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Gremlin-2 is a novel tumor suppressor that negatively regulates ID1 in breast cancer

Jiwoo Jung^{1†}, Na Hui Kim^{2†}, Jayeon Park¹, Dayeon Lim¹, Minji Kwon¹, World Gil³, Suyeon Jung³, Minjeong Go³, Chaeon Kim³, Ye Hwang Cheong⁴, Mee-Hyun Lee⁵, Hee Sun Park⁶, Yong-Bin Eom^{1,3} and Sin-Aye Park^{1,3*}

Abstract

Background Breast cancer is one of the most common cancers in women and is closely associated with obesity. Gremlin-2 (GREM2), an antagonist for bone morphogenetic proteins (BMPs), has been considered an inhibitor of adipogenic differentiation in adipose-derived stromal/stem cells. However, the role of GREM2 in breast cancer cells remains largely unknown, and its signaling mechanism has yet to be clarified.

Methods Bioinformatics analysis was conducted using public databases. Breast cancer cells overexpressing mock or GREM2 were used for in vitro and in vivo studies. Cell viability, colony formation, migration, and animal studies were performed to investigate the role of GREM2 in breast cancer cells. Screening of target genes affected by GREM2 overexpression in breast cancer cells was performed through RNA sequencing (RNA-seq) analysis.

Results The expression level of GREM2 mRNA was significantly reduced in both breast cancer tissues and cell lines. Kaplan-Meier analysis showed that low expression of GREM2 and high methylation of the GREM2 promoter were each associated with poor patient survival. The low mRNA expression of GREM2 in breast cancer cells was increased by the demethylating agent decitabine. Breast cancer cells overexpressing GREM2 decreased cell proliferation when compared to control cells, both in vitro and in vivo. Through comparison of RNA-seq analysis between cell lines and tissue samples, gene ontologies that were consistently upregulated or downregulated by GREM2 in breast cancer were identified. In particular, the expression of inhibitor of DNA-binding-1 (ID1) was repressed by GREM2. BMP2 is one of the upstream regulators that increases the expression of ID1, and the expression of ID1 reduced by GREM2 was restored by overexpression of BMP2. Also, the migration ability of breast cancer cells, which had been suppressed by GREM2, was restored by BMP2 or ID1.

Conclusions Low expression of GREM2 in breast cancer cells is associated with hypermethylation of the GREM2 promoter, which may ultimately contribute to poor patient survival. GREM2 participates in regulating the expression of various genes, including ID1, and is involved in suppressing the proliferation of breast cancer cells. This suggests that GREM2 has the potential to act as a novel tumor suppressor in breast cancer.

Keywords Gremlin-2, Breast cancer, Hypermethylation, RNA sequencing, Inhibitor of DNA-binding-1, Tumor suppressor

[†]Jiwoo Jung and Na Hui Kim contributed equally to this work.

*Correspondence:
Sin-Aye Park
sappark@sch.ac.kr

Full list of author information is available at the end of the article



Background

Breast cancer is the most common malignancy in women worldwide and occurs across a variety of age groups, including women with early onset [1]. Typically, breast cancer is divided into subtypes based on the presence or absence of three receptors: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [2]. Although several studies have endeavored to discover novel molecular targets for each subtype of breast cancer, the range of treatments accessible to breast cancer patients remains primarily limited to those that target conventional receptors. Therefore, to propose promising treatments applicable to breast cancer, it is essential to thoroughly investigate new molecular targets for breast cancer and closely analyze their exact mechanisms.

Gremlin-2 (GREM2) is a well-known paralog of gremlin-1 (GREM1). It has a C-terminal cystine knot structure and acts as an antagonist to bone morphogenetic proteins (BMPs). Both gremlins play important roles in embryonic development, organ development, and tissue differentiation through the regulation of BMPs [3, 4]. However, studies have shown that GREM1 and GREM2 have different functions. While GREM1 has been widely reported to promote diseases like organ fibrosis [5, 6] and cancer [7–10], GREM2 has been primarily found to inhibit the differentiation of adipose [11], skin [12], or bone marrow [13]-derived stem/progenitor cells. GREM2 reduces the negative effects of inflammation after a myocardial infarction by inhibiting BMP signaling [14]. The direct role of GREM2 in cancer biology is not yet well known, but some studies suggest a tumor suppressor function of GREM2. Treatment with GREM2 protein inhibits the growth of endometrial cancer cell lines [15]. Interestingly, miR-423-5p promotes chemotherapy resistance in prostate cancer by suppressing GREM2 expression [16]. MiR-103a-3p suppresses GREM2 expression and contributes to the proliferation, migration, and invasion of colon [17] or liver [18] cancer cells. In our previous study, we demonstrated that GREM2 inhibits the proliferation and metastasis of breast cancer cells by preventing the differentiation of adipocytes. Overexpression of GREM2 in adipocytes not only inhibits adipogenic differentiation but also inhibits the expression and secretion of several adipokines, including IL-6, ultimately participating in suppressing breast cancer progression [19]. More interestingly, these functions of GREM2 are associated with the inhibition of BMP signaling [14] or the augmentation of Wnt/ β -catenin [11, 19, 20] signaling.

Inhibitor of DNA-binding-1 (ID1) is a member of the helix-loop-helix (HLH) transcription factors. ID1, which lacks a DNA binding domain, forms heterodimers with other HLH transcription factors and inhibits their transcriptional activity [21]. ID1 is one of the

key factors regulating cellular differentiation, lineage commitment, and development [22]. ID1 is also overexpressed in various cancers and has been found to be associated with several characteristics of cancer, such as cancer cell growth, migration, and invasion [23, 24]. In particular, ID1 is involved in cancer cell invasion [25], metastasis [25, 26], and stemness [27, 28] of cancer stem cells in breast cancer. Also, ID1 is associated with worse prognosis in breast cancer patients [29]. ID1 has been found to be significantly correlated with various signaling pathways such as BMP, K-Ras, MYC, etc [23]. Human ID1 gene contains a BMP-2-responsive element and the expression of ID1 is known to be mediated by BMP-2 and Smad1/4 [30]. ID1 cooperates with Ras to subvert the cellular senescence response and drive metastatic breast carcinoma [26]. Additionally, the MYC-ZNF148-ID1/3 axis has been reported to induce cancer stem cell characteristics in aggressive breast cancer [28].

This study demonstrates that GREM2 expression is reduced in breast cancer tissues and cells. Moreover, low levels of GREM2 mRNA expression in breast cancer cells are associated with its promoter hypermethylation and poor patient survival. Our *in vitro* and *in vivo* experiments reveal that GREM2 can inhibit the proliferation of breast cancer cells. Through RNA sequencing (RNA-seq) analysis, it was confirmed that GREM2 affects the expression of specific genes, including ID1. These results suggest that GREM2 is a novel tumor suppressor and may be a new target for breast cancer diagnosis or treatment.

Methods

Cell culture and reagents

Human breast cancer cell lines MDA-MB-453, SKBR3, and mouse breast cancer cell line MTV/TM-011 cells were originally obtained from Korean Cell Line Bank (Republic of Korea). The cells were incubated with RPMI (Corning Inc., USA) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) and 1% penicillin/streptomycin (Corning Inc.). Cells were maintained at 37 °C in air humidified with 5% CO₂/95% air. Rabbit polyclonal GREM2 antibody was purchased from Abcam (UK). Anti-cyclin A2, anti-cyclin B1, anti-cyclin D1, anti-cyclin E2, anti-CDK2, anti-CDK4, anti-CDK6, and anti- β -actin were obtained from Cell Signaling Technology (USA).

