainly by radiation biologists (Sutherland and Durand, 1976; Sutherland et al., 1981). It is only more recently that the potential benefits of this culture system have been widely appreciated. Consequently, spheroids models have lead to an increased understanding of

differentiation, tissue organization and homeostasis. In addition, it has been commonly accepted that strong similarities exist in morphology and many functional characteristics between spheroid cultures and in vivo solid tumors.

The benefits of using spheroids cell culture to study the complex mechanisms involved in cell-cell adhesion are quite obvious. Without attachment to matrix, the cells cultured as spheroids in suspension only interact with each other, which is a more defined system of cell-cell adhesion than monolayer culture. For example, the colon carcinoma cell line LIM 1863, a spontaneous spheroid cell line, has been used to investigate cellular mechanisms involved in the maintenance of organic structure (Hayward and Whitehead, 1992). Furthermore, numerous recent studies have concluded that cell adhesion and cell death are closely related. However, many of these studies were performed on monolayer cell cultures which, in many cases, cannot model realistically the processes in living tissues. This is especially true in the study of apoptosis resistance of tumor cells since solid tumors do not grow two-dimensionally, but rather in a three-dimensional array. Thus, three-dimensional cell culture like multicellular spheroids, which mimic many of the intercellular relationships in solid tumors, maybe more useful for the study of tumor cell survival.

OUTLINE OF PRESENT STUDY

In this study, we used a three-dimensional multicellular model of SCC to study the mechanism of E-cadherin-mediated anoikis resistance. In the first part of the study, we examined expression pattern of c-erbB family members in different SCC cells and performed experiments to confirm that EGFR is activated upon E-cadherin-mediated cell-cell contact formation in SCC cells using both immunofluorescence staining and Western blot. In second part of the study, we first confirmed that activation of EGFR and the downstream MAPK pathway is required for E-cadherin–mediated anoikis resistance in SCC cells using specific pharmacologic inhibitors. Next, we transfected E-cadherin into E-cadherin-negative oral SCC cells and found that exogenous E-cadherin expression induced EGFR activation upon cell-cell adhesion and rescued E-cadherin-negative oral SCC cells from anoikis. Furthermore, we found that EGFR and MAPK activation is required for maintaining high level of Bcl-2 expression in SCC MCAs. In third part of the study, we analyzed the EGFR activation following cell-cell adhesion and found that EGFR activation is E-cadherin and actin cytoskeleton organization dependent. In search of an underlying mechanism(s) by which E-cadherin transactivates EGFR, we provided evidence that E-cadherin-mediated EGFR activation is ligand-independent and is related to E-cadherin/EGFR complex formation and clustering. Taken together, our results suggest that E-cadherin-mediated cell-cell adhesion promotes cell survival through ligand-independent activation of EGFR and the downstream MAPK pathway.

CHAPTER TWO: MATERIALS AND METHODS

Materials

Human recombinant epidermal growth factor (EGF) and Human recombinant hepatocyte growth factor (HGF) was purchased from Invitrogen. Pharmacologic inhibitors of EGFR (tyrphostin AG1478) were from Calbiochem, MEK1/2 inhibitor (U0126) from Promega, PI3K inhibitor (LY294002) from Cell Signaling Technology, and actin polymerization inhibitor (cytochalasin D) from Sigma. A mouse anti–E-cadherin monoclonal antibody (mAb) (HECD-1; obtained from M. Takeichi, Kyoto University, Japan) was used in immunoprecipitation, immunofluorescence staining, and antibody inhibition and clustering experiments. A rat anti–E-cadherin mAb (E9; obtained from C. Damsky, University of California, San Francisco) was used in Western blotting. Another rat anti-E-cadherin mAb (Decma-1; Sigma) was used in immunofluorescence staining. mAbs against EGFR or tyrosine-phosphorylated EGFR (activated EGFR) (clones 13 and 74; Transduction Laboratories) were used in Western blotting and immunofluorescence staining. Antibodies raised against the extracellular domain of EGFR (Ab-11; NeoMarkers) were used in Western blotting. Antibodies raised against phosphorylated EGFR tyrosine 1068, 1086, 1148 or 1173 (pY1068 EGFR, pY1086 EGFR, pY1148 EGFR, pY1173 EGFR; Biosource) were used in Western blotting. Antibodies against phospho-MAPK (phospho-p44/42-Thr202/Tyr204; Cell Signaling Technology) and MAPK (anti-pan-ERK mAb; Transduction Laboratories) were used in Western blotting. Monoclonal antibody (DO24) against the extracellular domain of the human c_Met receptor (Upstate Biotechnology) was used for immunoprecipitation. Polyclonal antibody (C-28) against the C-terminal peptide of the c-Met receptor (Santa

Cruz Biotechnology) was used for Western blot analysis. Mouse monoclonal antibody to phosphotyrosine (PY20; Transduction Laboratories) was used for Western blot analysis. A mouse anti–Bcl-2 mAb (ascites; Dako) was used in immunoprecipitation and Western blotting. A polyclonal antibody to Bax (Ab-1; Oncogene) was used in Western blotting. For loading control, a mouse anti-tubulin mAb (Ab-4; NeoMarkers) was used in Western blotting.

Cell Culture

The human oral SCC cell lines HSC-3 and HOC-313 clones (D1 and C8) have been described previously {Kantak, 1998 #10}. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) (Gimini). HOC-313 cells were cloned by serial dilution of a single-cell suspension plated in 96-well plates. Single-cell clones were randomly selected for further culture.

Cell monolayer cultures were prepared by seeding 6×10^5 cells in tissue culture dishes (10 cm; Falcon). For the culture of HSC-3 and HOC-313 cells in suspension, monolayers were trypsinized the day before and then briefly treated with 5 mM EDTA to prepare single cells. To generate multicellular aggregates (MCAs), cells were then plated on polyhydroxylethyl-methacrylate (poly-HEMA)–coated 10-cm dishes (2×10^6 cells/dish) in the presence of Dulbecco's modified Eagle's medium supplemented with 0.5% FBS. To produce suspended single cell cultures, cells were suspended in semisolid medium consisting of 0.5% FBS/Dulbecco's modified Eagle's medium containing 1.5% methylcellulose (Sigma) at 6×10^5 cells per 10-cm poly-HEMA–coated dish.

Recombinant Adenovirus Infection

The infection of HSC-3 cells by recombinant adenoviral vectors was performed as described previously (13). Briefly, the recombinant adenoviral vectors carrying EGFR-CD533 or green fluorescent protein (GFP) (gifts from K. Valerie, Virginia Commonwealth University, Richmond) were added to HSC-3 cells at a multiplicity of infection of 100. Cells were then incubated for 24 h before further experiments to ensure adequate expression of the genes of interest.

Transfection

To generate the E-cadherin or Bcl-2 expression vector, the full-length mouse E-cadherin cDNA or human Bcl-2 cDNA was subcloned into the pcDNA3 vector (Invitrogen). For constitutive expression of E-cadherin or Bcl-2, 1×10^6 E-cadherin–negative HOC-313 C8 clonal cells or HSC-3 cells were transfected with 4 μg of pcDNA3/E-cadherin vector or pcDNA3/Bcl-2 vector, respectively, using the Lipofectamine Plus kit (Gibco-BRL) according to the manufacturer's protocol. Stable transfected cells were selected in 800 μg/ml of G418 (Gibco-BRL) for 2 weeks.

Immunofluorescence Staining

HSC-3 cells were plated as MCA culture for the indicated times and then transferred onto poly-L-lysine–coated glass coverslips and incubated for 30 min at 37°C. After washing with 0.1 mM Na_3VO_4 in PBS, cells were fixed and permeabilized for 15 min at room temperature with 4% paraformaldehyde and 0.1% Triton X-100. Cells were stained with primary antibodies and counterstained by either fluorescein- or rhodamine-

labeled secondary antibodies. Analysis was performed with a confocal microscope (Bio-Rad).

Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting were performed as described previously (14). Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% NP-40, 0.1% SDS, 5 mM EDTA, 2 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 1 mM sodium vanadate). Cell lysates were processed for immunoprecipitation with specific antibody. The immunoprecipitates or total cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis, transferred to Immobilon membranes, and probed with appropriate antibodies. Immunoreactive bands were visualized using ECL (Amersham Pharmacia Biotech).

Antibody Clustering Assay

HSC-3 cells grown to near confluence were serum-starved overnight. For antibody-mediated clustering, cells were harvested from culture with 5 mM EDTA, washed with PBS, and plated on poly-HEMA–coated dishes. The cells in suspension were incubated for 30 min at 4°C with a predetermined saturating concentration of HECD-1 antibody (20 μg/ml) in serum-free medium. The cells were then incubated with 5 μg/ml goat anti-mouse IgG for various times at 37°C to induce clustering of E-cadherin. At the end of each time period, the cells were washed with ice-cold PBS containing 10 mM NaF, 10 mM $Na_4P_2O_7$, and 0.5 mM Na_3VO_4, and lysed with RIPA buffer, followed by immunoblotting.

Apoptosis and Viability Assays

To determine cell death, a DNA-laddering assay and a FACS-based TUNEL assay were used. For intranucleosomal DNA laddering, genomic DNA was extracted using a Suicide Track DNA Ladder Isolation Kit (Oncogene Research). Samples were then analyzed in a 1.5% agarose gel. For the TUNEL assay, cells were collected from poly-HEMA dishes by pipetting or from tissue culture dishes by scraping into the medium in which they had been incubated. In monolayer cultures, floating cells were collected and combined with the attached cells before DNA extraction. Cells (2×10^6) were fixed for 15 min in 1% paraformaldehyde in PBS, followed by 30-min fixation in 70% ethanol. The fixed cells were then washed, stained with FITC-dUTP, and treated with propidium iodide/RNase using an APO-DIRECT kit (Pharmingen), followed by flow cytometric analysis using a FACScan Analyzer (Becton Dickinson).

To assess the proportion of cells that not only survived but also regained proliferative ability after suspension, cells from suspension cultures were replated on tissue culture plates at low cell density in Dulbecco's modified Eagle's medium supplemented with 10% FBS. The clonogenic and proliferative ability of cells that reattached after replating was determined 3 days later by crystal violet staining and counting of colonies.

***In Vivo* Growth Assay**

The effect of EGFR blockade on tumor growth *in vivo* was tested in a xenograft model in athymic nude mice as described previously {Busse, 2000 #56}. Briefly, five- to

six-week-old female Balb/c nu/nu mice (Charles River Laboratories) were injected subcutaneously with HSC-3 cells (5×10^6). Beginning three days after implant, eight mice per group were randomly assigned to receive intraperitoneal injection of either 50 mg/kg AG1478 in dimethyl sulfoxide (DMSO) or DMSO alone, once every two days for 28 days. Tumor diameters were measured with calipers, and tumor volumes (in mm^3) were calculated by the following formula: volume = $(width^2 \times length)^2$.

CHAPTER THREE: RESULTS

PART 1: EGFR Activation in SCC cells Cultured as Multicellular Aggregates

It is well known that in most oral SCCs and in cell lines established from these tumors EGFR is overexpressed. Recently, increased level of other c-erbB receptor family members like erbB2 and erbB3 have also been found in some SCC cell lines. In the present study, we analyzed the expression of c-erbB family members in several oral SCC cell lines and evaluated the activation status of these receptors.

Expression pattern of c-erbB family members in oral SCC cell lines

To establish the expression pattern of c-erbB family members in oral SCC cell lines, we analyzed EGFR, erbB2, and erbB3 expression level using immunoblotting. As shown in Fig. 1.1A, all the cell lines expressed relatively high level of EGFR, although not as high as that of A431, which is the epidermal SCC cell line traditionally known for its high EGFR overexpression. HSC-3 cells, which was the cell line mostly used in this study, had the highest EGFR expression compared to all the other oral SCC cell lines we tested. The expression level of EGFR in HSC-3 cells was around 60% of that of A431 cells. ErbB2 was also expressed in HSC-3 cells, although the level was relatively low and less than that of SCC 10A or A431 cells (Fig. 1.1B). In contrast, we did not find any erbB3 expression in either HSC-3 or SCC-10A cells.

E-cadherin–mediated cell-cell adhesion activates EGFR

We have previously shown that survival following loss of attachment to the extracellular matrix requires E-cadherin–mediated intercellular adhesion. In this study, we first examined the dynamics of E-cadherin and EGFR distribution, since the two

receptors have been reported to be associated as a complex in SCC cells (Hoschuetzky et al., 1994; Pece and Gutkind, 2000). When HSC-3 cells were plated on poly-HEMA–coated dishes, the cells gradually formed E-cadherin–mediated aggregates, beginning with single cells that subsequently formed small aggregates by 6 h, collected into large, irregular clumps of cells by 12 h, and condensed into compact MCAs between 15 and 24 h (Fig. 1.2A). In freshly detached cells, both E-cadherin and EGFR were diffusely distributed. As intercellular adhesion proceeded, the receptors gradually colocalized at the cell-cell boundaries by as early as 6 h. Interestingly, as E-cadherin and EGFR codistributed at sites of cell-cell adhesion, they disappeared from cell surface areas that were devoid of cell-cell contact (Fig. 1.2B, left panel). This indicates that both E-cadherin and EGFR are swept to junctional areas following intercellular adhesion.

That EGFR was rapidly concentrated at cell-cell contacts in MCAs prompted us to investigate whether E-cadherin–mediated intercellular adhesion induces EGFR activation. After cells started to form MCAs, activated EGFR (p-EGFR), visualized with specific antibodies, accumulated at cell-cell adhesion sites as early as 6 h (Fig. 1.2B, right panel). To further confirm that EGFR was undergoing transactivation at cell-cell junctions, we analyzed the level of activated EGFR during the formation of aggregates by immunoblotting with antibodies specific for tyrosine-phosphorylated EGFR (Fig. 1.3A). HSC-3 cells were cultured as MCAs in medium contained 0.5% FBS to minimalize the effect of growth factors. At the time of plating, activated EGFR was at basal levels. Following the gradual formation of cell aggregates, there was a parallel induction of EGFR activation evident at 6 h and persisting at even higher levels at the 12- and 24-h time points. In contrast, phosphorylated EGFR in suspended single cells remained at low

levels over these time periods. We also cultured HSC-3 cells as aggregates or single cells culture in medium contained 1% ITS and obtained similar results of EGFR phosphorylation pattern. As a complementary approach, we used a calcium switch method to examine EGFR activation in monolayer cultures. We found that, whereas treatment with EGTA reduced activated EGFR to below its basal level, the cell-cell adhesion that re-formed after addition of calcium induced a rapid elevation of activated EGFR (data not shown). To examine whether EGFR was activated in other cell lines cultured as MCAs, we tested SCC 10A and SCC 10B cells, which had relatively high level of EGFR expression (Fig. 1.1A). 24 h after aggregates formation, EGFR activation was detected using immunoblotting in both cell lines (Fig. 1.3B). These results indicate that cell-cell adhesion leads to EGFR activation at sites of E-cadherin engagement.

Since erbB2 is expressed in HSC-3 cells and is the preferred heterodimerization patterner with EGFR (Graus-Porta et al., 1997), next we checked whether erbB2 was activated in HSC-3 MCAs. 24 h after aggregates formation, phosphorylation level of erbB2 was not increased compared to the basal phosphorylation level at 0 h (Fig. 1.4A), suggesting cell-cell adhesion did not induce erbB2 activation. However, the level of phosphorylated erbB2 was relatively high following EGF treatment.

c-Met, another tyrosine kinase receptor is also overexpressed in some oral SCC cell lines (unpublished data). It has been shown that c-Met can be activated by cell attachment (Wang et al., 1996), which prompted us to investigate whether c-Met is activated following cell-cell adhesion. However, when HSC-3 MCAs cultured for different time periods were probed by immunoblotting, no phosphotyrosine residues was detected in the anti-c-Met immunoprecipitates (Fig. 1.4B). In contrast, c-Met was

strongly activated following HGF treatment in monolayer cultures. Together, these results suggest that EGFR but not erbB2 or c-Met is activated following E-cadherin-mediated cell-cell adhesion.

