Expression of NgBR Is Highly Associated with Estrogen Receptor Alpha and Survivin in Breast Cancer

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Abstract

NgBR is a type I receptor with a single transmembrane domain and was identified as a specific receptor for Nogo-B. Our recent finding demonstrated that NgBR binds farnesylated Ras and recruits Ras to the plasma membrane, which is a critical step required for the activation of Ras signaling in human breast cancer cells and tumorigenesis. Here, we first use immunohistochemistry and real-time PCR approaches to examine the expression patterns of Nogo-B and NgBR in both normal and breast tumor tissues. Then, we examine the relationship between NgBR expression and molecular subtypes of breast cancer, and the roles of NgBR in estrogen-dependent survivin signaling pathway. Results showed that NgBR and Nogo-B protein were detected in both normal and breast tumor tissues. However, the expression of Nogo-B and NgBR in breast tumor tissue was much stronger than in normal breast tissue. The statistical analysis demonstrated that NgBR is highly associated with ER-positive/HER2-negative breast cancer. We also found that the expression of NgBR has a strong correlation with the expression of survivin, which is a well-known apoptosis inhibitor. The correlation between NgBR and survivin gene expression was further confirmed by real-time PCR. In vitro results also demonstrated that estradiol induces the expression of survivin in ER-positive T47D breast tumor cells but not in ER-negative MDA-MB-468 breast tumor cells. NgBR knockdown with siRNA abolishes estradiol-induced survivin expression in ER-positive T47D cells but not in ER-negative MDA-MB-468 cells. In addition, estradiol increases the expression of survivin and cell growth in ER-positive MCF-7 and T47D cells whereas knockdown of NgBR with siRNA reduces estradiol-induced survivin expression and cell growth. In summary, these results indicate that NgBR is a new molecular marker for breast cancer. The data suggest that the expression of NgBR may be essential in promoting ER-positive tumor cell proliferation via survivin induction in breast cancer.

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Introduction

Breast cancer is the most common carcinoma in women and the second most common cause of cancer death in females [1]. Early detection in conjunction with screening programs and the advent of more efficacious and targeted adjuvant systemic therapy have contributed to the decrease in breast cancer mortality [1]. The effectiveness of pathway-specific targeted and patient-tailored therapeutics demands the need for continued advances in our understanding of the molecular biology of breast cancer progression and discovery of new prognostic markers [1].

The ductal and lobular subtype constitute the majority of all breast cancers worldwide, with the ductal subtype accounting for 40–75% of all diagnosed cases [2–5]. Nearly 80% of all diagnosed in situ and invasive breast cancers are of ductal origin [1,6]. In 2012, an estimated 229,060 new cases of breast cancer were expected to be diagnosed and approximately 39,920 deaths were expected to occur in the United States alone [7]. Breast cancer is the most common malignant disease in Western women, and distant metastasis are the main cause of death [6]. Here, we reveal a new potential diagnosis marker for breast invasive ductal carcinoma (IDC).

The Nogo isoforms-A, -B and -C are members of the reticulon family of proteins. Nogo-A and Nogo-C are highly expressed in the central nervous system (CNS), with Nogo-C also uniquely found in skeletal muscle, while Nogo-B is found in most tissues [8,9]. Nogo-A (also called RTN4-A) binds its specific receptors, such as NgR and LINGO1, and acts as a negative regulator of axon sprouting [10–13]. Nogo-B was previously identified as a protein that is highly expressed in caveolin-1 enriched micro-domains of endothelial cells [EC] [14]. The amino terminus
(residues 1–200) of Nogo-B (AmNogo-B) serves as a chemotactic for EC [14]. Mice deficient in Nogo-A/B show exaggerated neointimal proliferation, abnormal remodeling [14] and a deficit in ischemia induced angiogenesis and angiogenesis [15]. NgBR was identified as a receptor specific for AmNogo-B by an expression cloning approach [16]. High affinity binding of AmNogo-B to NgBR is sufficient for AmNogo-B mediated chemotaxis and tube formation of endothelial cells [16]. We have previously demonstrated that NogoB-NgBR ligand-receptor pair is necessary for in vivo angiogenesis in zebrafish [17]. Genetic knockdown of NogoB or NgBR by antisense morpholinos abolished intersomitic vessel (ISV) formation during developmental angiogenesis [17]. Our recent studies further demonstrated that NgBR is essential for Ras activation in breast tumor cells (unpublished data). However, there is no information regarding the roles of Nogo-B and NgBR in any kind of cancers, including breast cancer. Here, we demonstrate the expression patterns of Nogo-B and NgBR, their relationships with different molecular subtypes of breast cancer, and their possible roles in promoting tumor cell growth in breast cancer.

Materials and Methods

Tissue Microarray Slides

Three cohorts for a total of 656 breast tumor tissues and 15 normal breast tissues on tissue microarray (TMA) slides were used in this study. The first cohort, composed of 190 breast tumors and 15 normal breast tissues with duplicate cores for each case, was purchased from Shanghai Biochip Co [18]. The second cohort composed of 210 breast tumors with a single core for each case was obtained from the breast tissue bank at the Baylor College of Medicine. The third cohort composed of 256 breast tumor tissues with pathological information was purchased from BioChain (Newark, CA). All these breast cancer cases were histopathologically re-evaluated on hematoxylin and eosin-stained slides by two pathologists (BW and JH). These breast tissue specimens are anonymous and have institutional IRB (Institutional Review Board for Baylor College of Medicine) exemption.

