EphA2 Overexpression Decreases Estrogen Dependence and Tamoxifen Sensitivity

Ming Lu, Kathy D. Miller, Yesim Gokmen-Polar, Meei-Huey Jeng, and Michael S. Kinch²

Department of Basic Medical Sciences, Purdue University Cancer Center, West Lafayette, Indiana 47907 [M. L.]; Division of Hematology and Oncology, Indiana University, Indianapolis, Indiana 46202 [K. D. M., Y. G.-P., M.-H. J.]; and MedImmune, Inc., Gaithersburg, Maryland 20878 [M. S. K.]

ABSTRACT

The EphA2 receptor tyrosine kinase is found at low levels on nontransformed adult breast epithelial cells but is frequently overexpressed on aggressive breast cancer cells. Recent studies have documented an inverse relationship between EphA2 and estrogen receptor expression in breast cancer cell lines. In our present study, we demonstrate that overexpression of EphA2 decreases estrogen dependence as defined using both in vitro and in vivo criteria. The EphA2-transfected cells demonstrate increased growth in vitro and form larger and more aggressive tumors in vivo. EphA2 overexpression also decreases the ability of tamoxifen to inhibit breast cancer cell growth and tumorigenesis. These effects of EphA2 overexpression can be overcome by antibody-based targeting of EphA2. In particular, certain EphA2 antibodies can resensitize EphA2-overexpressing breast tumor cells to tamoxifen. These results have important implications for understanding the molecular basis underlying estrogen dependence and provide further evidence that EphA2 may provide a much-needed therapeutic target for breast cancer.

INTRODUCTION

Breast cancer remains a devastating disease that affects the lives of 180,000 women and results in over 40,000 deaths in the United States each year (1). Although an expanding arsenal of active agents is available for the treatment of metastatic disease, overall survival has changed little during the last half century (1, 2). Further advances will require new therapeutic strategies that are firmly rooted in a basic understanding of the factors that regulate breast cancer cell growth, metastasis, and estrogen dependence (2, 3).

EphA2 (ECK) is a receptor tyrosine kinase that is found at low levels on nontransformed adult breast epithelial cells (4). In nontransformed cells, EphA2 localizes to sites of cell-cell contact, where it can interact with its membrane-anchored ligands (ephrin-A proteins; Ref. 5). Much recent evidence indicates that the cellular consequences of ligand binding include negative regulation of cell growth, migration, and invasion (5–10).

EphA2 is frequently overexpressed in breast cancer (8, 11). In addition, malignant cells often have decreased cell-cell interactions, and EphA2 becomes diffusely distributed on the surface of tumor cells (5, 6, 12, 13). The resulting changes in ligand binding and subcellular localization impart dramatic changes in function, which cause EphA2 to favor (rather than inhibit) malignant cell growth, migration, and invasion (6, 8). Indeed, we recently demonstrated that ectopic overexpression of EphA2 promotes a tumorigenic and metastatic phenotype and that these changes relate to decreased ligand binding (8).

In the search for the causes of altered EphA2 expression and function in breast cancer, emerging evidence suggests an intimate interplay between EphA2 and ER. First, the levels of EphA2 expression in breast cancer cells relate inversely to ER expression (8, 12). Furthermore, E2 treatment of mammary epithelial cells is sufficient to negatively regulate EphA2 expression (12). Whereas these findings indicate that estrogen regulates EphA2 expression, less is known regarding whether EphA2 contributes to the increased growth or invasiveness of ER-deficient breast cancer cells.

In the present study, we demonstrate that ectopic overexpression of EphA2 increases the malignant character of ER+ breast cancer cells. The growth-promoting effects of EphA2 promote tumor cell growth in the absence of estrogen and decrease the inhibitory actions of tamoxifen. Finally, we show that antibody targeting of EphA2 reverses the effect of EphA2 overexpression and resensitizes these cells to tamoxifen. These findings have implications for understanding the progression toward an estrogen-independent phenotype and suggest potential opportunities for therapeutic intervention.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells were cultured in MEM (Cellgro; Mediatech, Inc., Herndon, VA) with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml fungizone, and 2 mM l-glutamine. MCF-7/EphA2 cells were cultured under the same conditions. Estrogen-deficient media were prepared with charcoal-stripped, phenol-red free MEM (Cellgro; Mediatech, Inc.).

