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Mammaglobin 1 Promotes Breast Cancer Malignancy And Confers Sensitivity To Anticancer Drugs

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Mammaglobin 1 (MGB1), a member of the secretoglobin family, is expressed in mammary epithelial tissues and is overexpressed in most mammary carcinomas. Despite the extensive research correlating MGB1 expression profiles to breast cancer pathogenesis and disease outcome, the biological significance of MGB1 in cancer processes is still unclear. We have thus set out to conduct a functional evaluation of the molecular and cellular roles of MGB1 in breast cancer processes leading to disease progression. Using a series of breast cancer cell models with conditional MGB1 expression, we demonstrate that MGB1 promotes cancer cell malignant features. More specifically, loss of MGB1 expression resulted in a decrease of cell proliferation, soft agar spheroid formation, migration, and invasion capacities of breast cancer cells. Concomitantly, we also observed that MGB1 expression activates signaling pathways mediated by MAPK members (p38, JNK, and ERK), the focal adhesion kinase (FAK), matrix metalloproteinases (MMPs) and NFκB. Moreover, MGB1 regulates epithelial to mesenchymal (EMT) features and modulates Snail, Twist and ZEB1 expression levels. Interestingly, we also observed that expression of MGB1 confers breast cancer cell sensitivity to anticancer drug-induced apoptosis. Together, our results support a role for MGB1 in tumor malignancy in exchange for chemosensitivity. These findings provide one of the first descriptive overview of the molecular and cellular roles of MGB1 in breast cancer processes and may offer new insight to the development of therapeutic and prognostic strategies in breast cancer patients.

Key words: MGB1; metastasis; EMT; MAPK; FAK; NFκB; chemosensitivity

INTRODUCTION

In more than 90% of breast cancer cases, patient death is associated to the presence of metastasis [1,2]. Although the mechanisms driving breast cancer metastasis are not completely understood, there is a general agreement that cancer metastasis encompasses alterations in cell phenotypic features which grant mammary epithelial cells the capacity to invade other tissues to establish metastatic tumors. These processes are also known as: 1) the epithelial-mesenchymal transition (EMT) where tumor cells express invasive properties to enter the circulatory system and; 2) the mesenchymal-epithelial transition (MET) where circulating cancer cells reboot an epithelial program to enter and colonize metastatic niches in distant organs [3–5]. This tightly regulated process is an important step in the evolution of cancer aggressivity. The identification of genes involved in breast cancer malignancy is thus essential to mitigate the morbidity and mortality associated with the disease progression.

Mammaglobin 1 (MGB1) is a protein expressed in mammary epithelial tissues and is over expressed in most breast carcinomas [6]. Because of its tissue-specific expression profile, MGB1 is considered a prominent biomarker for the detection of circulating breast cancer cells [7,8]. Accordingly, MGB1 is now used as a clinical biomarker to detect breast cancer metastasis bigger than 0.2 mm^2 (i.e., GeneSearchTM Breast Lymph Node (BLN) Assay) [9]. Despite the strong association of MGB1 expression profiles with mammary cancer cell pathogenesis, the biological significance of MGB1 in cancer processes is still ill-defined.

Abbreviations: MGB1, Mammaglobin; MAPK, Mitogen Activated Protein Kinase; FAK, Focal Adhesion Kinase; MMP, Matrix Metalloproteinases; NFκB, Nuclear Factor of kappa B cells; EMT, Epithelial to Mesenchymal Transition; MET, Mesenchymal to Epithelial Transition; BLN, Breast Lymph Node Assay; shRNA, Short Hairpin RNA; qRT PCR, Quantitative Reverse-Transcription-Polymerase Chain Reaction; MGB1 05, MGB1-deficient MB231 cells; IC_{50}, Concentration Inhibiting 50% of Cell Function; MRP1, Multidrug Resistance protein-1; Pgp, P-Glycoprotein; ABCG2, ATP-Binding Cassette Sub-Family G Member 2.

Conflict of interest: None.

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There are many conflicting studies that correlate MGB1 expression with cancer behavior and disease outcome. On one hand, studies associate elevated expression levels of MGB1 in breast cancer to better prognostic value and less aggressive phenotypes. These findings are supported by the observations that primary tumors with elevated expression levels of MGB1 entail lower tumor grades, suppressed expression of hormones receptors, nuclear diploid content, low Ki67 proliferation labeling index and absence of auxiliary nodal invasion [10–12]. It has also been demonstrated that elevated MGB1 levels in primary tumors is associated with a longer relapse-free survival period and greater overall survival [13]. On the other hand, others report that MGB1 expression is associated with poor prognostic outcome. The latter studies have demonstrated that elevated MGB1 levels in peripheral blood of breast cancer patients is associated with larger tumor size, higher Ki67 index and greater risk of developing metastasis [14–16].

It is still unclear whether aberrant MGB1 expression is a cause or effect of breast cancer disease. Despite the mounting data of clinical correlations between MGB1 expression signatures and cancer cell malignancy, there is a total lack of studies defining the specific role of MGB1 in breast cancer processes. We therefore set out to elucidate the molecular and cellular implications of MGB1 in various breast cancer processes (i.e., proliferation, apoptosis, anchorage-independent growth, invasion, and migration). Through the use of an aggressive breast cancer cell model with attenuated MGB1 expression, we observed that loss of MGB1 expression resulted in a less aggressive cancer phenotype. To our knowledge, this is the first mechanistic evaluation demonstrating a role for MGB1 in breast cancer malignancy and progression.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections

The MCF7 (ductal carcinoma, HTB-22); T47D (ductal carcinoma, HTB-133); MCF12A (epithelial, CRL-10782); MCF10A (epithelial, CRL-10317); MDA-MB231 (ductal carcinoma, HTB-26); MDA-MB415 (gland adenocarcinoma, HTB-128); MDA-MB468 (gland adenocarcinoma, HTB-132); BT474 (ductal carcinoma, HTB-20); and SKBR3 (adenocarcinoma, HTB-30) mammary cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA).

Cells were cultured in DMEM medium supplemented with 10% foetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). T47D were cultured in RPMI 1640 medium supplemented with 15% FBS, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml), sodium pyruvate (1 mM), and bovine insulin (0.01 mg/ml). MCF12A, and MCF10A were maintained in DMEM/F12 medium supplemented with 5% FBS, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml), sodium pyruvate (1 mM), bovine insulin (10 μg/ml), EGF (20 ng/ml), cholera toxin (100 ng/ml), and hydrocortisone (500 ng/ml). SKBR3 cells were maintained in McCoy's medium with 10% FBS, L-glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). Culture media and reagents were obtained from Life Technologies (Burlington, ON, Canada) except for the FBS which was provided by PAA Laboratories (ON, Canada).

MGB1 suppression was obtained using either transient transfection with a pool of MGB1-directed siRNA (SMARTpool, Dharmacon, ON, Canada); or, stable transfections with shRNA cloned into the Open Biosystems Expression Arrest GIPZ Lentiviral shRNAmir (Thermo Scientific, Waltham, MA). In this system, the pGIPZ plasmid contains either one of the five different shRNAmir sequences (clones 01–05) targeting the MGB1 transcript; or, an irrelevant non-silencing shRNAmir sequence used for a negative control. Stable transfections were performed using Lipofectamin (Life Technologies) following the fabricant instructions. 48 h post-transfection, the selective agent, 1 μg/ml puromycin, was added to the media to select stable mixed populations of transfected cells. Transient knock-in transfections were performed with MGB1 recombinant expression vector constructs. PCR products of MGB1 cDNA were obtained from T47D cells with the forward 5′ CAGCGGCTTCCTTGATCCTT 3′ and reverse 5′ GCAATCCGTAGTTGGTTTCTC 3′ primers and cloned into the pcDNA3.1 vector. Constructs were then verified by sequencing and alignment according to the MGB1 sequence (NM_002411).

