Ephrin-B2 inhibits cell proliferation and motility *in vitro* and predicts longer metastasis-free survival in breast cancer

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Abstract. The tyrosine kinase receptor EphB4 and its ligand ephrin-B2 interact through cell-to-cell contacts. Upon interaction, EphB4 transmits bidirectional signals. A forward signal inside EphB4-expressing cells is believed to suppress tumor growth, while inside the ephrin-expressing cells, an oncogenic reverse signal arises. In breast cancer cells with a high EphB4 receptor expression the forward signal is low, in part due to the low expression of the ligand ephrin-B2. Therefore, we hypothesized that by re-introducing the ligand in EphB4-positive cells, tumor suppression could be induced by the stimulation of the forward signal. This question was addressed *in vitro* by the stable lentiviral infection of breast cancer cells with either wild-type *EFNB2* or with a mutant *EFNB2-5F*, unable to transmit reverse signaling. Furthermore, we investigated ephrin-B and EphB4 protein expression in 216 paraffin-embedded tumors using immunohistochemistry. The *in vitro* results indicated that ephrin-B2 expression was associated with a lower cell proliferation, migration and motility compared with the control cells. These effects were more pronounced when the cells lacked the ability to transmit the reverse signal (B2-5F). In clinical material, ephrin-B protein expression was associated with a positive estrogen receptor (ER) status, a low HER-2 expression and was negatively associated with Nottingham histologic grade (NHG) III. Ephrin-B expression indicated a good prognosis, whereas EphB4 expression was associated with a shorter metastasis-free survival in univariate and multivariate analysis. Furthermore, the prognostic value of *EFNB2* and *EPHB4* was confirmed at the gene expression level in public datasets. Thus, on the whole, the findings of this study suggest that ephrin-B2 expression is associated with less proliferation and lower motility of breast cancer cells and with a longer patient survival in breast cancer.

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Introduction

Few markers, such as receptor tyrosine kinases (RTKs) from the epidermal growth factor receptor (EGFR) family, are currently used in clinical practice to estimate patient prognosis or determine the appropriate treatment course. However, some patients continue to develop metastasis, thus indicating the need for novel clinical markers and therapeutic targets.

Members of the Eph (from the erythropoietin-producing hepatocellular cell line where it was first cloned) receptors have been found to be overexpressed in screens of RTKs in cancer (1,2). The Eph receptor was first identified in an erythropoietin-producing human hepatocellular carcinoma cell line (ETL-1) (3) in an effort to discover novel tyrosine kinase receptors. This family now constitutes the largest family of RTKs participating in cell-to-cell communications and tissue integrity during embryogenesis, as well as in cell proliferation, survival and motility, which are crucial steps towards the development of metastases (1,4).

The Eph receptors and their ligands, eph receptor interacting proteins (ephrins) interact upon cell-to-cell contacts triggering bidirectional signals both inside the Eph (so-called, forward signal) and the ephrin-expressing cells (so-called, reverse signal). The forward signal is believed to suppress the tumor, while the reverse signaling is thought to promote tumor growth. Thus, Eph receptor and ephrin signaling is complex, leading to paradoxical results both *in vitro* and *in vivo* (1).

EphB4 and EphA2 are some of the most extensively studied Eph receptor family members in breast cancer. EphA2

is related to a poor breast cancer prognosis and resistance to trastuzumab (5) and tamoxifen (6-9). EphB4 overexpression has been shown to be associated with a poor patient outcome and may be a survival factor for breast cancer cells (6,10,11). However, the results are still controversial: EphB4 could be highly expressed in breast cancer cell lines compared to non-transformed epithelial cells (12); however, in clinical samples, the receptor has been shown to be associated with a low histological grade and it is expressed at lower levels in invasive carcinomas compared to normal breast tissue (13).

The tumorigenic properties of EphB4 may manifest in the absence of its preferred ligand, ephrin-B2, as suggested by EphB4 upregulation in mammary epithelial cells, where the expression of the ligand ephrin-B2 seems to be lost (14). Previous results have indicated that stimulation with a soluble ephrin-B2-Fc ligand inhibits tumor formation and growth in a breast cancer xenograft model (12).

Therefore, in this study, we wished to address the question whether the re-expression of ephrin-B2 in breast cancer cells, where the EphB4 receptor is present, could inhibit the tumorigenic properties of these cells. To examine the effects of EphB4 and ephrin-B2 co-expression *in vitro*, we used two lentiviral vectors to stably infect MCF7 cells with a wild-type *EFNB2* (B2-WT) or a mutant *EFNB2* (B2-5F) which is unable to transmit reverse signaling. We found that the *EFNB2*-expressing cells (MCF7-B2) exhibited a lower proliferation, formed less foci and had an impaired cell motility. The MCF7-B2 cells were also less responsive to growth factor-induced migration. In particular, the mutant B2-5F cells, only able to transmit the forward signal, exhibited a decreased expression of several genes involved in cell motility.

Of note, similar results were observed in our patient material where ephrin-B and EphB4 protein expression were investigated by immunohistochemistry in paraffin-embedded tissues from 216 patients. Ephrin-B2, but not EphB4 expression indicated a longer metastasis-free survival in both univariate and multivariate analyses compensating for known clinical markers. Moreover, a validation survey in public datasets confirmed that a high *EFNB2* gene expression was associated with a longer distant recurrence-free survival, whereas a high *EPHB4* expression indicated a poor prognosis, particularly for the group of patients whose tumors expressed *EPHB4* in the absence of *EFNB2*. Altogether these results suggest that ephrin-B2 may have clinical value in breast cancer.

Materials and methods

Cell culture and lentiviral infection. MCF7 cells were purchased from the American Type Culture Collection (ATCC® No. HTB-22), which also validated the cell identity as Luminal A. In addition, a PCR Mycoplasma Test kit I/C from PromoKine (PromoCell GmbH) was used to periodically examine the cells for mycoplasma infection. The cells were routinely cultured in Eagle's MEM medium (Gibco, Thermo Fisher Scientific) containing 2 mM L-glutamine and further supplemented with Earle's Balanced Salt Solution, 1.5 g/l bicarbonate, 0.1 mM non-essential amino acids, 1 mM Na pyruvate, 10 μg/ml bovine insulin, 10% FBS and antibiotics (12). For lentiviral infection, the MCF7 cells were seeded at a density of $60x10^3$ cells/well in a 24-well plate. Following overnight

incubation at 37˚C in a CO_2 incubator, the cells were infected with the following multiplicities of infection (MOI): The *GFP* (MOI=5), *GFP-EFNB2-WT* (MOI=7) or *GFP-EFNB2-5F* (MOI=10). Lentiviral vectors were added in the presence of polybrene (3 μg/ml) (Millipore) and the supernatant was removed 16 h post-infection. Green fluorescent cells, i.e., successfully transduced cells, of similar intensity were sorted by FACS and maintained in culture for use in further experiments. Ephrin-B2 expression was periodically controlled by fluorescence microscopy using a Radiance Multiphoton Laser Point Scanning Confocal Microscope or by immunoblotting.