Adhesion-mediated EGFR activation requires receptor kinase activity

EGFR activation includes several steps involving receptor dimerization, activation of the cytoplasmic tyrosine kinase domain, and autophosphorylation of the receptor (Downward et al., 1984).To test whether cell-cell adhesion induces intrinsic receptor kinase activity, we used tyrphostin AG1478, a specific inhibitor of EGFR kinase activity (Levitzki et al., 1995). Incubation with 1 μM AG1478 abolished EGFR activation in aggregates at different time points (Fig. 1.3B). These results suggest that EGFR kinase activity is required for adhesion-mediated EGFR activation.

FIGURE LEGENDS

Fig. 1.1. **Expression pattern of c-erbB family members in SCC cells.** ***A***, immunoblot analysis of EGFR expression in different SCC cell lines. ***B***, immunoblot analysis of erbB2 expression in different SCC cell lines.

Fig. 1.2. **EGFR/E-cadherin colocalization following cell-cell adhesion.** ***A***, HSC-3 cells were cultured as MCAs for different time periods as indicated and then transferred onto glass coverslips. After fixation, EGFR and E-cadherin were immunostained with rabbit anti-EGFR polyclonal antibodies (green) and mouse anti-E-cadherin mAb (red). Specimens were analyzed by confocal microscopy, and representative images are shown. ***B***, Confocal microscopy images of 6h MCAs immunolabeled for EGFR with rabbit anti-EGFR polyclonal antibodies (green), and E-cadherin with mouse anti-E-cadherin mAb or p-EGFR with mouse mAb anti-activated, phosphorylated EGFR (red). Representative images are shown.

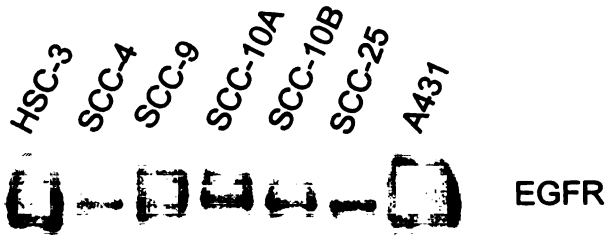
Fig. 1.3. **E-cadherin–mediated cell-cell adhesion induces EGFR activation.** ***A***, immunoblot analysis of activated EGFR during HSC-3 MCA formation. As controls, HSC-3 cells in monolayer (ML) were serum-starved overnight and then treated with 15ng/ml EGF for 5 min or cultured in suspension as single cells (SC) for 24 h. Relative protein densities of phosphorylated EGFR were determined; EGFR kinase activity is expressed as fold induction relative to the HSC-3 MCA at 0 h. Data are shown as the mean ± SD of three independent experiments. ***B***, immunoblot analysis of activated EGFR

in SCC-10A and SCC-10B MCAs. As controls, HSC-3 cells were serum-starved overnight and then treated with 15ng/ml EGF for 5 min.

Fig. 1.4. **Analyses of erbB2 and c-Met activation in HSC-3 MCAs.** ***A***, immunoblot analysis of activated erbB2 in HSC-3 MCAs. As controls, HSC-3 cells were serum-starved overnight and then treated with 15ng/ml EGF for 5 min. ***B***, HSC-3 cells were cultured as MCAs for the indicated time periods before cell lysates were processed for immunoprecipitation with anti–c-Met mAb followed by immunoblotting with anti-phosphotyrosine mAb or anti-c-Met polyclonal antibody. As controls, HSC-3 cells were serum-starved overnight and then treated with HGF (10 ng/mL) for 15 min.

Fig.1.5. **Adhesion-induced EGFR activation requires receptor kinase activity**. HSC-3 cells were cultured as MCAs for different time periods as indicated in the presence or absence of 1 μM tyrphostin AG1478. Equivalent protein was immunoblotted with antibodies to p-EGFR and tubulin.

A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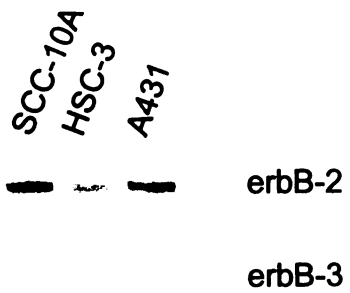
SCC-9
SCC-10A
SCC-10B
SCC-25
A431
EGFR

B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HSC-3
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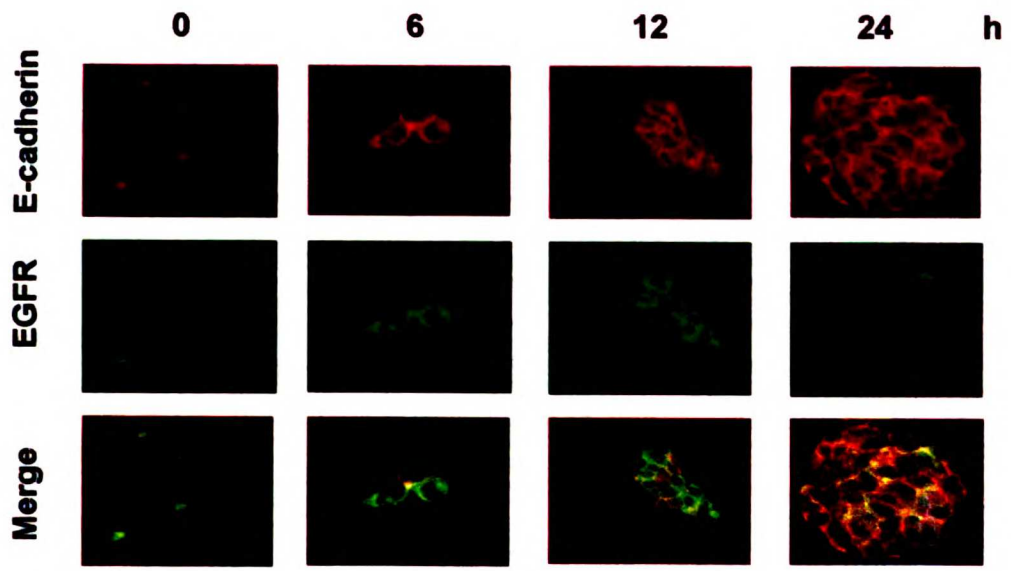
erbB-2
erbB-3

Figure 1.1

A



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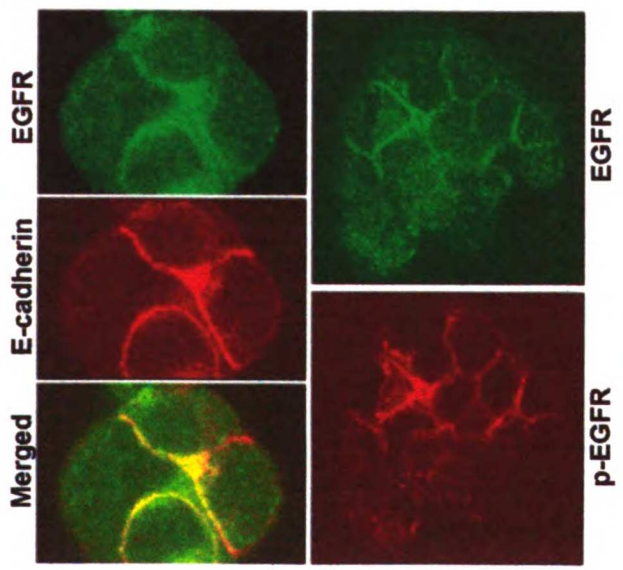

Figure 1.2

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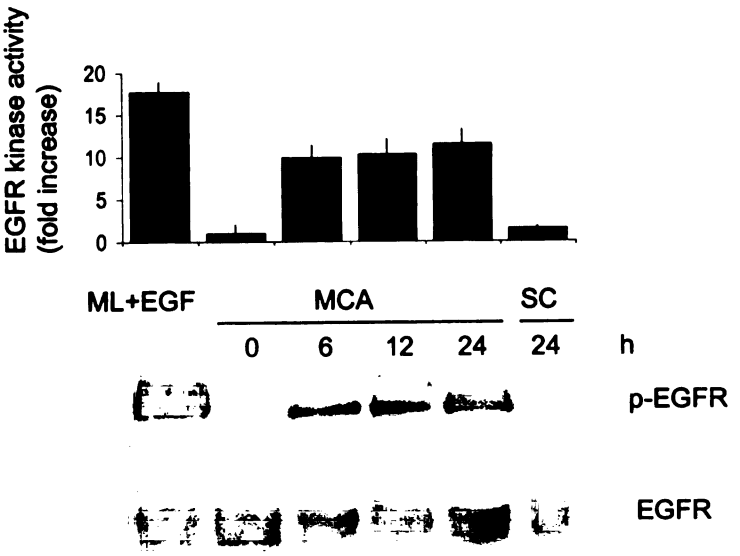
EGFR kinase activity
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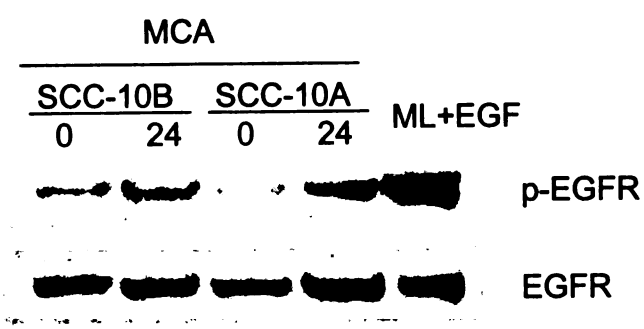
10
5
0
ML+EGF
MCA
SC
0
6
12
24
24
h
p-EGFR
EGFR

B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SCC-10A
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0
24
p-EGFR
EGFR

Figure 1.3

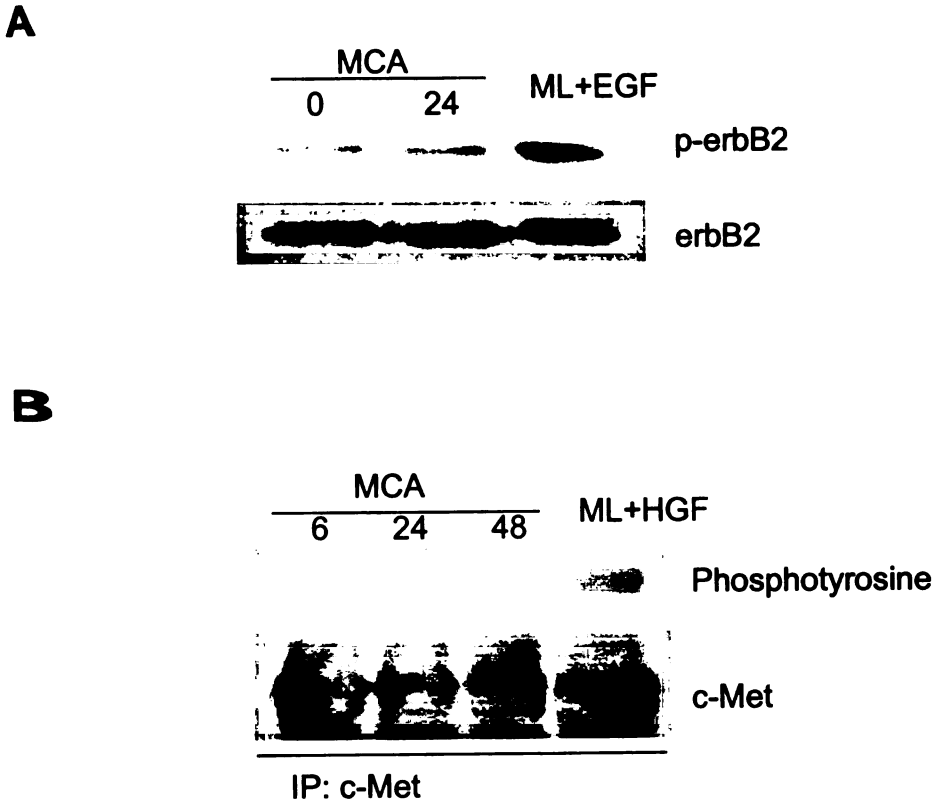
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Figure 1.4

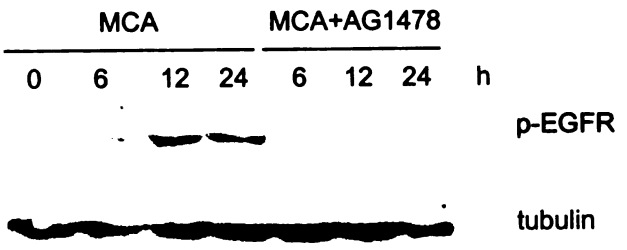

Figure 1.5

PART 2: EGFR and Downstream MAPK Activation is Required for E-cadherin-mediated Anoikis Resistance

In a previous study (Kantak and Kramer, 1998), it was reported that E-cadherin–mediated cell-cell adhesion could protect oral SCC cells from anoikis. However, the exact mechanism of E-cadherin–mediated anoikis resistance is unclear. In the present study, we evaluated the role of activated EGFR and downstream pathways in E-cadherin-mediated SCC cell survival. We confirmed that activation of EGFR and the downstream MAPK pathway is required for E-cadherin–mediated anoikis resistance. We also demonstrated that EGFR and MAPK activation is required for maintaining high level of Bcl-2 expression in SCC MCAs, which is related to their survival.

E-cadherin–mediated EGFR activation promotes anoikis resistance

MCAs of HSC-3 cells were resistant to apoptosis, whereas suspended single cells rapidly underwent anoikis, as detected by the extensive DNA laddering typical of apoptotic intranucleosomal cleavage (Fig. 2.1A, lane 1 and 4). To test whether E-cadherin–dependent EGFR activation provides the survival signal in aggregates, we incubated cells with tyrphostin AG1478. EGFR blockade with 1 μM AG1478 induced apoptosis in HSC-3 cell aggregates cultured for 48 h, indicated by DNA laddering (Fig. 2.1A, lane 3). The DNA laddering induced by AG1478 was similar to that of HSC-3 single cells cultured in suspension (Fig. 2.1A, lane 4). By contrast, AG1478-treated HSC-3 monolayer cells showed no evidence of apoptosis (Fig. 2.1A, lane 5). Next, we analyzed DNA fragmentation to identify apoptotic cells by TUNEL assay. Again, AG1478 treatment dramatically increased the percentage of TUNEL-positive cells in

HSC-3 MCAs at 48 h (Fig. 2.1B). Control monolayer HSC-3 cells showed low levels of TUNEL-positive cells, whereas, after suspension as single cells for the same time period, over 40% of the cells were apoptotic. These results suggest that EGFR kinase activity is required for the survival of HSC-3 cells in suspension culture.

To determine whether a similar mechanism occurs *in vivo*, EGFR blockade was tested on subcutaneous HSC-3 xenografts implanted in athymic nude mice. Although tumors still grew subcutaneously, daily intraperitoneal injections of 50 mg/kg AG1478 greatly suppressed HSC-3 tumor growth when compared with controls that received only DMSO (Fig. 2.2A, B). After tumors from both groups were harvested, DNA laddering assay was performed (Fig. 2.2C). Tumor cells inside the AG1478-treated xenografts apparently underwent apoptosis, suggesting that at least part of the delay in tumor growth was due to excessive cell death. Similarly, control HSC-3 MCAs incubated with AG1478 *in vitro* showed extensive DNA laddering. There was no sign of apoptosis in either *in vivo* or *in vitro* DMSO-treated groups. The results of these experiments suggest that EGFR kinase activity is also required for the survival of HSC-3 cells *in vivo*.

