

## Dual targeting of EphA2 and ER restores tamoxifen sensitivity in ER/EphA2-positive breast cancer

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Received: 26 March 2010 / Accepted: 17 June 2010 / Published online: 3 July 2010  
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**Abstract** Overexpression and altered function of EphA2 receptor tyrosine kinase are critical in the progression of breast cancer and provide a target for breast cancer therapy. We have previously demonstrated that EphA2 overexpression decreases estrogen dependence and Tamoxifen sensitivity both in vitro and in vivo. EA5, a novel monoclonal antibody that mimicks the binding of ephrin A to EphA2, reverses the effect of EphA2 overexpression and restores Tamoxifen sensitivity in EphA2-transfected MCF-7 cells in vitro. To explore the role of EphA2 overexpression on ER-dependent mechanisms, we used two different ER+/EphA2-transfected cell line models (MCF-7<sup>neo</sup>/MCF-7<sup>EphA2</sup>

and T47D<sup>neo</sup>/T47D<sup>EphA2</sup>). EA5 inhibits primary tumor growth and restores Tamoxifen sensitivity in the MCF-7<sup>EphA2</sup> xenografts. Using the T47D<sup>EphA2</sup> in vitro model, we verified that EphA2 decreases ER activation in response to E2 stimulation consistent with our earlier results in MCF-7<sup>EphA2</sup> model. We found no direct interaction between ER and EphA2 and no difference in expression of canonical ER-dependent proteins or ER co-regulators. However, E2 stimulation phosphorylates FAK<sup>Tyr925</sup> only in ER+/EphA2+ cell lines. Treatment of T47D<sup>EphA2</sup> cells with EA5 and Tamoxifen leads to dephosphorylation of FAK<sup>Tyr925</sup> in response to E2. Our data demonstrate that dual targeting of EphA2 and ER is a promising approach for delaying resistance to Tamoxifen. The data support our hypothesis that EphA2 impacts ER function via a FAK dependent pathway.

**Electronic supplementary material** The online version of this article (doi:10.1007/s10549-010-1004-y) contains supplementary material, which is available to authorized users.

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**Keywords** EphA2 · Estrogen receptor · Breast cancer ·  
Tamoxifen sensitivity

### Introduction

The Eph receptor tyrosine kinase family, the largest known family of receptor tyrosine kinases (RTKs) comprising at least 14 receptors and eight ligands, plays an important role in diverse biological processes including malignancy, cell migration and angiogenesis [1–3]. Among the Eph receptors, EphA2 (ECK) is unique, as it is found primarily in adult human epithelial cells rather than expressed during development [4]. EphA2 overexpression has been reported in many solid tumors including melanoma, prostate, ovarian and breast cancer [5]. EphA2 in malignant cells functions differently than in nonmalignant epithelial cells [6, 7]. In normal cells, EphA2 is localized to sites of cell–cell

contact where it interacts with its ligands (ephrin A proteins), leading to a decrease in cell growth, motility, and angiogenesis. In contrast, EphA2 is not in proper position in malignant cells to interact with the ephrins.

EphA2 is overexpressed and functionally altered in breast cancer cell lines and breast cancer specimens [6, 8–10]. Expression of EphA2 is low in non-transformed mammary epithelial cells, where it is tyrosine phosphorylated and enriched within cell–cell contacts. In contrast, EphA2 is grossly overexpressed in metastatic breast cancer cells, where it is not phosphorylated and is diffusely localized over the cell membrane. Non-tumorigenic breast cell lines express both ER and EphA2 at low levels. In fully transformed and tumorigenic cell lines with high levels of ER, EphA2 is not detected. Conversely, most tumorigenic cell lines that lack ER express EphA2 at high levels [6, 8]. EphA2 staining was also increased in breast carcinomas (an average of 87%) as compared with benign mammary epithelial cells (an average of 3%) [6]. In another study, 82% of the invasive breast cancer cases showed EphA2 overexpression, while the expression of EphA2 was found to be low in normal breast tissues. Surprisingly, 90% of ER+ cases were found to overexpress EphA2, while only 60% of ER-cases overexpress EphA2 [10]. Increased EphA2 levels are also associated with poor patient prognosis in ER+ tumors when comparing a set of 19 genes down-regulated during acinar morphogenesis to two independent breast cancer microarray datasets [11, 12].

EphA2 increases malignant behavior in breast cancer. Overexpression of EphA2 is sufficient to confer malignant transformation and tumorigenic potential on nontransformed (MCF-10A) mammary epithelial cells [6]. EphA2 overexpression also correlates with aggressiveness and metastatic potential in preclinical breast cancer models [6, 8, 13, 14]. In MMTV-Neu mice, loss of EphA2 impaired both tumor initiation and lung metastasis [14].

The role of EphA2 in ER+ breast cancers is not well established. Although EphA2 overexpression is inversely correlated with ER expression in breast cancer cell lines, EphA2 has broad relevance and is important in both ER+ and ER-human breast cancer specimens. In mammary epithelial cells, estrogen negatively regulates EphA2 overexpression [8]. We further demonstrated that ectopic overexpression of EphA2 increased the malignant character of ER+ breast cancer cells [15]. In addition, EphA2 overexpression decreased estrogen dependence and Tamoxifen sensitivity both in vitro and in vivo without altering ER protein levels. We have produced a monoclonal antibody (EA5) that binds to EphA2 and mimics ligand binding, inducing phosphorylation and degradation of the EphA2 receptor [16]. EA5 restores normal EphA2 function, inhibiting breast cancer growth and restoring hormone

dependence and Tamoxifen sensitivity in vitro [15]. In this study, we further explored the efficacy of EphA2 antibody (EA5) therapy on in vivo tumor growth in ER+ breast cancer models. We also investigated the role of EphA2 overexpression in ER-dependent mechanisms using two different ER+/EphA2-transfected cell line models. Here, we report the mechanistic relevance of targeting EphA2 in ER/EphA2 breast cancer.