Cell Viability and Apoptosis Assays

5×10^3 cells were seeded in 96 well plates and analyzed at the indicated time points for cellular viability and apoptosis using a multiplex assay formed with the CellTiter Blue® and Apo-ONE® assay kits (Promega, Madison, WI) as described previously [17]. In brief, cells were seeded in 100μl/well with complete medium for the indicated time points. Thereafter, 20 μl of CellTiter Blue® substrate was added to the 100 μl of media containing the cells and incubated at 37°C for 1 h. The plates were analyzed on a fluorescence microplate reader (FLUOstar Optima, BMG Lab technologies, $544_{Ex}/590_{Em}$). Apoptosis was also measured on the same microplate by removing 80 μl of the total media and adding 40 μl of the Apo-ONE® substrate. Plates were next incubated at room temperature for 1h on a plate shaker and analyzed by fluorescence reading ($485_{Ex}/520_{Em}$).

Reverse Transcription and Quantitative PCR

Total RNA was isolated from cells using TriZol reagent (Life Technologies) as described previously [17]. Reverse transcription was performed on total

RNA using SuperScript III reverse transcriptase (Life Technologies). In short, for 5 μg of RNA, we added 1 μl of Oligo dT (1 mg/ml) and RNase free water to a final volume of 20 μl. The mix was then heated at 65°C for 5 min and cooled on ice for 5 min followed by the addition of 8 μl of the strand buffer (5X), 1.5 μl of the SuperScript III (200 U/μl), 2 μl of DTT 0.1M, 1.6 μl of dNTP (25 mM), 1 μl of RNAse inhibitor and 5.9 μl of RNAse free water to make a final volume of 40 μl. The mix was then incubated at 55°C for 1 h and 72°C for 15 min. Next, 2 μl of RNAse H (10 mg/ml) were added followed by incubation at 37°C for 5 min.

Quantitative gene expression analysis was performed using the SYBR® Green FastMix for iQ (Quanta, Biosciences Inc., Gaithersburg, MD). The reaction was carried out in a 25 μl volume containing 12.5 μl of 2X SYBR® Green, 2.5 μl of each of the forward and reverse primers (3 μM) listed in Table 1, 5.5 μl H_2O and 2 μl of cDNA. The RT-qPCR assays were performed on a Realplex Real Time PCR apparatus (Eppendorf, ON, Canada). Samples were heated for 2 min at 95°C followed by 40 PCR cycles of 30 s at 95°C, 30 s at 58°C and 30 s at 72°C. Subsequently, a melt curve analysis from 60°C to 95°C was performed. Relative expression levels were calculated using the ΔΔCt method and normalized to hypoxanthine ribosyltransferase (HPRT) [18].

Western Blot Analysis

Cells (6×10^5) were lysed using 0.1 ml of 2X whole cell lysate (WCL) buffer (0.125 M Tris pH 6.8, Glycerol 0.2 g/ml, 4.0% SDS) along with freshly added 10 μl phenylmethylsulfonyl fluoride (PMSF, 10 mg/ml), 5 μl of protease inhibitor cocktail set III (Calbiochem Gibbstown, NJ), 10 μl sodium orthovanadate (100 mM) (Sigma–Aldrich, St. Louis, MO) and 1 μl phosphatase inhibitor cocktail 1 (Sigma–Aldrich). Cells treated with the complete WCL solution were scraped and passed 5 times through a 26G syringe. Protein concentration was then determined with a bicinchoninic acid (BCA) quantification assay (Thermo Scientific). Next, 20 μg of protein was mixed in 2X laemmli buffer, heated at 95°C for 5 min and separated on a 10% polyacrylamide gel. Proteins were then transferred to a PVDF (polyvinylidene fluoride) membrane (Millipore, Billerica, MA). As previously described [17], the membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated thereafter with either anti-MGB1 (ab83499, Abcam, Toronto, ON, Canada), anti-p38 (#9212, Cell Signaling, Boston, MA), anti-phospho-p38 (#9216, Cell Signaling), anti-ERK (#9107, Cell Signaling), anti-phospho-ERK (#9106, Cell Signaling), anti-JNK (#9252, Cell Signaling), anti-phospho-JNK (#9251, Cell Signaling), anti-PI3K (#ab109006, Abcam), anti-PTEN (#ab133254, Abcam), anti-AKT (#9272, Cell Signaling), anti-NFκB p65 (#622602, Biolegend, San Diego, CA), anti-IκBα (ab32518, Abcam), anti-Snail (#3879, Cell Signaling), anti-Twist (#07–909, Millipore), anti-E-cadherin (#MABT26, Millipore), anti-integrin-α5 (#ab6131, Abcam), anti-fibronectin (#ab2413, Abcam), anti-FAK (#05–537, Millipore), anti-phospho-FAK (#AF4528, R&D Systems, Minneapolis, MN), anti-MMP2 (#4022, Cell Signaling), anti-MMP9 (#ab38898, Abcam) or anti-G3PDH (#2275-PC-100, Trevigen, Gaithersburg, MD) antibodies overnight at 4°C. Next, horseradish peroxidase (HRP)-conjugated secondary antibodies (Pierce, Thermo Scientific) were incubated with the membrane for 1 h at room temperature and the signal was revealed by chemiluminescence according to the manufacturer's protocol (SuperSignal West Dura, Thermo Scientific).

Reporter Gene Assays

Transient transfections of cells were performed using the X-tremeGENE -9 DNA transfection reagent

Table 1. Mammaglobin 1 Promotes Breast Cancer Malignancy and Confers Chemosensitivity Picot et al., 2014

	Forward primers (5'→3')	Reverse primers (5'→3')
HPRT	TGACACTGGCAAAACAATGCA	GGTCTTTTTCACCGACAAGCT
MGB1	CAAGACAATCAATCCACAAGTGTCTAAGAC	CAGAGTTTCATCCGTTTGGTTAAGAAAACATTC
Fibronectin	CCGCCGAATGTAGGACAAGA	TGCCAACAGGATGACATGAAA
Vimentin	CAACCTGGCCGAGGACAT	ACGCATTGTCAACATCCTGTCT
E-cadherin	CAAGCTATCCTTGCACCTCAG	GCATCAAGAGAACTCCTATCTTG
Snail	GCTGCAGGACTCTAATCCAGAGTT	GACAGAGTCCCAGATGAGCATTG
Twist	GGAGTCCGCAGTCTTACGAG	TCTGGACCTGGTAGAGG
Zeb1	GCCAATAAGCAAACGATTCTG	TTTGGCTGGATCACTTTCAAG
MRP1	TCTACCTCCTGTGCCTGAATCTG	CCGATTGTCTTTGCTCTTCATG
ABCG2	TGACCTGAAGGCATTTACTG	GGTAGAAAGCCACTCTTCAG
Pgp	AAATTGGCTTGACAAGTTGTATATGG	CACCAGCATCATGAGAGGAAGTC