Antibodies, siRNAs and vectors. Primary antibodies used for immunoblotting and/or immunoprecipitations were as follows: Mouse anti-EphB4 (clone 3D7/G8, cat. no. 37-1800, Zymed/Invitrogen), mouse anti-pan ephrin-B (clone 2D3E6 cat. no. 37-8100, Zymed), anti-phosphotyrosine-conjugated with HRP (4G10, cat. no. 05-321, Millipore), mouse anti-GAPDH (cat. no. ab185059, Abcam), rabbit anti-GFP (cat. no. GTX20290, GeneTex), rabbit anti-signal transducer and activator of transcription 3 (STAT3), purchased from Cell signaling Technology (cat. no. 87685 and anti-SH3 and PX domains 2A (SH3PXD2A), obtained from Nordic Biosite (cat. no. AP16560a). Secondary polyclonal antibodies conjugated with horseradish peroxidase (HRP) goat anti-rabbit (cat. no. P0448) and goat anti-mouse (cat. no. P0447) were purchased from Dako (Dako Denmark) For the EphB4 antibody validation the following siRNAs were used: FlexiTube EphB4 GS0250 (#1; SI00063748, #2; SI00288596, #3; SI00063791, #4; SI00288589. Further, we used the AllStar Negative Control (SI03650318, Qiagen) and the Silencer Control GAPDH Positive Control (Life Technologies, Thermo Fisher Scientific). Plasmids and lentiviral vectors were as follows: Mouse ephrin-B2 (GenBank accession no. NM_010111.5) with an N-terminal eGFP tag inserted between a signal peptide and the mature coding sequence (15,16) was cloned into the pCCLsin.PPT.hPGK.GFP pre-lentiviral vector (17) replacing the eGFP insert of the vector. A mouse tyrosine phosphorylation-deficient ephrin-B2 (ephrin-B25F), in which tyrosine residues at 307, 314, 319, 333 and 334 were substituted with phenylalanine was modified by PCR-based techniques. The pCCLsin.PPT.hPGK.GFP pre-lentiviral vector encoding eGFP was used as a control. All PCR-amplified and mutated cDNAs were verified by sequencing (18). The viral vectors were a gift from Dr Ombretta Salvucci (Basic Research Laboratory, Laboratory of Cellular Oncology, NIH/NCI, Bethesda, MD, USA).

Immunoblotting and immunoprecipitations. MCF7 cells were seeded in complete medium into 60-mm plates ($5x10^5$ cells/plate) for immunoprecipitation and immunoblotting. The cells were lysed in cold RIPA buffer supplemented with a protease inhibitor cocktail (Cat. no. P2850, Sigma). The total protein concentration was calculated by BCA assay (Pierce, Thermo Fisher Scientific) and the samples were adjusted to the same concentration prior to further experiments. Total cell lysates were diluted with 2X Laemmli sample buffer and boiled for 5 min at 95˚C prior to electrophoresis. For immunoprecipitation, cell lysates were further diluted to 1 ml in RIPA buffer, added to the tubes with the

primary antibody and the beads (gamma bind Plus Sepharose, GE Healthcare) and rocked for 1 h at 4˚C. The supernatant was discarded after repeated centrifugation steps at 14,000 rpm at 4˚C and the beads remaining in the pellet were washed and boiled at 95˚C for 5 min. For SDS-PAGE, equal amount of protein was loaded/sample in a gradient TGX gel 4-15%. Primary antibodies (at the indicated dilutions) were incubated overnight at 4˚C to detect ephrin-B (1:200), total phosphotyrosines (1:1,000), GAPDH (1:5,000) or GFP (1:1,000) in the total lysates or the immunoprecipitates. HRP-conjugated secondary antibodies were diluted 1:2,000 in 5% milk blocking buffer and incubated for 1 h at room temperature. The free software ImageJ v1.4 (N.I.H, USA) was used to estimate the relative band intensity in the blots. Briefly, the same region of interest (ROI) was selected for all the bands and the corresponding background below each band for both, the protein of interest and the loading control. The pixel density (intensity) of each band and its corresponding background was measured. Net values after subtracting the background were used to calculate the relative ratio protein of interest/loading control.

Immunofluorescence. The MCF7 cells were seeded in coverslips coated with 10 μg/ml human fibronectin (Millipore) and fixed in 3.7% formaldehyde/PBS for 15 min. The slides were prepared with anti-fading mounting medium containing DAPI and examined in an Inverted Nikon Eclipse TE300. Images were analyzed with the software SPOT advanced version or in a LM Zeiss inverted confocal microscope at x600 magnification.

Cell proliferation, spheroid and colony formation assays. Prior to each experiment, the cells were counted and adjusted to the indicated cell density: For cell proliferation $5x10^3$ cells were plated in 100 μl complete medium/well in 96-well plates in quadruplicate and harvested daily for up to 5 days. Cell proliferation was estimated by MTT assay following the protocol recommendations. Briefly, 15 μl of MTT stock solution at 0.5 mg/ml were added to each well and incubated for 4 h in a CO_2 incubator at 37˚C. The amount of MTT product, dissolved in 0.2 ml DMSO, was estimated at 570 nm.

For the spheroid formation assay, the cells ($1x10^3$/100 μl) were seeded in ultra-low cluster 96-well plates (Costar, Corning 7007). The spheroid formation was initiated by centrifuging the plates at 1,000 x g for 10 min at room temperature using a centrifuge with swinging buckets. The plates were incubated for up to 11 days and the medium was renewed every second day. The spheroid formation was monitored after 1, 3, 7 and 11 days. Each time the fluorescent area occupied by the spheroids from 10 wells was calculated with the free software Image J v.1.4.

The ability of the cells to form colonies was investigated using a colony formation assay where 10^3 cells were plated in 2 ml/well in 6-well plates and supplemented by replacing 1 ml medium every other day for up to 11 days. Fluorescent colonies were photographed in an Inverted Nikon Eclipse TE300 equipped with a camera coupled to the microscope and the images analyzed with the software SPOT advance version on the last day before removing the culture medium. Subsequently, the cells were washed and fixed with 3.7% paraformaldehyde for 15 min at room temperature. The cells were then stained with 1% crystal violet solution for 30 min. The excess of dye was washed away and the plates were scanned. In order to quantify the colonies, the cells were dissolved in 1% SDS/PBS solution and the supernatants were transferred to 96-well plates to determine the optical density at 570 nm.

Transwell assay. Transwell assay was used to measure the ability of the cells to migrate through a membrane toward a gradient of growth factors. Cell inserts with 8 μm pore membrane (cat.# 353182, BD Bioscineces) were coated with human fibronectin (10 μg/ml) and introduced into the wells of a 12-well plate. Following overnight incubation at 4˚C, the inserts were washed and blocked for 1 h with 1% BSA/PBS. The cells were detached with the enzyme acutase (Sigma) and centrifuged for 5 min at 1,200 rpm at room temperature to wash the pellet in starvation medium (medium w/o insulin and 0.5% FBS). The cells were placed in the insert at a density of $1x10^5$ cells/0.2 ml starvation medium. A growth factor gradient was created in the wells by first adding a mixture of 5 ng/ml heregulin-β1 (HRGβ1) and epidermal growth factor (EGF) at the bottom of the wells and then filling up with 1.2 ml starvation medium. Cell migration was assessed after 20 h of incubation in 5% CO_2 and 37˚C. The cells remaining at the top of the insert membrane were removed with cotton tip applicators, repeating the operation at least 5 times. The remaining cells in the lower part of the insert membrane were fixed with 3.7% formaldehyde, permeabilized with 0.1% triton and stained with Hoechst (1 μg/ml) for 10 min. The membranes were cut and mounted with the bottom facing up with prolong anti-fading mounting medium (Molecular Probes). Images of 6-10 random fields were acquired at x100 magnification using an inverted fluorescence microscope Zeiss Axiovert A1.

Wound-healing assay. Two-dimensional invasion assays were performed to assess lateral migration. Silicon culture inserts inside a 35-mm dish were used (ibidi). The growth area/well was 0.22 cm², allowing up to 70 μl culture medium. The inserts were filled with $8x10^4$ cells to allow confluence. Upon cell attachment, the cell monolayer was washed twice with PBS and starved overnight. The following day, the inserts were removed leaving a 500±50 μm wound and the dish was washed to remove unattached cells and then refilled with 2 ml complete medium. Images at x40 magnification of two different areas of the wound were acquired at several time points between 0 h (control) and 45 h. The wounded area between the two migrating layers of fluorescent cells was calculated using public software Image J v1.4 (NIH).