Establishment of stable cell line

MDA-MB-453, SKBR3, and MTV-TM-011 cells were cultured in RPMI containing 10% FBS to reach 50% confluence. The next day, MDA-MB-453 and SKBR3 cells were transfected with mock or GREM2 ORF plasmid (Sino Biological, Cat#. HG10283-CY, China) using lipofectamine (Thermo Fisher Scientific) and incubated for 72 h. Stable cell lines were selected by 300 μ g/ml

hygromycin (InvivoGen, USA). MTV/TM-011 cell lines stably expressing either mock or Grem2 were established using a lentiviral transduction system. Briefly, mouse Grem2 lentiviral vector (EX-Mm06243-Lv122) was obtained from GeneCopoeia (USA). The lentiviruses were packaged in 293T cells using Lenti-Pac™ HIV expression packaging kit (GeneCopoeia). After 72 h transfection, the viral supernatant was collected, filtered and used for the transduction of MTV/TM-011 cells in the presence of 8 µg/ml polybrene (Merck Millipore, USA). Stable cell lines were selected by 1 µg/ml puromycin (InvivoGen).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells using TRIzol® (Thermo Fisher Scientific). Reverse transcription of total RNA was performed using M-MLV reverse transcriptase (Enzymomics, Republic of Korea) according to the manufacturer's protocol. Quantitative PCR (qPCR) was performed using TOP real™ qPCR 2X Pre-MIX (Enzymomics) and StepOnePlus Real-Time PCR (Thermo Fisher Scientific). ACTB or GAPDH was used as an internal reference. Primer sequences are listed in the Supplementary Table 1.

Western blot analysis

Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were used to analyze the expression of various proteins. Cells were lysed in the lysis buffer (Cell Signaling Technology) containing protease inhibitors and phosphatase inhibitors (Roche, Switzerland). The quantitative protein concentration was determined by BCA Protein Assay Kit (Thermo Fisher Scientific) and equal amounts of protein were loaded on 8–12% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Merck Millipore) and subjected to immunoblotting using various antibodies overnight at 4°C, followed by further incubation with the secondary antibody (AbFrontier, Republic of Korea) at room temperature for 1 h. Visualization of protein bands was detected with Westsave Gold detection reagents (AbFrontier).

Wound healing assay

Cells were seeded in 12-well culture dishes and wounded by manually scraping the surface with a 1 ml pipette tip. The scratched surface was washed with PBS to remove cell debris. These cells were treated with medium containing 10% FBS and then cultured for 18 to 72 h. Using an inverted microscope with a 4x magnification, phase contrast images of the wound area were taken at 0 h (control) and 18–72 h after culture. Image J software was used to quantify the wound area.

Colony formation

Cells were seeded in 6-well plates at densities of 2.0×10^4 cells/well, respectively. The existing medium was replaced with medium containing 10% FBS every two days for 7 to 10 days. Cells were then fixed with methyl alcohol, stained with 0.5% crystal violet solution, and rinsed with PBS. After washing with PBS, the cells were examined under a microscope and photographs were taken in several fields. Cells stained with crystal violet were dissolved in dimethyl sulfoxide, and the absorbance was measured and quantified.

In vivo mouse model

Female BALB/c mice, 5 weeks of age (weight 18–20±1–2 g) were purchased from Orient Bio Inc. (Republic of Korea). Mice were controlled in specific pathogen free conditions: 20–24 °C, 12/12 h of dark/light cycle, 60±5% humidity, and plastic cage (4 mice/cage). For the syngeneic breast cancer mouse model, MTV/TM-011-mock or MTV/TM-011-Grem2 cells were inoculated into the flanks of anesthetized mice by isoflurane inhalation. For the orthotopic breast cancer mouse model, MTV/TM-011-mock or MTV/TM-011-Grem2 cells were inoculated into the fourth mammary fat pad of anesthetized mice by isoflurane inhalation. Both the volume of the tumors and the body weight of mice were measured twice a week. At the end of the experiment, mice were euthanized by CO₂ inhalation and each tumor was removed.

Immunofluorescence staining

Before staining fixed paraffin-embedded tissues, we followed the standard protocol including the steps of deparaffinization, antigen retrieval, and permeabilization. For immunofluorescence, detection of primary antibodies was done using fluorescent conjugates of Alexa Fluor® 488 antibody (Thermo Fisher Scientific) along with Pro-Long® Gold Antifade Reagent with DAPI (Thermo Fisher Scientific).

RNA sequencing (RNA-seq) and data analysis

Sequencing libraries were generated and sequenced by Macrogen (Republic of Korea). Total RNA was purified using RNeasy mini Kit (Qiagen, Germany). Purified RNA was randomly fragmented and synthesized into cDNA. After PCR amplification, an insert size of 200–400 bp was secured through a size selection process. The analysis results were mapped to the reference genome using the HISAT2 program, and the expression level obtained through transcript quantification of the sample was extracted as FPKM (Fragments Per Kilobase of transcript per Million mapped reads)/RPKM (Reads Per Kilobase of transcript per Million mapped reads) and TPM (Transcripts Per Kilobase Million) values. Genes or transcripts

with statistically differentially expressed values of two or more groups under different conditions were selected. Additionally, functional annotation analysis was performed for these genes based on gene ontology (GO).

Statistical analysis

Data was expressed as the mean \pm SD of results obtained from at least three independent experiments. Significant differences were determined by a Student's *t*-test or one/two-way ANOVA. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

The mRNA level of GREM2 is suppressed in breast cancer tissues/cells and is associated with poor survival of breast cancer patients

By analyzing public databases such as GEPIA and TNMplot, we discovered that the mRNA level of GREM2 was significantly suppressed in breast cancer

tissues (Fig. 1A) and further reduced in metastatic tumors (Fig. 1B). The Expression Atlas database analysis revealed a significant decrease in GREM2 expression compared to MKI67, a cancer marker, in all 20 representative breast cancer cell lines (Fig. 1C). Subsequently, we investigated the correlation between GREM2 expression and the overall survival (OS) of breast cancer patients. Notably, low expression of GREM2 was associated with reduced OS in all four categories of breast cancer, although the *p*-value for the three positive (ER+, PR+, and HER2+, *p*-value=0.15) group was not significant (Fig. 1D). The low expression of GREM2 was strongly associated with worse OS, particularly in breast cancer patients who were double negative (ER-, PR-, and HER2+) (GREM2 low vs. high expression patients: hazard ratio (HR) of survival=0.09, 95% confidence interval (CI): 0.01–0.82, *P*=0.0068) and triple negative (ER-, PR-, and HER2-) (GREM2 low vs. high expression patients: HR of survival=0.32, 95% CI: 0.11–0.93, *P*=0.028) (Fig. 1D).