(Roche Applied Science, Laval, QC, Canada). Briefly, 24 h before transfection, cells were seeded into 96-well white plates (2.1×10^4 cells/well) where they subsequently received a total of 100 ng of plasmid DNA complexed with the provided transfection reagent. Reporter gene assays were conducted using the Dual-Luciferase SystemTM (Promega). Transfected DNA also included 25 ng of *Renilla* luciferase vector (ptk-RL) (Promega) as a control. Luciferase-based reporter genes constructs include: NFκB-luc (Promega); Snail–luc [19] (generously provided by Dr. Antonio Garcá de Herreros, IMIM-Hospital del Mar, Barcelona, Spain); Twist-luc [20] (generously provided by Dr. Mien–Chie Hung, MD Anderson Cancer Center, Houston, TX); ZEB1-luc [21] (generously provided by Dr. Harikrishna Nakshatri, IU Simon Cancer Center Indiana University, Indianapolis, IN); and FAK–luc [22] (generously provided by Dr. Vita Golubovskaya, Roswell Park Cancer Institute, Buffalo, NY). 48 h post-transfection, cells were lysed and then analyzed for luciferase activity with a luminometer (BMG Fluostar Optima) as previously described [17]. Relative reporter activity was calculated and normalized based on *Renilla*-luciferase levels which reflected transfection efficiency. Each assay was performed in biological and experimental triplicates.

Migration and Invasion

For the migration assay, cells were starved in DMEM containing only 0.1% FBS for 16 h previous to the seeding into tissue culture transwell inserts (Greiner Bio-One, NC). The inserts were first coated with 250 μl of gelatin 0.1% (Sigma–Aldrich). Following an incubation at 37°C for 2 h, excess coating was then removed and 200 μl of suspended cells (50,000 cells/well) in DMEM 0.1% FBS were added to the inserts. The transwells were then placed in a well containing DMEM 0.1% FBS and incubated at 37°C for 1 h. Next, they were moved into wells containing DMEM 20% and incubated for 9 h at 37°C. The negative control transwells were moved into wells containing DMEM 0.1%. After incubation, cells that passed through the membrane were harvested with trypsin and submitted to a cell viability assay as described previously for quantification and comparison.

For the invasion assay, each transwell insert was coated with 50 μl of matrigel 5 mg/ml (BD Biosciences, ON, Canada) in DMEM 0.1% and incubated for 16 h at 37°C. Cells (40,000) were added to each well (200 μl) and starved in DMEM 0.1% FBS for 16 h. Transwells were then transferred to DMEM 20% FBS and incubated at 37°C for 6 h. After the incubation, invading cells were quantified as described in the migration assay.

Soft Agar Assays

In six well plates, we added 2 ml of a solution composed of 1.2% low melting agarose (Affymetrix /USB Corporation, Cleveland, OH) in 2X DMEM supplement with 20% FBS, 4 mM L-glutamine, 200 units/ml penicillin and 200 μg/ml streptomycin. Next, 3×10^5 cells were added to a 1.5 ml solution containing 0.3% agarose in 2X DMEM which was then added on top of each treated well. After solidification, 250 μl of complete DMEM media was added on top of the agarose. Cells were incubated at 37°C and 5% CO_2 for 9 days where complete media was changed every 3 days. Pictures were taken at the indicated time points with an inverted microscope at 40X and 100X magnifications.

RESULTS

Profiling of MGB1 Expression in Breast Cancer Cell Models

Given the paucity of research on the functional role of MGB1 in breast cancer pathogenesis, we first examined and compared MGB1 expression in a series of commonly used breast epithelial cell models (non-cancerous and cancerous). Using RT-qPCR, we evaluated MGB1 transcript levels in MCF12A, MCF10A, MB231, MCF7, MB468, T47D, SKBR3, MB415, and BT474 cells (Figure 1A). As expected, we observed that MGB1 mRNA expression was higher in cancerous breast cell lines in comparison to non-cancerous cell models (MCF12A and MCF10A). Consistent with clinical observations, we found that breast cancer cell lines mimic the correlated MGB1 upregulation in a cancerous setting, thus providing an adequate study model for MGB1 mechanistic evaluation of breast cancer pathogenesis.

MGB1 Downregulation Inhibits Breast Cancer Cell Growth

To properly assess the role of MGB1 in cancer cell processes, we generated breast cancer cell models with attenuated MGB1 expression. A series of MGB1-targeting shRNAs (shRNA 01–05) were stably transfected into the MB231 aggressive breast cancer cell line and validated for suppressed MGB1 mRNA and protein levels using qRT-PCR and Western blotting, respectively (Figures 1B and 1C). We observed that the majority of the shRNAs targeting different regions of the MGB1 transcript resulted in attenuated MGB1 protein expression levels in comparison to control cells (i.e., non-transfected cells, G3PDH-targeting shRNA, and a non-silencing scrambled shRNA). We thus selected the MB231 stably expressing the shRNA #05 (MGB1 05) for subsequent analyses of MGB1-mediated breast cancer processes.

We first assessed the effects of MGB1 suppression on the proliferation rates of MB231 cells. A viability assay was thus performed in time (0–7 d) on the MGB1 05 cell population (MB231 cells with attenuated MGB1 expression) and compared to control cells which include: 1) non-transfected (NT) MB231 cells and, 2) MB231 cells stably transfected with a non-targeting scrambled shRNA (non-silencing/NS) (Figure 2A). All tested MB231 cell populations proliferated over time. However, cells with suppressed MGB1 levels

