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Our funded project allowed us to identify a receptor tyrosine kinase, EphA2, which is overexpressed and functionally altered in breast cancer cells. EphA2 overexpression identifies an aggressive subset of breast tumor and we find that EphA2 directly contributes to breast cancer metastasis. We also have shown that EphA2 functions as a powerful oncoprotein when overexpressed in human mammary epithelial cells. Importantly, the effects of EphA2 overexpression can be reversed by treating tumor-derived or EphA2-transformed cells with specific antibodies or artificial forms of ligand. Our studies have potentially important implications for breast cancer diagnosis and treatment. EphA2 overexpression appears to apply to a large number of breast cancer patients and importantly, identifies cells with metastatic potential. Moreover, our studies suggest that unique properties of EphA2 (*e.g.*, aberrant ligand binding) may render malignant cells susceptible to future therapeutic strategies that could redirect EphA2 so that it blocks the growth or invasiveness of malignant breast cancer cells

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INTRODUCTION

It is generally understood that cancer is a disease of aberrant signal transduction. In particular, the expression levels or function of receptor tyrosine kinases are frequently elevated in malignant cells. The purpose of our funded project was to identify one particular receptor tyrosine kinase, which was identified using D7 monoclonal antibodies. Our interest in the D7 antigen centered upon its differential expression in benign and malignant cells. Once identified, we sought to overexpress the gene that encodes for the D7 antigen in D7-deficient mammary epithelial cells and test for changes in cell growth and invasiveness.

BODY

Identification of the D7 Antigen (Objectives 1A and 1B)

In the search for the causes of breast cancer, many different lines of investigation have identified elevated levels of tyrosine kinase in breast cancer cells (Cance and Liu, 1995). However, the tyrosine kinases that are responsible for this signaling remain largely unknown. Our laboratory became interested in tyrosine kinase activity in breast cancer cells upon noting that elevated levels of tyrosine phosphorylation cause a dramatic reorganization of cellular adhesions and thereby promote a malignant phenotype (Kinch and Burridge, 1995; Kinch et al., 1995). We had also demonstrated that general inhibitors of tyrosine kinase activity (*e.g.,* herbimycin, genistein) could revert the adhesive phenotype of malignant cells to that of non-transformed mammary epithelial cells. To identify the specific tyrosine kinases that were responsible for this phenotype, we generated a panel of monoclonal antibodies that recognized tyrosine kinases in oncogene-transformed epithelial cells (Kinch et al., 1998). We developed and applied new methodologies to generate monoclonal antibodies tyrosine kinases in cancer cells.

Our antibody study yielded a large number of different antigens, but we focussed upon one particular kinase, recognized by D7 antibodies. This decision was based upon evidence that the D7 antigen was differentially expressed in non-transformed and malignant breast cancer cells. Moreover, we found that the phosphotyrosine content and subcellular localization of the D7 antigen differed when comparing non-transformed and tumor-derived cells (See Appended Manuscript) (Zantek et al., 1999). However, the identity and nature of enzymatic activity (tyrosine or serine/threonine activity) of the D7 kinase was unknown.

The first goal of our DoD-funded project sought to identify the D7 antigen. To accomplish this, we synthesized a cDNA expression library (in lambda-GT11 bacteriophage) from breast cancer cells that overexpressed the D7 antigen. We then probed this library with D7 antibodies and isolated three independent phage clones. DNA sequencing revealed that each phage encoded for the EphA2 receptor tyrosine kinase. We then confirmed the specificity of D7 antibodies for EphA2 using known EphA2 antibodies (generously provided by Dr. R. Lindberg, Amgen).

At the time that we identified the D7 antigen, rather little was known of EphA2. EphA2 is a 130 kDa transmembrane receptor tyrosine kinase of the Eph family of receptor tyrosine (Lindberg and Hunter, 1990) (Figure 1). Eph kinases are unique in that they bind ligands that are attached to the cell surface and this feature will be important below for understanding the functional alterations of EphA2 in breast cancer (Eph Nonmenclature Committee (Flanagan et al., 1997; Gale and Yancopoulos, 1997; van der Geer et al., 1994). Most Eph kinases are expressed only in the developing nervous system. By contrast, 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). This expression pattern, along with a lack of available monoclonal antibodies, had severely limited study of EphA2.

EphA2 and E-cadherin (Objectives 1C and 2)

Immunolocalization studies of the D7 antigen in non-transformed and malignant breast epithelial cells revealed dramatic differences in EphA2 subcellular distribution. In non-transformed cells, EphA2 co-distributed with E-cadherin into cell-cell contacts. In contrast, EphA2 in malignant cells was either diffusely distributed or found within membrane ruffles at the leading edge of cell invasion. Notably, the differential subcellular localization of EphA2 was found to relate to E-cadherin expression and the phosphotyrosine content of EphA2. This relationship was intriguing since E-cadherin is a potent tumor suppressor in breast cancer and we asked whether E-cadherin regulates EphA2 function.

As detailed in the Appended Manuscript (Zantek et al., 1999), we have demonstrated that E-cadherin regulates EphA2 subcellular localization and function. Specifically, we found that E-cadherin stabilizes cell-cell contacts and thereby allows EphA2 to interact with its ligands, which are anchored to the cell membrane. Consequently, the EphA2 in non-transformed cells is tyrosine phosphorylated whereas the EphA2 in malignant cells is not tyrosine phosphorylated. Interestingly, we also have demonstrated that the EphA2 in non-transformed and malignant cells has comparable enzymatic activity and that EphA2 enzymatic activity is independent of its phosphotyrosine content. Rather than directing the magnitude of enzymatic activity, our results suggest that ligand-mediated tyrosine phosphorylation of EphA2 controls substrate specificity or links EphA2 to downstream signaling pathways (via interactions with proteins that contain SH2 domains). This regulation has important biological implications for breast cancer since we also demonstrated that ligand-mediated activation of EphA2 blocks the growth and invasiveness of breast cancer cells.

EphA2 Expression in Breast Cancer (Objective 3A)

As demonstrated in the Appended Manuscript (Zelinski et al.,) (Zelinski et al., 2000), our studies have found differential expression of EphA2 in benign and malignant breast epithelial cells. Western blot analysis reveals that the highest

levels of EphA2 are consistently found in breast cancer cell models that are metastatic *in vivo* (i.e., xenograft analyses). Moreover, we have begun to measure EphA2 levels in clinical specimens of breast cancer. Immunohistochemical analyses of formalin-fixed paraffin-embedded specimens of benign and malignant breast tissues confirm elevated EphA2 levels in breast cancer specimens. As a follow-up study, we are currently preparing to increase the size of the study to determine if EphA2 levels relate to disease grade or outcome.

EphA2 Is a Powerful Oncoprotein (Objective 3B)

Since the highest levels of EphA2 consistently related to a metastatic phenotype, we have begun to overexpress EphA2 in cells with little or no endogenous EphA2. As detailed in the Appended Manuscript (Zelinski et al., 2000), we overexpressed EphA2 cDNAs in non-transformed MCF-10A cells and have found that this is sufficient to induce malignant transformation as defined using standard *in vitro* and *in vivo* analyses. These results are exciting since no other oncogene (*e.g.,* Ras, HER2, TC21, Rac, Rho) has been able to confer tumorigenic or metastatic potential upon MCF-10A cells. Thus, these results suggest that EphA2 overexpression is particularly relevant to breast cancer. Ongoing studies in our laboratory are dissecting the mechanisms by which EphA2 overexpression can induce malignant transformation of MCF-10A cells.

EphA2 overexpression alters the adhesive interactions of MCF-10A cells. In particular, EphA2 destabilizes cell-cell attachments while increasing cell interactions with extracellular matrix (ECM proteins). This directly impacts EphA2 function by destabilizing its interactions with its cell-anchored ligands. Consequently, the EphA2 in these cells is not tyrosine phosphorylated. However, we have found that the EphA2 in transformed MCF-10A cells remains responsive to stimulation by monoclonal antibodies or soluble forms of ligand (Zantek et al., 1999; Zelinski et al., 2000). These reagents can activate EphA2 (defined as increased phosphotyrosine content) and thereby block the growth and invasiveness of breast cancer cells (*e.g.,* MDA-MB-231, EphA2-transformed MCF-10A cells). These results suggest a possible mechanism by which EphA2 might serve as a therapeutic target in breast cancer cells and form the basis for our future research.