NgBR and Nogo-B Antibody Generation

The peptide (AHHRMRWRADGRSLEK, residues from 81–96 of NgBR) was used to immunize rabbits (Epitomics, Burlingame, CA). The antiserum was purified using the same peptide-conjugated SulfoLink Coupling Gel (Pierce, Rockford, IL). The peptide recognizing epitope 14 to 30 of human Nogo-B was peptide-conjugated SulfoLink Coupling Gel (Pierce, Rockford, IL). NgBR rabbit monoclonal antibody (Clone ID: EPR8668) was also generated by Epitomics as a collaboration project and was used for Western blot analysis.

Immunohistochemistry

Sections of 4 μm thickness were dried, deparaffinized and rehydrated. For all the antibodies, heat-mediated antigen retrieval was performed using steamer treatment for 20 minutes in Target Retrieval Solution (Dako S1699) before immunohistochemistry (IHC). IHC was performed using pre-diluted antibodies such as NgBR (1:50), Nogo-B (1:3000), survivin (1:200), estrogen receptor alpha (ER, Dako, 1:100), progesterone receptor (PR, Dako, 1:50), Her2 (Dako, 1:50) and CK5/6 (Dako, 1:100). The detection system used was ImmPRESS Reagent and ImmPACT NovaRED (Vector Laboratories, Burlingame, CA). Slides were counterstained using hematoxylin. Detailed information of antibodies is shown in Table 1. To confirm the specificity of NgBR and Nogo-B IHC staining, incubation of the preabsorbed NgBR and Nogo-B antibodies using their corresponding epitope peptide-conjugated beads were considered as negative controls.

Table 1. Details of antibody and dilution.

| Antibody     | Clone | Source        | Dilution |
|--------------|-------|---------------|----------|
| NgBR         | 671   | Epitomics     | 1:50     |
| Nogo B       | IMG-5346A | Imgenex     | 1:3000   |
| Survivin     | NB500-201 | Novus      | 1:200    |
| ER           | SP1   | Dako         | 1:100    |
| PR           | PgR 636 | Dako         | 1:50     |
| Her2         | TAB250 | Invitrogen   | 1:50     |
| CK 5/6       | D5/16 B4 | Dako       | 1:100    |

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Cytoplasmic and membranous staining for NgBR, Nogo-B, plasma membrane staining for Her2, nuclear reactivity for ER, PR, cytoplasmic and nuclear staining for survivin were considered positive. Quantitative scoring of NgBR, Nogo-B and survivin IHC staining was performed following previously published methods [19]. The percentage of positive cells was assigned a score from 0(0%), 1(1–10%), 2(11–25%), 3(26–50%), 4(51–75%) and 5(>75%) and the staining intensities within the respective subcellular locations were noted as 0 = negative, 1 = weak, 2 = moderate and 3 = strong, NgBR, Nogo-B and survivin staining were expressed as the score calculated by combining the staining intensity and percentage of positive cells. They were scored as negative (−, IHC score 0 to 4), weak (+, IHC score 5 to 6) and strong (+++, IHC score 7 to 8). Qualitative scoring of both ER and PR was performed using ASCO/CAP criteria, i.e. 1% cell with weak staining considered as positive for ER and PR. Her2 was qualitatively/semi-quantitatively scored using ASCO/CAP guidelines, i.e., Her2 scored as negative (IHC score 0 and 1+) and positive (IHC score 2+ and 3+).

Cell culture. MCF-7, T47D and MDA-MB-468 breast tumor cells from ATCC were grown in DMEM (Invitrogen) containing penicillin (100 U/ml), streptomycin (100 mg/ml), and 10% (v/v) fetal calf serum (HyClone) that was changed to 10% charcoal stripped FBS (GIBCO) when performing estradiol treatment.

siRNA transfection. NgBR siRNA1 (S1 forward: GGA-AUAACUAAGACCUCUA, S1 reverse: UUGAGGCUAU-GUAUUCU), NgBR siRNA2 (S2 forward: CCAGAAUUUG-CAAAAGUA, S2 reverse: UACAUUUUGCAAUUGCGG) oligonucleotides with 3’ dTdT overhangs were synthesized by QIAGEN (Valencia, CA). The specificity of NgBR siRNA has been validated in our previous publication [16,17]. NgBR siRNA1 was used in all of NgBR knockdown experiments and NgBR siRNA2 was only used in experiments shown in the Figure S2. Control siRNA in experiments refers to a non-silencing siRNA (NSF: UUCUCGCCAAGUGCCAGCU, NSR: AGCUGAGAC-GUCCGG AGAA) designed and synthesized by QIAGEN. MCF-7, T47D and MDA-MB-468 cells were transfected with siRNA using Oligofectamine (Invitrogen). Cell growth assay and examination of cell signaling were performed at 48–72 hours after transfection.

Cell growth assay. MCF-7 and T47D cells were subcultured to each well of 12 wells plate. After overnight culture, cells were transfected with non-silencing siRNA (NS, negative controls) or siRNA specifically targeting NgBR (siNgBR). The next day after transfection, cells were treated with 10 nM b-estradiol. 

Association of NgBR with Survivin in Breast Cancer

| Antibody     | Clone | Source        | Dilution |
|--------------|-------|---------------|----------|
| NgBR         | 671   | Epitomics     | 1:50     |
| Nogo B       | IMG-5346A | Imgenex     | 1:3000   |
| Survivin     | NB500-201 | Novus      | 1:200    |
| ER           | SP1   | Dako         | 1:100    |
| PR           | PgR 636 | Dako         | 1:50     |
| Her2         | TAB250 | Invitrogen   | 1:50     |
| CK 5/6       | D5/16 B4 | Dako       | 1:100    |
Specificity of Nogo-B and NgBR IHC Staining

To confirm the specificity of NgBR and Nogo-B IHC, we performed IHC staining in human IDC tissue sections and used primary antibodies preabsorbed with their corresponding epitope peptide-conjugated beads as negative controls. As shown in Figure S1, expression of both NgBR and Nogo-B proteins were observed only in the cytoplasm or in membrane/cytoplasm of cancer cells, and smooth muscle cells or endothelial cells of blood vessels. The expression of NgBR in smooth muscle cells was much stronger than in endothelial cells. On the contrary, the expression of Nogo-B in endothelial cells was stronger than in smooth muscle cells. As negative controls, the same primary NgBR and Nogo-B antibodies were preabsorbed using their corresponding epitope peptide-conjugated beads. There are no specific staining signals in cancer cells, smooth muscle cells as well as endothelial cells of blood vessels. IHC staining using preabsorbed NgBR and Nogo-B antibodies confirmed the specificity of NgBR and Nogo-B IHC staining.

