Tumorigenesis and Neoplastic Progression

Receptor Tyrosine Kinase EphB4 Is a Survival Factor in Breast Cancer

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EphB4, a member of the largest family of receptor tyrosine kinases, is normally expressed on endothelial and neuronal cells. Although aberrant expression of EphB4 has been reported in several human tumors, including breast cancer, its functional significance is not understood. We report here that EphB4 is expressed in 7 of 12 (58%) human breast cancer specimens and 4 of 4 (100%) breast tumor cell lines examined. Overexpression of EphB4 in breast cancer cells was driven by gene amplification and by the erbB family of receptors via activation of Janus tyrosine kinase-signal transducers and activators of transcription and protein kinase B. The aberrantly expressed receptor was phosphorylated by its natural ligand, EphrinB2, and signaled via the protein kinase B pathway. Targeted knockdown of EphB4 expression by small interference RNA (and antisense oligodeoxynucleotides (ODNs)) led to dose-dependent reduction in cell survival, increased apoptosis, and sensitization to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Antisense ODN-mediated EphB4 knockdown resulted in reduced tumor growth in a murine tumor xenograft model. Antisense ODN-treated tumors were 72% smaller than control tumors at 6 weeks, with an 86% reduction in proliferating cells, 15-fold increase in apoptosis, and 44% reduction in tumor microvasculature. Our data indicate that biologically active EphB4 functions as a survival factor in breast cancer and is a novel target for therapy. (*Am J Pathol* 2006, 169:279–293; DOI: 10.2353/ajpath.2006.050889)

cases per 100,000 population (U.S. Cancer Statistics Working Group. United States Cancer Statistics: 1999–2001 Incidence and Mortality Web-based Report Version. Atlanta (GA): Department of Health and Human Services, Centers for Disease Control and Prevention, and National Cancer Institute; 2004. Available at: <http://www.cdc.gov/cancer/npcr/uscs>. Accessed March 31, 2005). A significant body of evidence links the erbB family of receptor tyrosine kinases to breast cancer and poor outcome.¹ The most extensively studied of these receptors, erbB2 or HER-2/neu, is overexpressed in 30% of breast cancers and results in a highly malignant phenotype.² HER-2/neu expression correlates with poor tumor grade, estrogen receptor negativity, and the presence of distant metastasis.^{3,4} ErbB1 or epidermal growth factor receptor (EGFR) expression is seen in 36% of breast cancers,⁵ is also associated with poor histological grade, negative estrogen receptor status, larger tumor size, and the development of distant metastases,⁴ and it predicts early recurrence of and death from breast cancer.⁶

EphB4 is also a protein tyrosine kinase, a member of the largest family of receptor tyrosine kinases, and plays an important role in angiogenesis and vascular network assembly.^{7–11} EphB4 is normally expressed on venous endothelial cells, while its ligand, EphrinB2, is expressed on arterial endothelial cells as a transmembrane protein as well.¹² Interaction between receptor and ligand leads to bidirectional signaling.¹³ EphB4 activation leads to a number of downstream effects that influence cell attachment, migration, and interaction with ligand-expressing cells.^{9,12,14–19} Reverse signaling via EphrinB2 stimulates migration and sprouting angiogenesis.²⁰ Growing evidence points to the expression of several Eph receptors in different cancer types.^{21–26} The biological significance of this aberrant expression, however, is controversial.

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Breast cancer is the most common malignancy in women in the United States, with an estimated incidence of 135

Analysis of human breast cancer specimens reveals that EphB4 expression correlates with histological grade and stage and is associated with DNA aneuploidy.²⁷ Transgenic expression of EphB4 in mouse mammary epithelium does not initiate transformation. However, in the context of neuT, EphB4 accelerates the development of breast cancers, and promotes metastasis.²⁸ A recent report by Noren et al²⁹ suggests that EphB4 expressed on breast cancer cells engages endothelial cell EphrinB2 to promote tumor vascularization, thereby enhancing tumor growth. Paradoxically, these authors also reported that stimulation of tumor cell EphB4 (with EphrinB2) results in a loss of tumor cell viability, suggesting that the proangiogenic effects of tumor cell EphB4 can mask its direct antisurvival effects.

We sought to characterize the biological significance of EphB4 in breast cancer. We show here that EphB4 is expressed in a majority of human breast cancer specimens and breast cancer cell lines that we analyzed. EphB4 expression is regulated by signaling via members of the erbB family of receptors and by amplification of the *EPHB4* gene locus. Knocking down EphB4 expression in breast tumor cell lines with small interference RNA (siRNA) (and antisense oligonucleotides) results in loss of cell viability, activation of caspase-8, and induction of apoptosis by sensitizing cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Finally, EphB4 knockdown with specific antisense oligonucleotides in a murine breast cancer xenograft model results in significantly smaller tumors, induction of apoptosis, and reduction in tumor vascularity. EphB4 is thus a critical survival factor in breast cancer cells and is a novel therapeutic target in breast cancer.

Materials and Methods

Reagents

Media and fetal bovine serum were from Invitrogen (Carlsbad, CA). Antibodies against EGFR, EphrinB2 (P20), and CD31 (M20) were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-mouse EphB4 was from R&D Systems, Inc. (Minneapolis, MN), anti-protein kinase B (Akt) and anti-p-Akt were from Cell Signaling Technology (Beverly, MA), and anti-human Fc was from Jackson Laboratories (Bar Harbor, ME). Monoclonal antibody against phosphotyrosine (clone 4G10) was from Upstate (Lake Placid, NY), anti-Ki-67 was from DAKO (Carpenteria, CA), anti-Bcl-2 and anti-Bcl-x_L were from Calbiochem (San Diego, CA), anti-Mcl-1 was from BD Pharmingen (San Diego, CA), anti-FLIP was from Alexis Corp. (San Diego, CA), and anti-actin was from Sigma-Aldrich (St Louis, MO). Monoclonal EphB4 antibodies used in immunoprecipitation, immunofluorescence, immunohistochemistry, and immunoblotting and monoclonal EphrinB2 antibody used in immunohistochemistry were kindly provided by VasGene Therapeutics, Inc. (Los Angeles, CA). AG1478, AG490, PP2, SB203580, and SH5 were from Calbiochem, wortmannin and PD 98059 were from Sigma-Aldrich, EGF and EphrinB2/Fc chimeric protein were from R&D Systems, Inc., and human IgG Fc fragment was from Jackson Laboratories. Soluble human TRAIL was ob-

tained from Santa Cruz Biotechnology. The MACSelect 4.1 transfected cell selection kit was from Miltenyi Biotec (Auburn, CA).

Cell Lines and Culture

The cell lines MCF-7, ZR75, T47D, SKBR3, and A549 were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Roswell Park Memorial Institute (RPMI)-1640 media containing 10% fetal bovine serum, 5 mmol/L L-glutamine, and penicillin/streptomycin. Primary normal breast epithelial cells harvested from normal human breast tissue obtained at the time of breast reduction surgery were cultured in the above medium. Human umbilical vein endothelial cells were obtained from Clonetics (San Diego, CA) and cultured as described before.³⁰ Parent mouse fibroblast cells (3T3) stably transfected with either the empty vector or HER-2/neu were maintained in 10% Dulbecco's modified Eagle's medium supplemented as above.

