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Nogo-B receptor increases the resistance of estrogen receptor positive breast cancer to paclitaxel

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Abstract

Intrinsic or acquired chemoresistance is a hurdle in oncology. Only 7%–16% of estrogen receptor α (ER α) positive breast cancer cases achieve a pathological complete response (pCR) after neo-adjuvant chemotherapy. Nogo-B receptor (NgBR) is a cell surface receptor that binds farnesylated Ras and promotes Ras translocation to the plasma membrane. Here, we demonstrate NgBR as a potential therapeutic target for ER α positive breast cancer patients to attenuate paclitaxel resistance. NgBR knockdown enhanced paclitaxel-induced cell apoptosis by modulating expression of p53 and survivin in ER α positive breast cancer cells via NgBR-mediated PI3K/AKT and MAPK/ERK signaling pathways. NgBR knockdown attenuated either 17 β -estradiol or

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epidermal growth factor stimulated phosphorylation of ER α at Serine 118 residue. The CHIP-PCR assay further demonstrated that NgBR knockdown decreased ER α binding to the estrogen response element (ERE) of the ER α target gene and increased the binding of p53 to the promoter region of survivin to attenuate survivin transcription. In summary, our data suggest that NgBR expression is essential to promoting ER α positive breast cancer cell resistance to paclitaxel. Findings from this study implicate a novel therapeutic target for treating ER α positive breast cancer in neo-adjuvant/adjuvant chemotherapy.

Keywords

Nogo-B receptor; survivin; p53; paclitaxel; estrogen receptor; breast cancer

1. Introduction

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among women [1, 2]. Approximately 70–75% of human breast cancers are estrogen receptor α (ER α) positive, and most breast cancer deaths occur in ER α positive patients [3–5]. Chemotherapy is an important treatment strategy in the management of patients with advanced breast cancer or who desire breast-conserving surgery [5, 6]. However, pathological complete response (pCR) rates after neo-adjuvant chemotherapy is only achieved in a minority of patients with ER α positive breast cancer [7]. A randomized phase III trial showed that only 7%–16% of ER α positive breast cancer cases achieved their pCR [8]. Many studies indicate that current chemotherapy has much less efficacy in ER α positive breast cancer because estrogen induces chemoresistance by promoting cell proliferation, inhibiting apoptosis, and stimulating both metastasis and angiogenesis [9–13]. Thus, ER α mediated resistance to chemotherapy has become a great challenge to clinical treatment. Taxanes, such as paclitaxel and docetaxel, are among the most commonly used cytotoxic drugs for the treatment of breast cancer [14, 15]. Although it is efficacious for many cancers, including ovarian cancer, small cell lung cancer, and many other malignancies [16], the de-novo or acquired resistance to this drug class in ER α positive breast cancer remains a challenge in oncology.

NgBR is a type I receptor with a single transmembrane domain and was identified as a specific receptor for Nogo-B. Our recent findings demonstrated that 1) NgBR binds farnesylated Ras and recruits Ras to the plasma membrane, which is a critical step required for receptor tyrosine kinases (RTKs)-mediated activation of Ras signaling in human breast cancer cells and tumorigenesis [17]; 2) increased NgBR expression is highly associated with survivin (an apoptosis inhibitor) expression in ER α positive breast cancer [18]; 3) NgBR is highly expressed in chemo-resistant human hepatocellular carcinoma and involved in p53-dependent resistance to 5-fluorouracil [19]. Findings from this study add to the story by elucidating the molecular mechanism by which highly expressed NgBR in ER α positive breast tumor cells enhances the ER α -mediated signaling and resistance to paclitaxel.

2. Material and Method

2.1 Antibodies, reagents and siRNA

A peptide (AHHRMRWRADGRSLEK, residues from 81–96 of NgBR) was used to immunize rabbits (Epitomics, Burlingame, CA). Antiserum was purified using the same peptide-conjugated SulfoLink Coupling Gel (Pierce, Rockford, IL). Purified NgBR rabbit polyclonal antibody was used for immunostaining. The peptide recognizing epitope 14 to 30 of human Nogo-B was used to immunize rabbits (IMG-5346A, Imgenex, San Diego, CA). Antibodies for NgBR (#ab168351) and Estrogen Receptor alpha (phospho S118) (#ab32396) were purchased from Abcam (Cambridge, MA). Antibodies for phos-Akt (S473 and T308, #9271), phos-p42/44 ERK (#9101), total Akt (#4691), total Erk (#4595) and survivin (#2808) were purchased from Cell Signaling Technology (Beverly, MA). We also used antibodies for p53 (#10442-1-AP) and Hsp90 (13171-1-AP) from Proteintech (Rosemont, IL). Estradiol (#E2758), paclitaxel (#T7191), doxorubicin (#44583) and EGF (#E5036) were purchased from Sigma-Aldrich (St. Louis, MO). NgBR siRNA (forward: GGAAAUACAUAAGACCUACA, reverse: UGUAGGUCUAUGUAUUUCC) oligonucleotides with 3' dTdT overhangs were synthesized by QIAGEN (Valencia, CA). The specificity of NgBR siRNA has been validated in our previous publication [20, 21]. Control siRNA in experiments refers to a non-silencing siRNA (NSF: UUCUCCGAACGUGUCACGU, NSR: ACGUGACACGUUCGGAGAA) designed and synthesized by QIAGEN. Lipofectamine RNAiMAX reagent (Invitrogen, Carlsbad, CA, USA) was used for the transfection of siRNA according to the manufacturer's instructions.