Cell motility PCR array. RNA was extracted from $5x10^6$ cells with mini RNeasy kit (Quiagen). Cell lysis was performed in 0.5 ml lysis buffer supplied by the kit. The RNA concentration was calculated using a nanodrop spectrophotometer and the RNA quality using the Bioanalyzer (Agilent) with the RNA 6000 Nano kit. The RNA concentration was adjusted to 250 ng/μl. A 96-well plate format of the human cell motility RT² Profiler™ PCR Array (Qiagen) was used to analyze the expression of 84 genes involved in cell motility. PCR reactions were performed on ABI Prism 7900.

For data analysis, the Ct and baseline were calculated and exported to the online tool RT^2 profiler PCR array data analysis v3.5 (Qiagen). The housekeeping genes in the experiment were *B2M*, *HPRT1*, *RPL13A*, *GAPDH* and *ACTB*. The formula used to calculate the relative gene expression level [2^(-ΔCt)] was the following: ΔCt=Ct (gene of interest)-avg. Ct (housekeeping genes). The formula 2^(-ΔΔCt) was used to calculate fold changes between the test samples (cells expressing ephrin-B2) and control samples (GFP-expressing cells). The experiment was performed in triplicate in 3 independent occasions. Differential expression ≥2 was reported in the figures at a significance level of P<0.05.

Patient material. Tumor samples were collected within the Stockholm clinical trial (1976-1990) (19). The trial included pre-menopausal and post-menopausal women with a unilateral, operable breast cancer. Only pre-menopausal patients were included in this study. The surgical procedure was modified radical mastectomy. Further inclusion criteria were either histologically verified lymph node metastasis or a tumor diameter, exceeding 30 mm, measured on the surgical specimen. Patients were randomized to receive either adjuvant chemotherapy or radiotherapy. Patients in the chemotherapy group received 12 courses of cyclophosphamide, methotrexate, 5-fluorouracil according to the original Milan protocol (100 mg/m^2 cyclophosphamide orally at days 1-14, 40 mg/m^2 methotrexate and 600 mg/m^2 5-fluorouracil intravenously on days 1 and 8). However, during the first 18 months of the trial, 10-15 mg chlorambucil was administered orally on days 1-8 instead of cyclophosphamide and to avoid dose reductions up to 18 months treatment time was allowed for the last 12 courses. Patients randomized to radiotherapy, received a dose of 46 Gy with 2 Gy per fraction 5 days a week. Total treatment time was approximately 4.5 weeks and the target volume included the chest wall, the axilla, the supraclavicular fossa and the internal mammary nodes. Estrogen receptor (ER) (19) and human epidermal growth factor receptor 2 (HER2) protein level (20) clinical variables were obtained from previous studies. The initial material included 547 pre-menopausal patients. Formalin-fixed and paraffin-embedded tissues from 216 patients were available on tissue microarray (TMA) and included in this study (Fig. 1). Patient characteristics did not significantly differ from the ones included in the larger Stockholm trial (21).

Immunohistochemistry. The patient material used to explore EphB4 and ephrin-B protein expression was included in TMA slides. Breast cancer samples from 216 patients from the Stockholm II trial were analyzed as previously reported (21). Briefly, the slides were baked for 2 h at 60°C before the PT-Link system (Dako) was used at pH 6.0 for 20 min at 97°C for deparaffinization, rehydration and antigen retrieval. Primary antibodies were diluted 1:50 (mouse anti-EphB4) or 1:200 (mouse anti-pan ephrin-B) with overnight incubation at 4°C. The HRP-conjugated secondary antibody (Envision+System-HRP Labelled-Polymer anti mouse, Dako, Ref#4002) was used with incubation for 30 min. Cell nuclei were counterstained with Mayer's Hematoxylin prior to stepwise dehydration in an ethanol gradient. Images were acquired with the Aperio Scanscope AT Turbo (Leica Biosystems) with
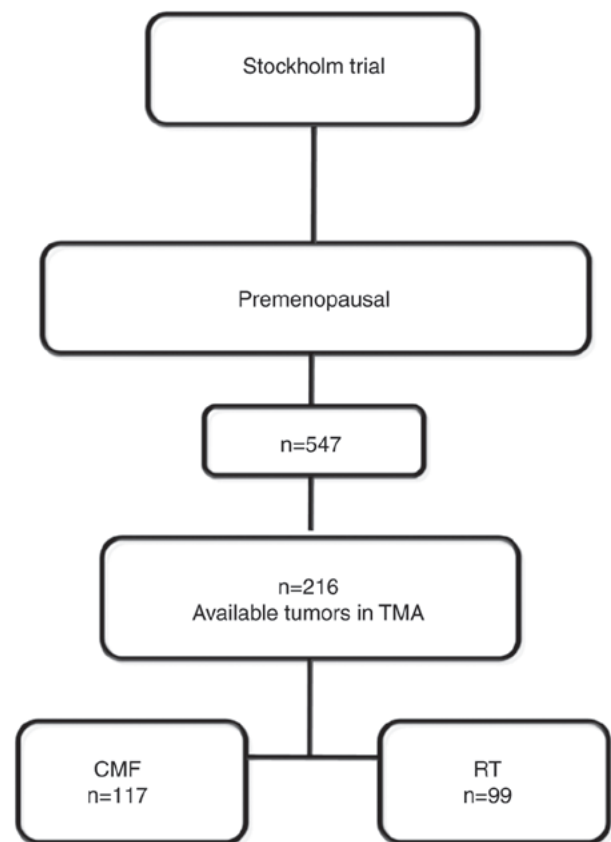

Figure 1. Patient material from the randomized Stockholm trial. Available tumors from 216 premenopausal patients from the Stockholm trial (n=547) were embedded in paraffin and tissue cores were arrayed in TMA slides. Out of these, 117 patients were randomized to receive chemotherapy (CMF) and 99 patients, received radiotherapy (RT).

20x/0.75 NA Plan Apo at x20 magnification. The software Aperio ImageScope v.11 was used for image analysis and the free software ImageJ v.1.440 (NIH, USA) was used to quantify the intensity of the bands when validating the EphB4 antibody by immunoblotting.

Public gene expression datasets. The exploratory study was performed in the following gene expression datasets: Karolinska Institute (KI) (GSE1456, n=159) (22) and van de Vijver (n=295) (23) and http://bioinformatics.nki.nl/data.php. For the statistical analysis, the *EFNB2* gene expression data were divided into quartiles (q) where q1 was defined as low expression and q2-4 was high expression. When several probes were used to detect the mRNA expression (KI) and the probes were positively correlated, the average of the gene expression data was used for the analysis. For *EPHB4*, q1-3 was low and q4 was high expression.