## Materials and methods

### Orthotopic xenograft model

MCF-7<sup>neo</sup> or MCF-7<sup>EphA2</sup> cells ( $5 \times 10^6$ ) were implanted into the mammary fat pad of athymic nude mice as described previously [15]. Once tumors were established, mice were started treatment with the saline control, EA5 (MedImmune, Gaithersburg, MD), Tamoxifen (Sigma, St. Louis, MO) or EA5 in combination with Tamoxifen as indicated in the Fig. 1a or b. Tumor volume was calculated as  $L \times W^2/2$ , where  $L$  is length and  $W$  is width. All animal experiments were done under a protocol approved by the Indiana University Institutional Animal Care and Use Committee.

### Cell culture, stable transfection of EphA2 and verification by western blot

MCF-7<sup>neo</sup> or MCF-7<sup>EphA2</sup> cell lines were generated as described previously [15]. T47D cell line was maintained as recommended by the manufacturer (American Type Culture Collection, Manassas, VA). T47D cells were transfected with pcDNA3.1 vector or pcDNA3.1-EphA2 construct (gift of D. Knapp), at a 3:1 ratio, using FuGENE<sup>®</sup> 6 (Roche Diagnostics, Indianapolis, IN). Stable transfected clones were established by selection with 500  $\mu\text{g/ml}$  G418 (Sigma, St. Louis, MO). Out of four clones, we confirmed the best clone for EphA2 overexpression using western blot analysis.

### Gene reporter assays

T47D<sup>neo</sup> or T47D<sup>EphA2</sup> cells were seeded at  $6 \times 10^4$ /well in 12-well plate in phenol red-free MEM with 5% charcoal stripped fetal calf serum (Cellgro, Mediatech Inc., Manassas, VA). After 48 h incubation, they were transfected with ERE-luciferase construct or TK-luciferase construct (gift of M-H Jeng.) using FuGENE<sup>®</sup> 6 at a 3:1 ratio. Cells were treated as indicated in Fig. 2b, and luciferase activity was performed using Dual-Luciferase Reporter System (Promega, Madison, WI) according to the manufacturer's instructions.

For the assessment of the coactivators/corepressors effect, the cells were also transfected with control, coactivators (SRC-1 or AIB1; gift of Dr. M-H Jeng) or corepressors (NCOR1 and SMRT; gift of H. Nakshatri) and ERE luciferase assay was performed as above.

RNA preparation, Agilent whole human genome 4×44K oligo microarrays and data analysis

Cells were cultured in phenol red-free MEM with 5% charcoal-stripped fetal calf serum for 48 h. Cell pellets (quadruplets for each cell line) were shipped to Miltenyi Biotec Inc. (Auburn, CA) on dry ice according to their instructions. RNA isolation and Agilent Whole Human Genome Oligo Microarrays 4×44K (two-color) of cell lines were performed by Miltenyi Biotec Inc. (Auburn, CA) according to the Agilent Technologies protocols (Santa Clara, CA).

The output signal intensities, after median normalization, were analyzed using significance analysis for microarrays (SAM) [17] to identify differentially expressed genes. For genes shown in the Venn diagram, the parameter delta in SAM is chosen to be 3 for T47D cell line and 2 for MCF7 cell line to have roughly similar number of genes from the two cell lines. For genes with multiple probes, fold-changes are averaged.

Validation of the chosen genes by quantitative real-time PCR (qPCR)

RNA was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). We validated the mRNA level for the genes of interest by real-time qPCR using TaqMan<sup>®</sup> Custom Array designed and performed on an ABI Prism 7900 platform according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). TaqMan Human Endogenous Control Array was used to choose the right endogenous control. *GAPDH* was chosen as the endogenous reference gene for normalization. The relative quantification of the gene expression changes was analyzed using the Applied Biosystems's DataAssist<sup>™</sup> Software v1.0.

Western blot analysis

Cells were lysed in RIPA buffer and equal amounts of protein were subjected to SDS-PAGE and western blot analysis as described previously [18]. Blots were incubated with antibodies against EphA2 (clone D7, Millipore, Bedford, MA), FHL2, and FZD7 (Abcam, Cambridge, MA), SOX2, and p-FAK<sup>Tyr925</sup> (Cell Signaling; Danvers, MA), total FAK (BD Transduction Lab, San Jose, CA) or GAPDH (Fitzgerald Industries, Concord, MA) as indicated.

Invasion assay

Boyden in vitro invasion assay was performed as described previously [19]. Briefly, cells were plated in phenol red-free MEM with 5% charcoal stripped fetal calf serum for 48 h and switched to 1% serum media for 24 h. Cells were transferred to the upper chamber of the phenol red free Matrigel-coated transwell plates and were treated with vehicle (ethanol), EA5 (3 µg/ml), Tamoxifen (4-OH-Tam; 10<sup>-6</sup> M) or in combination with EA5 and Tamoxifen and allowed to migrate towards E2 (10<sup>-10</sup> M) in the lower chamber for 72 h. Cell invasion was evaluated after Diff-Quick staining by counting cells in four randomly chosen fields of filters. The results are representatives of three individual experiments done in triplicates.