Exogenous expression of E-cadherin rescues receptor-negative cells from anoikis

To further test the idea that E-cadherin engagement can induce EGFR activation and rescue cells from apoptosis, we exogenously expressed E-cadherin in E-cadherin-negative HOC313 cells, another oral SCC cell line with a mixed population of both high and low expressers of E-cadherin. Previously we were able to isolate individual receptor-negative and receptive-positive single-cell clones with different abilities in forming MCAs, including D1 clone which has high level of E-cadherin and C8 clone which has

almost no detectable signal for the receptor (Kantak and Kramer, 1998). The E-cadherin–transfected C8 (C8/E-cad) cells expressed high levels of the receptor (Fig. 2.3A). To determine whether the exogenously transfected E-cadherin colocalizes with EGFR at cell-cell boundaries, MCAs of C8/E-cad cells were processed for immunofluorescence analysis. Similar to what was observed with HSC-3 MCAs (Fig. 1.2B), we found that in C8/E-cad MCAs the EGFR was also colocalized with E-cadherin and that significant level of phosphorylated EGFR was detected by immunostaining at cell-cell junctions (Fig. 2.3B). Next EGFR activation was assessed by immunoblotting for EGFR tyrosine phosphorylation in the C8/E-cad cells after they were plated as MCAs for 24 h (Fig. 2.3C). While low level of total EGFR was expressed in E-cadherin–negative C8 cells, no EGFR activation was detected in C8 MCAs. In MCAs from C8/E-cad cells, strong phosphorylation of EGFR was detected and the level of EGFR activation was nearly as intense as that generated in D1 cells, a positive control. Interestingly, the level of total EGFR also increased in C8/E-cad cells. Next, we analyzed the effect of ectopically expressed E-cadherin on survival in suspension by the TUNEL assay. The C8/E-cad cells showed enhanced resistance to anoikis (Fig. 2.3D). Whereas about 33% of C8 cells cultured in suspension were TUNEL positive, only 9.6% of the C8/E-cad cells were apoptotic. Again, this enhanced anoikis resistance of C8/E-cad cells was reversed by EGFR kinase blockade with tyrphostin AG1478 (22.7%). Therefore, ectopic expression of E-cadherin is able to induce EGFR activation in C8 MCAs and partially rescue the C8 cells from anoikis.

E-cadherin–mediated adhesion leads to ERK/MAPK activation

Among downstream pathways of EGFR, MEK/MAPK (ERK1/2) and PI3K/Akt pathways are the main pathways involved in cell survival (Datta et al.,1999). We tested whether E-cadherin–dependent phosphorylation of EGFR activates these two pathways. Following induction of cell-cell adhesion, there was a progressive and sustained elevation of phosphorylated ERK1/2 that was evident as early as 6 h and peaked around 24 h (Fig. 2.4A). This time-course of ERK1/2 activation closely paralleled EGFR activation following aggregate formation. Moreover, inhibition of EGFR kinase activity with 1 μM tyrphostin AG1478 abolished ERK1/2 phosphorylation, indicating that ERK1/2 activation in MCAs was induced by EGFR transactivation (Fig. 2.4B). To determine whether downstream MAPK phosphorylation is required for the survival of MCAs in suspension, we used U0126, a specific MEK inhibitor and measured DNA fragmentation by TUNEL assay to quantify apoptotic cells. As shown in Fig. 4B, treatment of 10 μM U0126 for 24 h blocked ERK1/2 phosphorylation in HSC-3 cell aggregates. At this concentration, U0126 also induced significant apoptosis in HSC-3 aggregates at 48 h (Fig. 2.4C). These findings demonstrate that the MEK/MAPK pathway is involved in E-cadherin–mediated anoikis resistance via EGFR activation.

In contrast to the robust ERK1/2 activation following intercellular adhesion, phosphorylated Akt was not detected in MCAs (Fig. 2.5A). However, Akt activation was readily observed in substrate-adherent HSC-3 monolayer cells, and the level of activated Akt was strongly elevated following EGF treatment. The apparent lack of a role for Akt in cell-cell adhesion–induced survival is suggested by the effect of the specific PI3K inhibitor LY294002, which had very low effect on MCA cell survival measured by both TUNEL assay (Fig. 2.4C) and DNA laddering assay (Fig. 2.5B). In contrast, treatment of

MCAs with AG1478 to block EGFR, or with U0126 to block MAPK, induced apoptosis, as evidenced by extensive DNA laddering. These findings imply that MCA cell survival depends primarily on the E-cadherin–EGFR–MEK/MAPK signaling pathway, and not on the PI3K/Akt pathway.

Adhesion-dependent EGFR and MAPK activation regulates Bcl-2 expression

Previously we found that the level of anti-apoptotic Bcl-2 protein is elevated in response to E-cadherin–mediated adhesion (Kantak and Kramer, 1998). As shown in Fig. 2.6A, Bcl-2 level was high in HSC-3 monolayer and MCA cultures but decreased in single cell culture. These results indicated that susceptibility to apoptosis in suspended single cells is correlated with loss of Bcl-2 expression. To test whether E-cadherin–mediated adhesion regulates Bcl-2 expression via the EGFR/MAPK pathway, we treated HSC-3 aggregates with either EGFR inhibitor AG1478 or MAPK inhibitor U0126. As expected, both monolayers and MCAs exhibited high Bcl-2 levels. After treatment with either AG1478 or U0126, the level of Bcl-2 decreased drastically, whereas the level of Bax remained relatively constant (Fig. 2.6B). The Bcl-2/Bax protein ratio decreased by more than threefold in MCAs following blockade of EGFR or MAPK.

To establish whether elevated Bcl-2 is linked to HSC-3 cell survival, we stably transfected HSC-3 cells with Bcl-2. Overexpression of Bcl-2 in HSC-3 cells was then confirmed by Western blotting (Fig. 2.7A). Compared with control cells, Bcl-2 protein levels were much higher in both monolayer and single-cell suspension culture of Bcl-2–transfected cells. Importantly, in viability assays, when both cell lines were subjected to single-cell suspension for 48 h and then replated onto culture dishes, no visible colonies

of control cells survived (Fig. 2.7B). However, in the Bcl-2–transfected cells, a significant number of cells survived and proliferated to form colonies after replating. Similarly, mock-transfected HSC-3 cells were unable to survive (data not shown). Next, we used a TUNEL assay to analyze the effect of Bcl-2 overexpression on anoikis. As shown in Fig. 2.7C, in contrast to control cells, which progressed to apoptosis as single cells, cells overexpressing Bcl-2 exhibited a marked reduction in anoikis. Taken together, these results suggest that EGFR and MAPK activation is required for an elevated Bcl-2/Bax protein ratio in cell aggregates and that Bcl-2 appears to be sufficient to provide protection from anoikis.

FIGURE LEGENDS

Fig. 2.1. **Cell-cell adhesion-induced EGFR activation suppresses anoikis.** ***A***, DNA fragmentation of HSC-3 cells. HSC-3 cells were plated as MCA culture in the absence or presence of 1 μg/ml AG1478 for 48 h (*lanes 2 and 3*). Suspended single cells (SC) alone (*lane 4*) and monolayers (ML) cultured for 48 h in the presence of 1 μM AG1478 (*lane 5*) were used as controls. The DNA laddering assay was performed as described in Experimental Procedures. *Lane 1*, standard 100-bp DNA ladder.
B, TUNEL analysis of AG1478-treated HSC-3 cells. HSC-3 cells were plated as ML, MCA or SC culture for 48 h before TUNEL analysis. 1 μM AG1478 was added to the culture as indicated. Values represent the apoptotic cell fractions that stained positive with FITC-dUTP.

Fig. 2.2. **EGFR-dependent survival of HSC-3 cells *in vivo*.** ***A***, growth inhibition of HSC-3 xenografts in nude mice. Mice (8 per group) were injected subcutaneously with HSC-3 cells. Five days after injection the animals were treated with AG1478 in DMSO or DMSO alone as described in Experimental Procedures. Each data point represents the mean tumor volume ± SE of eight animals. Asterisks indicate a significant difference between AG1478- and DMSO-treated mice ($P \leq 0.01$; Student's t test). ***B***, Representative tumor samples from mouse group treated with AG1478 in DMSO (right) or DMSO alone (left). ***C***, AG1478- and DMSO-treated HSC-3 tumors were harvested at day 21 and processed for DNA laddering assay in an agarose gel. Each lane represents a

single tumor following treatment (left) or HSC-3 MCAs treated with or without 1 μM AG1478 for 48 h (right).

Fig. 2.3. **Ectopic expression of E-cadherin rescues E-cadherin–negative cells from anoikis.** ***A***, FACS analysis of E-cadherin level in E-cadherin–transfected C8 (C8/E-cad) cells. E-cadherin–negative C8 cells are shown as a control. ***B***, immunoblot analysis of activated EGFR in E-cadherin–transfected C8 MCAs at 24 h. E-cadherin–positive D1 and E-cadherin–negative C8 MCAs are shown as controls. ***C***, C8/E-cad cells were cultured as MCAs for 6 h and then transferred onto glass coverslips and immunostained for EGFR with rabbit anti-EGFR polyclonal antibodies (green), and E-cadherin with rat anti-E-cadherin mAb or p-EGFR with mouse mAb anti-activated, phosphorylated EGFR (red). Specimens were analyzed by confocal microscopy, and representative images are shown. ***D***, TUNEL analysis of E-cadherin–transfected C8 MCAs at 48 h. D1 and C8 MCAs are shown as controls.

Fig. 2.4. **EGFR-dependent ERK/MAPK activation in HSC-3 cell aggregates.** ***A***, ERK1/2–MAPK activation pattern in HSC-3 MCAs as described in Experimental Procedures. ***B***, effect of AG1478 on ERK1/2 phosphorylation. HSC-3 cells were plated as MCA culture in the absence or presence of 1 μM AG1478 for 24 h before protein extraction. The MEK inhibitor U0126 at 1 and 10 μM was used as the control. ***C***, TUNEL analysis of 10 μM U0126- or 50 μM LY294002-treated HSC-3 MCAs at 48 h as described in Experimental Procedures.

Fig.2.5. **Analysis of PI3K/Akt pathway in HSC-3 MCAs.** ***A***, Akt activation pattern in HSC-3 MCAs. ***B***, DNA fragmentation of HSC-3 cells. HSC-3 cells were plated as MCA culture in the presence of 50 μM LY294002, 10 μM U0126 or 1 μg/ml AG1478 for 48 h.

Fig. 2.6. **Analysis of Bcl-2 in HSC-3 cell aggregates.** ***A***, HSC-3 cells were cultured as monolayers, MCAs or single cells for the indicated time periods before cell lysates were processed for immunoprecipitation and immunoblotting with anti–Bcl-2 mAb. ***B***, HSC-3 cells were plated as MCA culture in the presence of 1 μM AG1478 or 10 μM U0126 or 50 μM LY294002 for 24 h before cell lysates were processed for immunoprecipitation and immunoblotting with anti–Bcl-2 mAb. Equivalent protein was also immunoblotted with anti-Bax antibody, and the relative density of the Bcl-2/Bax protein ratio was determined. Data are shown as the mean ± SD of three independent experiments.

Fig. 2.7. **Ectopic expression of Bcl-2 rescues suspended single cells from apoptosis.** ***A***, immunoblot analysis of Bcl-2 in Bcl-2–transfected HSC-3 cells cultured as monolayers (ML) or as suspended single cells (SC). Controls were normal HSC-3 cells cultured as monolayers or as suspended single cells. ***B***, clonal growth of Bcl-2–transfected HSC-3 cells recovered after 48 h of single-cell suspension culture. ***C***, TUNEL analysis of Bcl-2–transfected HSC-3 cells cultured as suspended single cells for 48 h. Normal HSC-3 monolayers and suspended single cells were used as controls.