Immunohistochemistry and Immunofluorescence

Human normal breast and breast cancer specimens were obtained under Institutional Review Board-approved protocols. Sections (5 μ m) of fresh frozen human breast tumor tissues were fixed in 4% paraformaldehyde and washed in phosphate-buffered saline. Sections (5 μ m) of formalin-fixed, paraffin-embedded tissues were deparaffinized and hydrated. Antigen epitope retrieval was performed by heating slides in 10 mmol/L sodium citrate buffer (pH 8.5) at 80°C for 20 minutes. Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ in phosphate-buffered saline for 10 minutes, followed by blocking of nonspecific sites with SuperBlock blocking buffer (Pierce, Rockford, IL) for 1 hour both at room temperature. Sections were incubated with primary antibody overnight at 4°C and, after three washes in phosphate-buffered saline, with appropriate secondary antibody for 1 hour at room temperature. Antibody binding was localized with ABC staining kit from Vector Laboratories (Burlingame, CA) according to the manufacturer's instructions, and peroxidase activity was detected using 3,3'-diamino benzidine substrate solution (Vector). Sections were counterstained with Harris hematoxylin for 45 s, dehydrated, and mounted in xylene. For immunofluorescence, secondary antibody incubation was followed by incubation for 30 minutes at room temperature with 2 μ g/ml fluorescein isothiocyanate-labeled avidin (Vector). After three washes in phosphate-buffered saline, sections were counterstained with 4',6-diamidino-2-phenylindole and mounted in immunofluorescence medium (Vector). Routine negative controls included deletion of primary and secondary antibody, premixing primary antibody with blocking peptide and substitution of normal IgG isotope for primary antibody. When using mouse anti-human Ki-67 antibody, an MOM kit (Vector) was used to block nonspecific binding to mouse tissue. For *in vivo* tumor specimens, the number of cells staining positively was counted by a blinded observer in five random high power fields.

Immunoblotting

Cell lysates were prepared using Cell Lysis Buffer (GeneHunter, Basgavukke, TN) supplemented with protease inhibitor cocktail (Pierce). Total protein was determined using the DC reagent system (Bio-Rad, Hercules, CA). Typically, 20 μ g of whole cell lysate was run on 4 to 20% Tris-glycine gradient gel (Invitrogen). The samples were electrotransferred to polyvinylidene difluoride membrane (Bio-Rad), and nonspecific binding was blocked in TBST (Tris-buffered saline with 0.1% Tween-20) buffer containing 5% non-fat milk (Bio-Rad). Membranes were first probed with primary antibody for 2 hours, washed, and probed with the secondary antibody for 1 hour, and developed. Membranes were then stripped with Restore Western Blot stripping buffer (Pierce) and reprobed with β -actin to confirm equivalent loading and transfer of protein. Signal was detected using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Bands were scanned and signal intensity was normalized to β -actin using ImageJ software (version 1.32j; National Institutes of Health, Bethesda, MD).

Quantitative PCR

Gene amplification was analyzed by quantitative polymerase chain reaction (PCR). DNA was extracted from peripheral blood monocytes of a normal donor and the breast cancer cell lines using the Blood and Cell Culture DNA Midi kit from Qiagen (Valencia, CA) according to the manufacturer's instructions. Quantitative PCR was performed on 50 ng of DNA on the MX3000P real-time PCR system (Stratagene, La Jolla, CA) using SYBR Green I Brilliant Mastermix according to the manufacturer's instructions with thermal profile of 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute, and then 72°C for 1 minute. The primers were EphB4-forward, 5'-TCC TGC AAG GAG ACC TTC AC-3'; EphB4-reverse, 5'-CAG AGG CCT CGC AAC TAC AT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-forward, 5'-GAG GGG TGA TGT GGG GAG TA-3'; GAPDH-reverse, 5'-GAG CTT CCC GTT CAG CTC AG-3'; β -actin-forward, 5'-GTC TTC CCC TCC ATC GTG-3'; and β -actin-reverse, 5'-ACA CGC AGC TCA TTG TAG-3'. The amplification signal for EphB4 was normalized to GAPDH, and the gene copy number was normalized to healthy donor peripheral blood monocytes. Amplification of β -actin was also examined as an additional control. For assessing urokinase-type plasminogen activator (uPA) mRNA levels, total mRNA was extracted from cultured cells using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). First strand cDNA was synthesized from 5 μ g of total RNA using SuperScript III (Invitrogen). Quantitative PCR was then performed as above. Optimized reactions for uPA were 150 nmol/L each of the forward primer, 5'-ACT-GGC-TTG-AAG-ATC-ACC-AG-3', and reverse primer, 5'-CCC-TCT-CAC-AGC-TCA-TGT-CT-3', with a thermal profile of 95°C for 10 minutes followed by 40 cycles of 95°C for 30 seconds, 57°C for 1 minute, and then 72°C for 1 minute. The specificity of the amplification was confirmed by the presence of a single dissociation peak. All reactions were performed in triplicate and with no reverse transcriptase and no template as negative controls.

Receptor Stimulation Analyses

EphrinB2/Fc or Fc fragment alone was clustered by incubating with 1:1000 dilution anti-human Fc antibody for 1 hour at 4°C. MCF-7 cells grown to 80% confluence were serum-starved overnight and treated for a specified time with 1 μ g/ml clustered EphrinB2/Fc or Fc alone. Protein lysates were prepared, and 100 μ g of each lysate was used for immunoprecipitation of EphB4 using 2 μ g/ml anti-EphB4 monoclonal antibody overnight at 4°C. Antigen-antibody complexes were absorbed on 10 μ l of protein A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours at 4°C. Pellets were immunoblotted with antiphosphotyrosine antibody to detect phosphorylation status. Efficiency of immunoprecipitation was examined on duplicate membranes with EphB4-specific monoclonal antibody.

Cell Viability Assay

MCF-7 cells were seeded in 48-well plates at a density of 1×10^4 cells/well in a total volume of 500 μ l. Medium was changed after cells were attached, and triplicate samples were treated as described in Results. Cell viability was assessed by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.³¹ For studying sensitivity of cells to TRAIL-induced apoptosis, appropriate concentration of ligand was added to the supernatant 16 hours before MTT assay.

