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PRECLINICAL STUDY

GGNBP2 acts as a tumor suppressor by inhibiting estrogen receptor α activity in breast cancer cells

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Abstract Gametogenetin-binding protein 2 (GGNBP2) is encoded in human chromosome 17q12-q23, a region known as a breast and ovarian cancer susceptibility locus. GGNBP2, also referred to ZFP403, has a single C2H2 zinc finger and a consensus LxxLL nuclear receptor-binding motif. Here, we demonstrate that *GGNBP2* expression is reduced in primary human breast tumors and in breast cancer cell lines, including T47D, MCF-7, LCC9, LY2, and MDA-MB-231 compared with normal, immortalized estrogen receptor α (ERα) negative MCF-10A and MCF10F breast epithelial cells. Overexpression of *GGNBP2* inhibits the proliferation of T47D and MCF-7 ERα positive breast cancer cells without affecting MCF-10A and MCF10F. Stable GGNBP2 overexpression in T47D cells inhibits 17β-estradiol (E_2)-stimulated proliferation as well as migration, invasion, anchorage-independent growth in vitro, and xenograft tumor growth in mice. We further demonstrate that GGNBP2 protein physically interacts with ERα, inhibits E_2-induced activation of estrogen response element-driven reporter activity, and attenuates ER target gene expression in T47D cells. In summary, our in vitro and in vivo findings suggest that GGNBP2 is a novel breast cancer tumor suppressor functioning as a nuclear receptor corepressor to inhibit ERα activity and tumorigenesis.

Keywords GGNBP2 · Breast cancer · ERα · Tumorigenesis · Tumor suppressor

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Introduction

Previous genetic studies identified that allelic loss in familial breast and ovarian cancer patients is observed on human chromosome 17 q12-q23, in which the well-known tumor suppressor breast cancer 1 (*BRCA1*) is located [1–4]. However, mutations in *BRCA1* account for only ~10 % of all breast cancers [5, 6]. While numerous breast cancer susceptibility genes, i.*e*., *BRCA1*, *BRCA2*, *PTEN*, *TP53*, *ATM, NBN*, *CHEK2*, and *PALB2* have been identified, the genes responsible for the remaining ~50 % of all breast tumors are not yet established [7].

In silico analyses of 693 genes encoded in human chromosome 17q12-q23 region revealed a gene ~6.3 MB upstream of *BRCA1*-encoding gametogenetin-binding protein 2 (GGNBP2), also known as zinc finger protein 403 (*ZFP403*) with additional alias *ZNF403*, *DIF3*, *LZK1*,

LCRG1 [8]. GGNBP2 was originally identified as a dioxin-induced factor 3 (DIF3) in mouse embryonic stem cells in 2001 [9]. GGNBP2′s name comes from its interaction with a testicular protein gametogenetin [10]. GGNBP2 encodes a single C2H2 [Cystine–Cystine–Histidine–Histidine] zinc finger and has a consensus nuclear receptor (NR) binding box LxxLL [Leucine-x-x (any amino acid)-Leucine-Leucine] [11, 12]. LxxLL motifs are also found in other known breast cancer susceptibility genes, *i.e.*, *BRCA1*, *NCOA1* (also known as steroid receptor coactivator 1, SRC-1), and *NCOA3* (also known as steroid receptor coactivator 3, SRC-3) [5, 13–15]. Therefore, we hypothesized that *GGNBP2* may be a putative NR coregulator and a candidate breast cancer susceptibility gene.

GGNBP2 is evolutionarily conserved, sharing 87 % homology in nucleotide sequence and 96 % homology in amino acid sequence between humans and mice. Its mRNA transcripts have been detected in a number of tissues including testis, heart, brain, lung, liver, kidney, pancreas, placenta, and skeletal muscle [8, 9]. *GGNBP2* encodes a full-length protein composed of 740 amino acids. The protein was reported to localize to the nucleus [9, 10]. In humans, a shorter form of GGNBP2 containing 288 amino acids at the N-terminus has been reported and named as laryngeal carcinoma-related gene 1 (LCRG1) because of its downregulation in primary laryngeal carcinomas. It has been suggested that LCRG1 may play a role in laryngeal tumorigenesis [8, 16, 17]. More recently, reduced GGNBP2 was observed in chemotherapeutic-resistant ovarian cancer cell lines [18]. However, the function of GGNBP2 in breast cancer and steroid hormone action remains unknown. Here, we provide evidence that GGNBP2 inhibits breast cancer cell growth through modulating estrogen receptor α (ERα) action.

Materials and methods

Multiple human tissue expression array assays

Multiple human tissue expression cDNA arrays from nine different adult normal tissues and pairwise tumor patient samples including the normal and breast cancer tissues of 50 individuals were purchased from BD Biosciences Clontech (San Jose, CA, USA) and hybridized with a random primed cDNA probe of human *GGNBP2*. The cDNA probe was labeled using the T7QuickPrime Kit (Amersham, Pittsburgh, PA, USA) and α-^{32}P-dCTP according to the instructions of the manufacturer. The probe was purified using ProbeQuant G-50 columns (Amersham). After hybridization and washing, the hybridized membranes were scanned using a Molecular Dynamic Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) to capture the phosphoimages and detect the signal intensity at each spot. The membranes were then boiled in 0.1 % SDS to strip off hybridized *GGNBP2* probe and re-hybridized with an α-^{32}P-dCTP labeled human glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) cDNA probe. Following background correction, *GGNBP2* signal intensity of each sample on the membranes was normalized to the housekeeping gene reference *GAPDH* and then the signal intensity was calculated for each spot on the arrays.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time quantitative PCR (qPCR)

Total RNA was extracted from human breast cell lines and xenograft tumor tissues using TRIZOL Reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. RNA samples from mammary glands of wild-type C57 mice at virgin and pregnancy day 4.5, 6.5, 12.5, and 18, and from normal mammary gland and mammary tumor of *Wnt1* transgenic mice were generously provided by Dr. John Lydon (Baylor College of Medicine, Houston, TX, USA). Total RNA was adjusted to a concentration of approximately 1.0 μg/μL. Two microgram total RNA was reverse transcribed into cDNA with random primers (Invitrogen) and avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA). The cDNA was amplified by PCR with the primer sets of human or mouse *GGNBP2* and *GAPDH*, respectively. PCR primers, as listed below, were designed according to the sequences obtained from GenBank using the Vector NTI 12.0 program (Invitrogen) and synthesized by Operon Technologies (Alameda, CA, USA). Mouse *Ggnbp2*: 5′-CTCATTGGTGAACTTGACTGC-3′ (forward) and 5′-TCACTGCTTTCTCGTCTGCGGTG-3′ (reverse). Mouse *Gapdh*: 5′-TGCAGTGGCAAAGTGGAGAT-3′ (forward) and 5′-TTTGCCGTGAGTGGAGTCATA-3′ (reverse). For determination of the expression of exogenous *GGNBP2* and amphiregulin (*AREG)* in stable cell lines, RT-PCR was performed using following primers (a forward primer 5′-CACCATGGGTTCTCATCATCATCATCATC-3′, which recognizes the exogenous *His C* tag and a reverse primer 5′-AAACATCCATCCAAC ATCCTC-3′, which recognizes the human *GGNBP2* cDNA). Human *AREG*: 5′-GTCTTCAGGGAGTGAGATTTCC-3′ (forward) and 5′-GTGAGGATCACAGCAGACATAA-3′ (reverse). All primers were designed to amplify the products that covered more than one exon. Each PCR-cycle consisted of denaturation for 45 s at 94 °C, annealing for 1 min at 57 °C, and extension for 1 min at 72 °C. The amplified products were separated by electrophoresis in agarose gels and stained using ethidium bromide.