Expression of Nogo-B and NgBR in Invasive Ductal Carcinoma

Figure 1 (panel D-F) demonstrates representative IHC images of NgBR, Nogo-B and survivin in breast invasive ductal carcinoma (IDC). The overall expression of NgBR and Nogo-B in tumor cells is much stronger than in normal breast epithelial cells. The breakdown of the distribution of NgBR in breast tumors was as follows: 36.5% negative staining, 30.3% weak and 33.3% strong. Based on the scoring system previously described in the methods section, we analyzed the relationship of Nogo-B and NgBR expression with survivin expression as well as other well known breast cancer molecular subtype markers, such as ER, PR, HER2 and CK5/6. The statistical analysis results (Table 2) showed that higher expression of Nogo-B is frequently detected in ER-positive, and HER2-negative IDC. The expression pattern of survivin is also consistent with higher NgBR expression, namely in ER-positive, and HER2-negative IDC. Based on molecular subtypes of IDC, NgBR is highly expressed in non-triple negative breast cancer, particularly in luminal A subtype (ER-positive and/or PR-positive, HER2-negative) of breast cancer. Although there is a strong correlation between Nogo-B and NgBR, the presence of Nogo-B as determined by IHC staining is higher than NgBR in IDC. The breakdown of the distribution of Nogo-B in IDC tumors was as follows: 8.2% negative staining, 23.5% weak and 68.2% strong. As shown in Table 2, expression of Nogo-B only has correlation with survivin, but does not have significant correlation with ER, PR, HER2 and any molecular subtypes. We further analyzed the association of NgBR and survivin expression with the progression of breast cancer. As shown in Table 3, the score of NgBR and survivin IHC staining increased in the later stages of breast cancer and the correlation of their expression in different stages of breast invasive ductal adenocarcinoma is statistically significant.

To further confirm the correlation of NgBR and survivin expression in breast cancer, we used a real-time PCR approach to determine the copy number of NgBR and survivin transcripts in normal and different stages of breast cancers. Three human breast tumor qPCR panels (BCRT101, BCRT102, BCRT104) were used (Origene). The panels contained a total of 136 normalized cDNAs prepared from pathologist-verified human breast tumor specimens, including 16 normal breast tissue samples, and 120 ductal adenocarcinoma tissue samples. Accompanying pathology reports were used to categorize the 120 ductal adenocarcinoma specimens into four different disease stages from I to IV. Real-time PCR results (Figure 2A) show that NgBR expression is significantly higher in Stage II (53 samples) and Stage III-IV (44 samples) ductal adenocarcinoma specimens when compared with both normal breast samples (16 samples) and Stage I ductal adenocarcinoma samples (23 samples). Consistent with NgBR expression pattern, survivin expression (Figure 2B) is significantly higher in Stage II (53 samples) and Stage III-IV (44 samples) ductal adenocarcinoma specimens when compared with normal breast samples.
samples (16 samples) and Stage I ductal adenocarcinoma samples (23 samples). We also found that expression of NgBR and survivin has statistically significant correlations in the Stage II (correlation = 0.448, \( p < 0.05 \)) and Stage III–IV (correlation = 0.386, \( p < 0.05 \)) of ductal adenocarcinoma samples, but there are no statistically significant correlations in normal and Stage I groups (Table 4). Combined with IHC staining results, our data clearly demonstrated that NgBR expression is strongly associated with survivin expression in later stages of ductal carcinomas.

Roles of NgBR in Regulating Survivin Expression and Cell Growth in ER-positive Breast Cells

To determine the roles of NgBR in regulating survivin expression, we chose two breast tumor cell lines. One is estrogen receptor alpha positive breast tumor cell line T47D, and the other one is estrogen receptor alpha negative breast tumor cell line MDA-MB-468. We knocked down NgBR in both T47D and MDA-MB-468 cells with validated NgBR siRNA [16,17] and examined the estradiol-induced expression of survivin. As shown in Fig. 3A and 3B, estradiol (10 nM) treatment for 48 hours increased the survivin protein levels by 5.87 fold in non-silencing (NS) siRNA treated T47D cells and 2.35 fold in NgBR knockdown T47D cells (NS: 5.869 ± 0.402 vs siNgBR: 2.351 ± 0.290, \( n = 3 \), \( p < 0.05 \)). However, as shown in Fig. 3C and 3D, estradiol (10 nM) treatment for 48 hours did not significantly increase the survivin expression either in NS siRNA treated MDA-MB-468 cells or in NgBR knockdown MDA-MB-468 cells (NS: 1.054 ± 0.008 vs siNgBR: 1.103 ± 0.021, \( n = 3 \), \( p = 0.092 \)). To further determine the potential roles of NgBR in regulating survivin expression and breast tumor cell growth, we knocked down NgBR in MCF-7 cells, which are a typical ER alpha positive breast carcinoma cell line, and examined the survivin expression and cell growth in response to estradiol treatment. As shown in Figure 4A and 4B, estradiol (10 nM) treatment for 6 or 48 hours increased the survivin protein