Statistical analyses. The statistical analysis for protein expression, in the clinical material, was performed using Statistica 64 version 12.0 software (StatSoft. Inc.). The association with known clinical variables in breast cancer was tested with the Pearson's Chi-square or Spearman's rank correlation tests. Cox regression was used in univariate and multivariate analyses to examine whether there was an independent association between EphB4 and ephrin-B protein
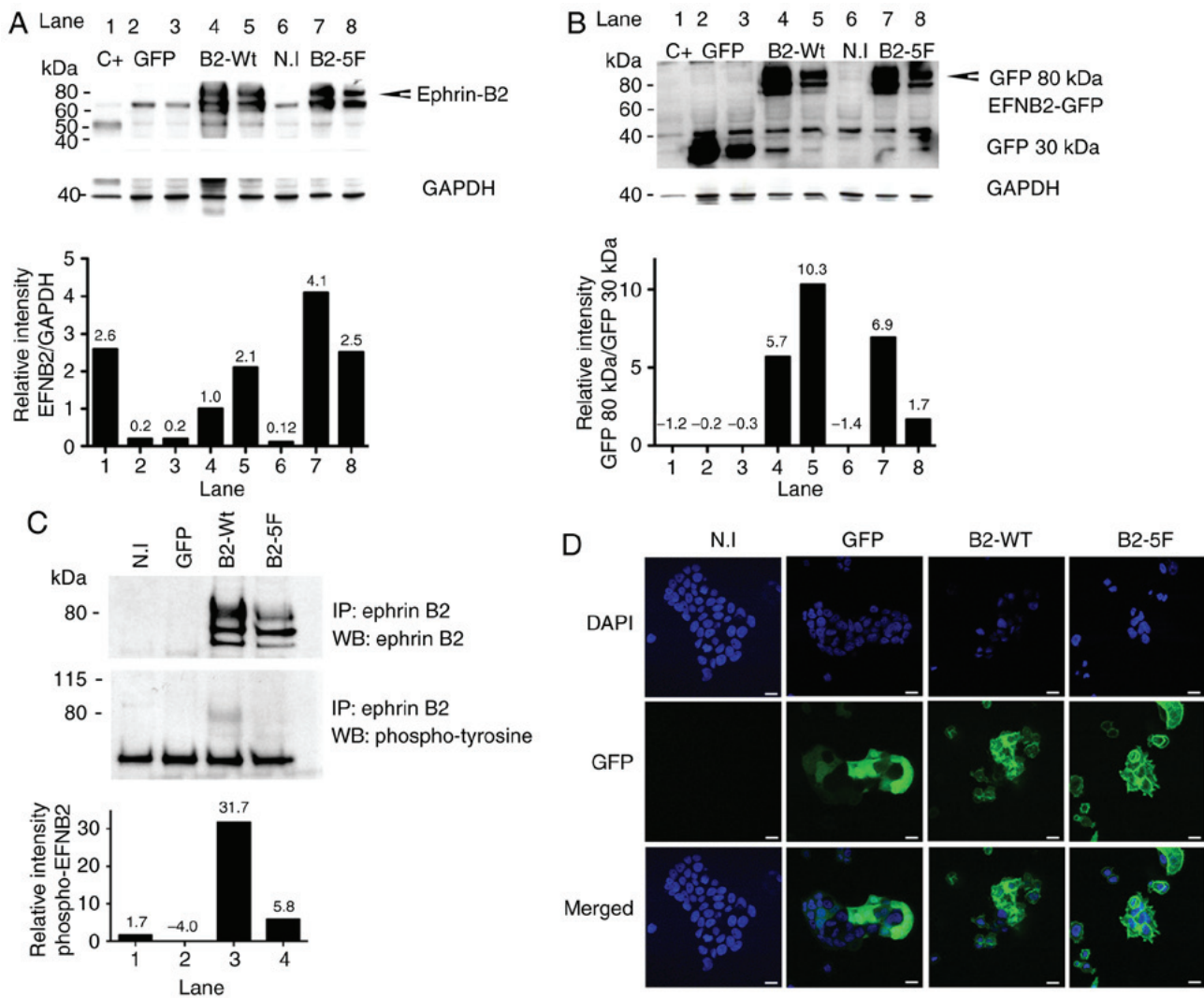
Figure 2. Generation of MCF7-B2 cells. Infected cells with viral vectors expressing GFP (lanes 2 and 3), ephrin-B2-WT (B2-WT) (lanes 4 and 5) and ephrin-B2-5F (B2-5F) (lanes 7 and 8) were treated with two multiplicities of infection (MOI): MOI 25 (lanes 2, 4 and 7) and MOI 1.5 (lanes 3, 5 and 8). Total lysate from SKBR3 cells infected with human ephrin-B2 (EX-M0409-Lv105, Genecopoeia) (~50 kDa) was used as a positive control (C+). Non-infected MCF7 cells (N.I) were used as a negative control. GAPDH was used as a loading control. Ephrin-B2 detected with the anti-pan (A) ephrin B antibody or (B) anti-GFP antibody and visualized as an 80 kDa GFP-fusion protein indicated by arrowheads. Ephrin-B2 activation was verified by (C) immunoprecipitation and column charts, from this representative experiment, and representative immunoblots are shown. Ephrin-B2 expression was visualized by confocal microscopy at x600 magnification. (D) Cell nuclei are indicated with DAPI staining. Scale bars, 30 µm.

expression and the presence of distant metastases, local metastasis or death due to breast cancer. The survival analysis to estimate probabilities for distant recurrence-free survival (time from surgery until distant metastasis was detected), loco regional recurrence-free survival (time from surgery until loco-regional recurrence was detected) and breast cancer-free survival (period from surgery until death due to breast cancer) was performed by comparing survival in multiple samples and represented with the Kaplan-Meier plots with the indicated hazard ratios, calculated with the Cox proportional hazards model. The statistical analysis for the *in vitro* part was carried out using software Prism from GraphPad Software. Statistically significant differences between the controls and B2-expressing cells were assessed by ANOVA followed by Bonferroni's multiple comparison post hoc test. Otherwise, the unpaired t-test was used when comparing 2 groups. The experiments were repeated at least 2 times and each experiment included >3 replicates.

Results

Ephrin-B2 expression in MCF7 cells. Previous research has demonstrated that breast cancer cell lines, in particular MCF7 cells, express low levels of ephrin-B2 in the presence of high EphB4 receptor levels (12). Lentiviral vectors encoding GFP fusion proteins with either wild-type ephrin-B2 (B2-WT) or a phosphotyrosine-deficient ephrin-B2 (B2-5F) were used to overexpress ephrin-B2 in MCF7 breast cancer cells. Ephrin-B2 expression was monitored by immunoblotting (Fig. 2A and B) and by immunoprecipitation (Fig. 2C).