EphB4 siRNAs and Antisense Oligodeoxynucleotides

Various EphB4-specific anti-sense phosphorothioate-modified oligodeoxynucleotides (ODNs) and siRNA were synthesized from Qiagen (Valencia, CA). The most active antisense ODN and siRNA that knock down EphB4 expression in the transiently transfected 293T cell line were chosen (data not shown). The antisense ODN used, AS-10, spanned nucleotides 1980 to 1999 with a sequence 5'-ATG GAG GCC TCG CTC AGA AA-3'. To eliminate cytokine responses, the cytosine at the CpG site was methylated (AS-10M) without any loss in EphB4 knock-down efficiency (data not shown). Scrambled ODNs containing random nucleotide sequence and a similar CpG site, 5'-TAC CTG AAG GTC AGG CGA AC-3', was used as control. siRNA 465 corresponding to the sequences 5'-GGU GAA UGU CAA GAG GCU GUU-3' and 3'-UUC CAC UUA CAG UUC UGC GAC-5' was used for RNA interference. Control siRNA was generated by mutating three bases in this sequence to effectively abrogate EphB4 knockdown. This mutated siRNA (siRNA Δ) had the sequences 5'-AGU UAA UAU CAA GAC GCU GUU-3' and 3'-UUU CAA UUA UAG UUC UGC GAC-5'. Additionally, siRNA directed against green fluorescent protein with sequences 5'-CGC UGA CCC UGA AGU UCA TUU-3' and 3'-UUG CGA CUG GGA CUU CAA GUA-5' was also used as a negative control.

Transient Transfection and Cell Sorting

Cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, transfection complexes containing 5 μ l of Lipofectamine per 250 μ l of reduced serum Opti-MEM medium and appropriate concentration siRNA diluted in same medium were added to antibiotic-free culture medium. Cells were cultured in this medium for 6 hours, after which they were returned to the original culture medium. All experiments were performed 48 hours after transfection.

For sorting transfected cells, the MACSelect 4.1 transfected cell selection kit was used as per the manufacturer's instructions. In brief, cells were cotransfected with expression vector containing the plasmid of interest and pMACS 4.1 plasmid. After 36 hours, cells were harvested with 5 mmol/L ethylenediamine tetraacetic acid and incubated with MACSelect 4 Microbeads for 15 minutes at 4°C. The cell suspension was then passed via an MS+ column in a magnetic field. After three washes, the column was removed from the field, and selected cells were eluted in culture medium. Selection efficiency was confirmed by fluorescence-activate cell sorting analysis of sorted cells with fluorescent EphB4 monoclonal antibody (data not shown).

Invasion and Migration Assays

Chemotaxis was assessed using a modified Boyden chamber assay. For invasion studies, MCF-7 cells were appropriately treated, and 0.5×10^5 cells were transferred into 8 μ l Matrigel-precoated inserts (BD Bioscience, Palo Alto, CA). The inserts were placed in companion wells containing 700 μ l of RPMI supplemented with 5% fetal bovine serum and 5 μ g/ml fibronectin as a chemoattractant. Following 12 hours of incubation, the inserts were removed, and noninvading cells on the upper surface were scraped with a cotton swab. The cells on the lower surface of the membrane were fixed in 100% methanol for 15 minutes, air dried, and stained with Giemsa stain for 2 minutes. The cells were counted in five individual high power fields, and the percentage of reduction was calculated with respect to mutated siRNA or scrambled ODN. Assays were performed in triplicate for each treatment group. For migration studies, the same experiment was performed for 8 hours, except that the inserts were not coated with Matrigel.

Gelatin Zymography

MCF-7 cells were cultured until 80% confluent. Cells were transfected with EphB4 siRNA or mutated siRNA (50 nmol/L) and cultured for 24 hours. Conditioned medium from equal cell numbers was collected, concentrated, and electrophoresed through a 10% Zymogram gel (Criterion Zymogram; Bio-Rad). The gel was washed three times over 1 hour with 2.5% Triton X-100 to remove the SDS and incubated for 24 hours at 37°C in collagenase buffer containing 50 mmol/L Tris, 200 mmol/L NaCl, and 10 mmol/L CaCl_2 , pH 7.5. Gelatinolytic activity was visualized by staining the gel in 0.5% Coomassie Blue.

Apoptosis Assay

Apoptosis was studied *in vitro* using the Cell Death Detection ELISA^{PLUS} Kit (Roche Applied Science, Piscataway, NJ) according to the manufacturer's instructions. Briefly, MCF-7 cells were cultured in 24-well plates to 80% confluence and treated with test compounds at various concentrations. Cells were harvested at 36 hours, and 1×10^4 cells from each well were incubated in 200 μ l of lysis buffer. Nuclei were pelleted by centrifugation, and 20 μ l of supernatant containing the mono- or oligonucleosomes was incubated with anti-histone-biotin and anti-DNA-peroxidase in streptavidin-coated 96-well plate for 2 hours at room temperature. Color was developed with peroxide substrate, and absorbance at 405 nm was read in a microplate reader (Molecular Devices, Sunnyvale, CA). Apoptosis was detected in deparaffinized sections of animal tumors by terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL) assay using an *in situ* cell death detection kit (Roche Applied Science) according to manufacturer's instructions.

Caspase Activity Assay

Caspase activity was measured by colorimetric assay (R&D Systems, Inc.) according to the manufacturer's instructions. Briefly, MCF-7 cells appropriately treated were lysed in lysis buffer and clarified by centrifugation. The clarified lysate was incubated with reaction buffer and the appropriate colorimetric substrate at 37°C for 2 hours. Color development was quantified by measuring absorbance at 405 nm. Routine controls included deletion of cell lysate and substrate.

Murine Breast Tumor Xenograft Model

MCF-7 cells were propagated, collected by trypsin digestion, and resuspended in serum-free medium. 5×10^6 cells were injected in the area of the right breast tissue in 10- to 12-week-old female Balb/C athymic mice. Mice were implanted with 1.7 mg of 17 β -estradiol pellets as an exogenous estrogen source. Tumor size was measured every other day, and volume was estimated as $0.52 \times a \times b^2$, where a and b are the largest and smallest lengths of the palpable tumor. On day 4 after cell implantation, tumor volumes were calculated to ensure uniformity in size, and animals were randomly divided into three groups ($n = 6$ mice per group). Each group was administered daily intraperitoneal injection AS-10M or scrambled ODN at a dose of 10 mg/kg or vehicle alone (sterile normal saline, pH 7.4). Animals were sacrificed, and tumors and normal organs were harvested after 6 weeks. Harvested tissue was fixed in formalin for paraffin embedding and histological analysis. All procedures were approved by our Institutional Animal Care and Use Committee and performed in accordance with the Animal Welfare Act regulations.

Statistical Analysis

Data are presented as mean $\pm$ SE. Differences in tumor volume *in vivo* and number of cells staining posi-

tively were analyzed by Students' *t*-test, and significance was set at $P < 0.05$.