Figure 1. Immunohistochemical (IHC) staining of NgBR, Nogo-B and survivin in non-neoplastic breast epithelium and invasive ductal carcinoma (IDC). Staining was developed using NovaRed as described in methods. Images were taken using an Olympus microscope with x20 lens. (A–C) IHC staining of NgBR, Nogo-B and survivin in normal breast tissues. Few epithelial cells are positive for NgBR (A) and Nogo-B (B), and the majority of myoepithelial cells express NgBR (A) and Nogo-B (B). Few epithelial and myoepithelial cells are weak positive for survivin (C). (D–F) IHC staining of NgBR, Nogo-B and survivin in luminal A IDC with strongly positive staining of NgBR (D), Nogo-B (E) and survivin (F). The positive staining was localized in the cytoplasm of tumor cells with few tumor cells demonstrating membrane staining of Nogo-B and survivin. (G–I) IHC staining of NgBR, Nogo-B and survivin triple negative IDC with negative staining of NgBR (G), Nogo-B (H) and survivin (I) in all tumor cells.

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Table 2. Protein expression in invasive ductal carcinoma (N = 400).

| Protein Level of staining N (%) | NgBR | Nogo-B | Survivin |
|---------------------------------|------|--------|----------|
| +146 (36.5)                    |      |        |          |
| −                               | 29 (7.3) | 3 (0.8) | 1 (0.3) | 0.000 |
| +                               | 47 (11.8) | 33 (8.3) | 14 (3.5) |        |
| +                              | 70 (17.5) | 85 (21.3) | 118 (29.5) |      |
| 6                               |        |        |          |
| −                               | 59 (14.8) | 5 (1.3) | 0 (0) | 0.000 |
| +                               | 59 (14.8) | 66 (16.5) | 24 (6.0) | 11 (2.8) | 37 (9.3) | 101 (25.3) |    |
| ++                              | 28 (7.0) | 50 (12.5) | 109 (27.3) | 3 (0.8) | 32 (8.0) | 152 (38.0) |  |
| 6                               |        |        |          |
| ER negative                     | 84 (21.0) | 52 (13.0) | 37 (9.3) | 0.000 |
| positive                        | 62 (15.5) | 69 (17.3) | 96 (24.0) | 16 (4.0) | 55 (13.8) | 154 (38.5) | 24 (6.0) | 82 (20.5) | 121 (30.3) |  |
| PR negative                     | 107 (26.8) | 87 (21.8) | 69 (17.3) | 0.000 |
| positive                        | 39 (9.8) | 34 (8.5) | 64 (16.0) | 9 (2.3) | 29 (7.3) | 99 (24.8) | 12 (3.0) | 46 (11.5) | 79 (19.8) |  |
| Her2 negative                   | 117 (29.3) | 89 (22.3) | 88 (22.0) | 0.031 |
| positive                        | 29 (7.3) | 32 (8.0) | 45 (11.3) | 4 (1.0) | 22 (5.5) | 80 (20.0) | 6 (1.5) | 29 (7.3) | 71 (17.8) |  |
| CK5 negative                    | 132 (33.0) | 111 (27.8) | 114 (28.5) | 0.257 |
| positive                        | 14 (3.5) | 10 (2.5) | 19 (4.8) | 5 (1.3) | 10 (2.5) | 28 (7.0) | 3 (0.7) | 18 (4.5) | 22 (5.5) |  |
| Molecular subtype               |        |        |          |
| Triple negative                 | 59 (12.5) | 31 (7.8) | 19 (4.8) | 0.001 |
| positive                        | 52 (13.0) | 31 (7.8) | 19 (4.8) | 0.000 |
| Non-triple negative             | 96 (24.0) | 90 (22.5) | 114 (28.5) | 22 (5.5) | 71 (17.8) | 207 (51.8) | 44 (1.10) | 109 (27.3) | 147 (36.8) |  |

The IHC staining levels of NgBR, Nogo-B and survivin were expressed as the score calculated by combining the staining intensity and percentage of positive cells. They were scored as negative (−, score 0 to 4), weak (+, score 5 to 6) and strong (+++, score 7 to 8). N: case number; (%): percentage of total case number.

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Table 3. Correlation analysis of NgBR and survivin in different stages of breast invasive ductal adenocarcinoma.

| Stage | NgBR Score | Survivin Score | Correlation p-value |
|-------|------------|---------------|---------------------|
| I     | 3.43 (0.515) | 5.35 (0.412) | 0.68* 0.000         |
| II    | 143 (55.9) | 4.74 (0.213) | 5.615 (0.178) 0.73* 0.000 |
| III   | 72 (28.1) | 5.167 (0.264) | 5.708 (0.266) 0.861* 0.000 |
| IV    | 13 (5.1) | 6.769 (0.231) | 6.615 (0.266) 0.714* 0.015 |
| Total | 256 (100) | 4.912 (0.149) | 5.669 (0.128) 0.746* 0.000 |

*p<0.05; N: case number; (%): percentage of total case number.