Ephrin-B2 was tyrosine-phosphorylated in the B2-WT cells, but not in the B2-5F cells as was expected. B2-5F cells contain 5 phenylalanine residues instead of tyrosines in the carboxyterminal domain, which renders the protein unable to transduce phosphotyrosine-dependent-reverse signaling (see antibodies, siRNAs and vectors). Ephrin-B2 expression was also visualized by immunofluorescence allowing for the
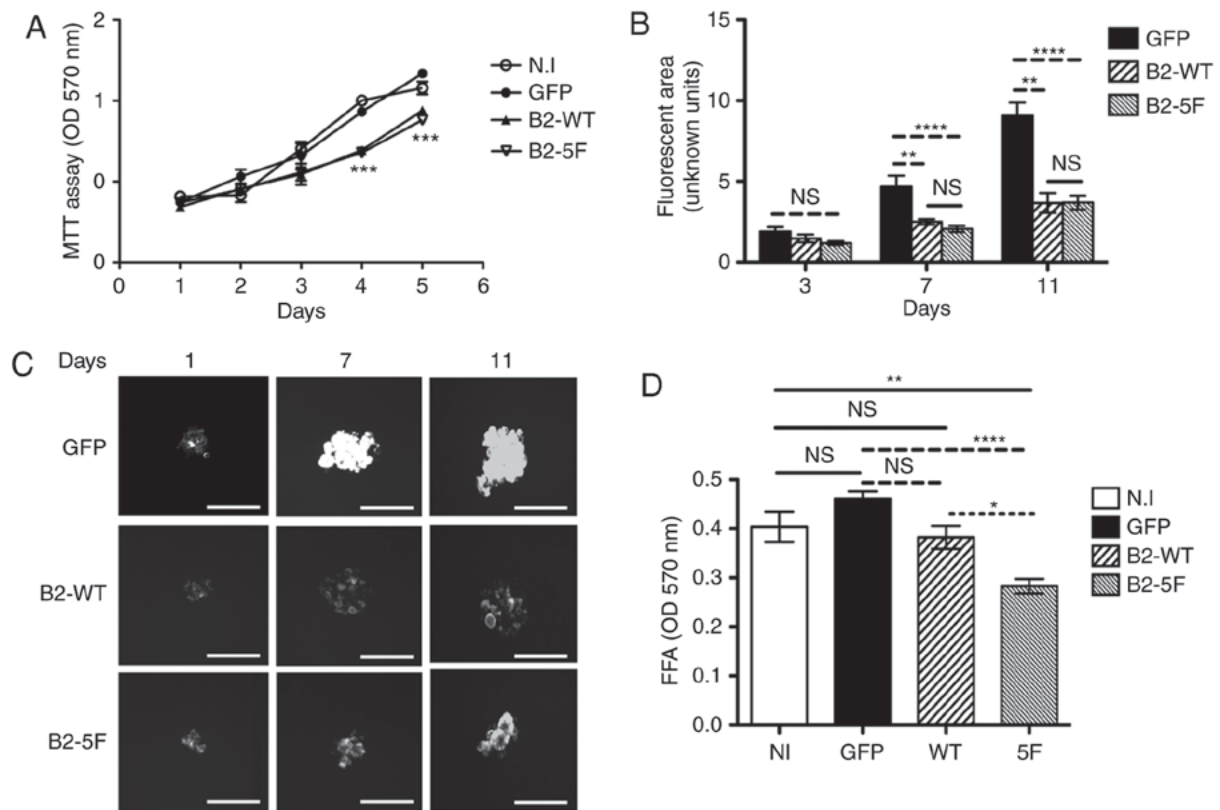
Figure 3. Cell proliferation and growth assays. MTT assay showing significant differences in cell proliferation for B2-WT and B2-5F cells compared to control cells (A) (n=3 with quadruplicates). (B) Spheroid assay showing that B2-WT and B2-5F cells formed smaller spheroids compared to the control cells with (C) provided images (n=3 with 10 replicates). Scale bars, 100 μm. (D) Focal formation assay (FFA) showing that B2-5F expressing cells exhibited impaired foci formation compared with the control and B2-WT cells (n=3 with duplicate wells). Statistical differences calculated by one- or two-way ANOVA followed by the Bonferroni's multiple comparison post hoc test. Error bars represent standard error of the mean (SEM). The level of significance was set to P<0.05 (*P<0.01, **P<0.001, ***P<0.0001 and ****P<0.00001). N.S, not significant.

tracking of the ephrin-B2 protein to the cell membrane in contrast to the GFP control, that was scattered throughout the cells (Fig. 2D).

After the first screening, the MCF7-infected cells were sorted by flow cytometry and used in all the experiments thereafter.

Cell proliferation, spheroid and focal formation assays. The B2-WT and B2-5F cells exhibited a lower proliferation compared to the control cells (Fig. 3A) with statistically significant differences after 3 days in culture. However, no differences were observed between the B2-WT and the B2-5F cells. The B2-WT and B2-5F cells formed smaller spheroids under low attachment conditions compared to the control cells (Fig. 3B and C). Statistical differences between B2-WT and B2-5F compared to control cells, were appreciated after 7 days in culture. However, no marked differences in spheroid size were identified between the B2-WT and B2-5F cells. By contrast, the B2-5F cells exhibited a decreased colony formation ability compared to the B2-WT and control cells. However, no marked differences were observed as regards colony formation between the B2-WT and control cells (Fig. 3D).

Migration assays. The results of Transwell migration assay presented in Fig. 4A revealed that, in the absence of growth factors (-), no significant differences in migration were

observed between the cells. However, when challenged with EGF and HRG-b1, which stimulate migration, the scattering and proliferation of MCF7 control cells (24,25), the B2-WT and B2-5F-expressing cells migrated less compared to the control cells. The results presented in Fig. 4B revealed that while the treated control cells migrated more compared to the untreated cells, the B2-WT cells did not exhibit a significant difference in migration and only the migration of the treated B2-5F cells was significantly inhibited compared to the untreated counterparts. The lateral migration assay presented in Fig. 4C and D, reveled similar results: Ephrin-B2 expression contributed to defective migration into the wounded area. since both the B2-WT and B2-5F-expressing cells exhibited lower lateral migration compared with the control cells.

These results suggested that the B2-WT and B2-5F-expressing cells had a delayed cell motility compared to the control cells. Of note is that the defect in cell motility was significant in the B2-5F cells already after 21 h compared to 45 h that it required for the B2-WT cells. This delay in cell motility may be due to perturbations in the movement direction, as suggested in Videos S1-3.

Cell motility array. The human cell motility array, including 84 genes, was performed to identify the relevant genes behind the impaired movements of the B2-WT and B2-5F cells. Table SI summarizes the genes that were altered >2-fold between the
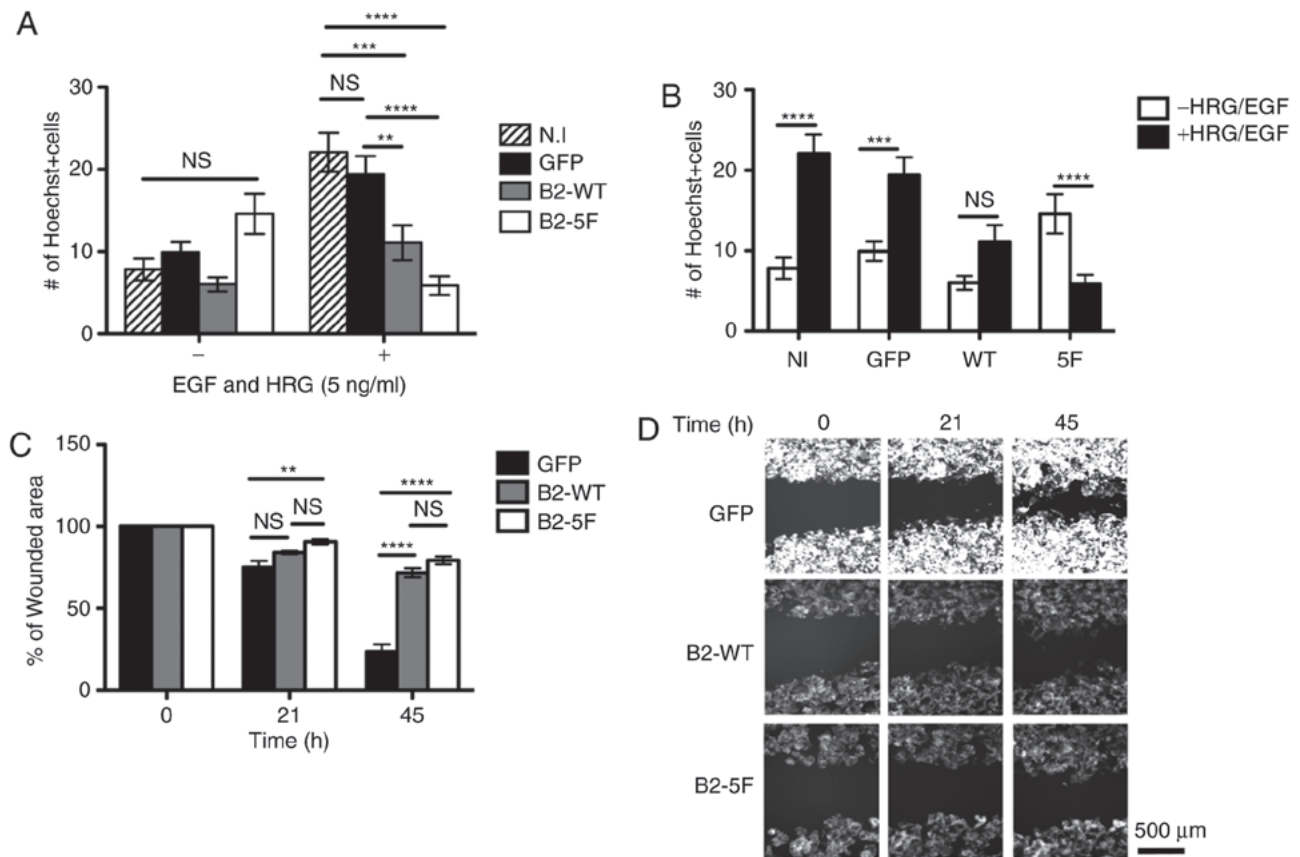
Figure 4. Migration assays. The Transwell assay at 20 h revealed no differences in migration in absence of EGF and HRGβ1 (-). (A) However, the presence of HRGβ1 and EGF (+) led to greater migration of the MCF control cells compared with B2-WT and B2-5F cells. (B) A different representation of the growth factor's effect for each particular group, showed that the control cells migrated more in the presence of growth factors, while B2-5F cell migration was inhibited (n=3, images acquired from 6-10 fields). (C and D) Wound-healing assay showing less cell motility of the ephrin-B2-cells compared with GFP-control cells (n=3 with duplicates). Scale bar, 500 mm. Statistical differences between the groups were identified by two-way ANOVA followed by the Bonferroni's multiple comparison post hoc test or t-test for comparisons between 2 groups. Error bars represent standard error of the mean (SEM). The level of significance was P<0.05 (**P<0.001, ***P<0.0001 and ****P<0.00001). N.S, not significant.