2.2 Obtaining breast tissue samples

Treatment outcomes of breast cancer patients receiving chemotherapy were retrieved from the surgical pathology archives at the Froedtert Hospital & Medical College of Wisconsin and from retrospective chart review. Thirty-eight breast cancer cases were selected for this study. After receiving approval from the Institutional Review Board for the Medical College of Wisconsin, de-identified samples (pre- and post-treatment) were obtained.

2.3 Immunohistochemistry (IHC) staining and scoring

Breast cancer pathological diagnosis was confirmed with hematoxylin and eosin staining of paraffin-embedded tissue sections. Immunohistochemical analysis for Nogo-B and NgBR status was performed on 4- μ m sections using respective antibodies from Imgenex (Nogo-B, clone IMG-5346A) and Epitomics (clone 671). The detection system used was 3,3'-diaminobenzidine (DAB) from DAKO (Santa Clara, CA). Slides were counterstained using hematoxylin. Either cytoplasmic or membranous staining for Nogo-B and NgBR was considered positive. Quantitative scoring of NgBR and Nogo-B immunostaining was performed following previously published methods [18, 19]. The percentage of positive cancer cells was assigned a score from 0(0%), 1 (1–10%), 2 (11–25%), 3 (26–50%), 4 (51–75%) and 5 (>75%) and was defined as negative (–, IHC score 0 to 1), low (+, IHC score 2 to 3) and, high (+++, IHC score 4 to 5). Associations between NgBR, Nogo-B status and clinical parameters were assessed by a chi-square test. All breast cancer cases were histopathologically re-evaluated on hematoxylin and eosin-stained slides by two pathologists (BW and IA).

2.4 Cell culture

T47D breast tumor cells from ATCC were grown in RPMI-1640 (Life Technologies) containing penicillin (100 U/ml), streptomycin (100 mg/ml), 10% (v/v) fetal calf serum (Sigma-Aldrich). MCF-7 breast tumor cells from ATCC were grown in DMEM (Life Technologies) containing penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% (v/v) fetal calf serum (Sigma-Aldrich). T47D and MCF-7 cells were changed to either 10% or 5% charcoal stripped FBS (Sigma-Aldrich) in phenol red free medium when performing 48 hours of 17 β -estradiol (E2) treatment. Both cells were changed to 2% charcoal stripped FBS (Sigma-Aldrich) in phenol red free medium when performing 24 hours of epidermal growth factor (EGF) treatment. Both cells were changed to serum free, phenol red free medium when performing short time E2 or EGF treatment. All cell lines were cultured in a 37°C - humidified atmosphere containing 95% air and 5% CO₂.

2.5 Clonogenic survival assay

Cells were seeded in triplicate into a 60 mm culture dish (1000 cells/well). Cells were transfected with no-silencing (NS) control siRNA or NgBR siRNA. At 24 hours after transfection, cells were cultured in medium containing E2 or EGF in the absence or presence of paclitaxel at the indicated doses for 48 hours. Then, the cells were maintained for 2 weeks. The cell colonies were washed three times with phosphate buffered saline buffer (PBS), fixed in cold methanol for 15 minutes, and stained with Crystal Violet (Sigma-Aldrich) for 15 minutes at room temperature.

2.6 Apoptosis assay by AO/EB staining

The cells cultured in 24-well plates were cultured in medium containing E2 or EGF in the absence or presence of the paclitaxel for 48 hours. After indicated treatment times, the cells were stained with acridine orange (AO, 100 μ g/mL) and ethidium bromide (EB, 100 μ g/mL) purchased from Sigma Aldrich, and were observed under a fluorescence microscope (OLYMPUS). The normal cells and early apoptotic cells were stained by AO to display bright green fluorescence, while the late apoptotic cells were stained by EB to display orange fluorescence.

2.7 Apoptosis measured by annexin V-FITC/propidium iodide (PI) staining

An Annexin V-FITC/PI apoptosis kit (BD Biosciences) was used to quantify the percentage of cells undergoing apoptosis. Cells cultured in 60mm culture dishes were cultured in medium containing either E2 or EGF in the absence or presence of paclitaxel for 48 hours. Cells were then harvested and stained with 5 μ L Annexin V-FITC and 5 μ L PI in 500 μ L of apoptosis reaction solution at room temperature in the dark for 15 min. BD LSRII flow cytometer was used to detect apoptotic cells. Cell population in different quadrants was calculated statistically.

2.8 Nuclear protein isolation

A nuclear extraction kit (#10009277, Cayman chemical) was used to extract nuclear protein. Cells were washed twice with cold PBS and suspended in 500 μ L complete hypotonic buffer. After incubation for 15 min on ice, 100 μ L 10% NP-40 was added to the suspension. The

suspension was spun for 30 s at $14,000 \times g$ at 4°C with a Microfuge, and the supernatant was removed. The pellet was re-suspended in 100 μl complete nuclear extraction buffer and kept on ice for 15 min with vigorous 15 s vortex for two times. The mixture was spun again for 10 min at $14,000 \times g$ at 4°C . The supernatant was collected as nuclear extract and kept at -80°C for analysis.