Statistical analyses

Statistical analyses for xenograft, gene reporter and invasion assays were performed using Student's t test (Graph-Pad Software, San Diego, CA).  $P \leq 0.05$  was defined as significant.

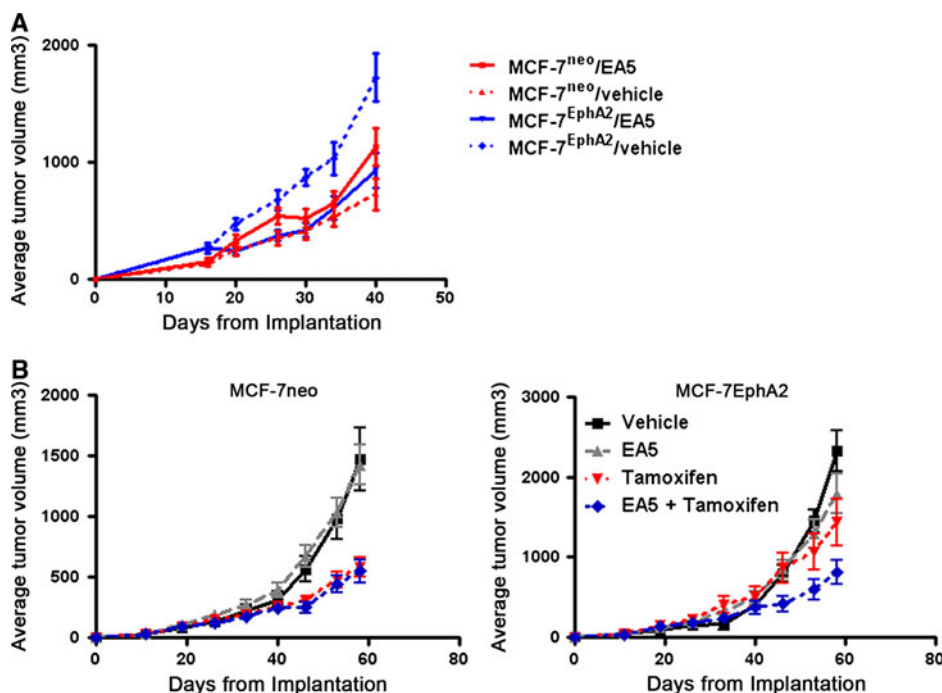
## Results

EA5 inhibits growth of MCF-7<sup>EphA2</sup> xenografts and increases Tamoxifen sensitivity in vivo

To determine the impact of EA5 on EphA2-overexpressing tumors, we employed orthotopic tumor implantation into mammary fat pads of either MCF-7 control (MCF-7<sup>neo</sup>) or EphA2-transfected cells (MCF-7<sup>EphA2</sup>). MCF-7<sup>EphA2</sup> xenografts grew faster and formed larger tumors ( $P = 0.0011$ ) than MCF-7<sup>neo</sup> xenografts (Fig. 1a). Treatment with EA5 alone reduced tumor volume significantly in MCF-7<sup>EphA2</sup> xenografts ( $P = 0.0055$ ), compared with vehicle-treated tumors, while no significant impact of EA5 was observed between EA5 or vehicle-treated MCF-7<sup>neo</sup> cells ( $P = 0.0863$ ).

We have shown that MCF-7<sup>EphA2</sup> xenografts were less sensitive to Tamoxifen ( $P = 0.01$ ) as compared with control (MCF-7<sup>neo</sup>) xenografts [15]. Therefore, we assessed the in vivo potential of EA5 in combination with Tamoxifen in mice bearing MCF-7<sup>EphA2</sup> tumors versus MCF-7<sup>neo</sup> tumors. Combined therapy with EA5 and Tamoxifen reduced the tumor growth in MCF-7<sup>EphA2</sup> xenografts significantly ( $P = 0.0001$ ) when compared with vehicle-treated group (Fig. 1b). As expected, Tamoxifen alone was less effective ( $P = 0.0295$ ) in MCF-7<sup>EphA2</sup> xenografts in comparison to vehicle-treated xenografts. In contrast, the sensitivity of MCF-7<sup>neo</sup> xenografts to Tamoxifen remained similar, when the combination of EA5 and Tamoxifen ( $P = 0.0015$ ) or

**Fig. 1** Impact of EA5 on in vivo tumor growth and tamoxifen sensitivity. **a** EA5 inhibits growth of MCF-7<sup>EphA2</sup> xenografts, but does not impact growth of MCF-7<sup>neo</sup> tumors. Mice bearing MCF-7<sup>neo</sup> or MCF-7<sup>EphA2</sup> xenografts were treated with vehicle (saline control) or EA5 (7 mg/kg) twice weekly by intra-peritoneal injection (i.p.) (20 mice per group). **b** EA5 increases Tamoxifen sensitivity in vivo. Mice bearing MCF-7<sup>neo</sup> or MCF-7<sup>EphA2</sup> xenografts were treated with vehicle, EA5 (5 mg/kg/twice weekly/i.p.), Tamoxifen (1 mg/M-F/gavage), or in combination with EA5 and Tamoxifen (note the different scale for tumor volumes). Dose is within the range of effective concentration (data not shown). Specific dose is driven by concentration of the availability of the drug supply

