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13. ABSTRACT ***(Maximum 200 Words)***

We identified the D7 antigen in a screen for kinases that are overexpressed in Ras-transformed breast epithelia. We generated monoclonal antibodies specific for tyrosine phosphorylated proteins in breast cancer cells and focussed upon one particular antigen that was overexpressed in transformed epithelia. We also linked the function of D7 with the E-cadherin tumor suppressor. Our first objective was to identify the D7 antigen, determine the basis of its enzymatic activity (as a tyrosine or serine/threonine kinase), and determine if D7 interacts with E-cadherin. Our second objective was to measure the phosphorylation and enzymatic activity of this kinase in normal and transformed epithelia. Our final objective was to measure D7 expression in breast cancer cells and determine if D7 (EphA2) overexpression altered cell growth, migration or invasiveness. In this progress report, we demonstrate that the D7 antigen is the EphA2 receptor tyrosine kinase. We find that E-cadherin stabilizes the binding of EphA2 to its cell-bound ligands. We also demonstrate gross overexpression of EphA2 in malignant breast cancer cells as confirmed both with cellular models and in archival specimens of breast cancer tissues. Altogether, our results demonstrate extraordinary potential of EphA2 as a marker and target of malignant breast cancer.

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INTRODUCTION:

Cancer often involves alterations in signaling pathways that result in a plethora of tyrosine phosphorylated substrates. However, most of the tyrosine kinases that are responsible for this signaling are unknown. The long-term goal of our research is to identify and establish the functional relevance of tyrosine kinases that are overexpressed in cancer cells. Specifically, we have sought to identify novel kinases responsible for the elevated tyrosine phosphorylation in breast cancer cells. We focussed our attention upon one particular tyrosine kinase that was recognized by D7 antibodies, which were generated against tyrosine phosphorylated protein in breast cancer cells. We demonstrate here that the D7 antigen is the EphA2 receptor tyrosine kinase.

BODY:

Identification of Tyrosine Kinases in Malignant Cells

We originally identified the D7 antigen in a screen for tyrosine kinases and their substrates that are overexpressed or functionally altered in cancer cells. Our basis for this investigation was the fact that many different studies had linked tyrosine kinases with metastatic transformation (Parsons and Parsons, 1993). It was also understood that tyrosine kinases initiate intracellular signaling pathways that are necessary for the growth, migration and survival of cancer cells. However, the identity of most tyrosine kinases that are overexpressed or activated in cancer cells was unknown.

To identify tyrosine kinases that are overexpressed or functionally altered in breast cancer, we generated a panel of monoclonal antibodies that were specific tyrosine kinases and their substrates in transformed epithelial cells (Kinch et al., 1998). We developed new methodologies to generate monoclonal antibodies against tyrosine phosphorylated proteins in cancer cells. Specifically, we isolated tyrosine phosphorylated proteins from Ras-transformed human breast epithelial cells and used this as the antigen. Improvements in antigen delivery, B-cell isolation and hybridoma culture greatly improved the sensitivity and breadth of antigen recognition. Preliminary screening identified approximately 400 different antigens whose expression or function was altered in cancer cells. From this large pool of initial candidates, we focussed upon the antigen recognized by D7 antibodies, as this antigen was both overexpressed and functionally altered in malignant cells. We report here that the D7 antigen is the EphA2 receptor tyrosine kinase (Objectives IA and IB).

As a brief background, EphA2 is a 130 kDa transmembrane receptor tyrosine kinase of the Eph family, the largest known family of tyrosine kinases (14 members to date) (Lindberg and Hunter, 1990) (Figure 1). One critical feature that is important for understanding both the nomenclature and function of Eph kinases is that they bind ligands that are attached to the cell surface (rather than soluble ligands) (Eph Nonmenclature Committee (Flanagan et al., 1997; Gale and Yancopoulos, 1997; van der Geer et al., 1994). The ligand binding properties reveal a broad dichotomy of Eph kinases. EphA kinases (EphA1 to EphA8) bind ligands that are attached to the cell surface via a GPI anchor (known as the Ephrin-A family) whereas EphB kinases (EphB1

to EphB6) bind transmembrane ligands (known as the Ephrin-B family) (Eph Nonmenclature Committee (Flanagan et al., 1997; Gale and Yancopoulos, 1997). Each Eph kinase can bind all the ligands within its group. Thus, EphA2 can bind and become activated by any of the five Ephrin-A family ligands. The unique feature of having a cell-bound ligand will be important for understanding how EphA2 function is deregulated in metastatic breast cancers.

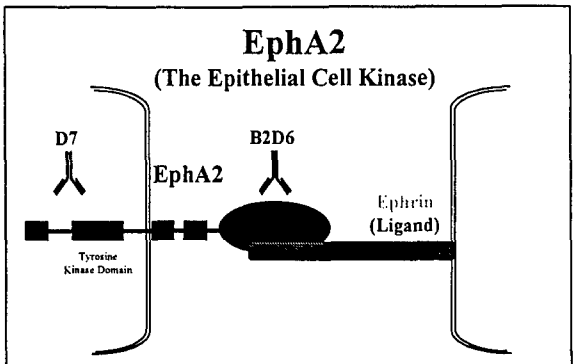


Fig. 1. Shown is a diagram of the EphA2 receptor tyrosine kinase. Eph family members are unique in that they have cell-bound ligands. Also shown are two EphA2 antibodies (D7 & B2D6) that we have isolated and characterized.

EphA2 is the only Eph family member that is expressed predominantly in adult epithelial cells (indeed, its original name was the Epithelial Cell Kinase or ECK) (Lindberg and Hunter, 1990). While other Eph family members have been of intense interest to neurobiologists, the unique expression pattern of EphA2 within epithelial cells has otherwise limited its study.

EphA2 Overexpression in Malignant Tumor Cells

We originally identified EphA2 as a tyrosine kinase that was overexpressed in Ras-transformed MCF-10A human mammary epithelial cells (Kinch et al., 1998). We subsequently confirmed this using other Ras-transformed cell model systems (e.g., Ras-transformed 267B1 prostatic epithelial cells, 10T1/2 fibroblasts or 3T3 fibroblasts). Signaling by the Ras pathway is frequently increased in breast cancer, either because of overexpression, oncogenic mutation, or activation of upstream signals (e.g., growth factor receptors) (Clark and Der, 1995). We also found that EphA2 was highly expressed in breast cancer cells using numerous cell models and neoplastic tissues. Interestingly, EphA2 was found to be grossly overexpressed in cells with metastatic potential (See Figure 2). The most prominent overexpression of EphA2 (greater than 100-fold) was found in tumor cells that were derived from metastatic sites or in cells that formed distant metastases in athymic mice (Objective 3A). Notably, differentiated breast tumor cells (*e.g.,* ZR-75-1) had lower levels of EphA2 expression than non-transformed epithelia. We found that this represented suppression of EphA2 expression by a molecular signaling network initiated by estrogen (Figure 3). Indeed, loss of ER is associated with breast cancer progression and we found that loss of estrogen-mediated suppression of EphA2 contributed to the overexpression of EphA2 expression in ER-deficient breast cancer cells (not shown).

While cell lines can provide valuable insight into mechanisms and markers of malignant transformation, one must consider that cell lines have been subjected to selection processes because of culturing conditions or multiple passages. We therefore have begun to measure EphA2 expression in benign and malignant breast tissues using immunohistochemistry (for protein levels) and *in situ* hybridization (for mRNA levels) (Objective 3A; Figure 4). Our studies used "sausage" slides, onto which multiple benign

and malignant breast specimens have been mounted onto a single slide. This provides internal controls for staining intensity. Immunostaining revealed virtually undetectable levels of EphA2 immunoreactivity in normal breast. EphA2 staining of benign cells was restricted to a small number of luminal epithelial cells; there was no staining of myoepithelial cells or stromal elements (not shown). EphA2 immunoreactivity was significantly higher in breast carcinomas as compared to benign mammary gland (Figure 4), with an average staining intensity score of 2.1 (range 0-3; $p < 10^{-8}$). In carcinoma cells, EphA2 was diffusely distributed with prominent cytoplasmic staining. Increased staining intensity was accompanied by a larger percentage of cells (an average of 60%) that stained positive for EphA2 in breast carcinomas as compared with benign epithelium. Identical results were confirmed using multiple EphA2 antibodies and with EphA2 mRNA probes (data not shown).

Taken together, we have demonstrated overexpression of EphA2 in a variety of different malignant carcinoma cells. Future studies will begin to determine if EphA2 overexpression is a cause or an effect of a malignant phenotype.

Altered Tyrosine Phosphorylation of EphA2 in Malignant Cells

Despite the gross overexpression of EphA2 in malignant cells, this EphA2 has a much-reduced phosphotyrosine content as compared with EphA2 in benign epithelial cells (Objective 2A). This does not reflect differences in EphA2 enzymatic activity since kinase assays reveal comparable levels of EphA2 activity in benign and malignant cells (Objective 2B; data not shown). In this respect, EphA2 differs from many other tyrosine kinases (*e.g.*, cErbB2, EGF receptor, PDGF receptor, Src), whose phosphorylation increases in cancer cells (Cance and Liu, 1995; Press et al., 1990; Murphy et al., 1988). For these kinases, phosphorylation elevates tyrosine kinase activity, triggering signal transduction cascades that promote cell proliferation. With EphA2, the amount of EphA2 kinase activity in a cell is directly proportional to EphA2 protein expression levels, and is independent of its phosphotyrosine content.

Nor does the decreased phosphotyrosine content of EphA2 represent a mutation of the kinase. Kinase assays reveal a comparable level of EphA2 autophosphorylation in

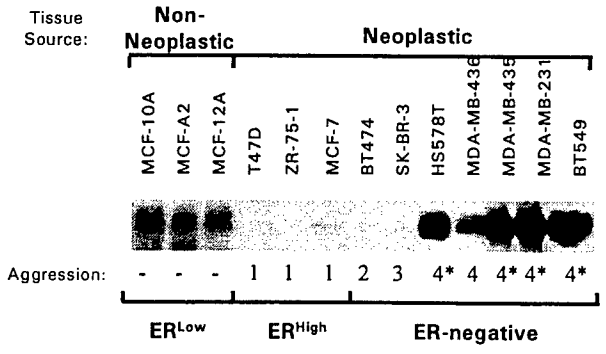


Fig. 2. EphA2 Overexpression in Invasive Breast Cancer Cells. Western blot analysis revealed that EphA2 expression correlated with ER expression. For BT474 and SKBR3, see Fig. 4. (An asterisk denotes cells that form metastatic tumors in athymic mice.

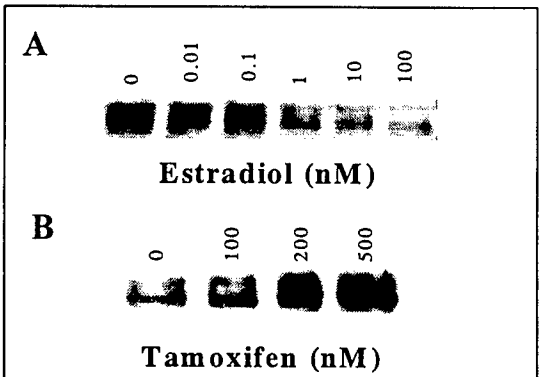


Fig. 3. Estrogen Represses EphA2 Expression. (A) EphA2 expression in MCF-10A cells is repressed by estradiol (E2). (B) Tamoxifen blocks the ability of E2 (5 nM) to repress EphA2 expression.

benign and malignant cells (See appended manuscript; (Zantek et al., 1999)). More importantly, mapping of the phosphorylated sites reveals identical patterns in benign and malignant cells, thus confirming that the same sites can be phosphorylated in the two cell types. Consistent with this, we can restore tyrosine phosphorylation of EphA2 in malignant cells using a soluble form of ligand that has been engineered to activate EphA2 in suspension. We find that this B61-IgG fusion protein induces rapid EphA2 phosphorylation.