2.9 Real time RT-PCR

Total RNA was extracted from cell samples using RNeasy kit (QIAGEN). The cDNA was synthesized from 2 μg total RNA by reverse transcription (RT) using iScript cDNA synthesis kit (Bio-Rad, Los Angeles, CA). Real time PCR was performed with Bio-Rad MyiQ detection system and normalized by the corresponding beta-actin mRNA levels. The sequences of real-time PCR primers were described in our previous publication [18].

2.10 Western blotting

To extract total cellular protein, cells were lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5, 1% NP40, 0.1% SDS, 0.1% sodium deoxycholate, 0.1 mM EDTA, 0.1 mM EGTA, protease and phosphatase inhibitor cocktail (Roche)). The cellular lysate was centrifuged for 10 min at 10,000 rpm at 4°C , and the supernatant was collected as total cellular extract. To determine protein expression by western blotting, 40 μg protein from each sample was loaded and separated on a 12% SDS-PAGE gel and transferred onto a nitrocellulose membrane (Bio-Rad). The membrane was blocked with TBST containing 5% non-fat dry milk for 1 h at room temperature (RT), and then incubated with primary antibody overnight at 4°C followed by washing for 3×10 min with TBST. The blot was re-blocked and incubated with horseradish peroxidase conjugated donkey anti-rabbit IgG for 1 h at RT. After washed for 3×10 min with TBST, the membrane was incubated in western blotting chemiluminescence reagents followed by exposure to film.

2.11 Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed on MCF-7 and T47D cells using the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) (#9005, Cell signaling technology, Danvers, MA). Cells were treated with E2 after being transfected with NS or NgBR siRNA for 3h. DNA-ER α complexes or DNA-p53 complexes were immunoprecipitated using antibodies against ER α (#8644) or p53 (#2527) (Cell signaling technology). DNA, after de-crosslinking and purification, was subjected to PCR with EmeraldAmp GT PCR Master 2xMix (SD2489, Takapa, Mountain View, California). PCR products were resolved on 2% agarose gels and visualized after staining with ethidium bromide. ESR1 (#9673) and satellite (#4486) primers are purchased from Cell Signaling Technology. The sequence of survivin forward and reverse primers is 5'-CACGCGTTCTTTGAAA-GCAGTCGA-3' and 5'-CGCGATTCAAATCTGGCGGTTA-3', respectively.

2.12 Kaplan–Meier survival analyses

NgBR (NUS1) mRNA expression values were retrieved from a ER positive, Her-2 negative breast cancer profiling metaset deposited in a Kaplan-Meier Plot (225071)[22]. The cumulative survival probability was evaluated using the Kaplan-Meier method.

2.13 Statistical analysis

SPSS 19.0 software was used for all statistical analysis. The relationship between NgBR, Nogo-B status and clinical parameters was analyzed using a chi-square test. The statistical significance of quantitative assays was analyzed using either two-tailed Student t test or ANOVA analysis for more than two groups. Data are presented as mean $\pm$ the standard error of the mean (SEM). A P-value < 0.05 defined statistical significance.

3. Results

3.1 NgBR expression levels in the response of ER α positive breast cancer patients to neoadjuvant chemotherapy

We recently showed that E2 induces the expression of survivin in ER α positive T47D breast cancer cells but not in ER α -negative MDA-MB-468 breast cancer cells. NgBR knockdown with siRNA abolishes E2-induced survivin expression in T47D cells but not in MDA-MB-468 cells [18]. We also showed that E2 increases the expression of survivin and cell growth in ER α positive MCF-7 and T47D cells whereas knockdown of NgBR reduces E2-induced survivin expression and cell growth [18]. Thus, we hypothesized that NgBR could be involved in regulating E2-induced resistance in ER α positive breast cancer patients.

To examine the roles of NgBR in regulating the response of ER α positive breast cancer to neoadjuvant chemotherapy, we randomly collected breast tissue samples from 38 ER α -positive breast cancer cases and performed immunostaining to examine NgBR and Nogo-B expression levels in breast tumor sections before and after neo-adjuvant chemotherapy (Fig. 1). All patients received Taxane-based (either paclitaxel or docetaxel regimen) treatment. Thirty-two cases did not demonstrate or partially demonstrated a response to neoadjuvant chemotherapy. Among these 32 cases, 22 cases show increased or unchanged NgBR expression after chemotherapy and 26 cases showing increased or unchanged Nogo-B expression after chemotherapy (Table 1). Due to the small sample size, the association between NgBR/Nogo-B expression and pathologic response was not statistically significant (Table 2–3). Thus, we further examined the association of NgBR expression with the outcome of ER α positive breast cancer patients. NgBR (NUS1) mRNA expression data were retrieved from a gene-expression profiling dataset (225071 from Kaplan–Meier Plot database) of 428 ER α positive and Her-2 negative breast tumors [22]. Subsequent Kaplan–Meier analysis revealed a significantly reduced Recurrence Free Survival (RFS, $p=0.00084$, Fig. S1) for 211 patients with high NgBR-expression in tumors as compared to 217 patients with low NgBR-expression in tumors.

