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Golgi Membrane Protein 1 (GOLM1) Promotes Growth and Metastasis of Breast Cancer Cells via Regulating Matrix Metalloproteinase-13 (MMP13)

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Breast cancer (BC) is the leading cause of death in women worldwide. Golgi membrane protein 1 (GOLM1) has been identified as novel regulator in carcinogenesis, but its function in BC is unclear.

Material/Methods: The expression of GOLM1 in BC tissues and cell lines was detected by using qRT-PCR assay. CCK-8 and colony-formation assays were used to evaluate BC cell growth *in vivo*. Wound-healing and Transwell assays were used to detect cell migration and invasion. To investigate GOLM1 functions *in vivo*, we established a xenograft mice model and a lung metastasis model. The level of epithelial-to-mesenchymal transition (EMT)-related markers was analyzed by immunofluorescent staining.

Result: GOLM1 was overexpressed in BC cell lines and tissues. Overexpression of GOLM1 induced EMT and promoted proliferation, migration, and invasion of BC cells. Furthermore, overexpressing of GOLM1 markedly promoted the tumorigenicity and metastasis of BC cells *in vivo*, whereas knock-down of GOLM1 caused the opposite outcomes. Furthermore, we proved that GOLM1 promoted BC cell aggressiveness by regulating matrix metalloproteinase-13 (MMP13).

Conclusions: Our results prove that GOLM1 facilitates the growth and metastasis of breast cancer cells.

MeSH Keywords: Breast Neoplasms • Matrix Metalloproteinase 13 • Neoplasm Metastasis

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Background

Breast cancer (BC) is a life-threatening malignancy with a high incidence and metastasis rate among woman [1,2]. Many studies have identified various epigenetic signatures that are involved in tumorigenesis and tumor progression [3]. Recently, the survival of patients with BC has improved because of the development of therapeutic strategies. Unfortunately, tumor metastasis is still one of the main causes of death. Metastasis is a complex multistep process that involves cancer cell dissemination, invasion, survival, and growth in distant tissues [4]. Although certain oncogenes and tumor-suppressors have been identified to play vital roles in the metastasis of cancer, the exact molecular mechanisms underlying BC metastasis are unclear.

Golgi membrane protein 1 (GOLM1), which is a resident cis-Golgi membrane protein, consists of a single N-terminal transmembrane domain, an extensive C-terminal, and coiled-coil domain [5,6]. Previous investigations indicate that GOLM1 is associated with distant metastasis and poor prognosis of patients with HCC through interacting with epidermal growth factor receptor (EGFR) [7,8]. In addition, GOLM1 can serve as a significant predictor of prostate cancer progression. In prostate cancer, GOLM1 functions as an oncogene by inducing prostate cancer cell growth, migration, and invasion, and inhibiting cell apoptosis, mainly by regulating the PI3K/AKT/mTOR signaling axis [9,10].

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases associated with several pathological and physiological processes [11,12]. MMP13 is overexpressed in esophageal squamous cell carcinoma (ESCC), and overexpression of MMP13 promotes cancer cell aggressiveness [13]. In addition, MMP13 is overexpressed in nasopharyngeal cancer (NPC), and exosomes purified from conditioned medium of nasopharyngeal cancer cells or NPC patients' plasma and MMP13-containing exosomes promote NPC progression [14]. Moreover, high levels of MMP13 are correlated with lymph node metastasis and advanced tumor staging of oral squamous cell carcinoma (OSCC) [15]. These findings indicate that MMP13 might serve as a prognostic marker and a target for cancer treatment.

In the present study, we revealed that GOLM1 is overexpressed in BC tissues and cell lines. GOLM1 promotes BC cell growth and metastasis by regulating the expression of MMP13. Collectively, our results prove that GOLM1 is a vital factor for BC progression, and suggest that GOLM1 is a potential target for combating breast cancer.

Material and Methods

Cell culture and BC tissues

Breast cancer cell lines MCF-7, MDA-MB-231, SK-BR3, and MDA-MB-468 and the mammary epithelial cell line MCF-10A were cultured with DMEM or 1640 containing 10% FBS at 37°C in 5% CO_2. A breast cancer microarray containing 100 human breast cancer tissues and 10 adjacent non-tumor tissues (BC081120b) were obtained from Xi'an Alina Biotechnology Co. (Xi'an, Shaanxi, China).