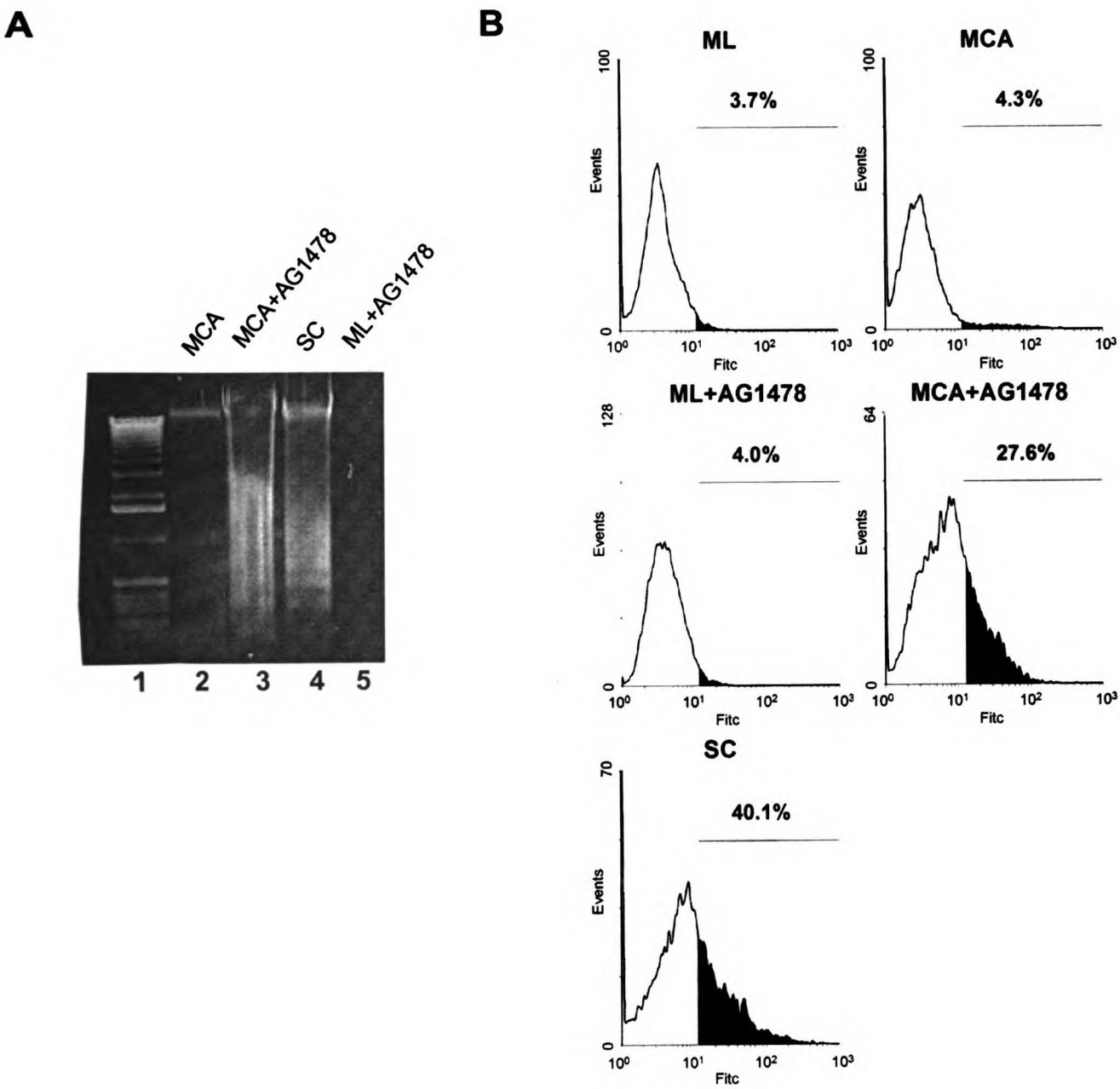
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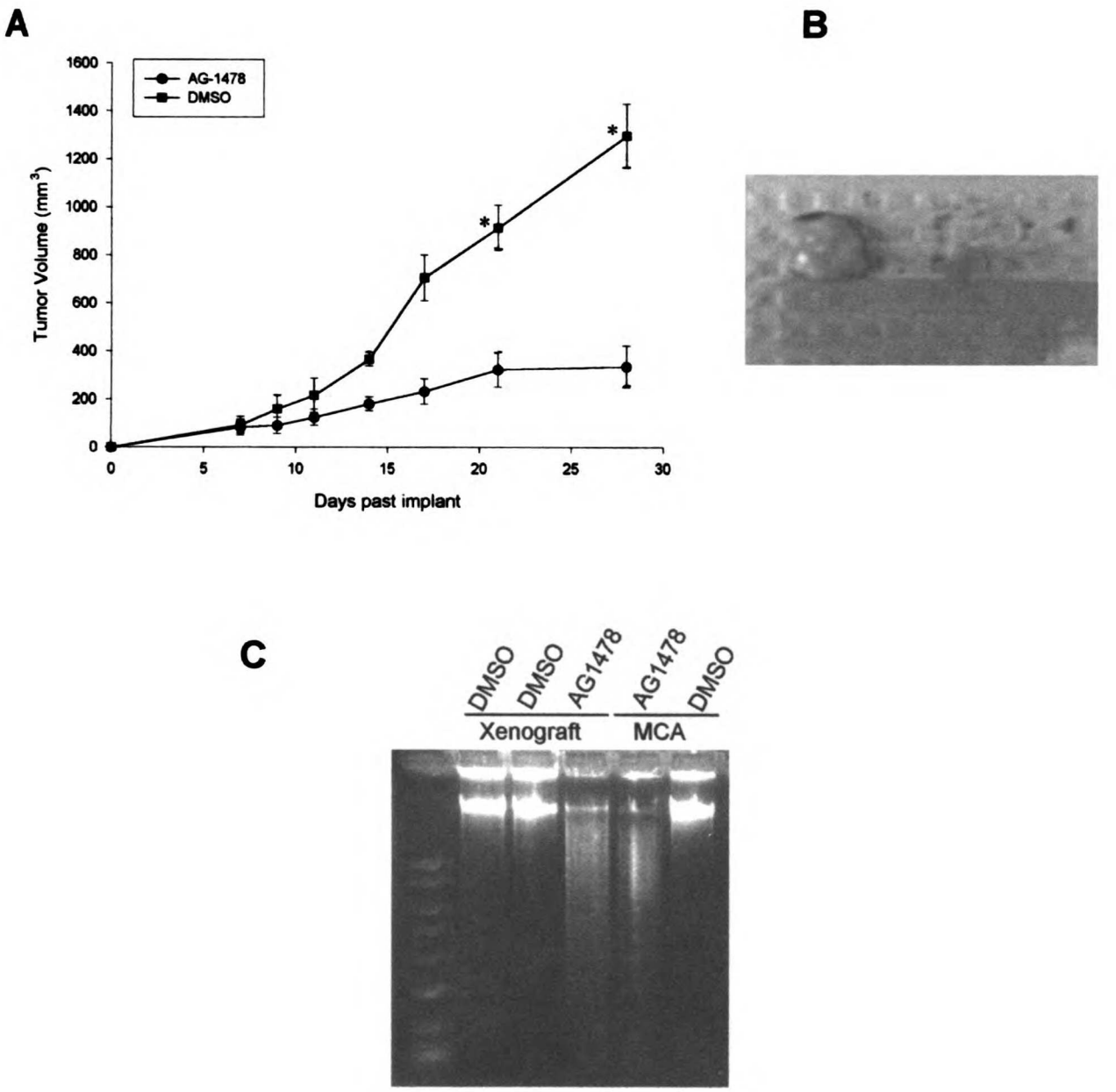
Figure 2.1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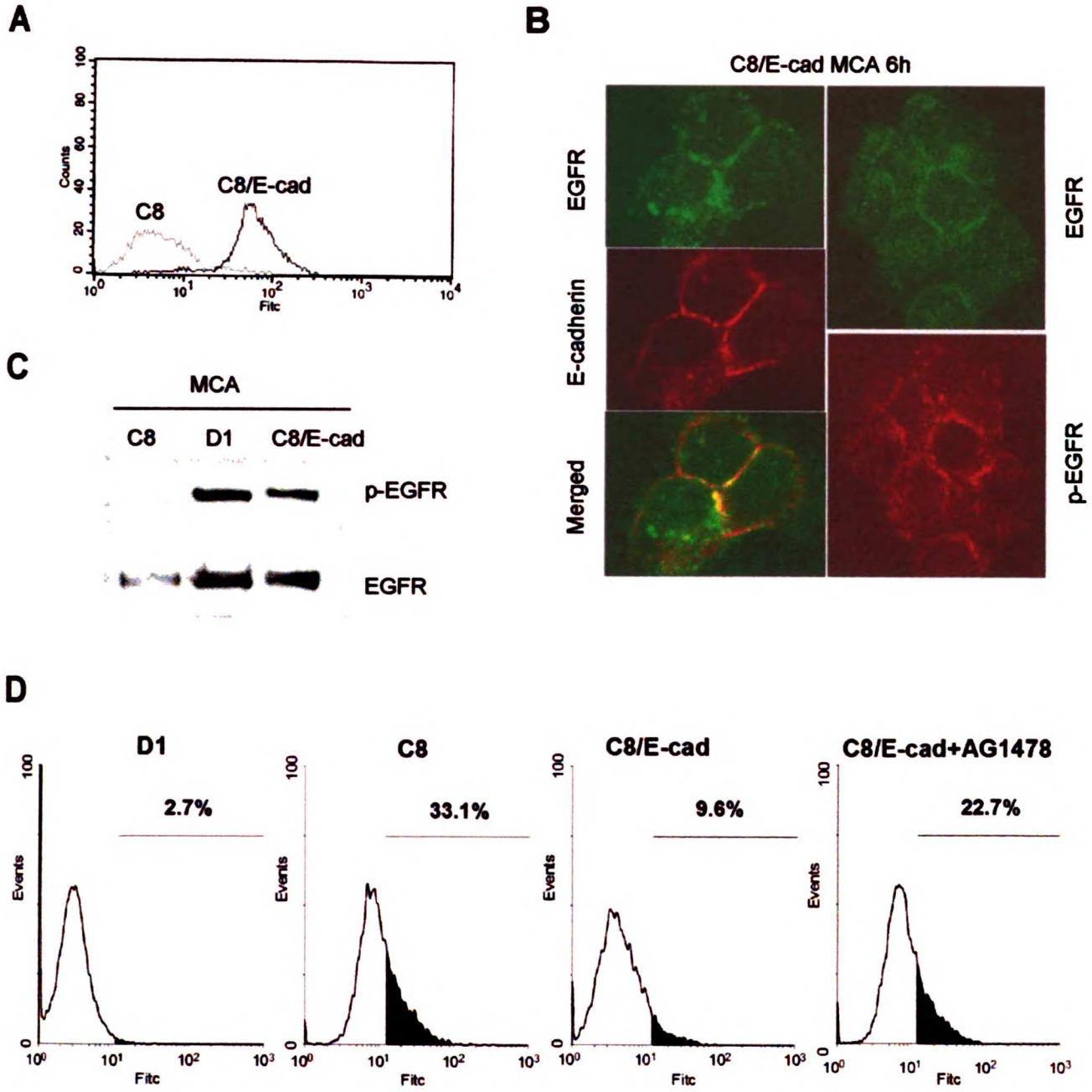
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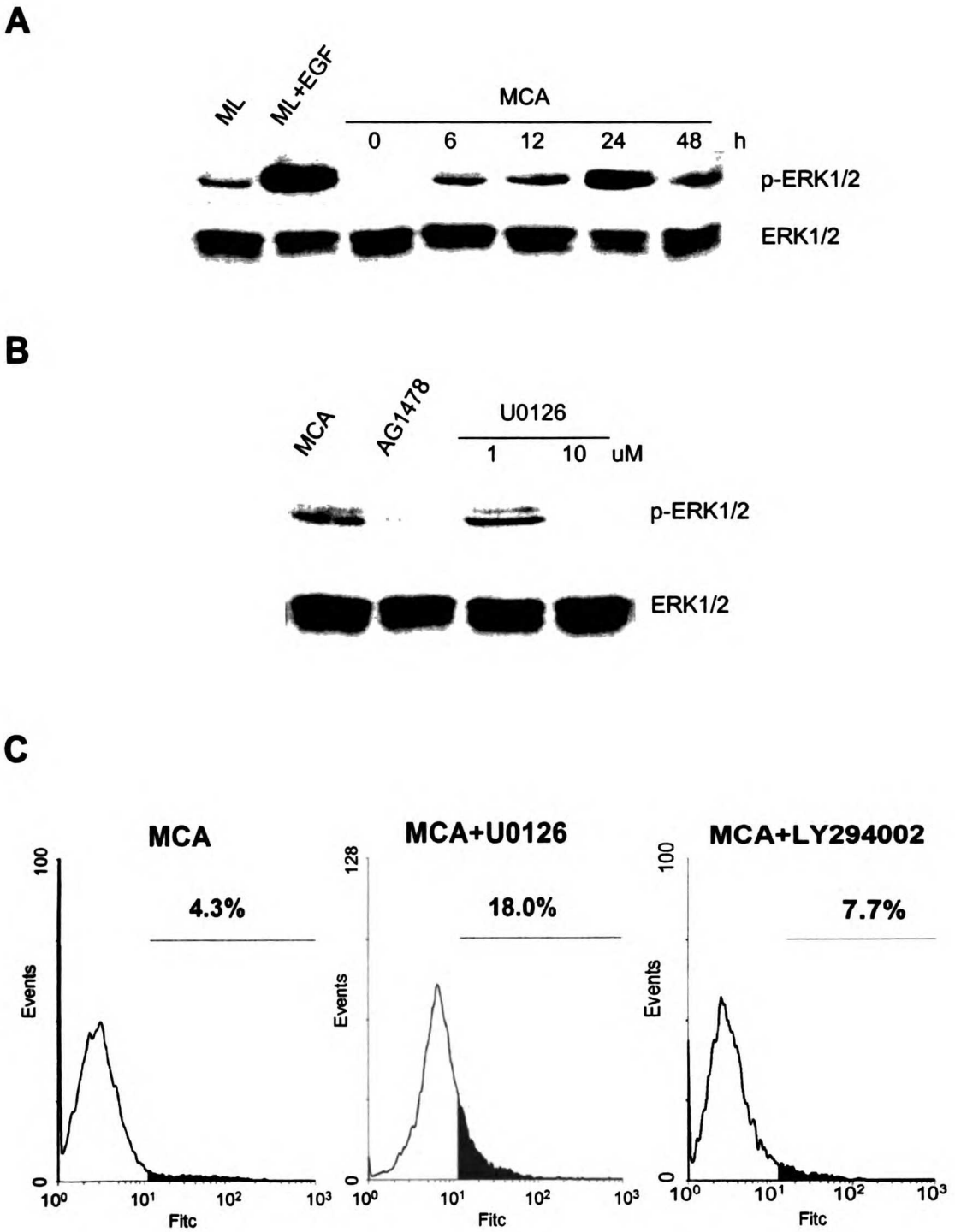
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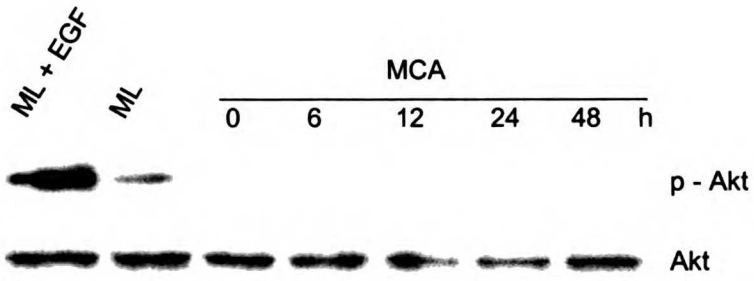
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p - Akt
Akt

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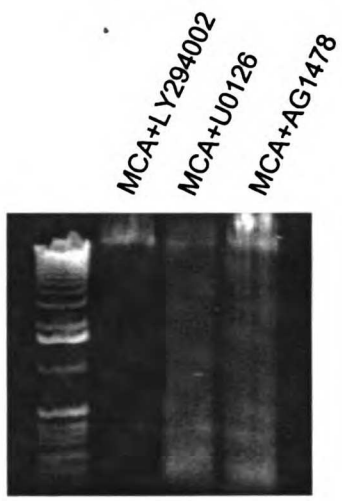
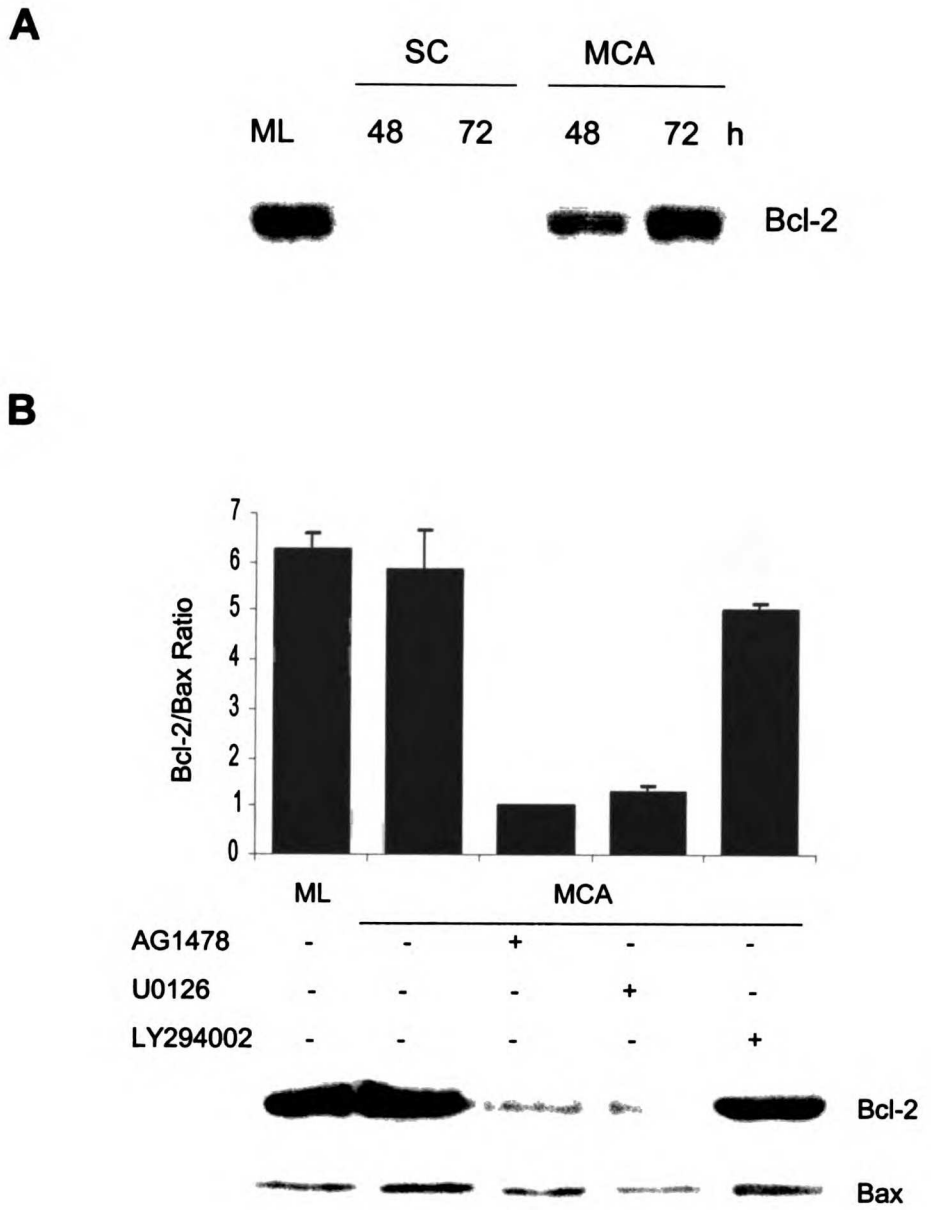

MCA+LY294002
MCA+U0126
MCA+AG1478

Figure 2.5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B
Bcl-2/Bax Ratio
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2
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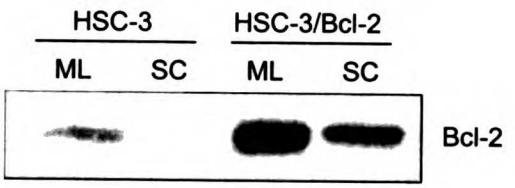
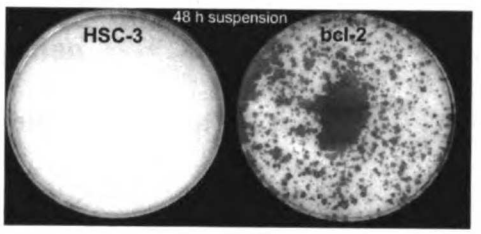
4
5
6
7
ML
MCA
AG1478
U0126
LY294002
Bcl-2
Bax

Figure 2.6

A



B



C

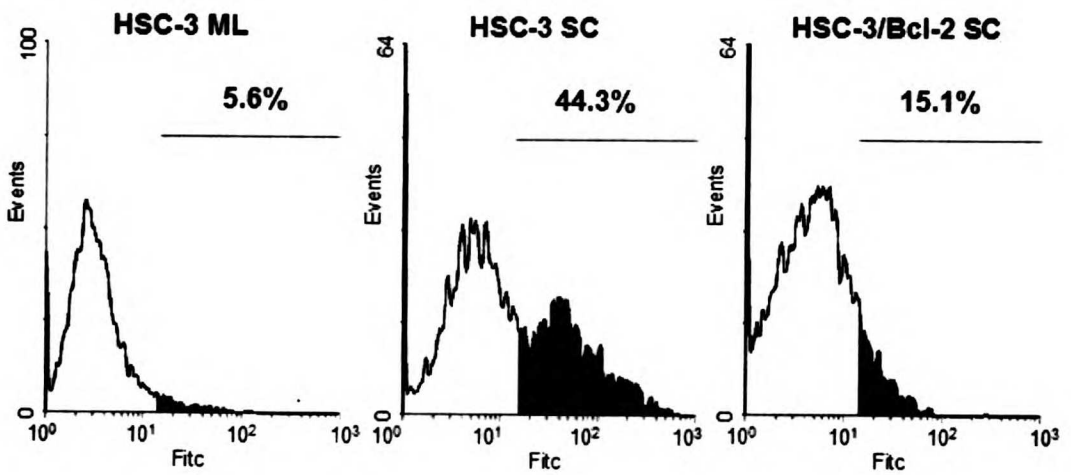


Figure 2.7

PART 3: Mechanism of EGFR Transactivation Following Cell-cell Adhesion

In the second part of this study, we focused on the function of activated EGFR in SCC cell survival and found that activated EGFR and downstream MAPK are required for E-cadherin-mediated anoikis resistance. In the third part of this study, we explored the possible mechanisms of EGFR activation following cell-cell adhesion.