3.2 NgBR knockdown increases paclitaxel-induced apoptosis and decreases ER α -mediated resistance of breast cancer cells to paclitaxel

Previously published data have shown that estrogen and ER α are involved in signaling pathways required for anti-apoptosis and proliferation of tumor cells [12, 23]. To determine the contribution of NgBR to the resistance of ER α positive breast cancer to paclitaxel, we knocked down NgBR in T47D cells using validated siRNA as reported in our previous publication [20]. Cells were treated with E2 and paclitaxel as show in Figure 2A. Forty-eight hours later, cell survival was measured by a viable cell counting assay as described in the

Methods. Consistent with previous reports [24, 25], E2 increases cell viability and paclitaxel decreases cell viability (Fig. 2B). Also, E2 prevents the decrease of cell viability caused by paclitaxel, and NgBR knockdown further decreases viability of T47D cells treated with E2 and paclitaxel. Similarly, NgBR knockdown in MCF-7 cells also reduced E2-caused resistance and increases the sensitivity to paclitaxel (Fig S2).

The capability of in vitro tumorigenesis was determined by clonogenic survival assay. T47D cells receiving E2 treatment show larger colonies and higher colony number (Fig. 2C). Paclitaxel treatment significantly reduced the colony number and size, and E2 treatment restored the colony formation capability of T47D cells treated with paclitaxel. As compared to cells treated with non-silencing control siRNA, clonogenic capability of NgBR knockdown cells was significantly decreased under the condition of paclitaxel treatment alone or along with E2 stimulation (Fig. 2C).

To determine if NgBR expression is required for preventing paclitaxel-induced apoptosis of ER α positive breast cancer cells, we examined the effects of NgBR knockdown on paclitaxel-induced apoptosis using two different methods. As shown in the representative images of AO/EB staining in Figure 2D, we appreciated that E2 treatment reduces the paclitaxel-induced apoptosis in T47D cells. NgBR knockdown attenuates the acquired resistance from E2 treatment. We further used the Annexin V-FITC/propidium iodide (PI) staining-based flow cytometry approach to determine cell apoptosis (Fig 2E). Consistently, E2 treatment attenuated paclitaxel-induced apoptosis of T47D cells, but NgBR knockdown restores the sensitivity of T47D cells to paclitaxel by showing increased apoptosis. These results indicate that ER α positive breast cancer cells acquired resistance to paclitaxel after exposure to E2, but NgBR knockdown with siRNA reduces E2-induced resistance of ER α positive breast cancer cells to paclitaxel.

As shown in Figure S3, E2 treatment also increased the survival of T47D cells and attenuated doxorubicin-induced cell death determined by cell viability assay and AO/EB staining, respectively. NgBR knockdown significantly reduced E2-promoted T47D cell survival and re-sensitized the cells to doxorubicin treatment.

3.3 NgBR regulates the expression of p53 and survivin in ER α positive breast cancer cells as well as E2-induced phosphorylation of Akt and ERK

To determine the molecular mechanism by which NgBR increases ER α -mediated paclitaxel resistance, we examined NgBR-mediated downstream signaling pathways and altered the expression of apoptosis regulators, such as survivin and p53. T47D cells were treated with E2 for 48 h after NgBR knockdown using siRNA. Consistent with our previous reports, NgBR knockdown diminished survivin expression (Fig. 3A). Interestingly, we found that p53 expression in NgBR knockdown T47D cells is upregulated as compared to cells treated with NS siRNA and E2 (Fig 3A). We also found that E2 treatment induces the expression of NgBR and survivin at the protein level (Fig 3A) and mRNA level (Fig. S4). In addition, we observed that NgBR knockdown also reduced E2-induced phosphorylation of Akt and ERK in T47D cells (Fig 3B).

3.4 NgBR knockdown attenuates E2-induced phosphorylation of ER α at Serine 118 residue

Phosphorylation of ER α Serine 118 (S118) residue has been reported as an indicator of ER α activation [26]. Many kinase pathways such as MAPK, IGF, E2 and EGF stimulation, can induce phosphorylation of ER α S118 and enhance tumor resistance to therapy [26]. To determine if NgBR is required for the activation of ER α , we determined the effects of NgBR knockdown on E2-stimulated phosphorylation of ER α by Western blot analysis. As shown in Figure 3C, E2 stimulation increased the phosphorylation of ER α S118 residue. NgBR knockdown attenuated E2-stimulated phosphorylation of ER α S118 residue. However, NgBR knockdown did not change the total protein levels of ER α under the short stimulation period.

3.5 NgBR modulates p53 binding to the promoter region of survivin gene

Based on our results that E2 induces the expression of survivin and that NgBR knockdown down-regulates survivin expression in ER α positive breast cancer cells (Fig 3A), we hypothesized that NgBR may be involved in regulating ER α mediated-transcription activity. To test this hypothesis, we performed ChIP-PCR assay to determine how NgBR is involved in ER α -mediated gene transcription. Chromatin DNA extract was immunoprecipitated from either T47D (Fig. 4A/4B) or MCF-7 (Fig. 4C/4D) cells using anti-ER α or anti-p53 antibody, respectively. As shown in Figure 4A and 4C, PCR results of ER α ChIP assay showed that E2 stimulation enhanced the binding of ER α to the estrogen response element (ERE) of ESR1 gene, and NgBR knockdown decreased the E2-increased binding activity. Survivin is one of the p53 target genes [11, 27, 28]. Additionally, PCR results of the p53 ChIP assay showed that NgBR knockdown enhanced the binding of p53 to the promoter region of survivin in the presence of E2 stimulation (Fig. 4B/4D).