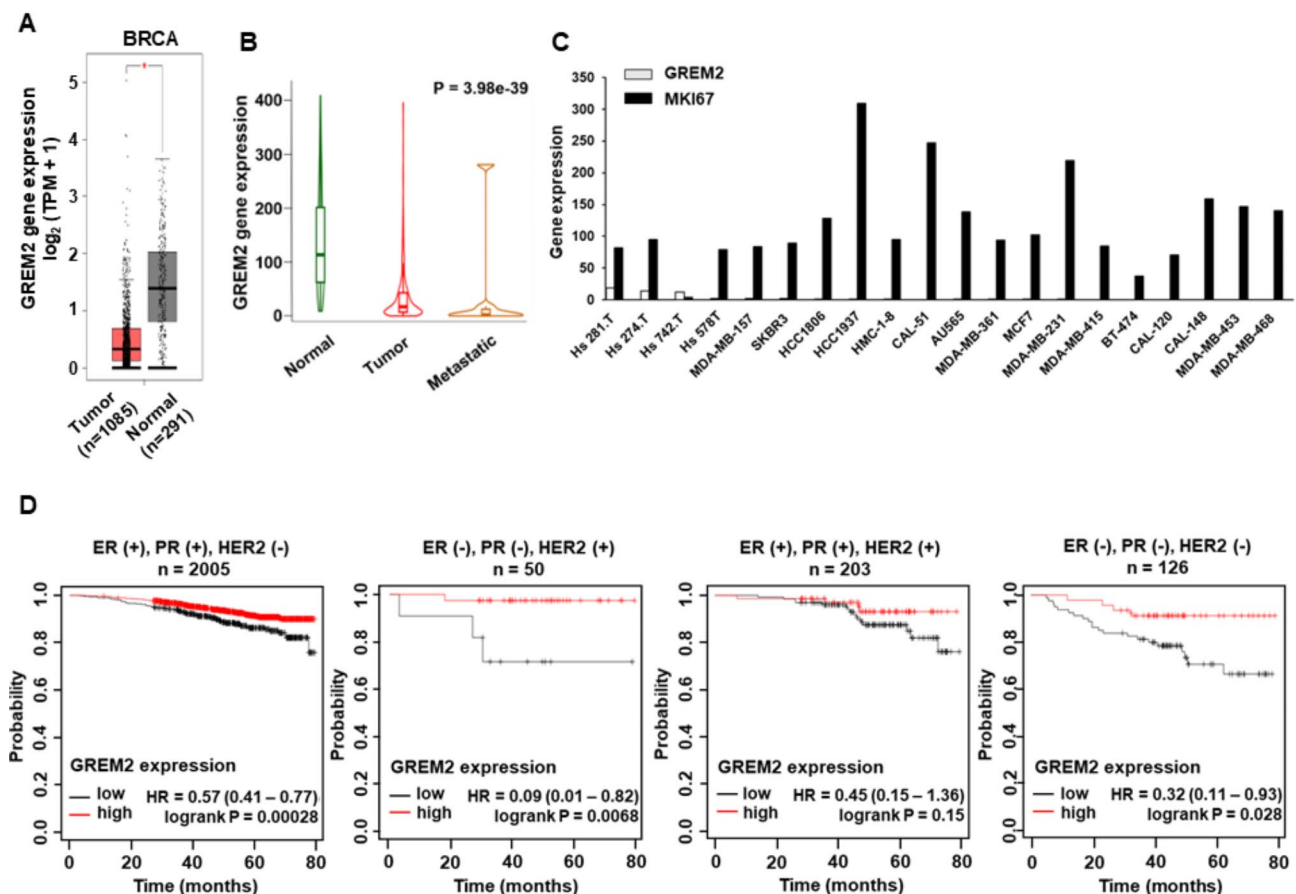


Fig. 1 Low expression of GREM2 in breast cancer tissues/cells is associated with poor patient survival. **A** GREM2 expression was analyzed between TCGA breast invasive carcinoma tissues ($n = 1085$) and TCGA normal + GTEx normal ($n = 291$) tissues using GEPIA. **B** Expression of GREM2 in tumor ($n = 1097$) and metastatic tissues ($n = 7$) of breast cancer patients was compared with normal tissues ($n = 113$) using RNA-Seq TNMplot database. **C** The Expression Atlas database was used to analyze the mRNA levels of GREM2 and MKI67 in breast cancer cell lines. **D** Kaplan–Meier analysis (Kaplan–Meier Plotter) of overall survival by low or high GREM2 mRNA expression was performed in each indicated category of breast cancer patients. HR, hazard ratio

These findings contradict our previous results [8], which showed no significant correlation between GREM2 expression and breast cancer patient survival. In the previous study, only patient groups based on the presence or absence of ER expression were analyzed in the gene chip-based database provided by Kaplan-Meier plotter. However, the current results were based on a recently updated RNA-seq database provided by Kaplan-Meier plotter and analyzed considering the presence or absence of ER, PR, and HER2. Based on these findings, we suggest that GREM2 is significantly downregulated in breast cancer cells and that a low level of GREM2 predicts poor prognosis in breast cancer patients.

Hypermethylated GREM2 promoter is associated with poor survival of breast cancer patients

We utilized the GSCA database to investigate the potential correlation between low-level GREM2 expression in breast cancer cells and hypermethylation of the

GREM2 promoter. As shown in Fig. 2A, there was a significant increase in methylation of the GREM2 promoter (cg02577267_GREM2) in breast cancer tumors compared to normal tissues. MethSurv survival analysis demonstrated a noteworthy association between higher methylation of the GREM2 promoter and poor OS in breast cancer. The Cancer Genome Atlas (TCGA) breast cancer data showed that 8 out of 23 CpGs in the GREM2 promoter showed a significant correlation between methylation levels and patient survival (Supplementary Table 2). Additionally, higher methylation of each of the eight GREM2 promoter CpGs was associated with lower patient survival (Fig. 2B, Supplementary Fig. 1). To test whether high methylation mediates low levels of GREM2 expression, three breast cancer cell lines (MDA-MB-453, SKBR3, and MTV/TM-011) were treated with demethylating agent decitabine. Decitabine significantly increased the mRNA expression of GREM2 in these cells (Fig. 2C), indicating that hypermethylation of the GREM2

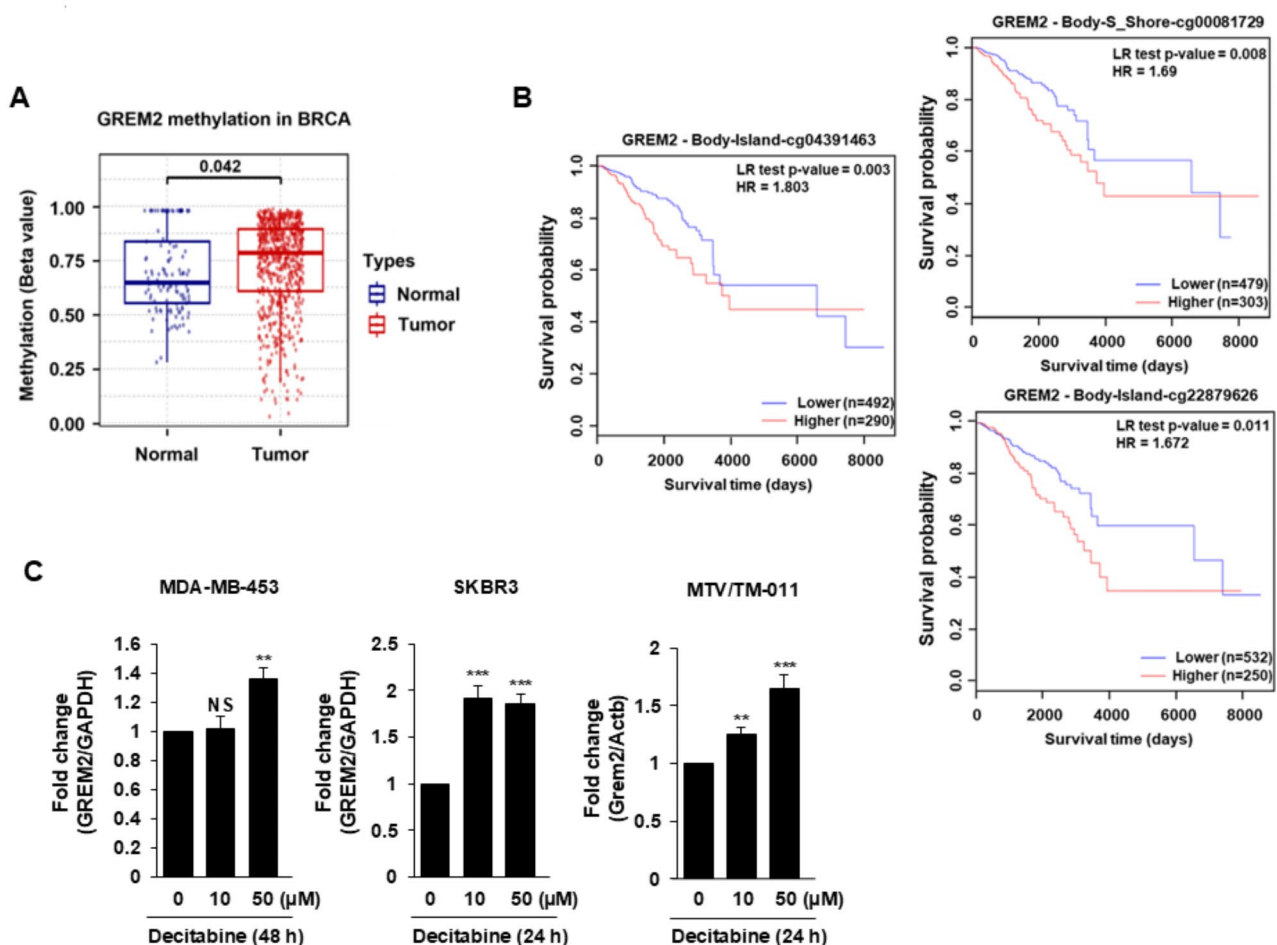


Fig. 2 Hypermethylation of the GREM2 promoter is associated with poor survival in breast cancer patients. **A** Methylation levels of the GREM2 promoter were compared between TCGA breast invasive carcinoma patients and normal tissues in the GSCA database. **B** The survival rate of breast cancer patients according to the methylation level of each CpG site of GREM2 was analyzed using MethSurv. **C** Three breast cancer cell lines were treated with decitabine (10 or 50 μM) for 24–48 h and the mRNA level of GREM2 was quantified by qPCR analysis. Two-sided *t*-test. **, $p < 0.01$; and ***, $p < 0.001$. NS: not significant

promoter may be associated with transcriptional repression of GREM2 in breast cancer cells.