Results

EphB4 Is Expressed by Human Breast Cancer Tissue and Breast Cancer Cell Lines

Human breast cancer specimens were obtained under Institutional Review Board-approved protocols. In a pilot study to evaluate expression of EphB4 in human breast cancer specimens we examined, by immunofluorescence, 12 unselected tumor specimens (11 invasive ductal cancers and one ductal carcinoma *in situ* (DCIS)) for which frozen sections were available. Serial sections were stained using hematoxylin and eosin EphB4-specific monoclonal antibody (Figure 1A) and EphrinB2-specific monoclonal antibody and polyclonal anti-CD31 antibody (Figure 1B). Tumor-specific EphB4 expression was seen in 7 of 12 specimens (58%). Both DCIS and invasive cancer specimens showed membrane-localized expression of EphB4 (Figure 1A). EphB4 expression was restricted to tumor tissue and was absent in adjacent normal stroma (seen in hematoxylin and eosin staining of a serial section in DCIS, and invasive cancer cases 1 and 5). EphB4 also selectively marked small tumor deposits within inflammatory cells separate from the main tumor tissue (Figure 1A, case 5). We confirmed the specificity of the signal by preincubating the antibodies with blocking peptide and demonstrating loss of signal in a serial section (right panels in DCIS and invasive cancer case 1). EphB4 expression was also seen in blood vessels, presumably of venous lineage (arrow in DCIS and invasive cancer cases 4 and 5 in Figure 1A, see serial section stained with CD31 antibody in Figure 1B, second row). Normal ductal epithelium had no demonstrable expression of EphB4 (G in middle panel, second row, Figure 1B). Some tumor cells also weakly expressed EphrinB2 (Figure 1B, top row, right panel). In addition, EphrinB2 expression was also seen as expected in blood vessels (Figure 1B, arrowheads, second row, see serial section stained with CD31 antibody).

We then studied expression of EphB4 in different breast cancer cell lines by immunoblotting. All of the four breast cancer cell lines examined showed relatively high levels of protein expression with highest amount in MCF-7 cells (Figure 1C). No expression was detectable in a primary culture of normal breast epithelium. Human umbilical vein endothelial cells and A549 lysates were included as positive and negative controls, respectively. Using quantitative PCR, *EphB4* gene copy number was amplified (more than four copies after correction for expression of β -actin and GAPDH) in two of four cell lines examined with the highest amplification in T47D cells. Protein expression of the ligand EphrinB2 was detectable by immunoblotting in T47D cells and in very low levels in MCF-7 cells. EphrinB2 expression was not detectable in the other two cell lines (Figure 1C, second row).

Regulation of EphB4 Expression by erbB Family of Receptors

We sought to determine whether erbB receptors regulate the expression of EphB4. EGFR is expressed at high levels by SKBR3 cells and to a small extent by MCF-7 and T47D cells (Figure 1C, third row). The ZR75 cell line does not express EGFR. Similarly, HER-2/neu is also expressed at high levels by SKBR3 cells and minimal expression was noted in MCF-7 and T47D cells with no expression in ZR75 cells (Figure 1C, fourth row). Thus, SKBR3 cells were treated with an EGFR-kinase-specific inhibitor, AG1478, to study the effect on EphB4 (Figure 1D). A dose-dependent inhibition of EphB4 expression was seen with an IC_{50} at 500 nmol/L. At a dose of 1 μ mol/L AG1478, EphB4 expression was nearly completely inhibited at 36 hours (Figure 1D, left panel). AG1478 had no effect on ZR75 cells that do not express EGFR (Figure 1D, right panel). At a dose of 1 μ mol/L, AG1478 resulted in a 65% inhibition of EphB4 in MCF-7 cells (data not shown). T47D, the cell line with highest amplification in the gene locus of EphB4, showed no change in EphB4 protein levels after treatment with AG1478 (data not shown). To confirm these findings, induction of EphB4 via EGFR signaling was examined. Serum-starved SKBR3 cells were treated with increasing doses of EGF for 36 hours (Figure 1E, left panel). A dose-dependent increase in EphB4 expression was seen following treatment with EGF.

To elucidate the signaling molecules downstream to EGFR that regulate EphB4 expression, serum-starved SKBR3 cells were treated with 100 ng/ml EGF in the presence of various inhibitors of specific downstream molecules. The Janus kinase-2 inhibitor AG490 (4 μ mol/L) completely abrogated EphB4 expression. The phosphatidylinositol 3-kinase or phosphoinositide 3-kinase (PI3K) inhibitor wortmannin (50 nmol/L) also abolished EphB4 expression, whereas a 79% reduction in expression levels was seen with the Akt inhibitor SH-5 (100 nmol/L). There was also a 75% reduction in levels of EphB4 with the Src inhibitor PP2 (500 nmol/L). The mitogen-activated protein kinase kinase (MEK) inhibitor PD8059 (200 nmol/L) resulted in a 31% reduction in expression, whereas the p38 MAPK inhibitor SB203580 (5 μ mol/L) had no effect on EphB4 levels (Figure 1E, right panel). Thus, EGFR signaling regulates EphB4 expression via different downstream pathways, with the Janus tyrosine kinase (JAK)-signal transducers and activators of transcription (STAT) and PI3K-Akt pathways playing a predominant role.

Based on these findings, we proposed that erbB2 may have similar effects on EphB4 expression. We treated SKBR3 cells with herceptin under conditions known to result in endocytosis and degradation of the receptor. A 76% reduction in HER-2/neu levels with 20 μ g/ml herceptin at 48 hours correlated with a 76% reduction in EphB4 expression (Figure 1F, left panel). Herceptin had no effect on EphB4 expression in ZR75 (Figure 1F, right panel) or T47D cells (data not shown) and resulted in a 60% inhibition in EphB4 levels in MCF-7 cells (data not shown). Direct evidence for the role of HER-2/neu in the regulation of EphB4 was ob-

A

DCIS

Infiltrating Ductal Ca

Case 1

Case 2

Case 3

Case 4

Case 5

B

C

D

E

F

G

Figure 1. EphB4 is expressed in breast tumor specimens and breast cancer cell lines and is regulated by erbB family of receptors. Fresh frozen sections of human breast cancer tissues were evaluated by immunohistochemistry for expression of EphB4 (A) and EphrinB2 and CD31 (B). Tumor-specific expression was evaluated by H&E staining of a serial section (DCIS and infiltrating ductal cancer cases 1 and 5), specificity of signal was confirmed by loss of signal following preincubation with blocking peptide (BP, DCIS, and invasive cancer case 1), and vessel staining was confirmed by staining a serial section for CD31 (B, second row). **Arrow** in DCIS and cases 4 and 5 shows positive EphB4 signal in a blood vessel. **Arrow** in B, second row indicates a CD31-positive blood vessel that also expresses EphB4, whereas the **arrowhead** indicates a CD31-positive blood vessel that also expresses EphrinB2. T, tumor; S, stroma; G, normal breast gland/duct. The bar in the bottom right panel of B represents 75 µm in DCIS, case 1, top row of case 5, and top row of B, and 200 µm in cases 2, 3, and 4, bottom row of case 5, and bottom row of B. **C:** Immunoblot of 20 µg of cell lysates from various breast cancer cell lines and normal breast epithelial cell primary culture (Br. Epithelium) was serially analyzed for expression of EphB4, EphrinB2, EGFR, HER-2/neu, and β-actin. A549 cells and human umbilical vein endothelial cells were loaded as negative and positive controls, respectively, for EphB4 expression. Quantitative PCR was performed with total cellular DNA using primers for EphB4, β-actin, and GAPDH as described in Materials and Methods. The number of *EPHB4* gene copies (from triplicate experiments with the scale set to reflect the number of copies in peripheral blood monocytes from normal subjects at 2 and normalized for expression of β-actin and GAPDH) is shown under each cell line. SKBR3 and ZR75 cells were treated for 36 hours with varying doses of an EGFR-specific kinase inhibitor, AG-1478, and cell lysates were analyzed by immunoblotting for expression of EphB4 (D). Serum-starved SKBR3 cells were treated for various doses of EGF for 36 hours as shown and EphB4 levels in lysates analyzed on immunoblotting (E, left panel). Serum-starved SKBR3 cells were treated with 100 ng/ml EGF in the presence of different kinase inhibitors for 36 hours and EphB4 levels in lysates analyzed similarly (E, right panel). **F:** SKBR3 and ZR75 cells were treated for 24 hours with varying doses of erbB2 antibody trastuzumab (Herceptin), and cell lysates were analyzed by immunoblotting for the proteins shown. **G:** Cell lysates from parent mouse fibroblasts (3T3 cells) stably transfected with empty vector or HER-2/neu were analyzed for expression of EphB4 and β-actin. Band intensity relative to β-actin is shown below the blots in D–F.