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levels by 1.70 or 4.18 fold in non-silencing (NS) siRNA treated MCF-7 cells, respectively. However, NgBR knockdown in NgBR siRNA treated MCF-7 cells reduced the estradiol-induced survivin expression (6 hr estradiol treatment, NS: 1.698±0.294 vs siNgBR: 1.184±0.114, n = 3, p = 0.178; 48 hr estradiol treatment, NS: 4.562±0.145 vs siNgBR: 3.319±0.134, n = 3, p<0.05). To confirm the specificity of NgBR siRNA, we used a second siRNA (siNgBR2) targeting the coding region of NgBR to confirm both siNgBR1 and siNgBR2 can efficiently knock down NgBR and specifically abolished the estradiol-induced expression of survivin in MCF-7 breast tumor cells (Fig. S2). In addition, we used real-time PCR approach to examine the change of survivin gene expression. As shown in the Figure S3A, estradiol treatment increases the survivin gene expression in ER-alpha positive MCF-7 and T47D breast tumor cells but not in ER-alpha negative MDA-MB-468 cells. As shown in the Figure S3B, estradiol increases the survivin gene expression in MCF-7 cells (24 hour: 1.490±0.084 fold increase) and NgBR knockdown reduces estradiol-induced survivin gene expression (24 hour: 1.021±0.096 fold increase). As shown in Fig. 3A and 4A, NgBR knockdown did not reduce estrogen receptor expression. However, estradiol treatment caused the decrease of ER-alpha levels because of ER-alpha recycling [20, 21]. In addition, we also examined of NgBR knockdown on estradiol-stimulated cell growth. As shown in Figure 4C, estradiol treatment increases the growth of MCF-7 cells by 11.8% at 24 hours and 29.1% at 48 hours (n = 3, p<0.05), respectively, and NgBR knockdown abolishes the estradiol-stimulatory effects (24 hour: NS: 1.550±0.014 vs NS+2:1.733±0.037×10^5 cells vs NS+2:1.733±0.037×10^5 cells vs siNgBR+2:1.447±0.068×10^5 cells, n = 3, p<0.05; 48 hour: NS: 1.807±0.015×10^5 cells vs NS+2:3.33±0.023×10^5 cells, n = 3, p<0.05; NS+2:3.33±0.023×10^5 cells vs siNgBR+2:1.800±0.0405×10^5 cells, n = 3, p<0.05). Similarly, NgBR knockdown diminishes the estradiol-induced growth of...
T47D breast tumor cells (24 hour: NS: 7.95±0.550×10^5 cells vs NS+E2:12.733±0.521×10^5 cells, n = 3, p<0.05; NS+E2:12.733±0.521×10^5 cells vs siNgBR+E2:9.777±0.665×10^5, n = 3, p<0.05). As shown in Figure 4C and Figure S4A, NgBR knockdown does not reduce the basal growth of both MCF-7 and T47D breast tumor cells, respectively. These results demonstrate that NgBR is essential for estrogen-dependent survivin induction and ER positive breast tumor cell growth.

**Discussion**

Although Nogo-B and NgBR have been shown to play important roles in regulating endothelial cell migration and blood vessel formation [14,16,17], the roles of Nogo-B and NgBR in cancer cells and cancer progression are still unclear. Nogo-B (also known as ASY) was previously identified as one of the apoptosis-inducing genes in human cancer [22]. Ectopic expression of the Nogo-B/ASY gene led to extensive apoptosis, particularly in cancer cells [22]. It was further demonstrated that Nogo-B/ASY overexpression contributes to endoplasmic reticulum stress and induces apoptosis through Ca^{2+} depletion in endoplasmic reticulum [23]. However, at the same time, stable transfectants overexpressing high levels of Nogo-B/ASY are resistant to endoplasmic reticulum stress associated stimuli, which implies that Nogo-B/ASY overexpression activates a protective response to endoplasmic reticulum stress [23]. In addition, the osteosarcoma SaOS-2 cell lines and the CHO cell lines have been shown to express high levels of endogenous Nogo-B. Overexpression of Nogo-B in both SaOS-2 and CHO cell lines do not differ significantly from the respective parental wild-type or control cell lines both in respect to cell proliferation and to spontaneous apoptosis or cell death induced by staurosporine and tunicamycin [24]. These conflicting studies have caused the uncertainty about the precise role of Nogo-B in modulating the apoptosis of cancer cells.

Our preliminary results show that overexpression of the amino-terminal domain of Nogo-B (AmNogo-B) does not cause any significant effects on tumor cell growth and cell survival (data not shown). As shown in Figure 4C and Figure S4A, knockdown of NgBR also does not affect the growth and survival of MCF-7 and T47D cells, typical estrogen receptor alpha positive breast tumor cells, under baseline growth conditions. However, NgBR knockdown reduces estradiol-induced MCF-7 and T47D cell growth (Fig. 4C and Fig. S4A), respectively. Further comparison of ER-alpha and NgBR expression in both MCF-7 and T47D cells as well as their response to estradiol stimulation show that T47D has lower ER-alpha expression and higher NgBR expression than MCF-7 cells (Fig. S4B), but T47D has more remarked response to estradiol-induced expression of survivin (Fig. S3A) as well as cell growth as compared to MCF-7 cells (Fig. S4A vs. Fig. 4C). It indicates that higher expression of NgBR may enhance the ER-alpha-mediated signaling. Our recent studies demonstrated that NgBR acts as a scaffold protein required for Ras plasma membrane translocation and Ras signaling in tumor cells [unpublished data]. Our findings suggest that NgBR may recapitulate the oncogene function of Ras and coordinate with ER to promote estrogen response. This detailed molecular mechanism needs further investigation.

Given these findings, we sought to demonstrate the significances of Nogo-B and NgBR in specific types of breast cancer. ER, PR, HER2 are three well-characterized tumor markers that are typically expressed and are strongly associated with prognosis in breast cancer. To distinguish the heterogeneity of this disease, breast cancer has been categorized as four distinct subtypes based on gene expression profiling, including luminal A (ER-positive

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**Table 4. Correlation analysis of NgBR and survivin transcripts in different stages of ductal adenocarcinoma.**

| Stage          | N (%) | Correlation | p-value |
|----------------|-------|-------------|---------|
| Normal breast tissue | 16(11.7) | 0.077 | 0.776 |
| Breast cancer stage |       |             |         |
| I              | 23(16.9) | 0.279 | 0.198 |
| II             | 53(39.0) | 0.448* | 0.000 |
| III-IV         | 44(32.4) | 0.386* | 0.007 |
| Total          | 136(100) | 0.432* | 0.000 |

*p<0.05; N: case number; (%): percentage of total case number.