EphA2 and E-cadherin

While determining molecular mechanisms that regulate EphA2 phosphorylation, we have focussed upon a hypothesis in which E-cadherin promotes ligand-mediated activation (Objective 2B). An early clue to this was provided by the diffuse or membrane ruffle localization of EphA2 in malignant cells (See appended manuscript; (Zantek et al., 1999). Neither distribution would facilitate ligand binding whereas localization within cell-cell contacts (as occurs in benign epithelia) would favor interactions with cell-bound ligands. We investigated factors responsible for these changes and soon thereafter focussed upon E-cadherin. E-cadherin is the primary cell-cell adhesion molecule in epithelial cells (Geiger and Ayalon, 1992) and co-distributed with EphA2 into sites of cell-cell contact in benign epithelia (See appended manuscript; (Zantek et al., 1999). This was confirmed using epi-fluorescence microscopy and confocal microscopy (Objective 1C).

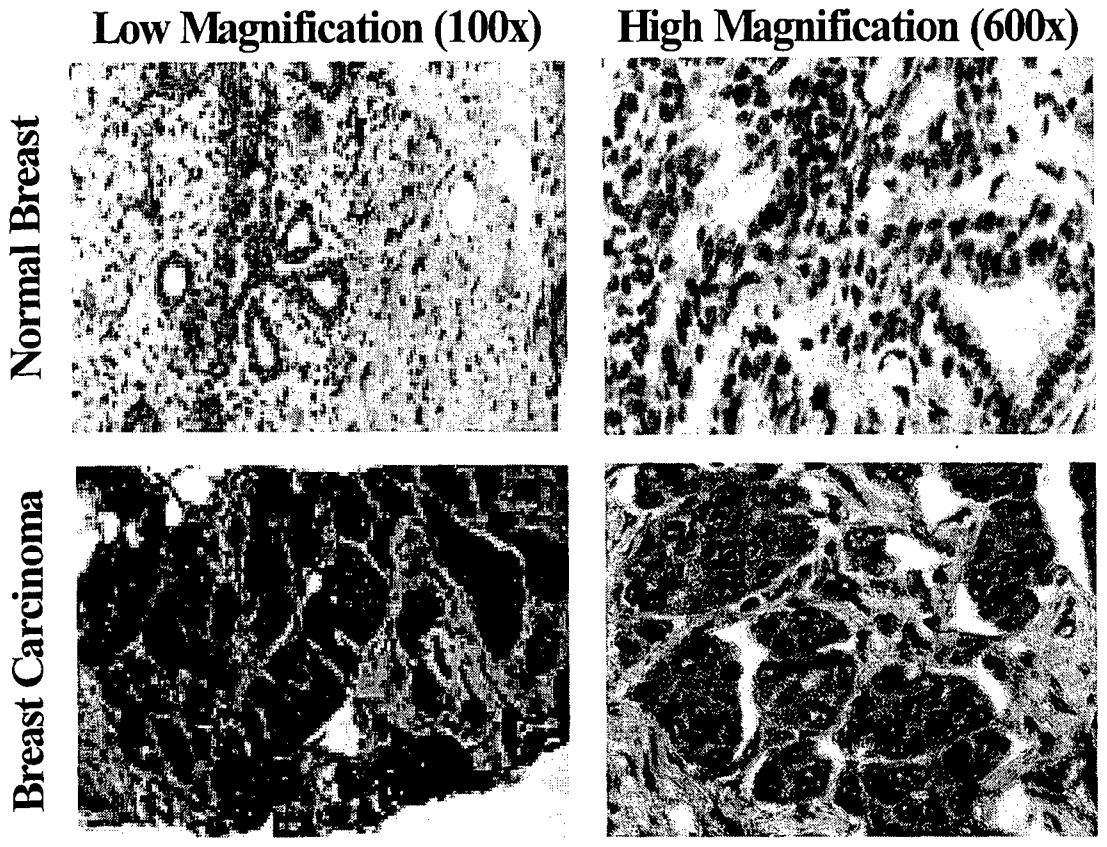
To test if E-cadherin functions to regulate EphA2 activation, we manipulated E-cadherin function in benign and malignant cells. Conditions that blocked E-cadherin function in benign cells (*e.g.,* antibodies, peptides, decreased calcium levels) also abrogated EphA2 phosphorylation whereas conditions that favored E-cadherin adhesion restored EphA2 phosphorylation (See appended manuscript; (Zantek et al., 1999). In particular, highly invasive cancer cells often lose expression of E-cadherin and we could restore EphA2 phosphorylation by transfecting malignant cells with E-cadherin cDNAs. The EphA2 in cells transfected with E-cadherin redistributed from membrane ruffles (as in control cells) into sites of cell-cell contact. At present, we have no evidence for or against a direct interaction between E-cadherin and EphA2. The two proteins are expressed in overlapping patterns but we have not been able to co-immunoprecipitate EphA2 and E-cadherin (Objective 1C). However, *direct interaction between the two molecules would not be necessary if E-cadherin primarily serves to stabilize cell-cell contacts and thereby promotes ligand binding or prevents a phosphatase from acting upon EphA2.*

EphA2 Phosphorylation Regulates Cell Migration and Growth

Although EphA2 activation decreases ECM adhesion, cell growth and migration, the molecular mechanism is poorly understood. *We hypothesize that tyrosine phosphorylation links EphA2 to downstream signaling pathways that block cell growth and migration.* In this scenario, phosphorylated and unphosphorylated EphA2 would be expected to behave differently. Indeed, overexpression of unphosphorylated EphA2 in malignant cells correlates with increased cell growth and invasiveness. By contrast, phosphorylation of EphA2, induced either by E-cadherin or soluble ligand (B61-IgG),

blocks cell growth and migration (See appended manuscript; (Zantek et al., 1999). These seemingly contradictory results could be resolved if EphA2 behaves differently in benign and malignant cells. We will test this in Objective 3B. For example, tyrosine phosphorylation of EphA2 could create binding sites for adapter, cytoskeletal or signaling proteins and thereby change EphA2 signaling.

EphA2 Overexpression in Pathologic Specimens of Breast Cancer



EphA2 Expression in Breast Cancer Specimens

	EphA2 Staining (Score)			
	0	1	2	3
Normal Gland:				
Number	3/3	0	0	0
% Cells	<5%			
Fibroadenoma				
Number (%)	5/9	4/9[M]	0	0
% Cells	<5%	10-20%		
Breast Cancer:				
Number (%)	1/10	0	6/10[D]	3/10[D]
% Cells	<5%	0	50-95%	>95%

	EphA2 Staining			
	0	1	2	3
COLON:				
Normal:				
Number (%)	4/5	1/5 (M)	0	0
% Cells	<5%	20-50%		
Polyp:				
Number (%)	2/5	0	2/5 (M)	1/5 (D)
% Cells	<5%		50-95%	>95%
Carcinoma:				
Number (%)	0	0	6/9 (D)	3/9 (D)
% Cells			50-95%	>95%
PROSTATE:				
Differentiated Tumors (Well-Organized Ducts)				
Number (%)	6/8	2/8 (M)	0	0
% Cells	<5%	10-20%		
Poorly Differentiated Tumors				
Number (%)	0	1/20 (D)	10/20 (D)	9/20 (D)
% Cells		50-95%	50-95%	>95%

Figure 4. EphA2 expression was measured using immunohistochemical analyses of formalin-fixed, paraffin-embedded breast, colon and prostate specimens. Note that EphA2 was overexpressed in carcinoma cells as compared with benign cells. We also observed a change in EphA2 localization from membrane (M) to diffuse (D) staining as indicated.

KEY ACCOMPLISHMENTS:

- Identification of the D7 antigen as the EphA2 receptor tyrosine kinase (Objective 1A and 1B)
- Demonstration that EphA2 is grossly overexpressed in malignant breast cancer cell lines and tissues (Objective 3A)
- Co-localization of EphA2 and E-cadherin into sites of cell-cell contact (Objective 1C)
- Demonstration that E-cadherin does not interact directly with EphA2 but promotes ligand-mediated activation (Objective 1C)
- Demonstration that the phosphotyrosine content of EphA2 in benign and malignant cells differs while enzymatic activity remains constant (Objectives 2A and 2B)
- Demonstration that E-cadherin expression and function control EphA2 activation (Objective 2B)

REPORTABLE OUTCOMES:

Manuscript (in press):

Zantek, N.D., Azimi, M., Fedor-Chaiken, M, Brackenbury, R. and Kinch, M.S. E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. ***Cell Growth and Differentiation***. 1999. *In press.*

Presentations:

Invited Speaker, EphA2 Overexpression in Breast Cancer. ASCB National Conference, Washington, DC, December 1998.

CONCLUSIONS:

Our results reveal that the EphA2 tyrosine kinase is overexpressed and functionally altered in malignant cells. We find that the E-cadherin tumor suppressor protein stabilizes the binding of EphA2 to its ligands, and thereby controls the subcellular localization and tyrosine phosphorylation of EphA2. The consequences of ligand-mediated EphA2 activation include decreased cell growth and migration. It therefore follows that EphA2 overexpression in malignant cells, combined with loss of E-cadherin function, contributes to the invasive characteristic of malignant breast cancer cells. Our studies in the next year will evaluate whether EphA2 overexpression increases the growth and invasiveness of breast cancer cells (see Objective 3C in the proposal). We will also evaluate whether EphA2 serves as a diagnostic or prognostic marker of breast cancer. Ultimately, gross overexpression of EphA2 on breast cancer cells may provide a target at the surface of malignant cells for specific antibodies in a manner that is analogous to the targeting of HER2 in some breast cancer cells.

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APPENDICES:

Publication

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IN PRESS
CELL GROWTH & DIFF

E-Cadherin Regulates the Function of the EphA2 Receptor Tyrosine Kinase[1]

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[3.] Abbreviations used are: EphA2, epithelial cell kinase; BrdU, bromodeoxyuridine; P-Tyr, phosphotyrosine; SDS, sodium dodecyl sulfate; ECM, extracellular matrix; EGTA, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid.

ABSTRACT

EphA2 is a member of the Eph family of receptor tyrosine kinases, which are increasingly understood to play critical roles in disease and development. We report here the regulation of EphA2 by E-cadherin. In non-neoplastic epithelia, EphA2 was tyrosine phosphorylated and localized to sites of cell-cell contact. These properties required the proper expression and functioning of E-cadherin. In breast cancer cells that lack E-cadherin, the phosphotyrosine content of EphA2 was decreased and EphA2 redistributed into membrane ruffles. Expression of E-cadherin in metastatic cells restored a more normal pattern of EphA2 phosphorylation and localization. Activation of EphA2, either by E-cadherin expression or antibody-mediated aggregation, decreased cell-ECM adhesion and cell growth. Altogether, this demonstrates that EphA2 function is dependent on E-cadherin and suggests that loss of E-cadherin function may alter neoplastic cell growth and adhesion via effects on EphA2.

INTRODUCTION

Protein tyrosine phosphorylation generates powerful signals necessary for the growth, migration, and invasion of normal and malignant cells (1). A number of tyrosine kinases have been linked with cancer progression (2) and increased tyrosine kinase activity is an accurate marker of cancer progression (3; 4). EphA2 (Epithelial Cell Kinase or ECK) is a 130 kDa member of the Eph family of receptor tyrosine kinases (5), which interact with cell-bound ligands known as ephrins (1; 6; 7). While EphA2 and most other Eph kinases are expressed and well studied in the developing embryo (8), in the adult, EphA2 is expressed predominantly in epithelial tissues (5). The function of EphA2 is not known, but it has been suggested to regulate proliferation, differentiation and barrier function of colonic epithelium (9); stimulate angiogenesis (10); and regulate neuron survival (11). Little is known of EphA2's role in cancer, although recent studies demonstrate EphA2 expression in human melanomas (12), colon cancers (9), and some oncogene-induced murine mammary tumors (13).

