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PRECLINICAL STUDY

GRB7 is required for triple-negative breast cancer cell invasion and survival

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Abstract Triple-negative breast cancer (TNBC) is a heterogeneous disease that is usually associated with poor prognosis, and frequently associated with the basal-like breast cancer gene expression profile. There are no targeted therapeutic modalities for this disease, and no useful biomarkers. High *GRB7* RNA expression levels are associated with an elevated risk of recurrence in patients with operable TNBC treated with standard adjuvant anthracycline and taxane therapy. To determine whether GRB7 is involved in the pathobiology of TNBC, we evaluated the biological effects of GRB7 inhibition in a panel of triple-negative cell lines—MDA-MB-468, MDA-MB-231, HCC70, and T4-2. We found GRB7 inhibition reduced cell motility and invasion of these cell lines and promoted cell death by apoptosis in 3D culture. These data suggest that GRB7 itself, or GRB7-dependent pathways, may prove to be important therapeutic targets in this disease.

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Introduction

Breast tumors are classified in clinical practice based on the expression of estrogen receptor (ERα), progesterone receptor (PR), and the overexpression/amplification status of HER2/ERBB2. Cancers lacking expression of ER, PR, and HER2 have been referred to as "triple-negative breast cancer" (TNBC), and are associated with a poor prognosis. TNBC is a heterogeneous disease, although about 80% of all TNBC are associated with a "basal" pattern of gene expression [1]. TNBCs are not sensitive to targeted therapeutics used for HER2-positive (Trastuzumab and Lapatinib) and ERα-positive (Tamoxifen and aromatase inhibitors) breast disease [2]. A better understanding of the biology of this disease will be essential to improve clinical outcomes.

Elevated *GRB7* mRNA expression has been recently identified as an adverse prognostic factor in women with stage I–III TNBC treated with adjuvant doxorubicin-containing chemotherapy, suggesting that it may be a useful biomarker in this disease [3], however, the extent to which TNBC cells are dependent upon GRB7 remains unclear. GRB7 is a multidomain protein that relays signals from its interacting partners to their downstream signaling pathways. The SH2 domain of GRB7 mediates its association with tyrosine kinases such as the focal adhesion kinase (FAK) and members of the ERBB family [4]. Other prominent domains with clear relevance to cancer include the Ras-Association domain, which binds to small GTPases and the PH domain which interacts with phospholipids [5].

This multidomain organization allows GRB7 to potentially integrate signals from multiple upstream sources and propagate them using its diverse effector proteins, e.g., Ras [6], PLCγ1 [7], or FHL2 [8].

In this study, experimental inhibition of GRB7 was achieved using a cell-penetrating non-phosphorylated peptide inhibitor, the potency and specificity of which has been previously described in extensive detail [9, 10]. This peptide (G7-18NATE) binds to the SH2 domain of GRB7 and inhibits its association with several different tyrosine kinases, including ERBB family members and FAK, preventing GRB7 phosphorylation [9]. This highly specific peptide does not interfere with other closely related SH2-containing adapter proteins, including GRB2, GRB10 and GRB14 [9, 10]. In order to facilitate internalization of the GRB7 inhibitor, it is conjugated to a Penetratin carrier peptide [11]. For all experiments, the effect of the penetratin-conjugated GRB7 inhibitor (G7-18NATE) is compared to the unconjugated penetratin peptide. Specificity of the inhibitor was confirmed by using two independent siRNAs against GRB7. We examined the effect of GRB7 inhibition on the ability of TNBC cell lines to migrate, invade and form colonies in 3D culture, each of which mimics an important aspect of in vivo tumor biology and report that each of these processes requires the activity of GRB7-dependent pathways.

Materials and methods

Cell culture

A panel of four basal-like breast cancer cell lines was cultured as follows: HCC70 in RPMI 1640 (Cellgro) with 10% FBS (Hyclone); MDA-MB-468 in L-15 (Hyclone) with 10% FBS; MDA-MB-231 in DMEM (Cellgro) with 10% FBS, and T4-2 in H14 medium (DMEM/F12 supplemented with 5 μg/ml prolactin, 250 ng/ml insulin, 1.4×10^{-6} M hydrocortisone, 2.6 ng/ml sodium selenite, 10 μg/ml transferrin, and 10^{-8}M β-estradiol as previously described [12]. These particular cell lines were chosen from the larger panel in Fig. 1 because they were known to grow well in 3D culture [13]. The HCC70, MDA-MB-468, and MDA-MB-231 cell lines were obtained from ATCC which validates their correct identity. The identity of the T4-2 cell line (which is available from Sigma) was further confirmed by detecting the presence of the specific mutation in codon 179 of p53, previously reported for this cell line [14]. The estrogen receptor positive MCF7 and T47D cell lines were also obtained from ATCC.