GREM2 inhibits breast cancer cell proliferation in vitro and in vivo

To investigate the effect of GREM2 on breast cancer cell growth, we introduced GREM2 overexpression plasmids into three different breast cancer cell lines and established stable cell lines for each. Significant increases in the mRNA (Fig. 3A) and protein (Fig. 3C) levels of GREM2 were observed in all three cell lines. The overexpression of GREM2 effectively suppressed the proliferation of breast cancer cells (Fig. 3B), as well as the expression levels of various cell cycle-related proteins when compared to the control group (Fig. 3C). Additionally, GREM2 overexpression inhibited the formation of cell colonies (Fig. 3D and E) and the migration of breast cancer cells (Fig. 3F and G). Although the ER status of MTV/TM-011 cell lines is unknown, MDA-MB-453 and SKBR3 are representative ER-negative cell lines. We further investigated the effect of GREM2 overexpression on cell proliferation in representative ER-positive cell lines, T47D (ER⁺/HER2⁻) and BT474 (ER⁺/HER2⁺). As shown in Supplementary Fig. 2, both cell viability and colony proliferation were inhibited in T47D and BT474 cell lines overexpressing GREM2 compared with the control.

Next, to determine the effect of GREM2 on tumor growth of breast cancer cells, MTV/TM-011-mock or MTV/TM-011-Grem2 cells were injected into the left or right flank of mice, respectively. Our results showed that overexpression of GREM2 in breast cancer cells significantly reduced tumor volume compared to controls (Fig. 4A-C). Furthermore, the expressions of CDK4, cyclin B1, and cyclin D1 were reduced in Grem2-overexpressing tumors compared with control tumors (Supplementary Fig. 3A). As shown in Fig. 4D and E, mice injected with MTV/TM-011-Grem2 cells into the mammary fat pads developed smaller primary tumors than mice injected with MTV/TM-011-mock cells, resulting in a dramatic reduction in tumor volume [mean ± SD (mm³): 1116.11 ± 267.72 (MTV/TM-011-mock) vs. 443.73 ± 87.84 (MTV/TM-011-Grem2), four mice/each group]. Consistent with the growth outcome of the primary tumor, mice injected with MTV/TM-011-Grem2 had less lung metastases than mice injected with MTV/TM-011-mock (Fig. 4F). Additionally, the mRNA expression levels of Mmp2, Mmp3, Mmp9, Mmp11, and Mmp13 were significantly lower in primary breast cancer tissues of the MTV/TM-011-Grem2 group compared to the control group (Supplementary Fig. 3B). Altogether, these findings suggest that GREM2 plays an important role in suppressing the proliferation and metastasis of breast cancer cells.

Differentially expressed genes (DEGs) are identified between GREM2 overexpression group and control group in breast cancer cells

To investigate the role of GREM2 in breast cancer and its impact on tumor suppression, we identified potential target genes affected by GREM2. Gene expression levels between breast cancer cells overexpressing GREM2 (MDA-MB-453-GREM2, SKBR3-GREM2; *n*=2 for each cell line) and control cells (MDA-MB-453-mock, SKBR3-mock; *n*=2 for each cell line) were compared using RNA-seq analysis. Initially, we examined the differentially expressed genes (DEGs) using a fold change (FC) threshold of ≥ 1.5 and a significance level of *p* < 0.05 in the RNA-seq analysis. Subsequently, genes commonly upregulated or downregulated by GREM2 in both MDA-MB-453 and SKBR3 cell lines were identified. As shown in Fig. 5A and Supplementary Tables 3, 147 genes were upregulated and 105 genes were downregulated in both breast cancer cell lines by GREM2 overexpression.

The identified DEGs were classified based on their biological processes (BP), molecular functions (MF), and cellular components (CC) using the David database. In each category, the top 10 statistically significant GOs were selected and presented in a diagram (Fig. 5B and C, supplementary Fig. 4), ordered by gene count. For the BP category, representative GOs upregulated by GREM2 overexpression included inflammatory response, cell adhesion, and immune response. In the MF category, the representative GOs were receptor binding, iron ion binding, and integrin binding. Finally, for the CC category, the representative GOs were integral components of membrane, plasma membrane, and membrane. On the other hand, representative GOs downregulated by GREM2 overexpression included translation, cytoplasmic translation, and rRNA processing in the BP category; protein binding, RNA binding, and structural constituent of ribosome in the MF category; and cytosol, cytoplasm, and nucleus in the CC category.

Based on the RNA-seq results, qPCR analysis was further performed to verify the upregulation or downregulation of some target genes by GREM2. In GREM2-overexpressing breast cancer cells, the mRNA expression levels of the following genes were significantly increased: TP53AIP1 (p53-regulated apoptosis-inducing protein 1), TPST1 (tyrosylprotein sulfotransferase 1), MSMO1 (methylsterol monooxygenase 1), and SCARA3 (scavenger receptor class A member 3) (Fig. 5D). Conversely, the mRNA expression levels of PRKAR2B (protein kinase CAMP-dependent type II regulatory subunit beta), EIF4EBP1 (eukaryotic translation initiation factor 4E binding protein 1), ABLIM3 (actin binding LIM protein family member 3), and PDE8B (phosphodiesterase 8B) genes were significantly reduced in GREM2-overexpressing breast cancer cells compared to control