Transfection and Selection. MCF-7 cells were cotransfected with pNeoMSV-EphA2 (generously provided by Dr. T. Hunter; Scripps Institute) and pBABE-Puro, at a 7:3 ratio, using LipofectAMINE Plus (Life Technologies, Inc., Grand Island, NY). As a vector control, a parallel transfection was performed using pNeoMSV and pBABE-Puro. Transfected cells were selected using 1 μg/ml puromycin (Sigma, St. Louis, MO), and EphA2 overexpression was confirmed by Western blot analyses (5). All experiments used bulk culture transfectants, and identical results were confirmed using at least two separate transfections. Parental cells and cultures transfected with pBABE-Puro provided additional controls.

Antibodies. EphA2 monoclonal antibodies (clone D7) were purchased from Upstate Biologicals, Inc. (Lake Placid, NY). Monoclonal antibodies for β-catenin were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies for ERα were purchased from Chemicon (Temecula, CA; catalogue number MAB463). Polyclonal antibodies for ERβ were purchased from Chemicon (catalogue number AB1410). Monoclonal antibody EA2 was generated and purified by the Kinch laboratory as detailed previously (9).

In Vitro E2 and Tamoxifen Treatment. Cells were treated with E2 (Sigma, stock concentration of 1 mg/ml in absolute ethanol) at 37°C. For tamoxifen treatment experiments, cells were treated with 4-hydroxy-tamoxifen in <0.1% chloroform (Sigma) at 37°C.

Western Blot Analysis. All Western blot analyses were performed as described previously (5). All samples were normalized for total protein (10 μg/sample), and blots were stripped and reprobed with β-catenin antibodies to confirm equal loading.

Cell Proliferation Assays. MCF-7neo or MCF7/EphA2 cells were seeded in 96-well plates. Cell growth was measured with Alamar blue (Biosource International, Camarillo, CA) following the manufacturer’s suggestions. Colony formation in soft agar was performed as described previously (8) and scored microscopically, defining clusters of at least three cells as a positive. The data

Received 12/10/02; accepted 4/16/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

3 Supported in part by National Cancer Institute Grant CA91318 (to M. S. K.), Department of Defense Grant DAMD17-01-1-0380 (to M. S. K.), American Cancer Society Grant CRTG-00-199-01-CCE (to K. D. M.), and a Career Development Award from the American Society of Clinical Oncology (to K. D. M.).

4 To whom requests for reprints should be addressed, at MedImmune, Inc., 35 West Watkins Mill Road, Gaithersburg, MD 20878. E-mail: kinchm@medimmune.com.

The abbreviations used are: ER, estrogen receptor; HPF, high-power field; E2, 17β-estradiol; CAT, chloramphenicol acetyltransferase; ERE, estrogen response element; TK, tyrosine kinase.
as a control. Fresh media including the appropriate drugs were added after 24 h of transfection. Cells were harvested after 24 h, and CAT activity was evaluated as described previously (14).

Statistical Analyses. All statistical analyses were performed using Student’s t test and Microsoft Excel (Seattle, WA), and P ≤ 0.05 was defined as significant. In vivo tumor growth analyses were performed using GraphPad Software (San Diego, CA).

RESULTS

Ectopic Overexpression of EphA2 Confers a More Aggressive Phenotype. To evaluate the consequences of EphA2 overexpression in estrogen-sensitive breast cancer cells, human EphA2 cDNAs were stably overexpressed in MCF-7 cells. MCF-7 cells were selected because they require estrogenic activity for optimal growth and survival (15). Western blot analyses confirmed the ectopic overexpression of EphA2 in transfected cells relative to matched controls (Fig. 1). Subsequent stripping and reprobing of the membranes with β-catenin antibodies verified equal sample loading.