There is much interest in how tyrosine kinases like EphA2 regulate cell growth and differentiation. One often-unappreciated mechanistic hint is the observation that substrates of tyrosine kinases are found almost exclusively within sites of cellular adhesion (14). In epithelial cells, for example, tyrosine phosphorylated proteins are predominantly located in E-cadherin-associated adherens junctions (14; 15). E-cadherin mediates calcium-dependent cell-cell adhesions through homophillic interactions with E-cadherin on apposing cells (16; 17). In cancer cells, E-cadherin function is frequently destabilized, either by loss of E-cadherin expression (18) or by disruption of linkages between E-cadherin and the actin cytoskeleton (19-23). Restoration of E-cadherin function, either by E-cadherin transfection (24; 25) or treatment with pharmacological reagents (21), is sufficient to block cancer cell growth and induce epithelial differentiation. However, the mechanisms by which E-cadherin imparts these tumor suppressor functions are largely unknown. While E-cadherin-mediated stabilization of cell-cell contacts

undoubtedly is involved, there is recent evidence that E-cadherin also generates intracellular signals that could contribute to tumor suppression (15; 26; 27).

Previous studies by our laboratory have linked E-cadherin with signaling by tyrosine phosphorylation. E-cadherin aggregation into assembling adherens junctions initiates a signaling cascade involving tyrosine phosphorylation that may contribute to E-cadherin's tumor suppressor function (28). In addition, we have demonstrated that transformed epithelial cells have elevated levels of tyrosine phosphorylation that destabilize E-cadherin function (21). To identify tyrosine kinases and their substrates in breast cancer, we recently generated monoclonal antibodies specific for tyrosine phosphorylated proteins in Ras-transformed breast epithelial cells (15). Using these antibodies, we identified the EphA2 tyrosine kinase as a protein that is tyrosine phosphorylated upon E-cadherin-mediated adhesion. We also show that E-cadherin regulates the functioning of EphA2.

MATERIALS AND METHODS

Cell Lines and Antibodies.

Human breast carcinoma cells and non-transformed human mammary epithelial cell lines were cultured as described (29; 30). We purchased antibodies specific for E-cadherin (Transduction Laboratories, Lexington and DECMA-1, Sigma, St. Louis, MO), phosphotyrosine (PY20, ICN, Costa Masa, CA; 4G10, Upstate Biotech. Inc, Lake Placid, NY; polyclonal antibodies, Transduction Laboratories), and fluorescein-conjugated bromodeoxyuridine (BrdU) (Harlan Sera-Lab Ltd., Loughborough, UK). Monoclonal antibodies specific for EphA2 (clones D7 and B2D6) were produced in the laboratory as described (15) or purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Rabbit polyclonal antibodies for EphA2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). EK166B monoclonal EphA2 antibodies

were generously provided by R. Lindberg (Amgen, Thousand Oaks, CA). Paxillin-specific antibodies were obtained from K. Burridge (University of North Carolina). To visualize f-actin, fluorescein-conjugated phalloidin was purchased from Molecular Probes (Eugene OR).

Western Blot Analysis.

Unless noted otherwise, all experiments utilized confluent cell monolayers that were extracted in a buffer containing 1% Triton X-100 or in RIPA buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS for 6 minutes on ice as previously described (21). After measuring protein concentrations by Coomassie staining (Pierce, Rockford, IL) or BioRad D_C Protein Assay (Hercules, CA), equal amounts of protein were resolved by SDS-PAGE, transferred to nitrocellulose (Protran; Schleicher and Schuell, Keene, NH,) and Western blot analysis performed as previously described (21). Antibody binding was detected by enhanced chemiluminescence (ECL) as recommended by the manufacturer (Pierce). To reprobe, blots were stripped as described (21).

Immunofluorescence and Confocal Microscopy.

Immunostaining was performed as described previously (21). In brief, cells were grown on glass coverslips to visualize individual cells. Cells were observed at both high cell density (approximately 70% confluence) and low cell density (approximately 20% confluence) by seeding $1x10^6$ cells onto either a 3.5 or 10 cm tissue culture plate overnight at 37°C. At high cell density, extensive overlapping of neoplastic cells precludes accurate subcellular visualization. The samples were fixed in 3.7% formaldehyde solution, extracted in 0.5% Triton X-100 and stained. Immunostaining was visualized using rhodamine-conjugated donkey anti-mouse antibodies (Chemicon, Temecula, CA) and FITC-conjugated donkey anti-rabbit (Chemicon) and epi-fluorescence microscopy (Olympus BX60, 600x, Lake Success, NY) and recorded onto T-Max 400 film (Eastman-Kodak, Rochester, NY). For confocal microscopy, samples were viewed

on a Nikon Diaphot 300 outfitted with a Bio-Rad MRC 1024 UV/Vis System and Coherent Innova Enterprise Model 622 - 60 mW output water-cooled lasers.

Immunoprecipitation.

Immunoprecipitation experiments were performed as described (21) for 1.5 hours at 4°C with the appropriate EphA2-specific monoclonal antibodies (D7 or B2D6) and rabbit anti-mouse (Chemicon) conjugated Protein A-Sepharose (Sigma, St Louis, MO). Immunoprecipitates were washed three times in lysis buffer, resuspended in SDS sample buffer (Tris buffer containing 5% SDS, 3.8% DTT, 25% glycerol, 0.1% bromphenol blue) and resolved by 10% SDS-PAGE.

***In Vitro* Kinase Assays**

For *in vitro* autophosphorylation assays, immunoprecipitated EphA2 was washed in lysis buffer, and incubated in 10mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid]), 3mM $MnCl_2$, 5mM PNPP (Sigma 104 phosphatase substrate, Sigma), 1mM $NaVO_4$, 1μM ATP, and 10μCi [γ-^{32}P]-ATP (New England Nuclear, Boston, MA) at 25°C for the times shown. The reactions were terminated by the addition of 5X Laemmli sample buffer at multiple time points before saturation. After resolving samples by 10% SDS-PAGE, the gel was transferred to nitrocellulose (Schleicher and Schuell) or Immobilon P (Pierce) and incorporated material was detected by autoradiography. To hydrolyze phosphoserine/threonine, the membranes were treated with 1N KOH, 65°C for 1 hour and reassessed by autoradiography. After several half lives, Western blot analysis was performed to determine EphA2 loading.

Cross-linking of EphA2 Receptors.

For antibody cross-linking experiments, cells grown as a monolayer were incubated at 4°C for 20 minutes with 4 μg/mL EphA2 antibody (either clone EK166B or B2D6) or purified fusion protein of ephrin-A1 fused to IgG (B61-IgG) (10). Primary antibody alone, rabbit anti-

mouse IgG alone and control fusion proteins were used as controls. The samples were washed with media, incubated with 20 μg/mL rabbit anti-mouse IgG in conditioned media at 4°C for 10 minutes and warmed to 37°C for 10 minutes before extraction and immunoprecipitation. To determine the optimal time for activation, the plates were incubated in the presence of cross-linking antibody at 37°C for 0-120 minutes.

EGTA and Antibody Treatments

"Calcium switch" experiments were performed as described previously (28). Monolayers of MCF-10A cells were grown to approximately 80% confluence. EGTA was added to growth media to a final concentration of 4mM and the cells were incubated at 37°C for 30 minutes. The media was removed and calcium concentrations restored with normal growth media. To block E-cadherin function, the media was supplemented with E-cadherin antibodies (1:100 dilution; DECMA-1; Sigma) or 10 μg/mL of peptide corresponding to the E-cadherin HAV sequence (YTLFS**HAV**SSNGN). Controls include isotype control antibodies (rat anti-HA antibody, Boehringer Mannheim) and matched, scrambled peptides (SGATNSLHNFSVY). The Purdue Laboratory for Macromolecular Structure synthesized peptides. Cells were then incubated for the indicated times at 37°C and extracted for Western blot analysis and immunoprecipitation. Cell monolayers grown on glass coverslips were treated in the same manner and immunostained for EphA2.

E-Cadherin Expression and Function.

MDA-MB-231 cells were co-transfected with pBATEM2, a mouse E-cadherin expression vector (31) and pSV2neo (32) using FuGENE™ 6 Transfection Reagent (Boehringer Mannheim, Indianapolis, IN) following the manufacturer's instructions. Transfected cells were selected in growth media supplemented with 400μg/mL G418. Immunostaining and Western blot analysis with specific antibodies confirmed E-cadherin expression.

Proliferation Assay.

Cells were plated onto glass coverslips and cultured overnight in growth media. EphA2 antibodies (EK166B or B2D6, extracellular or D7, intracellular) or ligand fusion protein (B61-IgG) were added to the media at 1 μg/mL and incubated at 4°C for 20 minutes, washed with media, and incubated with 20μg/mL rabbit anti-mouse plus 3μg/mL BrdU at 37°C for 4 hours. Cells were fixed in cold methanol for 8 minutes, extracted with 2N HCl at 37°C for 30 minutes and stained with a BrdU antibody to indicate proliferating cells and Hoechst dye to label the nuclei of all cells on the coverslip. A minimum of six random fields were selected in a double blind study and at least 150 cells were assessed in each sample. Each experiment was repeated at least three times.

Statistical Methods.

All statistical analyses were done using SAS System for Windows, version 6.12. An analysis of variance model was used to compare the percentage of cells that grew in each field, within each specimen, in the control group to the percentage of cells that grew in each field, within each specimen, in the experimental group. Group (control vs. experimental) was treated as a fixed effect and specimen within each group was treated as a random effect. A normal probability plot of the residuals was used to assess the homogeneity of the variances of the mean percent cell growth for the control and experimental groups. A probability value of $p < 0.05$ was considered as statistically significant.

RESULTS

Regulation of EphA2 Expression in Breast Cancer Cells.

We measured EphA2 expression levels in breast epithelial cell lines derived from non-neoplastic epithelia (*e.g.* MCF-10A, MCF-12A, MCF10-2) (30; 33) and metastatic breast cancer (*e.g.* MDA-MB-231, MDA-MB-435) (34; 35). EphA2 was found to be expressed in non-transformed mammary epithelial and metastatic breast cancer cell lines tested (Figure 1A and data not shown), with 2-5 fold more EphA2 in neoplastic cells as determined by Western blot analysis using multiple EphA2 antibodies and by Northern blot analysis (data not shown).

Despite its overexpression, EphA2 in metastatic cells displayed a much-reduced phosphotyrosine content. For these studies, EphA2 was immunoprecipitated from confluent monolayers of either non-neoplastic or metastatic cells and Western blot analysis performed with phosphotyrosine specific antibodies. This revealed prominent phosphorylation of EphA2 in non-neoplastic cells while the EphA2 from metastatic cells was not tyrosine phosphorylated (Figure 1C). The decreased phosphotyrosine content was confirmed using different EphA2 antibodies (D7, B2D6, rabbit polyclonal) for immunoprecipitation and with multiple phosphotyrosine antibodies (PY20, 4G10, rabbit polyclonals) for Western blot analysis (data not shown). Decreased EphA2 phosphorylation was also observed in other metastatic breast cancer cell lines as well as invasive tumor cell lines derived from colon, pancreatic, ovarian and lung cancers (data not shown).