Cell transfection

PCR-amplified full-length GOLM1 cDNA (GOLM1) or MMP13 cDNA (MMP13) was cloned into pMSCV retrovirus plasmid to increase GOLM1, and GOLM1 shRNA lentiviral transduction particle (shGOLM1) was cloned into pSuper-retro-puro to decrease the expression of GOLM1. The shRNA sequence was: CCGGGTGAATAACATCACCACAGGTC TCGAGACCTGTGGTGATGTTATTCACTTTTTTG (GenePharma, Shanghai, China). shRNA targeting MMP13 was synthesized by GenePharma and the targeted sequence was: sense, 5'-GGAGAUAUGAUGAUACUAAdTdT-3'; antisense, 5'-UUAGUAUCAUCAUAUCUCCdTdT-3'. Scrambled shRNA (Santa Cruz Biotechnology) was used as a negative control. Cells were transfected with GOLM1 cDNA, shGOLM1, or shMMP13 using Lipofectamine 2000 reagent.

Cell proliferation and colony formation

MDA-MB-231 cell was transfected with GOLM1 or shGOLM1, and then seeded into 96-well plates and cultured for 24 h, 48 h, 72 h, or 96 h. After that, Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was added to the 96-well plates. OD value was detected by a micro-plate reader. In the colony-formation assay, MDA-MB-231 cells were seeded into 6-well plates. After 14 days, colonies were stained with crystal violet.

Cell migration

MDA-MB-231 cells were seeded into 6-well plates. After 24 h, the cell monolayer was scratched by using a 100-μL pipette tip. Cells were continually cultured with DMEM without serum. The wound healing was photographed at 0 h and 48 h using an inverted microscope (Carl Zeiss, Hallbergnoos, Germany) [16].

Cell invasion

We seeded 3×10^4 MDA-MB-231 cells into the upper Transwell chamber (8-μm, Corning, NY, USA) with an insert pre-coated with Matrigel (BD Bioscience). DMEM containing 20% FBS was placed into the lower chamber. After 1 day, the invaded

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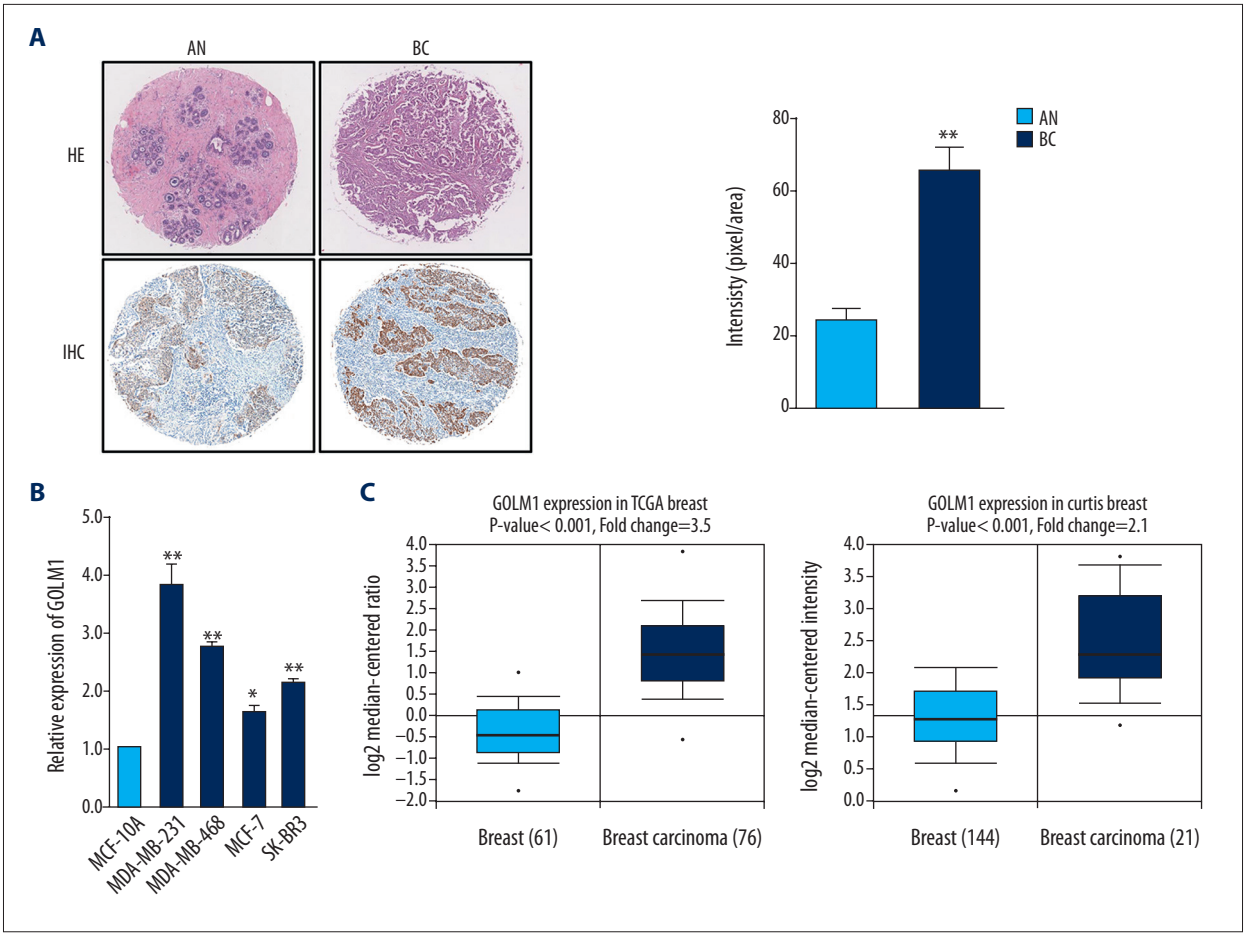