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and/or PR-positive, HER2-negative, low Ki67 index), luminal B (ER-positive and/or PR-positive, HER2-positive or HER2 negative, higher Ki67 index), HER2 enriched (HER2-positive and ER-negative/PR-negative) and triple-negative/basal like (ER-negative, PR-negative and HER2-negative) [1,25–27], [28]. Luminal A and luminal B are the most common subtype, usually representing low- to intermediate-grade tumors characterized by the expression of genes that are commonly expressed by normal ductal epithelial cells [1]. The luminal A subtype is well-differentiated and associated with lobular histology and more frequent co-expression of both ER and PR than the luminal B subtype. Most cases of luminal B presented as grade II or III carcinoma showing HER2 overexpression and a higher Ki67 index [28]. The HER2 enriched subtype usually represents high-grade tumors with strong HER2 expression. The triple-negative/basal like subtype usually represents high-grade tumors displaying necrosis, prominent lymphocytic infiltration and a pushing border, carrying a poor prognosis [1,25–27]. Our results suggested that high expression of NgBR is positively associated with ER-positive and HER2 negative breast cancers. Our results further indicate that high expression of NgBR in ER positive breast cancer may promote tumor cell growth and division by increasing the expression of survivin via an estrogen-dependent manner. These data strongly suggest that there is a close relationship among ER alpha, NgBR, survivin and their associated signaling pathways in breast cancer. Further experiments are needed to confirm this hypothesis.

Our results clearly demonstrated that both NgBR and survivin are highly expressed in ER positive IDC (Fig. 1 and Table 2). NgBR knockdown reduced the estradiol-induced expression of survivin in ER positive breast tumor cells but not in ER-negative breast tumor cells (Fig. 3 and Fig. 4). Survivin was first identified as a baculovirus anti-apoptotic protein and is a member of the inhibitor of apoptosis proteins (IAP) family, which specifically inhibits caspases 3, 7 and 9 [31–33] and is involved in acquiring resistance to apoptosis. It has been shown that survivin inhibits apoptosis, regulates cell division and enhances angiogenesis [33]. Survivin is rarely expressed in terminally differentiated adult tissues, however high expression of survivin is found in most cancers [33–35]. High expression of survivin has been found to be related to poor survival in breast cancer patients [36,37] and progression of breast cancer [38]. Survivin is also associated with resistance to chemotherapy and hormone therapy, and predicts a poor clinical outcome in breast cancer [37,39]. Recent meta-analysis of survivin expression in breast cancer patients also demonstrated a significant association between positive survivin expression and a poor overall survival consequence in breast cancer patients [40]. Decreased survivin expression was found to

Figure 3. NgBR regulates estradiol-induced survivin expression in estrogen receptor positive breast tumor cells. T47D is an estrogen receptor positive breast tumor cell line. MDA-MB-468 is an estrogen receptor negative breast tumor cell line. NgBR was knockdown in both T47D and MDA-MB-468 cells using siRNA as described in methods. Both tumor cells were treated with 10 nM estradiol for 48 hours. Protein levels of NgBR, ER-alpha and survivin were determined by Western blot analysis. Beta-Actin is applied as a housekeeping protein. The density of each band was measured using NIH ImageJ and presented as relative intensity of survivin after normalized with beta-actin housekeeping protein. (A) NgBR knockdown diminished estradiol-induced survivin expression in T47D breast tumor cells. (B) Quantitative analysis of survivin protein level change in T47D cells by measuring intensity of survivin western blot band. Data is presented as fold changes of estradiol treatment group as compared to the non-treatment group (n = 3; * siNgBR vs NS p < 0.05). (C) NgBR knockdown has no effect on survivin expression in MDA-MB-468 breast tumor cells. (D) Quantitative analysis of survivin protein level change in MDA-MB-468 cells by measuring intensity of survivin western blot band. Data is presented as fold changes of estradiol treatment group as compared to the non-treatment group. (n = 3; siNgBR vs NS p = 0.092). doi:10.1371/journal.pone.0078083.g003
increase sensitivity to chemotherapy drugs [41,42] and ionizing radiation [43]. It has been shown that estrogen upregulates the expression of survivin in ER positive MCF-7 breast cancer cells [44]. This finding might suggest that there is a positive association between ER and survivin expression in breast cancer. In the context of our pathological findings in 656 specimens of breast cancer patients and in vitro results, high expression of NgBR in ER-positive breast cancer may contribute to the survivin induction caused by estrogen stimulation. Our findings further indicate that the signaling to control tumor growth may be partially mediated through the ER-NgBR-survivin pathway in ER-positive breast cancer. This pathway may serve as a potential target for directed therapy.

**Figure 4. NgBR is essential for estradiol-induced survivin expression and cell growth of MCF-7 breast tumor cells.** MCF-7 is an estrogen-dependent breast cancer cell line. NgBR was knocked down in MCF-7 cells using siRNA. (A) NgBR knockdown diminished estradiol-induced survivin expression in MCF-7 breast tumor cells. MCF-7 cells were treated with 10 nM estradiol for 6 and 48 hours. Protein levels of NgBR and survivin were determined by Western blot analysis. Beta-Actin is applied as a housekeeping protein. The density of each band was measured using NIH ImageJ and presented as relative intensity of survivin after normalized with beta-actin housekeeping protein. (B) Folds of survivin increase were determined by measuring relative western blot intensity of survivin. Data is presented as fold changes of estradiol treatment group as compared to the non-treatment group (n = 3; 6 hrs estradiol treatment, NS vs siNgBR p = 0.178; * 48 hrs estradiol treatment, NS vs siNgBR p < 0.05). (C) NgBR knockdown impaired estradiol-stimulated cell growth of MCF-7 breast tumor cells. MCF-7 cells were treated with 10 nM estradiol for 24 and 48 hours. Viable cell numbers were counted using the Bio-Rad TC10™ Automated Cell Counter. Data is presented as mean ± SEM (n = 3, # 24 hrs or 48 hrs estradiol treatment vs baseline p < 0.05; * siNgBR vs NS p < 0.05); E2: estradiol.