EphA2 overexpression increased malignant growth. Whereas matched MCF-7 control cells were largely unable to colonize soft agar (an average of 0.1 colony/field), EphA2-transfected cells formed larger and more numerous colonies (4.7 colonies/field; P < 0.01) that persisted for at least 3 weeks (Fig. 2A; data not shown). Despite increased colonization of soft agar, the growth of EphA2-overexpressing cells in monolayer culture did not differ from that of matched controls (Fig. 2B), thus indicating that the growth-promoting activities of EphA2 were most apparent using experimental conditions that model anchorage-independent (malignant) cell growth.

Consistent with increased soft agar colonization, orthotopically implanted EphA2-overexpressing cells formed larger, more rapidly growing tumors in vivo. In the presence of supplemental estrogen, the EphA2-overexpressing cells demonstrated a 2-fold increase in tumor volume relative to matched controls (Fig. 3A). EphA2-overexpressing tumors differed phenotypically from control tumors in that they were

![Graphs](image_url)
EphA2 Overexpression Decreases Estrogen Dependence. To further investigate the consequences of EphA2 overexpression, we measured the sensitivity of EphA2-transfected cells to tamoxifen. Tamoxifen reduced soft agar colonization of control MCF-7 cells by at least 60%. The inhibitory actions of tamoxifen on MCF-7EphA2 cells were less pronounced (25% inhibition; Fig. 5A). Notably, excess E2 overcame the inhibitory effects of tamoxifen, which provided additional evidence for the specificity of this finding (Fig. 5A). Similarly, the tumorigenic potential of MCF-7EphA2 cells was less sensitive to tamoxifen as compared with control (MCF-7neo) cells (Fig. 5B).

Because tamoxifen sensitivity often relates to ER expression (16–19), we asked whether EphA2 overexpression altered ER expression or activity. Western blot analyses revealed comparable levels of ERα and ERβ in control and EphA2-overexpressing cells (Fig. 6A). Furthermore, comparable levels of ER activity were detected in control and EphA2-transformed cells, and this enzymatic activity remained sensitive to tamoxifen (Fig. 6, B and C). These results indicate that the ER in MCF-7EphA2 cells is expressed and remains sensitive to tamoxifen, thus suggesting that the defect that renders MCF-7EphA2 less dependent on estrogen lies downstream of ER signaling.

Targeted Intervention against EphA2 Decreases Growth and Restores Tamoxifen Sensitivity. To determine whether targeted intervention against EphA2 could reverse the phenotype of MCF-7EphA2, we used a panel of monoclonal antibodies (e.g., EA2) that induce EphA2 degradation (9). We first confirmed that antibody

more vascular and locally invasive at the time of resection (data not shown). To confirm that these tumors expressed EphA2, whole cell lysates of resected tumors were subjected to Western blot analyses with EphA2-specific antibodies (Fig. 3B). The membranes were then stripped and reprobed with β-catenin antibodies to verify equal sample loading. The relative amount of EphA2 was higher in tumor samples than in the input cells (before implantation), suggesting that tumors arose from cells with high levels of EphA2. Comparable findings with in vitro and in vivo models indicate that EphA2 overexpression results in a more aggressive phenotype.

EphA2 Overexpression Decreases Estrogen Dependence. To determine whether EphA2 overexpression could alter the estrogen sensitivity of MCF-7 cells, parallel studies were performed in the absence of exogenous estrogen. Experimental deprivation of estrogen amplified differences between the cellular behaviors of control and EphA2-overexpressing cells. Whereas EphA2-transfected MCF-7 cells continued to colonize soft agar more efficiently than matched controls (Fig. 4A), these cells did grow in the absence of exogenous estrogen (Fig. 4B). In contrast, supplemental estrogen was required for monolayer growth of control cells (Fig. 4B).