Figure 1. GOLM1 is overexpressed in BC cells. (**A**) Representative image of GOLM1 staining in BC tissues and corresponding non-cancerous tissues. ** P<0.01 compared with AN. (**B**) The expression of GOLM1 in BC cell lines and human mammary epithelial cell line MCF-10A was determined by using qRT-PCR assay. ** P<0.01 compared with MCF-10A. (**C**) The expression of GOLM1 mRNA in BC tissues *vs.* normal tissues in Oncomine database.

MDA-MB-231 cells were stained with 1% crystal violet. The invaded MDA-MB-231 cell were photographed using an inverted microscope (Carl Zeiss) [17].

Immunofluorescence assay

Cells were fixed with 4% paraformaldehyde and incubated with antibodies against E-cadherin or N-cadherin overnight at 4°C, and then were incubated with FITC-conjugated secondary antibody (Santa Cruz, CA, USA). Nuclei were stained with DAPI (Bioworld, Nanjing, Jiangsu, China).

Quantitative real-time PCR (qRT-PCR) assay

RNA was extracted using TRIzol (Invitrogen, Carlsbad, USA). The SYBR Green Master Mix Kit (TaKaRa Bio, Tokyo, Japan) was used for quantification of genes. GAPDH was selected as the internal control. The sequence was: GOLM1 RT primer, 5'-CACAGGCGAGAAGCTCATTC-3'; GOLM1 forward primer, 5'-CTATCCGCTCGTCACACTGTT-3'; GOLM1 reverse primer, 5'-CAGTGCGTGTCGTGGAGT-3'; MMP13 forward primer, 5'-CTTCTTCTTGTTGAGCTGGACTC-3'; MMP13 reverse primer, 5'-CTGTGGAGGTCACTGTAGACT-3'; GAPDH forward primer, 5'-ATGGGACGATGCTGGTACTGA-3'; GAPDH reverse primer, 5'-TGCTGACAACCTTGAGTGAAAT-3'. The comparative cycle threshold (Ct) method was applied to quantify the expression of genes through calculating the $2^{(-\Delta\Delta Ct)}$ method.

Immunoblotting analysis

We loaded 25 μg total proteins on 10% SDS-PAGE and then transferred it onto PVDF membranes. Next, membranes were incubated with antibody against GOLM, MMP13, or GAPDH (1: 1000, Bioworld) followed by incubation with horseradish peroxidase-conjugated IgGs (Bioworld). Bands were detected by using the ECL system (Millipore, Braunschweig, Germany).