3.6 NgBR knockdown attenuates EGF-mediated resistance of ER α breast cancer cells to paclitaxel

We recently demonstrated that NgBR binds farnesylated Ras and promotes the translocation of Ras to the plasma membrane [17]. The localization of Ras in the plasma membrane is required in order to be activated by receptor tyrosine kinases, such as epidermal growth factor (EGF) receptor (EGFR) and insulin like growth factor 1 receptor (IGF-1R) [29]. NgBR knockdown inhibits EGF-stimulated Ras signaling and diminishes tumorigenesis in vitro and in vivo [17, 29]. Therefore, we further determined if NgBR is involved in EGF-induced phosphorylation of ER α and EGF-promoted resistance in ER α positive breast cancer cells.

Like E2, EGF treatment increased the survival of T47D cells and attenuated paclitaxel-induced cell death (Fig. 5). NgBR in T47D cells was knocked down using validated siRNA and the cells were treated with EGF and paclitaxel as shown in Figure 5A. NgBR knockdown significantly reduced EGF-promoted T47D cell survival and re-sensitized the cell to paclitaxel treatment (Fig 5B). Clonogenic assay results showed that cells receiving EGF treatment showed larger colonies and higher colony number, and increased the resistance to paclitaxel (Fig 5C). As compared to cells treated with non-silencing control siRNA, clonogenic capability of NgBR knockdown cells was significantly decreased under the condition of paclitaxel treatment alone or along with EGF enhancement (Fig. 5C). As

shown in the representative images of AO/EB staining (Fig. 5D), we appreciated that EGF treatment reduces the paclitaxel-induced apoptosis of T47D cells. NgBR knockdown attenuates the acquired resistance from EGF treatment. We further used Annexin V-FITC/propidium iodide (PI) staining-based flow cytometry approach to determine cell apoptosis (Fig 5E). Consistently, EGF treatment decreased paclitaxel-induced apoptosis of T47D cells, but knockdown of NgBR restored sensitivity of T47D cells to paclitaxel by showing increased apoptosis (Fig. 5E).

As shown in Figure S5, EGF treatment increased the survival of T47D cells and attenuated doxorubicin-induced cell death determined by cell viability assay and AO/EB staining, respectively. NgBR knockdown significantly reduced EGF-promoted T47D cell survival and re-sensitized the cells to doxorubicin treatment.

3.7 NgBR modulates EGF-induced expression of survivin as well as EGF-induced phosphorylation of Akt and ERK

T47D cells were treated with EGF for 24 h. The protein levels of survivin were determined by Western blot. As shown in Figure 6A, EGF increased the expression of survivin and NgBR knockdown decreased EGF-induced expression of survivin, as compared to cells treated with NS siRNA and EGF (Fig 6A). Furthermore, EGF induced NgBR and survivin expression at protein (Fig. 6A) and mRNA levels (Fig. S6). Finally, we observed that NgBR knockdown reduced EGF-induced phosphorylation of Akt and ERK in T47D cells (Fig 6B).

3.8 NgBR knockdown attenuates EGF-induced phosphorylation of ER α at Serine118 residue and nuclear translocation of phosphorylated ER α

As shown in Figure 6C, EGF stimulation increased the phosphorylation of ER α S118 residue in T47D cells. However, NgBR knockdown attenuated EGF-stimulated phosphorylation of ER α S118 residue. One previous study demonstrated that EGF stimulation promotes the translocation of phos-Ser118-ER α to the nucleus, which is a critical step for initiating the transcription of ER α -target genes [30]. We isolated nuclear protein from T47D cells and examined the amount of phos-Ser118-ER α in the nucleus by Western blot analysis. Consistent with the previous report [30], EGF stimulation promotes the translocation of phos-Ser118-ER α (Fig. S7). However, NgBR knockdown diminished EGF-stimulated nuclear translocation of phos-Ser118-ER α . Taken together, these data indicate that NgBR knockdown also inhibits EGF-stimulated phosphorylation of ER α in ER α positive breast cancer cells.

4. Discussion

In this study, we found NgBR was highly expressed in the residual tumor cells in tissue sections of ER α positive breast cancer patients receiving chemotherapy (Fig. 1 and Table 1). NgBR knockdown decreased E2-induced expression of survivin by increasing the expression of p53 (a tumor suppressor) (Fig. 3) and binding it to the promoter region of survivin (Fig. 4). Consequently, the results of cell viability and apoptosis assays clearly demonstrate that NgBR knockdown attenuates the E2-induced resistance to paclitaxel (Fig. 2) and doxorubicin (Fig. S3). In addition, NgBR knockdown attenuates the EGF-induced resistance

to paclitaxel (Fig. 5) and doxorubicin (Fig. S5) by decreasing EGF-stimulated phosphorylation of ER α and EGF-induced expression of survivin (Fig. 6). As shown in Figure S8, our study elucidated the important roles of NgBR in promoting the acquired resistance of ER α positive breast cancer to paclitaxel and doxorubicin.

Estradiol-induced proliferation and anti-apoptosis are well-known causal factors resulting in drug resistance of ER α positive breast cancer [12, 32]. ER α uses dual strategies to promote ER α positive abnormal cellular proliferation by enhancing the transcription of ERE-containing pro-proliferative genes and repressing the transcription of p53-responsive anti-proliferative genes [33], such as p21 [24], survivin and MDR1 [32]. ER α and p53 have an inverse relationship affecting both expression and function [33]. One previous study has shown that E2 enhanced ER α binding to p53 and repressed the transcription of p53-responsive antiproliferative genes [33]. In our study, we used the well-known ER α target gene ESR1 to demonstrate that estradiol treatment enhanced the binding of ER α to the ERE-containing gene and NgBR knockdown attenuated the binding of ER α to the ER α target gene (Fig. 4A and 4C). In addition, NgBR knockdown enhanced the binding of p53 to the promoter region of survivin and resulted in decreased survivin expression (Fig. 4B and 4D). This result suggests the mechanism by which NgBR regulates ER α and p53 mediated gene transcription. However, the NgBR-dependent gene regulation profile, in the context of ER α and p53-dependent regulation, requires further ChIP-seq and RNA-seq experiments, as planned in our future investigation.