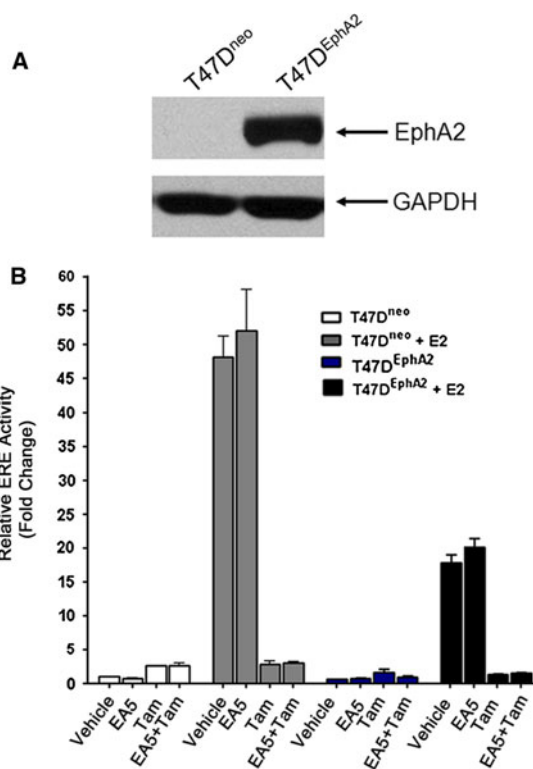


Tamoxifen alone ( $P = 0.0015$ ) was compared with vehicle-treated xenografts. Thus, targeting both EphA2 and ER increased the sensitivity of cells to Tamoxifen in ER/EphA2+ tumors.

EphA2 overexpression decreases ER activation in ER+ breast cancer cell lines in vitro

We have demonstrated that MCF-7<sup>EphA2</sup> cells decreased ER activation in response to E2 treatment [15]. To verify this, we also established T47D breast cancer cells overexpressing human EphA2 cDNA (T47D<sup>EphA2</sup>), confirmed by western blot analysis (Fig. 2a).

To assess the activation of ER in response to E2 and Tamoxifen in both T47D<sup>neo</sup> and T47D<sup>EphA2</sup> cells, we transiently transfected the cells with an ERE-luciferase construct or TK-luciferase construct (normalization control) and treated them as indicated in Fig. 2b. ER activity was increased about 48-fold ( $P = 0.0001$ ) in T47D<sup>neo</sup> with E2 compared with the T47D<sup>neo</sup> cells without E2 (Fig. 2b). Transfection of EphA2 induced ER activity only 17.82-fold with E2 compared with T47D<sup>neo</sup> cells without E2. When compared with T47D<sup>neo</sup> cells with E2, T47D<sup>EphA2</sup> cells were 2.69-fold ( $P = 0.0001$ ) less dependent on E2 stimulation. These results in T47D cells are consistent with our earlier observation with MCF-7<sup>EphA2</sup> and show that overexpression of EphA2 decreases ER activation in response to E2 stimulation. Based on higher levels of expression of EphA2, T47D<sup>EphA2</sup> cell line was used for subsequent analysis of ER-EphA2 mechanisms.



**Fig. 2** Overexpression of EphA2 decreases ER activation in T47D cells in vitro. **a** EphA2 expression of MCF-7<sup>neo</sup> or MCF-7<sup>EphA2</sup> cells was determined by Western blot analysis. **b** cells were transfected with ERE-luciferase construct or TK-luciferase construct as described in “Materials and methods” section. After treating them with vehicle (ethanol), EA5 (3  $\mu$ g/ml), Tamoxifen (4-hydroxy-Tam;  $10^{-6}$  M; Sigma, St. Louis, MO) or in combination with EA5 and Tamoxifen in the absence or presence of E2 ( $10^{-10}$  M; Sigma, St. Louis, MO) for 24 h, ERE luciferase activity was performed. The data are representative of three individual sets done in triplicates

### Interaction of EphA2 with ER is not direct

To assess whether EphA2 exerts a direct effect by binding to ER, we performed GST-pull-down assay using GST-control and GST-ER $\alpha$  full-length constructs. GST-ER $\alpha$  failed to bind to [<sup>35</sup>S]methionine-labeled EphA2 regardless of E2 treatment (data not shown). We also tested the association between ER $\alpha$  and EphA2 by co-immunoprecipitation assays (data not shown). Immunoprecipitation of ER $\alpha$  followed by Western analysis confirmed the observation with GST-pulldown assay, suggesting that no direct binding between EphA2 and ER was observed.

### EphA2 does not affect ER coregulator expression or function

Several coregulators of ER have been identified that activate or repress the transcription of ER $\alpha$ -responsive genes [20, 21]. Among them, the p160 coactivators (SRC-1 and AIB1) and nuclear receptor corepressors (NCOR1 and SMRT) have been well studied in breast cancer. Crosstalk between ER and growth factor signaling leads to alterations in the coregulator expression levels and in response or resistance to endocrine therapy [22]. We next asked whether the decrease in estrogen dependence in EphA2 overexpressing cells affects the expression levels of SRC-1, AIB1, NCOR1 and SMRT in relation to E2 ( $10^{-10}$  M). No differential protein expression of SRC-1, AIB1, NCOR1 and SMRT was observed (data not shown). Furthermore, we transiently co-transfected SRC-1, AIB1, NCOR1 or SMRT plasmids with the ERE luciferase and assessed the ER activity in T47D<sup>neo</sup> and T47D<sup>EphA2</sup> cells with or without E2 stimulation. No consistent relationship was observed between ERE luciferase activity and SRC-1 or AIB1 expression in T47D<sup>neo</sup> and T47D<sup>EphA2</sup> cells. Co-transfection of NCOR1 or SMRT failed to repress the ER activity in T47D<sup>neo</sup> and T47D<sup>EphA2</sup> cells. E2 stimulation did not alter this observation (data not shown). These results suggest that EphA2 overexpression does not alter coregulator expression or activity in T47D model. In addition, SRC-1, AIB1, NCOR1 and SMRT were not significantly altered at the mRNA level in both T47D<sup>EphA2</sup> and MCF7<sup>EphA2</sup> models (Supplemental Table S1).