3D laminin-rich extracellular matrix cultures [15] were prepared by trypsinization of cells from tissue culture plastic, seeding of single cells on top of a thin layer of growth factor-reduced Matrigel (BD Biosciences), and the addition of a medium containing 5% Matrigel. The cell lines were seeded at the following densities; T4-2: 1.0×10^4 cells per cm^2; MDA-MB-468 and HCC70: 2.1×10^4 cells per cm^2; and MDA-MB-231: 1.6×10^4 cells per cm^2. All 3D cell cultures were performed in H14 medium with 1% fetal bovine serum, with the exception of T4-2, which was cultured in H14 medium without serum. 3D cultures were maintained with media changes every second day.

The G7-18NATE inhibitor peptide was made cell permeable by conjugating it to the penetratin carrier peptide [11]. The unconjugated penetratin peptide was used at the same concentration as the inhibitory peptide (10 μM) as a negative control in all experiments. Penetratin-treated cells were not significantly different from cells treated with another negative control (DMSO, data not shown). Penetratin and the penetratin-coupled form of G7-18NATE were prepared as previously described [9, 10].

Suppression of GRB7 expression by siRNA was achieved using two independent siRNAs with the following sequences: siRNA I: GGGUGCAGCUGUACAAGAAUU ACCA, siRNA II: UGAAUGCUGGCAGCUUUCCUGA GAU. The Stealth RNAi siRNA negative control (Invitrogen) was used to control for non-specific effects. siRNAs were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions at a final concentration of 100 nM.

Expression studies

GRB7 gene expression was analyzed using our previously published microarray data [13]. GRB7 protein expression was evaluated by western blotting and immunofluorescence using a rabbit anti-GRB7 (N-20) antibody (Santa Cruz Biotechnology). Also used were antibodies against phospho-ERK, total ERK and GAPDH (Cell Signaling Technology), and actin (Sigma).

Monolayer wound healing assay

Cells were grown to confluence in 12-well plates (BD Falcon) and scraped with a sterile micropipette tip. The wound was marked and imaged immediately after wounding and 24 h later. The area of the wound was measured at the 0 and 24 h time points and results are presented as percentage wound closure at 24 h.

Transwell invasion assay

For the transwell invasion assay, cell culture inserts (8 μm pore, 12-well format; BD Falcon) were coated with 100 μl dilute Matrigel (0.5 mg protein/ml) in serum-free medium