EGFR activation following cell-cell adhesion requires E-cadherin and actin cytoskeleton organization

To further establish the role of E-cadherin in EGFR activation, HSC-3 cells were treated with inhibiting anti-E-cadherin antibody HECD-1 (50 ug/ml) prior to culturing as MCAs. In the presence of HECD-1, the formation of the large, compact aggregates was severely inhibited at (Fig. 3.1A). As shown in Fig. 2.1B, hindering E-cadherin engagement with blocking antibodies ablated EGFR activation. This result was consistent with the phenomenon showed before that incubation with E-cadherin blocking antibodies induced apoptosis in MCAs (Kantak and Kramer, 1998).

E-cadherin is linked to actin cytoskeleton via catenins which bind to its cytoplasmic tail. To test that actin cytoskeleton integrity is required for adhesion-mediated EGFR activation, HSC-3 cells were treated with cytochalasin D (0.5 uM), a known inhibitor of actin polymerization, prior to plating as MCAs. In the presence of cytochalasin D, the compaction of aggregates was inhibited. Western blotting showed that cytochalasin D reduced the phosphorylation of EGFR in MCAs (Fig. 3.2A). At this concentration, cytochalasin D also induced significant apoptosis in HSC-3 aggregates at 48 h as measured by TUNEL assay (Fig. 3.2B). These findings indicate that the

activation of EGFR following cell-cell adhesion formation is E-cadherin dependent and also requires intact actin cytoskeleton organization.

Artificial cross-linking of E-cadherin activates EGFR

To test the role of E-cadherin in EGFR activation directly, we artificially induced E-cadherin cross-linking by antibody-mediated clustering assay (Fig. 3.3). In these experiments, HSC-3 cells in single-cell suspension culture were first treated with E-cadherin mAb, followed by incubation with a secondary cross-linking antibody. Treatment of suspended single cells with anti–E-cadherin antibodies induced a rapid EGFR activation that was first evident by 15 min after cross-linking and plateaued by 45 min. These findings clearly show that the activation of EGFR following cell-cell adhesion formation can occur in the absence of cell-cell adhesion when E-cadherin is artificially ligated with specific antibody. In contrast, HECD-1 mAb or anti-mouse IgG alone failed to trigger EGFR activation. These findings clearly show that the activation of EGFR can occur in the absence of cell-cell adhesion when E-cadherin is artificially ligated and crosslinked with specific antibody. Obviously this approach does not mimic the complexity that occurs at cell-cell junctional assemblies, but does suggest a mechanism dependent on receptor crosslinking and that this event is sufficient to induce EGFR transactivation.

Adhesion-induced activation of EGFR is ligand independent

The finding that artificial cross-linking of E-cadherin directly induces EGFR activation suggests that adhesion-induced EGFR activation occurs through a process that

does not involve the interaction of EGFR with a ligand. Since it has been shown that EGFR transactivation can occur through cleavage of EGF-like precursors (Carpenter, 2000), it was important to establish in the current system that EGFR activation proceeds via a ligand-independent event. To test whether such a mechanism was occurring in MCAs, we first employed EGFR function-blocking antibodies. EGFR mAb clone 225 (Ab-3) has been widely used to block EGFR ligand binding and to consequently inhibit EGFR activation (Blakely et al., 2000). As expected, when HSC-3 monolayer cells were preincubated with Ab-3 for 30 min, the response to EGF stimulation was largely abolished (Fig. 3.4). However, when HSC-3 cells in suspension were similarly treated with a saturating level of Ab-3 antibodies, EGFR activation in cell aggregates did not diminish. This suggests that ligand-receptor binding is not responsible for EGFR activation in MCAs. However, even though antibody was added as aggregates formed, it is possible that access to the interior area of MCAs was hindered.

To further establish that adhesion-induced EGFR activation is ligand independent, we transduced HSC-3 cells with a recombinant adenoviral vector containing EGFR-CD533. This EGFR truncation mutant, which lacks the cytoplasmic COOH-terminal 533 amino acids that include the kinase domain, functions as a dominant-negative (Kashles et al., 1991; Reardon et al., 1999). At high levels of expression, this mutant should not only compete for ligands but also should abrogate EGFR activation. HSC-3 cells transduced with EGFR-CD533 were shown to express this mutant at high level by Western blotting with an antibody specific to the extracellular domain of EGFR (Fig. 3.5A). Un-transduced and control GFP-transduced monolayer cells were fully responsive to EGF, but cells overexpressing the EGFR mutant became unresponsive to ligand. This

demonstrated that EGFR-CD533 was acting as an effective dominant-negative. In contrast, when similarly transduced cells were cultured as MCAs, the expression of this dominant-negative EGFR had no effect on the progressive increase of EGFR phosphorylation following MCA formation (Fig. 3.5B). These results strongly suggest that EGFR activation following cell-cell adhesion is ligand independent and is functionally different from ligand-induced receptor activation.

E-cadherin-mediated adhesion leads to phosphorylation of specific tyrosine residues on EGFR

As shown above, adhesion-induced EGFR phosphorylation is quantitatively lower than that induced by ligands such as EGF (Fig. 1.3A). We also showed that adhesion-induced EGFR activation is ligand-independent. To analyze the possible difference in phosphorylation of specific tyrosine residues on EGFR between ligand- and adhesion-induced EGFR activation, we used specific antibodies against different EGFR tyrosine residues. As shown in Fig. 3.6, tyrosine 1068 was strongly phosphorylated as early as 6h after cell-cell adhesion and the extent of phosphorylation was even higher at 24h, which was about 80% of that of induced by EGF. Similarly, tyrosines 1086, 1148 and 1173 were also phosphorylated after adhesion, indicating that all major tyrosine residues on EGFR were phosphorylated in response to E-cadherin-mediated cell-cell adhesion. However, the extent of phosphorylation of residues 1086, 1148 and 1173 was only about 50-65% of that induced by EGF, respectively. These results suggest that E-cadherin-mediated adhesion leads to phosphorylation of all major tyrosine residues corresponding to the sites previously shown to be phosphorylated by EGF. The extent of

phosphorylation was residue-specific, and for all but tyrosine 1068, lower than that of induced by EGF.

EGFR forms complexes with E-cadherin at cell-cell junctions

Previous studies have shown that EGFR can be linked via β-catenin to the cytoplasmic tail of E-cadherin (Hoschuetzky et al., 1994). To investigate whether E-cadherin and EGFR form stable complexes in multicellular aggregates, MCA cell lysates were processed for immunoprecipitation with anti–E-cadherin mAb, followed by immunoblotting with anti-EGFR antibodies. EGFR was effectively recovered in the anti–E-cadherin immunoprecipitates (Fig. 3.7) indicating that E-cadherin and EGFR can physically associate. Importantly, while the amount of EGFR coimmunoprecipitating with E-cadherin was low in monolayer cells or when the suspended cells were initially plated, the level of EGFR-cadherin complex formation gradually increased as MCAs were forming (6 to 12 h) and became maximal at 24 to 48 h. The increase in EGFR coimmunoprecipitating with E-cadherin paralleled the increase in activated EGFR coimmunoprecipitating with E-cadherin, suggesting that EGFR activation correlates with E-cadherin–EGFR complex formation at cell-cell contacts. The phospho-EGFR/EGFR protein ratio confirmed that the specific activity of the receptors remained fairly constant over 48 h. Reblotting with anti–E-cadherin antibody after stripping of the membrane confirmed that the differences in EGFR association were not caused by the variation in the amount of E-cadherin recovered from the immunoprecipitates. When the total cell lysates were analyzed by immunoblotting, the relative level of E-cadherin was found to

increase during MCA formation whereas the EGFR level remained relatively constant.

Activated EGFR in MCAs is preferentially complexed with E-cadherin

To measure the distribution of activated EGFR between cadherin-bound and cadherin-free pools under different culture conditions, cell lysates were first exhaustively immunoprecipitated with anti–E-cadherin mAb, followed by sequential immunoprecipitation with anti-EGFR antibodies. The E-cadherin and EGFR immunoprecipitates were then blotted with EGFR–specific antibodies. For EGF-stimulated, subconfluent monolayer cells, the majority of the activated EGFR was not associated with E-cadherin but rather was recovered in the E-cadherin–depleted cell lysate fraction. Similarly, the low level of activated EGFR in the control subconfluent monolayer cells was partitioned in the E-cadherin–depleted fraction (Fig. 3.8). In contrast, nearly all the activated EGFR in MCAs was complexed with E-cadherin, with only a minor fraction recovered in the E-cadherin–depleted fraction. Consistent with this finding was an increase in total EGFR recovered in the E-cadherin immunoprecipitates from MCAs. It was found that the amount of EGFR/E-cadherin complex represented only a small fraction of total cellular EGFR (~13%) even when the cells had formed mature aggregates (Fig. 3.8). In contrast, cultures of control monolayers or EGF-treated monolayers, EGFR /E-cadherin complexes represented only ~7% of the total cellular pool of EGFR. These results support the conclusion that the cellular distribution of adhesion-induced and ligand-independent EGFR activation is distinct from that of EGF-induced activation and that as cell-cell adhesion proceeds, the subset of EGFR complexed

with E-cadherin is transactivated.

EGFR cytoplasmic domain is required for association with E-cadherin

As stated above, the dominant-negative EGFR mutant EGFR-CD533 was unable to inhibit EGFR activation in MCAs. To test whether EGFR-CD533 could complex with E-cadherin as the wild-type receptor does, HSC-3 cells were transduced with the cytoplasmic domain-truncated mutant and permitted to form MCAs. EGFR is known to associate with the cytoplasmic tail of E-cadherin via β-catenin {Hoschuetzky, 1994 #14}, presumably through a region in the EGFR cytoplasmic domain. This seems to be the case, since only wild-type EGFR, not EGFR-CD533, could be recovered in anti–E-cadherin immunoprecipitates (Fig. 3.9).

Assembly and colocalization of EGFR/E-cadherin complex is independent of EGFR kinase activity

In order to further evaluate the potential role of EGFR activation in EGFR/E-cadherin complex formation and its colocalization at cell-cell junctions, we used tyrphostin AG1478 to specifically block EGFR kinase activity and then analyze the receptor distribution by both immunolabeling and immunoprecipitation. In the presence of AG1478 we found that HSC-3 cells were able to form compacted cell aggregates to the same extent as control cells. Importantly, the condensation of EGFR and E-cadherin at MCA cell-cell boundaries in the presence or absence of AG1478 was comparable (Fig. 3.10A, left panel). The extensive EGFR phosphorylation at cell-cell junctions of MCAs readily detected in control aggregates was effectively inhibited in the presence of

AG1478 (Fig. 3.10A, right panel). Next, we found that the amount of EGFR co-immunoprecipitated with E-cadherin was similar in control and AG1478-treated MCAs (Fig. 3.10B). However, the level of phosphorylated EGFR associated with E-cadherin was severely reduced in AG1478-treated MCAs. These results strongly suggest that kinase activity of EGFR is not required for 1) its ability to complex with E-cadherin or 2) to localize at cell-cell junctions.

FIGURE LEGENDS

Fig.3.1. **EGFR activation following cell-cell adhesion requires E-cadherin.** ***A***, effect of anti–E-cadherin mAb on HSC-3 MCA formation. HSC-3 cells were plated as MCA culture in the absence or presence of 50 μg/ml anti–E-cadherin mAb HECD-1 for 24 h. ***B***, HSC-3 cells were plated as MCA culture in the presence of 50 μg/ml anti–E-cadherin mAb HECD-1 or mouse IgG for 24 h. Equivalent protein was immunoblotted with mAbs to phospho-EGFR and total EGFR.

Fig.3.2. **EGFR activation following cell-cell adhesion requires actin cytoskeleton organization.** ***A***, effect of cytochalasin D treatment on EGFR activation in MCAs. HSC-3 cells were plated as MCA culture in the presence or absence of 0.5 uM cytochalasin D for indicated time period. Equivalent protein was immunoblotted with mAbs to phospho-EGFR and total EGFR. ***B***, TUNEL analysis of cytochalasin D -treated HSC-3 cells. HSC-3 cells were plated MCA culture for 48 h before TUNEL analysis. 0.5 uM cytochalasin D was added to the culture as indicated. Values represent the apoptotic cell fractions that stained positive with FITC-dUTP.

Fig. 3.3. **EGFR activation induced by E-cadherin cross-linking.** HSC-3 cells were detached with 5 mM EDTA and kept in suspension in poly-HEMA–coated dishes. After incubation for 1 h at 4°C with saturating concentration of anti–E-cadherin HECD-1 mAb (50 μg/ml), surface clustering of E-cadherin receptors was induced by further incubation with goat anti-mouse IgG (5 μg/ml) for the indicated times at 37°C. Equivalent protein

was then immunoblotted with antibodies to activated EGFR (p-EGFR) and total EGFR. Relative protein densities of activated EGFR were determined; EGFR kinase activity is expressed as fold increase relative to the HSC-3 cells in suspension. Data are shown as the mean ± SD of three independent experiments.

Fig. 3.4. **Effect of EGFR-blocking antibodies on EGFR activation in HSC-3 MCAs.** Cells were plated for MCA formation in the presence of anti-EGFR mAb (Ab-3, 50 ng/ml) for the indicated times before protein extraction. As controls, monolayer cells serum-starved overnight were left untreated (ML), stimulated with 15 ng/ml EGF for 5 min (ML+EGF), or preincubated with 50 ng/ml Ab-3 (ML+EGF+Ab-3) for 30 min before incubation with 15 ng/ml EGF for 5 min.

Fig.3.5. **Effect of dominant-negative EGFR on EGFR activation in HSC-3 MCAs.** ***A***, dominant-negative EGFR-CD533 blocked EGF-induced EGFR activation in HSC-3 monolayer. HSC-3 cells were infected with recombinant adenoviruses to express either EGFR-CD533 or control GFP. Twenty-four hours after initial infection, cells were serum-starved overnight and stimulated with 15 ng/ml EGF for 5 min. Data reported here are representative of three independent experiments. ***B***, dominant-negative EGFR-CD533 failed to inhibit EGFR activation in HSC-3 MCAs. HSC-3 cells were infected with recombinant adenoviruses to express either EGFR-CD533 or control GFP. Twenty-four hours after initial infection, cells were plated as MCA culture for the indicated times. Data reported here are representative of three independent experiments.

Fig.3.6. **Phosphorylation of specific EGFR tyrosines by E-cadherin-mediated cell-cell adhesion.** HSC-3 cells were cultured as MCAs for different time periods as indicated. As controls, HSC-3 cells were serum-starved overnight and then treated with 15ng/ml EGF for 5 min. Equivalent protein was immunoblotted with antibodies that specifically recognize phosphorylated tyrosine 1068 (pY1068), 1086 (pY1086), 1148 (pY1148) or 1173 (pY1173). The same blots were reblotted with antibodies to total phosphorylated EGFR (p-EGFR). Relative protein densities of each experiments were determined and shown on the right.