Survivin, as a member of the inhibitor of apoptosis protein family (IAP), is involved in different types of cancer cell division, apoptosis and tumor resistance to paclitaxel [27–29]. A previous study has suggested survivin is a predictive biomarker of a cPR to neoadjuvant chemotherapy in patients with stage II and III breast cancer [34]. The association between NgBR expression and survivin in ER α positive breast cancer suggests a potential role of NgBR in chemotherapy resistance. As shown in Figure 3A and 6A, increased expression of both NgBR and survivin was observed in T47D cells treated with either E2 or EGF. NgBR knockdown reduced the amount of survivin in ER α positive breast cancer cells and increased the efficacy of paclitaxel in inducing apoptosis of these tumor cells (Fig. 2, 5 and S2). Furthermore, our ChIP-PCR results (Fig. 4) elucidated the molecular mechanism by which NgBR regulates the p53-mediated transcription of survivin [12, 32]. Our data further demonstrated that NgBR knockdown not only attenuates ER α binding to the ERE of ER α target gene but also enhanced the binding of p53 to the promoter region of survivin to attenuate the transcription of survivin (Fig. 4). These results suggest that NgBR is a potential therapeutic target to reduce acquired resistance to paclitaxel.

Ras is a well-known oncogene that has been shown to cause tumorigenesis and drug resistance by activating downstream kinases such as phosphatidylinositol-3-OH kinase (PI-3K)/Akt and Raf-1 kinase/ERK [35–37] [38, 39]. Although Ras mutations rarely occur in breast cancer (less than 10%) [40], oncogenic Ras can contribute to the tumorigenic and invasive potential of breast epithelial cells [40]. Therefore, upregulation of normal Ras activity by RTKs, such as the EGFR and IGF1-R, has been shown in ER α positive breast cancer [41–43]. The classical mechanism of E2 action is mediated by the nuclear ER α (nER α) that regulates transcription of target genes containing the consensus ERE in their

promoter region [41–43]. In addition, E2 can also exert its action through membrane ER α (mER α) in conjugation with the signaling complex including EGFR, IGF-1R, adaptor protein Shc/Grb2 as well as RasGEFs (SOS1 and RasGEF3) to activate Src/Ras-dependent activation of Raf1-MARK/PI3K-Akt pathways [41–43]. This pathway promotes estrogen-dependent tumor resistance [44]. Our recent publication demonstrated that NgBR binds farnesylated Ras and is required for keeping Ras at the plasma membrane [17]. Therefore, NgBR is essential for the Ras-mediated signaling pathway [17]. Previous reports have shown that EGF induces the phosphorylation of ER α at the serine 118 residue [45], which is the confirmed signal involved in ER α -mediated resistance to chemotherapy [46]. Our data further demonstrated that either E2 or EGF treatment increases the resistance of ER α breast cancer cells to paclitaxel and doxorubicin, and NgBR knockdown not only impairs E2 or EGF-stimulated phosphorylation of ER α (Fig. 3C and 6C) but also attenuates E2 or EGF-caused resistance to paclitaxel and doxorubicin (Fig. 2, 5, S3, S5). This data further supports that NgBR is a potential therapeutic target for blocking concurrent chemoresistant signaling.

Chemotherapy has been used therapeutically for recurrent ER α positive breast cancer that has failed endocrine therapy, and in patients who demonstrate a high risk of recurrence [47]. Chemotherapy is also used in the neoadjuvant setting for patients with locally advanced tumors or who desire breast-conserving surgery [47, 48]. However, after neoadjuvant chemotherapy, ER α positive breast tumors have a much lower pCR rate (7–16%) as compared to ER α negative breast tumors (30%–50%) [49–51]. The resistance of ER α positive breast cancer to chemotherapy has become a challenge for clinical treatment both in the neoadjuvant and adjuvant settings [14, 15]. Our immunostaining data showed an increased population of NgBR and Nogo-B positive tumor cells in residual tumors after chemotherapy although the association was not statistically significant due to a limited sample size (Fig. 1 and Table 1). The larger human study results from the KM-Plot demonstrated a significant correlation between increased expression of NgBR and higher patient mortality (Fig. S1). The detailed mechanism by which increased levels of NgBR expression are associated with increased mortality need further investigation.