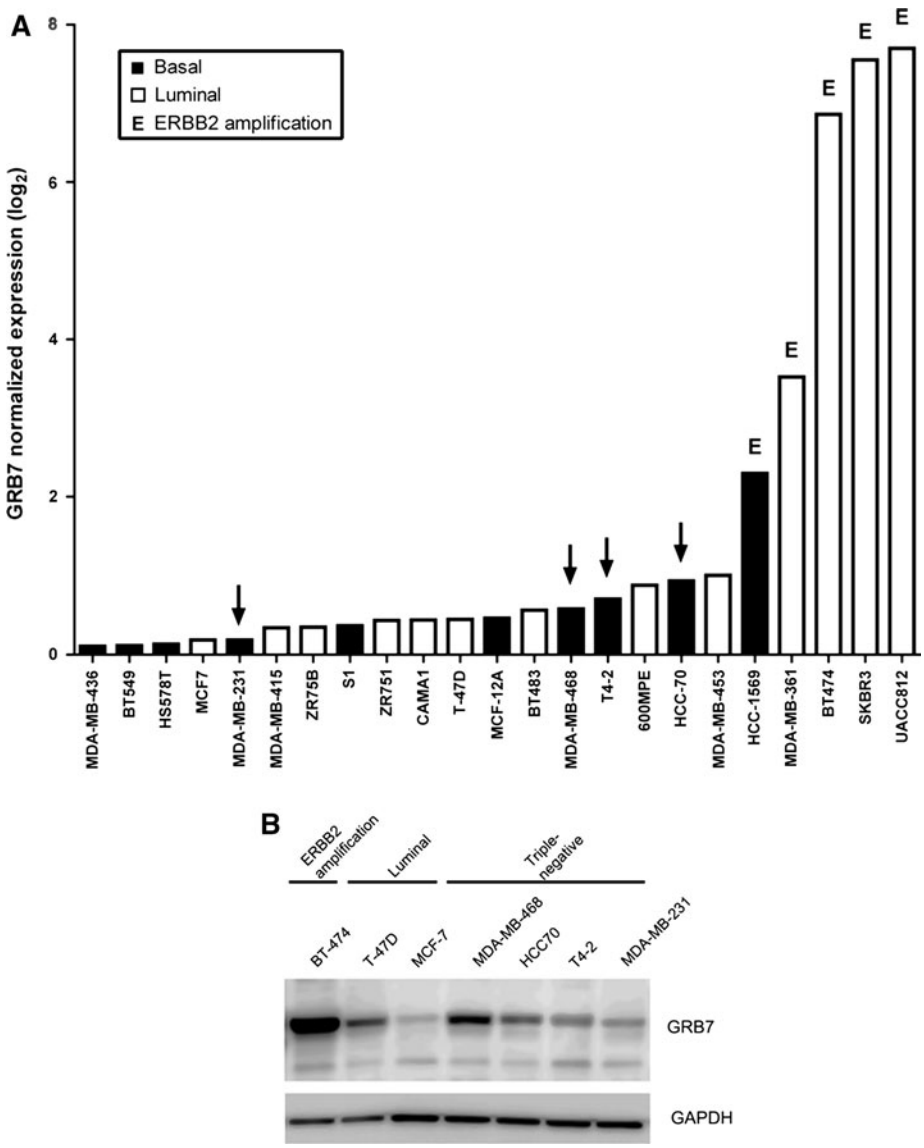

Fig. 1 GRB7 expression in TNBC cell lines. **a** Microarray analysis of *GRB7* mRNA levels in 23 breast cancer cell lines. *Black* breast cancer cell lines with basal-like gene expression profile; *White* breast cancer cell lines with luminal gene expression profile; Cell lines with reported amplification of *ERBB2/HER2* are indicated by "E"; *arrows* TNBC cell lines utilized in this study. **b** Western blot analysis for GRB7 in total cell lysates from four TNBC cell lines and representative examples of other breast cancer subtypes

which was allowed to solidify overnight in a humidified cell culture incubator and was rehydrated with warm serum-free medium for 3 h prior to the experiments. Cells were grown to 75% confluence and then starved for 24 h in growth medium containing 0.1% FBS. 1.0×10^5 cells were seeded in 1 ml serum-free medium in the upper chamber and the lower chamber was filled with 1 ml of growth medium with 10% FBS as a chemoattractant. The cultures were maintained for the following time periods which were empirically determined for each cell line due to inherent differences in relative invasive ability: MDA-MB-231: 5 h; T4-2: 24 h; and MDA-MB-468 and HCC70: 48 h. Invaded cells were fixed and visualized by staining with 0.2% crystal violet and counted using a 4× objective in each of three randomly chosen fields. Each experiment was performed in duplicate.

Proliferation and apoptosis assays

Colonies were extracted from the 3D Matrigel cultures with one volume of ice-cold PBS/5 mM EDTA, followed by 30 min incubation with several volumes of ice-cold PBS [15] and gentle centrifugation ($100\times g$). Cells in S-phase were labeled with BrdU for 6 h prior to harvest and incorporated BrdU was detected using the BrdU Labeling and Detection Kit I (Roche Diagnostics, Mannheim). Apoptosis was analyzed using the DeadEnd^{TM} Fluorometric TUNEL System (Promega, Madison).

Statistics

All data analysis was performed using GraphPad Prism version 5.03. The non-parametric Kruskal–Wallis test was

used to test differences between median cross-sectional area of colonies in 3D culture. Student's t test was used for all other comparisons.

Results

Triple-negative/basal breast cancer cell line models

We examined the mRNA expression level of *GRB7* in 23 breast cancer cell lines by microarray analysis and, as expected from the location of GRB7 within the HER2 amplicon [16], we found that the highest levels were present in *HER2*-amplified cell lines (Fig. 1a). Among the basal/triple-negative group, we selected four cell lines for our experiments: HCC70, MDA-MB-231, MDA-MB-468, and T4-2. Each cell line has an expression profile similar to basal breast cancers and lacks expression of ER, PR, and HER2 [13, 17]. Compared to HER2-amplified cell lines, these four basal breast cancer cell lines expressed relatively low levels of *GRB7* mRNA, however western blot analysis confirmed expression of GRB7 protein (Fig. 1b). Examples of HER2-amplified (BT474) and estrogen receptor positive (MCF7 and T47D) cell lines are included for comparison. Although it is commonly held that the most optimal therapeutic targets are those which are either mutated or overexpressed, our analysis of TNBC patient samples [3] led us to hypothesize that GRB7 may be a key mediator of signals in this tumor subtype and, as such, may be a useful therapeutic target.