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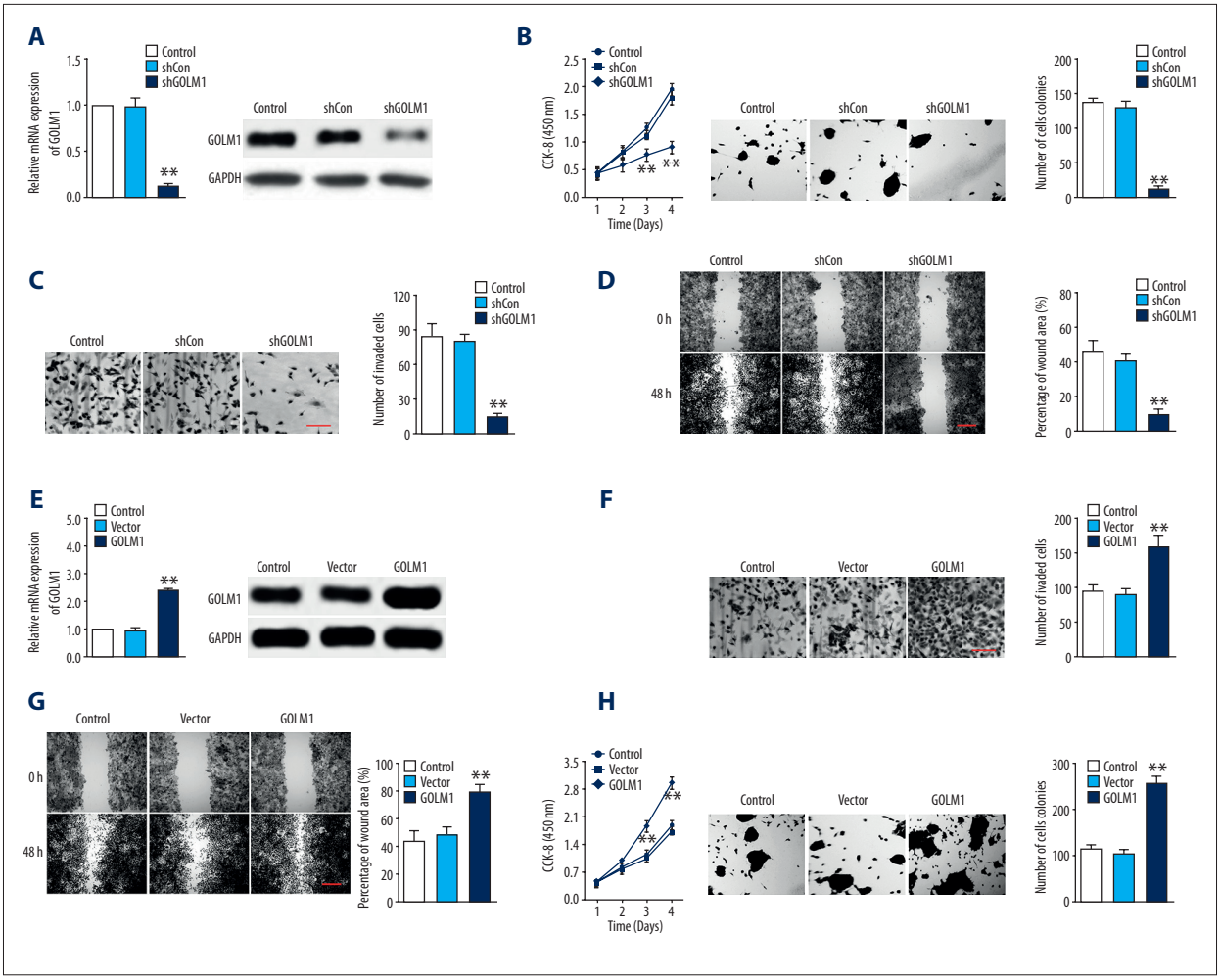

Figure 2. The role of GOLM1 in the proliferation and metastasis of BC cells. (**A**) shGOLM1 was transfected into MDA-MB-231 cells, and the expression of GOLM1 was detected by Western blotting and qRT-PCR. (**B**) MDA-MB-231 cells were transfected with shGOLM1 and subjected to CCK-8 and colony-formation assays. (**C**) MDA-MB-231 was transfected with shGOLM1, and the invasion of cell was determined by Transwell invasion assay. (**D**) The migration of MDA-MB-231 cells was examined by wound-healing assay. (**E**) MCF-1 cells were transfected with GOLM1 cDNA, and the level of GOLM1 was determined by Western blotting and qRT-PCR. (**F**) Transwell invasion analysis of MCF-7 cells transfected with GOLM1 cDNA. (**G**) Wound-healing assay showed the enhanced migration of MCF-7 cells transfected with GOLM1 cDNA. (**H**) Overexpression of GOLM1 increased MCF-7 cell proliferation and colony formation. ** P<0.01 compared with control.

Tumor xenograft

Male athymic nude mice were purchased from Weitonglihua Biotechnology (Beijing, China). We subcutaneously inoculated 1×10^6 cells (re-suspended in 100 µL PBS) into nude mice. Tumor size was measured and the tumor volume was calculated using the formula: length×(width)^2×0.5. The mice were observed for 6 weeks for tumor formation. For lung metastasis analysis, BC cells (5×10^5) were injected into mice via the lateral tail vein. Mice were sacrificed 4 weeks after inoculation and lungs were removed and stained with hematoxylin and eosin (HE). Animal experiments were approved by the Animal Care Committee of Tongji Medical College, Huazhong University of Science and Technology.

Statistical analysis

Analyses were completed using GraphPad prism 5 software. P<0.05 was considered statistically significant. Differences between 2 groups were evaluated by using either one-way ANOVA followed by post hoc Dunnett's test or by using the two-tailed *t* test.