Real-time qPCR analysis was also performed to determine endogenous *GGNBP2* expression in MCF-10A,

MCF-7, T47D, LCC9, LY2, and MDA-MB-231 cells and *CCND1* and *TFF1* expression in T47D cell xenograft tumor tissues using the predesigned TaqMan probes and primers (Invitrogen).

Cell lines

Human breast cell lines T47D, MCF-7, MCF-10A, MCF-10F, and MDA-MB-231 cells, and HeLa cells were purchased from ATCC (Manassas, VA, USA). LCC9 and LY2 cell lines were derived from MCF-7 cells by cultivation with the antiestrogens ICI 182,780 (Fulvestrant) and LY 117018, respectively, and were graciously provided as a gift by Dr. Robert Clarke, Georgetown University [19]. All the cells were maintained in the recommended culture media: RPMI-1640 medium (Sigma, St Louis, MO, USA) for T47D cells; ATCC-formulated Eagle's Minimum Essential Medium (DMEM, ATCC) for MCF7, LCC9, and LY2 cells; MEGM mammary epithelial cell growth medium from Lonza (Allendale, NJ, USA) for MCF10A cells, L-15 for MDA-MB-231 cells (Sigma); and a mixture of DMEM and F12 medium (Sigma) for MCF-10F and HeLa cells, respectively. All culture media were supplemented with 10 % fetal bovine serum (FBS, Sigma) and antibiotic–antimycotic solution (Invitrogen). All cells were cultured at 37 °C in a 5 % CO_2 in air atmosphere.

DNA transfections and generation of cell lines with stable overexpression of exogenous *GGNBP2*

The human *GGNBP2* cDNA fragment was obtained by PCR using human testis cDNA as the template and validated by DNA sequencing. The correct full-length human *GGNBP2* cDNA was cloned into *pcDNA3–6His*C vector (Invitrogen) to construct the mammalian expression plasmid *pcDNA3–HisC tagged-GGNBP2 (pcDNA3–GGNBP2)*. To generate stable *GGNBP2* overexpression cells, T47D, MCF-7, MCF-10A, and MCF-10F cells were cultured in their perspective culture medium to approximately 50 % confluence in a 12-well plate and were transfected with 1 μg of either *pcDNA3–GGNBP2* or *pcDNA3–HisC* control plasmid using Lipofectamine 2000 (Invitrogen). The cells were then cultured in the same medium containing 200 μg/mL G418 (Cellgro molecular genetics, Manassas, VA, USA) for 21 days. Monoclonal resistant cells were obtained using limiting dilution assay, maintained in medium supplemented with G418 (200 mg/L) for amplification, and subcultured for subsequent experiments. The stable transfection and expression of *GGNBP2* in the T47D, MCF-7, MCF-10A, and MCF-10F cells were verified by RT-PCR analysis.

Cell proliferation assays

The in vitro cell proliferation assays were performed by cell counting using a hemocytometer under the microscope, and by the measurement of the absorbance at 490 nm using the CellTiter 96 AQueous One Solution cell proliferation assay (MTS) kits following the protocol recommended by the manufacturer (Promega). For cell-counting studies, equal number of control T47D (T47D-*HisC*) and *GGNBP2*-overexpressing cells (T47D-*GGNBP2* clones #12 and #15) were seeded on a 24-well plate (2.5×10^4 cells/well), and cultured for 2, 4, 6, 8, and 10 days. Media were changed with fresh media every other day. At the denoted days of culture, cells were harvested using trypsin solution, and cell numbers were determined using a hemocytometer under an Olympus light microscope. Eight samples per each group were counted at each time point. To determine the absorbance at 490 nm, stably transfected T47D, MCF-7, MCF-10A, and MCF-10F cells were seeded in 96-well plates at an initial density of 8×10^3 cells/well suspended in their perspective media and cultured for 4 days. Then, these cells were incubated with CellTiter 96 AQueous One Solution at 37 °C for 1 h and the absorbance at 490 nm was determined using a Bio-Rad microplate reader (Hercules, CA, USA).

For the study of estradiol effects on T47D cell proliferation, equal numbers (8×10^3 cells/well in the 96-well plate) of control T47D-*HisC* and *GGNBP2*-overexpressing cells (clone #15) were cultured in medium supplemented with charcoal-extracted FBS (Sigma) for 24 h and then treated with various concentrations of 17β-estradiol (E_2, Sigma) or ethanol. The medium was changed every 48 h. Cell proliferation assays were performed on day 5 after E_2 treatment using a CellTiter96 AQueous One Solution Cell Proliferation kit (Promega).

Three-dimensional (3-D) cultures of T47D cells

Three-D Matrigel cell cultures were conducted as described previously [20]. In brief, parental T47D cells and *GGNBP2*-overexpressing T47D cells clone #15 were resuspended in chilled 100 % Matrigel (BD Biosciences, San Jose, CA, USA), and seeded on 24 well culture plates (5000 cells/well). These cells in the Matrigel were cultured in RPMI-1640 medium supplemented with 10 % FBS, and media were carefully replaced with fresh media every other day. At day 2, 4, and 6 after culture, the morphology of cultured cells in the matrigel was captured using an inverted phase-contrast microscope, and the cells were harvested by trypsinization. Cell number was determined using a hematocytometer under an Olympus light microscope.

Colony formation on soft-agar plates

Colony formation on soft-agar plates was performed as described previously [21]. Briefly, equal numbers of T47D-*GGNBP2* cell clones #12, #15, and T47D-*HisC* cells (2×10^4 cells/35 mm dish) were plated in 0.2 % agar onto 0.5 % solidified agarose in RPMI-1640 media supplemented with 10 % FBS and then cultured at 37 °C. Culture medium was changed every 3 days. After 17 days, the plates were fixed with methanol at −20 °C for 5 min and then stained with crystal violet. The resulting colonies were counted under an Olympus dissecting light microscope.

Wound-healing cell migration analysis

Wound-healing cell migration analyses were performed as described previously [22]. Briefly, T47D-*GGNBP2* cells (Clone #12 and #15) and T47D-*HisC* control cells were seeded in 6-well plates at a concentration of 2×10^6 cells/mL in RPMI-1640 supplemented 10 % FBS. Cells were allowed to form a confluent monolayer before wounding. After 12 h culture, a strip of confluent cells was removed across the monolayer using a rubber policeman and then cultured in the same medium for 5 days. Images were taken under an Olympus inverted phase-contrast light microscope, and the gap distances were measured using Olympus MicroSuite software to determine the cell migration rates.

Matrigel chamber invasion assay

The ability of T47D-*GGNBP2* cell clones #12 and #15, parental T47D, T47D-*HisC* cells for passing through matrigel was measured using BD BioCoat Matrigel Invasion chambers according to the manufacturer's suggested protocol (BD Biosciences). In brief, equal numbers of parental T47D, T47D-*HisC,* and T47D-*GGNBP2* cells (2.5×10^4 cell/chamber) suspended in 500 μL serum-free RPMI-1640 medium were loaded to the upper chambers. The upper chamber is a 6.5-mm diameter insert with 8 μm pores of Matrigel-coated polyethylene terephthalate membrane. The inserts were placed in 24-well plates containing the same medium supplemented with 10 % FBS as the chemoattractant. The chambers were incubated for 12 h at 37 °C. At the end of incubation, the cells in the upper surface of the membrane were carefully removed with cotton swabs, and cells invaded across the matrigel to the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Five randomly selected fields (×10 objective) were photographed with an Olympus inverted light microscope, and the total numbers of invaded cells on the lower surface of the membrane were counted under an Olympus inverted light microscope.