Further comparison of EphA2 in non-neoplastic and metastatic cells revealed other changes in EphA2 distribution and function. Immunofluorescence staining with EphA2-specific antibodies revealed that EphA2 in non-neoplastic cells was mostly found within sites of cell-cell contact (Figure 2), with little staining of membrane that was not in contact with neighboring cells. By contrast, EphA2 in metastatic cells was absent from sites of cell-cell contacts. Instead, the EphA2 in these cells was either diffusely distributed or enriched within membrane ruffles at the leading edge of migrating cells. The enrichment within membrane ruffles was confirmed by co-localization of EphA2 with f-actin (data not shown). This localization within membrane ruffles

was not observed in non-transformed epithelia, even at low cell density. These differences in subcellular distribution were confirmed using three different EphA2-specific antibodies (D7, B2D6 and rabbit polyclonal). The correlation between EphA2 localization and phosphotyrosine content forms the basis for much of the remainder of this manuscript.

EphA2 Enzymatic Activity in Metastatic Cells

Tyrosine phosphorylation of a kinase often regulates enzymatic activity. To test the effect of differences in EphA2 phosphorylation on kinase activity, we measured EphA2 autophosphorylation by using *in vitro* kinase assays with immunoprecipitated material (Figure 3). Despite the low phosphotyrosine content of EphA2 in metastatic cells, this EphA2 demonstrated enzymatic activity that was comparable to, or higher than, the activity of EphA2 isolated from non-neoplastic cells. This activity was unaffected by the basal phosphotyrosine content of EphA2 since unlabeled phosphate was rapidly exchanged with labeled phosphate during the autophosphorylation assays as previously described (36; 37). KOH treatment of the membranes prior to autoradiography did not significantly reduce the level of phosphorylation, indicating that the observed enzymatic activity represented mostly phosphorylation on tyrosine residues. It is also notable that the phosphotyrosine content of EphA2 (Figure 1B) was not predictive of its enzymatic activity (Figure 3).

Receptor Aggregation Induces EphA2 Tyrosine Phosphorylation in Metastatic Cells

EphA2 in neoplastic cells retained the capacity to become activated. For example, EphA2 tyrosine phosphorylation was induced by aggregation of EphA2 with a soluble form of ephrin-A (B61-IgG, a chimera of the EphrinA1 extracellular domain fused to immunoglobulin heavy chain; also known as a "ligand-body") (10; 38) (Figure 4C). By contrast, a control chimera (Ctrl-IgG) did not alter EphA2 phosphorylation. Clustering EphA2 at the cell surface with specific antibodies (EK166B or B2D6) also induced levels of EphA2 activation comparable to non-neoplastic cells (Figure 4A). Receptor aggregation, and not simply antibody binding, was

necessary for EphA2 phosphorylation as incubation with anti-EphA2 (1^{o}) alone did not increase EphA2 phosphorylation relative to matched controls. This effect was specific for EphA2 as neither secondary (2^{o}) antibodies alone or clustering of isotype matched control antibodies (which recognize an inaccessible cytoplasmic epitope on EphA2) did not induce tyrosine phosphorylation of EphA2 (data not shown). Analysis of the timing of EphA2 phosphorylation revealed EphA2 phosphorylation within 2 minutes after cross-linking, with optimal phosphorylation detected after 5 minutes (Figure 4B).

E-cadherin Regulates EphA2 in Non-transformed Epithelia

Tyrosine phosphorylation of EphA2 correlates with its localization within sites of cell-cell contact. Because Eph receptors become activated by ligands that are attached to the surface of neighboring cells (39), we reasoned that stable cell-cell adhesions might be necessary for EphA2 activation. Adhesions mediated by E-cadherin generate the most stable interactions between epithelial cells (16) and we noted that EphA2 was not phosphorylated and was absent from intercellular contacts in cells lacking E-cadherin. These include metastatic cancer cells as well as non-transformed fibroblasts (*e.g.*, NIH 3T3, REF-52, C3H10T$^{1}/_{2}$) and myoepithelial cells (HBL-100) (data not shown). We therefore tested whether E-cadherin might regulate EphA2 phosphorylation.

As both EphA2 and E-cadherin are found at sites of cell-cell contact, we first examined if the two proteins co-localize using two-color immunofluorescence microscopy. This revealed an overlapping distribution of EphA2 and E-cadherin along the lateral membranes of epithelial cells and specifically within sites of cell-cell contact (Figure 5). Vertical sectioning by confocal microscopy confirmed co-localization of E-cadherin and EphA2 within sites of cell-cell contact (data not shown).

To test if the co-localization of EphA2 and E-cadherin might indicate a functional link between the two proteins, we disrupted calcium dependent E-cadherin-mediated adhesion by supplementing the cell culture media with 4 mM EGTA, a calcium-chelating agent. EGTA treatment caused EphA2 dephosphorylation (Figure 6A) and induced either a diffuse or membrane ruffle pattern of staining (Figure 6C), which was reminiscent of EphA2 in metastatic cells. Subsequent restoration of normal levels of extracellular calcium restored normal levels of EphA2 phosphorylation and cell-cell localization within 5 minutes (Figure 6A,C).

While results with EGTA-treated samples implicate cell-cell adhesion with the control of EphA2 phosphorylation and subcellular localization, we sought to determine if E-cadherin contributed to this regulation. For this, we supplemented the cell culture medium with function-blocking E-cadherin antibodies and peptides (DECMA-1 antibodies or HAV peptides) (40; 41). When inhibitors of E-cadherin function were added to the medium concomitant with the restoration of extracellular calcium, EphA2 did not become tyrosine phosphorylated (Figure 7A) and remained diffuse or present within membrane ruffles (Figure 7C). By contrast, isotype-matched control antibodies and scrambled peptides did not prevent EphA2 phosphorylation or localization within intercellular junctions. Specific inhibition of E-cadherin with these inhibitors also blocked EphA2 phosphorylation and cell-cell localization upon treatment of confluent cell monolayers (data not shown), thus confirming that EphA2 phosphorylation and localization are sensitive to the functioning of E-cadherin.

EphA2 is Responsive to E-cadherin Expression in Metastatic Cells

To examine further the link between EphA2 and E-cadherin, we transfected MDA-MB-231 cells with E-cadherin (231-E-cad) and selected for levels of E-cadherin expression that were equivalent to MCF-10A cells. As controls, we transfected cells with empty vector (231-neo). EphA2 in 231-neo was not phosphorylated and was enriched within membrane ruffles (Figure 9). By contrast the EphA2 in 231-E-cad redistributed into sites of cell-cell contacts and had levels of

phosphotyrosine that were comparable to MCF-10A cells (Figure 8A). These changes in EphA2 phosphorylation and localization increased with cell density (data not shown), consistent with an idea that E-cadherin function regulates EphA2 phosphorylation and localization.

EphA2 Regulates Cell Adhesion and Proliferation.

Microscopic analysis revealed that E-cadherin expression altered the adhesive profile of MDA-MB-231 cells (Figure 9). Whereas parental and 231-neo cells were mesenchymal in appearance and readily grew atop one another, the E-cadherin-transfected cells had more prominent cell-cell adhesions and grew as single-cell monolayers. Analysis of cell-ECM attachments by staining with paxillin-specific antibodies revealed numerous focal adhesions in control MDA-MB-231 cells while 231-E-cad cells had fewer focal adhesions. The decrease in focal adhesions was most prominent in 231-E-cad cells within colonies (Figure 9, bottom right) while individual cells had focal adhesions that were comparable to controls (data not shown).

EphA2 activation contributes to the decreased cell-ECM adhesion. To activate EphA2 in MDA-MB-231 cells, we aggregated EphA2 at the cell surface with specific antibodies (as described above) and found that this caused a rapid loss of focal adhesions within 5 minutes. This was confirmed by paxillin staining (Figure 10) and by interference reflection microscopy (data not shown). Similar results were obtained in other neoplastic cell lines (not shown). By contrast, treatment with either primary or secondary antibodies alone did not alter focal adhesions.

Focal adhesions are sites of intracellular signaling that promote cell growth (42; 43). Since EphA2 activation blocks focal adhesions, we questioned whether EphA2 activation would impact cell growth. To test this, we activated EphA2 with specific antibodies or B61-IgG "ligand-bodies" (as described above). Concomitant with receptor cross-linking, we included BrdU in the culture medium and measured DNA synthesis over the following four hours. As

shown in Table I, EphA2 activation decreased the proliferation in MDA-MB-231 cells (31% reduction $p<0.001$) whereas control conditions (primary or secondary antibodies alone and isotype controls) did not change cell growth. The short duration of EphA2 signaling that is induced by antibody aggregation (Figure 4B) likely underestimates EphA2's growth inhibitory potential. A similar decrease in cell growth was obtained following EphA2 activation in other cell types, including MDA-MB-435 cells (22% reduction, $p<0.0005$) and MCF-10A cells (16% reduction, $p<0.01$). For experiments with MCF-10A, we plated cells at low cell density and scored individual cells (to preclude cell-cell contacts that might otherwise activate EphA2).

DISCUSSION

The major findings of this study are that the localization and phosphorylation of EphA2 in mammary epithelial cells are dependent on E-cadherin-mediated adhesion and that loss of E-cadherin in metastatic tumor cells causes alterations in EphA2 localization and phosphorylation. In addition, we found that experimental induction of EphA2 phosphorylation decreases cell-ECM attachment at focal adhesions and negatively regulates the proliferation of metastatic cells.

Decreased EphA2 Phosphorylation in Metastatic Cells

We originally identified EphA2 using antibodies that recognize tyrosine phosphorylated proteins in Ras-transformed MCF-10A-neoT cells (15). MCF-10A-neoT cells express E-cadherin (21) and consequently, EphA2 is tyrosine phosphorylated (data not shown). Notably, EphA2 was tyrosine phosphorylated in non-neoplastic mammary epithelial cell lines but not in metastatic cell lines. In this respect, EphA2 differs from many other tyrosine kinases (*e.g.,* cErbB2, EGF receptor, PDGF receptor, Src), whose phosphorylation increases in cancer cells (2; 44; 45). For these kinases, phosphorylation elevates tyrosine kinase activity, triggering signal transduction cascades that promote cell proliferation.

The phosphotyrosine content of EphA2 does not relate to its intrinsic enzymatic activity in mammary epithelial cells. *In vitro* assays revealed that despite its low phosphotyrosine content, the enzymatic activity of EphA2 in metastatic cells is comparable to or increased over the activity of phosphorylated EphA2 in non-neoplastic epithelial cells. This is consistent with evidence that the phosphorylation of EphB2 also has little effect on its kinase activity (46). Our results suggest that rather than controlling enzymatic activity, the phosphotyrosine content of EphA2 might influence the choice or availability of substrates and interacting proteins. In addition, changes in the phosphotyrosine content of EphA2 might provide signals that are independent of EphA2 enzymatic activity, which is supported by recent reports that other Eph kinases (VAB-1 and EphB2) have kinase-independent functions (47; 48). This suggests that protein interactions, localization, phosphotyrosine content and enzymatic activity all contribute to Eph receptor function.

There are several possible explanations for the loss of EphA2 phosphorylation in metastatic cells. The primary sites of receptor autophosphorylation are not mutated since the sites that become autophosphorylated *in vitro* are the same in non-transformed and neoplastic cells (M.S. Kinch, unpublished results). Consistent with this, EphA2 tyrosine phosphorylation was restored by cross-linking EphA2 with antibodies or by transfection with E-cadherin. Another possible cause for decreased EphA2 phosphorylation could be loss of EphA2 ligands (ephrin-A class molecules). However, our ability to restore EphA2 phosphorylation in E-cadherin-transfected cells appears to exclude this possibility. A third possibility is that the phosphotyrosine content of EphA2 is repressed by an associated tyrosine phosphatase. Consistent with this, treatment of neoplastic cells with tyrosine phosphatase inhibitors restores normal levels of EphA2 tyrosine phosphorylation (N. Zantek, unpublished result). However, the identity of the phosphatases responsible for this is presently unknown.