GRB7 inhibition attenuates in vitro cell motility

To assess the requirement of TNBC cells for GRB7 for motility, monolayer wound healing assays were performed (Fig. 2a). Representative data for one cell line (MDA-MB-468) are presented throughout our report and quantitative analysis of all cell lines evaluated is shown. For this particular assay, HCC70 was not evaluated as this cell line does not grow to a confluent monolayer and therefore cannot be experimentally wounded. Penetratin-treated cells (10 μM) repaired the wound within 24 h after scraping, however, the addition of 10 μM of the G7-18NATE peptide inhibited cell migration by at least 60% for each of the three cell lines (Fig. 2a), demonstrating that GRB7 activity is required for TNBC cell motility.

Inhibition of GRB7 attenuates invasion through basement membrane

Having established that inhibiting GRB7 could interfere with cell motility in a relatively crude assay, we next determined whether blocking GRB7 could inhibit cell invasion through basement membrane, a more complex multifactorial process (requiring locomotion, chemotaxis and proteolytic activity) with more relevance to physiological cancer cell invasion in vivo. The invasive potential of the cell lines was assessed in a Boyden chamber coated with extracellular matrix (Matrigel) and using 10% fetal bovine serum as chemoattractant. As each cell line is different in its inherent invasive capacity, assays were allowed to proceed for a time empirically determined to be optimal for each cell line. Normalizing the invasion to the penetratin-treated control for each cell line, the GRB7 inhibitory peptide clearly resulted in a marked reduction of in vitro invasion (Fig. 2b).

Inhibition of GRB7 impairs colony formation in a 3D microenvironment

In addition, we tested the ability of the GRB7 inhibitor to block clonal cell growth in 3D extracellular matrix culture (Fig. 2c). The cell lines were seeded as previously described [13, 15]. Inhibiting GRB7 resulted in a significant reduction ($P < 0.001$) in colony size compared to the vehicle control in each of the four TNBC cell lines tested (Fig. 2c). Therefore, in addition to a requirement for migratory and invasive phenotypes, GRB7 function is also necessary for colony formation by TNBC cells. To test whether this inhibition of colony formation was specific to the TNBC cell lines or was a more general phenomenon, we tested the effects of the GRB7 inhibitor on the colony formation of the estrogen receptor positive MCF7 and T47D cell lines and found no significant inhibition (Fig. 2d).

Validation studies on the G718-NATE inhibitor

Although the specificity of the GRB7 inhibitor has been already described in detail in a series of model systems in previous publications [9, 10], we performed several additional control experiments to confirm that suppression of GRB7 activity underlies the data in Fig. 2. We tested the effect of the GRB7 inhibitor on a known GRB7-dependent function and also evaluated siRNA-mediated GRB7 suppression to confirm that this alternative targeting approach yielded similar outcomes. First, we evaluated the effect of G718-NATE on EGFR signaling and found that pretreatment of the MDA-MB-468 cell line with this inhibitor impaired the ERK phosphorylation that occurs in this cell line in response to EGFR stimulation with EGF (Fig. 3a, b). Using two independent siRNAs against GRB7 (Fig. 3c, d), we confirmed that targeting GRB7 in this way resulted in impaired migration, invasion and colony formation in both MDA-MB-231 and MDA-MB-468 (Fig. 3e).