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therapy. These results suggest that NgBR may play an important role in ER-positive breast cancer growth via increasing survivin expression.

In summary, our study is the first to investigate the expression and localization of Nogo-B protein and NgBR receptor in human breast cancer. The findings from this study demonstrate: (a) NgBR is highly expressed in ER positive and Her2 negative IDC breast cancer, whereas Nogo-B is ubiquitously expressed in IDC; (b) expression of NgBR is correlated with survivin expression in IDC as well as in later stages of breast cancer; (c) NgBR is essential for estradiol-induced survivin expression in ER positive breast tumor cells; and (d) finally, NgBR is also required for estradiol-stimulated ER positive breast tumor cell growth. Although we need further investigation to reveal the molecular mechanism by which NgBR promotes survivin expression in ER-positive breast cancer cells and the potential roles of NgBR in ER-positive breast cancer progression, current findings suggest high expression of NgBR may be a novel diagnosis marker or a potential therapeutic target for ER-positive breast cancer.

Supporting Information

Figure S1 Immunohistochemical (IHC) staining of NgBR and Nogo-B in invasive ductal carcinoma (IDC). Staining was developed using NovaRed as described in methods. Images were taken using an Olympus microscope with x20 lens. (A, B) To confirm the specificity of NgBR and Nogo-B IHC staining, we performed IHC staining in human IDC tissue sections and used primary antibodies preabsorbed with their corresponding epitope peptide-conjugated beads († peptide conjugated beads) as negative controls. (TIF)

Figure S2 NgBR regulates estradiol-induced survivin expression in MCF-7 cells. NgBR was knocked down in MCF-7 cells using two different siRNA targeting NgBR (siNgBR1, and siNgBR2). Protein levels of NgBR, ER-alpha and survivin were determined by Western blot analysis. Beta-Actin is applied as a housekeeping protein. (TIF)

Figure S3 NgBR regulates estradiol-induced survivin gene expression in estrogen receptor positive breast tumor cells. (A) Estradiol increases survivin gene expression in estrogen-receptor positive breast tumor cell lines (MCF-7, T47D) but not in estrogen-receptor negative cell line (MDA-MB-468). All these three cell lines were treated with 10 nM estradiol for 24 hours. Survivin gene expression was determined by real-time PCR and is normalized with beta-actin. All groups are compared to MCF-7 no estradiol treatment group. (B) NgBR regulates estradiol-induced survivin gene expression in MCF-7 cells. NgBR was knocked down in MCF-7 cells using siRNA as described in methods. The cells were treated with 10 nM estradiol for 24 hours. Survivin gene expression was determined by real-time PCR and is normalized with beta-actin. All groups are compared to NS no estradiol treatment group. E2: estradiol. (TIF)

Figure S4 (A) NgBR knockdown impairs estradiol-stimulated growth of T47D breast tumor cells. Fifty thousand T47D cells were sub-cultured to each well of 12 wells plate. T47D cells were knocked down by siRNA targeting NgBR (siNgBR) and treated with 10 nM estradiol for 24 hours. Viable cell numbers were counted using the Bio-Rad TC10Tm Automated Cell Counter. Data is presented as mean±SEM (n = 3, # p<0.05; * siNgBR vs NS p<0.05). E2: estradiol. (B) Protein expression levels of survivin, ER-alpha and NgBR in MCF-7 and T47D breast tumor cells determined by Western blot analysis. (TIF)

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Author Contributions

Conceived and designed the experiments: JH QRM. Performed the experiments: BW BZ. Analyzed the data: BW BZ PN JH QRM. Contributed reagents/materials/analysis tools: PN AK JH QRM. Wrote the paper: AK JH QRM.