The EphA2-overexpressing cells retained tumorigenic potential in the absence of supplemental estrogen. Whereas control MCF-7 cells rarely formed palpable tumors, the EphA2-transfected cells formed tumors that persisted for more than 12 weeks (Fig. 4C; data not shown). Thus, both in vitro and in vivo assay systems confirm that EphA2 overexpression decreases the need for exogenous estrogen.

EphA2 Overexpression Decreases Tamoxifen Sensitivity. To further investigate the consequences of EphA2 overexpression, we measured the sensitivity of EphA2-transfected cells to tamoxifen. Tamoxifen reduced soft agar colonization of control MCF-7 cells by at least 60%. The inhibitory actions of tamoxifen on MCF-7EphA2 cells were less pronounced (25% inhibition; Fig. 5A). Notably, excess E2 overcame the inhibitory effects of tamoxifen, which provided additional evidence for the specificity of this finding (Fig. 5A). Similarly, the tumorigenic potential of MCF-7EphA2 cells was less sensitive to tamoxifen as compared with control (MCF-7neo) cells (Fig. 5B).

Because tamoxifen sensitivity often relates to ER expression (16–19), we asked whether EphA2 overexpression altered ER expression or activity. Western blot analyses revealed comparable levels of ERα and ERβ in control and EphA2-overexpressing cells (Fig. 6A). Moreover, comparable levels of ER activity were detected in control and EphA2-transformed cells, and this enzymatic activity remained sensitive to tamoxifen (Fig. 6, B and C). These results indicate that the ER in MCF-7EphA2 cells is expressed and remains sensitive to tamoxifen, thus suggesting that the defect that renders MCF-7EphA2 less dependent on estrogen lies downstream of ER signaling.

Targeted Intervention against EphA2 Decreases Growth and Restores Tamoxifen Sensitivity. To determine whether targeted intervention against EphA2 could reverse the phenotype of MCF-7EphA2, we used a panel of monoclonal antibodies (e.g., EA2) that induce EphA2 degradation (9). We first confirmed that antibody

more vascular and locally invasive at the time of resection (data not shown). To confirm that these tumors expressed EphA2, whole cell lysates of resected tumors were subjected to Western blot analyses with EphA2-specific antibodies (Fig. 3B). The membranes were then stripped and reprobed with β-catenin antibodies to verify equal sample loading. The relative amount of EphA2 was higher in tumor samples than in the input cells (before implantation), suggesting that tumors arose from cells with high levels of EphA2. Comparable findings with in vitro and in vivo models indicate that EphA2 overexpression results in a more aggressive phenotype.

EphA2 Overexpression Decreases Estrogen Dependence. To determine whether EphA2 overexpression could alter the estrogen sensitivity of MCF-7 cells, parallel studies were performed in the absence of exogenous estrogen. Experimental deprivation of estrogen amplified differences between the cellular behaviors of control and EphA2-overexpressing cells. Whereas EphA2-transfected MCF-7 cells continued to colonize soft agar more efficiently than matched controls (Fig. 4A), these cells did grow in the absence of exogenous estrogen (Fig. 4B). In contrast, supplemental estrogen was required for monolayer growth of control cells (Fig. 4B).

The EphA2-overexpressing cells retained tumorigenic potential in the absence of supplemental estrogen. Whereas control MCF-7 cells rarely formed palpable tumors, the EphA2-transfected cells formed tumors that persisted for more than 12 weeks (Fig. 4C; data not shown). Thus, both in vitro and in vivo assay systems confirm that EphA2 overexpression decreases the need for exogenous estrogen.

EphA2 Overexpression Decreases Tamoxifen Sensitivity. To further investigate the consequences of EphA2 overexpression, we measured the sensitivity of EphA2-transfected cells to tamoxifen. Tamoxifen reduced soft agar colonization of control MCF-7 cells by at least 60%. The inhibitory actions of tamoxifen on MCF-7EphA2 cells were less pronounced (25% inhibition; Fig. 5A). Notably, excess E2 overcame the inhibitory effects of tamoxifen, which provided additional evidence for the specificity of this finding (Fig. 5A). Similarly, the tumorigenic potential of MCF-7EphA2 cells was less sensitive to tamoxifen as compared with control (MCF-7neo) cells (Fig. 5B).