Regulation of EphA2 activation by E-cadherin

We focused on the possibility that decreased stability of cell-cell contacts inhibits tyrosine phosphorylation of EphA2 in metastatic cells. Both Eph family receptor tyrosine kinases and their ephrin ligands are bound to the cell surface (1; 6; 7), so cells must be in close contact to facilitate Eph-ephrin interactions. Little is known, however, about the nature of these contacts and their precise effects on Eph-ephrin interactions.

Because many breast tumors lack E-cadherin and have unstable cell-cell junctions (18; 29), we investigated how expression of E-cadherin affects EphA2 phosphorylation in mammary epithelial cells. We found inhibition of E-cadherin function either by removal of Ca^{2+} or with function-blocking antibodies or peptides reduced EphA2 phosphorylation and caused EphA2 to redistribute into membrane ruffles. Conversely, expression of E-cadherin in MDA-MB-231 cells restored EphA2 phosphorylation and localization to sites of cell-cell contact. The simplest explanation for these results is that E-cadherin stabilizes cell-cell contacts and thereby facilitates interactions between EphA2 and its ligands.

At present, there is no evidence for or against a direct interaction between E-cadherin and EphA2. The two proteins are expressed in overlapping patterns but we have not been able to co-immunoprecipitate EphA2 and E-cadherin (N. Zantek, unpublished results). EphA2 also does not co-cluster with E-cadherin at the cell surface in response to antibody-mediated aggregation of either molecule (M. Fedor-Chaiken and M.S. Kinch, unpublished information), which is consistent with our biochemical evidence. We cannot exclude that experimental conditions used for protein extraction dissociate such interactions or that a small fraction of activated EphA2 co-clusters with E-cadherin. Direct interaction between the two molecules may not be necessary if E-cadherin primarily serves to stabilize cell-cell contacts and thereby promote interactions between EphA2 and its ligands. Other aspects of E-cadherin function, such as signaling (28),

cytoskeletal association (49), or junction formation (16) might also target EphA2 to sites of cell-cell contact.

EphA2 Regulates Cell-ECM Adhesion and Growth

An immediate consequence of EphA2 activation is decreased cell-ECM contact at focal adhesions. Focal adhesions are sites of membrane-cytoskeletal interaction that provide anchorage for cell migration and invasion (50). Focal adhesions also play critical roles in signal transduction, where they organize intracellular signals that control cell growth and survival (42; 43). We propose that E-cadherin-mediated stabilization of ligand binding induces EphA2 to block focal adhesions. Consistent with this, it is understood that epithelial cells balance their cell-cell and cell-ECM adhesions and that this is linked with the proper functioning of E-cadherin (51; 52). Individual epithelial cells have more focal adhesions than cells within colonies whereas cells with decreased E-cadherin function have increased cell-matrix adhesion, regardless of cell density (21). Although the molecular mechanisms responsible for this are unknown, many proteins that interact with Eph kinases regulate cell adhesion or cytoskeletal organization, including the p85 subunit of PI_3 kinase, Src, Fyn and Ras-GAP (38; 53-55).

Focal adhesions initiate signals that promote cell growth and it follows that loss of these structures may contribute to decreased cell growth following EphA2 activation. By inference, loss of EphA2 activation might contribute to deregulated growth of neoplastic cells by increasing signals from focal adhesions. This would be consistent with evidence that neoplastic cells have increased signaling by focal adhesion proteins (*e.g.,* FAK) (56). Although EphA2 activation decreases cell growth, the expression pattern of EphA2 does not fit the classic pattern of a tumor suppressor. Most tumor suppressors are inactivated either because of decreased expression or loss of enzymatic activity. By contrast, neoplastic cells express high levels of EphA2 which, although non-phosphorylated, retains comparable levels of enzymatic activity. An alternative

explanation is that EphA2 positively regulates cell growth but that this signaling is reduced in non-transformed epithelia. Support for this includes evidence that EphA2 is overexpressed in neoplastic cells and is supported by the fact that other Eph kinases (*e.g.*, EphA1) are oncogenic (57). In this scenario, EphA2 "activation" by E-cadherin or receptor aggregation might decrease EphA2 function, perhaps by reducing EphA2 expression levels. It is intriguing that the lowest levels of EphA2 are found in cells where it is phosphorylated and that ligand-mediated aggregation decreases EphA2 expression levels. A third possibility is that EphA2 functions very differently in normal and neoplastic epithelia. The phosphotyrosine content and subcellular localization of EphA2 differ in normal and neoplastic cells, and either property could alter substrate specificity or availability. Indeed, tyrosine phosphorylated EphA2 (but not unphosphorylated EphA2) interacts with the PI_3 kinase and the SLAP adapter protein (58). SLAP was recently shown to negatively regulate cell growth (59), which is supportive of our evidence that EphA2 also regulates cell proliferation. Future studies will be necessary to define EphA2's role as a positive and/or negative regulator of cell growth and to determine if these properties differ between normal and neoplastic epithelia.

Conclusions

Loss of E-cadherin in carcinomas promotes invasion (18; 60), cell motility (27), and cell proliferation (26). In the present study, we have identified the receptor tyrosine kinase EphA2 as one protein that is phosphorylated after cell-cell contact and demonstrated that both the phosphorylation and localization of EphA2 are sensitive to changes in E-cadherin function and expression. We also find that EphA2 activation negatively regulates cell-ECM adhesion and cell growth. These findings raise the possibility that important effects of E-cadherin on tumor cell behavior may occur via effects on EphA2.

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Table I. EphA2 Activation Inhibits Cell Proliferation

	Treatment	% BrdU Uptake	SEM	Statistical Analysis[1]
MDA-MB- 231	**No Rx**	43.8	2.0	--
	1° Ab Alone	44.1	2.2	$p > 0.43$
	2° Ab Alone	39.7	2.3	$p > 0.21$
	1° + 2°	**30.4**	1.7	**$p < 0.0001$**
	Control- IgG + 2°	43.0	2.1	$p > 0.44$
	B61-IgG +2°	**29.1**	3.1	**$p < 0.01$**[2]
MDA-MB-435	**No Rx**	52.8	5.1	--
	1° Ab Alone	52.6	3.4	$p > 0.25$
	2° Ab Alone	52.8	6.3	$p > 0.39$
	1° + 2°	**39.6**	3.0	**$p < 0.00005$**
MCF-10A (Low Density)	**No Rx**	53.6	1.8	--
	1° Ab Alone	53.9	0.8	$p > 0.43$
	2° Ab Alone	55.1	0.5	$p > 0.22$
	1° + 2°	**45.0**	1.4	**$p < 0.01$**

Table I. EphA2 Activation Inhibits Cell Proliferation BrdU uptake into newly synthesized DNA was measured for four hours after cross-linking of EphA2 at the cell surface with specific antibodies. The data represent at least three independent, double-blinded experiments. Cell growth was determined in at least 100 cells from each experimental and control and the results shown compare with DNA synthesis with untreated (No Rx) samples. None of the differences between or among individual negative controls (No Rx, 1° Ab alone, or 2° Ab alone) were significant ($p > 0.05$). [1]Statistical analyses compared the experimental to No Rx for each sample. [2]For the fusion proteins, there was also a significant difference ($p<0.02$) between the control and B61 fusion proteins.

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Figure Legends

Figure 1. Decreased EphA2 Phosphorylation in Metastases EphA2 from (**A**) whole cell lysates or (**B**) immunoprecipitated from monolayers of non-neoplastic (MCF-10A, MCF10-2, MCF-12A) and metastatic (MDA-MB-231, MDA-MB-435) breast cancer cell lines was resolved by SDS-PAGE and Western blot analysis performed with EphA2 antibodies. (**C**) The blot from (**B**) was stripped and reprobed with phosphotyrosine-specific (PY20) antibodies. Note the absence of tyrosine phosphorylated EphA2 in metastatic breast cancer cells.

Figure 2. Altered EphA2 Localization in Metastatic Cancer Cells. The subcellular distribution of EphA2 in non-transformed mammary epithelial cells (MCF-10A) and metastatic breast cancer cells (MDA-MB-231) was assessed by immunostaining with EphA2-specific antibodies. The cells were plated at either high (top panels) or low (bottom panels) cell density to emphasize the localization of EphA2 within cell-cell contacts or membrane ruffles of non-transformed or invasive cells, respectively. Bar = 10 μm.

Figure 3. EphA2 Enzymatic Activity. The enzymatic activity of EphA2 was measured using an *in vitro* autophosphorylation assays. At the times shown, the *in vitro* reaction was terminated, and resolved by SDS-PAGE. The blot shown was treated with KOH to hydrolyze phosphoserine and phosphothreonine prior to autoradiography. After several half lives, Western blot analysis was performed with EphA2 antibodies to confirm equal sample loading (data not shown).

Figure 4. Antibody-Mediated Aggregation Induces EphA2 Phosphorylation in Metastatic cells. (A) Immunoprecipitated EphA2 was subjected to Western blot analysis with phosphotyrosine antibodies (PY20) following aggregation of cell surface EphA2 for 5 minutes at 37°C with specific antibodies (1°+2°). Note that simple engagement of anti-EphA2 (1° Alone) or anti-mouse (2°) alone was insufficient to induce tyrosine phosphorylation above basal levels (No Rx). The blot was then stripped and reprobed with EphA2 antibodies as a loading control. **(B)**

The time course of EphA2 phosphorylation was measured after cross-linking (1°+2°) EphA2 in MDA-MB-231 cells for 0-60 minutes by Western blot analysis of immunoprecipitated EphA2 with phosphotyrosine-specific antibodies (PY20). (**C**) EphA2 was aggregated using a soluble ligand fusion protein (B61-IgG). A control fusion protein (Ctrl-IgG) serves as a negative control and B2D6-mediated aggregation as a positive control for activation.

Figure 5. Co-Localization of EphA2 and E-cadherin. The subcellular distribution of EphA2 (left panel) and E-cadherin (right panel) was evaluated in MCF-10A cells using two-color immunofluorescence microscopy. Note the overlapping distribution of EphA2 and E-cadherin within sites of intercellular junctions.