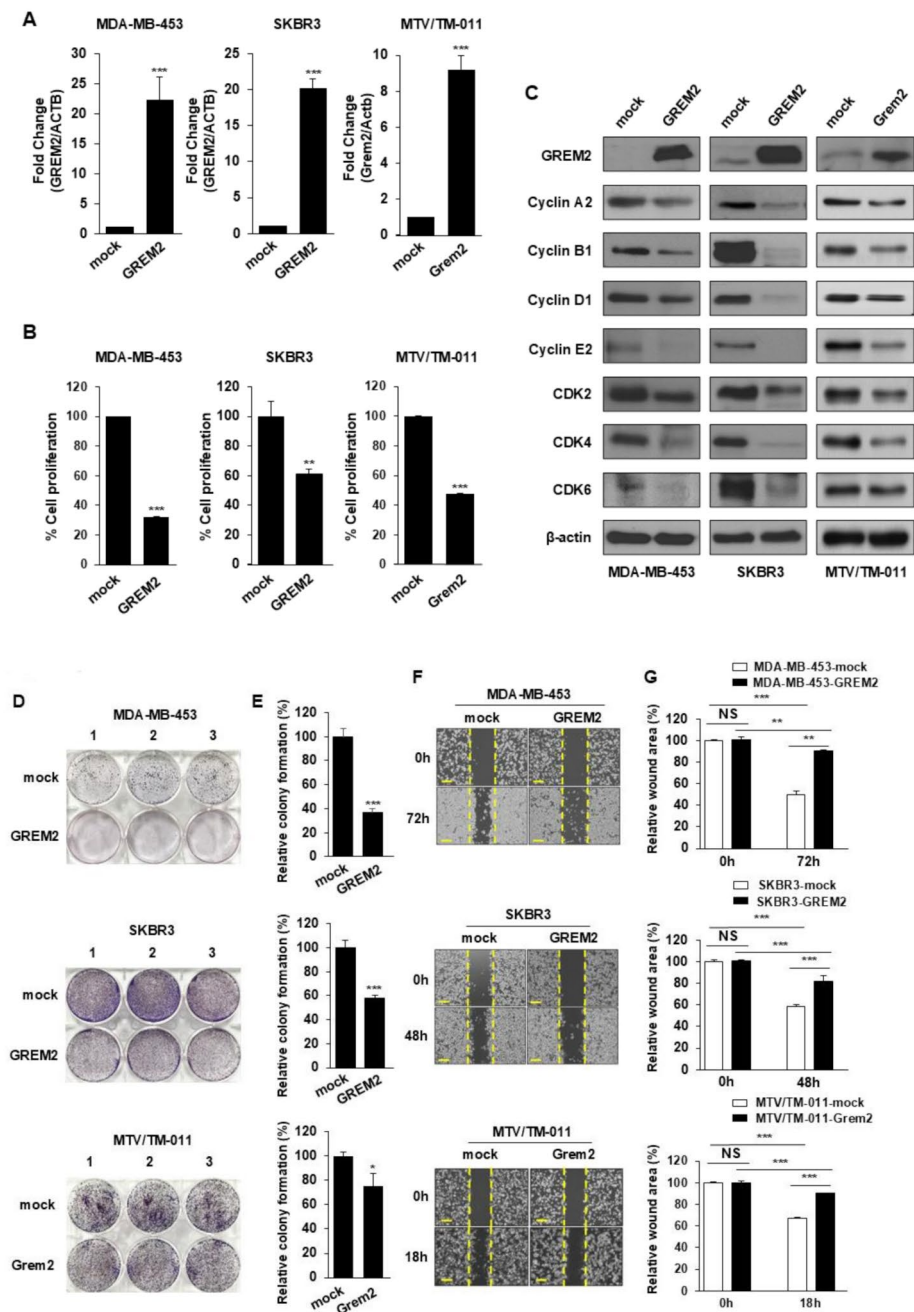


Fig. 3 Overexpression of GREM2 inhibits proliferation of breast cancer cells. **A** Each breast cancer cell line was established using a mock or GREM2 ORF plasmid. Expression level of GREM2 was confirmed by qPCR analysis. **B** Cells were seeded 96-well plates and incubated for 48 h, followed by the cell proliferation assay. **C** Proteins were isolated from each stable cell line, and lysates were immunoblotted with the indicated antibodies. **D, E** Breast cancer cells were seeded in 6-well plates and cultured for 7 to 10 days. Colony formation was analyzed and quantified using crystal violet staining. **F, G** Cells were seeded in 12-well plates and wounded to monitor the extent of wound healing at specified time points. Scale bar = 200 μ m. **A, B, E** Two-sided *t*-test. **G** Two-way ANOVA. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. NS: not significant

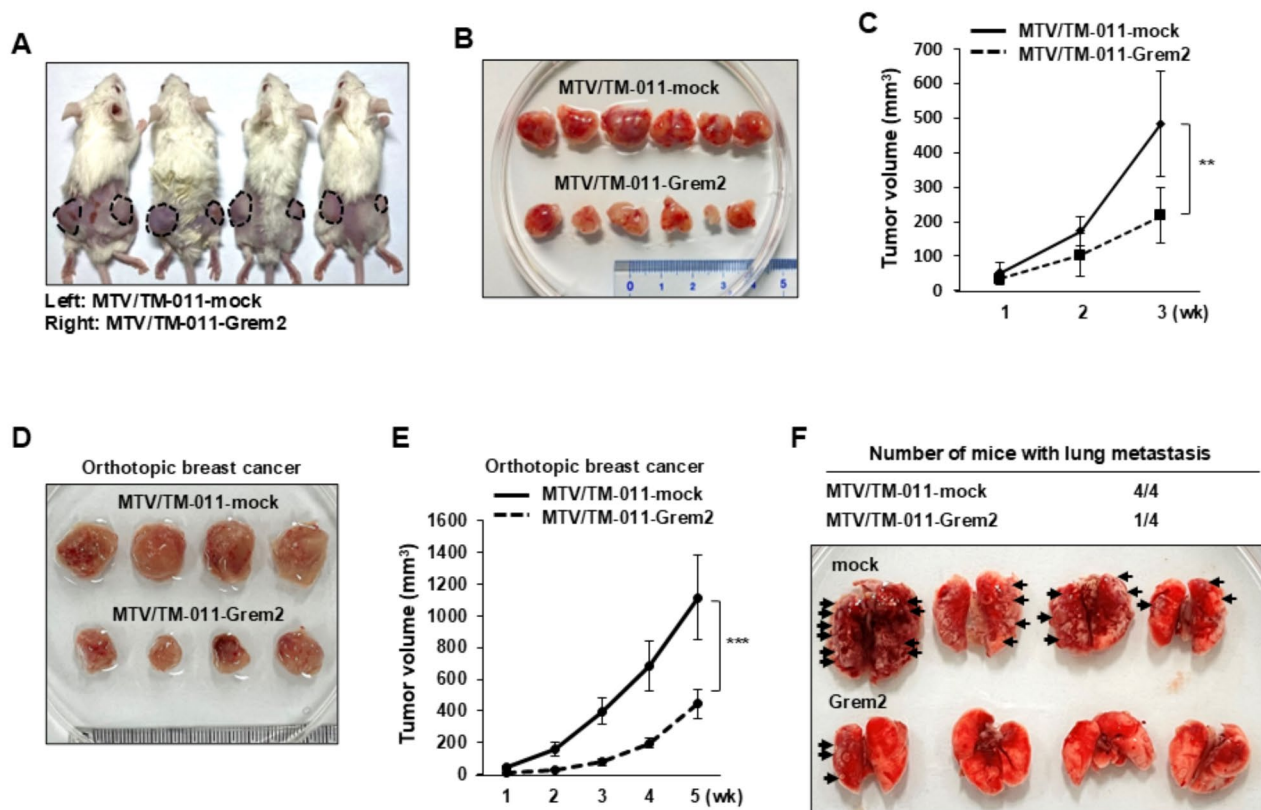


Fig. 4 GREM2 suppresses tumor growth of breast cancer cells in vivo. **A-C** MTV/TM-011-mock or MTV/TM-011-Grem2 cells were inoculated subcutaneously into the flanks of female nude mice (left: mock, right: Grem2, $n=6$). Representative images of mice (**A**), dissected tumors (**B**), and tumor volume (**C**). **D-F** MTV/TM-011-mock or MTV/TM-011-Grem2 cells were injected into mammary fat pads of mice. Representative images of primary tumors (**D**), tumor volume (**E**), and lung metastatic foci (**F**). Black arrowheads indicate prominent lung metastatic foci. Two-sided *t*-test. **, $p < 0.01$ and ***, $p < 0.001$

cells (Fig. 5E). Additionally, the association between these genes and GREM2 was identified in the human breast tissue database. Analysis of the GTEx breast tissue database revealed that each of these genes exhibited a significant positive correlation (supplementary Fig. 5A) or negative correlation (supplementary Fig. 5B) with GREM2 expression.

ID1 is one of the potent target genes of GREM2 in breast cancer

To further understand the downstream signaling pathways regulated by GREM2 in breast cancer, we conducted a comparison between the results of cell-based RNA-seq analysis (Fig. 5) and human breast tissue-based RNA-seq analysis. Using RNA-seq data from breast invasive carcinoma patients provided by TNMplot, we identified 3357 genes that showed a positive correlation with GREM2 ($0.2 < r < 1.0$) and 657 genes that showed a negative correlation with GREM2 ($-1.0 < r < -0.2$). We then performed DAVID analysis on these genes (Fig. 6A). For genes positively or negatively correlated with GREM2 in breast cancer tissues, the top 10 GO categories were identified in BP, MF, and CC (Fig. 6B and C, and supplementary

Fig. 6). In each category of BP, MF, and CC, the top 10 GOs (by number of genes) from RNA-seq for GREM2 overexpressing cell lines were compared with the top 50 GOs (by number of genes) from RNA-seq for human breast cancer tissues. Interestingly, many GO pathways that showed positive or negative correlation with GREM2 overlapped between the two analyses. While a wide variety of GOs showed positive correlations with GREM2, GOs showing negative correlations with GREM2 were mainly concentrated in limited pathways associated with translation, ribosomes, and protein binding (Fig. 6D).