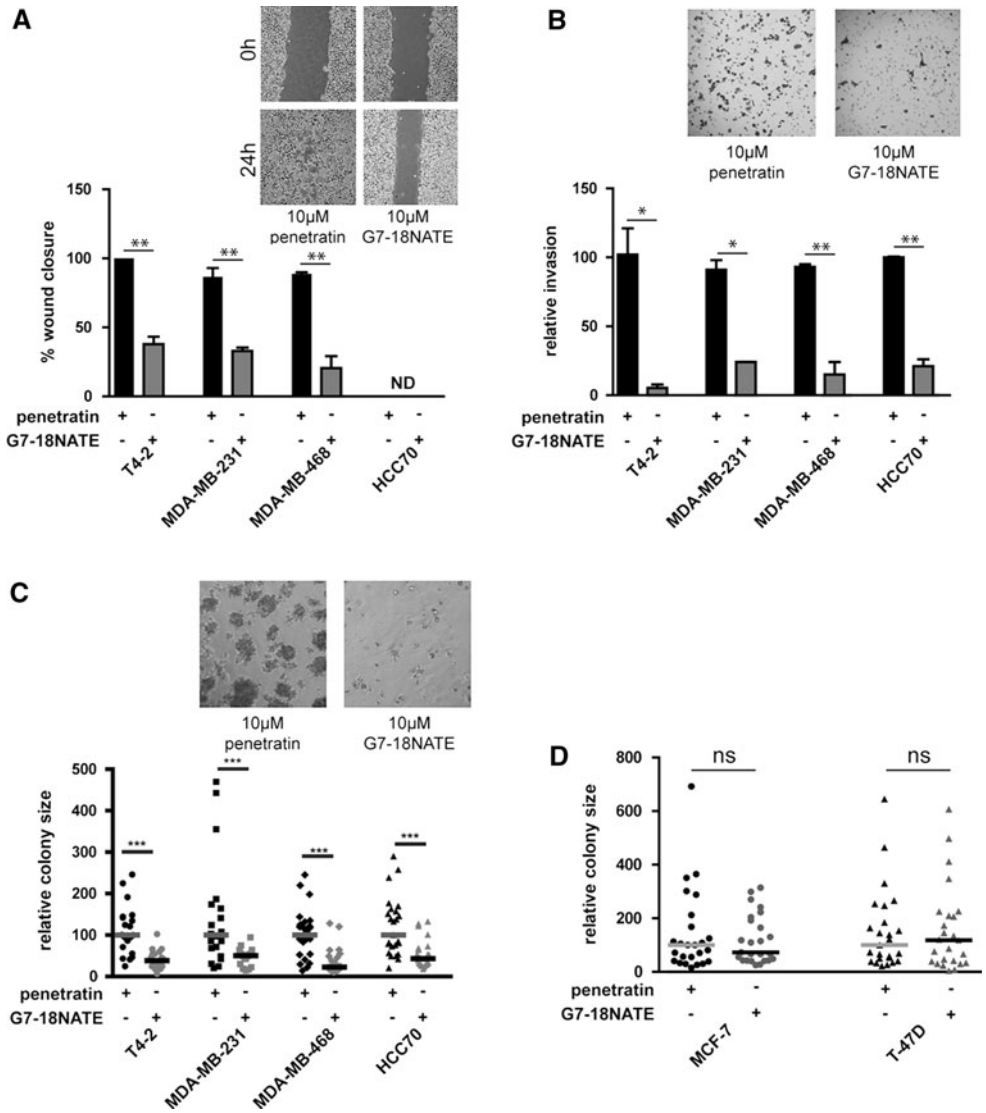

Fig. 2 GRB7 inhibition attenuates TNBC cell motility, invasion and 3D culture colony formation. **a** (*Upper*) Representative images of the monolayer wound healing assay utilizing the MDA-MB-468 cell line immediately after scratching, and 24 h later. The cells were treated with 10 μM of the control peptide (penetratin) or the GRB7-inhibitor peptide (G7-18NATE). (*Lower*) Quantitative analysis of wound healing assay for T4-2, MDA-MB-231 and MDA-MB-468 cell lines. Because HCC70 does not grow to form a confluent monolayer, it was unsuitable for analysis in this assay. **b** (*Upper*) Representative images of the transwell invasion assay utilizing the MDA-MB-468 cell line, demonstrating that 10 μM G7-18NATE inhibits invasion through the Matrigel-coated filters. (*Lower*) Quantitative analysis of invasion assays for each breast cancer cell line. *Columns* mean of duplicate experiments, each normalized to the level of invasion detected in the relevant Penetratin-treated control; *bars* standard error. **c** (*Upper*) Representative images of colonies formed by the MDA-MB-468 cell line in 3D cell culture in the presence of 10 μM GRB7 inhibitory peptide or the Penetratin control. (*Lower*) Quantitative analysis of colony size formed by each cell line in the presence of the G7-18NATE inhibitor or the control peptide. *Bars* indicate the median colony size under each condition (***$P < 0.001$ Kruskal–Wallis test). **d** Quantitative analysis of colony size formed by two estrogen receptor positive cell lines in the presence of the G7-18NATE inhibitor or the control peptide

GRB7 inhibition causes apoptosis of TNBC cell lines in 3D extracellular matrix cultures

Because attenuation of colony growth in 3D culture may result from a combination of anti-proliferative and/or pro-apoptotic effects, we examined the relative contribution of these processes to the observed alterations in colony size. Bromodeoxyuridine analysis on day 5 (Fig. 4a, b) indicated that there was no significant difference in cell proliferation in the GRB7 inhibitor treated colonies. Conversely, MDA-MB-468 cells treated with the GRB7 inhibitor exhibited significantly increased apoptosis, as assessed by TUNEL assay (Fig. 4c). This finding was consistent in each of the TNBC cell lines in our panel (Fig. 4d). We confirmed that the peptide inhibitor had no effect on the proliferation or apoptosis of the two estrogen