KEY ACCOMPLISHMENTS:

- Identification of the D7 antigen as the EphA2 receptor tyrosine kinase (Objective 1A and 1B)
- Demonstration that E-cadherin regulates the function of EphA2 in benign mammary epithelial cells (Objective 1C)
- Demonstration of differential subcellular localization and function of EphA2 in benign and malignant mammary epithelial cells (Objective 2)
- Demonstration that EphA2 is grossly overexpressed in malignant breast cancer cell lines and tissues (Objective 3A)

- Demonstration that EphA2 overexpression is sufficient to induce malignant transformation (Objective 3B)

REPORTABLE OUTCOMES:

Manuscripts:

Zantek, N.D., Azimi, M., Fedor-Chaiken, M., Wang, B., Brackenbury, R., and Kinch, M.S. (1999). E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. Cell Growth Differentiation *10*, 629-638.

Zelinski, D.P., Zantek, N.D., Stewart, J., Irizarry, A., and Kinch, M.S. (2000). EphA2 overexpression causes tumorigenesis and metastasis of mammary epithelial cells. *Submitted*, Cancer Res.

Presentations:

Invited Speaker, EphA2 Overexpression in Breast Cancer. American Society for Cell Biology National Conference, Washington, DC, December 1998.

Presentation, Aberrant expression and function of the EphA2 tyrosine kinase in breast cancer. American Association for Cancer Research National Conference, San Francisco, CA, April, 2000.

Patents:

Kinch, M.S., 1999. Antibody treatment of metastatic disease. U.S. Provisional Patent Application No 60/149258.

Kinch, M.S. and Zantek, N., 1999. Antibodies as cancer diagnostic. U.S. Provisional Patent Application No 60/149259.

Degrees Supported:

Zantek, N.D. 1999. Ph.D., Purdue University.

Cell Line Development:

EphA2-transformed MCF-10A cells

Funding Applications as a Result of this Award:

Targeted intervention against EphA2 on cancer cells, NIH, July, 2000.

Antibody targeting of EphA2 on breast cancer cells, USA-MRMC, June, 2000.

Antibody targeting of EphA2, Susan G. Komen Breast Cancer Foundation, April, 2000.

Differential substrate profile of the EphA2 tyrosine kinase in non-transformed and malignant breast epithelia. USA-MRMC, February, 2000.

Protein tyrosine phosphorylation and cell adhesion. American Cancer Society, April, 2000.

CONCLUSIONS:

Our funded proposal allowed us to identify a receptor tyrosine kinase, EphA2, which is overexpressed and functionally altered in breast cancer cells.

EphA2 overexpression identifies an aggressive subset of breast tumor and we find that EphA2 directly contributes to breast cancer metastasis. EphA2 functions as a powerful oncoprotein when overexpressed in human mammary epithelial cells. Importantly, the effects of EphA2 overexpression can be reversed by treating tumor-derived or EphA2-transformed cells with specific antibodies or artificial forms of ligand.

Our studies have potentially important implications for breast cancer diagnosis and treatment. EphA2 overexpression appears to apply to a large number of breast cancer patients and importantly, identifies cells with metastatic potential. Moreover, our studies suggest that unique properties of EphA2 (*e.g.,* aberrant ligand binding) may render malignant cells susceptible to future therapeutic strategies that could redirect EphA2 so that it blocks the growth or invasiveness of malignant breast cancer cells.

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Appendices:

1. Zelinski, D.P., Zantek, N.D., Stewart, J., Irizarry, A., and Kinch, M.S. (2000). EphA2 overexpression causes tumorigenesis and metastasis of mammary epithelial cells. *Submitted*, Cancer Res.

2. Revised Statement of Work (Revision requested by USA-MRMC, August, 1999).

3. Zantek, N.D., Azimi, M., Fedor-Chaiken, M., Wang, B., Brackenbury, R., and Kinch, M.S. (1999). E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. Cell Growth Differentiation *10*, 629-638.

EphA2 Overexpression Causes Tumorigenesis and Metastasis of Mammary Epithelial Cells[1]

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ABSTRACT

Elevated levels of protein tyrosine phosphorylation contribute to a malignant phenotype, although the kinases that are responsible for this signaling remain largely unknown. Here, we report increased levels of the EphA2 protein tyrosine kinase in cell models and clinical specimens of breast cancer. We show that EphA2 overexpression is sufficient to confer malignant transformation and metastatic potential upon non-transformed (MCF-10A) mammary epithelial cells. The transforming capacity of EphA2 is related to its ability to control cellular adhesions and with the failure of EphA2 to interact with its cell-attached ligands. Interestingly, stimulation of EphA2 is sufficient to reverse the malignant growth and invasiveness of EphA2-transformed cells. Altogether, these results identify EphA2 as a powerful oncoprotein in breast cancer.

INTRODUCTION

Cancer is a disease of aberrant signal transduction. In the search for signals that cause breast cancer, many lines of investigation have linked cancer with elevated expression or altered function of receptor tyrosine kinases (1). Recent studies have identified overexpression of HER2 or epidermal growth factor receptor (EGFR) in some tumors and have used this knowledge to develop successful approaches for therapeutic targeting of cancer cells (2). However, overexpression of HER2 and EGFR is limited to a subset of tumors, which creates a need to identify other tyrosine kinases responsible for metastatic progression and pathogenesis.

When malignant cells metastasize to distant sites in the body, morbidity and mortality significantly increase (3). Metastatic cells have acquired the abilities to break away from the primary tumor, translocate to distant sites in the body, and colonize a foreign microenvironment. At the cellular level, metastatic cells have overcome restraints upon cell growth and migration that result from physical linkages and signals conveyed by cell-cell contacts (4). Malignant cells often have increased interactions with surrounding extracellular matrix (ECM) proteins, which provide linkages and signals that promote several aspects of metastasis (5).

Our previous studies revealed that the levels of protein tyrosine phosphorylation regulate a balance between cell-cell and cell-ECM adhesions in epithelial cells (6). Using oncogene-transformed mammary epithelial cells, we showed that elevated tyrosine kinase activity weakens cell-cell contacts and promotes ECM adhesions (6). To identify tyrosine kinases that control tumor cell adhesion, we developed novel technologies to generate monoclonal antibodies against tyrosine kinases in cancer cells (7). We focused upon one particular antigen that was functionally altered in oncogene-transformed epithelial cells. This antigen was identified as EphA2 (ECK). EphA2 is a 130 kDa receptor tyrosine kinase that is expressed on adult epithelia (8), where it is found at low levels and is

enriched within sites of cell-cell adhesion (9). The subcellular localization is important because EphA2 binds five different ligands, ephrinA1-5, which are attached to the cell membrane (10).

MATERIALS AND METHODS

Cells and Antibodies. All cells were cultured as described previously (9). Antibodies specific for β-catenin and phosphotyrosine (PY-20) were purchased from Transduction Laboratories (Lexington, KY). Antibodies specific for phosphotyrosine (4G10) and EphA2 were purchased from Upstate Biologicals, Inc. (Lake Placid, NY). EphrinA1-F_c was a generous gift from Dr. B. Wang (Case Western Reserve).

Western Blot Analysis and Immunoprecipitation. Western blot analyses were performed as described previously (9) and antibody binding was detected by enhanced chemiluminescence (Pierce) and autoradiography (Kodak X-OMAT; Rochester, NY). To confirm equal sample loading, the blots were stripped and reprobed with antibodies specific for β-catenin or vinculin.

Immunohistochemistry and Immunofluorescence Staining. Formalin-fixed, paraffin embedded "sausage" slides, each containing 15-30 breast cancer specimens, were kindly provided by B.J. Kerns (BioGenex) and stained and scored as described (11). Mean immunostaining intensity in benign and malignant breast were compared using Student's t-test with statistical software (SAS for Windows v.6.04 and Microsoft Excel '97), defining $P<0.05$ as significant. Staining of cell monolayers with EphA2 antibodies (clones D7 or B2D6) was performed as described previously (9).

Transfection and Selection. Monolayers of MCF-10A cells were co-transfected with the pNeoMSV-EphA2 (generously provided by Dr. T. Hunter, Scripps) and pBABE-Puro eukaryotic expression vectors, at a 4:1 ratio, using Lipofectamine Plus (GIBCO; Grand Island, NY). As a control for the transfection procedure, a parallel transfection was performed using pNeoMSV and pBABE-Puro. Puromycin-resistant cells were selected by supplementing the growth medium with 1μg/mL puromycin (Sigma, St. Louis, MO). EphA2 overexpression was confirmed by Western blot analysis with specific antibodies. All experiments were performed using bulk culture transfectants and identical results were obtained using cells from two separate transfections with EphA2 cDNAs. Parental cells and cultures transfected with pBABE-Puro were used as negative controls.