In the MF category, the GO term “protein binding” (GO:0005515) was most consistently negatively correlated with GREM2 in breast cancer cell lines and human breast cancer tissues (Fig. 6D). ID1 was one of the genes involved in “protein binding” that was significantly reduced in breast cancer cell lines overexpressing GREM2 (Supplementary Table 4). The mRNA level of ID1 was significantly decreased in two breast cancer cell lines that overexpressed GREM2 (Fig. 7A). A significant negative correlation was also identified between the mRNA expression of GREM2 and ID1 in human breast tissues (Spearman $r = -0.55$, $p = 1.9 \times 10^{-15}$) (Fig. 7B). Next, it

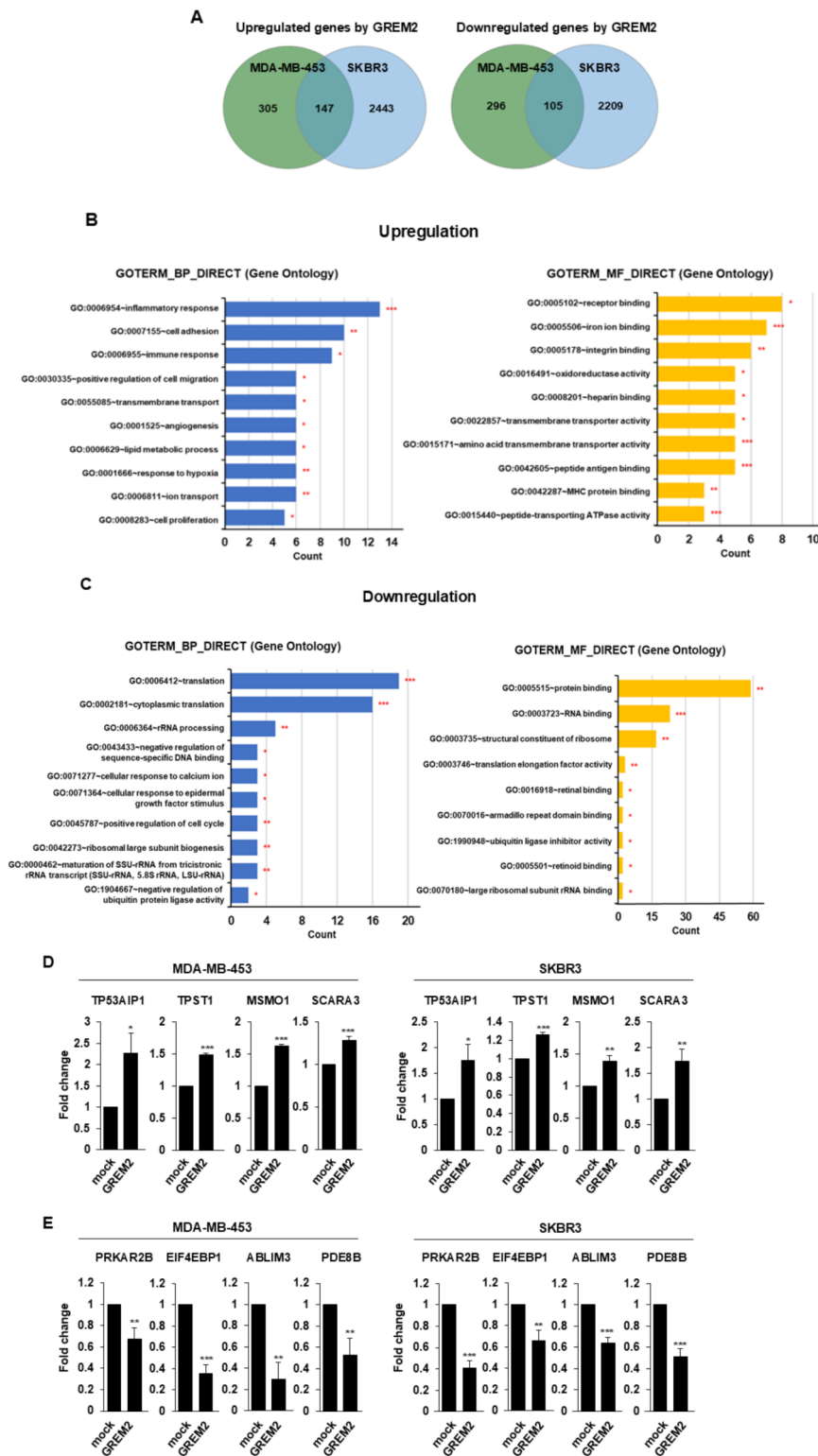


Fig. 5 GREM2 is involved in regulating the expression of various genes in breast cancer cells. **A** Venn diagram showing genes differentially expressed in GREM2-overexpressing breast cancer cells (MDA-MB-453-GREM2 and SKBR3-GREM2) compared to control cells (MDA-MB-453-mock and SKBR3-mock). Genes commonly increased (left) or decreased (right) by GREM2 in two breast cancer cell lines were analyzed. **B, C** GOs for genes up- (**B**) or downregulated (**C**) by GREM2 in breast cancer cells were analyzed using DAVID. BP, biological processes; MF, Molecular Function. **D, E** Some of the genes up- (**D**) or downregulated (**E**) by GREM2 were verified through qPCR analysis. Two-sided *t*-test. *, *p* < 0.05; **, *p* < 0.01; and ***, *p* < 0.001