tained by ectopic expression in the EphB4-negative mouse fibroblast cell line NIH-3T3. Stable transfection of HER-2/neu into these cells resulted in expression of EphB4 protein (Figure 1G), whereas no expression was observed in the vector-transfected cell line. Therefore, both EGFR/erbB1 and HER-2/neu/erbB2 can regulate EphB4 expression in breast cancer cell lines.

EphB4 on Breast Cancer Cells Is Biologically Active and Signals via the Akt Pathway

We next examined whether EphB4 on MCF-7 cells is functional by testing if the receptor is phosphorylated in response to stimulation by its cognate ligand, EphrinB2. There was minimal basal receptor phosphorylation following culture under serum-free conditions (Figure 2A). A significant increase in phosphorylation was seen after stimulation with 1 μ g/ml clustered EphrinB2/Fc but not Fc alone. Phosphorylation was seen as early as 10 minutes, peaked at 20 minutes, and began to decline by 60 minutes (Figure 2A, left panel). To determine downstream effects of EphB4 stimulation, we evaluated the status of Akt following EphB4 stimulation. EphB4 stimulation by clustered EphrinB2/Fc, but not Fc alone, resulted in a significant increase in phosphorylated Akt levels at 20 minutes, with a further increase noted after stimulation of EphB4 for 60 minutes (Figure 2A, lower two rows).

Prolonged Stimulation by EphrinB2 Leads to Loss of EphB4 and Reduced Cell Viability

We speculated that short term treatment with the ligand induces receptor activation, whereas prolonged treatment may lead to receptor down-regulation. If so, we could examine the biological effect of EphB4 in tumor cells. To this end, we treated MCF-7 cells with clustered EphrinB2/Fc for a prolonged period of time. We note for the first time that EphB4 levels begin to decline following 8 hours of stimulation with 10 μ g/ml ligand with an 86% reduction in EphB4 levels observed after 24 hours of stimulation (Figure 2B, upper panel). No change was observed following treatment with the Fc fragment alone (Figure 2B, lower panel). Receptor degradation was slower and less pronounced on stimulation with lower doses of ligand, and no significant change in level was observed at a ligand concentration of 1 μ g/ml (data not shown).

In parallel experiments, cell viability was studied in MCF-7 cells treated with varying doses of EphrinB2/Fc (Figure 2C). A dose-dependent reduction in cell viability was observed, and treatment with 10 μ g/ml ligand for 96 hours, but not Fc alone, resulted in 60% loss of viability. Prolonged exposure to high dose of the ligand thus produces an opposite effect leading to loss of receptor and cell viability.

EphB4 Knockdown Inhibits Cell Viability

To further clarify the role of EphB4 on cell viability, targeted EphB4 knockdown was achieved using EphB4-specific siRNA and antisense ODN. EphB4-specific

Figure 2. EphB4 signals via Akt in breast cancer cell lines and continued exposure to ligand leads to loss of receptor and reduced cell viability. MCF-7 cells were serum-starved overnight and stimulated for the various time periods shown with 1 μ g/ml clustered EphrinB2/Fc or Fc alone. EphB4 was immunoprecipitated from 100 μ g of whole cell lysates and phosphorylation status analyzed by anti-phosphotyrosine antibody immunoblotting (A, top row). A duplicate membrane was probed for EphB4 to document immunoprecipitation efficiency (A, second row). Whole cell lysates were probed for p-Akt (A, third row) and total Akt (A, fourth row) levels. MCF-7 cells were treated with 10 μ g/ml of clustered EphrinB2/Fc (B, upper panel) or Fc alone (B, lower panel) for the various time periods shown. Twenty μ g whole cell lysates was analyzed by immunoblotting for EphB4 and β -actin levels. Band intensity relative to β -actin is shown. 1×10^4 MCF-7 cells were plated in each well of a 48-well plate and treated for 96 hours with varying doses of clustered EphrinB2/Fc or Fc alone (C). Cell viability was assessed by MTT assay, and survival is expressed as percentage of absorbance relative to untreated cells. * $P < 0.05$.

siRNA, but not mutated siRNA Δ or siRNA directed against green fluorescent protein, caused a dose-dependent decrease in EphB4 protein levels (Figure 3A and data not shown). 48 hours after transfection of 100 nmol/L EphB4-siRNA, an 89% reduction in protein expression was observed. Concomitant with a fall in EphB4 levels, cell viability (measured at 48 hours of EphB4-siRNA treatment) declined in a dose-dependent manner. Treatment with 100 nmol/L EphB4-siRNA, but not siRNA Δ , reduced

cell viability by 77% (Figure 3B, left panel) in the EphB4-positive MCF-7 cell line, while no effect was seen in the EphB4-negative A549 cell line (Figure 3B, right panel). The IC_{50} for siRNA was ~ 50 nmol/L.

For application *in vivo*, we generated several antisense ODNs, and the most effective molecule, AS-10, was methylated at the CpG site (AS-10M). AS-10M treatment, but not scrambled ODN treatment, also resulted in a significant dose-dependent loss in EphB4 expression with an 84% loss in receptor expression at 72 hours of treatment (Figure 3C). Again, knockdown of EphB4 expression resulted in a significant reduction in cell viability with a 72% reduction in cell viability at 72 hours at a dose of 10 μ mol/L AS-10M (Figure 3D, left panel). The IC_{50} for AS-10M was at 6 μ mol/L. AS-10M had no effect on A549 cell line that does not express EphB4 (Figure 3D, right panel).

EphB4 Mediates Cancer Cell Migration and Invasion

The malignant phenotype of cancer cells also results from their ability to invade basement membrane and migrate to distant sites as metastases. We studied the effect of EphB4 knockdown on migration and invasion of MCF-7 cells immediately following transfection with a relatively lower dose of EphB4-siRNA (maximum siRNA dose of 50 nmol/L) after shorter time periods (8 to 12 hours), conditions that do not have a significant effect on cell viability. 50 nmol/L EphB4-siRNA reduced cell migration in a modified Boyden Chamber assay by 70% at 8 hours (Figure 3E, upper panel) and invasion into Matrigel-coated inserts by 80% at 12 hours (Figure 3E, lower panel), while siRNA Δ had no effect. Similarly, migration and invasion of MCF-7 cells was reduced by more than 65% after blocking EphB4 expression with AS-10M ODN (data not shown).