Because tamoxifen sensitivity often relates to ER expression (16–19), we asked whether EphA2 overexpression altered ER expression or activity. Western blot analyses revealed comparable levels of ERα and ERβ in control and EphA2-overexpressing cells (Fig. 6A). Moreover, comparable levels of ER activity were detected in control and EphA2-transformed cells, and this enzymatic activity remained sensitive to tamoxifen (Fig. 6, B and C). These results indicate that the ER in MCF-7EphA2 cells is expressed and remains sensitive to tamoxifen, thus suggesting that the defect that renders MCF-7EphA2 less dependent on estrogen lies downstream of ER signaling.

Targeted Intervention against EphA2 Decreases Growth and Restores Tamoxifen Sensitivity. To determine whether targeted intervention against EphA2 could reverse the phenotype of MCF-7EphA2, we used a panel of monoclonal antibodies (e.g., EA2) that induce EphA2 degradation (9). We first confirmed that antibody

more vascular and locally invasive at the time of resection (data not shown). To confirm that these tumors expressed EphA2, whole cell lysates of resected tumors were subjected to Western blot analyses with EphA2-specific antibodies (Fig. 3B). The membranes were then stripped and reprobed with β-catenin antibodies to verify equal sample loading. The relative amount of EphA2 was higher in tumor samples than in the input cells (before implantation), suggesting that tumors arose from cells with high levels of EphA2. Comparable findings with in vitro and in vivo models indicate that EphA2 overexpression results in a more aggressive phenotype.

EphA2 Overexpression Decreases Estrogen Dependence. To determine whether EphA2 overexpression could alter the estrogen sensitivity of MCF-7 cells, parallel studies were performed in the absence of exogenous estrogen. Experimental deprivation of estrogen amplified differences between the cellular behaviors of control and EphA2-overexpressing cells. Whereas EphA2-transfected MCF-7 cells continued to colonize soft agar more efficiently than matched controls (Fig. 4A), these cells did grow in the absence of exogenous estrogen (Fig. 4B). In contrast, supplemental estrogen was required for monolayer growth of control cells (Fig. 4B).

The EphA2-overexpressing cells retained tumorigenic potential in the absence of supplemental estrogen. Whereas control MCF-7 cells rarely formed palpable tumors, the EphA2-transfected cells formed tumors that persisted for more than 12 weeks (Fig. 4C; data not shown). Thus, both in vitro and in vivo assay systems confirm that EphA2 overexpression decreases the need for exogenous estrogen.
treatment of MCF-7EphA2 cells induced EphA2 activation and degradation. Decreased levels of EphA2 degradation were observed within 2 h, and EphA2 remained undetectable for at least the following 24 h (Fig. 7A).

We then asked whether these antibodies would alter the growth of control or EphA2-transfected MCF-7 cells. The soft agar colonization of control MCF-7 cells was sensitive to tamoxifen (Fig. 7B), and EphA2 antibodies did not further alter this response (because these cells lack of endogenous EphA2; see Fig. 1). The EphA2-transfected cells were less sensitive to tamoxifen (25% inhibition by tamoxifen) as compared with the matched controls (75% inhibition by tamoxifen). Whereas EphA2 antibodies decreased soft agar colonization (by 19%), the combination of EA2 and tamoxifen caused a much more dramatic (>80%) decrease in soft agar colonization. Thus, EA2 treatment restored a phenotype that was comparable with control MCF-7 cells. These findings suggest that antibody targeting of EphA2 can serve to sensitize the breast tumor cells to tamoxifen.