Western blot analysis

Cells and xenograft tumor tissues were homogenized in an ice-cold lysis buffer containing protease inhibitors (Roche, Indianapolis, IN, USA). The protein concentrations were measured using the Bradford method (Bio-Rad). An equal quantity of proteins were separated by 10 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with 3 % nonfat milk and incubated overnight with mouse anti-cyclin D1 (CCND1, 1:500), mouse anti-trefoil factor 1 (TFF1, 1:500; Invitrogen), mouse anti-ERα (1:200; Santa Cruz Biotech), and rabbit anti-GGNBP2 (1:3000; Pacific Immuno Corp, Ramona, CA, USA) antibodies, respectively. Peroxidase-conjugated anti-mouse IgG (1:2000, Vector Laboratories, Burlingame, CA, USA) was used as the secondary antibody. Immunoblotting signals were detected using the Amersham ECL plus Western blotting detection system (Amersham). All membranes were re-probed with anti-β-actin antibody (Sigma), which served as a loading control.

In vitro histidine pulled-down assay

TNT-coupled reticulocyte lysate system (Promega) was used for transcription/translation of His-tagged GGNBP2 and His-tagged LxxLL-deleted GGNBP2 ($GGNBP2^{LXL\Delta}$) protein. Briefly, the wild-type *pCNDA3-6HisC-GGNBP2* plasmid and QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) were used to delete LxxLL motif according to the protocol recommended by the manufacturer. The deletion of the sequence encoded for LxxLL motif was verified by DNA sequencing. Rabbit reticulocyte was mixed with 1 μg *pCDNA3-6His–GGNBP2* or $pCDNA3\text{-}6His\text{–}GGNBP2^{LXL\Delta}$ plasmid DNA and T7 RNA polymerase, and incubated at 30 °C for 90 min in a water bath according to the manufacturer's recommended protocol. ^{35}S-labeled human ERα and ERβ proteins were also synthesized using Promega's TNT-coupled rabbit reticulocyte lysate system in the presence of ^{35}S-methionine (MP Biochemicals, Santa Ana, CA, USA), and *pcDNA3-ERα* and *-ERβ* expression plasmids which do not contain a histidine-tag sequence in the expression vector (generously provided by Drs. Ray Wu and Bert W. O'Malley, Baylor College of Medicine). 10 μL of in vitro-translated His-tagged GGNBP2 protein was incubated with 10 uL of ^{35}S-labeled in vitro-translated human ERα or ERβ protein in the presence or absence of 10^{-6} M E_2 at 4 °C with constant rotation for 8 h. Histidine pulled-down assays were performed using the Dynabeads His-tag isolation and pull-down kit (Invitrogen) according to the

manufacturer's instructions. For each reaction, 50 μL Dynabeads were added and incubated on a roller at room temperature for 15 min. The protein-bead complexes were pulled-down by magnetic precipitation, washed, and then separated on 10 % SDS-PAGE gels. A 1/3 input of S^{35}-labeled ERα or ERβ was also loaded on SDS-PAGE gel as a positive reference. These SDS-PAGE gels were dried and exposed to X-ray films to visualize the binding of His-tagged GGNBP2 to ERα or ERβ.

Transfections and luciferase measurements

For transient transfections, T47D-*HisC*, T47D-*GGNBP2* clone #15, and HeLa cells were plated in 24-well plates. When the cells reached 50 % confluence, T47D-*HisC* and T47D-*GGNBP2* clone #15 cells were transfected for 10 h with 0.1 μg of a mixture of the estrogen response element (ERE)-firefly luciferase reporter and pCMV-*Renilla* luciferase constructs (Qiagen, Valencia, CA, USA) using Lipofectamine 2000 (Invitrogen) in serum-free RPMI-1640 medium. HeLa cells were transfected with ERE-luciferase reporter alone or cotransfected with both ERE-luciferase reporter and *pcDNA3-ERα* expression plasmids in serum-free DMEM medium. After transfection, the medium was replaced by fresh normal growth medium and culturing continued for another 24 h. On the day of hormonal treatments, the medium was changed to phenol red- and serum-free RPMI-1640 for T47D cells or DMEM for HeLa cells and the cells were treated with or without 10^{-9} M E_2 for 24 h. The luciferase reporter activities were measured using a dual-luciferase reporter assay kit and a luminometer from Promega according to the protocol provided by the manufacturer. To correct transfection efficiencies, the firefly luciferase activity was normalized to *Renilla* luciferase activity in the same cell lysates.

T47D cell xenograft

T47D-*GGNBP2* and parental T47D cells (1×10^5/injection) were suspended in 100 μL PBS containing 50 % matrigel (BD Biosciences) and injected into the mammary fat pad of 4–5 week-old female nude mice (Vital River Company, Beijing, China). Tumor sizes were measured every 5 days in two dimensions using a caliper, and the tumor volume was calculated with the following formula: tumor volume (mm^3) $= 0.5 \times ab^2$ (a & b being the longest and shortest diameters of the tumor, respectively) as described previously [23]. Mice were sacrificed and the tumors were collected by the 28th day after T47D cell inoculation. Real-time qPCR was performed to validate the expression of *GGNBP2* in the tumor xenografts. All mice were maintained as required under the National Institutes of Health guidelines for the care and use of laboratory animals. The use of animals in this study has been approved by the animal care and use committee of Tianjin cancer hospital.

Statistical analysis

Comparisons of means among more than two groups were performed by one-way analysis of variance (ANOVA). Student t test was used when comparing means of two groups. A p value <0.05 was considered as statistically significant.

Results

Ubiquitous expression of *GGNBP2* in human tissues with the highest expression in mammary glands

A multiple tissue expression array was used to examine *GGNBP2* expression in human tissues. Quantitative analyses by normalizing *GGNBP2* relative to the housekeeping gene *GAPDH* showed that *GGNBP2* expression was detected in all nine tissues examined with the highest levels in normal breast tissues and the lowest levels in normal kidney tissues (Fig. 1a). We also performed semi-quantitative RT-PCR analyses on multiple human cDNAs (purchased from Clontech) and found similar results (data not shown). RT-PCR also demonstrated that *Ggnbp2* is expressed in mouse mammary glands with the highest levels in late pregnant and lactating mammary glands (Fig. 1b).

Reduced *GGNBP2* expression in human and mouse breast cancer samples and human breast cancer cell lines

A cancer expression array containing 50 pairwise human normal and breast tumor tissues was hybridized with radiolabeled probes to examine *GGNBP2* expression in breast cancer samples (Fig. 1c). The reduction was also observed in metastatic cancer samples of the same patients (Fig. 1c). The hybridization intensity of *GGNBP2* was noticeably lower in 45 breast tumor samples among these 50 patients examined, suggesting that aberrant expression of *GGNBP2* in human breast cancer is fairly common. Quantitative analyses by normalizing to *GAPDH* showed that *GGNBP2* expression levels were significantly reduced (Fig. 1d). Compared to normal, immortalized human breast cell line MCF-10A, *GGNBP2* expression in six human breast cancer cell lines decreased at different degrees but significantly which included ERα positive cells lines MCF-7 and T47D, MCF-7-derived estrogen resistant cell lines LCC9 and LY2, and triple negative cell line MDA-MB-231