Fig. 3.7. **E-cadherin–EGFR complex formation correlates with EGFR activation during cell-cell adhesion.** HSC-3 cells were plated as ML for 24 h or as MCA for the indicated times. E-cadherin was immunoprecipitated (IP) from total cell lysates with HECD-1 mAb. The association of activated EGFR (p-EGFR) or EGFR with E-cadherin was detected with specific anti-p-EGFR or anti-EGFR mAb. The amount of EGFR and E-cadherin in total cell lysates by immunoblotting is also shown for comparison.

Fig.3.8. **Activated EGFR in MCAs is preferentially complexed with E-cadherin.** HSC-3 cells were plated as ML or MCA culture for 24 h. Cell extracts from EGF-treated HSC-3 monolayer cells and HSC-3 MCAs were exhaustively immunoprecipitated with anti–E-cadherin mAb to recover E-cadherin. Then the E-cadherin–depleted supernatants were immunoprecipitated with anti-EGFR antibodies. The anti-E-cadherin (top panel) and anti-EGFR (lower panel) immunoprecipitates were processed for immunoblotting with anti-p-EGFR mAb to access levels of activated EGFR. Membranes were stripped

and reblotted with anti–EGFR mAb and anti–E-cadherin antibodies (top panel only). The E-cadherin level in E-cadherin–depleted supernatants is also shown (lower panel). The ratio of the EGFR associated with E-cadherin/total cellular EGFR was determined by relative protein densities.

Fig.3.9. **EGFR cytoplasmic domain is required for association with E-cadherin.** HSC-3 cells were infected with recombinant adenoviruses to express either EGFR-CD533 or control GFP. Twenty-four hours after initial infection, cells were plated as ML or MCA culture. E-cadherin was immunoprecipitated from total cell lysates with HECD-1 mAb. The association of EGFR or EGFR-CD533 with E-cadherin was detected with specific anti-EGFR extracellular domain antibodies. The expression of both EGFR and EGFR-CD533 in EGFR-CD533–transduced cells is indicated for total cell lysates.

Fig.3.10. **Assembly and colocalization of the EGFR/E-cadherin complex is independent on EGFR kinase activity.** ***A.*** HSC-3 cells were cultured as MCAs for 6 h in the absence or presence of AG1478 (1 μM) and then transferred onto glass coverslips and immunolabeled for EGFR with rabbit anti-EGFR polyclonal antibodies (green), and E-cadherin with mouse anti-E-cadherin mAb or p-EGFR with mouse mAb anti-activated, phosphorylated EGFR (red). Specimens were analyzed by confocal microscopy, and representative images are shown. ***B.*** HSC-3 cells were plated as MCA for 12h in the absence or presence of AG1478 (1 μM). E-cadherin was immunoprecipitated (IP) from total cell lysates with HECD-1 mAb. The presence of activated EGFR (p-EGFR) or total

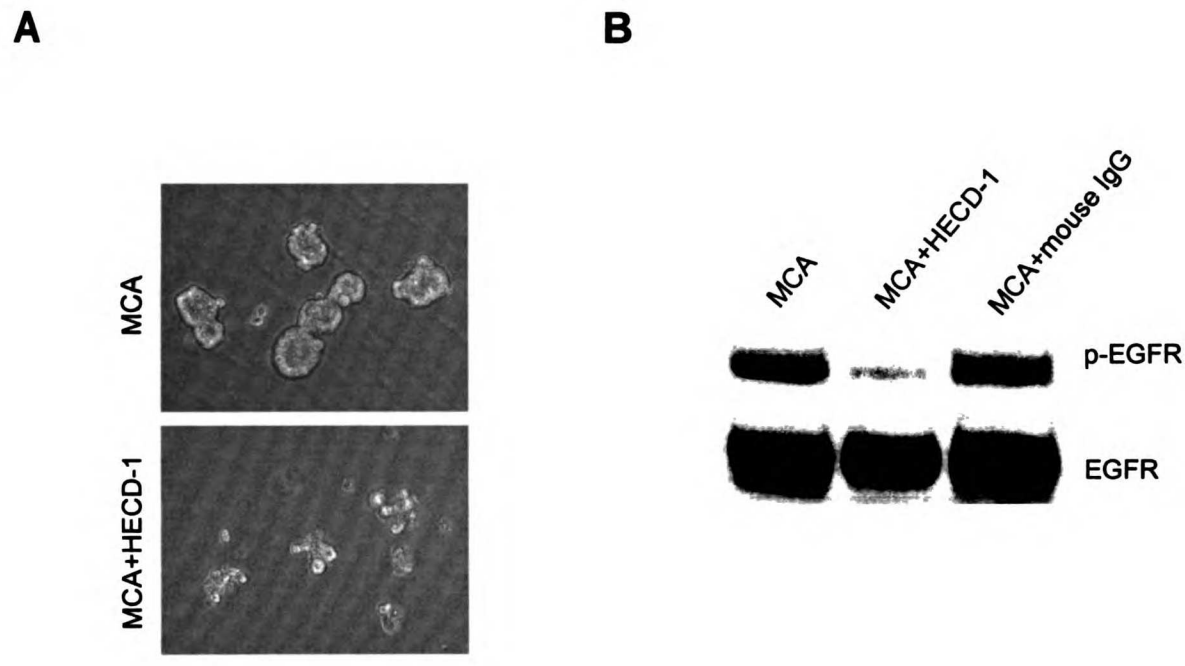
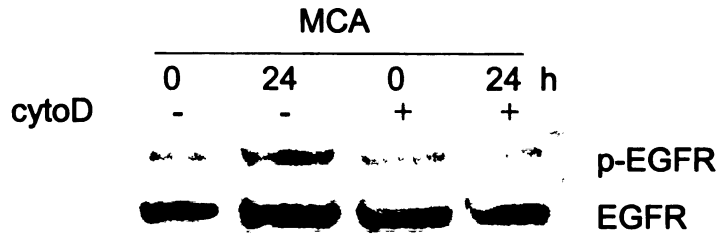


Figure 3.1

A


MCA
0
24
0
24 h
cytoD
-
-
+
+
p-EGFR
EGFR

B

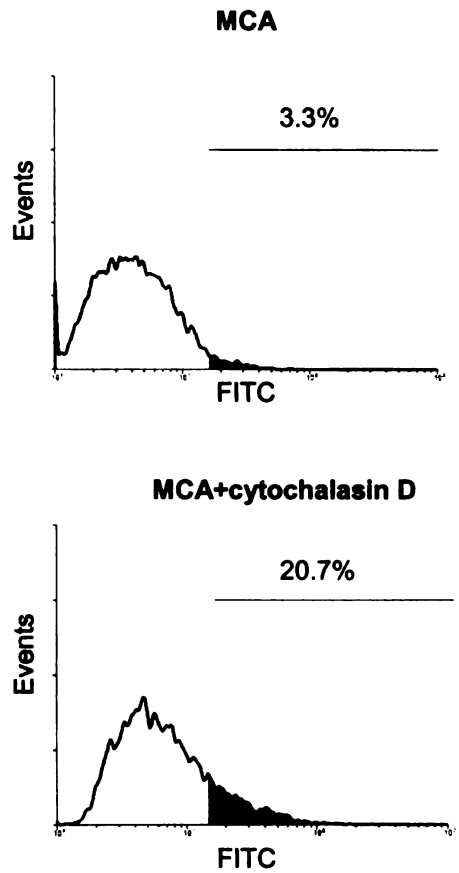

MCA
3.3%
Events
FITC
MCA+cytochalasin D
20.7%
Events
FITC

Figure 3.2

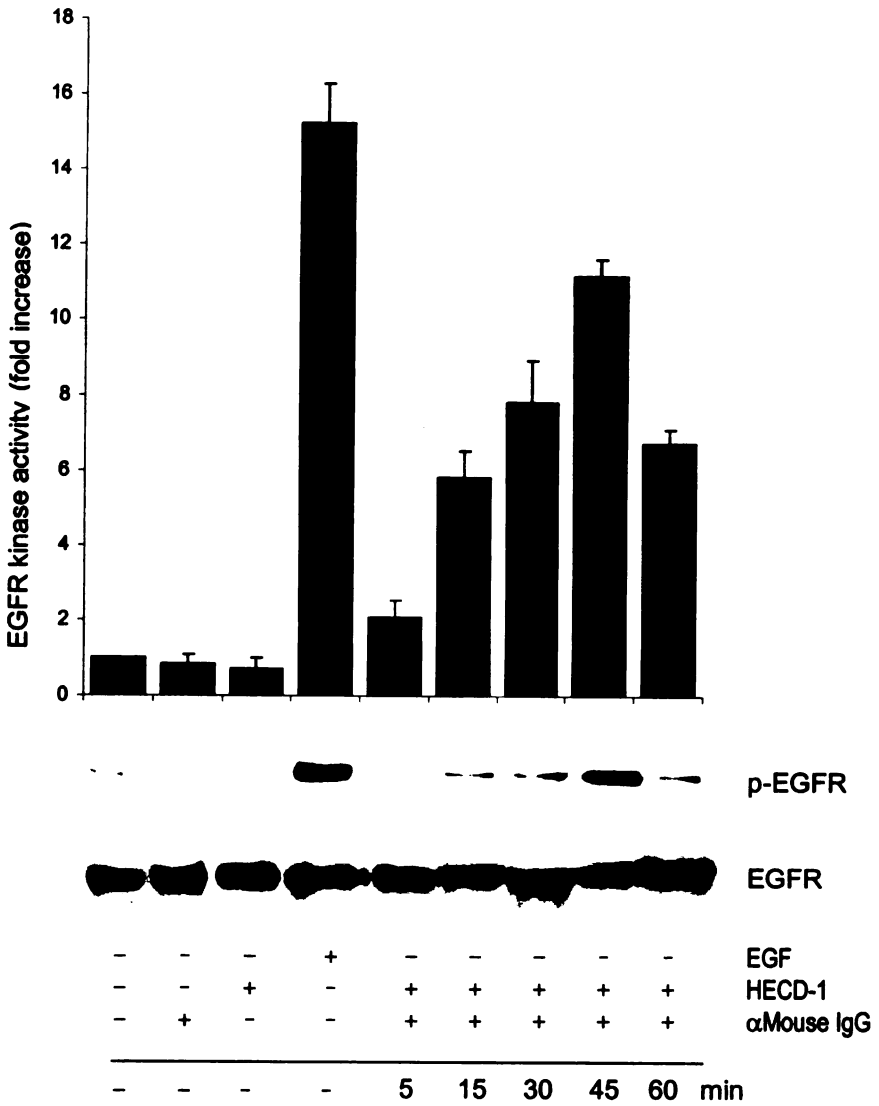

EGFR kinase activity (fold increase)
18
16
14
12
10
8
6
4
2
0
p-EGFR
EGFR
EGF
HECD-1
αMouse IgG
5 15 30 45 60 min

Figure 3.3

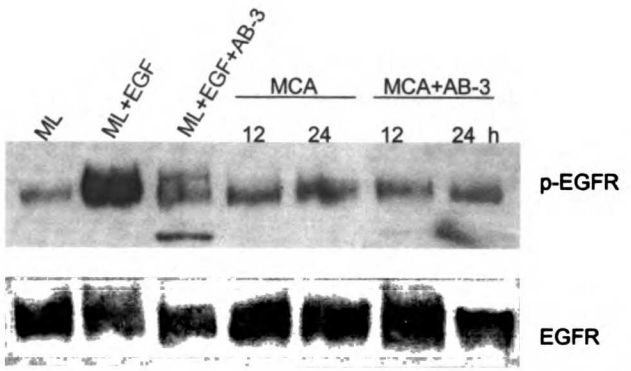


Figure 3.4

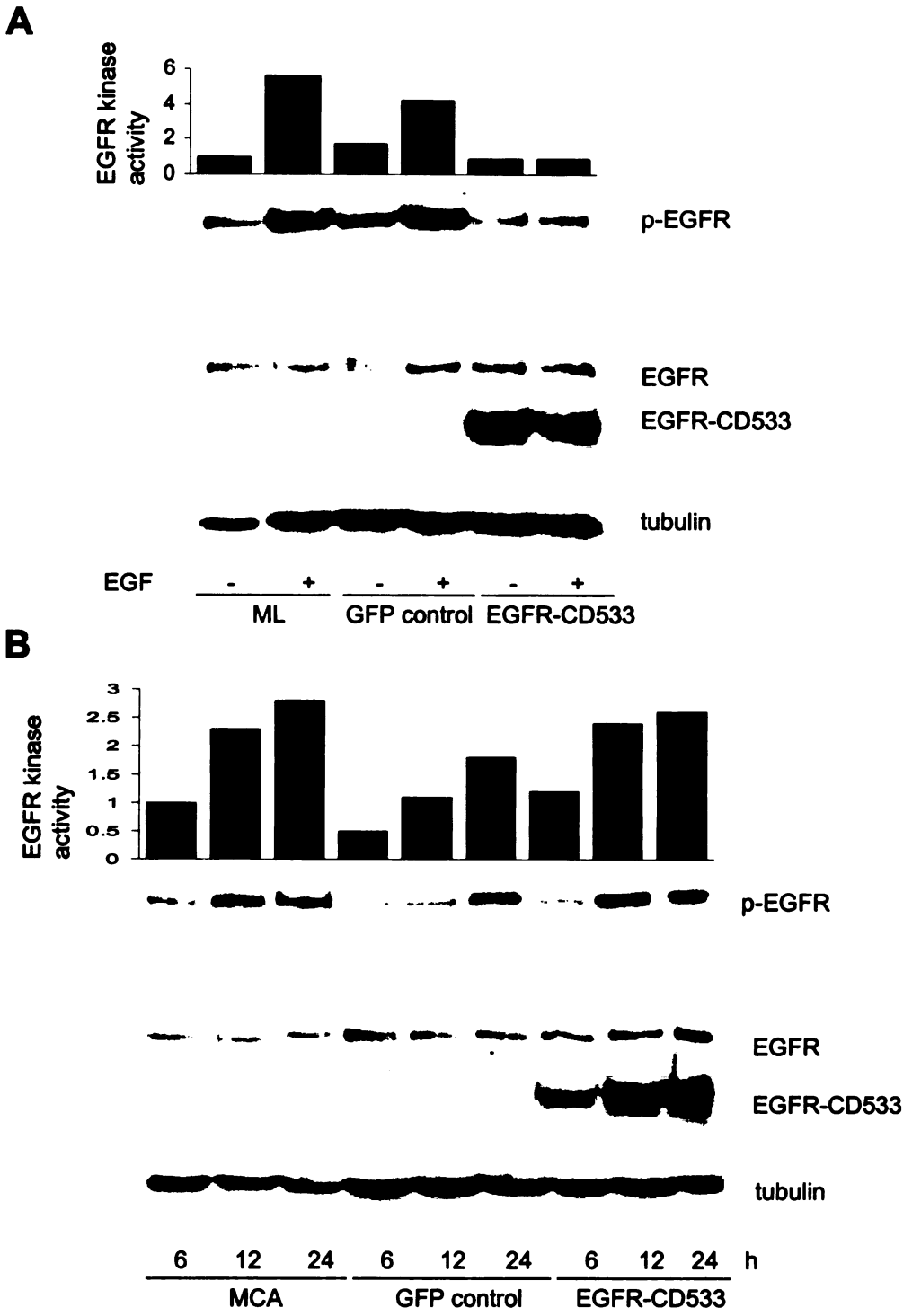
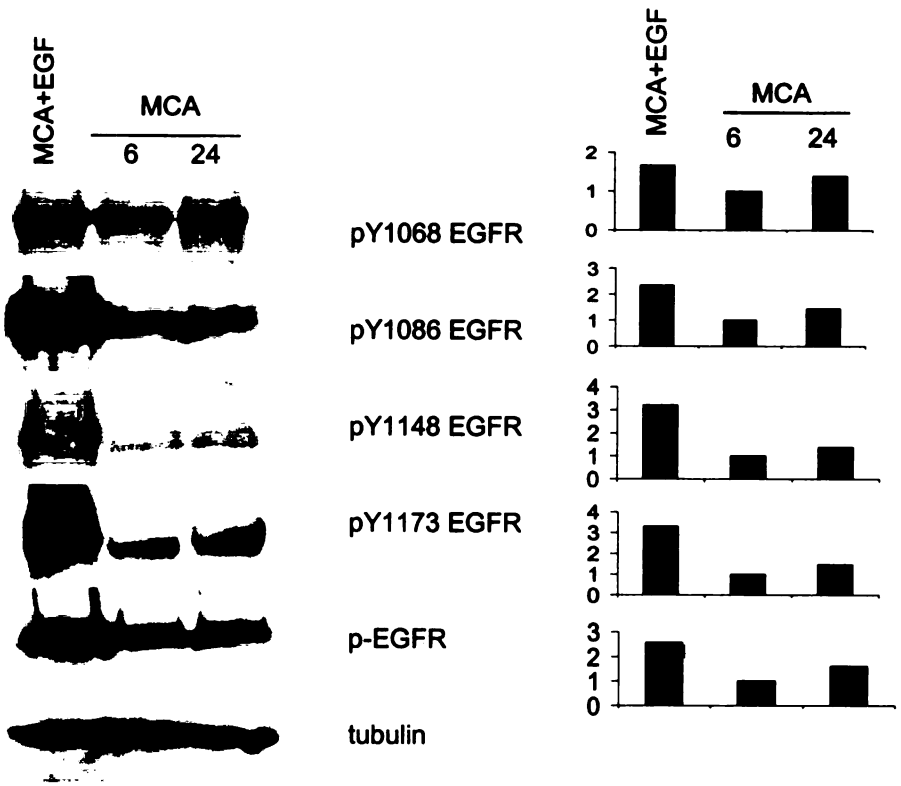