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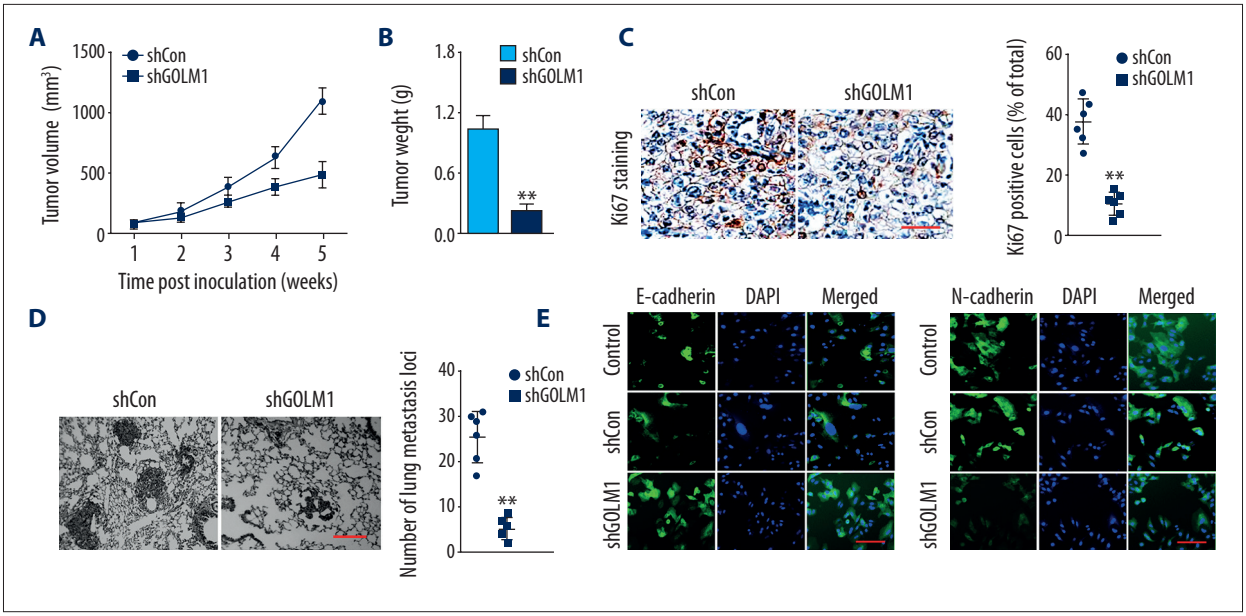

Figure 3. Underexpression of GOLM1 inhibits tumor progression of BC cells *in vivo*. (**A**) Growth curve showing that tumors derived from parental cells grew faster than cells in the MDA-MB-231-shGOLM1 group. (**B**) Tumor weight. (**C**) The expression of Ki67 in tumor mass was evaluated by IHC staining. (**D**) The number of metastatic foci in lungs was confirmed by HE staining. (**E**) Immunofluorescence staining of E-cadherin and N-cadherin in MDA-MB-231 transfected with shGOLM1. ** P<0.01 compared with shCon.

Results

GOLM1 is overexpressed in BC

The level of GOLM1 was detected in BC tissues and adjacent normal tissues by using immunohistochemistry (IHC) assay. As shown in Figure 1A, GOLM1 was significantly upregulated in breast cancer (BC) tissue compared to adjacent normal (AN) tissue. Furthermore, the level of GOLM1 was analyzed in breast cancer cells, including MCF-7, MDA-MB-231, SK-BR3, and MDA-MB-468. MDA-MB-231 cells exhibited higher expression of GOLM1 compared with the mammary epithelial cell line MCF-10A (Figure 1B). Furthermore, Oncomine analysis (TCGA breast statistics and Curtis breast statistics [18]) showed that the level of GOLM1 was higher in breast cancer tissue than in normal tissue (Figure 1C). These findings suggest that GOLM1 is overexpressed in BC and is associated with poor prognosis.