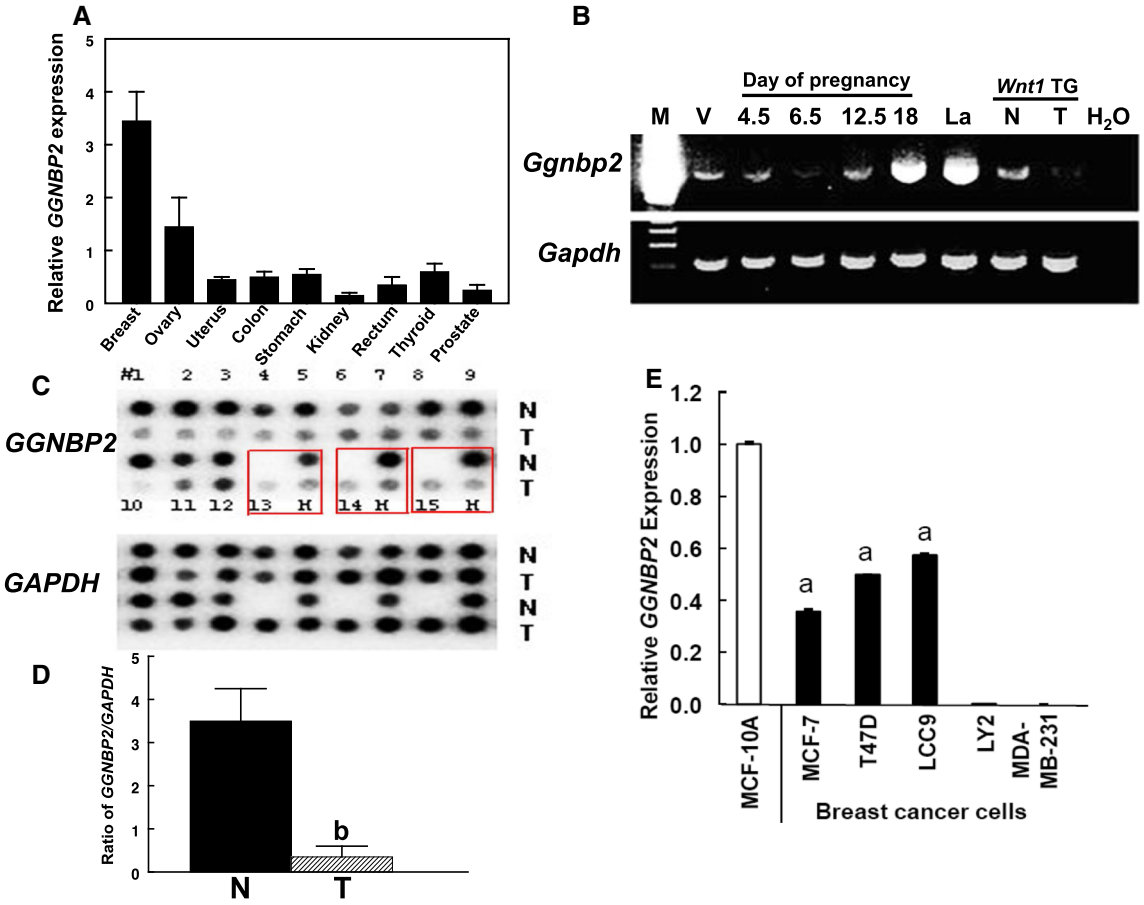

Fig. 1 *GGNBP2* expression is high in human breast, increased in mouse mammary gland in late pregnancy and lactation, and lower in breast tumors and cell lines than normal breast tissue and cell lines. **a** *GGNBP2* expression in normal human tissues by dot blot analyses on Clontech multiple human tissue expression arrays reveals the highest expression of *GGNBP2* in normal human breast tissue. Values are the mean ± SEM of 6 samples. **b** *Ggnbp2* expression in normal mammary glands of virgin (V, 5 week-old), pregnant, and lactating mice and mammary tumors from *Wnt1* transgenic (TG) mice by RT-PCR. *Gapdh* serves as an internal control and H_2O is used as a negative control for RT-PCR. The *Ggnbp2* blot is a longer exposure than the *Gapdh* blot to show the very low levels of *Ggnbp2* mRNA detected in day 6.5 pregnant and *Wnt1*TG (*Wnt1* transgenic) mouse mammary tumors. **c** Representative *GGNBP2* expression in 15 breast cancer patients (indicated by *numbers*). *N* normal; *T* tumor; *M* metastatic cancer samples of the same patient (*boxed*). **d** Quantitative results of *GGNBP2* mRNA levels in 50 paired normal and breast cancer tissues show a significant reduction of *GGNBP2* expression in human breast cancer samples. N, normal; T, tumor. In **a** and **d**, data are the normalized housekeeping gene *GAPDH* levels. Values are the Mean ± SEM of 50 samples. $^{b}P < 0.01$ compared to normal breast tissue samples. **e** qPCR using TaqMan primer/probe sets for determining relative endogenous *GGNBP2* mRNA levels was normalized to *18S*. Values are the average of triplicate determinations ± SEM of one experiment. CT values for *GGNBP2* in LY2 and MDA-MB-231 breast cancer cell lines were 'underdetermined'. $^{a}p < 0.05$ compared to MCF-10A

(Fig. 1e). The *Wnt1* transgenic mice are known to spontaneously develop mammary gland tumors [24]. RT-PCR revealed that *Ggnbp2* mRNA levels were dramatically reduced in the mammary tumors from *Wnt1* transgenic mice (Fig. 1b).

Overexpression of *GGNBP2* inhibited cell proliferation in ERα positive human breast cell lines

To address the potential antibreast tumor activity of GGNBP2 in vitro, we expressed exogenous His-tagged human *GGNBP2* in T47D, MCF-7, MCF-10A, and MCF-10F cells (Fig. 2a). Semi-quantitative RT-PCR analysis confirmed the expression of exogenous *GGNBP2* in these stably transfected cells (Fig. 2a). Western blot analysis showed that GGNBP2 protein levels were elevated in these stably transfected cells (Fig. 2a). Having generated these stable cells, we first examined the effect of overexpression of exogenous *GGNBP2* on cell proliferation. As shown in Fig. 2b, the number of cells of *GGNBP2*-overexpressing ERα positive T47D and MCF-7 cells but not ERα negative MCF-10A and MCF-10F cells [25–27] was significantly reduced after 4 days of culture, when compared to corresponding empty vector transfected (*HisC*) cells (Fig. 2b).