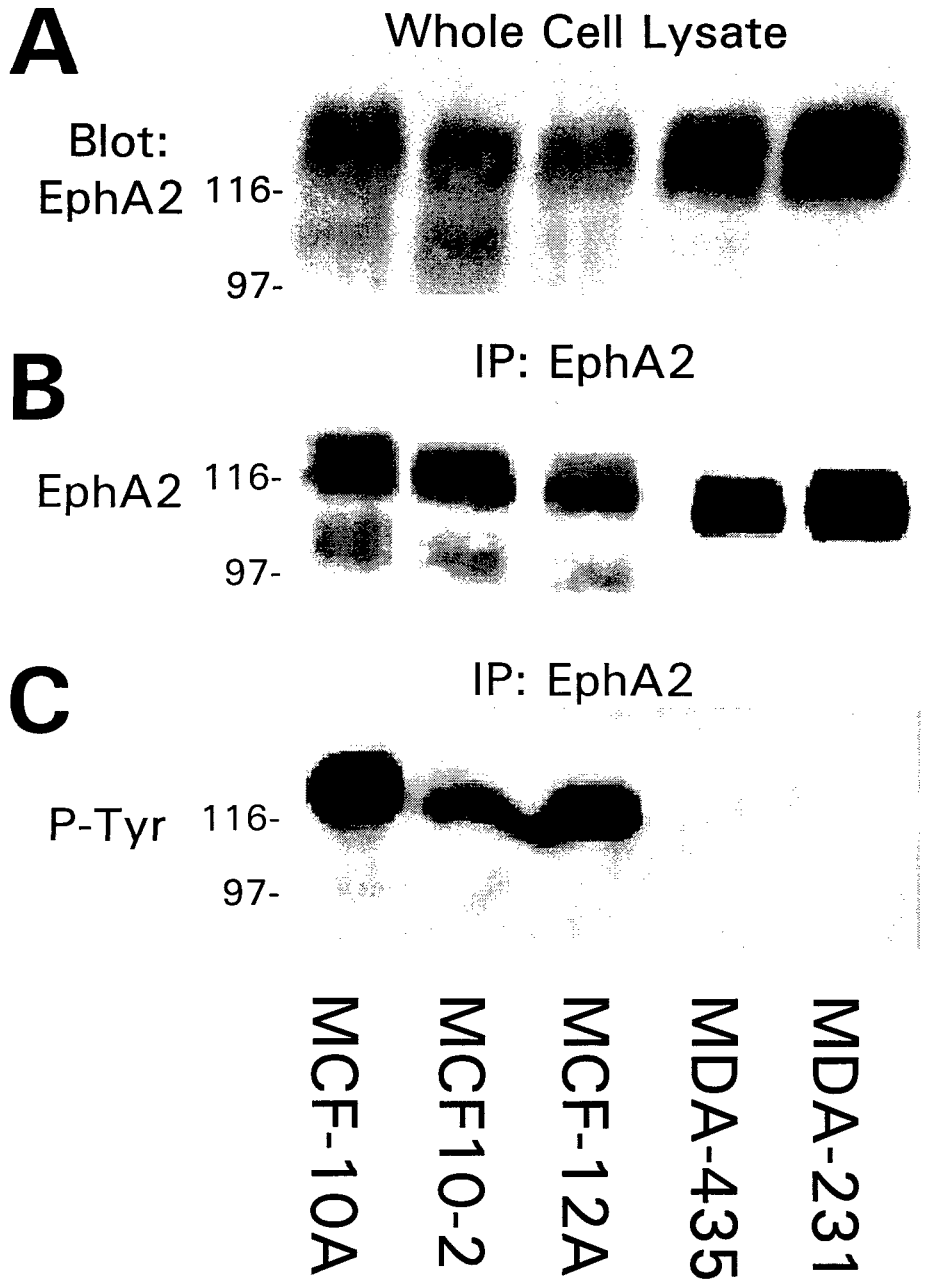
Figure 6. EphA2 Phosphorylation and Localization Require Stable E-Cadherin Adhesions. Stable cell-cell contacts in monolayers of MCF-10A cells were disrupted by the addition of EGTA (4 mM, 30 minutes, 37^{0}C) to the culture media. After removing the EGTA, normal growth media was returned for 0-120 minutes. (**A**) EphA2 was immunoprecipitated and Western blot analysis performed with phosphotyrosine-specific (PY20) antibodies. (**B**) The blot from (**A**) was stripped and reprobed with EphA2 antibodies as a loading control. (**C**) Staining with EphA2-specific antibodies assessed changes in the subcellular distribution of EphA2 before and after restoration of cell-cell adhesions.

Figure 7. Inhibition of E-cadherin Mediated Adhesion. Following treatment of MCF-10A cell monolayers with EGTA (EGTA), normal media conditions were restored in the absence (Control) or presence of function-blocking E-cadherin antibodies (α-E-cad) or peptides (HAV). Isotype control antibodies (Iso-Ctrl) and scrambled peptides (Pep-Ctrl) were included as matched negative controls. **(A)** Immunoprecipitated EphA2 was subjected to Western blot analysis with phosphotyrosine (PY20) antibodies. (**B**) The same blot as (**A**) was stripped and re-probed with EphA2 antibodies as a loading control. (**C**) EphA2 localization was determined after calcium restoration in the absence (Control) or presence of E-cadherin inhibitors.

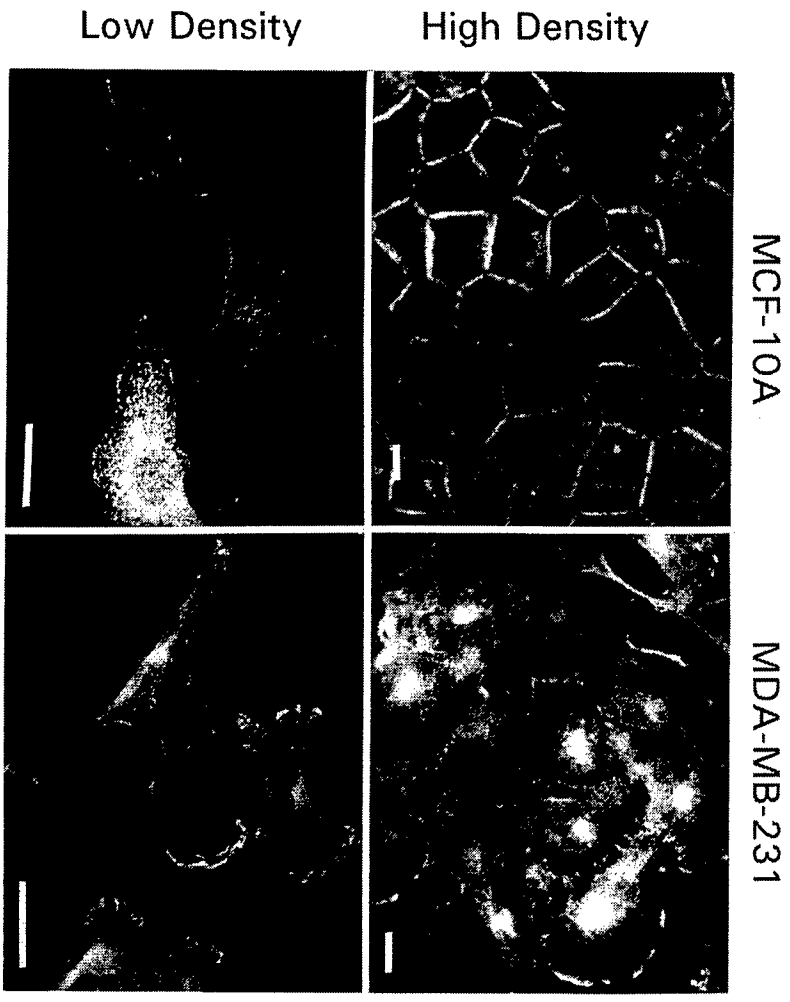
Figure 8. E-cadherin Expression Restores Normal EphA2 Function. (**A**) The phosphotyrosine content of immunoprecipitated EphA2 was measured by Western blot analysis following transfection of MDA-MB-231 cells with E-cadherin (231 E-cad) or a matched vector control (231 neo). MCF-10A is included as positive controls for EphA2 tyrosine phosphorylation. (**B**) The blot from (**A**) was stripped and reprobed with EphA2-specific antibodies as a loading control.

Figure 9. E-Cadherin Expression Directs EphA2 into Cell-Cell Contacts. The subcellular distribution of EphA2 and paxillin was assessed by immunofluorescence microscopy in control (231-neo) and E-cadherin transfected (231-E-cad) MDA-MB-231 cells. Note that E-cadherin promotes a redistribution of EphA2 into cell-cell contacts and decreases focal adhesions. Bar = 25 μm.

Figure 10. EphA2 Activation Decreases Cell-ECM Adhesion. The presence of focal adhesions was assessed by immunostaining for paxillin in MDA-MB-231 cells before and after activation of EphA2 by antibody-mediated aggregation. Note that incubation of cells with either primary (1°) or secondary (2°) antibodies alone did not alter the presence of focal adhesions whereas EphA2 aggregation dissipated focal adhesions. Bar = 25 μm



Zantek et al.,
Figure 1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High Density
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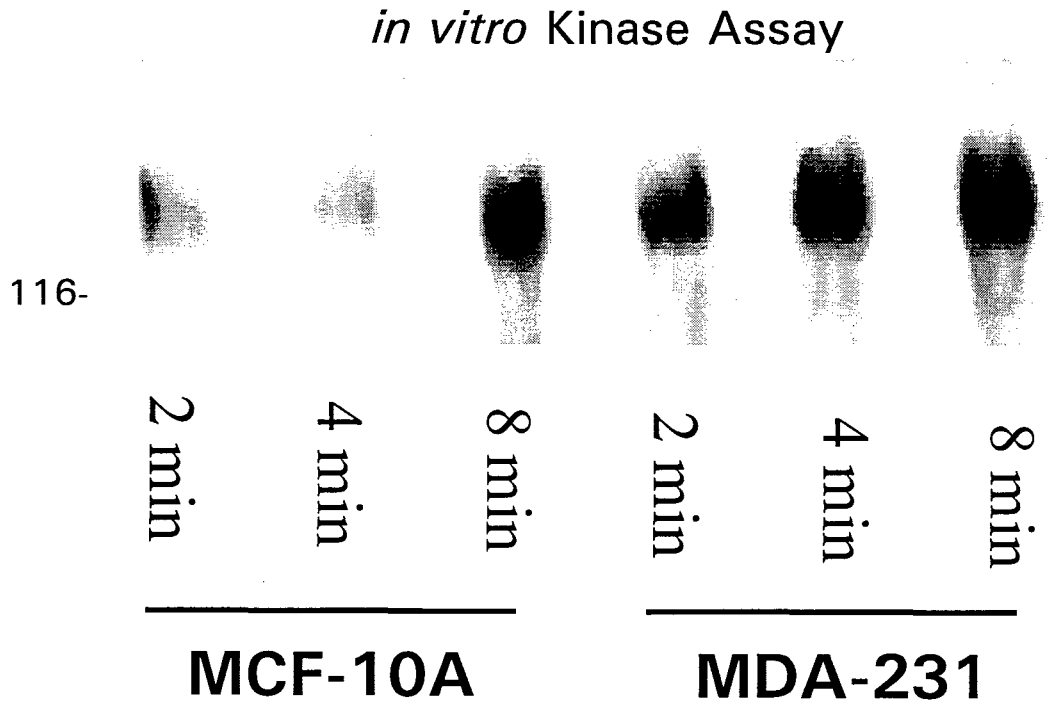