Cancer cells generally have an enhanced capacity to degrade and invade the surrounding matrix. Given that knock down of EphB4 inhibits tumor cell migration and invasion, we studied the effect of EphB4 knock down on matrix-degrading proteases. Following treatment of MCF-7 cells with 50 nmol/L EphB4-siRNA for 24 hours, a 60% reduction in MMP-9 activity was seen on Zymogram analysis (Figure 3F). Although expressed at very low levels in MCF-7 cells, MMP-2 activity was also down-regulated by 45% following EphB4 knockdown (Figure 3F). Similarly, EphB4 knockdown led to a 60% reduction in levels of uPA mRNA by quantitative reverse transcriptase (RT)-PCR. Thus, EphB4 regulates the activity of several matrix-degrading proteins, facilitating migration of tumor cells by increased degradation of surrounding matrix proteins.

EphB4 Knockdown Induces Apoptosis by Regulating Apoptotic Pathways

We speculated that loss of cell viability on knockdown of EphB4 with siRNA may be a consequence of apoptosis. Apoptosis was thus measured by quantitation of cytoplasmic nucleosomes in MCF-7 cells treated with siRNA. A dose-dependent increase in DNA fragmentation was

observed with EphB4-siRNA but not siRNA Δ (Figure 4A). Treatment with EphB4-siRNA at a dose of 100 nmol/L for 36 hours resulted in a 16-fold induction of apoptosis, while EphB4 siRNA Δ had no effect. We next determined if apoptosis was initiated at the cell membrane or the mitochondria by measuring activation of caspase-8 and caspase-9, respectively. EphB4-siRNA treatment led to a sevenfold increase in caspase-8 activity and a modest 2.5-fold increase in caspase-9 activity (Figure 4B), while EphB4 siRNA Δ had no such effect. Similar results were obtained with EphB4-antisense ODN (data not shown).

Overexpression of antiapoptotic proteins such as Mcl-1, Bcl-2, Bcl-x_L, and FLICE-inhibitory protein (FLIP) occurs commonly in cancers to protect tumor cells from death. We therefore evaluated the effect of EphB4 knockdown on these antiapoptotic proteins (Figure 4C). Concomitant to fall in EphB4 levels with EphB4-siRNA in MCF-7 cells, a reduction in levels of Mcl-1 and Bcl-x_L by 45% and 88%, respectively, was observed. EphB4 knockdown had no demonstrable effect on expression of FLIP and Bcl-2. EphB4 knockdown thus alters cell viability through induction of apoptosis pathways (caspase activation) and down-regulation of antiapoptotic proteins (Mcl-1 and Bcl-x_L).

EphB4 Expression Protects Cells from TRAIL-Induced Apoptosis

To ascertain if EphB4 modulates the effect of TRAIL on breast cancer cells, we studied TRAIL-induced apoptosis in MCF-7 cells with and without knockdown of EphB4. MCF-7 cells are relatively resistant to TRAIL-induced apoptosis with 50 ng/ml TRAIL causing 10% cell death (Figure 4D). Even at a dose of 500 ng/ml, TRAIL was also unable to kill 50% MCF-7 cells (data not shown). Introduction of EphB4-siRNA increased TRAIL-induced loss of cell viability in a dose-dependent fashion (Figure 4D, left panel). Whereas 50 nmol/L EphB4-siRNA alone resulted in 30% cell death and 50 ng/ml TRAIL alone in 10% cell death, a combination of 50 ng/ml TRAIL with 50 nmol/L EphB4-siRNA resulted in 60% cell death. Combination of TRAIL with EphB4 siRNA Δ , however, did not alter cell viability (Figure 4D, right panel). Addition of a caspase-8 inhibitor completely abolished cell death from EphB4 siRNA alone, TRAIL alone, and a combination of both (Figure 4D, left panel, top line). The increased sensitivity to TRAIL-induced apoptosis after a partial knockdown of EphB4 is consistent with a role of EphB4 in protection from TRAIL-induced apoptosis.

To further confirm this effect, we evaluated if forced expression of EphB4 protects cells against TRAIL-induced apoptosis. A549 cells do not express EphB4 (Figure 1C) and are partially sensitive to TRAIL-induced apoptosis. These cells were cotransfected with the truncated CD4 receptor (to allow sorting of transfected cells) and full-length EphB4 expression vector or null vector. Transfected cells were sorted using magnetic beads coated with anti-CD4 antibodies, and purity of transfected cells was cross-verified with fluorescent EphB4 monoclonal antibody to be 91% (data not shown). Cells transfected with EphB4, but not null

vector, expressed EphB4 protein (Figure 4E, right panel). We then studied the sensitivity of EphB4- or null vector-transfected cells to TRAIL-induced apoptosis (Figure 4E, left panel). Ectopic expression of EphB4 in A549 cells protected them from TRAIL-induced apoptosis. TRAIL at 100 ng/ml, for example, resulted in 40% loss of viability in vector-transfected cells, whereas EphB4 transfected cells were completely resistant to TRAIL-induced apoptosis.

Knockdown of EphB4 by Specific Antisense ODN Inhibits Tumor Growth *in Vivo*

Having confirmed that EphB4 functions as a survival factor in MCF-7 cells *in vitro*, we evaluated the effect of EphB4 knockdown *in vivo* on tumor growth in a murine breast cancer xenograft model. Mice ($n = 6$ per group, experiment repeated twice) were implanted with MCF-7 cells in the breast fat pad and supplemented with exogenous estrogen. Treatment was begun on day 4, intraperitoneally daily with 10 mg/kg EphB4-specific AS-10M, scrambled ODN, or vehicle alone. Treatment with AS-10M led to a significant reduction in tumor growth resulting in 72% smaller tumors at 6 weeks compared to control or scrambled ODN treatment (Figure 5A). The animals had no clinical evidence of toxicity, fed well, and maintained normal weight and activities. Gross and histological appearance of various organs at sacrifice showed no abnormalities (data not shown). In addition, spleen weights were comparable in the three groups at sacrifice, implying lack of induction of pleiotropic cytokine responses. Furthermore, serum levels of interleukin-12 and tumor necrosis factor- α were also comparable among animals in the three groups at sacrifice (data not shown). Immunoblot analysis of tumor extracts showed no expression of EphB4 in the AS-10M-treated tumors, whereas EphB4 was readily detectable in vehicle or scrambled ODN-treated tumors (Figure 5B). These data indicate that inhibition in tumor growth following AS-10M treatment was a direct result of EphB4 knockdown.

Immunohistochemical evaluation (Figure 5C) of AS-10M-treated tumors, in comparison to control tumors, showed an 86% reduction in Ki-67-positive cells, 15-fold increase in apoptosis by TUNEL assay, and 44% reduction in CD31-positive microvasculature. AS-10M had no effect *in vivo* on xenografts of SLK cells that do not express EphB4 (data not shown). Down-regulation of EphB4 expression, therefore, effectively and specifically inhibits tumor growth in EphB4 expressing breast cancer cells.