### EphA2 does not alter primary canonical ER target proteins

To determine whether EphA2 overexpression alters the protein levels of endogenous ER targets, we performed an E2 timecourse and assessed the protein levels of cyclin D1

and c-myc, two well-characterized ER-regulated genes. No difference was observed in expression of cyclin D1 and c-myc between T47D<sup>neo</sup> and T47D<sup>EphA2</sup> cells. In addition, E2 does not alter EphA2 expression in the transfected cells. As expected, there is no change in cell cycle progression with E2 stimulation (data not shown). Cyclin D1, c-myc and additional canonical ER targets were not significantly altered at the mRNA level in both T47D<sup>EphA2</sup> and MCF7<sup>EphA2</sup> models (Supplemental Table S2).

### Comparison of EphA2-associated gene expression profiles in T47D<sup>EphA2</sup> and MCF-7<sup>EphA2</sup> cell lines

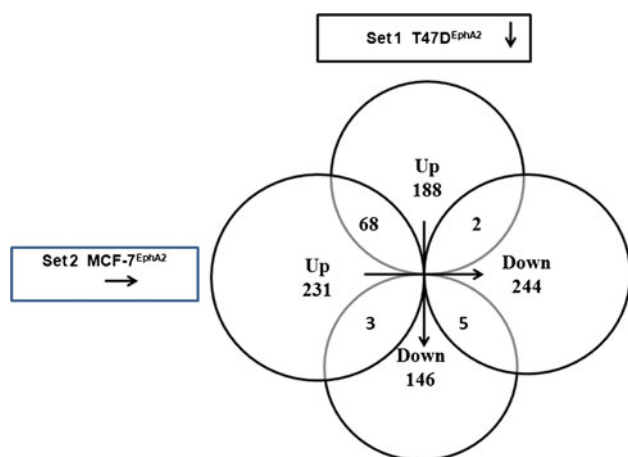
To identify the molecular mechanisms associated with the EphA2 overexpression, we compared gene expression profiles in T47D<sup>EphA2</sup> versus a T47D<sup>neo</sup> (Set 1) and MCF-7<sup>EphA2</sup> versus MCF-7<sup>neo</sup> (Set 2) cell lines using Agilent Microarrays. Differentially regulated genes were determined for commonly overlapping sets and each set alone. Figure 3 shows the comparisons of these genes in the Venn diagram based on the parameter delta in SAM. Of 258 genes upregulated in T47D<sup>EphA2</sup> compared to T47D<sup>neo</sup> cells (Set 1), 188 genes were unique to T47D<sup>EphA2</sup>; 68 genes were upregulated in MCF-7<sup>EphA2</sup> versus MCF-7<sup>neo</sup> cells (Set 2) while two genes were in opposite direction. 231 genes were uniquely upregulated in Set 2. Of 154 genes downregulated in T47D<sup>EphA2</sup> compared to T47D<sup>neo</sup> cells (Set 1), 146 were specific to T47D<sup>EphA2</sup>, while five genes were also downregulated in MCF-7<sup>EphA2</sup> versus MCF-7<sup>neo</sup> cells (Set 2). Three genes were in opposite directions. 244 genes were only downregulated in Set 2. Supplemental Table S3 shows the list of these genes.

### Wnt-pathway specific genes are altered in EphA2 overexpressing cell lines

The array analysis showed a number of genes from Wnt Pathway that were differentially regulated in T47D<sup>EphA2</sup> and MCF7<sup>EphA2</sup> cell line models. To validate the differential expression of potential Wnt genes, we performed qPCR analysis. Supplemental Table S4 summarizes the validation of the commonly altered Wnt-genes by qPCR in both models, while Supplemental Table S5 and S6 validate the expression of Wnt-pathway genes specific to set 1 (T47D<sup>EphA2</sup> only) and to set 2 (MCF-7<sup>EphA2</sup> only), respectively.

### Effect of estrogen on the selected Wnt-pathway specific genes at the protein level

We next examined the protein expression of a number of Wnt-pathway genes validated by qPCR in the absence and presence of E2 stimulation. Western blot analyses revealed



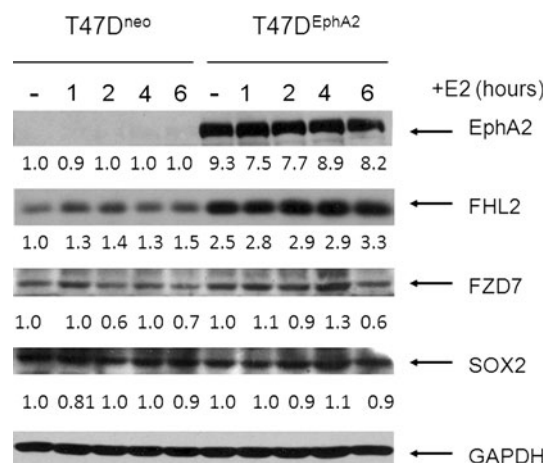
**Fig. 3** Comparison of gene expression profiles in ER/EphA2 over-expressing breast cancer cell lines. Venn diagram shows the numbers of upregulated and downregulated genes in T47D<sup>EphA2</sup> versus a T47D<sup>neo</sup> (Set 1) and MCF-7<sup>EphA2</sup> versus MCF-7<sup>neo</sup> (Set 2) cell lines

that expression of FHL2 markedly elevated in T47D<sup>EphA2</sup> compared to T47D<sup>neo</sup> control cells independent of the E2 time course (Fig. 4). In contrast, the protein levels of FZD7 and SOX2 expression remained similar in both T47D<sup>EphA2</sup> and T47D<sup>neo</sup> control cells. These data suggest that the expression of each Wnt family members was regulated differently in the absence and presence of E2 in EphA2-overexpressing cells at the mRNA and protein levels.