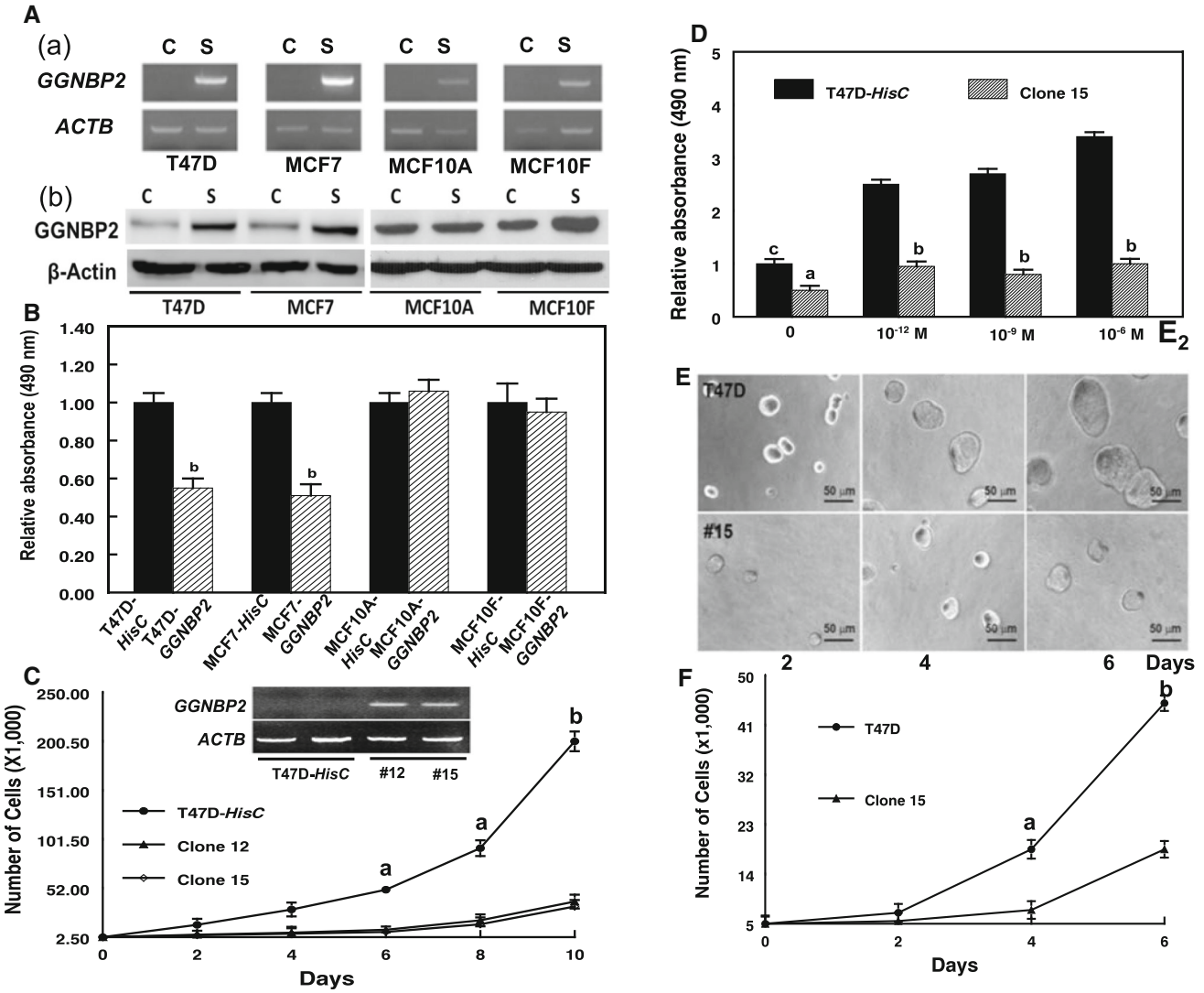

Fig. 2 Stable expression of exogenous *GGNBP2* inhibits the growth of human breast cell lines. **a** Representative RT-PCR (**a**) and Western blot (**b**) pictures of colonies formed in untransfected control (C) and stable (S) *GGNBP2*-transfected T47D, MCF-7, MCF-10A, and MCF-10F cells. The housekeeping gene β-actin (*ACTB*) serves as an internal control for RT-PCR and Western blotting. **b** Overexpression of *GGNBP2* significantly inhibits cell proliferation, measured by MTS assays, of T47D and MCF-7 cell lines but not MCF-10A and MCF-10F cell lines. $^{a}P < 0.05$ compared to corresponding parental cells. **c** Cell growth is inhibited by stable overexpression of *GGNBP2* in T47D human breast cancer cells clones #12 and #15. The *inset* shows semi-quantitative RT-PCR for *GGNBP2* in parental or *GGNBP2* stable cells with *ACTB* serving as an internal control for RT-PCR. $^{a}P < 0.05$ and $^{b}P < 0.01$ compared to T47D-*HisC* cells. **d** E_2-stimulated T47D cell proliferation is inhibited in T47D-*GGNBP2* clone #15 cells relative to parental T47D-*HisC* stable cells. $^{a}P < 0.05$ compared to T47D-*HisC* cells without E_2 treatment; $^{b,c}P < 0.01$ compared to T47D-*HisC* cells with E_2 treatments. **e** Representative images of T47D-*GGNBP2* and parental T47D cells grown in 3-D cultures. **f**. Cell growth of T47D-*GGNBP2* clone #15 is significantly diminished. $^{a}P < 0.05$ and $^{b}P < 0.01$ compared to parental T47D cells. For **b**, **c**, **d**, and **f**, values are the mean ± SEM of 3 separate experiments

The results also show that stable expression of exogenous *GGNBP2* significantly repressed T47D cell proliferation (Fig. 2c). To further determine whether GGNBP2 inhibited breast cancer cell proliferation by depending on ER, the proliferation of T47D cell stably overexpressing-*GGNBP2* and T47D-*HisC* control cells were compared with or without E_2 from 10^{-12} to 10^{-6} M (Fig. 2d). *GGNBP2* overexpression rendered the cells unresponsive to E_2-induced cell proliferation (Fig. 2d).

To examine if GGNBP2 can inhibit T47D cell growth in the context of extracellular matrix, T47D cells were cultured on Matrigel. Results showed that T47D-*GGNBP2* cells formed cell masses, similar to their parental T47D cells (Fig. 2e). However, the sizes in T47D-*GGNBP2* cell masses were smaller than those of parental T47D cells at days 4 and 6 of culture. These cell masses were then dispersed by trypsin digestion and the cell number was counted using a hematocytometer. As shown in Fig. 2f, the