Cell Adhesion Assays. Monolayers of MCF-10A cells transfected with empty vector or EphA2 were suspended using 4.5 mM EDTA. Cell-cell aggregation assays were performed by suspending 3×10^5 cells/mL in media for

30 minutes at 37°C and 5% CO_2. The average size of cell colonies was determined using light microscopy by dividing the total number of cells in each field by the number of particles (clusters containing one or more cells). To measure cell-ECM attachments, 5 x 10^5 suspended cells were plated into a 35mm tissue culture dish for 30 minutes at 37°C. Weakly adherent cells were detached by three vigorous washes and the remaining adherent cells were suspended with trypsin and counted using a hemacytometer. The average number of attached cells from at least four separate experiments is reported.

Colony Formation in Soft Agar. Colony formation in soft agar was performed as described (12). Colony formation was scored microscopically, defining clusters of at least three cells as a positive. For experiments with EphrinA1-F_c, 0.5 μg/mL of EphrinA1-F_c or a matched vehicle (50% Glycerol in PBS) was included in top agar solution and ligand was replenished daily with fresh media.

Cell Behavior in Matrigel. The behavior of cells in Matrigel was performed as described previously (13). Briefly, tissue culture dishes were coated with Matrigel (Collaborative, Bedford, MA) at 37°C before adding 1 x 10^5 vector or EphA2 transfected MCF-10A cells. The behavior of EphA2-overexpressing cells was assessed at 6 hour intervals using an inverted light microscope (Olympus IX-70). For experiments with EphrinA1-F_c, the culture medium was supplemented with 0.5 μg/mL of EphrinA1-F_c or an appropriately matched vehicle control. All images were recorded onto 35 mm film (Kodak T-Max-400).

Xenograft Analyses. Three to four week-old athymic (*nu/nu*) mice were purchased from Harlan Sprague Dawley (Indianapolis, IN) and Charles River (Wilmington, MA) and acclimated for 7-10 days. For subcutaneous implantation, $1x10^6$ or $5x10^6$ vector or EphA2 transfected MCF-10A cells were suspended in 100μL of fresh media and inoculated into the right craniolateral thorax (axilla) using a 23-gauge needle. For tail vein injections, $1x10^6$ cells were injected into the tail vein and mice were monitored for 7-28 days. At necropsy, primary tumors and all organs were evaluated macroscopically for the presence of tumors. Tissue samples of the primary tumor and organs were fixed in 10% buffered neutral formalin and embedded in paraffin. Tissue sections of the tumors and lung were stained with hematoxylin and eosin to assess morphology. Lung sections were stained with cytokeratin (AE1/AE3) or thrombin-specific antibodies (DAKO, Carpinteria, CA) to confirm the epithelial nature of lung metastases.

RESULTS

Elevated EphA2 Protein Levels in Breast Cancer Cells. To compare the levels of EphA2 protein in cell models of non-transformed (MCF-10A, MCF-12A, MCF-10-2) and aggressive (Hs578T, MDA-436, MDA-435, MDA-231, BT549) breast epithelia (14; 15), equal amounts of whole cell extracts were resolved by SDS-PAGE and subjected to Western blot analysis using EphA2-specific antibodies. Whereas lower levels of EphA2 protein were detected in non-transformed epithelial cells (Figure 1A, lanes 1-3), more EphA2 was detected in aggressive carcinoma cells (Figure 1A, lanes 4-8). Identical results were obtained when equivalent numbers of cells or equal amounts of protein were analyzed (data not shown). Increased levels of EphA2 in aggressive cell models were also confirmed using different EphA2 antibodies (D7, B2D6, EK166B; not shown), revealing that the differences in EphA2 levels did not reflect changes in a single epitope. The blots were stripped and re-probed with antibodies specific for β-catenin (Figure 1A) or vinculin (not shown), which confirmed equal sample loading.

The elevated levels of EphA2 in cell models prompted us to measure EphA2 in clinical specimens of breast cancer (Figure 1B). Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections revealed a low level of EphA2 immunoreactivity in benign mammary epithelia, with an average staining intensity of 0.1 (using a 0-3 scale to report staining intensity; Table I). EphA2 immunoreactivity was increased in breast carcinoma specimens, with an average staining intensity of 2.9. Increased staining intensity was accompanied by a larger percentage of carcinoma cells (an average of 87%) that stained positive for EphA2 as compared with benign mammary epithelial cells (an average of 3%).

EphA2 Overexpression Alters Cellular Adhesion. To assess the consequences of EphA2 overexpression, MCF-10A cells were transfected with human EphA2 cDNA (EphA2) or a vector control (vector) (Figure 2A). After establishing cultures of MCF-10A cells with stable overexpression of EphA2, microscopic evaluation revealed differences in the cell morphology as compared to vector-transfected control cells (Figure 2C, upper panels). Non-transformed MCF-10A cells displayed an epithelial morphology and interacted with one-another, even at low cell density. In contrast, EphA2-overexpressing MCF-10A cells (MCF^{EphA2}) adopted a fibroblast-like morphology and did not form cell-cell contacts, even at high cell density. To confirm that the mesenchymal morphology did not represent clonal variation, a separate sample of MCF-10A cells was transfected with EphA2 cDNAs and yielded identical results.

The morphological differences between vector and EphA2-transfected cells prompted us to measure cell-cell and ECM adhesions (Figure 2D). Cell-ECM adhesions were evaluated by incubating cells on ECM at 37°C for 30 minutes

before vigorous washing to remove weakly adherent cells. These assays revealed a 24-fold increase in ECM attachments in MCF^{EphA2} cells relative to vector-transfected controls ($P < 4x10^{-4}$). Cell-cell adhesions were assayed by incubating cells in suspension and counting the average size of cell colonies. Whereas vector-transfected MCF-10A cells interacted with one-another in colonies with an average size of 4.1 cells, the average colony size of MCF^{EphA2} cells was reduced to 1.3 cells ($P < 3x10^{-5}$) .

EphA2 Overexpression Decreases its Phosphotyrosine Content. Since stable cell-cell contacts cause EphA2 to become enriched within sites of cell-cell contact (9) we assessed EphA2 subcellular localization by immunostaining with specific antibodies (Figure 2C, lower panels). The EphA2 on non-transformed MCF-10A cells was restricted to a narrow line where adjacent cells came into direct contact, with little staining of membrane that was not in contact with neighboring cells. In contrast, the pattern of EphA2 staining on MCF^{EphA2} cells was diffuse, with little staining of cell-cell contacts.

The lack of EphA2 within cell-cell contacts in MCF^{EphA2} cells was intriguing since EphA2 is stimulated by ligands that are anchored to the cell membrane (10). To measure EphA2 stimulation, the phosphotyrosine content of immunoprecipitated EphA2 was measured by Western blot analysis with phosphotyrosine specific antibodies (Figure 2B). Whereas the EphA2 in vector-transfected MCF-10A cells was tyrosine phosphorylated, EphA2 was not tyrosine phosphorylated in MCF^{EphA2} cells. The decreased phosphotyrosine content was confirmed using multiple EphA2 antibodies for immunoprecipitation (D7, B2D6) and different phosphotyrosine-specific antibodies (4G10, PY20) for Western blot analyses.

To test if the EphA2 on MCF^{EphA2} cells could be stimulated by an exogenous ligand, we used EphrinA1-F_c, which consists of the extracellular domain of ephrinA1 linked to immunoglobulin heavy chain (16). Treatment of MCF^{EphA2} cells with 0.5 μg/mL EphrinA1-F_c increased the phosphotyrosine content of EphA2 (Fig 3A). Despite its inability to interact with its endogenous ligands, the EphA2 in MCF^{EphA2} cells could respond to exogenous stimuli.

EphA2 Overexpression Causes Malignant Transformation. The pattern of defects in cell adhesion, EphA2 subcellular distribution and phosphotyrosine content in MCF^{EphA2} cells were all reminiscent of metastatic cells (9), which prompted us to ask whether EphA2 overexpression induces malignant transformation. MCF^{EphA2} cells were found to colonize soft agar. Whereas vector-transfected MCF-10A cells formed 0.3 colonies per high-power field, MCF^{EphA2} cells displayed increased colony growth in soft agar, with an average of 3.0 colonies per high-power field ($P < 3x10^{-7}$) (Figure 3B). We then tested if the decreased ligand binding in MCF^{EphA2} cells related to colony formation in soft agar. To test this, MCF^{EphA2} cells were suspended in soft agar in the presence or

absence of 0.5 μg/mL EphrinA1-F_c. EphrinA1-F_c reduced colony formation in soft agar by 49% relative to vehicle-treated controls ($P < 5x10^{-6}$). Thus, EphA2 stimulation reversed the effects of EphA2 overexpression.