A
EGFR kinase activity
6
4
2
0
p-EGFR
EGFR
EGFR-CD533
tubulin
EGF
- + - + - +
ML
GFP control
EGFR-CD533
B
EGFR kinase activity
3
2.5
2
1.5
1
0.5
0
p-EGFR
EGFR
EGFR-CD533
tubulin
6 12 24 6 12 24 6 12 24 h
MCA
GFP control
EGFR-CD533

Figure 3.5


MCA+EGF
MCA
6
24
pY1068 EGFR
pY1086 EGFR
pY1148 EGFR
pY1173 EGFR
p-EGFR
tubulin
MCA+EGF
MCA
6
24
2
1
0
3
2
1
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4
3
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1
0
4
3
2
1
0
3
2
1
0

Figure 3.6

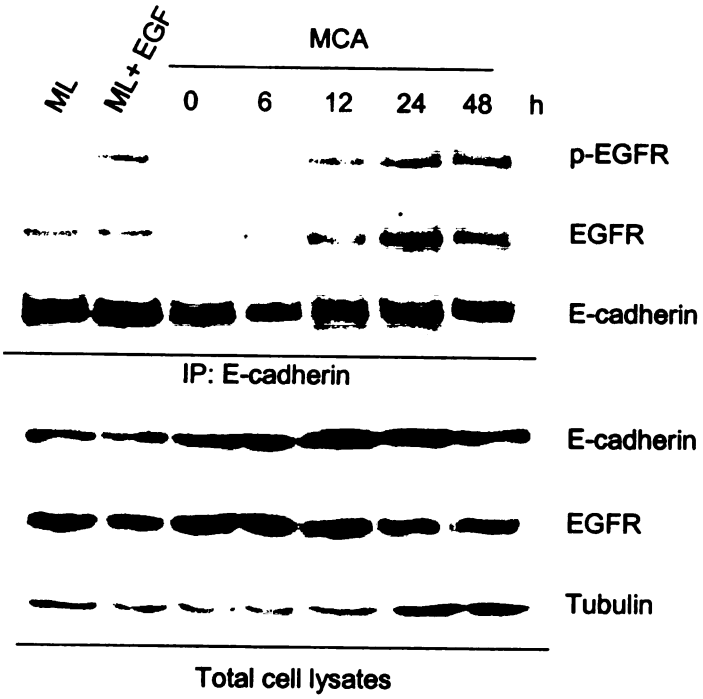

ML
ML+ EGF
MCA
0
6
12
24
48
h
p-EGFR
EGFR
E-cadherin
IP: E-cadherin
E-cadherin
EGFR
Tubulin
Total cell lysates

Figure 3.7

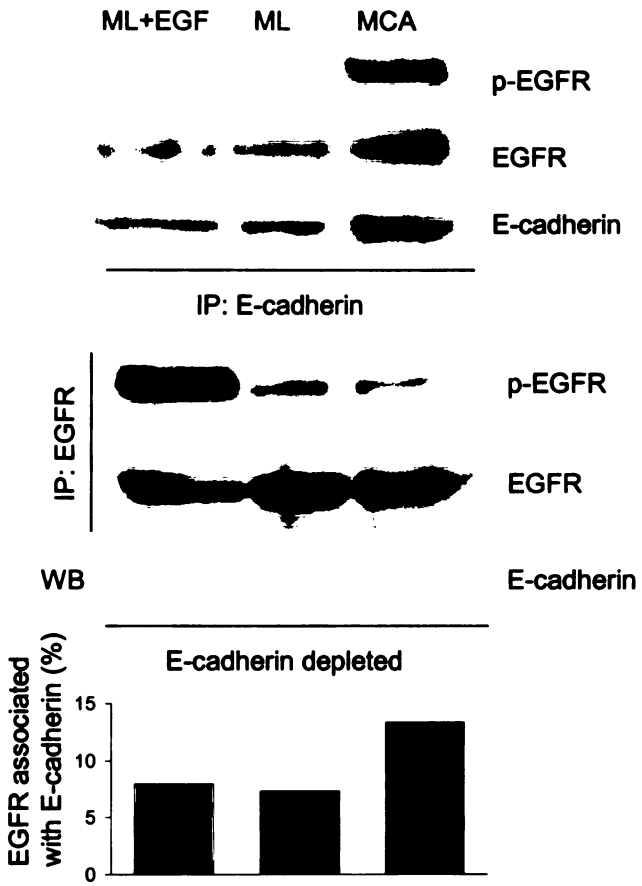

ML+EGF
ML
MCA
p-EGFR
EGFR
E-cadherin
IP: E-cadherin
IP: EGFR
p-EGFR
EGFR
WB
E-cadherin
E-cadherin depleted
EGFR associated with E-cadherin (%)
15
10
5
0

Figure 3.8

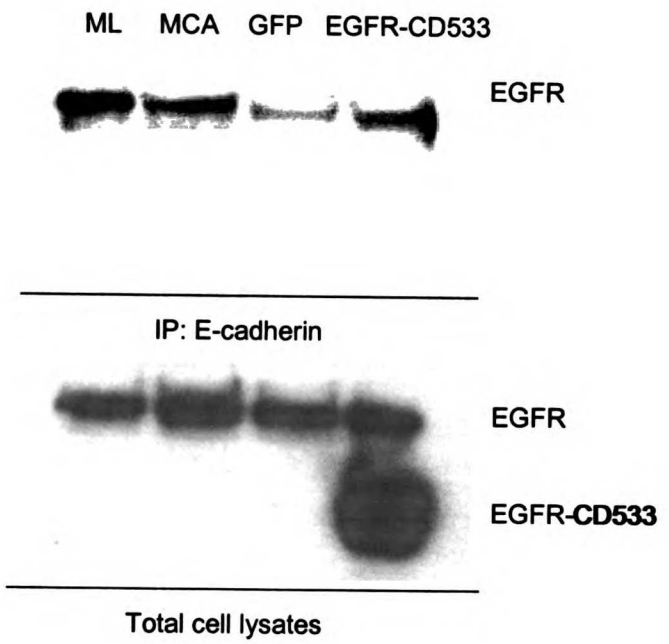

ML
MCA
GFP
EGFR-CD533
EGFR
IP: E-cadherin
EGFR
EGFR-CD533
Total cell lysates

Figure 3.9

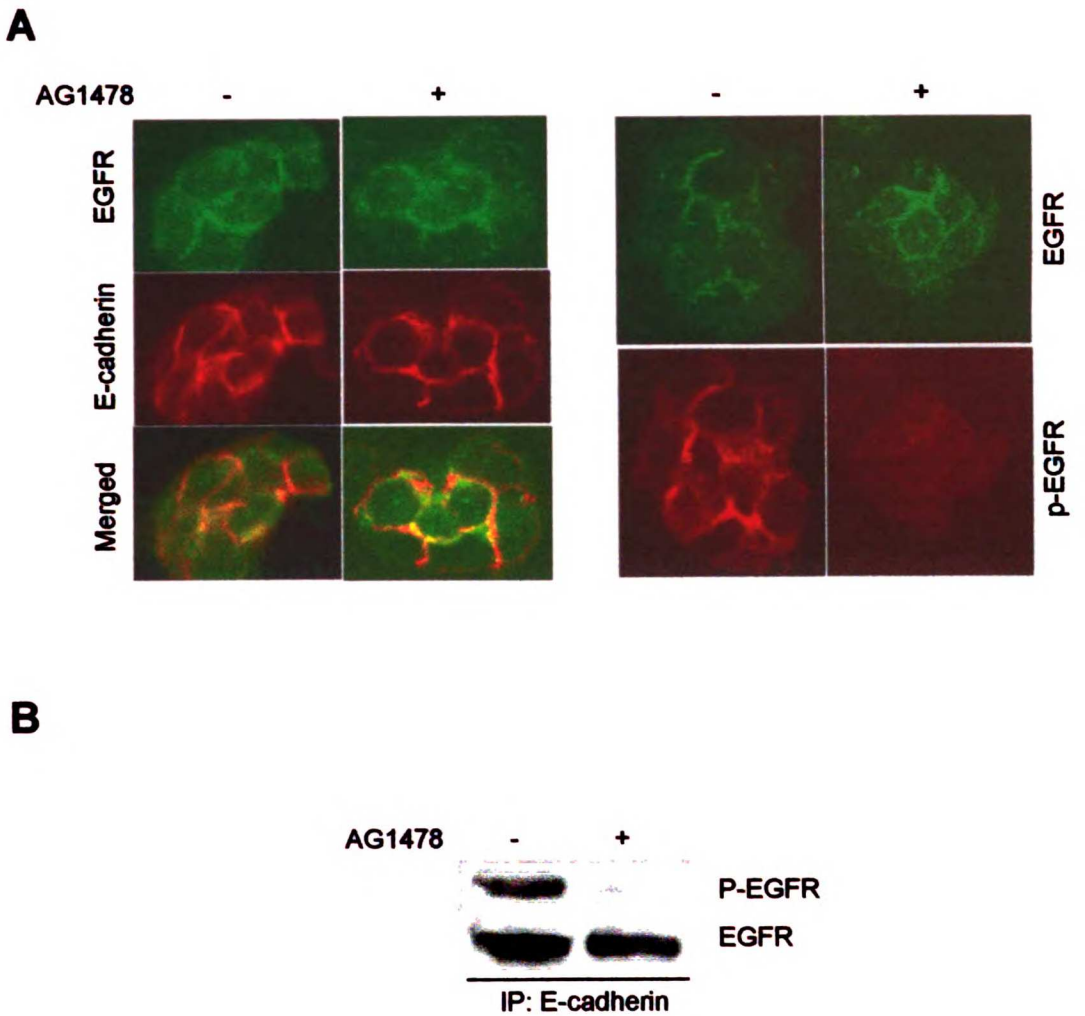
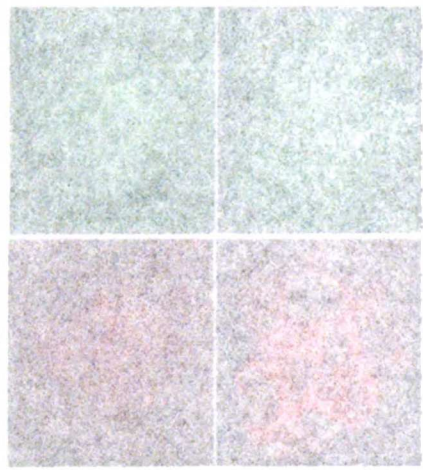

A
AG1478
-
+
-
+
EGFR
E-cadherin
Merged
EGFR
p-EGFR
B
AG1478
-
+
P-EGFR
EGFR
IP: E-cadherin

Figure 3.10



CHAPTER FOUR: DISCUSSION

We have shown that SCC cells cultured in suspension are able to avoid apoptosis when they form tissue-like multicellular aggregates. Thus, cell-cell contacts promote cell survival in the absence of interactions with the extracellular matrix and create a permissive environment for cell proliferation. This behavior is similar to how SCC cells are organized *in vivo,* where they proliferate suprabasally to form "nests" of three-dimensional aggregates with extensive cell-cell junctions. We use of the Greek term *synoikis* to describe this process of requiring neighboring associations of cells for survival (Fig. 4.1). The current data provide evidence that cell-cell adhesion triggers cadherin-dependent activation of EGFR and its associated downstream signaling pathways that promotes cell survival.

It seems that EGFR is the only tyrosine kinase receptor that is activated following cell-cell adhesion in our system. While other c-erbB family member like erbB2 is expressed in HSC-3 cells at a low level, it is not activated following cell-cell adhesion, which suggests that the expression level of the receptor may be important for its transactivaiton. Interestingly, the tyrosine kinase receptor c-Met is overexpressed in HSC-3 cells and is associated with E-cadherin (Hiscox and Jiang, 1999), we found no evidence of c-Met activation in multicellular aggregates.

We have used pharmacological inhibitors of EGFR and MEK to address the involvement of EGFR and the downstream MAPK pathway in E-cadherin-mediated cell survival. We used tyrphostin AG1478, a potent and highly specific tyrosine kinase inhibitor for EGFR. As shown by Levitzki and Gazit (1995), AG1478 inhibits EGFR tyrosine kinase *in vitro* with an IC_{50} = 3nM whereas the corresponding IC_{50} values for inhibition of other tyrosine kinases by this molecule are all greater than 50uM (HER2

IC_{50} > 100uM; PDGF IC_{50} > 100uM). In the present study, a concentration of 1uM AG1478 completely obliterated EGFR activation in HSC-3 MCAs and reversed E-cadherin-mediated anoikis resistance. For inhibiting ERK1/2 activation in the MAPK pathway, we used the specific MEK inhibitor U0126. This compound is known to effectively inhibit the kinase activity of both MAPK kinases, MEK-1 and -2, and hence activation of ERK1/2 (Gibson et al, 1999). In our study, U0126 at 10uM/ml almost totally blocked ERK1/2 phosphorylation in HSC-3 MCAs and induced apoptosis within 48h.

It has long been accepted that a cell's survival depends on signals provided by its surroundings. These signals can be generated by (a) soluble or membrane-bound ligands (e.g., growth factors, cytokines) that engage specific cell surface receptors, (b) cell-extracellular matrix interaction, or (c) intercellular adhesion between neighboring cells. As a cell surface receptor, EGFR has emerged as an important modulator of cell survival. A role for EGFR activation in epithelial cell survival was first suggested by the observation that treatment with EGFR-blocking antibody (C225) induces spontaneous apoptosis of the colorectal carcinoma cell line, DiFi (Wu et al., 1995). Furthermore, activation of EGFR confers resistance to anoikis in suspension cultures of normal epidermal keratinocytes (Rodeck et al., 1997) and mammary epithelial cells (Gilmore et al., 2002), suggesting that growth factor receptors can provide complementary survival signals to epithelial cells when cells are deprived of cell-matrix interaction. Consistent with these findings is the observation that EGF stimulates anchorage-independent growth of oral SCC cell lines (Lee et al., 1990). As the main intercellular adhesion receptor, cadherins have been implicated in regulating cell survival. A role for cadherins in

inhibition of apoptosis in granulosa cells (Peluso et al., 1996), oral SCC cells (Kantak and Kramer, 1998), and prostate carcinoma cells (Tran et al., 2002) has been demonstrated. In the current study, we were able to link two different kinds of survival signal generators—intercellular adhesion receptors and cell surface receptors—and we propose that different cell survival machineries can act synergistically to amplify survival signals in carcinoma cells.