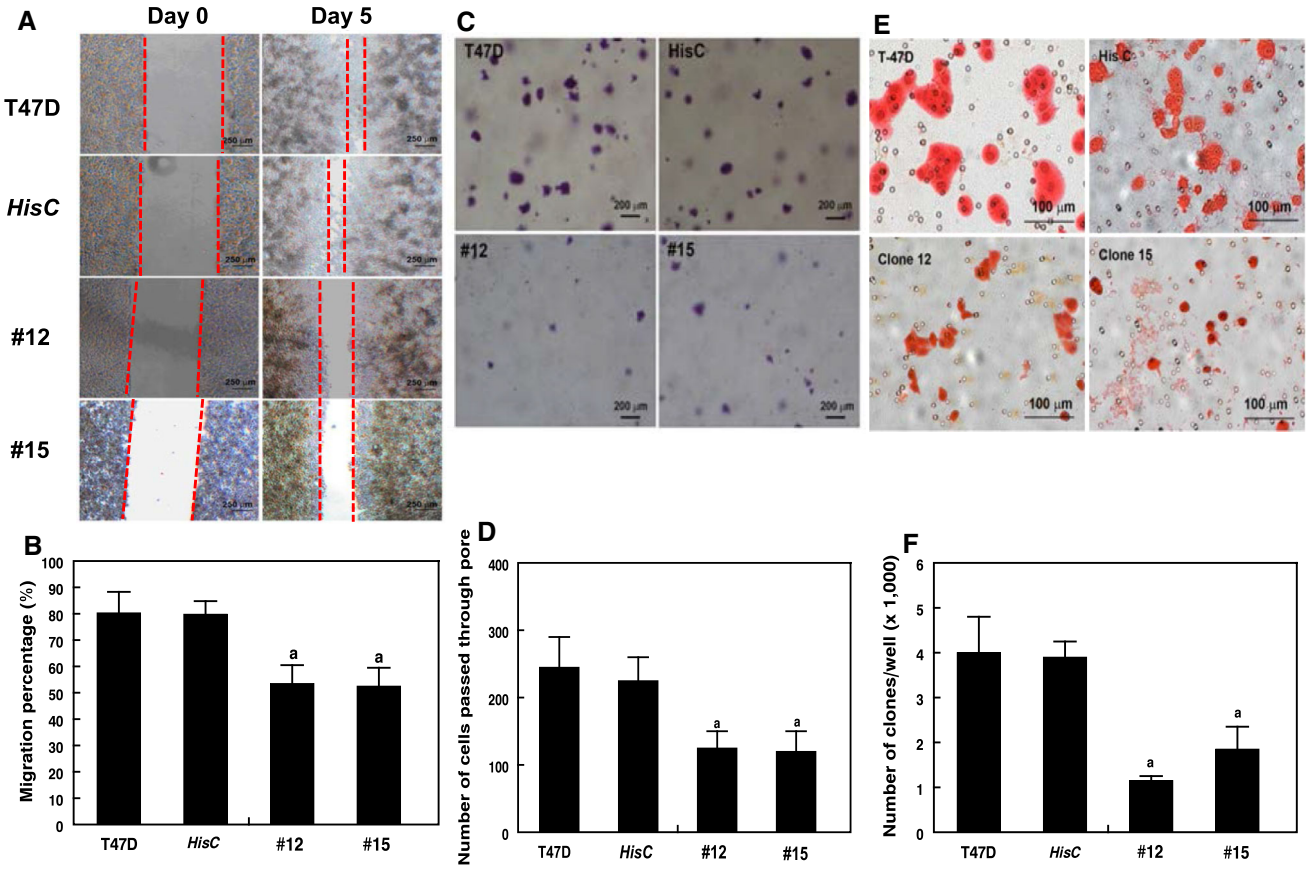

Fig. 3 *GGNBP2* overexpression inhibits T47D cell migration, anchorage-independent growth, and invasion. **a** and **b** Representative photos (**a**) taken at day 0 and after 5 days show that *GGNBP2* overexpression in clones #12 and #15 detains T47D cell migration in a wound-healing assay. Quantitative data (**b**) show that GGNBP2 overexpression significantly inhibits T47D cell migration. *Scale bar* 250 μm. **c** and **d**. Representative photos (**c**) and quantitative data (**d**) show that *GGNBP2* overexpression represses T47D cell invasion through the pores of membranes in chemotaxis chambers. *Scale bar* 100 μm, $^{a}P < 0.05$ compared to parental T47D and T47D-*HisC* cells. **e** and **f**. Representative photos of colonies formed (**e**) in parental T47D, T47D-*HisC* and T47D-*GGNBP2* cells on soft-agar plates after 17 days of culture. Quantitative data (**f**) indicate a significant reduction of cell invasion in T47D cells overexpressing *GGNBP2*. *Scale bar* 200 μm, $^{a}P < 0.05$ compared to either parental T47D and T47D-*HisC* cells. For **b**, **d**, and **e**, values are the mean ± SEM of 3 separate experiments

cell number of T47D-*GGNBP2* masses was significantly reduced after 4 days of culture.

Reduced migration and colony formation in T47D cells with stable *GGNBP2* expression

To test if GGNBP2 has a role in regulating cell migration, we performed wound-healing assays. As shown in Fig. 3a, parental T47D and T47D-*HisC* cells almost completely migrated into the empty spaces after 5 days. In contrast, only a few cells migrated to the empty spaces in T47D-*GGNBP2* clones #12 and #15 cells after 5 days (Fig. 3a). Quantitative analysis indicated that migration percentage was significantly reduced in T47D-GGNBP2 clones #12 and #15 cells after 5 days of culture (Fig. 3b). These results could be due to attenuated cell growth, reduced cell migration, or both by overexpression of *GGNBP2* in T47D cells. To further confirm that overexpression of *GGNBP2* can inhibit cell migration, we performed cell migration assays using Matrigel chemotactic chambers. As shown in Fig. 3c, d, there was a significant decrease in invaded cells in T47D-*GGNBP2* clones #12 and #15 when compared to either parental T47D or T47D-*HisC* control cells, suggesting that GGNBP2 inhibited cell migration. To examine if *GGNBP2* can inhibit anchorage-independent T47D cell growth, we performed soft-agar colony formation experiments. As shown in Fig. 3e, f, the total number of colonies in T47D-*GGNBP2* cells was significantly less than those in both parental T47D and T47D-*HisC* cells. Together, these results suggest that GGNBP2 inhibited the tumorigenic potential of T47D cells in vitro.

GGNBP2 interacted with ERα in vitro

Thus far our studies showed that *GGNBP2* overexpression inhibited cell proliferation in ERα positive T47D and MCF-7