Based on evidence linking the aggressiveness of tumor cells *in vivo* with their behavior in Matrigel (17)(13), vector and EphA2 overexpressing MCF-10A cells were allowed to interact with Matrigel (Figure 3C). Non-transformed MCF-10A cells rapidly organized into spherical colonies when cultured on Matrigel whereas MCF^{EphA2} cells adopted a stellate organization that was indistinguishable from the behavior of metastatic cells (*e.g.,* MDA-MB-231, MDA-MB,435) (not shown). To test if EphA2 stimulation could alter cell behavior on Matrigel, the MCF^{EphA2} cells were treated with 0.5 μg/mL EphrinA1-F_c, which restored a spherical phenotype that was comparable to non-transformed MCF-10A cells (Figure 3C, bottom right).

EphA2 Overexpression Confers Tumorigenic and Metastatic Potential. Since *in vitro* analyses of transformation do not always predict tumorigenic potential *in vivo*, control or EphA2-overexpressing MCF-10A cells were implanted into athymic (*nu/nu*) mice (Figure 4). Subcutaneous injection of MCF^{EphA2} cells caused the formation of palpable tumors within four days (Figure 4A) in 19 of 19 mice. The median volume of resulting tumors related to the number of implanted cells and reached an average of 300 mm^3 (for samples injected with $5x10^6$ cells) within 10 days (Table II). Necropsy revealed that the tumors were firmly attached to the underlying axillary muscle and surrounded by fibrous tissue (not shown). Histologically, the neoplastic cells were invasive and associated with fibrous connective tissue (Figure 4B,C). These neoplastic cells exhibited moderate cytoplasmic and nuclear pleiomorphism and formed dysplastic tubular and secreting structures. In control experiments, cells transfected with vector DNA failed to grow in athymic mice (0 of 13; Table II) and necropsy failed to identify any growth or invasion of these cells (not shown).

Since the highest levels of EphA2 were consistently found in breast cancer cells that are metastatic *in vivo* (15), $1x10^6$ control or MCF^{EphA2} cells were injected into the tail vein of athymic mice. Within seven days, necropsy revealed lung micrometastases within large vessels in 2 of 4 mice injected with MCF^{EphA2} cells (Table II). The metastases were generally found to occlude large blood vessels but did not breach the vessel wall (Figure 4D). Immunohistochemical staining with cytokeratin antibodies confirmed the epithelial nature of the thrombus and a lack of anti-thrombin staining revealed that the thrombus did not represent an abnormal or atypical outgrowth of endothelial cells (not shown). No lung colonization was observed in mice that had been injected with control MCF-10A cells (Table II).

DISCUSSION

The major findings of this study are that the EphA2 tyrosine kinase is overexpressed in many clinical specimens and cell models of breast cancer, where it functions as a powerful oncoprotein. We also show that EphA2 overexpression reorganizes cellular adhesions and prevents ligand-mediated stimulation of EphA2. However, restoration of ligand binding reverses the malignant phenotype of EphA2-overexpressing cells.

Consistent results with several cell models suggest that elevated levels of EphA2 are highly relevant to breast cancer. High levels of EphA2 are found in clinical specimens and aggressive cell models of breast cancer. Recent studies reveal that EphA2 overexpression may similarly apply to advanced melanoma (18), colon cancer (19) and prostate cancer (11). The fact that elevated EphA2 levels are found on multiple types of cancer suggests that EphA2 overexpression may be a common event in the metastatic progression of carcinoma cells.

Our results provide the first evidence that EphA2 is not merely a marker, but an active participant in tumorigenesis and metastasis. EphA2-overexpressing MCF-10A cells displayed the hallmarks of malignant transformation as defined *in vitro* and *in vivo*. MCF^{EphA2} cells formed tumors *in vivo* at a high frequency, which is remarkable given that other oncogenes (*e.g.,* Ras, HER2, TC21) are insufficient to convey tumorigenic or metastatic potential upon MCF-10A cells (13)(12). Thus, we suggest that EphA2 overexpression may be particularly relevant to metastatic progression.

EphA2 overexpression causes defects in cell adhesion that are characteristic of aggressive cancer cells. The weakened cell-cell contacts and increased ECM adhesions of MCF^{EphA2} cells resemble the adhesive phenotype of oncogene-transformed and tumor-derived epithelial cells (6). Consistent with this, the highest levels of EphA2 are consistently found on tumor-derived cell lines that display weak cell-cell contacts and increased ECM invasiveness (15). One possible explanation is that EphA2 phosphorylates adhesion or cytoskeletal proteins to alter the balance between cell-cell and ECM adhesions. This idea is supported by evidence linking EphA2 to adhesion and cytoskeletal proteins, including E-cadherin FAK, SLAP and PI 3-kinase (16)(20). Alternatively, EphA2 could alter the expression of important adhesion molecules. Future studies will be needed to identify the molecular targets of EphA2 in malignant cells.

The weakened cell-cell adhesions of EphA2-overexpressing cells are interesting since EphA2 binds a membrane-anchored ligand. We have shown that EphA2 in non-transformed epithelia is enriched within sites of cell-cell contact, where it interacts with ligand and is tyrosine phosphorylated (9). In contrast, EphA2 overexpression destabilizes cell-cell contacts, causes EphA2 to become diffusely distributed and prevents ligand-mediated tyrosine phosphorylation of EphA2. Similarly, the EphA2 in clinical specimens of breast cancer is diffusely distributed (See Figure 1B) and is not tyrosine phosphorylated (Kinch, unpublished observation), which suggests that these regulatory mechanisms are relevant *in vivo*.

EphA2 stimulation by an artificial ligand reverses the malignant behavior of EphA2-transformed cells. EphrinA1-F_c also blocks the growth and migration of malignant breast and prostate cancer cells (9)(16). The molecular basis of these inhibitory effects remains largely unknown, although tyrosine phosphorylation of EphA2 facilitates interactions with PI 3-kinase, SHP-2 and a Src-like adapter protein (SLAP), which is intriguing since each protein has been independently found to regulate cell growth or development (16; 20).

Overexpressed receptor tyrosine kinases can facilitate new and efficacious modalities for targeted intervention against cancer cells (2). A recent success arose from antibody targeting of HER2, a receptor tyrosine kinase that is overexpressed on some breast cancer cells (2). Unfortunately, HER2 overexpression is limited to one-third of breast carcinomas and is sporadic on other tumor types, which underscores the need for new targets. Our results suggest that EphA2 might provide a target for intervention against metastatic cancer. At minimum, EphA2 overexpression may identify a larger or different set of tumors than HER2. Strong EphA2 immunoreactivity was detected in 5 of 12 (~40%) breast cancer specimens whereas strong HER2 immunoreactivity was limited to 2 of the 12 samples (data not shown). Our evidence suggests that strategies that restore or mimic the effects of ligand could negatively regulate tumor cell growth and invasiveness (9)(16). This latter approach would redirect the function of an overexpressed oncoprotein so that it blocks tumor cell growth and invasiveness.

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Table I. EphA2 Immunoreactivity in Breast Cancer Specimens

	EphA2 Staining Intensity			
	0 (Negative)	1 (Weak)	2 (Moderate)	3 (Strong)
Benign Breast				
Sample Number	9	3	0	0
% Cells Positive	<5%	10-20%		
Breast Carcinoma				
Sample Number	1	0	6	5
% Cells Positive	<5%		50-100%	90-100%

Table I. Formalin-fixed, paraffin-embedded specimens of benign and malignant mammary tissues were stained with EphA2 antibodies and evaluated as described in the Materials and Methods. Statistical analyses revealed differences in EphA2 staining of benign and malignant samples ($P<1x10^{-6}$). See Figure 1 for an example of strong staining.

Table II. Tumorigenic and Metastatic Potential of EphA2-Transformed MCF-10A Cells

Cell	Site of Inoculation	# of Cells Injected	Incidence of Tumorigenicity	Tumor Volume (mm^3)
Ctrl	Subcutaneous	1×10^6	0/9	NA
EphA2		1×10^6	9/9	66 ± 20
Ctrl	Subcutaneous	5×10^6	0/4	NA
EphA2		5×10^6	10/10	293 ± 70
Ctrl	Tail Vein	1×10^6	0/4	
EphA2		1×10^6	2/4	

Table II. Tumorigenesis by MCF-10A cells (± EphA2) was evaluated following subcutaneous or tail vein injection. Please refer to Figure 4 for microscopic evaluation of implanted tumors. The significance of tumor formation was estimated to be $P < 1.3 \times 10^{-7}$ as determined by χ^2 analyses.

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Figure Legends

Figure 1. EphA2 Protein Levels in Breast Cancer Cells and Tissues
A, Whole cell lysates from cell models of non-transformed mammary epithelia (lanes 1-3) or aggressive breast cancers (lanes 4-8) were resolved by SDS-PAGE and Western blot analysis was performed using EphA2-specific (D7) antibodies. As a loading control, the membranes were stripped and reprobed with antibodies specific for β-catenin. The relative electrophoretic mobility of standards is shown on the left. *B,* EphA2 protein levels were assessed by immunohistochemical staining of formalin-fixed, paraffin-embedded specimens of benign (left) or malignant (right) breast specimens (Bar = 40 μm). The insets are higher-magnification images (Bar = 10 μm). Table I for details and data analysis. Note that the non-immunoreactive cytoplasm of benign epithelium (arrowheads) contrasts with the strong and diffuse immunoreactivity of malignant cells.