Cadherins may modulate cell survival through multiple mechanisms. In prostate and mammary epithelial cells, E-cadherin–mediated aggregation promotes cell survival through the Rb cell cycle control pathway (Day et al., 1999). In prostate carcinoma cells, N-cadherin stimulates the PI3K/Akt pathway (Tran et al., 2002), thereby upregulating Bcl-2. This process is supported by observation that E-cadherin activates the PI3K/Akt pathway (Pece et al., 1999). Our results indicate that E-cadherin promotes SCC cell survival through activation of EGFR and a downstream MAPK pathway. This mechanism is supported by a previous study using a calcium-switch approach in monolayer cultures, demonstrating that E-cadherin–mediated cell-cell contact formation activates the MAPK pathway (Pece and Gutkind, 2000). The finding that E-cadherin–mediated cell-cell adhesion induces activation of Gab-1, a docking protein that activates both ras/MAPK and PI3K/Akt pathways, suggests that both mechanisms may be operational (Shinohara et al., 2001). Although the specific mechanism by which E-cadherin modulates cell survival may be cell-type specific, the possible mechanisms are not mutually exclusive and may function simultaneously.

The normally low level of Akt activation observed in cell monolayer was strongly enhanced following EGF stimulation. However, we were unable to detect significant

levels of Akt phosphorylation in cells grown as MCA. Consistent with the apparent lack of Akt activation in cell aggregates, inhibition of the PI3K/Akt pathway with LY294002 had little effect on cell survival in MCA in contrast to inhibitions of EGFR or the ERK/MAPK pathway. We conclude that for squamous cell carcinoma cells, the primary signaling pathway essential to E-cadherin-mediated SCC cell survival is through EGFR activation of the MAPK pathway. Interestingly, inhibition of the ERK/MAPK pathway with specific inhibitors produced modest induction of anoikis compared to that induced by EGFR kinase inhibition. This suggests that additional signaling pathways downstream of EGFR activation may also be involved in SCC cell survival (e.g., STAT3, JNK and PLCγ) (Song et al., 2003; Lamb et al., 2003; Miao et al., 1997).

Because commitment to apoptosis is often decided by the relative balance between pro- and anti-apoptotic members of the Bcl-2 protein family, cadherin-mediated cell survival signaling is likely to lead to the regulation of members of this family. In our study, EGFR activation led to the elevation of anti-apoptotic Bcl-2, which is consistent with our previous report (Kantak and Kramer, 1998). In contrast, the levels of pro-apoptotic Bax remained constant. Thus, the Bcl-2/Bax ratio increases after E-cadherin–mediated adhesion. It is well known that cell survival is favored by a high Bcl-2/Bax protein ratio (Mackey et al., 1998). N-cadherin's upregulation of Bcl-2 through the PI3K/Akt pathway is carried out by phosphorylation of the pro-apoptotic protein Bad (Tran et al., 2002). In our study, the phosphorylation level of Bad remained stable after cell-cell adhesion (data not shown), although ERK/MAPK has been shown to increase the level of Bcl-2 through the phosphorylation of Bad (Scheid and Duronio, 1998). This suggests that the increase in Bcl-2 protein we observed is due to upregulation of the Bcl-2

gene by ERK/MAPK (Liu et al., 1999), although additional work is needed to define this process.

EGFR transactivation has been reported to take place in various systems by both ligand-dependent and ligand-independent mechanisms. For example, G-protein–coupled receptors activate EGFR through a ligand-dependent mechanism by promoting the cleavage of EGF-like precursors and the production of soluble ligand (Prenzel et al., 1999; Gschwind et al., 2003). In contrast, integrin adhesion receptor has been proposed to regulate EGFR activation through a ligand-independent activation pathway (Moro et al., 2002; Liu et al., 2002). Our results indicate that EGFR transactivation in multicellular aggregates following E-cadherin–mediated adhesion is ligand independent, consistent with the results of Pece and Gutkind with monolayer cells(2000).

It will be interesting to compare the details and consequences of ligand-dependent and ligand-independent activation of EGFR. In our study, as in others' (Moro et al., 1998), the level of EGFR phosphorylation was less intense than that produced by acute activation with EGF. Furthermore, Moro et al. (2002) found that integrins induce phosphorylation of EGFR on tyrosine residues 1068, 1086, and 1173, but not on residue 1148, a major site of phosphorylation in response to EGF. Although our results showed that all four tyrosine residues were phosphorylated following E-cadherin-mediated cell-cell adhesion, the extent of phosphorylation was much lower on tyrosine residues 1086, 1148 and 1173 compared to that of induced by EGF. Thus, it is possible that integrin and E-cadherin used different mechanisms to induce EGFR activation. In the ligand-independent model, the number or extent of phosphorylation sites in the EGFR autophosphorylation domain may differ from those produced in ligand-dependent model.

Furthermore, we showed that cell-cell adhesion-mediated EGFR activation, as well as downstream MAPK signaling, lasts as long as 24 to 48 h. These attenuated but sustained signals generated via adhesion receptors presumably yield a distinct set of signals that are different from the acute but transient activation induced by ligand. As previously reviewed by Marshall (1995), transient or sustained MAPK activation leads to different cellular responses (e.g., proliferation, differentiation), possibly through changes in gene expression. In our case, the attenuated but sustained MAPK signaling may activate anti-apoptosis pathways, resulting in enhanced cell survival.

We found several lines of evidences supporting that the activation of EGFR upon cell-cell contact formation is E-cadherin dependent. 1) Blocking the formation of E-cadherin-mediated adhesion using function-perturbing antibodies abolishes the elevation of EGFR phosphorylation in MCAs. 2) Antibody-mediated clustering of E-cadherins in suspended cells induced rapid activation of EGFR. 3) Exogenous expression of E-cadherin in E-cadherin-negative cells restored EGFR activation in MCAs. These findings suggest that E-cadherin ligation itself is both sufficient and necessary for ligand-independent activation of EGFR.

The requirement of cytoskeleton organization for E-cadherin-mediated signaling activity and survival is not surprising since previous evidence suggests that the suppression of apoptosis following attachment is not mediated solely by integrin/ECM interaction, but rather by the ability of the cells to spread and maintain an optimal cell shape (Chen et al, 1997). Cadherin-mediated cell-cell adhesion is just another mechanism to control cell shape and organization of the cytoskeleton. The involvement of cytoskeleton in cell survival has been summarized in several possible connections (Frisch

and Screaton, 2001), including: 1) JNK, an important survival regulator, is in turn regulated by the cytoskeletal protein filamin (Browne et al., 2000). 2) Two potently proapoptosis members of the Bcl-2 family, Bim and Bmf, are sequestered by cytoskeletal proteins DLC1 and DLC2, respectively (Puthalakath et al., 1999; Puthalakath et al., 2001). 3) Another cytoskeletal protein gelsolin prevents the Bax-stimulated release of cytochrome c, whereas caspase-cleaved gelsolin promotes apoptosis by an as yet unknown mechanism (Koya et al., 2000). In our experiments we found that the disruption of cytoskeleton organization lead to decrease of EGFR activation, which in turn leads to increase of apoptotic cells. However, it is possible that any of the above mechanisms can also be involved.

It remains unclear exactly how E-cadherin–mediated cell-cell adhesion transactivates EGFR. It is likely that activation of EGFR in cell aggregates proceeds via a two-step process that includes the initial linkage of the receptor with E-cadherin, which does not involve EGFR activation, followed by E-cadherin homodimerization. The formation of the EGFR/E-cadherin complex does not appear to be dependent on cell-cell adhesion, as it is present even in monolayer cells although at lower levels. This complex of EGFR and E-cadherin may be preexisting, possibly formed following synthesis of both receptors. When cells begin to assemble new junctional adhesions, E-cadherin alone and EGFR/E-cadherin complexes are somehow recruited to these forming zonula adherens junctions presumably by interactions with the cytoskeleton. More studies are needed to define the mechanism of this process. Apparently it is the juxtaposition and oligomerization of neighboring EGF receptors through catenin linkage to the cytoskeleton following recruitment of the EGFR/E-cadherin complex to junctional

contacts that triggers transactivation of EGFR. It is believed that ligand-induced dimer formation is required for EGFR activation and autophosphorylation of tyrosine residues in the carboxyl terminal of the receptor. It seems likely that cell-cell adhesion-induced oligomerization of EGFR leads to its concurrent activation and downstream signaling.

It is important to note that formation of the E-cadherin–EGFR complex is not sufficient for EGFR activation. In monolayer culture, the recovered complex contained low levels of p-EGFR. Also the process of EGFR activation is not distinctive from its capacity to form a complex with E-cadherin since even in monolayer cells where the relative level of EGFR/E-cadherin complex is relatively low, it does not increase functionally following EGF-induced activation. Since EGFR kinase activity does not appear to be required for the formation of an EGFR/E-cadherin complex, it is likely that an important event in the EGFR activation occurs following insertion of the complex at the cell-cell junction. In contrast, Moro et al. showed that EGFR kinase activity is required for the assembly of integrin-EGFR complex (Moro et al., 2002), which suggests that integrin and E-cadherin may use a different mechanism to transactivate EGFR. Taken together, these results suggest that E-cadherin by complexing with EGFR at nascent adherens junctions acts as an oligomerization scaffolding through interaction with the cytoskeleton. This is consistent with the junctional localization of activated EGFR in MCAs.

Previous studies have shown that, while human cancers such as SCCs are growing as multicellular masses *in vitro* or *in vivo,* cell-cell adhesion promotes resistance to treatment strategies, including traditional chemotherapy and radiotherapy (Green et al., 1999; St Croix et al., 1998b; Damiano et al., 2001). Our results may provide a clue to the

mechanism by which cell-cell adhesion renders tumor cells resistant to therapy since the major target is to induce apoptosis. Interestingly, in oral SCCs, modest E-cadherin expression is preserved in lesions advancing from premalignant to invasive and even metastatic stages (Bowie et al., 1993; Andrews et al., 1997; Ziober et al., 2001). We speculate that at least during the early stages of tumor progression, SCC may depend on cell-cell adhesion for suprabasal survival and proliferation and that E-cadherin–mediated cell survival via EGFR may render cancer cells resistant to treatment. Of note, some studies have shown that resistance to chemotherapy and radiotherapy can be pharmacologically reversed by using treatment that disrupts either cell-cell adhesion (St Croix et al., 1998a) or EGFR function (Raben et al., 2002; Bonner et al., 2002).

In summary, our results suggest a novel mechanism for adhesion-mediated cell survival that we call synoikis. E-cadherin–mediated cell-cell adhesion induces ligand-independent EGFR activation, which triggers the ERK/MAPK signaling module, leading to elevation of anti-apoptotic Bcl-2 (Fig.4.2). Thus, E-cadherin signals appears compensate for the loss of integrin signals, thereby suppressing anoikis. However, the exact mechanism of E-cadherin–induced EGFR transactivation is unclear, but it is likely correlated with EGFR–E-cadherin complex formation and recruitment to the cell-cell junctions. Although it has been shown that EGFR can be linked via β-catenin to the cytoplasmic tail of E-cadherin (Hoschuetzky et al., 1994), the exact domain of EGFR required for β-catenin binding is still unknown. It will also be interesting to see whether this mechanism is at work in other cell lines beside SCC cells and whether it can be fit together with other E-cadherin-mediated survival mechanisms such as Rb cell cycle control. Further studies are needed to resolve these issues.

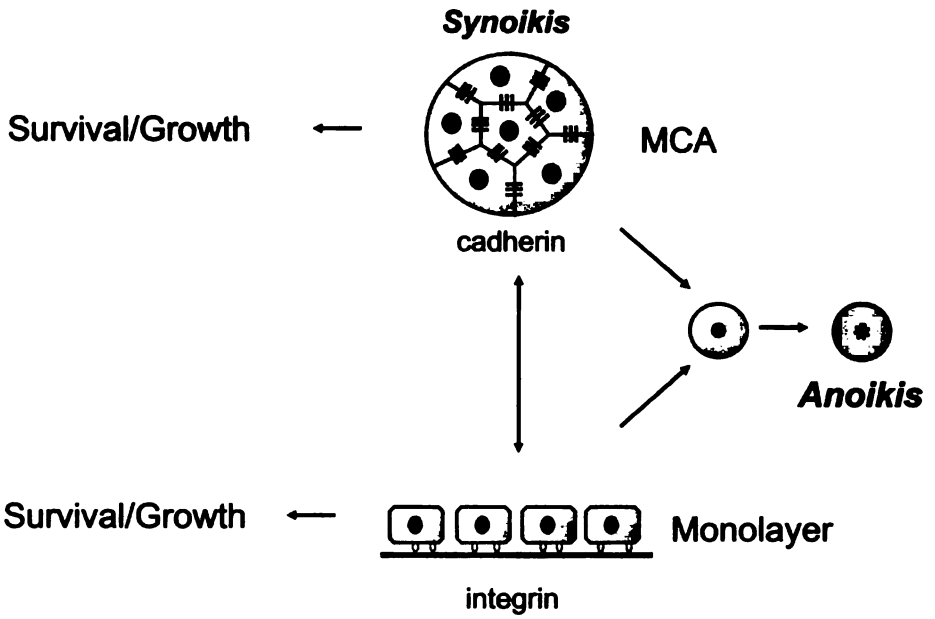


Fig.4.1. **Adhesion-mediated cell survival.** SCC cells require either integrin or cadherin receptor-based adhesion for survival but can switch from one to the other. However, loss of both leads to rapid onset of cell death through anoikis. We have termed the process of cells surviving together in a cooperative colony as ***"synoikis"***.

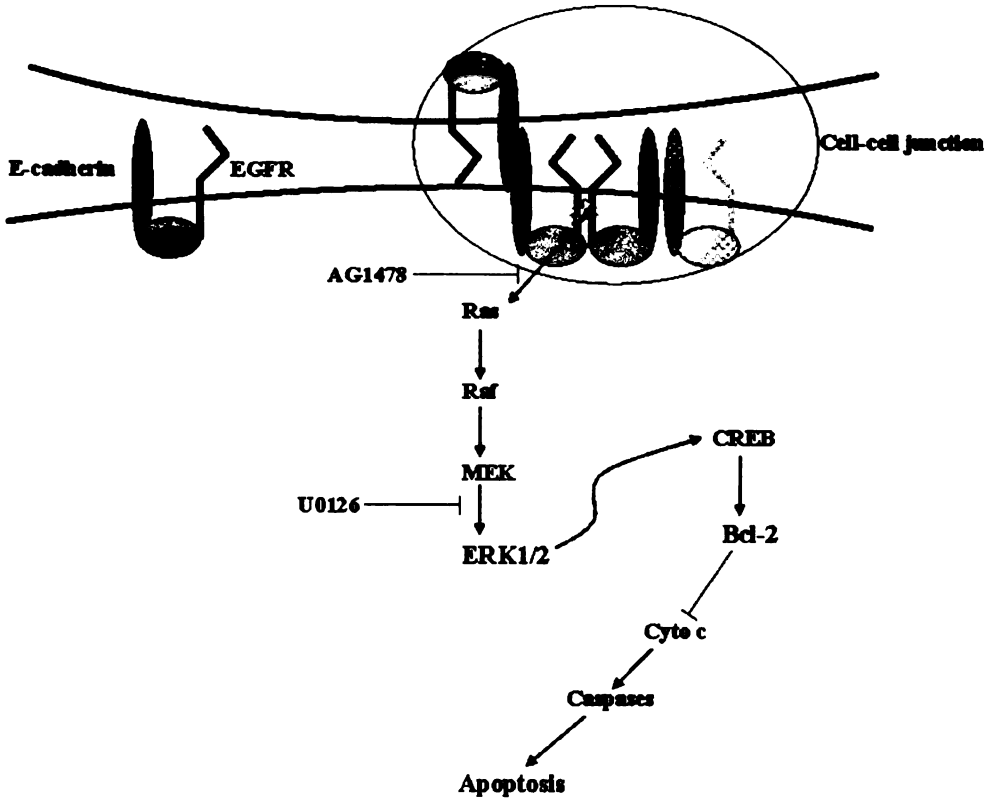


Fig.4.2. **Schematic view of E-cadherin-mediated cell survival via EGFR transactivation.** Complex of EGFR and E-cadherin through the linkage of β-catenin (β-Cat) are preexisting on cell membrane. During cell-cell adhesion formation, EGFR/E-cadherin complex are recruited to junctional contacts. EGFRs are transactivated by the juxtaposition and oligomerization of neighboring EGF receptors. EGFR kinase then triggers the ERK/MAPK signaling module, which can be blocked by EGFR kinase inhibitor AG1478 or MEK inhibitor U0126. Activation of ERK/MAPK leads to elevation of anti-apoptotic Bcl-2 and prevents apoptosis.

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