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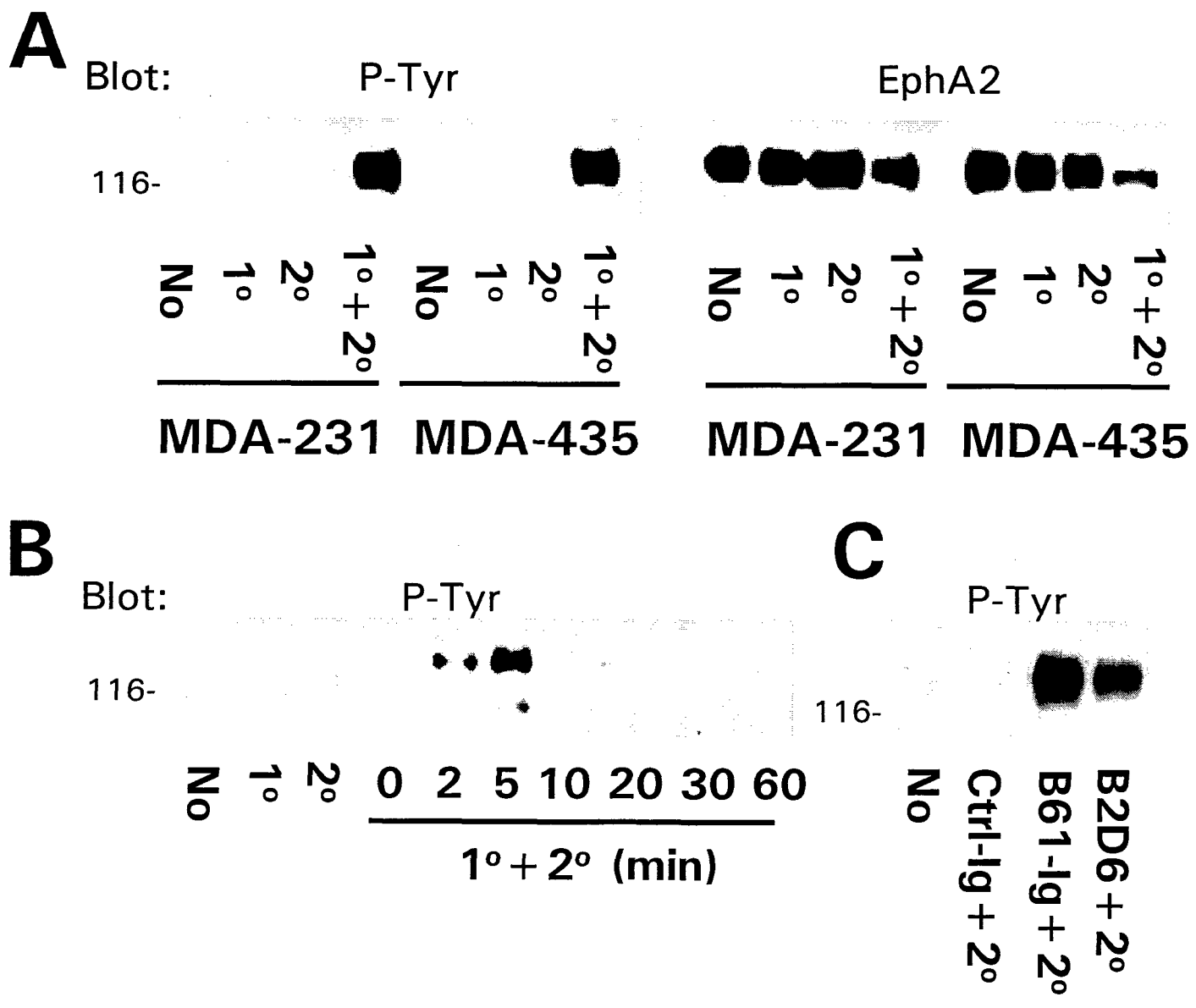
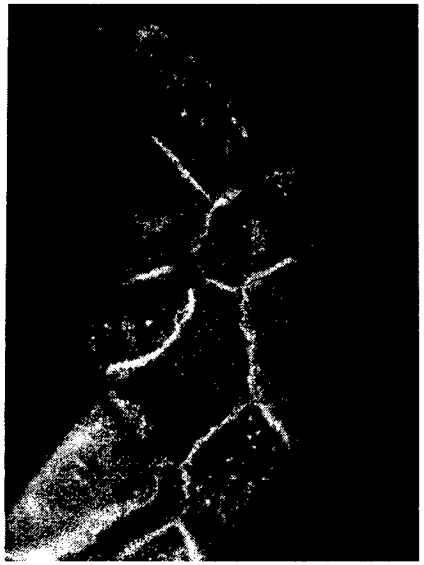
Figure 3