Figure 2. EphA2 overexpression causes malignant transformation
A, MCF-10A cells co-transfected with pBabe-puro and either pNeoMSV (vector) or pNeoMSV-EphA2 (EphA2). Western blot analysis of whole cell lysates, resolved by SDS-PAGE, was performed using EphA2-specific antibodies (top). The membranes were stripped and reprobed with β-catenin antibodies as a control for sample loading (bottom). The relative mobility of molecular mass standards is shown on the left. *B,* The phosphotyrosine content of EphA2 was determined by Western blot analyses of immunoprecipitated EphA2 (top). The blots were then stripped and reprobed with EphA2-specific antibodies (clone D7; bottom). The relative mobility of molecular mass standards is shown on the left. *C,* Upper Panels: The morphology of vector and EphA2-transfected cells was assessed by phase-contrast microscopy. Whereas control cells organize into colonies, EphA2-overexpressing cells resist interactions with one-another. Bar = 100 μm. Lower Panels: Monolayers of vector or EphA2-transfected MCF-10A cells were stained with EphA2-specific antibodies. Note that EphA2 was enriched within sites of cell-cell contact in vector-transfected controls but was diffusely distributed in EphA2-transfected cells. Bar = 50 μm. *D,* Assays of ECM attachments (left) were performed by incubating cells onto purified ECM for 30 minutes at 37°C. Shown is the fraction of cells that remained adherent after vigorous washing (left graph). Rosette assays measured the average size of cell-cell aggregates in suspension (right graph). Asterisks denote that EphA2 overexpressing cells had statistically significant increases in ECM contacts ($P < 4x10^{-4}$) and decreased cell-cell ($P < 3x10^{-5}$) adhesions.

Figure 3. The aggressive phenotype of MCF^{EphA2} cells is reversed by receptor activation.
A, MCF^{EphA2} cells were treated with 0.5μg/mL EphrinA1-F_c (EA1-F_c) for 8 minutes before immunoprecipitation of EphA2 with specific antibodies (clone

D7). Western blot analysis with phosphotyrosine (P-Tyr) specific antibodies (PY20 and 4G10) revealed that EphrinA1-F_c increased the phosphotyrosine content of EphA2. *B,* To measure anchorage-independent cell growth and survival, $1x10^4$ vector or EphA2-transfected MCF-10A cells were suspended in soft agar ± 0.5 μg/mL EphrinA1-F_c (EA1-F_c). After seven days, colony formation was scored microscopically, defining clusters containing at least three cells as a positive colony. MCF^{EphA2} cells demonstrated significant increases in anchorage independent growth (* $P < 4x10^{-7}$) whereas EphrinA1-F_c treatment significantly blocks the growth of MCF^{EphA2} cells (** $P < 5x10^{-6}$). *C,* The phenotype of control and EphA2-transformed MCF-10A cells was determined after incubation atop polymerized Matrigel ± 0.5 μg/mL EphrinA1-F_c (bottom panels) or an appropriately matched vehicle (top panels). Whereas control MCF-10A cells organized into spherical colonies, MCF^{EphA2} cells displayed a stellate growth pattern in Matrigel that mimicked the behavior of aggressive breast cancer cells (MDA-MB-231, not shown). Notably, treatment with 0.5 μg/mL EphrinA1-F_c caused the phenotype of MCF^{EphA2} cells to be indistinguishable from control MCF-10A cells. Bar = 100 μm.

Figure 4. EphA2 overexpression conveys tumorigenic and metastatic potential.

A-C, MCF^{EphA2} cells were implanted subcutaneously into the right craniolateral thorax (axilla) of athymic (*nu/nu*) mice. *A,* Within four days, the implanted cells formed palpable masses (arrowheads). *B,* The histologic appearance of the tumor revealed that these masses were almost entirely composed of moderately differentiated and invasive tumor cells (arrowheads) that formed dysplastic tubules with fluid-filled lumens (marked with an L). *C,* Neoplastic cells (arrowheads) invaded adjacent skeletal muscle fibers (m). *D,* MCF^{EphA2} inoculated intravenously into the tail vein of athymic mice colonized the lung and were enriched within large vesicles (arrowheads). Histologic appearance of pulmonary tumor thrombi in athymic mice revealed that tumor cells partially to totally obstructed intravascular spaces but did not invade the vessel wall. See Table II for details and data analysis. Bar = 40 μm.

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)

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

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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 nonneoplastic 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 was 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-extracellular matrix 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 the 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) is a M_r 130,000 member of the Eph family of receptor tyrosine kinases (5), which interact with cell-bound ligands known as ephrins (1, 6, 7). Whereas 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. Whereas 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 that are 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.

Results

Regulation of EphA2 Expression in Breast Cancer Cells. We measured EphA2 expression levels in breast epithelial cell lines derived from nonneoplastic epithelia (*e.g.*, MCF-

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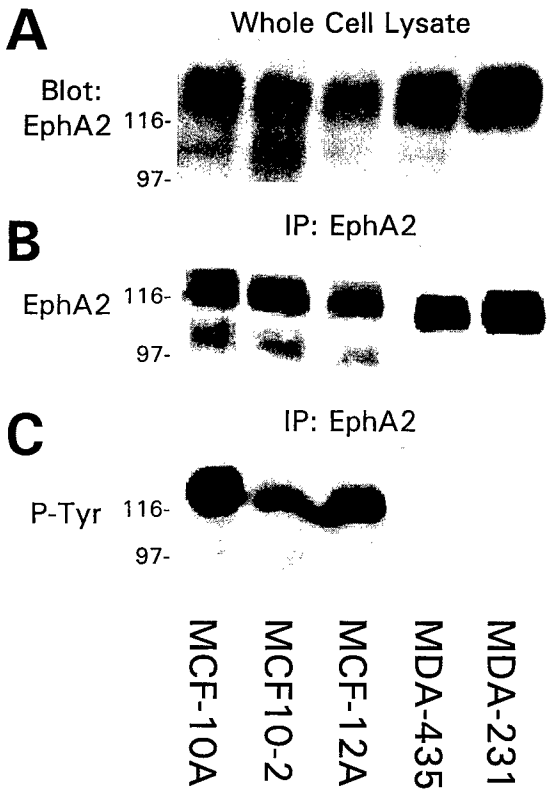

Fig. 1. Decreased EphA2 phosphorylation in metastases. EphA2 from whole cell lysates (*A*) or immunoprecipitated from monolayers of nonneoplastic (MCF-10A, MCF10-2, and MCF-12A) and metastatic (MDA-MB-231 and MDA-MB-435) breast cancer cell lines (*B*) was resolved by SDS-PAGE and Western blot analysis 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.

10A, MCF-12A, and MCF10-2; Refs. 29 and 30) and metastatic breast cancer (*e.g.*, MDA-MB-231 and MDA-MB-435; Refs. 31 and 32). EphA2 was found to be expressed in nontransformed mammary epithelial and metastatic breast cancer cell lines tested (Fig. 1*A* 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 nonneoplastic or metastatic cells and Western blot analysis performed with phosphotyrosine specific antibodies. This revealed prominent phosphorylation of EphA2 in nonneoplastic cells, whereas the EphA2 from metastatic cells was not tyrosine-phosphorylated (Fig. 1*C*). The decreased phosphotyrosine content was confirmed using different EphA2 antibodies (D7, B2D6, and rabbit polyclonal antibodies) for immunoprecipitation and with multiple phosphotyrosine antibodies (PY20, 4G10, and rabbit polyclonal antibodies) 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

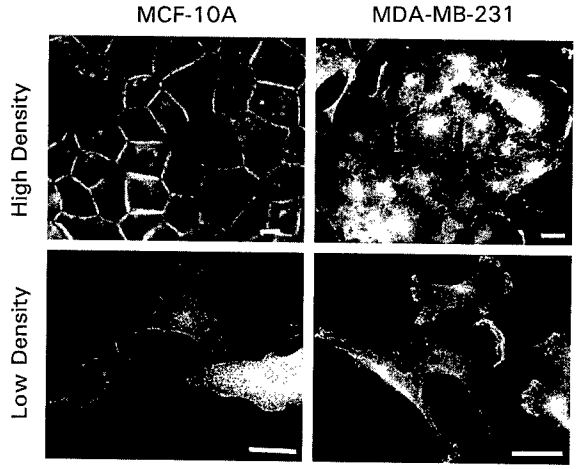

Fig. 2. Altered EphA2 localization in metastatic cancer cells. The subcellular distribution of EphA2 in nontransformed mammary epithelial cells (MCF-10A) and metastatic breast cancer cells (MDA-MB-231) was assessed by immunostaining with EphA2-specific antibodies. The cells were plated at either high (*top*) or low (*bottom*) cell density to emphasize the localization of EphA2 within cell-cell contacts or membrane ruffles of nontransformed or invasive cells, respectively. *Scale bars*, 10 μm.

cell lines derived from colon, pancreatic, ovarian, and lung cancers (data not shown).