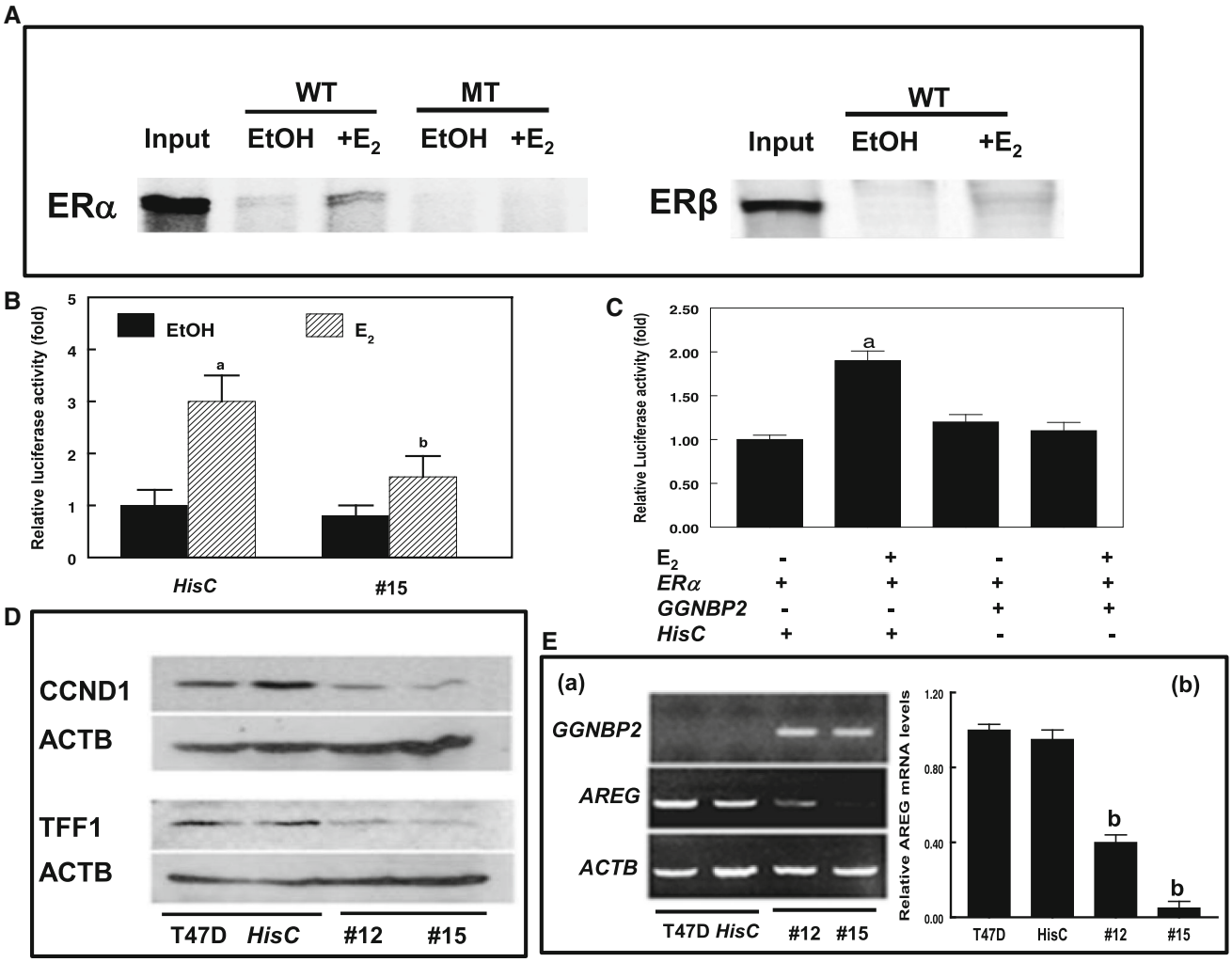

Fig. 4 GGNBP2 protein interacts with ERα and inhibits E_2-ERα transcriptional activity and target gene expression. **a** In vitro transcribed/translated His-GGNBP2 wild type (WT) or with the LxxLL deletion (MT) and ^{35}S-met-labeled ERα were incubated at 4 °C for 8 h with vehicle ethanol (EtOH) or 10^{-6} M E_2 and interactions assayed by histidine pull-down assay. Histidine pull-down assays show that ^{35}S-met-labeled ERβ does not interact with WT His-GGNBP2 either presence or absence of E_2. **b** Stable GGNBP2 overexpression significantly attenuates E_2-stimulated ERE-luciferase reporter activity in T47D cells (Clone #15). $^{a}P < 0.01$ compared to T47D-HisC cells treated with EtOH. $^{b}P < 0.05$ compared to T47D-HisC cells treated with 1 nM E_2. **c** GGNBP2 inhibits E2-ERα activity in transiently transfected HeLa cells. HeLa cells were transfected with an ERE-luciferase reporter, Renilla luciferase, and the indicated expression plasmids and treated with 10 nM E_2 for 24 h prior to dual luciferase assay. Data are mean ±SEM of 4 replicates in one experiment. **d** Western blot demonstrates that CCND1 (cyclin D1) and TFF1 (pS2) protein levels are reduced in both stable T47D-*GGNBP2* expression clones #12 and #15 compared to either parental T47D or T47D-*HisC* stable cells. Values are CCND1/ACTB relative to expression in T47D parental cells. **e** RT-PCR (**a**) and qPCR (**b**) of *AREG* mRNA expression shows decreased *AREG* in T47D-*GGNBP2* clones #12 and 15. $^{b}P < 0.01$ compared to parental T47D and T47D-*HisC* cells. For **b**, **c**, and **e**(*b*), values are the mean ± SEM of 3 separate experiments

human breast cancer cell lines and E_2-stimulated T47D cell proliferation. Given the putative conserved NR-binding motif in human GGNBP2, we hypothesized that GGNBP2 interacts with ER to modulate its activity. Thus we performed an in vitro protein–protein interaction assay using in vitro transcribed/translated His-GGNBP2 or His-GGNBP2$^{LXL\Delta}$ and ERα. As shown in Fig. 4a, ERα was pulled-down by His-tagged GGNBP2 but not His-tagged GGNBP2$^{LXL\Delta}$. However, His-tagged GGNBP2 did not pull-down ERβ (Fig. 4a). Interestingly, more ERα was pulled-down by His-tagged GGNBP2 in the presence of 1 μM E_2 than in the absence of E_2 (Fig. 4a). These results suggest that the LxxLL domain of GGNBP2 is critical for interaction with ERα and that this interaction is increased by E_2 in vitro.

GGNBP2 modulating ERα action in T47D and HeLa cells

To investigate whether GGNBP2 can modulate ERα transcriptional activity, we transiently transfected T47D cells

with *pcDNA3-HisC* control vector or *pcDNA3-GGNBP2* expression vector with an ERE-reporter plasmid and performed luciferase reporter assays. As expected, E_2-stimulated activation of the ERE-reporter was observed in T47D cells transfected with *HisC* control vector plasmid. However, transfection of *pcDNA3-GGNBP2* inhibited E_2-induced ERE activation by >50 % (Fig. 4b). The effect of GGNBP2 on ERα transcriptional activity was further verified in HeLa cells transfected with ERα. Transfection with the *GGNBP2* expression vector inhibited E2-induced ERE-reporter activity in the ERα-transfected HeLa cells (Fig. 4c).

Cyclin D1 (*CCND1*), Trefoil factor 1 (*TFF1*, also known as breast cancer estrogen inducible protein and pS2), and amphiregulin (*AREG*) are three well-known estrogen responsive genes in T47D cells, and *CCND1* is critical for breast cell proliferation [28–30]. To address whether GGNBP2 downregulates endogenous E2-regulated proteins/genes, we performed Western blot and RT-PCR analyses to examine their expression in stable control T47D-*HisC* cells and T47D-*GGNBP2* clone #15. As shown in Fig. 4d, e, the protein levels of both cyclin D1 and TFF1 and *AREG* mRNA levels were reduced in *GGNBP2*-overexpressing T47D cells. Taken together, these results suggest that GGNBP2 is an ERα modulator that can physically interact with ERα and repress its transcriptional activity.

Effect of *GGNBP2* on xenograft tumor growth of T47D cells in nude mice

The antiproliferative activity of *GGNBP2* in T47D cells in vitro led us to investigate whether its antitumor efficacy would be maintained in vivo. T47D-*GGNBP2* and parental T47D cells were injected into the mammary fat pad of intact adult female nude mice. As expected, tumors developed in both T47D-*GGNBP2* and parental T47D cells xenograft mice; however, *GGNBP2* overexpression substantially reduced tumor progression relative to parental T47D cell controls (Fig. 5a, b). The tumor sizes of T47D-*GGNBP2* xenografts were significantly smaller than the parental T47D cell xenografts (Fig. 5a, b). RT-PCR was performed to further validate that *GGNBP2* was indeed elevated in T47D-*GGNBP2* cells and their tumor xenografts. As shown in Fig. 5c, there was 4.3-fold increase of *GGNBP2* mRNA levels in *GGNBP2*-overexpressing cells than their parental T47D cells. Similarly, *GGNBP2* mRNA levels were markedly elevated in xenograft T47D-*GGNBP2* tumor tissues (~5-fold increase) compared to the parental T47D tumor tissues (Fig. 5d). Thus, *GGNBP2* stable expression was maintained in the T47D tumor xenograft. We examined ERα protein levels in xenograft tumor tissues of parental T47D and T47D-*GGNBP2* cells by Western blot in 5 tumors (Fig. 5e). ERα protein levels are significantly lower in T47D-*GGNBP2* xenograft tumors than parental T47D cell xenograft tumors (Fig. 5f). qPCR also demonstrated that the expression of ERα target genes *TFF1* (Fig. 5g) and *CCND1* (Fig. 5h) were significantly reduced in xenograft T47D-GGNBP2 tumor tissues. Together, these in vivo studies suggest that *GGNBP2* has an antibreast tumor activity.

Discussion

Loss of heterozygosity in human chromosome 17q12-q23 is frequently observed in breast and ovarian cancers [1–4]. Although the tumor suppressor gene *BRCA1* has been mapped to this region, *BRCA1* mutations account for a small portion of all breast cancers [5–7]. In this study, we demonstrated that *GGNBP2*, a gene located in the chromosome 17q12 region [8], is highly expressed in normal human breast and its expression is significantly reduced in the majority of human breast cancer samples and in the five human breast cancer cell lines examined. Further, overexpression of exogenous *GGNBP2* in ERα positive T47D human breast cancer cells results in a significant inhibition of their tumorigenic potential in vitro and in vivo, including a decrease in cell proliferation, anchorage-independent growth, migration and invasion, and tumor growth in nude mice. Together, these observations suggest that GGNBP2 is a candidate of breast tumor suppressor. Consistent with this suggestion that GGNBP2 is a tumor suppressor, a short form of human GGNBP2 known as LCRG1 was previously reported to be reduced in laryngeal carcinomas [8, 16, 31]. More recently, GGNBP2 expression was found to be downregulated in cisplatin- and carboplatin-resistant ovarian cancer cells [18]. Forced expression of *LCRG1* in HeLa cells suppressed proliferation, whereas knockdown of *LCRG1* by siRNA promoted multiplication [16, 32]. However, a study reported that down-regulation of the long form of human *GGNBP2* by shRNA but not the short form in Hela cells resulted in a repression of cell proliferation [17], suggesting that the different isoforms of human GGNBP2 might play different roles in regulation of cell proliferation. In this respect, future studies will be required to examine whether there is an isoform-dependent effect of GGNBP2 on breast cancer cell growth. Moreover, it will be important to determine whether genetic, including germline and somatic deleterious mutations, or epigenetic changes, including post-translational modifications on histone proteins, contribute to the repression of *GGNBP2* expression in breast tumors.