Discussion

Increased expression of EphB4 in two-thirds of human breast cancers has been previously reported,^{26,27} as well as an association between EphB4 expression and increased S phase fraction, DNA aneuploidy, and histological grade of the tumor.²⁷ Interestingly, in a subsequent report, Berclaz et al³² note a reduction in EphB4-positive cells on immunohistochemistry in breast carcinoma specimens compared to normal breast epithelium. These results contrast with their original report wherein strong expression of EphB4 transcripts was observed in the carcinomatous cells by *in situ* hybridization.²⁶ Such discrepancy can be a result of differences in sensitivity of the polyclonal antibodies used in these studies. In our preliminary analysis of 12 breast tumor specimens using EphB4-specific monoclonal antibodies, we observe tumor-specific EphB4 expression in 58% tumors, in agreement with the observations of Wu et al.²⁷ EphB4 staining can also reliably detect even a small number of tumor cells among other cell types. Normal breast ductal epithelium does not express EphB4, although positive staining is observed in adjacent vasculature. Preincubation with blocking peptide abolishes the signal further confirming the specificity of our monoclonal antibodies. A large prospective cohort study evaluating the expression and prognostic significance of EphB4 in breast cancer is currently being undertaken at our center.

We further show that EphB4 is overexpressed in a variety of breast cancer cell lines. In some proportion of breast cancers, EphB4 up-regulation can result from amplification of the gene locus. This is suggested by array comparative genomic hybridization studies of 14 breast cancer specimens in which the 7q22 site, which harbors the *EphB4* gene locus, was amplified in 29% cases.³³ Our *EphB4* gene amplification studies in tumor cell lines reveal amplification of the locus in two of four lines studied. Fluorescence *in situ* hybridization analysis of a large set of tumor tissues will provide the definitive frequency of gene amplification and its correlation with various clinical parameters.

Other possible pathways leading to up-regulation of EphB4 in breast cancer include signaling by the *erbB* family of receptors, *erbB1*/EGFR and *erbB2*/HER-2/neu, both of which play a prominent role in the transformation of breast epithelium and propagation of tumors. We pro-

Figure 3. EphB4 knockdown leads to decreased survival and tumor cell migration and invasion. MCF-7 cells were transiently transfected with Lipofectamine 2000 alone (control), EphB4-specific siRNA (EphB4-siRNA), or mutated EphB4 siRNA (EphB4-siRNA Δ). 48 hours later, 20 μ g of whole cell lysates were analyzed by immunoblotting for EphB4 and β -actin levels (A). 1×10^4 MCF-7 cells (EphB4-positive, B, left panel) or A549 cells (EphB4-negative, B, right panel) were transfected with Lipofectamine alone, mutated EphB4 siRNA Δ or native EphB4-siRNA and plated in a 48-well plate. Cell viability was assessed by MTT assay at 48 hours, and survival was expressed as percentage of absorbance relative to untreated cells. MCF-7 cells were treated with varying doses of scrambled ODN or methylated EphB4-specific ODN (AS-10M). 72 hours later, 20 μ g of whole cell lysates was analyzed by immunoblotting for EphB4 and β -actin levels (C). 1×10^4 MCF-7 cells (D, left panel) or A549 cells (D, right panel) were treated with scrambled or AS-10M ODN. Cell viability was assessed by MTT assay at 72 hours, and survival is expressed as percentage of absorbance relative to untreated cells. Effect of siRNA treatment on migration of MCF-7 cells was studied by modified Boyden chamber assay. Representative photomicrographs are shown (E, upper panel). Number of migrating cells was averaged over five high power fields and shown as a plot. Invasion of MCF-7 cells into Matrigel-coated inserts was studied as described in Materials and Methods. Cells invading the underside of the inserts in response to 5 μ g/ml fibronectin in the lower chamber were fixed and stained with Giemsa. Representative photomicrographs are shown (E, lower panel). Cell number was averaged over five random high power fields and shown as a plot. MCF-7 cells were treated with Lipofectamine alone or 50 nmol/L EphB4-siRNA or mutated EphB4-siRNA Δ . 24 hours later, MMP activity in clarified concentrated supernatants was analyzed by gelatin zymography. Intensity of the bands was quantitated for MMP-9 (F, left panel) and MMP-2 (F, middle panel) relative to Lipofectamine-treated cells. Total mRNA was extracted from the cells, and uPA mRNA levels were assessed by quantitative RT-PCR (F, right panel). Band intensity relative to β -actin is shown in A and C. * $P < 0.05$ between EphB4-siRNA-treated cells and control/mutated EphB4-siRNA-treated cells.

Figure 4. EphB4 protects cells from apoptosis. MCF-7 cells were transiently transfected with Lipofectamine alone (control) or EphB4-specific siRNA (EphB4-siRNA) or mutated siRNA (EphB4-siRNAΔ). **A:** Apoptosis was analyzed by enzyme-linked immunosorbent assay for cytoplasmic nucleosomes as detailed in Materials and Methods using whole cell lysates. **B:** Caspase-8 and caspase-9 activation was assayed colorimetrically and expressed as percent activity compared to Lipofectamine-treated cells. **C:** Expression levels of various anti-apoptotic proteins in 20 μ g of whole cell lysates were analyzed by immunoblotting. Band intensity relative to β -actin is shown below the blots. **D:** Transfected MCF-7 cells were exposed to various doses of TRAIL for 16 hours, and cell survival was assessed by MTT assay. C8i, caspase-8 inhibitor. A549 lung cancer cells were transfected with full-length EphB4 expression vector or null vector along with a truncated CD4 receptor that was used to sort transfected cells. Transfected and sorted cells were cultured in the presence of varying doses of TRAIL and cell survival assessed on day 3 (**E**, left panel). EphB4 expression in transfected cells was confirmed by immunoblotting (**E**, right panel).

Figure 5. EphB4-specific antisense ODN inhibits tumor growth in a murine orthotopic breast cancer xenograft model. 5×10^6 MCF-7 cells were implanted in the region of the right breast in 10- to 12-week-old, female Balb/C athymic mice supplemented with exogenous estrogen and tumor volume measured (**A**) as detailed in Materials and Methods. Mice were administered vehicle alone (control), or 10 mg/kg scrambled ODN (S) or methylated EphB4-specific antisense ODN (AS-10M) intraperitoneally daily starting day 4 after cell implantation. Animals were sacrificed 6 weeks later and tumors were harvested. **B:** 20 μ g of tumor lysates was analyzed by immunoblotting for EphB4 and β -actin levels. Band intensity relative to β -actin is shown. **C:** 5- μ m sections of formalin-fixed paraffin-embedded sections were stained with H&E and analyzed by immunohistochemistry for Ki-67 and CD31 expression. Apoptosis was evaluated by TUNEL with the in situ apoptosis staining kit. Number of cells staining positively was averaged over five random high power fields by a blinded observer and summarized in a plot (right panels in **C**). * $P < 0.005$ between AS-10M and control group. Scale bar in the bottom left panel in **C** represents 200 μ m in H&E and 75 μ m in the other photomicrographs.