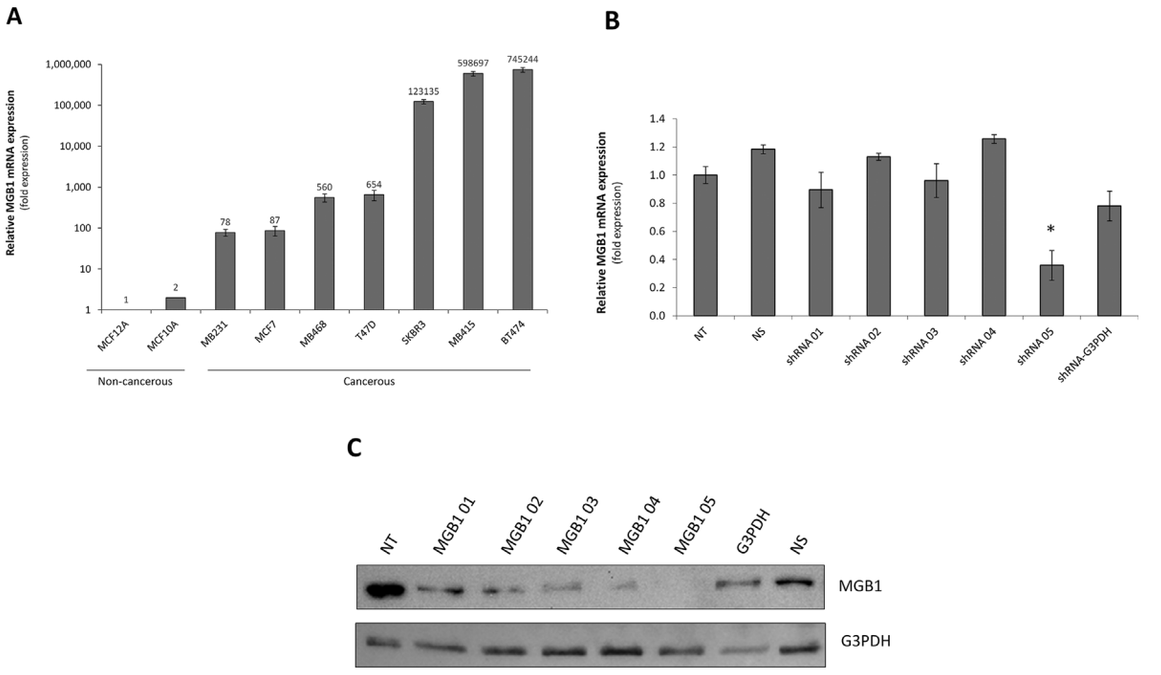

Figure 1. MGB1 expression profiles and cell models. (A) Expression of MGB1 transcripts were evaluated in a series of non-cancerous and cancerous mammary epithelial cell lines by qRT-PCR and plotted in relation to MGB1 basal mRNA levels found in MCF12A cells. Fold increases are represented above each sample column. (B) MGB1 mRNA and (C) protein expression levels were revealed by qRT-PCR and Western blot respectively in non-transfected (NT) MB231 breast cancer cells or stably transfected cells with either one of five MGB1-targeting shRNAs (MGB1 01–05) or control shRNA (including a non-specific non-silencing/NS shRNA and an anti-G3PDH shRNA). The mRNA levels were normalized against HPRT and plotted in relation to transcript levels from parental (NT) cells. The protein levels from G3PDH are also shown as a loading control. Results are representative of biological and experimental triplicates ($^{*}P < 0.001$).

(MGB1 05) demonstrated attenuated cell growth (52% lower of non-transfected parental MB231 cells and 37% lower than non-silencing shRNAs controls at day 7). These results suggest a role for MGB1 in breast cancer growth and tumorigenesis.

MGB1 Regulates Activation Pathways Supporting Breast Cancer Cell Growth and Survival

In order to elucidate the molecular mechanisms of MGB1-regulated growth, we looked at the expression and activation (phosphorylation) of the various Mitogen-Activated Protein Kinase (MAPK) pathways (i.e., p38, ERK and JNK) in our MGB1 05 cell line model using Western blotting (Figure 2B). Interestingly, the modulation of MGB1 expression in MB231 cells did not significantly alter the protein expression levels from p38, ERK1/2 and JNK members. However, upon our investigation of their respective phosphorylated (active) forms, we found that loss of MGB1 expression resulted in lower phosphorylation events on all three MAPK family members tested (p38, ERK1/2, and JNK) when compared to non-transfected parental and non-silencing controls. Immunodetection of G3PDH was also performed to indicate adequate loading of protein samples.

To pursue our elucidation of MGB1-regulated cell growth signaling cascades, we looked at the expression of Akt, PI3K, and PTEN (two modulators of the Akt pathway) in addition to NFκB signaling components by Western blot. First, we were unable to detect Akt protein expression in all MB321 cell populations tested. Nonetheless, we found that MGB1 05 cells exhibited a slight decrease in PI3K expression in comparison to control cells with no significant changes in PTEN protein levels (Figure 2C). On the other hand, when we assessed NFκB cascades, MGB1 05 cells exhibited greater p65 expression levels in addition to an increase of IκB-α (inhibitor of NFκB alpha) levels (Figure 2C). To ascertain the effect of MGB1 expression on NFκB transactivation, we performed reporter gene assays using a firefly-based luciferase gene under the transcriptional control of NFκB binding motifs. As depicted in Figure 2D, MGB1 05 cells displayed a significant inhibition (up to 47%) in NFκB transactivation potential over shRNA control MB231 cells. Our findings represent the first molecular evidence of a role for MGB1 in the modulation of MAPK and NFκB signaling cascades in breast cancer cells.

Loss of MGB1 Expression Attenuates Breast Cancer Cell Aggressivity

We pursued our investigation of the effects of MGB1 on key processes driving breast cancer