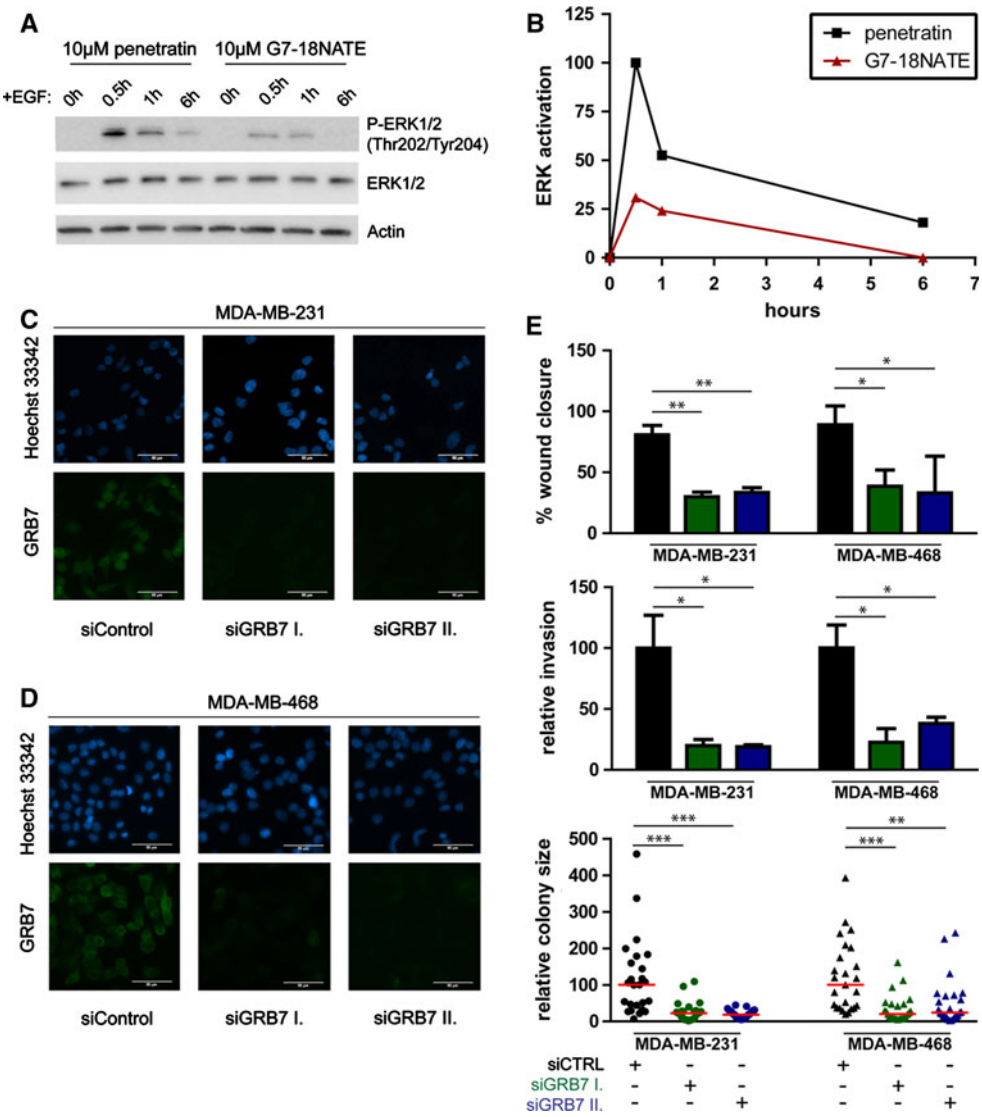

Fig. 3 Control experiments to demonstrate specificity of action of the G7-18NATE peptide inhibitor. **a** Pretreatment of MDA-MB-468 cells with 10 μM G7-18NATE significantly attenuated the activation of ERK in response to EGF stimulation of EGFR. **b** Densitometric quantification of the western blot in 3A. **c** Immunofluorescence analysis of GRB7 protein levels in MDA-MB-231 cells transfected with 100 nM of anti-GRB7 siRNAs or a negative control siRNA. **d** Immunofluorescence analysis of GRB7 protein levels in MDA-MB-468 cells transfected with 100 nM of anti-GRB7 siRNAs or a negative control siRNA. **e** Depletion of GRB7 by siRNA impairs MDA-MB-231 and MDA-MB-468 cell migration, invasion and colony formation

receptor positive cell lines in 3D culture (Fig. 4e). Using both siRNAs we confirmed that GRB7 depletion had no effect on proliferation but significantly increased apoptosis in the TNBC cell lines, but not in the estrogen receptor positive controls (Fig. 4f).

Discussion

Following our clinical findings demonstrating an association between high *GRB7* levels and poor outcomes in patients with TNBC [3], this study evaluated the requirement for GRB7 in TNBC cell lines. We found that GRB7 inhibition had pronounced effects on phenotypes highly relevant to breast cancer progression. Specifically, blockade of GRB7 reduced TNBC cell motility and invasiveness and promoted apoptosis in 3D culture. No inhibitory effects were observed in estrogen receptor positive breast cancer cell lines. The current study suggests that GRB7 is not merely a biomarker but an active participant in the disease process and provides proof-of-principle that inhibiting GRB7, or GRB7-associated pathways, may be a potentially valuable therapeutic option in TNBC.