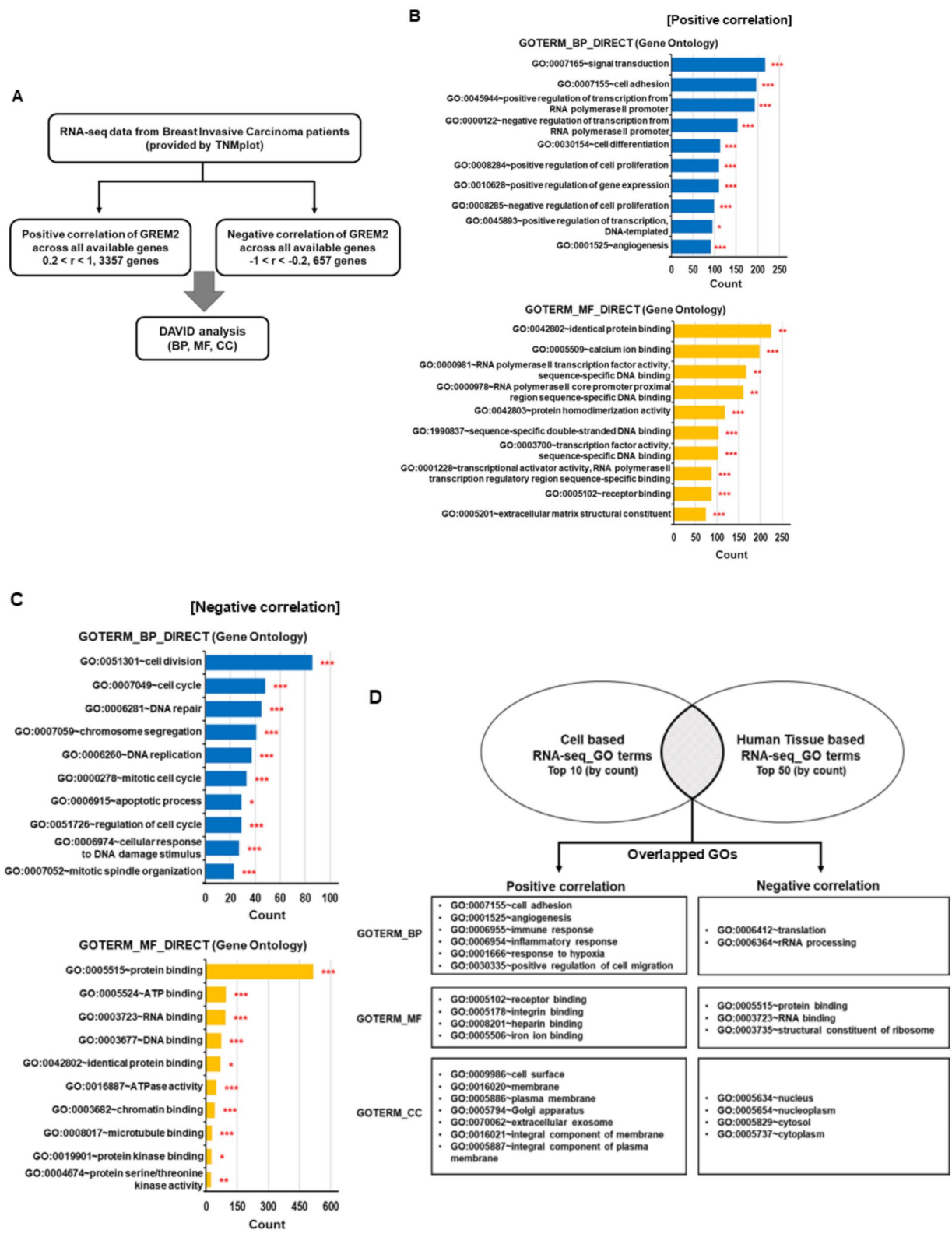


Fig. 6 Specific GO pathways showing positive or negative correlation with GREM2 in breast cancer patient tissues overlap with cell line-derived RNA-seq results. **A** Schematic of the filtering pipeline for genes showing positive or negative correlation with GREM2 in invasive breast carcinoma patients. **B, C** GOs for genes showing positive- (**B**) or negative correlation (**C**) with GREM2 in breast cancer patients were analyzed using DAVID. **D** GO pathways that were commonly positively or negatively correlated with GREM2 in both breast cancer cell lines and invasive breast cancer patient tissues were analyzed

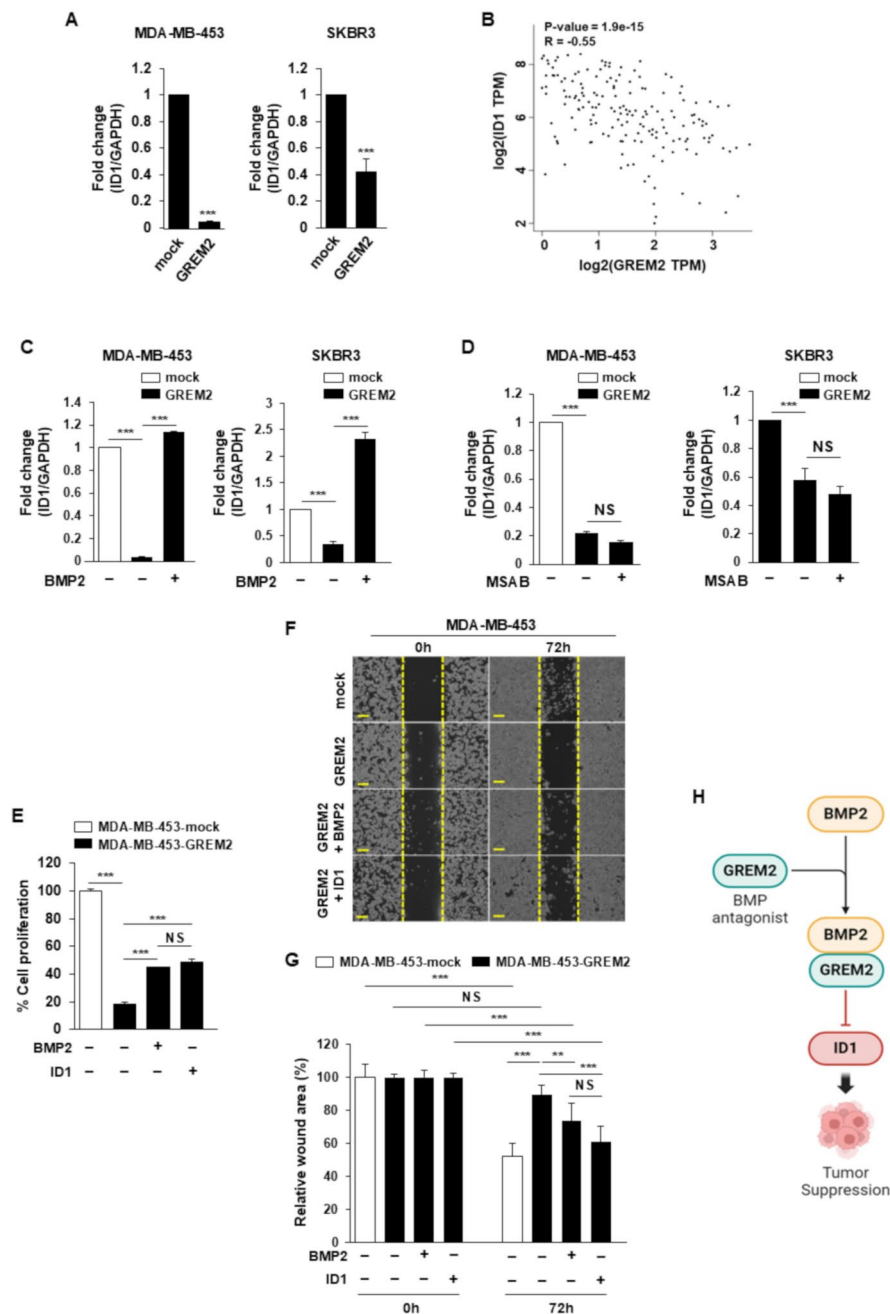


Fig. 7 GREM2 inhibits proliferation of breast cancer cells through negative regulation of BMP2-ID1. **A** The mRNA expression level of ID1 was analyzed through qPCR in each cell line. **B** The correlation between GREM2 and ID1 in GTEx human breast tissue database was investigated by using GEPIA. Correlation analysis was conducted using Spearman rank test. **C** Breast cancer cells overexpressing GREM2 were transfected with mock or BMP2 plasmid, and the mRNA expression level of ID1 was analyzed through qPCR. **D** Breast cancer cells overexpressing GREM2 were treated with or without MSAB (10 μM) for 24 h. RNA was isolated from each cell line and the mRNA expression level of ID1 was analyzed through qPCR. **E-G** Breast cancer cells overexpressing GREM2 were transfected with mock, BMP2, or ID1 plasmids, and then proliferation (**E**) or migration (**F, G**) analysis was performed for each cell line after 72 h. Scale bar = 200 μm. **H** Schematic diagram showing the proposed molecular mechanism of GREM2 in breast cancer. **A** Two-sided *t*-test. **C, D, E, G** Two-way ANOVA. **, *p* < 0.01; and ***, *p* < 0.001. NS: not significant

was confirmed whether the decrease in ID1 expression caused by GREM2 was affected by the antagonism of GREM2 against BMP2, one of the upstream regulators of ID1. As shown in Fig. 7C, the reduced mRNA expression

of ID1 in GREM2-overexpressing breast cancer cells was significantly restored by further increasing the expression of BMP2. In addition, it was confirmed whether the increase in Wnt/β-catenin signaling by GREM2 is

involved in the decrease in ID1 expression. However, the expression of ID1, which was repressed by GREM2, was not affected by treatment with MSAB, a Wnt/ β -catenin inhibitor (Fig. 7D). We investigated whether inhibition of ID1 or BMP2 by GREM2 was involved in the inhibition of proliferation of breast cancer cells. The decreased proliferation (Fig. 7E) and migration (Fig. 7F and G) ability of breast cancer cells by GREM2 was partially restored by increasing the expression of BMP2 or ID1. Collectively, these results demonstrate that GREM2 is a novel tumor suppressor in breast cancer that inhibits cell proliferation through negative regulation of BMP2-ID1 signaling (Fig. 7H).

Discussion

Our previous study demonstrated that GREM2, when overexpressed in preadipocytes, not only inhibits adipocyte differentiation but also suppresses the expression of various adipokines, including IL-6. Additionally, adipocytes overexpressing GREM2 play an important role in suppressing the growth, migration, and metastasis of breast cancer cells [19]. However, GREM2, a type of cytokine, has the potential to inhibit breast cancer growth independently of adipogenic differentiation and/or adipokine inhibition. In this study, we observed a significantly lower expression of GREM2 in both breast cancer patients and cells compared to the control group. Overexpression of GREM2 in breast cancer cells led to the inhibition of cell proliferation, migration, tumor growth and lung metastasis. RNA-seq analysis showed that GREM2 may affect the regulation of various gene expressions in breast cancer cells, particularly suppressing the expression of genes involved in translation, ribosomes, and protein binding. These findings suggest that GREM2 has the potential to serve as a novel tumor suppressor in breast cancer.