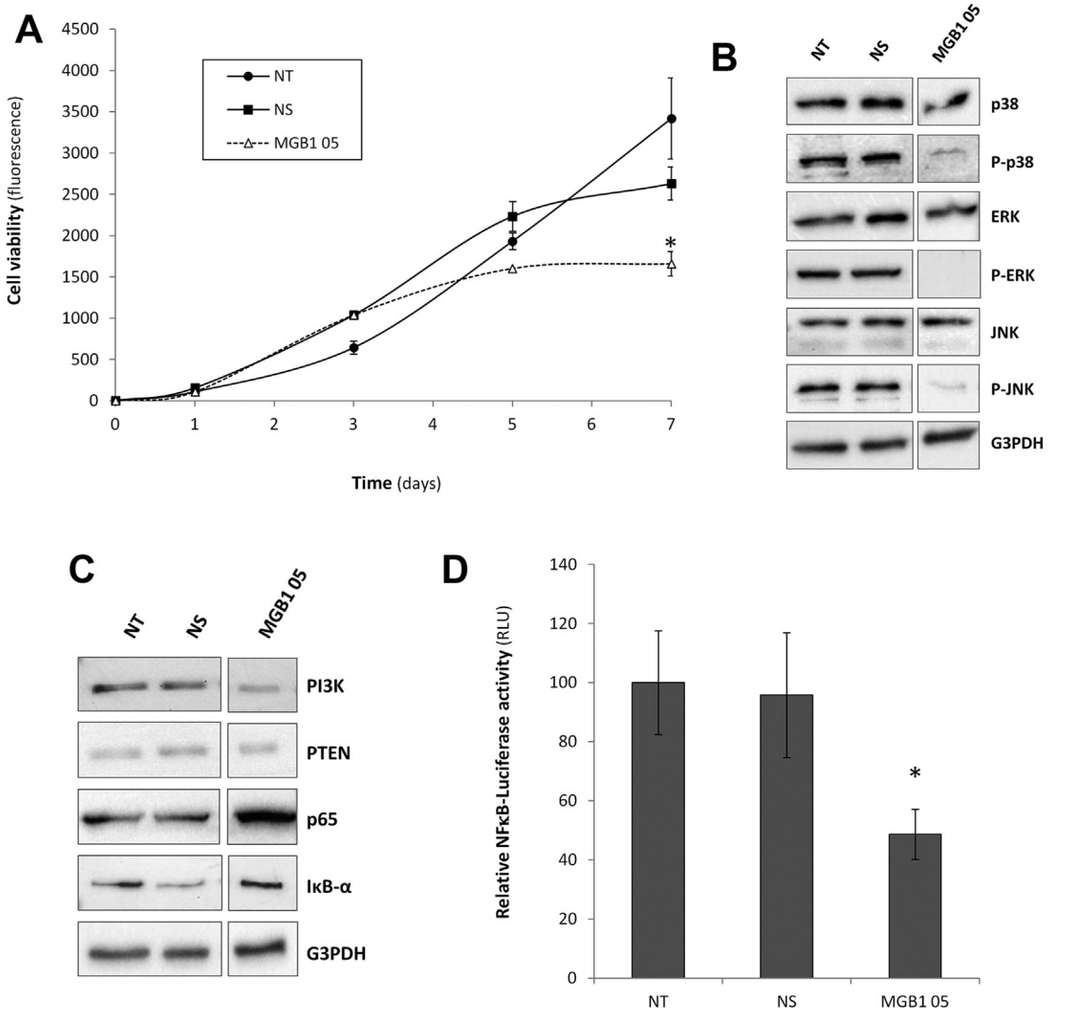

Figure 2. Loss of MGB1 inhibits breast cancer cell growth (A) Cellular growth was monitored over time using viability assays in MB231 cells either left non-transfected (NT); or, stably transfected with a non-silencing shRNA (NS) or MGB1-directed shRNA-05 (MGB1 05). (B) Protein expression levels of MAPKs (p38, JNK, and ERK), (C) PI3K, PTEN, and NFκB components (p65 and IκB-α) were assessed in MB231 stably transfected populations (NT, NS and MGB1 05) using Western blotting and compared to G3PDH as a loading control. (D) NFκB promoter activity was assessed using a luciferase-based reporter gene assay. Cells were co-transfected with a vector containing the NFκB promoter upstream of the luciferase gene in addition to a non-inducible Renilla luciferase vector to standardize transfection efficiency. Luciferase activity (relative light units/RLU) was normalized 48 h post-transfection and plotted in relation to parental cells (NT). The presented data is representative of biological and experimental triplicates. (*$P < 0.001$).

malignancy such as anchorage-independent growth (resistance to anoikis), cell migration and invasion events. To study anchorage independent growth, MB231 cell populations were mixed in soft agar suspensions and evaluated for cell colony formation in time. As expected, malignant MB231 cells proliferated in soft agar and formed cell spheroids over the course of 9 days (Figure 3A). However, MGB1-deficient MB231 cells (MGB1 05) in agar cultures presented smaller and fewer colonies in comparison to parental MB231 cells and non-silencing shRNA transfected controls. We next wanted to determine the role of MGB1 in breast cancer cell migration and invasion processes. Using culture transwell inserts, we found that MGB1 05 cells displayed slightly lower migration capacity (Figure 3B). However, MGB1 05 cells revealed a significant inhibition of invasion ability when compared to control samples (up to 87%) (Figure 3B).

To elucidate the molecular mechanisms by which loss of MGB1 expression results in attenuated aggressive features, we investigated key modulators (i.e., the focal adhesion kinase (FAK) [23] and matrix metalloproteinases (MMPs) [24]) known to induce and facilitate invasive behavior. Using Western blotting, we observed that MGB1 05 cells demonstrated a significant decrease in MMP-9 levels and slight attenuation of MMP-2 levels when compared to

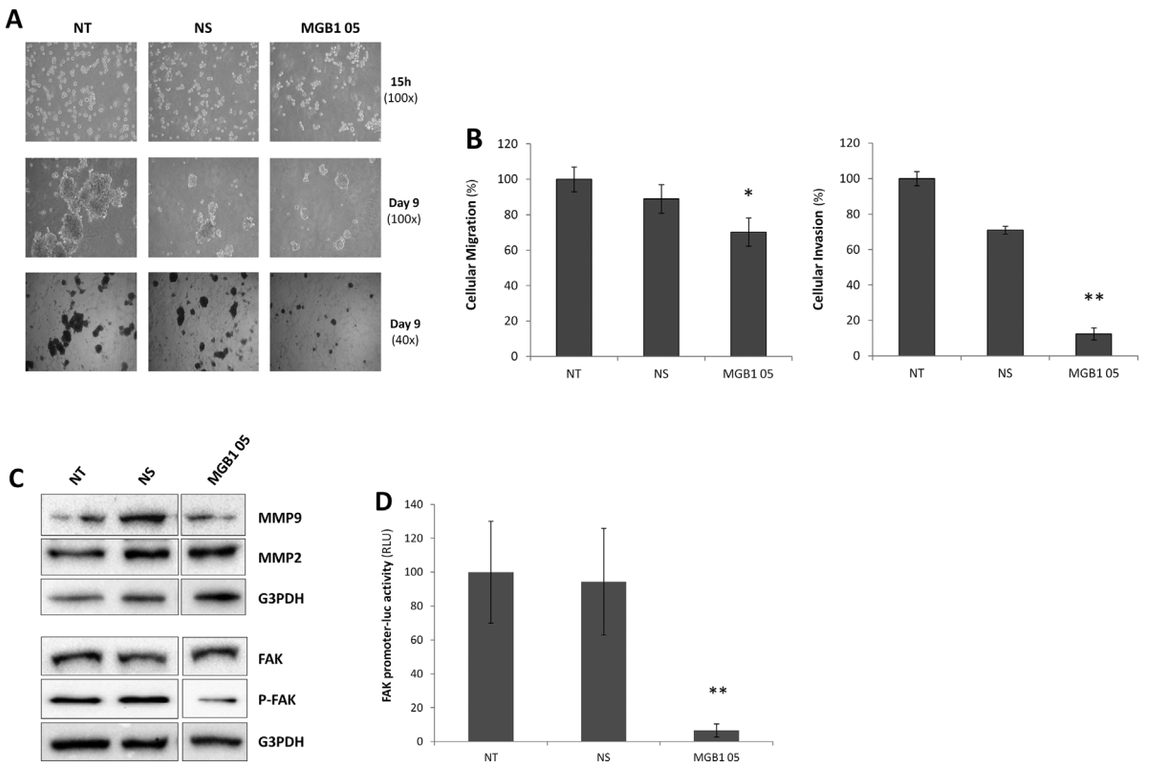

Figure 3. Loss of MGB1 expression inhibits breast cancer cell malignant features. (A) Anchorage-independent growth was monitored using a soft agar assays in non-transfected (NT) MB231 cells or stably transfected with either a non-silencing (NS) shRNA control or MGB1-targeting shRNAs (MGB1 05). Cells were cultured and photographed at 40X and 100X magnifications over time (15 h and 9 d post seeding). (B) Cell migration and invasion processes were evaluated in cell populations using invasion chambers and plotted in function to parental NT cells. Cells were also submitted to Western blots (C) to reveal the expression levels of MMPs (2 and 9), total and phophorylated FAK, and G3PDH protein. (D) FAK promoter activity was also evaluated in MB231 cell populations using a dual luciferase reporter system. The presented data is the calculated mean of three independent samples and is representative of three different experiments ($^{*}P < 0.05$) ($^{**}P < 0.001$).

G3PDH loading controls (Figure 3C). On the other hand, MGB1-deficient cells did not alter total FAK protein levels. However, MGB1 05 cells exhibited lower phosphorylated forms of FAK (activated FAK) especially when compared to shRNA-transfected controls (Figure 3C). To confirm MGB1-mediated suppression of FAK activity, we made use of a luciferase gene under the control of the human FAK promoter. We found that the FAK promoter activity is almost completely abrogated in MGB1 05 cells when compared to control samples (Figure 3D). Altogether, our results demonstrate that loss of MGB1 abolishes breast cancer malignant features (notably anchorage-independent growth and invasion). In addition, loss of MGB1 results in suppressed FAK activity and MMP expression levels.