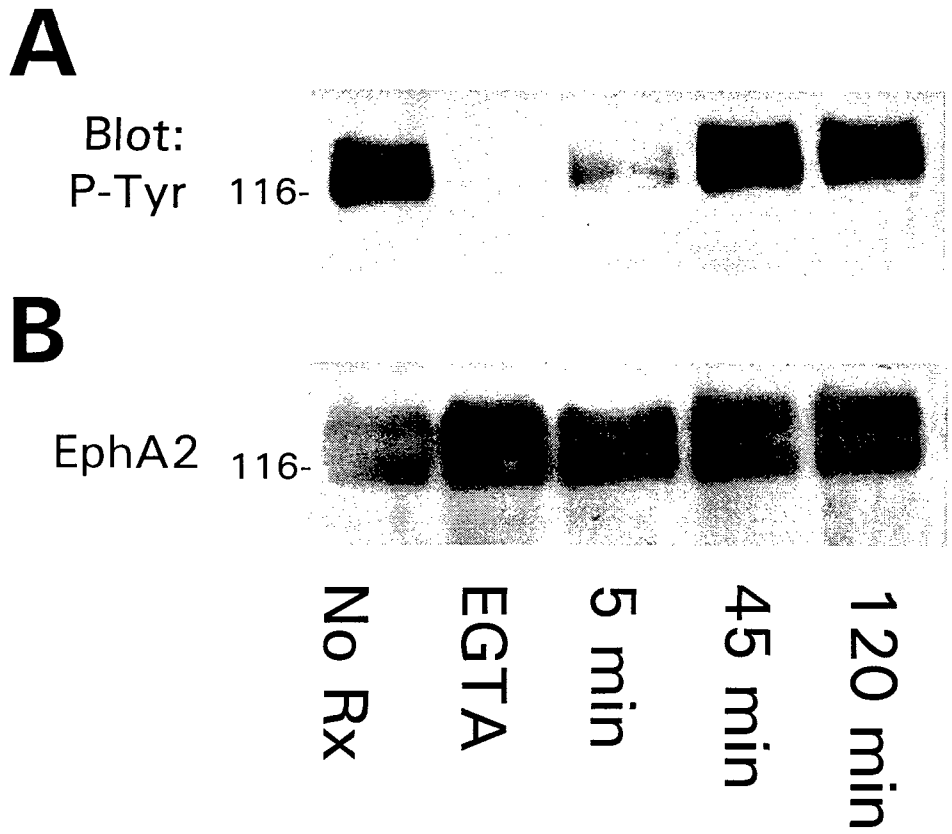


Zantek et al.,
Figure 4

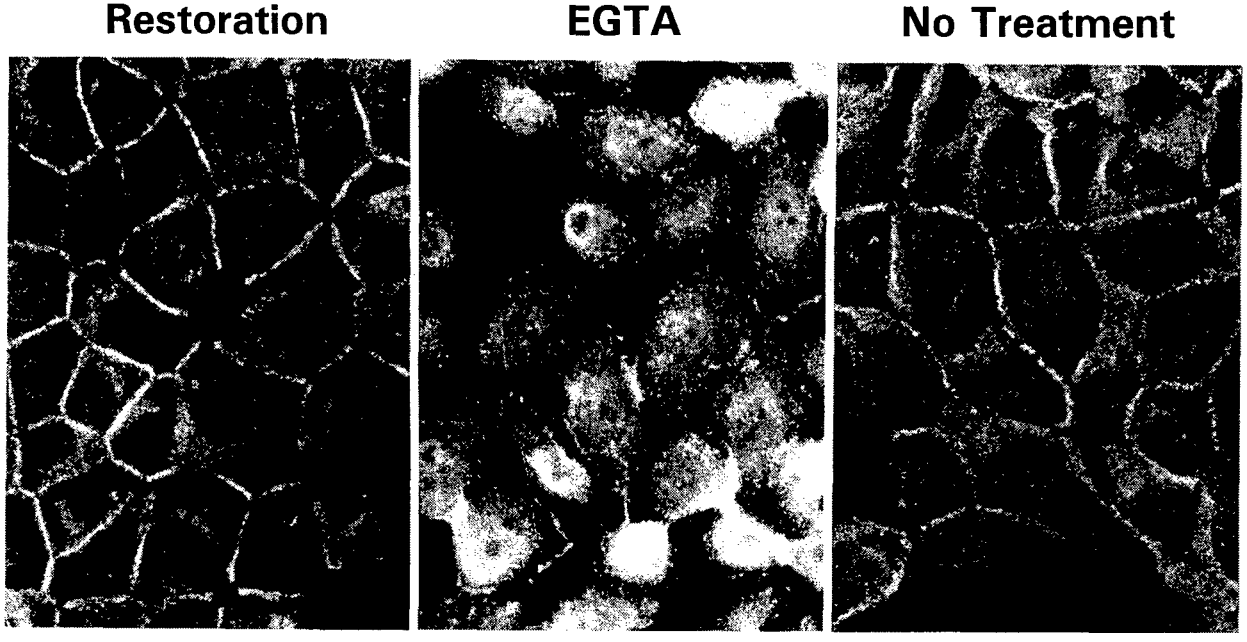
EphA2

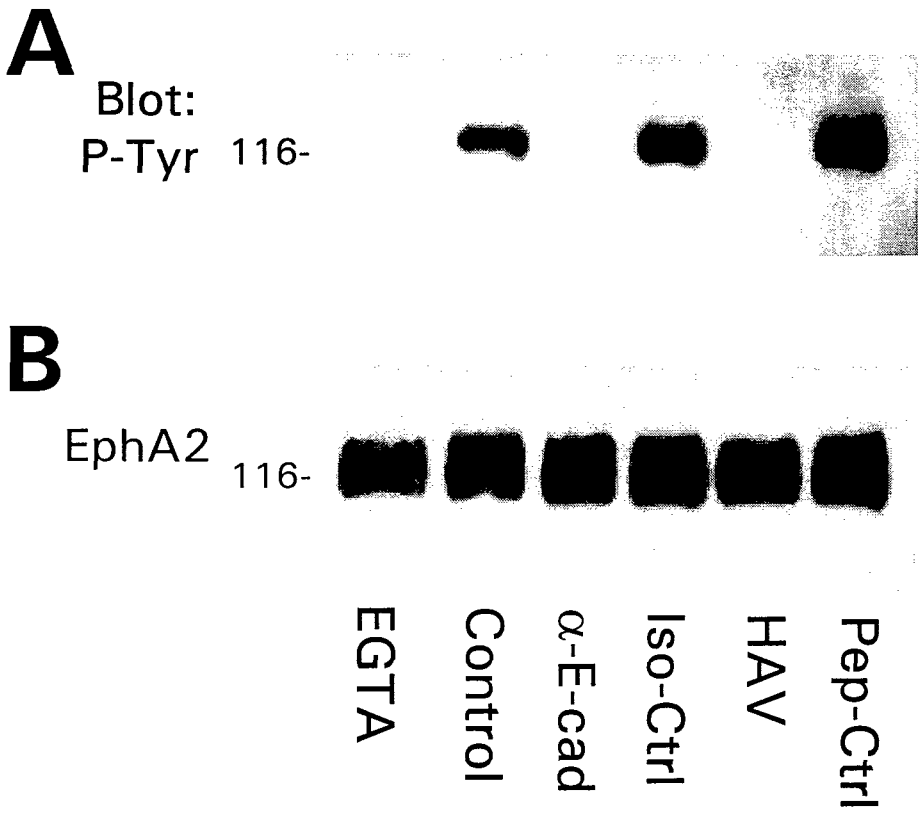
E-cadherin



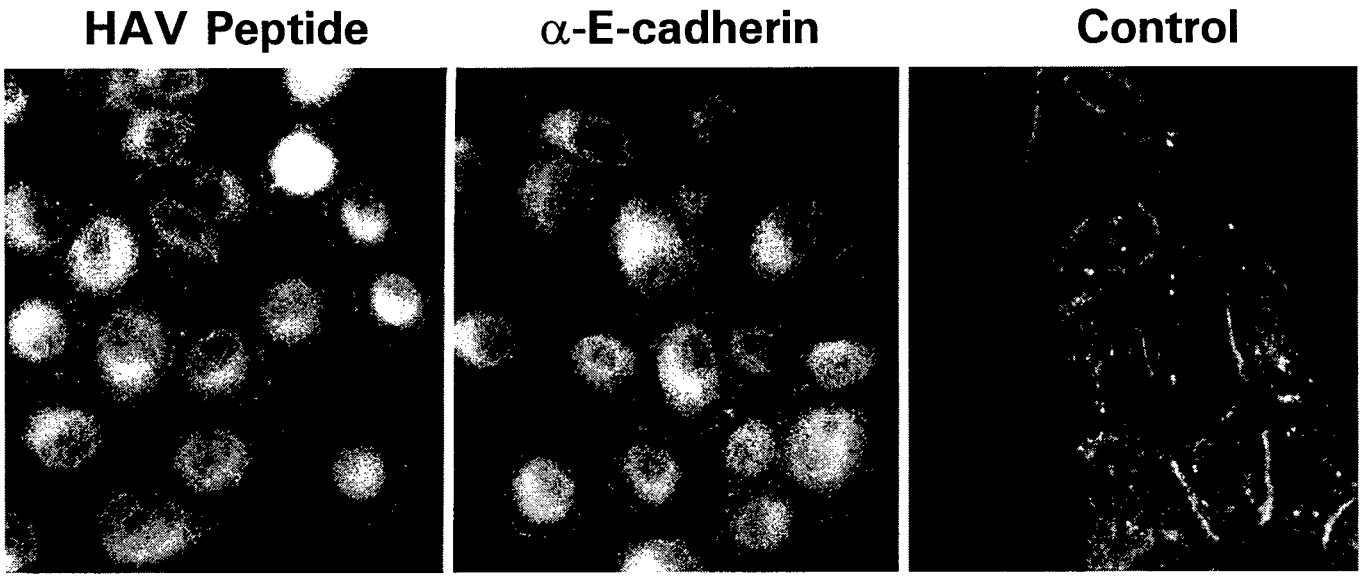


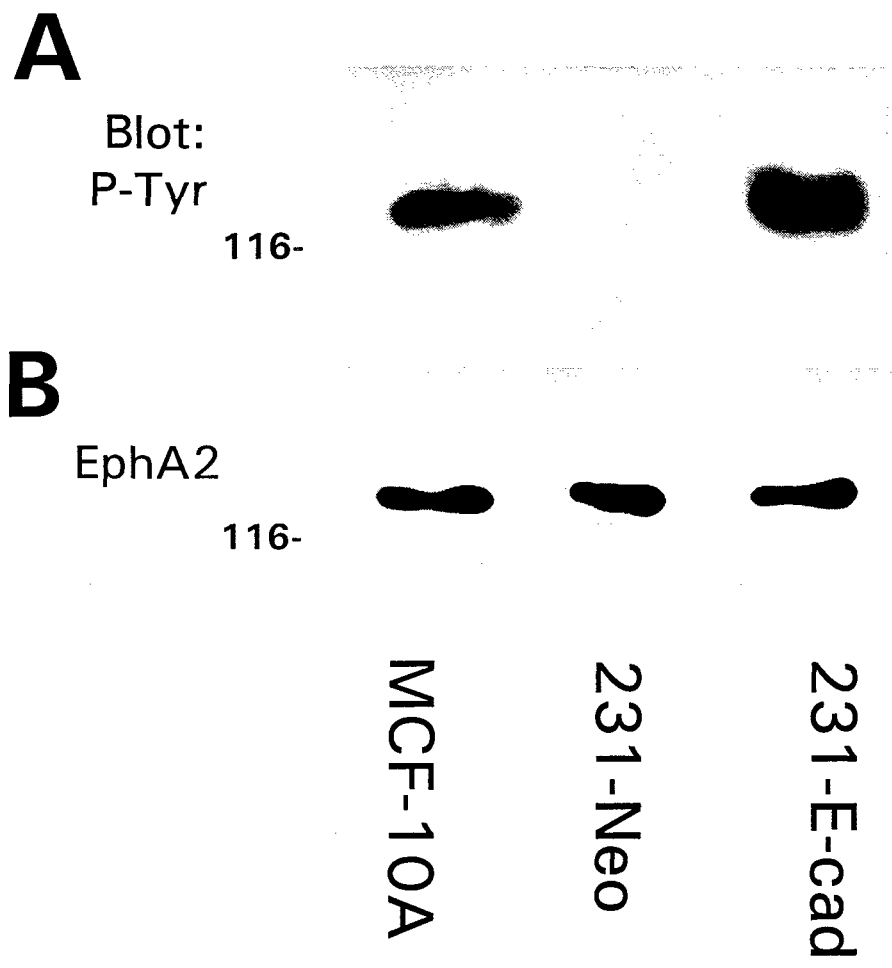
Zantek et al..,
Fig. 6A,B


Restoration
EGTA
No Treatment

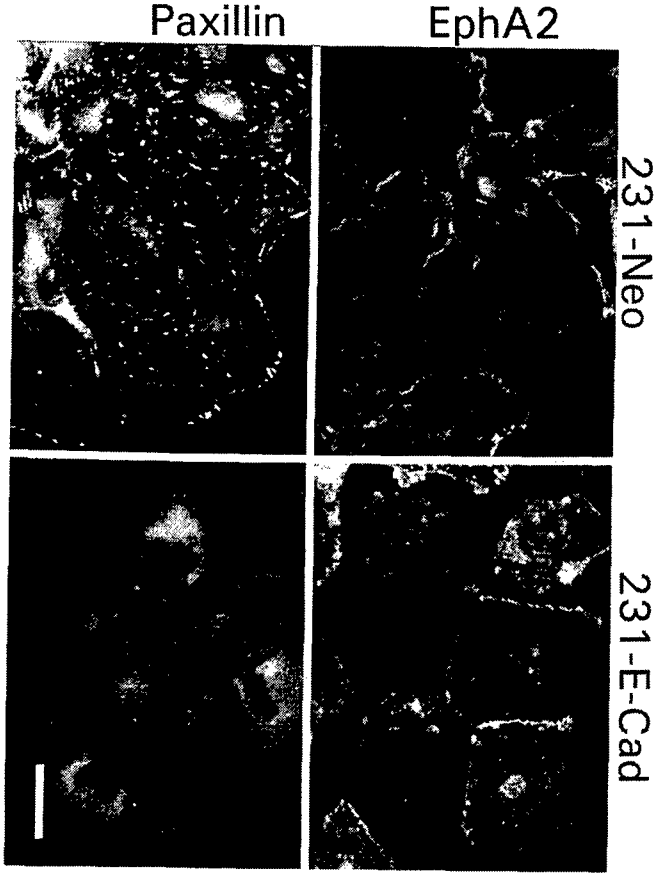


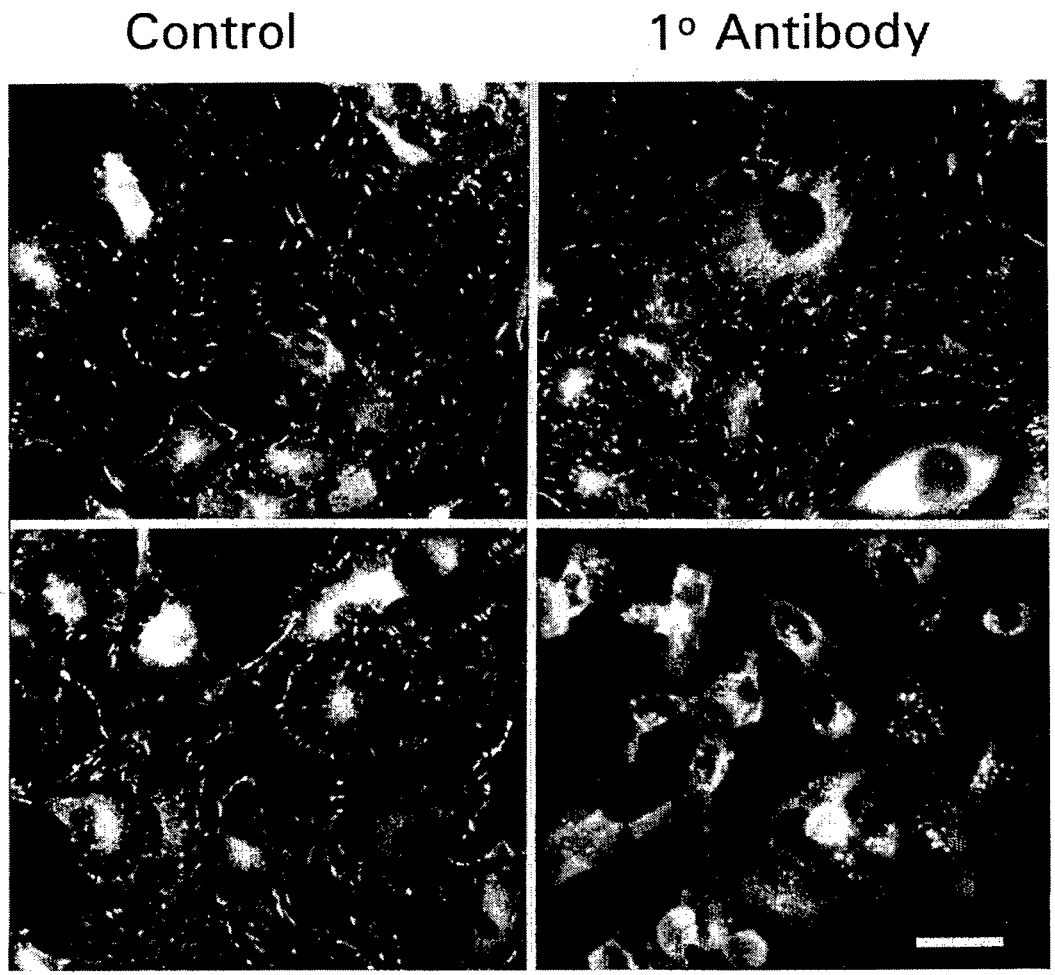
- Zantek et al..,
- Fig. 7A,B


HAV Peptide
α-E-cadherin
Control



Zantek et al.,
Figure 8


Paxillin
EphA2
231-Neo
231-E-Cad


Control
1° Antibody
2° Antibody
EphA2 Cross-Linked

Part II.F. Statement of Work

Characterization of an Adhesion-Associated Tumor Suppressor in Breast Cancer

Objective 1A: Identify the D7 antigen ***(months 1-6)***

Task 1. Affinity purification and microsequencing (months 1-3)
Task 2. cDNA isolation and sequencing (months 1-6)

Objective 1B: Determine the nature of D7's enzymatic activity ***(months 1-8)***

Task 1. Assess for possible interacting proteins (months 1-3)
-Concomitant with affinity purification (Objective 1A, Task 1)
Task 2. Determine whether D7 has serine/threonine or tyrosine kinase activity (months 6-8)

Objective 1C: Determine whether D7 is present in E-cadherin adhesion complexes ***(months 4-7)***

Task 1. Confocal microscopy for E-cadherin and D7 (month 4)
Task 2. Measure interactions using protein chemistry (months 4-7)

Objective 2A: Measure differences in D7 enzymatic activity between normal and transformed breast epithelia ***(months 5-10)***

Task 1. Measure tyrosine phosphorylation of D7 (month 5)
- Concomitant with Objective 1C, Task 2
Task 2. Compare kinase activity *in vitro* (months 6-10)

Objective 2B: Compare D7 enzymatic activity in normal and transformed breast epithelia ***(months 6-12)***

Task 1. Measure D7 activity during E-cadherin signaling in non-transformed cells (months 6-10)
-Concomitant with Objective 2A, Task 2
Task 2. Measure D7 activity during E-cadherin signaling in transformed cells (months 10-12)

Objective 3A. Begin to determine why D7 is lost in invasive cancers ***(months 13-18)***

Task 1. Analyze D7 in non-transformed and breast cancer cells by Northern blotting (months 13-16)
Task 2. Analyze D7 in non-transformed and breast cancer cells by RT-PCR (months 13-16)
Task 3. Measure D7 protein stability using pulse-label (months 17-18)

Objective 3B. Determine whether D7 suppresses a metastatic phenotype ***(months 17-36)***

Task 1. Construct expression vectors (months 17-19)
Task 2. Transfect (infect) and select ZR-75-1 with D7 (months 20-24)
Task 3. Analyze phenotype (immunofluorescence, cell growth, migration) (months 25-30)
Task 4. Transfect (infect) and select MCF-10-ST cell with E-cadherin (months 29-32)
Task 5. Analyze phenotype (immunofluorescence, cell growth, migration) (months 33-36)

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
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