**DISCUSSION**

The major finding of our present study is that EphA2 overexpression decreases the estrogen dependence of ER+ breast cancer cells. Consistent results with in vitro and in vivo systems indicate that EphA2 overexpression decreases the need for exogenous estrogen and renders breast cancer cells relatively resistant to tamoxifen. We also demonstrate that decreased estrogen sensitivity does not reflect loss of ER. Finally, we show that antibody targeting of EphA2 can overcome the consequences of EphA2 overexpression and increase the sensitivity of EphA2-overexpressing cells to tamoxifen.

Our present studies are unique in part because they demonstrate that EphA2 overexpression can decrease the need for exogenous estrogen. This finding is notable in light of recent evidence that the EphA2 expression is itself negatively regulated by estrogen (12). For example, the highest levels of EphA2 are generally found on ER-deficient tumor cells (8). Our present studies extend this observation by indicating that EphA2 overexpression can contribute to the more aggressive behavior that is generally understood to accompany loss of ER.

We propose the following model to summarize the interplay between EphA2 and ER. In estrogen-dependent tumors, estrogen maintains the low levels of EphA2 that have been reported using ER+ breast cancer cell models [e.g., MCF-7 and ZR-75-1 (12)]. As breast cancer progresses, ER expression or function is frequently decreased, which renders these cells less sensitive to estrogen and tamoxifen (16–19). The resulting loss of EphA2 transcriptional repression would be expected to upregulate EphA2 expression. Our present results are notable in that they suggest that increased EphA2 expression would in turn contribute to the increased growth and invasiveness of ER-deficient cells.

The mechanism by which EphA2 overexpression decreases the estrogen dependence of mammary epithelial cells remains largely unknown. Estrogen binding to its cognate receptors activates multiple pathways of intracellular and intercellular communication (19–21). For example, ER signaling regulates the expression of c-Myc, NHE-RF, MIP-1, PCDGF, and quinone reductase (19, 21). The evidence linking ER to MIP-1 is intriguing because MCF-7EphA2 cells overexpress this chemokine. Future studies could ask whether EphA2-mediated induction of these molecules contributes to more aggressive phenotype of EphA2-overexpressing cells.

Another outcome of our present study is that the consequences of EphA2 overexpression are most apparent when cells are grown under suboptimal conditions. For example, the growth-promoting actions of EphA2 are not apparent under standard monolayer conditions but are revealed in response to “stressors” such as estrogen deprivation or loss.

4 K. D. Miller, unpublished observations.
of cell-matrix anchorage. These findings are consistent with evidence that EphA2 does not appear to alter monolayer cell growth but has powerful effects on soft agar colonization and tumorigenicity (8, 9).

Our present studies may also have implications for the design and application of new therapeutic strategies. The use of antibodies targeting EphA2 is appealing because it directs the degradation of a molecule that is frequently overexpressed in breast cancer (9). We furthermore expect that EphA2 antibodies will have limited effect on normal cells because of low EphA2 expression and because the EphA2 in nontransformed epithelial cells is already engaged to its endogenous ligands and thus would be unavailable to therapeutic antibodies (5, 9). Finally, the overexpression of EphA2 on malignant cells, relative to normal epithelia, would be expected to amplify the negative regulatory signals that have been described previously (5–10). Taken together, these features suggest that EphA2 antibody therapy could selectively target malignant cells with minimal toxicity to normal tissues.

ACKNOWLEDGMENTS

We thank Jane Stewart, Min Hu, and Robert Foreman for expert technical assistance.

REFERENCES

EphA2 Overexpression Decreases Estrogen Dependency and Tamoxifen Sensitivity

Ming Lu, Kathy D. Miller, Yesim Gokmen-Polar, et al.


Updated version

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/63/12/3425

Cited articles

This article cites 21 articles, 10 of which you can access for free at:

http://cancerres.aacrjournals.org/content/63/12/3425.full.html#ref-list-1

Citing articles

This article has been cited by 7 HighWire-hosted articles. Access the articles at:

/content/63/12/3425.full.html#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.