Further comparison of EphA2 in nonneoplastic and metastatic cells revealed other changes in EphA2 distribution and function. Immunofluorescence staining with EphA2-specific antibodies revealed that EphA2 in nonneoplastic cells was mostly found within sites of cell-cell contact (Fig. 2), with little staining of membrane that was not in contact with neighboring cells. In 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 colocalization of EphA2 with f-actin (data not shown). This localization within membrane ruffles was not observed in nontransformed epithelia, even at low cell density. These differences in subcellular distribution were confirmed using three different EphA2-specific antibodies (D7, B2D6, and rabbit polyclonal antibodies). The correlation between EphA2 localization and phosphotyrosine content forms the basis for much of the remainder of this study.

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 (Fig. 3). Despite the low phosphotyrosine content of EphA2 in metastatic cells, this EphA2 demonstrated enzymatic activity that was comparable with or higher than the activity of EphA2 isolated from nonneoplastic cells. This activity was unaffected by the basal phosphotyrosine content of EphA2 because unlabeled phosphate was rapidly exchanged with labeled phosphate during the autophosphorylation assays as described previously (33, 34). KOH treatment of the membranes prior to autoradiography did not

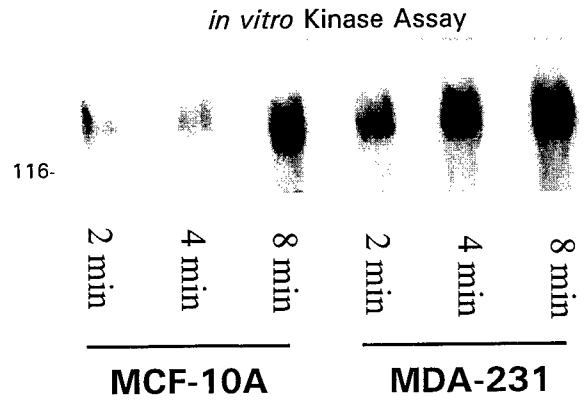

Fig. 3. EphA2 enzymatic activity. The enzymatic activity of EphA2 was measured using an *in vitro* autophosphorylation assay. At the times shown, the *in vitro* reaction was terminated and resolved by SDS-PAGE. The blot shown was treated with KOH to hydrolyze phosphoserine and phosphothreonine prior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

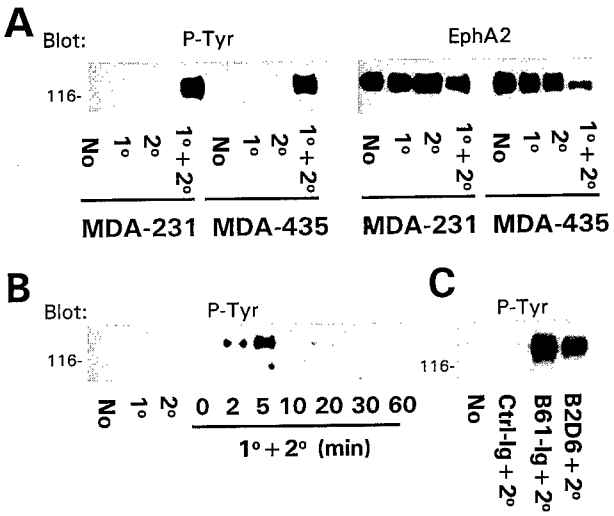

Fig. 4. Antibody-mediated aggregation induces EphA2 phosphorylation in metastatic cells. *A*, immunoprecipitated EphA2 was subjected to Western blot analysis with phosphotyrosine antibodies (PY20) following aggregation of cell surface EphA2 for 5 min at 37°C with specific primary and secondary antibodies (*1°+2°*). Note that simple engagement of anti-EphA2 (*1°*) or antimouse (*2°*) alone was insufficient to induce tyrosine phosphorylation above basal levels (*No*). 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 min 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) served as a negative control, and B2D6-mediated aggregation served as a positive control for activation.

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 (Fig. 1*B*) was not predictive of its enzymatic activity (Fig. 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"; Refs. 10 and 35; Fig. 4*C*). In 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 that were comparable with that nonneoplastic cells (Fig. 4*A*). Receptor aggregation, not simply antibody binding, was necessary for EphA2 phosphorylation as incubation with anti-EphA2 (Fig. 4, *1°*) alone did not increase EphA2 phosphorylation relative to matched controls. This effect was specific for EphA2 as neither secondary (Fig. 4, *2°*) 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 min after cross-linking, with optimal phosphorylation detected after 5 min (Fig. 4*B*).

E-Cadherin Regulates EphA2 in Nontransformed 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 (36), 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 nontransformed fibroblasts (*e.g.*, NIH 3T3, REF-52, and C3H10T½) and myoepithelial cells (HBL-100; data not shown). We, therefore, tested whether E-cadherin might regulate EphA2 phosphorylation.

Because both EphA2 and E-cadherin are found at sites of cell-cell contact, we first examined whether the two proteins colocalize 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 (Fig. 5). Vertical sectioning by confocal microscopy confirmed colocalization of E-cadherin and EphA2 within sites of cell-cell contact (data not shown).

To test whether the colocalization 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 medium with 4 mM EGTA, a calcium-chelating agent. EGTA treatment caused EphA2 dephosphorylation (Fig. 6*A*) and induced either a diffuse or membrane ruffle pattern of staining (Fig. 6*C*), 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 min (Fig. 6, *A* and *C*).

Although results with EGTA-treated samples implicate cell-cell adhesion with the control of EphA2 phosphorylation

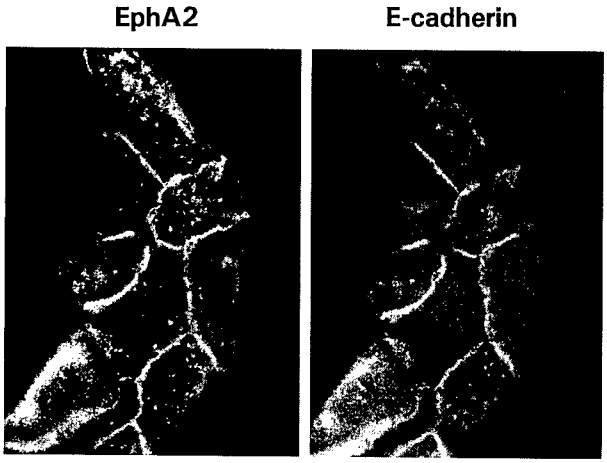

Fig. 5. Colocalization of EphA2 and E-cadherin. The subcellular distribution of EphA2 (*left*) and E-cadherin (*right*) was evaluated in MCF-10A cells using two-color immunofluorescence microscopy. Note the overlapping distribution of EphA2 and E-cadherin within sites of intercellular junctions.

and subcellular localization, we sought to determine whether 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; Refs. 37 and 38). When inhibitors of E-cadherin function were added to the medium concomitant with the restoration of extracellular calcium, EphA2 did not become tyrosine-phosphorylated (Fig. 7*A*) and remained diffuse or present within membrane ruffles (Fig. 7*C*). In 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 (Fig. 8). In contrast, the EphA2 in 231-E-cad redistributed into sites of cell-cell contacts and had levels of phosphotyrosine that were comparable with that of MCF-10A cells (Fig. 9*A*). 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 (Fig. 8). 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[3] attachments by staining with paxillin-specific antibodies revealed numerous focal adhesions in control MDA-MB-231 cells, whereas 231-E-cad cells had fewer focal adhesions. The decrease in focal adhesions was most prominent in 231-E-cad cells within colonies (Fig. 8, *bottom right*), whereas individual cells had focal adhesions that were comparable with 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 min. This was confirmed by paxillin staining (Fig. 10) and by interference reflection microscopy (data not shown). Similar results were obtained in other neoplastic cell lines (data not shown). In 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 (39, 40). Because 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 BrdUrd in the culture medium and measured DNA synthesis over the following 4 h. As shown in Table 1, 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 (Fig. 4*B*) 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-

[3] The abbreviations used are: ECM, extracellular matrix; BrdUrd, bromodeoxyuridine.