MGB1 Modulates Pro-Mesenchymal Phenotype Features

Given the potential of MGB1 to promote cancer cell malignancy, we set out to evaluate the capacity of MGB1 to modulate EMT–MET gene expression profiles. First, we studied the influence of MGB1 expression on epithelial phenotypic markers E-cadherin, Integrin-α5 and fibronectin in MB231 cells. Through the use of Western blotting, we did not detect E-cadherin expression in any MB231 cell populations tested. Similarly, MGB1 05 cells did not demonstrate any significant differences in neither Integrin-α5 nor fibronectin protein expression levels when compared to control cells (data not shown). We then focused our attention to the ability of MGB1 to mediate mesenchymal-related genes (i.e., Snail, Twist and ZEB1). Through the use of qRT-PCR, we found that MGB1 05 cells presented a marked decrease of Snail and Twist transcript levels but no variation in ZEB1 mRNA levels (Figure 4A). These observations were further validated using Western blots which revealed a slight decrease in both Snail and Twist protein expression (Figure 4B). We were however unable to detect a specific signal using the available commercial ZEB1 antibodies. To examine if MGB1 regulates Snail, Twist and ZEB1 promoter activity, we transfected our MGB1-suppressed MB231 cell model with luciferase reporter constructs under the control of Snail, Twist, and ZEB1 promoter regions (Snail-luc, Twist-luc, and ZEB1-luc, respectively). We observed that MGB1 05 cells

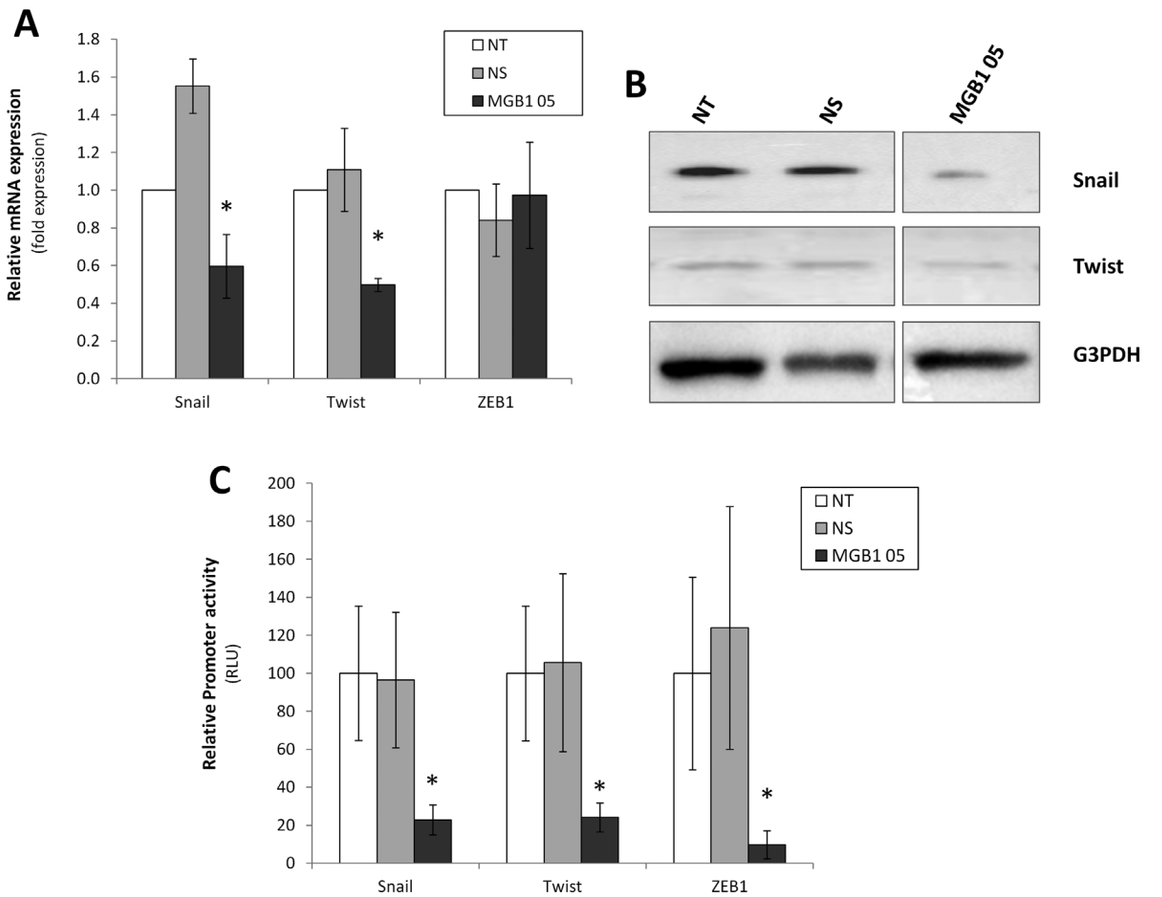

Figure 4. MGB1 sustains mesenchymal gene expression in breast cancer cells. MB231 cells (non-transfected/NT, non-silencing shRNA/NS and MGB1-deficient/MGB1 05) were examined for Snail, Twist and ZEB1 transcript (A) and protein levels (B) using qRT-PCR and Western blotting, respectively. The mRNA levels were normalized against HPRT and plotted in relation to transcript levels from parental NT cells. The protein levels were compared against G3PDH. (C) Snail, Twist, and ZEB1 promoter activities were also evaluated using a dual luciferase reporter system. Reporter activities were normalized and plotted in relation to their respective parental cells. The presented data is the calculated mean of three independent samples and is representative of three different experiments ($^*P < 0.001$).

displayed a significant inhibition of Snail, Twist and ZEB1 promoter activities in comparison to control cells (Figure 4C). Our results strongly suggest that MGB1 regulates mesenchymal gene expression profiles (i.e., Snail, Twist, and ZEB1) which are reminiscent of EMT in breast cancer cells.

To further explore the possibility that MGB1 may induce cancer cell tumorigenesis and malignancy, we transfected a non-cancerous (MCF10A) and a non-aggressive (MCF7) breast cell line with a MGB1 coding plasmid to study cellular growth and migration. To validate conditional recombinant expression of MGB1, qRT-PCR was performed in our transiently transfected models and we show an approximate 10^4 fold increase of MGB1 expression in both MCF7 and MCF10A cell lines over pcDNA empty vector transfected controls (Figure 5A). Using the latter transfected models, we observed that MGB1 induced a significant increase of MCF7 cell growth rates (up to 95%) and a slight augmentation in growth of MCF10A (up to 33%) cells over time (Figure 5). Upon our examination of MGB1-mediated migration properties, MGB1 recombinant expression induced a near twofold increase of migration events in MCF10A cells with no significant differences in the MCF7 model in comparison to vector control cells (Figure 5C). These results confirm a role for MGB1 in tumorigenesis and cancer malignancy and further validate MGB1 as a modulator of breast cancer processes.