Estrogens play a critical role not only in normal mammary gland development but also in the initiation and progression of breast cancers [33, 34]. Binding of estrogens to ERα induces conformational changes and nuclear

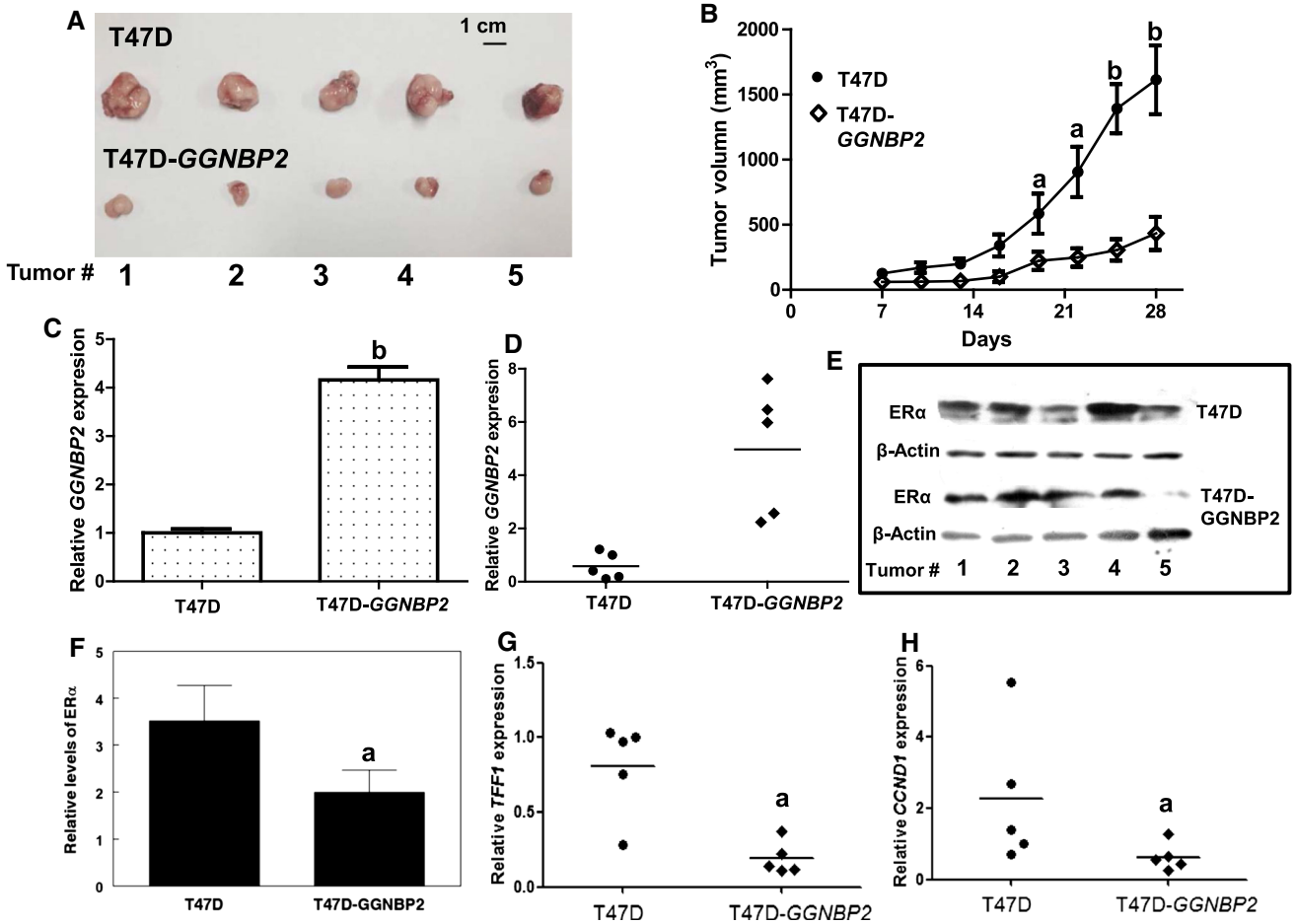

Fig. 5 *In vivo* tumor suppression effect of *GGNBP2* expression in T47D cells on xenografted nude mice. **a** Gross appearance of the tumors of xenografted parental T47D and T47D-*GGNBP2* cells after 28 days. *Scale bar* 1 cm. **b** Tumor growth curves of xenografted parental T47D and T47D-*GGNBP2* cells in nude mice. **c** and **d** Quantitative RT-PCR demonstrates that *GGNBP2* expression in T47D-*GGNBP2* cells before (**c**) and after (**d**) xenografting are significantly higher than parental T47D cells. **e** and **f** ERα protein levels in xenograft T47D and T47D-*GGNBP2* tumor tissues were measured by Western blot in 5 tumors (1–5) (**e**) and the results show that ERα protein levels are lower in the T47D-*GGNBP2* xenograft tumors than parental T47D xenograft tumors (**f**). **g** and **h** The expression of ERα target genes, *TFF1* (G) and *CCND1* (H) are significantly downregulated in xenograft tumor tissues of T47D-*GGNBP2* cells. $^{a}P < 0.05$ and $^{b}P < 0.01$ compared to parental T47D cells. For **b**–**d** and **f**–**h**, values are mean ± SEM of 5 mice in each group

translocation, ERE binding, and the recruitment of coregulators to regulate transcription of ERα target genes [35–38]. This hormone-driven genomic action of ERα is crucial in promoting proliferation of breast cancers [34, 39]. In this work, we demonstrate that *GGNBP2* overexpression markedly represses cell proliferation of ERα positive breast cancer cells but not ERα negative normal, immortalized breast epithelial cells. More importantly, GGNBP2 directly interacts with ERα, substantially impedes E_2-induced ERE activity and inhibited estrogen target gene expression in breast cancer cells. Our results also show that *GGBBP2* overexpression in T47D cells reduced ERα protein levels. Taken as a whole, our findings suggest that GGNBP2 functions as an ERα corepressor to influence the expression of its target genes, such as CCND1, TFF1, and AREG, and ultimately to control breast epithelial cell proliferation. In the light of our findings, more in-depth studies are warranted to further understand the molecular mechanisms by which GGNBP2 interplays with ERα to regulate ERα activity in breast cancers.

The LxxLL domain was initially identified in proteins that bind the activation function-2 region of nuclear receptor ligand-binding domains [40]. Subsequently, this conserved sequence is shown to interact with many NRs [41]. Deletion of the LxxLL domain abolished GGNBP2 binding to ERα, implying that the LxxLL domain has a key role in NR regulation. In addition to the regulation of the NR signaling, there are numerous examples protein–protein interactions involving LxxLL motifs [41]. This raises the possibility that GGNBP2 may also interact with other

NRs or transcription factors in addition to ERα to exert its cancer suppression action, which is required further investigation.

More than 75 % of all cases of breast cancers are ERα positive [42]. The identification and characterization of coregulators that modulate ERα activity are important steps in the understanding, not only of the underlying mechanisms that control the expression of estrogen target genes but also those that influence the initiation, progression, treatment, and clinical prognosis of breast cancers. In estrogen-dependent tumors, *GGNBP2* underexpression would be expected to result in an increase in ERα function and thus possibly render these cells more sensitive to low levels of estrogens. Thus, reduction or loss of *GGNBP2* would contribute to the increased growth and invasiveness of ERα positive cancer cells. Our findings suggest that GGNBP2 acts as an ERα corepressor and its downregulation in breast tumors could have clinical implications for prognosis and therapeutic options for a subset of breast cancer cases. Future studies will explore this speculation.

In summary, GGNBP2 was identified as a novel tumor suppressor which is downregulated in human breast tumors and cell lines. Our in vitro and in vivo results suggest that GGNBP2 may function as a nuclear receptor corepressor to inhibit ERα's transcriptional activity and consequently inhibit tumorigenic potential of breast cancer cells. Future studies will be required to determine whether genetic and/or epigenetic alternations of this gene associate with human breast cancers and test for clinical usefulness of this gene in conjunction with other known genetic markers for early detection of breast cancer risk and enhancement of current treatment strategies used in ERα-positive breast cancers.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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