control cells and B2-expressing cells. In order to identify the genes which are crucial for phosphotyrosine-mediated signaling, we analyzed the differences in gene expression between the B2-WT and B2-5F cells. The selected genes were those with >2-fold significant differences (P-value <0.05). WT-B2 expression in the MCF7 cells led to the dysregulation of several genes towards both up- and downregulation. However, the B2-5F cells consistently exhibited gene downregulation, in agreement with the results from the cell migration assays, where these cells had a significantly impaired motility. *STAT3* and the SH3 and PX Domains 2A (*SH3PXD2A*) were used to verify whether the gene expression changes were also present at the protein level. In agreement with the gene expression array, both proteins were downregulated in the B2-5F compared to B2-WT cells. STAT3 but not SH3PXD2A, was upregulated in B2-WT compared to the GFP control (Fig. S1).

Immunohistochemistry for protein expression. The obtained patient material was used to examine the association between protein expression and the prognostic relevance of ephrin-B and EphB4. The staining was evaluated in three separate core biopsies by 2 individual observers (JS and ZM) blinded to the clinical data. The sections were re-evaluated upon disagreement. EphB4 and ephrin-B were visualized in the

cell membrane, cytoplasm and nucleus. The cytoplasmic and membrane staining were based on intensity (0=negative/weak, 1=medium and 2=strong). We also observed that some samples exhibited strong and defined membranous staining around the nuclei (perinuclear staining). Thus, the cut off for positive cytoplasmic staining was >0 and for positive membranous staining =2. Nuclear staining was graded according to the frequency of positive cells: 0, 1-25%, >25-50%, >50%-75% and >75%. Positive EphB4 expression was defined as nuclear staining in >75% of the cells. Positive ephrin-B staining was defined as strong membrane staining and perinuclear staining (Fig. 5).

Positive ephrin-B expression negatively correlated with Nottingham histologic grade (NHG) III (r=-0.162) and HER2 expression (r=-0.140) and it positively correlated with ER (r=0.181) and EphB4 expression (r=0.162) (Tables I and SII). Univariate survival analysis revealed that a high ephrin-B expression indicated a longer distant recurrence-free survival, HR (95% CI) 0.65 (0.44-0.98), P=0.04, while nuclear EphB4 was associated with a shorter survival, HR (95% CI) 1.75 (1.18-2.61), P=0.006 (Fig. 6). Moreover, a combined variable ephrin-B/EphB4 revealed that those patients with ephrin-B-/EphB4+ tumors, survived for a significantly shorter time without distant recurrences in comparison with the other groups, HR (95% CI) 2.50 (1.55-4.04), P=0.0002 (Fig. 6C).
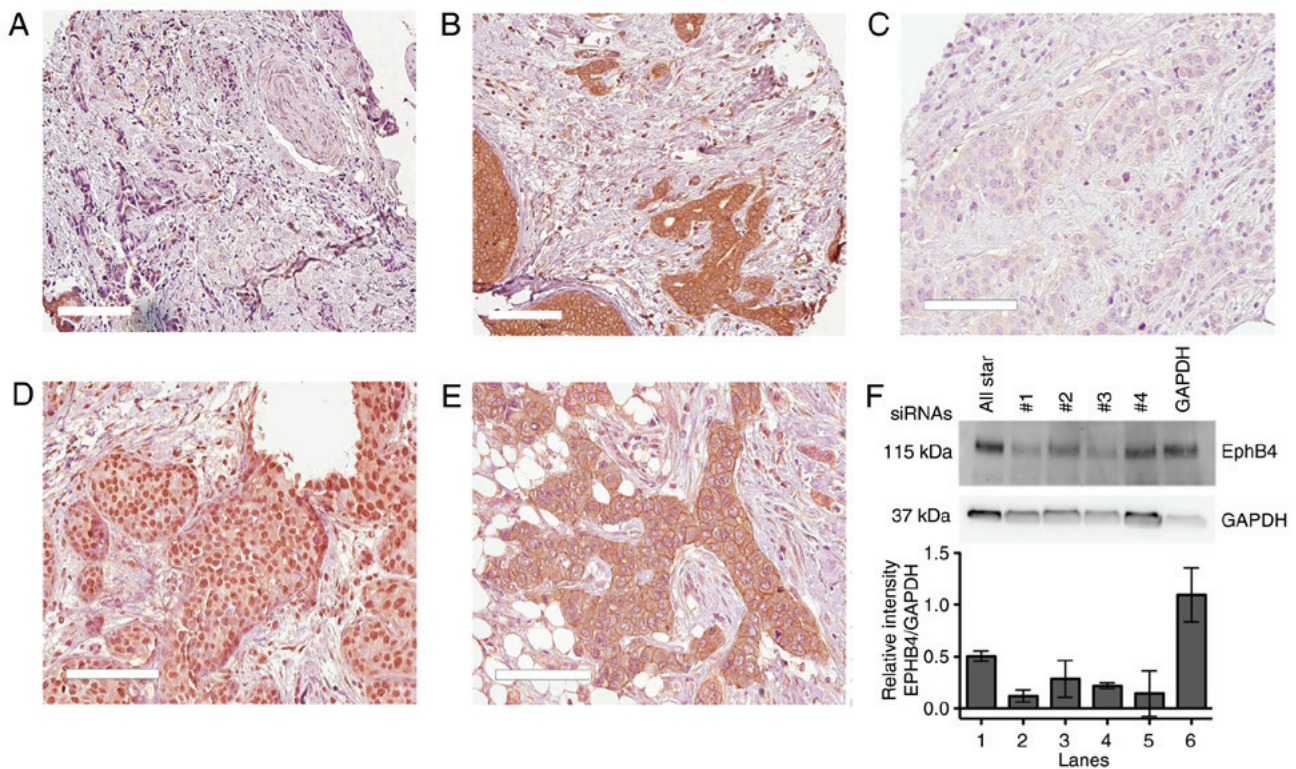
Figure 5. Immunohistochemistry was used to detect ephrin-B and EphB4 protein expression. Representative immunostaining of a (A) negative ephrin-B tumor, (B) membranous ephrin-B, (C) EphB4-negative, (D) nuclear EphB4 and (E) membranous EphB4. Scale bars, 100 μm. (F) EphB4 antibody validation in MCF7 cells was performed by immunoblotting and the intensity of the bands quantified (n=2). Error bars represent the standard error of the mean (SEM). The AllStar siRNA was used as negative control and GAPDH siRNA as positive control. The ephrin B antibody was the same used in all the *in vitro* experiments.