GOLM1 promotes proliferation, migration, and invasion of BC cells

To future explore the role of GOLM1 in BC, we constructed stably knocked-down expression of GOLM1 BC cell by transfected short hairpin RNA (shRNA) targeting GOLM1 (shGOLM1) into MDA-MB-231 cells (Figure 2A). Downregulation of GOLM1 markedly inhibited cell proliferation and decreased the colony formation *in vitro* (Figure 2B). Transwell assay results suggested that the invasion of MDA-MB-231 cells was remarkably inhibited by downregulation of GOLM1 (Figure 2C). Consistently, the wound-closure assay indicated that knockdown of GOLM1 dramatically decreased the migration ability of MDA-MB-231 cells (Figure 2D). Next, the BC cell line MCF-7 with low level of GOLM1 was transfected with GOLM1-cDNA to increase the expression of GOLM1 (Figure 2E). As shown in Figure 2F, 2G, GOLM1 overexpression markedly enhanced the migration and invasion of MCF-7 cells. We also found that overexpression of GOLM1 increased proliferation rate and colony formation of MCF-7 cells (Figure 2H). All these results reveal that GOLM1 promotes the proliferation, invasion, and migration of BC cells.

GOLM1 promotes BC cell growth and metastasis *in vivo*

Next, we constructed a subcutaneous xenograft model using MDA-MB-231-GOLM1 shRNA cells and their controls. Tumor growth curve results showed that tumors formed by MDA-MB-231-control cells grew faster than those formed by shGOLM1-transfected MDA-MB-231cells (Figure 3A). Five weeks after inoculation, the mice were sacrificed and weighed (Figure 3B). Expression of Ki67 in shGOLM1 was markedly decreased compared to that in the shCon group (Figure 3C). Next, we explored the lung metastasis of shGOLM1 MDA-MB-231 cells *in vivo* and found that the pulmonary metastasis was markedly inhibited in the MDA-MB-231 shGOLM1 group (Figure 3D). Finally, we showed that GOLM1 knock-down increased the expression of the epithelial marker E-cadherin but suppressed the level of the mesenchymal marker N-cadherin in MDA-MB-231 cells (Figure 3E).

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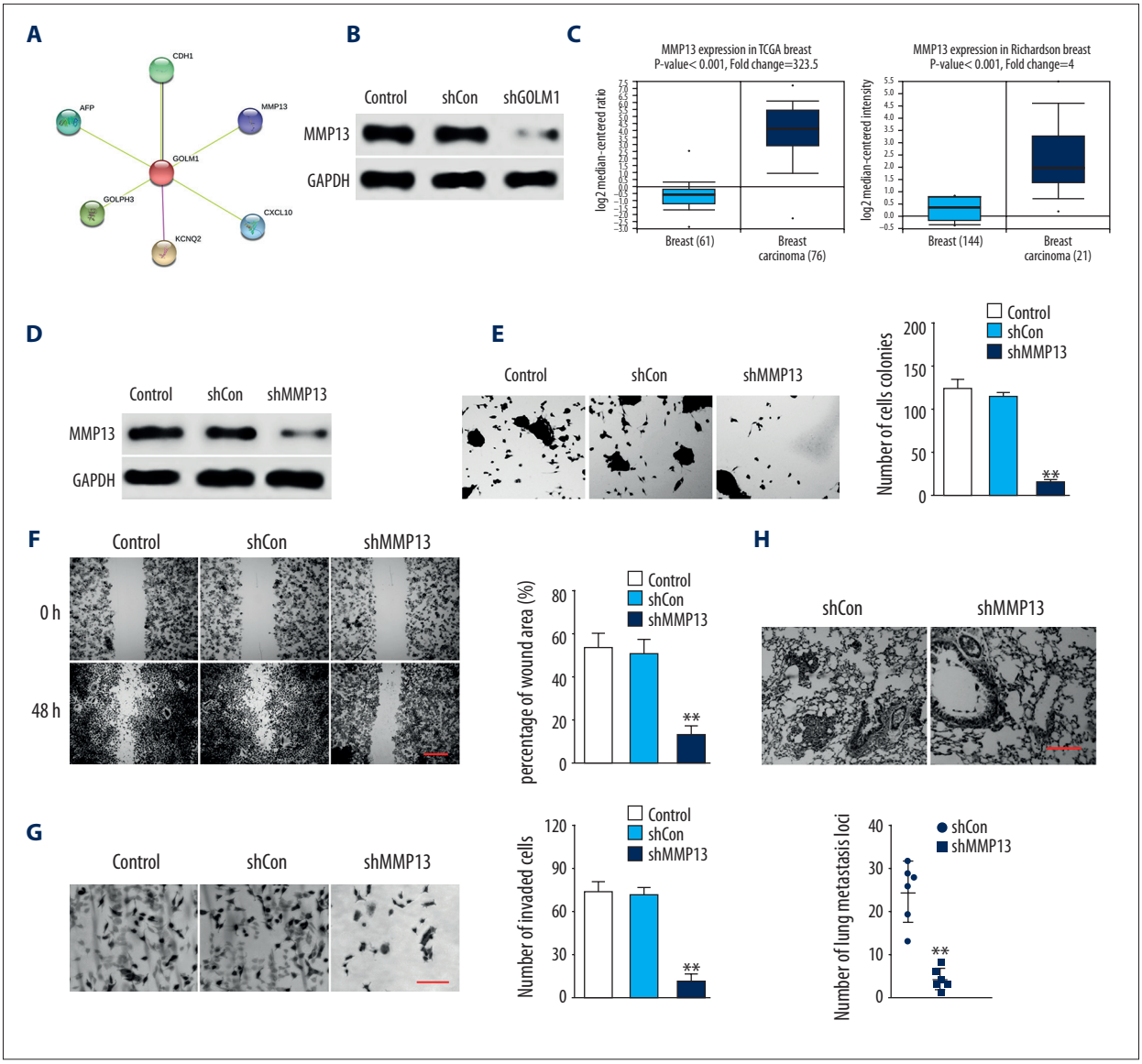