EphA2 overexpression does not activate TCF/ $\beta$ -catenin transcription in T47D<sup>EphA2</sup> cells

The Wnt signaling is a complex network that plays an important role in diverse cellular processes including cell fate determination, proliferation, control of cell movement, and invasion [23–26]. Many of the Wnt-regulated processes are mediated through well-studied canonical Wnt pathway, leading to the stabilization and nuclear translocation of  $\beta$ -catenin where it complexes with T-cell factor/lymphoid enhancer factor (TCF/LEF) family transcription factors and regulates the transcription of target genes [24]. In addition, the members of the Wnt family can stimulate  $\beta$ -catenin independent pathways that are involved with cytoskeletal organization and cell motility [23, 25], designated as noncanonical Wnt signaling.

To determine whether EphA2 overexpression induces the activation of canonical Wnt/TCF/ $\beta$ -catenin pathway, we measured the TCF-4 transcriptional activity by transiently expressing the luciferase TOPFlash TCF reporter plasmid in T47D<sup>EphA2</sup> and T47D<sup>neo</sup> control cells. We did not observe an increase in the TOPFlash luciferase reporter activity in T47D<sup>EphA2</sup> cells, indicating that  $\beta$ -catenin/TCF-4 complex is not active in EphA2-overexpressing cell line (data not shown). In addition, the levels



**Fig. 4** Protein levels of Wnt family members in T47D<sup>EphA2</sup> or T47D<sup>neo</sup> cells in response to E2 time course. After 48 h plating in phenol red-free media with 5% charcoal stripped fetal calf serum, cells were treated with vehicle (ethanol), EA5 (3  $\mu$ g/ml), Tamoxifen (4-OH-Tam;  $10^{-6}$  M) or in combination with EA5 and Tamoxifen in the absence or presence of E2 ( $10^{-10}$  M) for the indicated times and subjected to western blot analysis. The data are representative of three individual sets. The Western blots were scanned and quantified. Protein levels normalized for GAPDH, relative to T47D<sup>neo</sup> vehicle treatments, are indicated below the individual bands of the western blots

of  $\beta$ -catenin protein expression remained unchanged between T47D<sup>EphA2</sup> and T47D<sup>neo</sup> control cells (data not shown). These results are consistent with our observation that c-myc and cyclin D1, two targets of  $\beta$ -catenin/TCF transcriptional activation, are not upregulated in T47D<sup>EphA2</sup> cells, suggesting the involvement of noncanonical WNT pathways rather than the canonical pathways.

Dual targeting of EphA2 and ER inhibits phosphorylation of FAK at Tyrosine 925 residue (p-FAK<sup>Tyr925</sup>) in T47D<sup>EphA2</sup> cells

We next explored the role of focal adhesion kinase (FAK), a downstream target of EphA2 signaling [27, 28]. Increased FAK expression and tyrosine phosphorylation have also been associated with an invasive cell phenotype [29]. To examine the involvement of FAK in T47D<sup>EphA2</sup> cells in the absence and presence of E2, we assessed the phosphorylation levels of FAK by Western blotting using a phospho-FAK<sup>Tyr925</sup> specific antibody. We observed that EphA2 overexpression increases FAK phosphorylation at tyrosine 925 (Fig. 5a). This response is further augmented with E2 stimulation at 4 h. We further evaluated the effect of EA5, Tamoxifen alone or in combination on the inhibition of p-FAK<sup>Tyr925</sup> levels (Fig. 5b). EA5 and Tamoxifen in combination decreased the phosphorylation levels of FAK in T47D<sup>EphA2</sup> cells, suggesting a role for FAK signaling pathway in ER-EphA2 crosstalk and EphA2-mediated invasion.

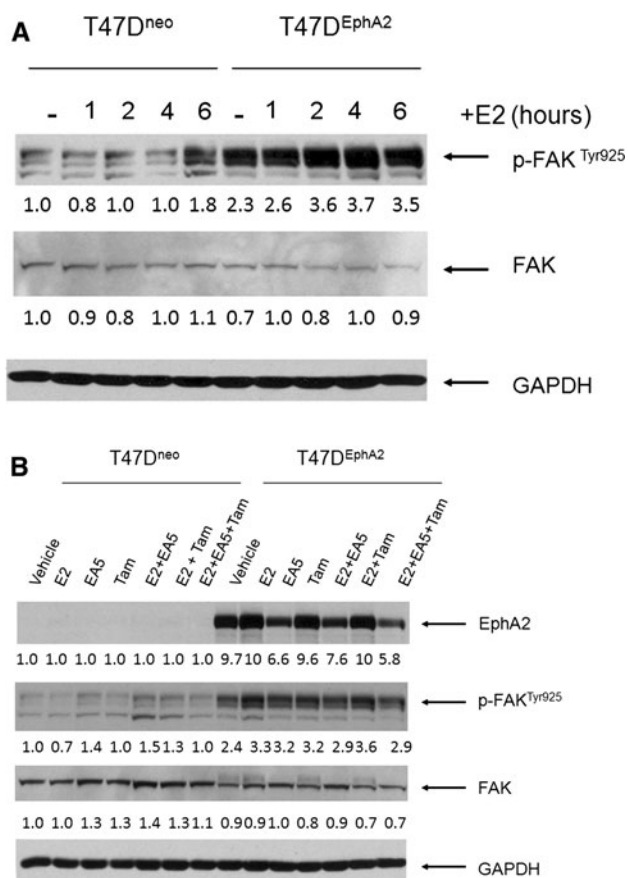
EphA2 overexpression increases the invasiveness of T47D cells, which can be blocked by dual inhibition of EphA2 and ER