This result was found at the protein level in our patient material and validated at the gene-expression level in the Van de Vijver dataset (23) (Fig. S2).

A multivariable model including EphB4, ephrin-B and other relevant variables, revealed that both ephrin-B and EphB4 were independent prognostic factors, particularly for distant recurrences, while only ephrin-B was an independent prognostic factor for loco-regional recurrences (Table II).

Furthermore, the clinical value of *EFNB2* was explored in two public gene expression datasets (22,23), where univariate analysis revealed that *EFNB2* was associated with a longer relapse-free survival in the Karolinska Institute (KI) dataset, HR (95% CI) 0.38 (0.17-0.87), P=0.02 and to longer distant recurrence-free survival in the Van de Vijver dataset, HR (95% CI) 0.47 (0.27-0.81), P=0.007, while *EPHB4* was an adverse prognostic factor, as already reported (6). Multivariate analysis in both datasets, revealed that *EFNB2* was an independent prognostic marker (Table SIII).

Discussion

The EphB4 receptor could both, promote and suppress tumor growth (26) depending on the presence of its ligand ephrin-B2, which is usually expressed in a different cell, but is also co-expressed (27-29). Upon EphB4-ephrin-B2 interaction, it is believed that the signal transmitted inside the EphB4-expressing cell or 'forward signal' is tumor suppressive (12), whereas the signal generated inside the ephrin-B2-expressing cell, the 'reverse signal', is oncogenic.

Since the EphB4 appears to be highly expressed in human breast cancer cells with a low ephrin-B2 expression, it can be deduced that EphB4-ephrin-B2 co-expression would lead to tumor suppression.

Upon ephrin-B2 expression in the cells, we found that ephrin-B2-WT, but not the mutated B2-5F, was tyrosine-phosphorylated, probably due to its interaction with the EphB4 receptor. However, we cannot disregard the involvement of other family members, since ephrin-B2 may have promiscuous interactions within the Eph family (30). Both ephrin-B2-WT and B2-5F were visualized in the cell membrane as expected.

Furthermore, we expected to find EphB4 activation, but noted that EphB4 was not constitutively phosphorylated nor activated as a result of ephrin-B2 expression or upon treatment with a soluble ephrin-B2-Fc (data not shown). These findings were in line with previous results where the EphB4 receptor was silenced by the co-expression of ephrin-B2 in MCF7 cells (18).

EphB4 and ephrin-B2 do not seem to play a main role in cell proliferation, but rather orchestrate directional movement by controlling cytoskeletal organization and cell adhesion (4). In this study, we observed that B2-expressing cells exhibited a lower proliferation compared to the control cells, although we did not observe differences in proliferation between the B2-WT and B2-5F cells. Consistent results were found when the cells were grown in 2D and 3D conditions. In addition, only the B2-5F cells were unable to form colonies suggesting a role for the ephrin-B2 phosphotyrosine-mediated signal during colony formation.

Table I. Ephrin B protein expression in relation to known clinical variables and EphB4 expression in breast cancer.

Variables	EFNB⁻ n (%)	EFNB⁺ᵉ n (%)	P-value
Lymph nodes			0.09
-	20 (15)	6 (8)	
+	111 (85)	74 (92)	
NHG[a]			**0.02**
I	25 (20)	21 (27)	
II	62 (49)	45 (57)	
III	39 (31)	13 (16)	
Tumor size			0.75
<20 mm	49 (38)	31 (40)	
≥20 mm	80 (62)	46 (60)	
ERα[b]			**0.012**
-	43 (37)	15 (20)	
+	73 (63)	60 (80)	
HER2[c]			**0.042**
-	106 (81)	73 (91)	
+	25 (19)	7 (9)	
Treatment			0.82
Chemotherapy	70 (53)	44 (55)	
Radiotherapy	61 (47)	36 (45)	
EphB4[d]			**0.019**
-	105 (81)	53 (67)	
+	24 (19)	26 (33)	

[a]Nottingham histologic grade, [b]ER⁺ cutoff ≥0.05 fmol/μgDNA determined by isoelectric focusing. [c]HER2⁺ following the HercepTest guidelines for membrane staining. [d]Nuclear EphB4 staining in >75% cells. [e]Positive Ephrin-B staining defined as strong membrane and perinuclear staining. Values in bold font indicate significant P-values.

Moreover, we found defective migration in cells overexpressing the ephrin-B2. MCF7-B2 cells exhibited a delayed lateral migration compared to the control cells. The fact that the B2-5F cells failed to fill the wound after 21 h suggests that the tyrosine-mediated signaling could also play an important role in cell motility. The doubling time for MCF7 in complete medium, according to ATCC recommendations, is approximately 38 h and therefore we could discard the possibility of inhibited cell proliferation at 21 h in the wound healing assay.

Impaired cell motility was also observed in a Transwell assay, where the both the B2-WT and the B2-5F-expressing cells migrated less in presence of growth factors, such as HRGβ1 and EGF compared with the control cells. These growth factors stimulate MCF7 cell proliferation and migration via the activation of HER-family members expressed by these cells (24,25). Herein, it can be speculated that in presence of HRGβ1 and EGF, the EphB4 receptor could be activated by crosstalk with other HER family members, for example the EGFR to induce cell motility. Instead, in the presence of ephrin-B2, the EphB4 receptor is inactivated and ephrin-B2 operates in a receptor-independent manner to inhibit cell motility. Notably, the phosphotyrosine-mediated signal seems to be important in this context since the B2-5F cells significantly migrated less in presence of growth factors.

A deficient ephrin-B2 expression *in vitro* has been coupled to poor spreading and increased non-directional motility in normal vascular cells (31). Another study demonstrated that ephrin-B2 expression in HUVECs contributed to a more rapid, but random migration (32) in contrast with our results, showing that ephrin-B2 expression and especially B2-5F, seems to be responsible for impaired cell movements. These observations were confirmed with the cell motility array revealing down regulation of gene expression in the B2-5F cells compared with the B2-WT and the GFP control cells with a correspondence between the downregulation of gene, and protein expression for the B2-5F cells.

Some of the affected genes in the B2-5F compared to the GFP control cells, were *CSF1*, *PLAUR*, *RDX*, *TGFβ1* and *WIPF1* (P≤0.01). The fact that *CSF1*, *PLAUR*, *PTK2B*, *RND3*, *SH3PXD2A* and *VASP* were downregulated in the B2-5F cells compared to the B2-WT cells indicated that these genes may be involved in tyrosine-mediated signaling.