Figure 4. Underexpression of MMP13 suppresses BC cell growth and metastasis. (**A**) Protein interaction network of MMP13, CDH1, CXCL10, CCL5, and GOLM1. The colored lines between the proteins indicate the various types of evidence demonstrating the interaction. (**B**) The protein expression of MMP13 in cells transfected with shGOLM1 was analyzed by Western blotting assay. (**C**) The expression of MMP13 in BC tissue *vs.* normal tissue in Oncomine database. (**D**) MDA-MB-231 cells were transfected with either the negative control shRNA (shCon) or shMMP13. The expression level of MMP13 was detected by Western blotting assay. (**E**) Colony-formation assay of MDA-MB-231 cell. (**F**) MDA-MB-231 cells were transfected with shMMP13, and the migration was determined by using wound-healing assay. Scale bar: 200 µm. (**G**) MDA-MB-231 cells were transfected with shMMP13, and its invasion capacity was detected. Scale bar: 200 µm. (**H**) shMMP13 cells or control cells were injected into nude mice via lateral vein. Representative pictures of lungs from mice were taken after 4 weeks. Numbers of lung metastases were quantified. ** P<0.01 compared with control or shCon.

Collectively, these results indicate that GOLM1 regulates tumor growth and tumor progression of BC cells *in vivo*.

MMP13 is regulated by GOLM1

To better explore the underlying mechanism of GOLM1 in cancer metastasis, we mapped GOLM1 onto STRING database (*http://string-db.org/*) to build a protein-protein interaction network [19]. As shown in Figure 4A, MMP13 acted as a bridge to connect GOLM1. Furthermore, the expression of MMP13 was remarkably inhibited by shGOLM1 (Figure 4B). Next, Oncomine analysis of cancer tissues *vs.* normal tissue (TCGA breast statistics and Richardson Breast Statistics [20]) suggested that MMP13 was markedly overexpressed in BC cells (Figure 4C).

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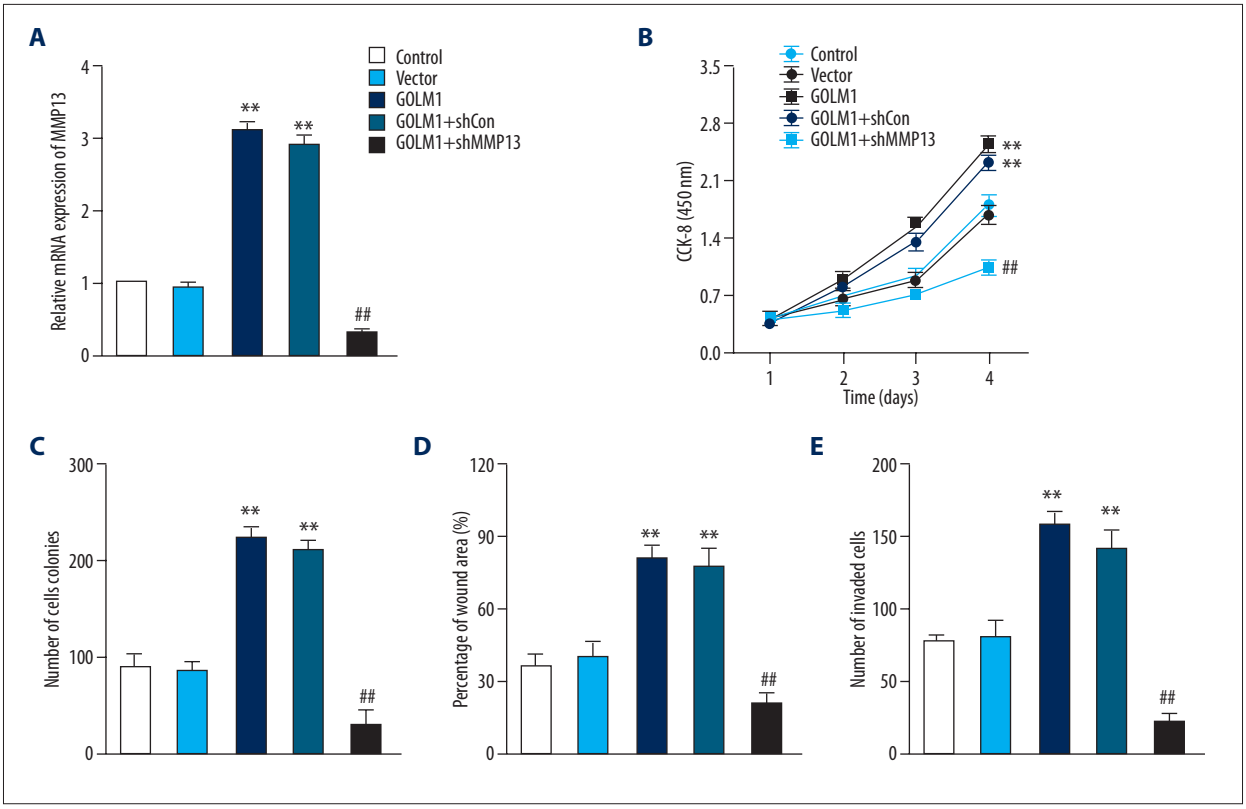