MGB1 Confers Breast Cancer Cell Sensitivity to Apoptosis

To pursue our functional characterization of MGB1 in hallmark cancer processes, we studied the effects of MGB1 on breast cancer cell resistance to apoptosis and anti-breast cancer drugs. Using MGB1 05 cells, we first monitored how loss of MGB1 expression would affect basal sensitivity to early apoptotic events by measuring caspases 3/7 activities over time (7 days). Interestingly, MGB1 05 cells displayed lower apoptotic events in comparison to transfected controls thus suggesting that loss of MGB1 confers cancer cell death resistance (Figure 6A). To extend our observations in a

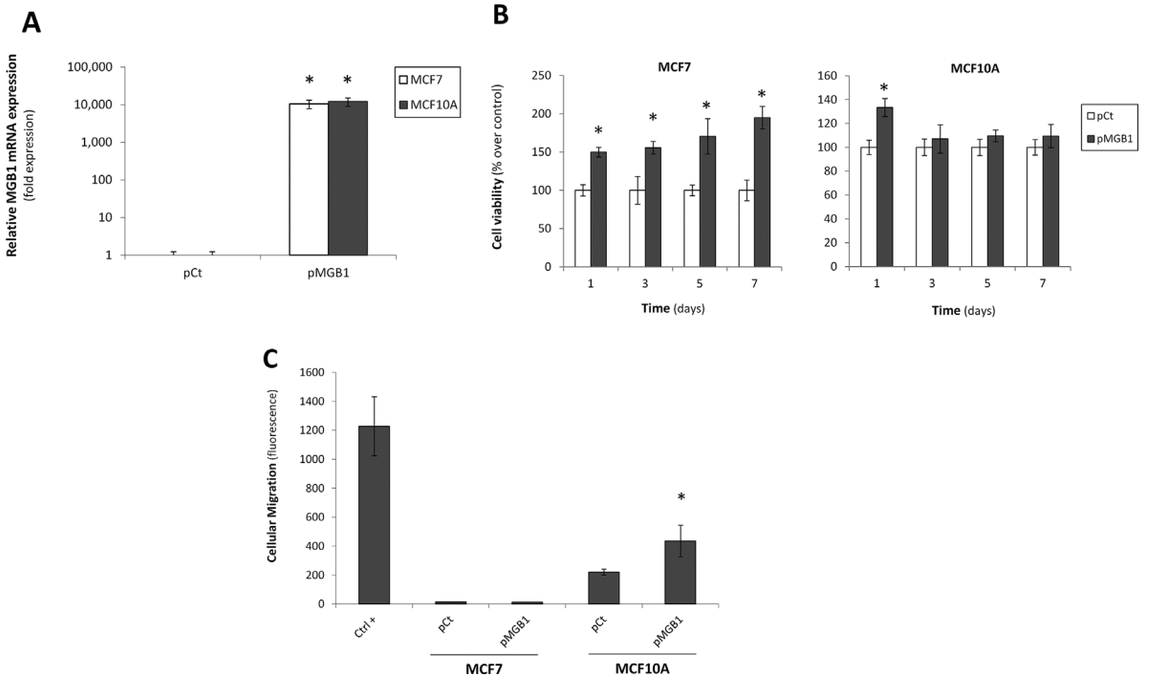

Figure 5. Recombinant expression of MGB1 promotes aggressive features. (A) MCF7 and MCF10A mammary cell lines were transiently transfected with a plasmid coding for MGB1 (pMGB1) or the empty vector alone (pCt) and validated by qRT-PCR. The mRNA levels were normalized against HPRT and plotted in relation to control cells (pCt). Cells were then submitted to (B) viability assays over time or (C) transwell migration assays. Positive control (Ctrl+) samples for cell migration are represented by MB231 cells. The presented data is the calculated mean of three independent samples and is representative of three different experiments ($^*P < 0.001$).

therapeutic setting, we evaluated the effects of MGB1 expression in chemotherapeutic agent-induced apoptosis. Anticancer drug concentrations capable of inhibiting 50% of cellular viability (IC_{50}) were calculated in MGB1 05 cells treated with increasing doses of paclitaxel, docetaxel, capecitabine, and doxorubicin over time. All of the drugs, except for capecitabine (data not shown), exerted cell growth inhibition on MB231 cells in a dose-dependent manner which provided a plotted IC_{50} value (Figure 6B). Surprisingly, MGB1-deficient MB231 cells were characterized with greater IC_{50} values for paclitaxel, docetaxel, and doxorubicin treatments. These observations suggest that MGB1 sensitizes breast cancer cells to chemical-based anticancer therapeutic regimes.

To examine the mechanism by which MGB1 confers sensitivity to drug-induced apoptosis, we evaluated the effects of MGB1 on common drug resistant genes known to mediate anticancer drug resistance. We thus assessed the expression levels of multi drug resistance protein-1 (MRP1), P-glycoprotein (Pgp) and ATP-binding cassette sub-family G member two (ABCG2) in MGB1 05 cells by qRT-PCR. Transcript levels of ABCG2 and Pgp were below detectible levels in all experimental settings. On the other hand, we observed that loss of MGB1 expression led to an approximate threefold increase of MRP1 transcript levels in comparison to transfected controls (Figure 6C). Altogether, these findings suggest that MGB1 can confer cancer drug sensitivity in breast cancer cells possibly through the modulation of drug resistant genes.

DISCUSSION

Despite the extensive research correlating MGB1 expression profiles to breast cancer pathogenesis, the biological significance of MGB1 in cancer processes is still unclear due to a paucity of data on MGB1 function. However, numerous studies have shown that MGB1 is overexpressed in breast cancer cells and have suggested that MGB1 may be implicated in tumor aggressivity [6,12,25,26]. In support of a role for MGB1 in cancer aggressiveness, a growing number of studies have also shown a role for MGB1 in gynecologic malignancies [27–30].

Through the use of a highly malignant breast cancer cell line (MB231), we show that the suppression of endogenously expressed MGB1 gene leads to a reduction of cancer cell growth. Likewise, we observed a growth rate increase in non-aggressive (MCF7) and non-cancerous (MCF10A) mammary cell lines upon recombinant MGB1 overexpression. These findings correlate with previous studies reporting that MGB1 can induce cell proliferation rates in both cervical and

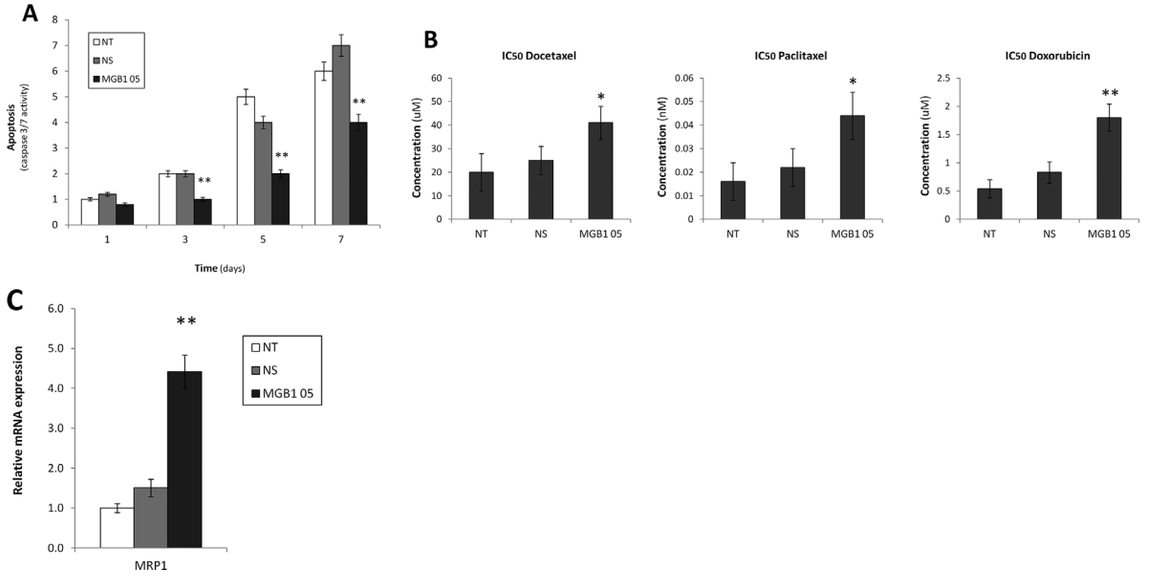

Figure 6. MGB1 sensitizes MB231 cells to apoptosis and anticancer drugs. (A) MB231 cell populations (non-transfected/NT, non-silencing/NS shRNA, and MGB1-deficient/MGB1 05) were examined for apoptosis via caspase 3/7 activity over time. (B) IC_{50} was calculated in cells submitted to serial drug dilutions for 72 h using normalized non-linear regression with Prism five software. (C) Transcript levels for MRP1 were obtained using qRT-PCR, normalized against HPRT and plotted in relation to transcript levels from parental cells. Results are representative of biological and experimental triplicates ($^{*}P < 0.05$) ($^{**}P < 0.001$).