References

1. Bombonati A, Sgroi DC (2011) The molecular pathology of breast cancer progression. J Pathol 223: 307–317.
2. Tavassoli FAD (2005) World Health Organization Classification of Tumours: Pathology and Genetics of Tumours of the Breast and Female Genital Organs. WHO: Geneva: 9–19.
3. Rakha EA, Putti TC, Abd El-Rehim DM, Paish C, Green AR, et al. (2006) Morphological and immunophenotypic analysis of breast carcinomas with basal and myoepithelial differentiation. J Pathol 208: 495–506.
4. Weigelt B, Geyer FC, Rei-Flies JA (2010) Histological types of breast cancer: how special are they? Mol Oncol 4: 192–206.
5. Weigelt B, Bachner FL, Rei-Flies JA (2010) The contribution of gene expression profiling to breast cancer classification, prognosis and prediction: a retrospective of the last decade. J Pathol 220: 263–210.
6. Wang Y, Huang H, Yin H, Zhao B, Wei H, et al. (2010) Nogo-A gene expression in breast cancer progression. J Cancer Res Ther 6: 79–84.
7. Vasa M, Dhanasekaran SM, Ramaswamy S, Sorlie T, Tamayo P, et al. (2002) Gene expression profiles of human breast cancers. Nat Genet 30: 39–45.
8. Huang H, Huang Y, Zhao B, Wei H, Yin H, et al. (2010) Nogo-A gene expression in breast cancer progression. J Cancer Res Ther 6: 79–84.
9. Vasa M, Dhanasekaran SM, Ramaswamy S, Sorlie T, Tamayo P, et al. (2002) Gene expression profiles of human breast cancers. Nat Genet 30: 39–45.
10. Wang Y, Huang H, Yin H, Zhao B, Wei H, et al. (2010) Nogo-A gene expression in breast cancer progression. J Cancer Res Ther 6: 79–84.
11. Weigelt B, Pettersen JE, van ’t Veer LJ (2005) Breast cancer metastasis: markers and models. Nat Rev Cancer 5: 591–602.
12. Siegel R, Naishadham D, Jemal A (2012) Cancer statistics, 2012. CA Cancer J Clin 62: 10–29.
13. Oertle T, Schwab ME (2003) Nogo and its PARTNers. Trends Cell Biol 13: 187–194.
14. Acevedo L, Yu J, Erdjument-Bromage H, Miao RQ, Kim JE, et al. (2004) A new role for Nogo as a regulator of vascular remodeling. Nat Med 10: 382–388.
15. Zhao B, Chun C, Liu Z, Homer DL, Llabre MM, et al. (2010) Nogo in breast cancer is essential for angiogenesis in zebrafish via Akt pathway. Blood 116: 5432–5433.
16. Zhao B, Chun C, Liu Z, Homer DL, Llabre MM, et al. (2010) Nogo in breast cancer is essential for angiogenesis in zebrafish via Akt pathway. Blood 116: 5432–5433.
17. Stoica GE, Franke TF, Moroni M, Mueller S, Morgan E, et al. (2003) Effect of Nogo-A on cerebral microvascular endothelial cell migration. Neuroreport 14: 2455–2458.
22. Li Q, Qi B, Oka K, Shimakage M, Yoshioka N, et al. (2001) Link of a new type of apoptosis-inducing gene ASY/Nogo-B to human cancer. Oncogene 20: 3929–3936.
23. Kuang E, Wan Q, Li X, Xu H, Zou T, et al. (2006) ER stress triggers apoptosis induced by Nogo-B/ASY overexpression. Exp Cell Res 312: 1933–1940.
24. Oertle T, Mertler D, Schwab ME (2003) Do cancer cells die because of Nogo-B? Oncogene 22: 1390–1399.
25. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, et al. (2000) Molecular portraits of human breast tumours. Nature 406: 747–752.
26. Sorlie T, Perou CM, Tibshirani R, Aas T, Geiser S, et al. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98: 10869–10874.
27. Sotiriou C, Neo SY, McShane LM, Korn EL, Long PM, et al. (2003) Breast cancer classification and prognosis based on gene expression profiles from a population-based study. Proc Natl Acad Sci U S A 100: 10933–10938.
28. Park S, Koos JS, Kim MS, Park HS, Lee JS, et al. (2012) Characteristics and outcomes according to molecular subtypes of breast cancer as classified by a panel of four biomarkers using immunohistochemistry. Breast 21: 50–57.
29. Voduc KD, Cheang MC, Tyllesley S, Gelmon K, Nielsen TO, et al. (2010) Breast cancer subtypes and the risk of local and regional recurrence. J Clin Oncol 28: 1694–1699.
30. Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, et al. (2010) Metastatic behavior of breast cancer subtypes. Clin Cancer Res 16: 3271–3277.
31. Devereaux QL, Reed JC (1999) IAP family proteins—suppressors of apoptosis. Genes Dev 13: 239–252.
32. Cheung HH, LaCasse EC, Korneluk RG (2006) X-linked inhibitor of apoptosis antagonism: strategies in cancer treatment. Clin Cancer Res 12: 3238–3242.
33. Duffy MJ, O’Donovan N, Brennan DJ, Gallagher WM, Ryan BM (2007) Survivin: a promising tumor biomarker. J Cell Physiol 219: 49–60.
34. Altieri DC (2003) Validating survivin as a cancer therapeutic target. Nat Rev Cancer 3: 46–54.
35. Li F (2003) Survivin study: what is the next wave? J Cell Physiol 197: 8–29.
36. Tanaka K, Iwamoto S, Gou G, Nohara T, Iwamoto M, et al. (2000) Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. Clin Cancer Res 6: 127–134.
37. Jha K, Shukla M, Pandey M (2012) Survivin expression and targeting in breast cancer. Surg Oncol 21: 125–131.
38. Singh M, Bleile MJ, Shroyer AL, Heinz D, Jarboe EA, et al. (2004) Analysis of survivin expression in a spectrum of benign to malignant lesions of the breast. Appl Immunohistochem Mol Morphol 12: 296–304.
39. Ralda EA, Reis-Filho JS, Ellis IO (2008) Basal-like breast cancer: a critical review. J Clin Oncol 26: 2568–2581.
40. Song J, Su H, Zhou Y, Guo L (2013) Prognostic value of survivin expression in breast cancer patients: a meta-analysis. Tumour Biol 34: 2053–2062.
41. Xiong H, Yu S, Zhuang L, Xiong H (2007) Changes of survivin mRNA and protein expression during paclitaxel treatment in breast cancer cells. J Huazhong Univ Sci Technolog Med Sci 27: 65–67.
42. Ho TF, Peng YT, Chuang SM, Lin SC, Feng BL, et al. (2009) Prodigiosin down-regulates survivin to facilitate paclitaxel sensitization in human breast carcinoma cell lines. Toxicol Appl Pharmacol 233: 253–260.
43. Asanuma K, Morai R, Yamada M, et al. (2000) Survivin as a radiosensitivity factor in pancreatic cancer. Jpn J Cancer Res 91: 1104–1109.
44. Frasor J, Danes JM, Kornm B, Chang KC, Lyttle CR, et al. (2003) Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144: 4562–4574.