It is important to assess the functional consequences of EphA2-driven signaling. Cell motility and invasion are two functional features that can be controlled by Wnt [26, 30], FAK [29, 31, 32], and EphA2-mediated signaling [33, 34]. To determine whether overexpression of EphA2 leads to increased invasiveness in T47D<sup>EphA2</sup> cells in relation to E2, we performed a Boyden chamber in vitro assay. As expected, T47D<sup>EphA2</sup> cells exhibited a significantly higher invasive capacity (326%;  $P = 0.0009$ ) compared to T47D<sup>neo</sup> cells (vehicle treated) which are normalized to 100% value (Fig. 6). The invasiveness of T47D<sup>EphA2</sup> cells further increased with E2 (509%) when compared with T47D<sup>EphA2</sup> cells without E2 (326%). However, this level did not reach statistical significance ( $P = 0.0741$ ). On the other hand, the invasiveness of T47D<sup>EphA2</sup> cells (326%) was dramatically inhibited (26%;  $P = 0.0001$ ) below the

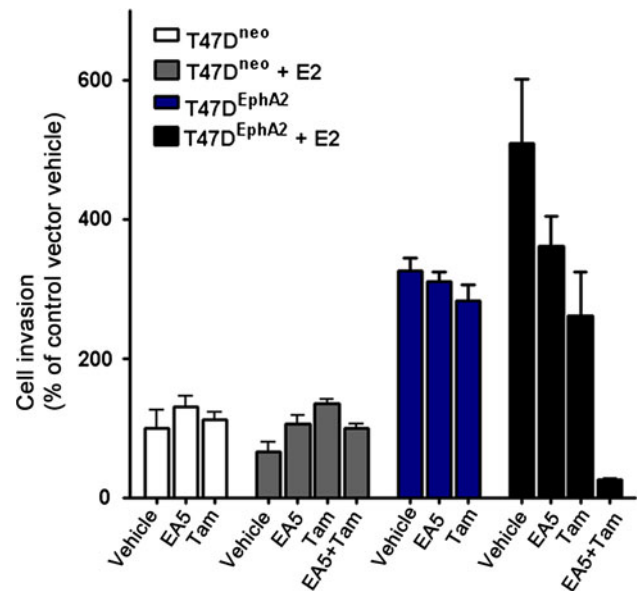
basal T47D<sup>neo</sup> control cells in combination of EA5 and Tamoxifen treatment, suggesting that EphA2 overexpression increases the invasiveness of T47D cells which can be blocked by dual inhibition of EphA2 and ER.

## Discussion

EphA2 functions as a key “molecular switch” to either stimulate or inhibit cell growth, metastasis and angiogenesis. In non-malignant cells, ligand binding causes EphA2 to negatively regulate cell growth and motility. In contrast, EphA2 fails to properly interact with its ligands and promotes uncontrolled tumor cell growth, angiogenesis, and invasion. The use of EphA2 monoclonal antibody EA5 is particularly attractive, as it mimics ligand binding and redirects the function of an overexpressed molecule, essentially flipping the ‘molecular switch’ back to the normal position [16]. Given the prevalence of EphA2 overexpression and altered function in breast cancer, targeting EphA2 using an EphA2 ligand mimicking antibody is a promising approach in breast cancer. We earlier reported that EphA2 decreases estrogen dependence and induces resistance to Tamoxifen [15]. We also demonstrated that antibody targeting of EphA2 reverses the effect of EphA2 overexpression and resensitizes these cells to Tamoxifen in vitro linking EphA2 to altered ER function and Tamoxifen resistance. However, the interplay between EphA2 and ER is complex.



**Fig. 5** Phosphorylation of p-FAK<sup>Tyr925</sup> occurs only in T47D<sup>EphA2</sup> cells and increases in response to E2 that can be inhibited in combination with EA5 and Tamoxifen. **a** T47D<sup>EphA2</sup> or T47D<sup>neo</sup> cells were subjected to E2 time course and Western blotting for the indicated antibodies. **b** Cells were treated as in Fig. 4)



**Fig. 6** EphA2 overexpression increases the invasiveness of T47D cells which can be blocked by dual inhibition of EphA2 and ER. To evaluate the invasion capacity, Boyden invasion assay was performed. Data is presented as percent of control invasion (T47D<sup>neo</sup> vehicle = 100%)

In this study, we first determined the efficacy of EA5 on *in vivo* tumor growth in ER+ breast cancer models. EA5 selectively inhibited the primary tumor growth of EphA2—overexpressing xenografts. Our results are consistent with our earlier reports that EphA2 antibodies can selectively inhibit malignant growth both *in vitro* and *in vivo* [16, 35]. As expected, EA5 also restored Tamoxifen sensitivity *in vivo* in agreement with our earlier *in vitro* data [15].