Because GRB7 interacts with many upstream kinases, it may function as a crucial node for integration and propagation of several signals. It is likely that the key upstream kinases may vary between different tumors but it seems that a requirement for GRB7 may be a feature common to several breast cancer subtypes. The frequent high expression of EGFR in TNBC [18], makes this protein an excellent candidate, a model supported by recent data delineating a pathway from EGFR to Ras/ERK signaling via GRB7 in HER2-overexpressing cells [6]. The high levels of apoptosis observed in 3D culture upon GRB7 inhibition also suggest that survival signals from the extracellular matrix, most likely via integrins and FAK, are

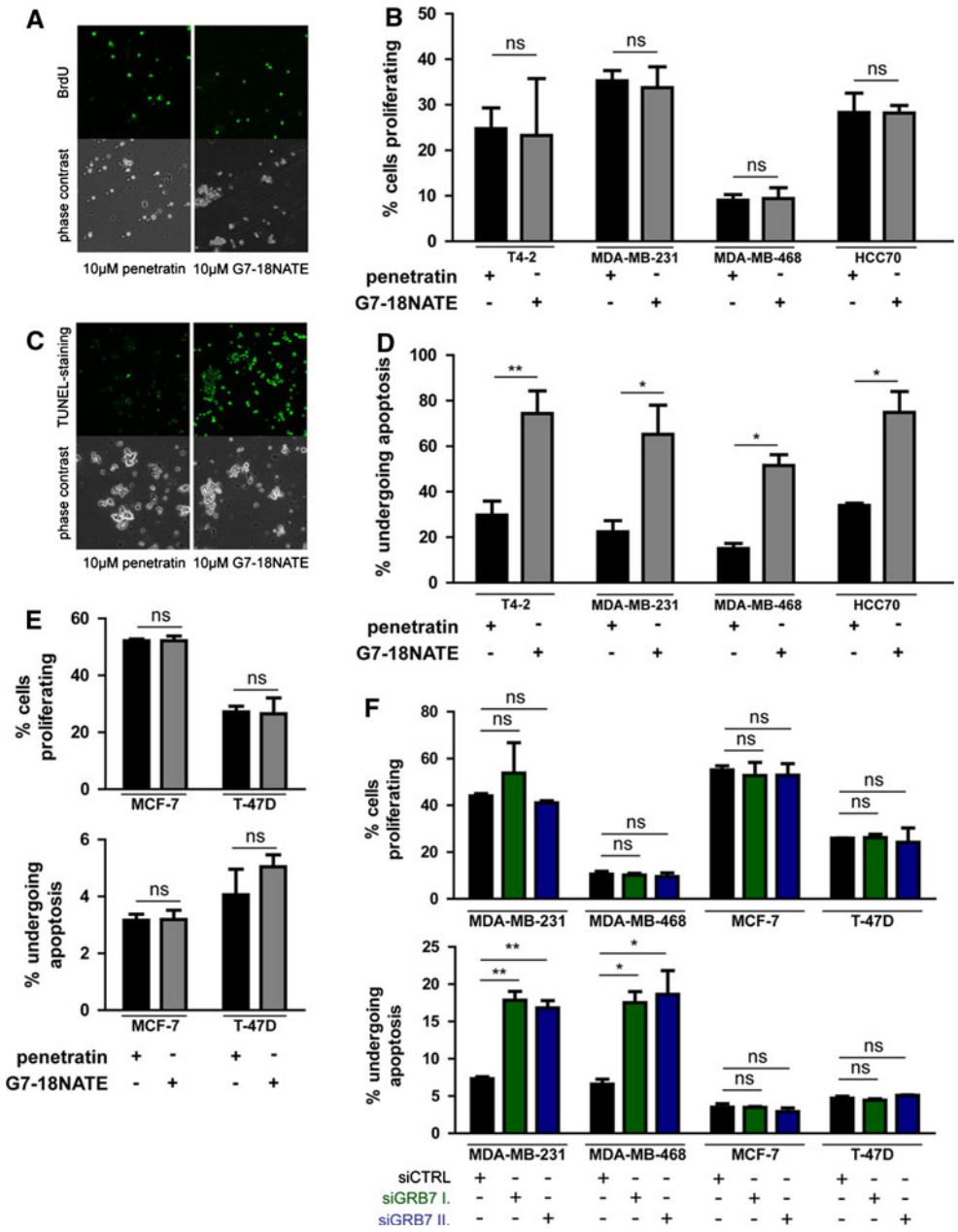

Fig. 4 Analysis of proliferation and apoptosis following GRB7 inhibition in 3D culture. **a** Representative images of MDA-MB-468 colonies labeled with BrdU. **b** Quantitative analysis of the percentage of cells in S-phase in the presence of 10 μM G7-18NATE or the penetratin control. **c** Representative images of MDA-MB-468 colonies analyzed for apoptosis using the TUNEL assay. **d** Quantitative analysis of apoptosis in all cell lines. **e** The Grb7 peptide inhibitor does not alter proliferation or apoptosis rates in MCF7 and T47D cell lines. **f** Confirmation using two siRNAs that the results obtained with the GRB7 peptide inhibitor in Fig. 4a–e are reproducible and are thus unlikely to be caused by off-target effects

being interrupted. If signals from diverse kinases are integrated through GRB7, then inhibiting this factor may allow us to intercept oncogenic signals emanating from multiple receptors. Given the emerging phenomenon of resistance to receptor tyrosine kinase inhibitors arising by upregulation of compensating kinases [19], inhibition of these pathways at the level of GRB7 may remove this selective pressure in cancer evolution and prevent resistance arising via this mechanism. More work is needed to define the key activators and effectors of GRB7 in the different breast cancer subtypes.

Although TNBC was our primary focus in this study, it is clear from the work of several groups that GRB7 also plays a prominent role in the pathobiology of other breast cancer subtypes and in other malignancies. The human *GRB7* gene is located on chromosome 17q12, immediately adjacent to *HER2* and is part of the *HER2* amplicon. GRB7 is often co-amplified and overexpressed with HER2 in breast cancer, and is associated with a poor prognosis in these tumors [16, 20]. In HER2-amplified cell lines, GRB7 was recently reported to be induced to even higher expression levels following treatment with Lapatinib [21] in a feedback loop that may promote Lapatinib resistance. In that study, silencing of GRB7 with siRNA enhanced Lapatinib sensitivity, suggesting that co-treatment with GRB7 inhibitors and HER2 inhibitors may be synergistic and may cut off one potential pathway of acquired resistance. That GRB7 is not merely a co-amplified