vide evidence by the direct application of EGF on EGFR-expressing cells, which shows a dose-dependent induction of EphB4. Similarly, introduction of erbB2/HER-2/neu in fibroblasts induces expression of EphB4. Conversely, chemical or protein inhibitors of receptor function or expression led to a decline in EphB4 expression in breast cancer cells. We have also defined the downstream mol-

ecules that orchestrate EphB4 regulation by EGFR and HER-2/neu. EGFR signals via several downstream pathways, including MAPK, JAK-STAT, PI3K, c-Jun N-terminal kinase, phospholipase C- γ , Src, and related proteins. Induction of EphB4 following activation of EGFR by EGF was attenuated by specific inhibition of JAK-STAT and PI3K-AKT pathways, whereas inhibition of p38 MAPK had

no effect. STAT3 is a downstream effector molecule that undergoes differential phosphorylation by JAK and MAPK. Whereas JAK phosphorylates a tyrosine residue at position 705, MAPK phosphorylates a serine residue at position 727. The differential effects of JAK inhibitor and p38 MAPK inhibitor suggest that the site of STAT3 phosphorylation plays an important role in the regulation of EphB4 expression by EGFR. PI3K signaling leads to activation of Akt and mammalian target of rapamycin, both of which regulate cell fate. Loss of phosphatase and tensin homolog, which is observed in nearly half of all cancers, allows unabated activity of PI3K and thus, downstream effects such as tumor cell survival. The complete abrogation of EphB4 expression with a PI3K inhibitor and Akt inhibitor provides evidence for a prominent role of this pathway in the regulation of EphB4. It is likely that JAK-STAT and PI3K pathways are codependent, because inhibition of either pathway led to a profound reduction in EphB4 levels. Further, it is tempting to speculate that EphB4 may participate in the biological effects of EGFR and Her2/neu in breast cancer.

Downstream signaling by EphB4 in tumor cells has not been studied, although activation of the PI3K pathway, including Akt phosphorylation has been observed in endothelial cells.¹⁷ We show similar EphB4 signaling effects in breast cancer cells. Phosphorylation of Akt occurred slower than that of EphB4 and persisted after EphB4 phosphorylation had begun to decline. Stimulation of Akt is consistent with a prosurvival role for EphB4 in breast cancer cells.

EphB4 also induces migration of tumor cells. Munarini et al²⁸ have previously shown that transgenic mice with mammary tumors resulting from targeted overexpression of neuT have localized tumors. In contrast, EphB4/neuT double transgenic mice frequently develop lung metastases.²⁸ Tumor cell migration is dependent on the degradation of matrix proteins. We show that two important enzymes in the matrix metalloprotease family, MMP2 and MMP9, are active at basal levels, and the activity declines significantly when cells are treated with EphB4 siRNA. Furthermore, EphB4 also regulates the mRNA levels of uPA, with a significant decline when EphB4 is knocked down with siRNA. Thus, EphB4 may promote tumor cell migration by inducing degradation of matrix proteins via regulation of MMPs and uPA. A recent report by Minn et al³⁴ has shown that up-regulation of matrix metalloproteases are crucial events that regulate the acquiring of a metastatic phenotype by breast cancer cells.

Several studies have suggested a role for EphB4 in breast cancer progression. EphB4 transgenic mice do not develop breast cancer per se, thus arguing against its role in breast cell transformation.²⁸ However, when these mice are crossed with transgenic mice expressing mammary tissue-specific neuT antigen, tumors form more rapidly. Thus, EphB4 promotes tumor growth/tumor cell survival. Noren et al²⁹ showed that EphB4 expression on breast cancer cells enhances tumor angiogenesis and tumor growth, even if EphB4 lacks kinase function, suggesting an interaction between EphB4 on tumor cells and EphrinB2 on vascular endothelial and smooth muscle cells. Alternatively, kinase-independent signaling may be

responsible for breast cancer growth. Interestingly, these authors reported that prolonged stimulation of breast cancer cells that express EphB4 with high dose EphrinB2 inhibits cell viability.²⁹ Ligand-induced receptor endocytosis and degradation is one of the feedback mechanisms that modulates receptor activation. Recently, Marston³⁵ and Zimmer³⁶ in independent studies showed that EphB receptors are internalized following ligand binding. We show that in MCF-7 cells prolonged exposure to high dose ligand results in loss of EphB4 with a concomitant fall in cell viability.

We then undertook a more direct approach to elucidate the role of EphB4 in tumor cells using specific siRNA. We show that EphB4 provides a survival advantage to tumor cells, even if the cells do not express EphrinB2. Thus, constitutive receptor activation from overexpression in tumor cells may be sufficient to gain cell survival signals. Furthermore, loss of EphB4 and activation of caspase-8, and to lesser extent caspase-9, indicate that inhibition of EphB4 expression permits intrinsic signals to induce cell death. The exact molecular mechanism by which EphB4 provides survival advantages has not been established. Early evidence is provided in the current study, showing that EphB4 prevents TRAIL-induced cell death via death receptor pathways that are known to activate caspase-8. Less prominent effects on the intrinsic pathway may also occur via the regulation of the bcl-2 family of antiapoptotic proteins, specifically Mcl-1 and Bcl-x_L.

EphB4 can, therefore, provide survival advantage to tumor cells in several ways. Overexpression of EphB4 on breast tumor cells induces tumor vasculature by binding to and activating EphrinB2 on vascular endothelial cells, leading to an angiogenic response. Secondly, EphB4 intrinsically provides survival advantage to breast tumor cells. Such events may result from self-aggregation and receptor activation due to high receptor density, in which case EphB4 can protect tumor cells from apoptosis in the absence of ligand. Alternatively, EphB4 may function in a signaling-independent mechanism, wherein interaction of EphB4 with certain components of death receptor pathway can interrupt apoptotic signals. Such a model has been demonstrated in liver cells in which Fas-L binding to Fas is blocked by the extracellular domain of the c-met receptor, and, thereby, apoptosis is inhibited.³⁷

The identification of novel molecular targets and understanding their interaction with multiple pathways that influence outcome is required to design rational therapies for breast cancer. In this report, we demonstrate that EphB4 is a novel therapeutic target in breast cancer by using antisense oligonucleotides that specifically block EphB4 expression in vivo. The specificity of ODN function was documented by loss of EphB4 in tumor tissue and lack of pleiotropic cytokine responses. Treatment with ODNs was highly effective with a 70% reduction in tumor size at 6 weeks, while control ODN had no effect. In vivo studies also support the suggested mechanisms, which include inhibition of tumor angiogenesis in addition to induction of apoptosis in tumor cells.

In summary, EphB4 is frequently expressed in breast cancer and driven by gene amplification or signaling by

erbB1 and erbB2. EphB4 provides cell survival advantage to tumor cells via attenuation of inherent cell death pathways and by up-regulation of anti-apoptotic proteins. Additional advantage to breast cancers in vivo results from the interaction between tumor cell EphB4 and vascular endothelial cell EphrinB2, which promotes tumor angiogenesis. EphB4 is thus a novel target for biological therapy in breast cancer.

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