We next asked whether these observations are MCF-7 cell line specific or generalized for other ER+ breast cancer models. To this end, we generated another EphA2-overexpressing ER+ cell line (T47D<sup>neo</sup>/T47D<sup>EphA2</sup> model). Our new data that EphA2 overexpression decreases ER activation in T47D<sup>EphA2</sup> model supports the concept that ER and EphA2 interplay exists. Direct binding of ER $\alpha$  to other cell surface receptors has been demonstrated [36]. Our data ruled out the direct interaction of EphA2 with ER. However, this may suggest the existence of an indirect relationship, such as EphA2 may affect ER coregulator expression or function. It is well-established that crosstalk between ER and growth factor signaling can alter the coregulator expression levels and response to endocrine therapy [22]. On the contrary, we did not find any correlation between EphA2 overexpression and protein levels of ER coactivators (SRC-1 and AIB1) or ER corepressors (NCOR1 and SMRT) in T47D<sup>neo</sup>/T47D<sup>EphA2</sup> model. We also did not observe any consistent relationship between the above coregulators and ER activity in this model even in the presence of E2 stimulation. We next investigated the effect of EphA2 on the canonical ER target genes, such as cyclin D1 and *c-myc*. We did not observe any significant cyclin D1 or *c-myc* expression in this model, suggesting the involvement of other noncanonical targets in ER and EphA2 crosstalk.

We next sought to identify novel potential targets that were differentially regulated in both ER/EphA2-overexpressing T47D<sup>EphA2</sup> and MCF-7<sup>EphA2</sup> cell line models. Comparative analysis revealed overlapping and distinct gene profiles (Supplemental Table S3), suggesting that several signaling pathways were differentially regulated in both models. One prominent observation from the gene expression analysis was the differential regulation of a group of genes associated with Wnt pathway in both cell lines (Supplementary Tables S4, S5, S6). Among these Wnt-genes, we validated the upregulation of *FHL2*, *CDH5* and *WNT6* genes and downregulation of *ARMCX2* at the mRNA levels in T47D<sup>EphA2</sup> cells. *FHL2* protein levels were also significantly upregulated in T47D<sup>EphA2</sup> cells regardless of E2 stimulation. It is intriguing that the role of Wnt/ $\beta$ -catenin pathways has been earlier shown in supporting estrogen-independent cell growth of MCF-7-fulvestrant resistant subline (MCF7-F), but not MCF7-Tamoxifen subline (MCF7-T) [37]. In addition, EphA2 was

upregulated in MCF-F subline and stayed unchanged in MCF7-T subline. As expected,  $\beta$ -catenin was up-regulated in both MCF7-T and MCF7-F, but only activated in MCF-7-F cells. In contrast, in our study, we did not observe any increase in  $\beta$ -catenin/TCF-4 complex using the T47D<sup>EphA2</sup> model, suggesting the involvement of noncanonical Wnt pathway players in our system.

The link between EphA2 and Wnt signaling is an underexplored area in cancer. EFN/EPH and Wnt signaling pathways network together during embryogenesis, tissue regeneration and carcinogenesis. Using comparative genomics analyses, *EFNB3* was identified as potential target gene of the Wnt/ $\beta$ -catenin signaling pathway [38]. Eph/EphrinB receptors are Wnt signaling target genes that might be associated with invasion and metastasis [39]. However, no data is available for the relationship between Wnt and EphA2 signaling. Wnt signaling is context-dependently transduced to at least two distinct pathways: the well-studied canonical Wnt/ $\beta$ -catenin pathway and the  $\beta$ -catenin independent noncanonical Wnt pathway [23, 39]. Noncanonical pathway is further divided into Wnt/Ca<sup>2+</sup> signaling and Wnt/planar cell polarity (PCP) signaling. Although the Wnt signaling is very complex and context dependent, accumulating evidence supports the role of PCP signaling in cancer progression, invasion, metastasis, and angiogenesis [39]. Further detailed studies are necessary to reveal the exact nature of interaction between EphA2 and Wnt signaling.

We next explored the role of FAK, a focal adhesion kinase downstream of EphA2 signaling. Interestingly, EphA2 overexpression increased FAK phosphorylation at tyrosine 925 which will be further enhanced by E2 stimulation. EA5 and Tamoxifen in combination decreased the phosphorylation levels of FAK in T47D<sup>EphA2</sup> cells, suggesting a role for FAK in E2-induced mechanisms and Tamoxifen resistance for the first time. FAK is involved in the regulation of Rho family GTPases which are also key mediators of Wnt signaling pathway [40]. Further studies are necessary to determine the connection of EphA2 and FAK to Wnt signaling.

A significant impact of targeting EphA2 and ER was the suppression of the invasive characteristics of the EphA2 overexpressing cell line T47D<sup>EphA2</sup> compared to T47D<sup>neo</sup> cell line. We demonstrated that EphA2 overexpression increases the invasiveness of T47D cells which can be blocked by dual inhibition of EphA2 and ER. Taken together, our findings confirm the functional importance of EphA2 overexpression in ER+ breast cancers. To our knowledge, this is the first study linking EphA2 overexpression to a more invasive type in ER+ breast cancers and Tamoxifen resistance. In addition, dual targeting of EphA2 and ER may provide a significant therapeutic intervention in ER+/EphA2+ breast cancer and improve the response to antiestrogen therapies.



**Acknowledgments** We thank Dr. D. Knapp (Purdue University) for EphA2 constructs; Dr. M-H Jeng (Indiana University) for ERE-luciferase, TK-luciferase, purified GST, GST-ER alpha, SRC-1 and AIB1 plasmids; Dr. H. Nakshatri (Indiana University) for NCoR1 and SMRT plasmids. We also thank Karen Coffman (MedImmune) for her assistance in providing EA5 antibody. Gene array was by Miltenyi Biotech Inc. Microarray Service. This work was supported by Susan G. Komen grant (BCTR0503531) to K.D. Miller.

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