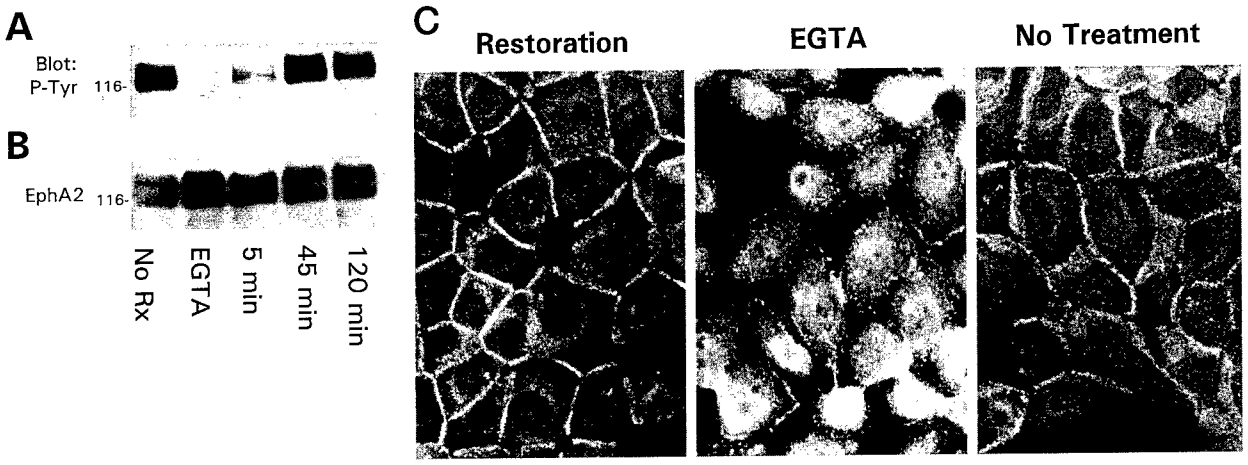

Fig. 6. EphA2 phosphorylation and localization require stable E-cadherin adhesions. Stable cell-cell contacts in monolayers of MCF-10A cells were disrupted by the addition of EGTA (4 mM, 30 min, 37°C) to the culture medium. After removal of the EGTA, normal growth medium was returned for 0–120 min. *A*, 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.

phosphorylated in nonneoplastic mammary epithelial cell lines but not in metastatic cell lines. In this respect, EphA2 differs from many other tyrosine kinases (*e.g.*, cErbB2, epidermal growth factor receptor, platelet-derived growth factor receptor, and Src), the phosphorylation of which increases in cancer cells (2, 41, 42). 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 with or increased over the activity of phosphorylated EphA2 in nonneoplastic epithelial cells. This is consistent with evidence that the phosphorylation of EphB2 also has little effect on its kinase activity (43). 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 (44, 45). 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 because the sites that become autophosphorylated *in vitro* are the same in nontransformed and neoplastic cells.[4] 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.[5] However, the identities of the phosphatases responsible for this are 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, 46), 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 coimmunoprecipitate EphA2 and E-cadherin.[5] EphA2

[4] M. S. Kinch, unpublished results.

[5] N. D. Zantek, unpublished results.

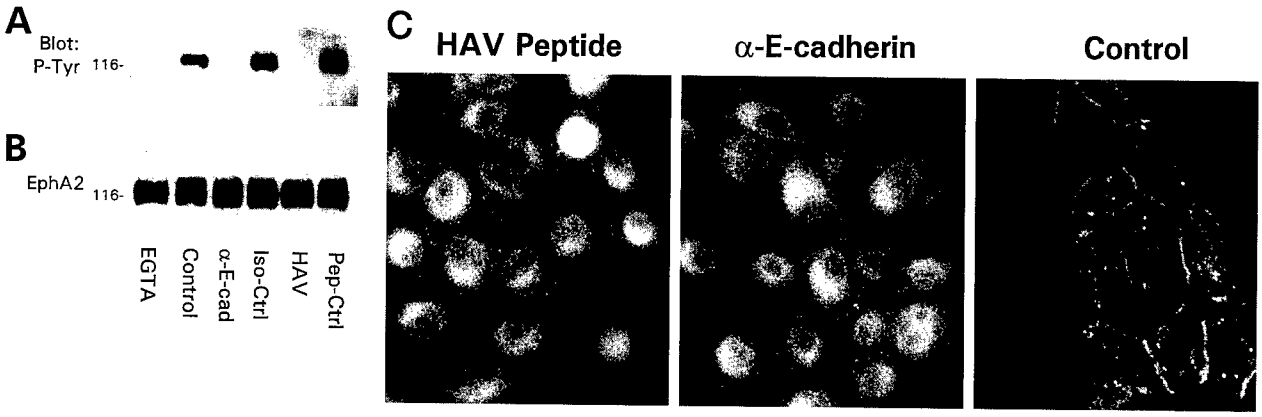

Fig. 7. Inhibition of E-cadherin-mediated adhesion. Following treatment of MCF-10A cell monolayers with EGTA, normal medium conditions were restored in the absence (*Control*) or presence of function-blocking E-cadherin antibodies (*α-E-cad*) or peptides (*HAV*). Isotype control antibodies (*Iso-Ctrl*) 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 in *A* was stripped and reprobed 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.

also does not cocluster with E-cadherin at the cell surface in response to antibody-mediated aggregation of either molecule,[6] 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 coclusters 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 (47), and 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 (48). Focal adhesions also play critical roles in signal transduction, where they organize intracellular signals that control cell growth and survival (39, 40). 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 (49, 50). 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 phosphatidylinositol 3′-kinase, Src, Fyn, and Ras-GAP (35, 51–53).

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; Ref. 54). 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. In contrast, neoplastic cells express high levels of EphA2, which, although nonphosphorylated, retains comparable levels of enzymatic activity. An alternative explanation is that EphA2 positively regulates cell growth but that this signaling is reduced in nontransformed 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 (55). 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 phosphatidylinositol 3′-kinase and the SLAP adapter protein (56). SLAP was recently shown to negatively regulate cell growth (57), 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 whether these properties differ between normal and neoplastic epithelia.

Conclusions. Loss of E-cadherin in carcinomas promotes invasion (18, 58), cell motility (27), and cell proliferation (26). In this study, we have identified the receptor tyrosine kinase EphA2 as one protein that is phosphorylated after cell-cell contact and demonstrated that both the phospho-

[6] M. Fedor-Chaiken and M. S. Kinch, unpublished results.

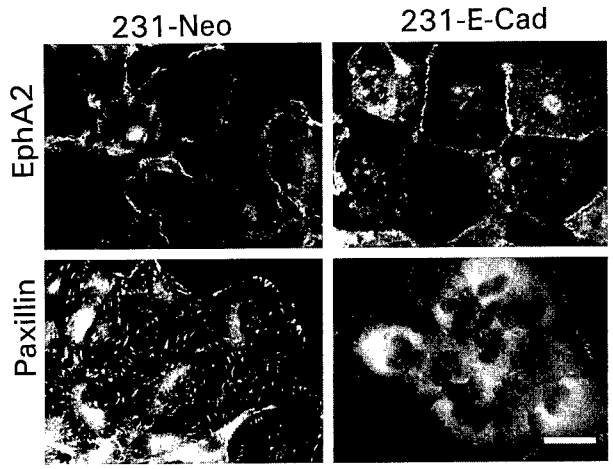

Fig. 8. E-Cadherin expression directs EphA2 into cell-cell contacts. The subcellular distribution of EphA2 and paxillin was assessed by immunofluorescence microscopy in control (*231-Neo*) and E-cadherin transfected (*231-E-Cad*) MDA-MB-231 cells. Note that E-cadherin promotes a redistribution of EphA2 into cell-cell contacts and decreases focal adhesions. *Scale bar*, 25 μm.

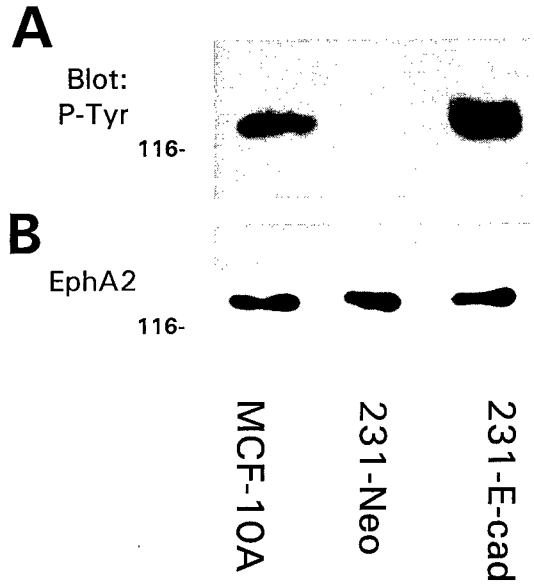

Fig. 9. E-cadherin expression restores normal EphA2 function. *A*, the phosphotyrosine content of immunoprecipitated EphA2 was measured by Western blot analysis following transfection of MDA-MB-231 cells with E-cadherin (*231-E-cad*) or a matched vector control (*231-Neo*). MCF-10A was included as a positive control for EphA2 tyrosine phosphorylation. *B*, The blot from *A* was stripped and reprobed with EphA2-specific antibodies as a loading control.

rylation 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.