breast cancer cell lines [30]. These findings are also consistent with evidence on other secretoglobin family members which regulate cell proliferation. More specifically, uteroglobin treatment of murine embryos has shown to promote cell growth and development [31]. Similarly, the uteroglobin-related protein (SCGB3A2) has demonstrated its potential as a growth factor in lung development [32]. On the other hand, our results contradict those obtained from Eun–Ha Koh et al. (2014) [33]. The latter study demonstrates that recombinant over expression of MGB1 in the MB231 cell line leads to lower cell viability while siRNA-mediated knock-down of MGB1 has no effect. We believe that technical variance and different study models may account for our divergent findings. Similar to Eun–Ha Koh et al. (2014), we have also made use of siRNA to transiently knock-down MGB1 expression in MB231 and observed that despite a reduction of MGB1 transcripts, MGB1 protein levels remained unchanged in a transient transfection system. Hence, we developed stable cell clones with MGB1-specific shRNAs to carry out our functional characterization.

In our molecular elucidation of MGB1-regulated proliferation, we observed that a loss of MGB1 expression led to an important decrease of MAPK (i.e., p38, ERKs, and JNK) and NFκB activation pathways. These findings were not surprising given the significance of NFκB signaling in cancer biology. In addition to its regulation of cellular growth and survival cascades, NFκB has recently been shown to mediate other cancer processes such as tissue invasion, migration, and metastasis [34]. A role for NFκB in cancer malignancy has been established by the observation that NFκB target genes are associated with the deployment of a mesenchymal phenotype, EMT features and breast cancer progression [21,35]. NFκB has also been shown to inhibit the expression of E-cadherin, a prominent marker of epithelialisation [21], most likely through the induction of E-cadherin repressors Snail [36], ZEBs [21], and Twist [37]. Accordingly, upon the inhibition of MGB1 expression, we observed a concomitant suppression of Snail, Twist, and ZEB1 promoter activities. Together, our findings strongly suggest that MGB1 promotes mesenchymal features that are reminiscent of EMT. Interestingly, we were unable to detect the induction of E-cadherin in our MGB1-deficient MB231 cell model. This phenomenon might be due to the fact that the MB231 cell line is a mesenchymal-dominant model unable to distinctively express epithelial features. Indeed, previous studies have reported that specific suppression of mesenchymal genes in the MB231 cell line do not necessarily correlate with E-cadherin induction and epithelial behavior reversion [38,39].

In pursuit of our characterization of MGB1 function in breast cancer cell malignancy, we found that loss of endogenous MGB1 expression resulted in the abrogation of breast cancer cell anchorage-independent growth, motility and invasion. Our results suggest that the loss in cancer aggressivity is partly due to the suppression of MMP expression and FAK activity in MGB1-deficient cells. Interestingly, MMPs and FAK

are also regulated by NFκB activation [22,40,41]. MMPs have been shown to play an important role in breast cancer metastasis by promoting the invasion of surrounding tissues by malignant cells [24] and have been correlated with tumor invasiveness [42,43] and poor prognosis [44]. Conversely, reports have previously shown that other secretoglobins, in particular uteroglobin and HIN-1 (SCGB3A1), suppress anchorage independent growth, extracellular matrix invasion and the production of MMP2 and MMP9 [45,46]. It's important to note however that the expression of the two latter secretoglobins is downregulated upon tumor development in contrast to MGB1 which is mostly induced during carcinogenesis [47,48]. Our results also show that MGB1 promotes the activation of FAK, a critical signaling pathway involved in mammary tumor malignancy [49,50]. FAK is a non-receptor tyrosine kinase which upon its phosphorylation regulates cell spreading, adhesion, migration, anoikis, invasion, and angiogenesis; processes all involved in cancer progression (reviewed in [51]). Consequently, FAK activity correlates with breast tumor metastatic potential and poor prognosis [51,52]. Mechanistically, it may be possible that MGB1 attenuates FAK activation through the intermediated suppression of NFκB, a pivotal regulator of FAK expression [22].

In our elucidation of MGB1 function in cancer hallmarks, we also evaluated the role of MGB1 in cell death resistance [53]. Surprisingly, we observed that breast cancer cells with suppressed MGB1 levels are not only more resistant to apoptotic events; but also, confer chemoresistance to paclitaxel, docetaxel and doxorubicin cytotoxic effects. These observations may be a direct result following the induction of MRP1, a prominent drug resistant gene in breast cancers [54,55]. If indeed elevated MGB1 levels entail chemosensitivity, our findings would have enormous implications in a clinical therapeutic setting given that multi drug resistance is still the main reason for chemotherapy resistance in breast cancers [56]. In addition to being a specific tumor biomarker, MGB1 could also predict prognostic value when considering chemo-based cancer treatment regimes. In support of our observations, a clinical study has recently shown that peripheral blood samples from breast cancer patients were initially positive for MGB1 mRNA expression [57]. However, following systemic chemotherapy, 90% of patient blood samples had no detectable MGB1 expression. Another report has shown that relapse time following surgery of patients with primary breast tumors characterized with elevated MGB1 levels is longer than that of patients with tumors bearing low MGB1 expression levels when patients are receiving adjuvant treatment [13]. These findings strongly suggest that tumor cells with elevated MGB1 expression are more sensitive to the cytotoxic effects of anticancer drugs and could therefore more likely be eradicated following chemo-based treatment regimes.

Still conflicted, numerous studies have attempted to correlate MGB1 expression levels to disease outcome. As a result, it was uncertain whether aberrant MGB1 is a cause or effect of breast cancer disease progression. This ambiguity is mostly due to a paucity of studies examining the molecular and cellular roles of MGB1 in breast cancer processes. Given our current findings, we strongly believe that the disparate clinical observations are also due to a lack of consideration to phenotypic transitioning programs (EMT–MET) supporting successful metastasis. For example, elevated levels of MGB1 in primary tumors samples have been correlated to good prognosis while studies examining MGB1 expression in secondary tumors or peripheral blood associate MGB1 to poor disease outcome [10–13,15,16]. The spatial and temporal expression of MGB1 would thus be important to scrutinize within the evolution of disease progression. Given the potential of MGB1 to regulate pivotal signaling of malignant breast cancer processes, in vivo experimentation are also warranted and would aid to decipher the roles of MGB1 in breast cancer metastasis and disease progression.

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