"passenger" in the HER2 amplicon is further emphasized by the report that it is an independent prognostic factor for adverse outcomes in breast cancer [22]. In ER+ breast cancer cell lines, it has been shown that GRB7 overexpression can confer tamoxifen resistance [23], while in patients with ER+ breast tumors, high GRB7 levels were predictive of reduced progression-free survival on tamoxifen [24]. The inclusion of *GRB7* in the Oncotype DX 21-gene signature further supports its importance in breast cancer [25]. More broadly, GRB7 overexpression has been implicated in carcinomas of the upper gastrointestinal tract [26], testicular germ cell tumors [27], and in chronic lymphocytic leukemia [28], suggesting numerous potential clinical uses for GRB7 inhibitors in addition to breast cancer.

Given the broad tissue distribution of GRB7, it is reasonable to be concerned about potential side effects of GRB7 inhibition in cancer therapy. It is encouraging to note that this peptide inhibitor has been shown to not have deleterious effects on mice over a 4 week period and to have anti-tumor efficacy in vivo in mouse models [10], suggesting that long term systemic treatment with small molecules targeting GRB7 may be feasible and tolerable in patients. Wilce and colleagues have identified a series of promising benzopyrazine derivatives which can act as GRB7 antagonists [29] and the recent report of the crystal structure of the GRB7 SH2 domain in a complex with the G7-18NATE peptide inhibitor by this group [30] is likely to facilitate the development of additional small molecules which can mimic this interaction. Our ongoing studies to identify the key TNBC cell pathways in which GRB7 participates will likely yield additional therapeutic targets which may ultimately prove to be more easily druggable.

Of the other GRB adaptor proteins, GRB2 is widely expressed in breast cancer [31] and plays a key role in coupling ERBB2 to its downstream signaling pathways [32]. The distribution of GRB10 in breast cancer has not been well characterized, although experimental studies suggest a role for this protein downstream of EGFR, IGF1R, and other receptor tyrosine kinases [33]. The GRB14 adapter protein blocks cell signaling downstream of insulin and FGF receptors [34, 35]. Our previous studies [9, 10] demonstrate that the GRB7 inhibitory peptide does not interact with these other GRB family members, and the siRNA experiments in this report confirm that the phenotypes observed in TNBC cell lines are specifically dependent on GRB7.

Our study highlights a hitherto unappreciated vulnerability of TNBC cells. Given the paucity of molecular targets in this disease and the poor prognosis for these patients, the identification of a key requirement for GRB7 may lead to the development of targeted therapies to interfere with GRB7 function or to block the key pathways in which it participates.

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