In summary, our findings show that targeting NgBR could re-sensitize ER α positive breast cancer to paclitaxel and doxorubicin by reducing both E2 and EGF-induced survivin expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. NgBR is highly expressed in estrogen receptor positive (ER+) breast cancer
2. Novel roles of NgBR in promoting the chemoresistance of ER+ breast cancer
3. NgBR is involved in phosphorylation of estrogen receptor
4. NgBR regulates the expression of survivin and p53 in ER+ breast cancer
5. NgBR depletion increases the binding of p53 to the promoter of survivin gene

Figure 1. NgBR expression increased in residual breast tissue of ERα positive breast cancer patients after chemotherapy

Immunohistochemical (IHC) staining was developed using DAB as described in methods. Images were taken using an Olympus microscope with x20 lens. **(A–F)** Representative H&E staining and IHC staining of NgBR and Nogo-B in breast tissues from one patient who showed partial response to neo-adjuvant chemotherapy. **(A–C)** Representative H&E staining and IHC staining of NgBR and Nogo-B in breast biopsy tissues before chemotherapy. **(D–F)** Representative H&E staining and IHC staining of NgBR and Nogo-B in breast resection tissues after chemotherapy. Scale bar: 100 μm

Figure 2. NgBR knockdown attenuates the estradiol-induced chemoresistance of T47D cells to paclitaxel

(A) Schematic illustration of the cell treatment protocol. T47D cells were treated with estradiol (E2) alone, paclitaxel (PTX) alone, or co-treated as depicted. Cells were seeded in growth medium, incubated 24 h, and then transfected with NS or NgBR siRNA overnight. Transfection medium was replaced with 10% charcoal stripped, phenol-red free medium. Six hours later, 10 nM E2 was added to the appropriate groups, and cells were incubated for an additional 4h. At that time, medium was supplemented with or without PTX (100 nM) and E2. Forty-eight hours later, cells were counted or stained for cell apoptosis analysis. (B) NgBR knockdown decreases E2-induced chemo-resistance of T47D cells to paclitaxel. Cell viability was analyzed by CCK8 assay in control, E2 (10 nM), paclitaxel (100 nM), and E2 pretreatment (4h) + paclitaxel treated T47D cells for 48 h. (C) NgBR knockdown decreases the clonogenicity of T47D cells. Clonogenic formation assay was used for measuring clonogenicity of T47D cells treated with E2 (10 nM), paclitaxel (2 nM), and E2 pretreatment (4h) + paclitaxel (left panel). The quantitative results show the average percentage of surviving colonies (right panel). (D) AO/EB staining was used for measuring apoptotic cell population in T47D cells treated with E2 (10 nM), paclitaxel (100 nM) and E2 pretreatment (4h) + paclitaxel (left panel). The quantitative results show the average percentage of apoptotic cells (right panel) (E) NgBR knockdown increases apoptosis of T47D cells induced by paclitaxel. Flow cytometry assay was used for measuring apoptotic cell

population in T47D cells treated with E2 (10 nM), paclitaxel (100 nM) and E2 pretreatment (4h) + paclitaxel (left panel). The apoptotic cells were detected by Annexin V-PI dual staining. Representative data from three independent experiments are shown in left panel. The total number of cells in the Q2 and Q4 quadrant was regarded as apoptotic cells. Percentages of apoptotic cells are shown in the bar graph (right panel). The statistical significance of quantitative assays was analyzed using ANOVA analysis. The data are means $\pm$ SEM of three independent assays. *: $P < 0.05$ vs. NS. **: $P < 0.05$ vs. NS CTL. #: $P < 0.05$ vs. paclitaxel. (n=3).

Figure 3. NgBR knockdown attenuates estradiol-induced expression of survivin as well as phosphorylation of Akt/ERK and ERα in T47D cells
(A) NgBR knockdown increases p53 expression and diminishes E2 -induced survivin expression in T47D cells (left). Quantitative analysis of p53 and survivin protein level change in T47D cells by measuring the intensity of western blot band (right). **(B)** NgBR knockdown reduces the E2-induced phosphorylation of Akt and ERK in T47D cells. After 24h NS or NgBR siRNA transfection, cells were arrested overnight in serum-free, phenol red-free medium and then stimulated with 10 nM E2 for 10 minutes. The E2-induced phosphorylation Akt and ERK were determined by western blot. Total Akt, total ERK, and Hsp90 protein levels were used as respective loading controls. **(C)** NgBR knockdown reduces the E2-induced phosphorylation of ERα in T47D cells. The phosphorylation of ERα at the residue S118 was determined by western blot analysis. The statistical significance of quantitative assays was analyzed using ANOVA analysis. The data are means $\pm$ SEM of three independent assays. *: $P < 0.05$ vs. NS; #: $P < 0.05$ vs. E2. (n=3).

Figure 4. NgBR knockdown attenuates the binding of ER α to ESR1 and increases the binding of p53 to the promoter region of survivin gene

Cells were grown in phenol red free medium with 10% charcoal stripped FBS for 4 days. Twenty-four hours after transfection with NS or NgBR siRNA, cells were treated with E2 at 10 nM for 3h. ChIP was performed using anti-ER α antibody to analyze ER α binding to ESR1 gene promoters in T47D cells (**A**) and in MCF-7 cells (**C**), and using anti-p53 antibody to analyze p53 binding to survivin gene promoters in T47D (**B**) and MCF-7 (**D**) cells. The amount of immunoprecipitated ESR1 and survivin chromatin DNA was determined by PCR.