Materials and Methods

Cell Lines and Antibodies. Human breast carcinoma cells and nontransformed human mammary epithelial cell lines were cultured as described previously (29, 46). We purchased antibodies specific for E-cadherin (polyclonal antibodies, Transduction Laboratories, Lexington, KY; and DECMA-1, Sigma Chemical Co., St. Louis, MO), phosphotyrosine (PY20, ICN, Costa Mesa, CA; 4G10, Upstate Biotechnology Inc., Lake Placid, NY; and polyclonal antibodies, Transduction Laboratories), and fluorescein-conjugated BrdUrd (Harlan Sera-Lab Ltd., Loughborough, United Kingdom). Monoclonal antibodies specific for EphA2 (clones D7 and B2D6) were produced in the laboratory as described (15) or purchased from Upstate Biotechnology Inc. 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, Chapel Hill, NC). To visualize f-actin, we used fluorescein-conjugated phalloidin, purchased from Molecular Probes (Eugene, OR).

Western Blot Analysis. Unless noted otherwise, all experiments used 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 min on ice, as described previously (21). After protein concentrations were measured by Coomassie Blue staining (Pierce, Rockford, IL) or Bio-Rad D_C Protein Assay (Hercules, CA), equal amounts of protein were resolved by SDS-PAGE and transferred to nitrocellulose (Protran, Schleicher & Schuell, Keene, NH), and Western blot analysis was performed as described previously (21). Antibody binding was detected by enhanced chemiluminescence as recommended by the manufacturer (Pierce). To reprobe, we stripped blots as described previously (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 (~70% confluence) and low cell density (~20% confluence) by seeding 1×10^6 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 antimouse antibodies (Chemicon, Temecula, CA) and FITC-conjugated donkey antirabbit (Chemicon) and epifluorescence microscopy (model BX60, ×600, Olympus 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 h at 4°C with the appropriate EphA2-specific monoclonal antibodies (D7 or B2D6) and rabbit antimouse (Chemicon) conjugated protein A-Sepharose (Sigma). Immunoprecipitates were washed three times in lysis buffer, resuspended in SDS sample buffer (Tris buffer containing 5% SDS, 3.8% DTT, 25% glycerol, and 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 10 mM PIPES, 3 mM $MnCl_2$, 5 mM PNPP (Sigma 104 phosphatase substrate; Sigma), 1 mM $NaVO_4$, 1 μM ATP, and 10 μCi of [γ-^{32}P]ATP (New England Nuclear, Boston, MA) at 25°C for the times shown. The reactions were terminated by the addition of 5× Laemmli sample buffer at multiple time points before saturation. After resolving samples by 10% SDS-PAGE, the gel was transferred to nitrocellulose (Schleicher & Schuell) or Immobilon P (Pierce), and incorporated material was detected by autoradiography. To hydrolyze phosphoserine/threonine, we treated the membranes with 1 N KOH at 65°C for 1 h and reassessed them 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 min with 4 μg/ml EphA2 antibody (either clone EK166B or B2D6) or purified fusion protein of ephrin-A1 fused to IgG (B61-IgG; Ref. 10). Primary antibody alone, rabbit antimouse IgG alone and control fusion proteins were used as controls. The samples were washed with medium, incubated with 20 μg/ml rabbit antimouse IgG in conditioned medium at 4°C

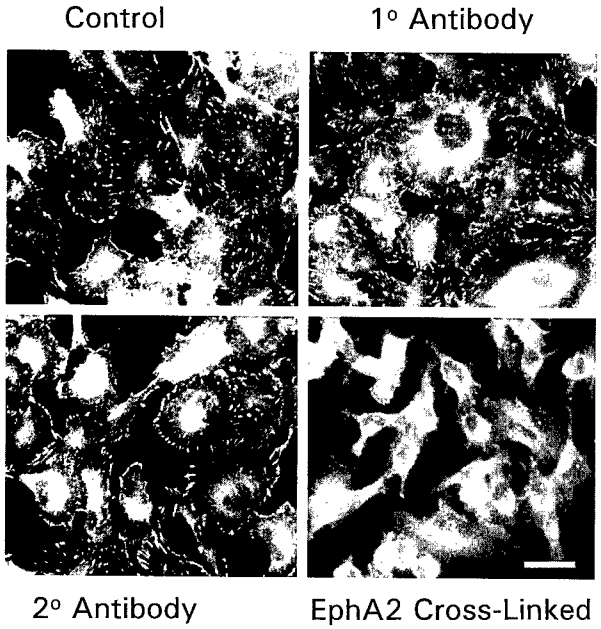

Fig. 10. EphA2 activation decreases cell-ECM adhesion. The presence of focal adhesions was assessed by immunostaining for paxillin in MDA-MB-231 cells before and after activation of EphA2 by antibody-mediated aggregation. Note that incubation of cells with either primary (*1°*) or secondary (*2°*) antibodies alone did not alter the presence of focal adhesions, whereas EphA2 aggregation dissipated focal adhesions. *Scale bar*, 25 μm.

Table 1 EphA2 Activation Inhibits Cell Proliferation[a]

Cell line	Treatment	% BrdUrd uptake (mean ± SE)	Statistical analysis[b] (*P*)
MDA-MB-231	Untreated	43.8 ± 2.0	
	Primary Ab[c] alone	44.1 ± 2.2	>0.43
	Secondary Ab alone	39.7 ± 2.3	>0.21
	Primary + secondary	30.4 ± 1.7	<0.0001
	Control-IgG + secondary	43.0 ± 2.1	>0.44
	B61-IgG + secondary	29.1 ± 3.1	<0.01[d]
MDA-MB-435	Untreated	52.8 ± 5.1	
	Primary Ab alone	52.6 ± 3.4	>0.25
	Secondary Ab alone	52.8 ± 6.3	>0.39
	Primary + secondary	39.6 ± 3.0	<0.00005
MCF-10A (low density)	Untreated	53.6 ± 1.8	
	Primary Ab alone	53.9 ± 0.8	>0.43
	Secondary Ab alone	55.1 ± 0.5	>0.22
	Primary + secondary	45.0 ± 1.4	<0.01

[a] BrdUrd uptake into newly synthesized DNA was measured for 4 h 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 are compared with DNA synthesis with untreated (untreated) samples. None of the differences between or among individual negative controls (untreated, primary antibody alone, or secondary antibody alone) were significant ($P > 0.05$).
[b] Statistical analyses compared the experimental to untreated for each sample.
[c] Ab, antibody.
[d] For the fusion proteins, there was also a significant difference ($P < 0.02$) between the control and B61 fusion proteins.

for 10 min, and warmed to 37°C for 10 min before extraction and immunoprecipitation. To determine the optimal time for activation, we incubated the plates in the presence of cross-linking antibody at 37°C for 0–120 min.

EGTA and Antibody Treatments. "Calcium switch" experiments were performed as described previously (28). Monolayers of MCF-10A cells were grown to ~80% confluence. EGTA was added to growth medium to a final concentration of 4 mM, and the cells were incubated at 37°C for 30 min. The medium was removed, and calcium concentrations restored with normal growth medium. To block E-cadherin function, we supplemented the medium with E-cadherin antibodies (1:100 dilution; DECMA-1; Sigma) or 10 μg/ml peptide corresponding to the E-cadherin HAV sequence (YTLFS*HAV*SSNGN). Controls include isotype control antibodies (rat anti-HA antibody; Boehringer Mannheim, Indianapolis, IN) 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 (59) and pSV2neo (60) using FuGENE 6 Transfection Reagent (Boehringer Mannheim), following the manufacturer's instructions. Transfected cells were selected in growth media supplemented with 400 μg/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 medium. 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 min, washed with medium, and incubated with 20 μg/ml rabbit antimouse plus 3 μg/ml BrdUrd at 37°C for 4 h. Cells were fixed in cold methanol for 8 min, extracted with 2 N HCl at 37°C for 30 min and stained with a BrdUrd 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 performed using the SAS System for Windows, Version 6.12. An ANOVA 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 *versus* 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 percentage cell growth for the control and experimental groups. $P < 0.05$ was considered statistically significant.

Acknowledgments

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