Furthermore, our results from the clinical material revealed a positive association between ephrin-B and ER, the representative marker for the less aggressive breast cancer subtype luminal A. In addition, a positive ephrin-B expression was associated with a low HER2 expression and negatively associated with NHG III. In line with these results, a high ephrin-B expression in our clinical material, indicated a good prognosis in both univariate and multivariate analyses. Although we cannot conclude that ephrin-B2 and EphB4 are co-expressed or co-localized in the tumor cells, the worse prognosis was registered among the subgroup of patients with high EphB4 in the absence of the ligand.

We also found that EphB4 was an adverse prognostic factor in agreement with other reports (6,33,34). Nevertheless, the clinical significance of ephrin-B in breast cancer is still poorly investigated in clinical material. An analysis of the ephrin-Bs mRNA expression in the Van de Vijver and the KI datasets, revealed that only *EFNB2* seemed to have prognostic value in comparison with *EFNB1* and *EFNB3* (Fig. S3). Otherwise, our recent search in the TCGA and METABRIC databases (35,36) revealed that *EFNB2* overexpression at the mRNA level had no prognostic significance. However, looking at the copy number alterations, normal or higher copy number of *EFNB2* indicated longer breast cancer survival (survival time from diagnosis to death due to breast cancer), HR (95% CI) 0.746 (0.63-0.88) P=0.0007 (METABRIC) and HR (95% CI) 0.58 (0.37-0.90) P=0.01 (TCGA). As regards EphB4 expression in the cell membrane, it was not associated with patient survival in this material. However, among those patients without lymph nodal infiltration (n=26), the presence of membranous EphB4 indicated good prognosis (data not shown), reminding of our previous results with the EphB2 receptor (21).

In conclusion, the findings of this study suggest that ephrin-B2 expression in EphB4 positive MCF7 cells inhibits cell proliferation, motility and migration. The phosphotyrosine mediated- signaling, in particular, seems to play a key role for cell motility and migration. These *in vitro* results could mirror the clinical situation where we found that ephrin-B protein expression was associated with a positive ER status, low grade and a low HER-2 expression.

Table II. Multivariable proportional Cox regression model considering distant recurrence-free survival (DRFS), breast cancer survival (BCS) and locoregional recurrence-free survival (LRRFS) as endpoints.

Endpoints	DRFS		BCS		LRRFS	
Variables	HR (95% CI)	P-value	HR (95% CI)	P-value	HR (95% CI)	P-value
Ephrin-B						
-	1.00	**0.03**	1.00	0.05	1.00	**0.02**
+	0.61 (0.38-0.96)		0.63 (0.39-1.01)		0.37 (0.17-0.84)	
EPHB4						
-	1.00	**0.002**	1.00	0.10	1.00	0.54
+	2.10 (1.31-3.38)		1.53 (0.92-2.55)		1.28 (0.58-2.81)	
ER						
-	1.00	0.34	1.00	0.09	1.00	0.44
+	0.79 (0.49-1.29)		0.65 (0.40-1.06)		0.75 (0.37-1.54)	
HER2						
-	1.00	0.91	1.00	0.55	1.00	0.56
+	1.03 (0.58-1.85)		1.20 (0.66-2.19)		1.29 (0.55-2.99)	
Nodes						
-	1.00	**0.01**	1.00	**0.008**	1.00	0.11
+	2.91 (1.24-6.84)		3.52 (1.40-8.89)		3.27 (0.76-14.15)	
Size						
-	1.00	**0.008**	1.00	**0.002**	1.00	0.32
+	1.86 (1.17-2.94)		2.16 (1.32-3.55)		1.44 (0.71-2.92)	
Therapy						
Chemotherapy	1.00	0.64	1.00	0.86	1.00	0.05
Radiotherapy	1.11(0.72-1.70)		0.95 (0.61-1.49)		0.47 (0.23-0.98)	

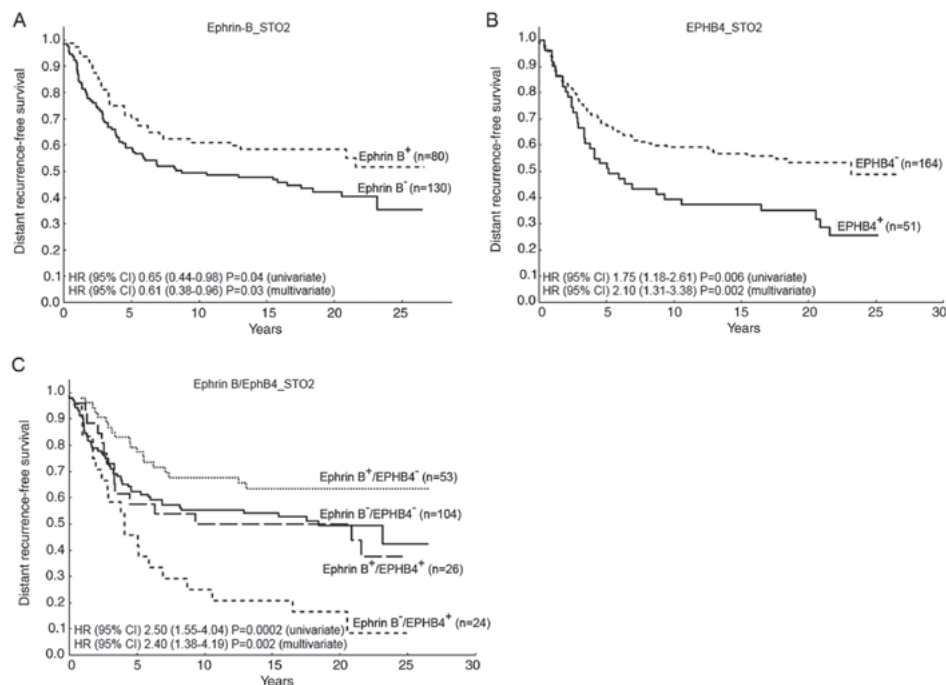Values in bold font indicate significant P-values.

Figure 6. Kaplan-Meier survival analysis of ephrin-B and EPHB4 protein expression in the Stockholm 2 breast cancer material. (A) A high ephrin-B (EFNB+) expression was coupled to longer distant recurrence-free survival and (B) a high nuclear EphB4 (EPHB4+) was related to shorter distant recurrence-free survival. (C) A combination variable *EFNB/EPHB4* revealed that patients with tumors expressing high EpB4 in absence of the ephrin-B ligand exhibited the shortest survival time without distant recurrences compared to the other groups.

In addition, ephrin-B expression was an independent prognostic factor for a longer distant recurrence-free survival and locoregional-free survival in multivariable analyses. On the contrary, a high EphB4 expression was an independent predictor of a shorter distant recurrence-free survival in multivariate analysis. These results were validated in two public datasets at the gene expression level and only *EFNB2*, among all ephrin-Bs, exhibited prognostic value. Since increased cell proliferation, motility and migration are characteristics of metastatic cells, and we found that these biological effects were mainly impaired in the B2-5F cells, we believe that inhibiting the ephrin-B phosphotyrosine mediated-signal could be a strategy to defy metastasis. However, further studies are warranted to continue exploring the ephrin-B2 and EphB4 interactions in single tumor cells, as well as to validate our results with other antibodies and in a larger clinical cohort.

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Availability of data and materials

Public datasets properly referred to in the text were used to generate part of the results. Patient data supporting the immunohistochemistry finding is not publicly available, but the authors are willing to conduct analysis on this upon reasonable request and with permission from Olle Stål.

Authors' contributions

GPT conceived and designed the experiments. GPT and ZM performed the experiments. GPT, ZM and JS analyzed the data. GPT and ZM wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The retrospective studies on tumor tissues have been approved by the Research Ethics Committee at the Karolinska Institute (dnr 97-451), with amendments.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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