Figure 5. NgBR knockdown attenuates EGF-induced resistance of T47D cells to paclitaxel (A) Schematic illumination of the cell treatment protocol. T47D cells were treated with EGF alone, paclitaxel (PTX) alone, or co-treated as depicted. Cells were seeded in growth medium, incubated 24 h, and then transfected with NS or NgBR siRNA overnight. Transfection medium was replaced with 2% charcoal stripped, phenol-red free medium. Six hours later, 100 ng/ml EGF was added to the appropriate groups, and cells were incubated for an additional 4 h. At that time, medium was supplemented with or without PTX (100 nM) and EGF. Forty-eight hours later, cells were counted or stained for cell apoptosis analysis. (B) EGF increases viability of paclitaxel-treated T47D cells and NgBR knockdown increases the response of T47D cells to paclitaxel. Viability of control, EGF (100 ng/ml), paclitaxel (100 nM), and EGF pretreatment (4h) + paclitaxel treated T47D cells were determined by CCK8 cell viability assay kit. The paclitaxel treatment was carried out for 48h. (C) EGF increases clonogenicity of paclitaxel-treated T47D cells and NgBR knockdown increases the response of T47D cells to paclitaxel. Clonogenic formation assay was performed to determine clonogenicity of T47D cells treated with EGF (100 ng/ml),

paclitaxel (2 nM) and EGF pretreatment (4h) + paclitaxel (left panel). The results show the average percentage of surviving colonies (right panel). **(D/E)** EGF attenuates the apoptosis of paclitaxel-treated T47D cells and NgBR knockdown increases the response of T47D cells to paclitaxel. **(D)** Apoptotic cells were determined by AO/EB staining. The results show the average percentage of apoptotic cell population of T47D cells treated with EGF (100 ng/ml), paclitaxel (100 nM) and EGF pretreatment (4h) + paclitaxel (left panel). The quantitative results show the average percentage of apoptotic cells (right panel). **(E)** The apoptotic cells were detected by Annexin V-PI dual staining. Flow cytometry assay was used for measuring apoptotic cell population of T47D cells treated with EGF (100 ng/ml), paclitaxel (100 nM) and EGF pretreatment (4h) + paclitaxel (left panel). Representative data from three independent experiments are shown in the left panel. The total number of cells in the Q2 and Q4 quadrants was regarded as apoptotic cells. Percentages of apoptotic cells are shown in the bar graph (right panel). The statistical significance of quantitative assays was analyzed using ANOVA analysis. The data are means $\pm$ SEM of three independent assays. *: $P < 0.05$ vs. NS. **: $P < 0.05$ vs. NS CTL. #: $P < 0.05$ vs. paclitaxel. (n=3).

Figure 6. NgBR knockdown attenuates EGF-induced expression of survivin and phosphorylation of Akt/ERK and ERα in T47D cells

(A) Protein levels were determined by western blotting. Quantitative analysis of survivin protein level change in T47D cells was performed by measuring the intensity of western blot band (right) (B) NgBR knockdown reduces the EGF-induced phosphorylation of Akt and ERK in T47D cells. At 24h after NS or NgBR siRNA transfection, cells were arrested overnight in serum-free, phenol red-free medium and then stimulated with EGF (100 ng/ml) for 5 minutes. The EGF-induced phosphorylation Akt and ERK were determined by western blotting. Total Akt, total ERK, and Hsp90 protein levels were used as respective loading controls. (C) NgBR knockdown reduces the EGF-induced phosphorylation of ERα (S118) in T47D cells. At 24h after NS or NgBR siRNA transfection, cells were arrested overnight in serum-free, phenol red-free medium and then stimulated with EGF (100 ng/ml) for 10 minutes. Total ERα and Hsp90 protein levels were used as respective loading controls. The statistical significance of quantitative assays was analyzed using ANOVA analysis. The data are means $\pm$ SEM of three independent assays. *: $P < 0.05$ vs. NS; #: $P < 0.05$ vs. EGF. (n=3).

Table 1

Baseline clinical characterisite of breast cancer patients

Characteristics		NgBR increase and stable	NgBR decrease	P value
Pathologic response	38	27(71.1)	11(28.9)	0.499
No response	5(13.2)	3(7.9)	2(5.3)	
Partial	27(71.1)	19(50.0)	8(21.1)	
Complete	2(5.3)	2(5.3)	0(0)	
N.A.	4(10.5)	3(7.9)	1(2.6)	
Characteristics		Nogo-B increase and stable	Nogo-B decrease	P value
Pathologic response	38	30(78.9)	8(21.1)	0.447
No response	5(13.2)	4(10.5)	1(2.6)	
Partial	27(71.1)	22(57.8)	5(13.2)	
Complete	2(5.3)	2(5.3)	0(0)	
N.A.	4(10.5)	2(5.3)	2(5.3)	

Clinical and pathologic details of the breast tumors.

Table 2

NgBR expression of breast cancer patients

NgBR expression before neo-adjuvant chemotherapy				
	Negative	Low	High	P value
Pathologic response	38	3(7.9)	32(84.2)	0.223
No response	5(13.2)	0(0)	5(13.2)	
Partial	27(71.0)	2(5.3)	23(60.5)	
Complete	2(5.3)	0(0)	1(2.6)	
N.A.	4(10.5)	1(2.6)	3(7.9)	

Pathologic details and NgBR expression of the breast tumors.

Table 3

Nogo-B expression of breast cancer patients

Nogo-B expression before neo-adjuvant chemotherapy				
	Negative	Low	High	P value
Pathologic response	38	1(2.6)	3(7.9)	34(89.5)
No response	5(13.2)	0(0)	0(0)	5(13.2)
Partial	27(71.0)	1(2.6)	2(5.3)	24(63.2)
Complete	2(5.3)	0(0)	1(2.6)	1(2.6)
N.A.	4(10.5)	0(0)	0(0)	4(10.5)

Pathologic details and Nogo-B expression of the breast tumors.