Tumor suppressor genes play a crucial role in regulating various cellular functions, including cell growth, cell cycle progression, DNA repair mechanisms, and apoptosis [31]. Mutations in key tumor suppressor genes such as TP53, PTEN, and RB lead to a loss of tumor suppressor function, increasing the likelihood of developing cancer [32–34]. Mutations in BRCA1 and BRCA2 genes, which are responsible for the DNA repair system, are well-known to contribute to the development and progression of breast cancer [35, 36]. In cancer cells, the expression of tumor suppressor genes is often suppressed through hypermethylation of gene promoters. PCDH10, which acts as a tumor suppressor in various types of carcinomas, has been reported to have low mRNA expression associated with promoter hypermethylation [37, 38]. Likewise, the mRNA expression of genes such as RAI2 [39, 40], DHRS3 [41], and HOXB9 [42] is also suppressed by promoter hypermethylation in various

carcinomas. Low expression of these genes is associated with increased proliferation of cancer cells and poor patient prognosis. Our results show that treatment with demethylating agent decitabine restored the suppressed expression level of GREM2 in breast cancer cells. This suggests that low levels of GREM2 expression in breast cancer cells are also likely associated with its promoter hypermethylation.

The low expression of GREM2 in breast cancer cells may be related to microRNA (miRNA) in addition to promoter hypermethylation. Recent reports have identified miRNAs that directly target GREM2 and inhibit its expression. For example, exosomal miR-423-5p secreted from cancer-related fibroblasts promotes chemotherapy resistance in prostate cancer by inhibiting GREM2 [16]. MiR-103a-3p is involved in colon cancer progression by regulating GREM2 expression [17]. Furthermore, empagliflozin could improve non-alcoholic fatty liver disease-related fibrosis by downregulating miR-34a-5p, which targets GREM2 [43]. These findings suggest the possibility that specific miRNAs may directly bind to the 3'UTR of GREM2 and inhibit its expression in breast cancer cells as well. However, more detailed studies are needed to fully understand this mechanism.

Our analysis revealed that GREM2 is involved in the upregulation of various molecular signaling pathways while downregulating pathways focused on translation, ribosomes, and especially protein binding. Some genes upregulated in breast cancer cell lines overexpressing GREM2 have been reported to have tumor suppressor functions in certain types of cancer. Among these genes, TP53AIP1 is an important gene for tumor suppressor p53-dependent apoptosis, and its overexpression promotes cell cycle arrest and apoptosis in breast cancer cells [44]. MSMO1 and SCARA3 were also genes upregulated by GREM2 and have been reported to suppress cancer progression in pancreatic cancer [45] and lung cancer [46], respectively. Meanwhile, among the protein binding-related genes significantly reduced by GREM2, PRKAR2B [47, 48], EIF4EBP1 [49–51], and ID1 [24, 52, 53] are known to be involved in the proliferation or progression of cancers. In particular, ID1 promotes proliferation and metastasis of breast cancer cells and is associated with poor prognosis of patients [25, 27, 29]. Our results indicate that reduced expression of ID1 mediated by GREM2 may ultimately contribute to the inhibition of breast cancer cell proliferation.

We propose that the up- and downregulation of various genes by GREM2 may be associated with changes in the activity of BMP or Wnt/ β -catenin signaling pathway. Gremlins (GREM1 and GREM2) are representative antagonists of BMPs and are well known to directly bind to BMPs and inhibit the BMP signaling pathway [54–56]. Interestingly, ID1 is one of the target genes of BMPs, and

its expression is increased by BMP2 [30, 57], BMP4 [58, 59], or BMP7 [58, 60]. In this study, the reduced expression of ID1 in GREM2-overexpressing cells was significantly restored by additional introduction of BMP2. This suggests that the reduction of ID1 expression by GREM2 may be related to the antagonism of GREM2 against BMPs in breast cancer cells. In addition, previous studies have shown that GREM2 inhibits adipogenesis in adipose-derived stromal/stem cells by activating the Wnt/ β -catenin signaling pathway [11, 20]. Activation of Wnt/ β -catenin signaling induced by GREM2 contributes to the reduction of IL-6 expression in adipocytes [19]. To determine whether the Wnt/ β -catenin signaling increased by GREM2 is also involved in the inhibition of ID1 expression by GREM2, GREM2-overexpressing cells were treated with MSAB, a Wnt/ β -catenin inhibitor. However, the expression of ID1 suppressed by GREM2 was not affected by inhibition of Wnt/ β -catenin signaling, suggesting that the Wnt/ β -catenin pathway is not involved in ID1 reduction by GREM2. Meanwhile, several studies have demonstrated that Wnt/ β -catenin negatively regulates the C/EBP family members [61, 62]. This suggests that enhancement of Wnt/ β -catenin signaling by GREM2 may reduce the expression of specific genes affected by the C/EBP pathway. Besides the C/EBP family, it is well-known that the NF- κ B [63, 64] or PI3K/Akt/mTOR [65, 66] signaling pathway interacts with Wnt/ β -catenin. Therefore, the expression levels of genes regulated by GREM2 in breast cancer cells may be affected not only by BMP or Wnt/ β -catenin signaling but also by changes in the activity of additional kinases and/or transcription factors.

This study suggests that GREM2 may be a novel tumor suppressor gene that can suppress breast cancer proliferation, but it is still difficult to conclude whether the function of GREM2 varies depending on the type of breast cancer. Further studies are needed to elucidate the role of GREM2 and its mechanism according to the subtype of breast cancer.

Conclusion

In this study, we found that the expression of GREM2 was significantly lower in cells and tissues of breast cancer patients, and this was associated with hypermethylation of the GREM2 promoter. Additionally, low expression of GREM2 and hypermethylation of its promoter are associated with poor prognosis in breast cancer patients. RNA-seq analysis showed that GREM2 is involved in regulating the expression of specific genes in breast cancer cells and ultimately inhibits cancer cell proliferation. In particular, GREM2, a BMP antagonist, contributed to suppressing the proliferation of breast cancer cells by reducing the expression of ID1, one of the target genes of BMP2. Based on our findings, we propose that GREM2

may function as a novel tumor suppressor gene in breast cancer and potentially serve as a new diagnostic marker and therapeutic target.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13058-024-01935-1>.

Supplementary Material 1

Author contributions

JJ and NHK are involved in study conception, collection and/or assembly of data, data analysis, and interpretation. JP, DL, MK, WG, SJ, MG, and CK are involved in collection and/or assembly of data and data analysis. YHC is involved in statistical processing and analysis. MHL, HSP, and YBE are involved in study conception and manuscript editing. SAP is involved in study conception and design, data interpretation, manuscript writing and editing, and financial support. All authors read and approved the final manuscript.

Funding

This work was supported by the Soonchunhyang University research fund, and the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 2022R1F1A1074226).

Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All animal experiments were performed in accordance with the guidelines for animal treatment of Soonchunhyang University. All experimental protocols in our study were conducted on protocols approved by the Institutional Animal Care and Use Committee of the Soonchunhyang University (SCH21-0014).

Consent for publication

All authors approved submission of the manuscript.

Competing interests

The authors declare no competing interests.

Author details

¹Department of Medical Sciences, Graduate School, Soonchunhyang University, Asan-si 31538, Republic of Korea

²Department of ICT Environmental Health System, Graduate School, Soonchunhyang University, Asan-si 31538, Republic of Korea

³Department of Biomedical Laboratory Science, College of Medical Sciences, Soonchunhyang University, Asan-si 31538, Republic of Korea

⁴Drug Discovery Research Laboratories, Dong-A ST Co., Ltd, Yongin 17073, Republic of Korea

⁵College of Korean Medicine, Dongshin University, Naju 58245, Republic of Korea

⁶Division of Pulmonology, Department of Internal Medicine, College of Medicine, Chungnam National University, Daejeon 35015, Republic of Korea

Received: 7 July 2024 / Accepted: 23 November 2024

Published online: 29 November 2024

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