Figure 5. MMP13 knock-down reversed the effects of GOLM1. (**A**) MCF-7 cells were transfected with GOLM1 alone or cotransfected with shMMP13 and GOLM1. The expression of MMP13 was determined by qRT-PCR. (**B**) MCF-7 cells were transfected with GOLM1 alone or cotransfected with shMMP13 and GOLM1. The CCK-8 assay was conducted in MCF-7 cells. (**C**) Colony-formation analysis of MCF-7 cells. (**D**) MCF-7 cells were subjected to wound-closure assay. Scale bar: 200 µm. (**E**) Transwell invasion assay. Scale bar: 200 µm. ** P<0.01 as compared to control, ## P<0.01 as compared to GOLM1 + shCon.

To investigate the functions of MMP13 in BC cells, MMP13 was knocked-down in MDA-MB-231 cells by transfection with shRNA targeting A MMP13 (shMMP13) (Figure 4D). As shown in Figure 4E, underexpression of MMP13 inhibited colony formation of MDA-MB-231 cells. Consistently, both migration and invasion of MDA-MB-231 cells were markedly inhibited by shMMP13 (Figure 4F, 4G). Finally, the function of MMP13 in BC cell metastasis was analyzed *in vivo*. As shown in Figure 4H, mice injected with silenced MMP13 MDA-MB-231 cells formed fewer metastasis *loci*. All these findings suggest that downregulation of MMP13 inhibits the growth and metastasis of BC cells.

Underexpression of MMP13 reverses the effects of GOLM1 in BC cell

We investigated whether MMP13 knock-down reverses the effects of GOLM1 on growth, migration, and invasion of MCF-7 cells. MCF-7 cells were transfected with GOLM1 alone or co-transfected with GOLM and shMMP13. The expression of MDA-MB-231 cells was verified by qRT-PCR assay (Figure 5A). Then, CCK-8 and colony-formation assays were performed to determine whether shMMP13 reversed the promotion effects of GOLM1 on MCF-7 cell growth. As shown in Figure 5B, 5C, underexpression of MMP13 reversed all the effects of GOLM1 overexpression on proliferation and colony formation of MCF-7 cells. Consistently, shMMP13 transfection suppressed the mobility and invasion induced by GOLM1 (Figure 5D, 5E). All these findings suggest that GOLM1 regulates the growth, migration, and invasion of BC cells by targeting MMP13.

Overexpression of MMP13 rescues the suppressive effects of GOLM1 downregulation

We explored whether MMP13 overexpression could rescue the suppressive effects of shGOLM1 on MDA-MB-231 cell proliferation, migration, and invasion. BC MDA-MB-231 cells were cotransfected with shGOLM1 and MMP13 overexpression plasmid. qRT-PCR analysis was applied to validate the MMP13 mRNA after transfection (Figure 6A). The CCK-8 assay results show that MMP13 rescued the inhibitory effect of shGOLM1 on MDA-MB-231 cell proliferation (Figure 6B). As shown in Figure 6C, shGOLM1 significantly inhibited colony formation of MDA-MB-231 cells, whereas overexpression of MMP13 rescued the colony formation ability. After cotransfection with

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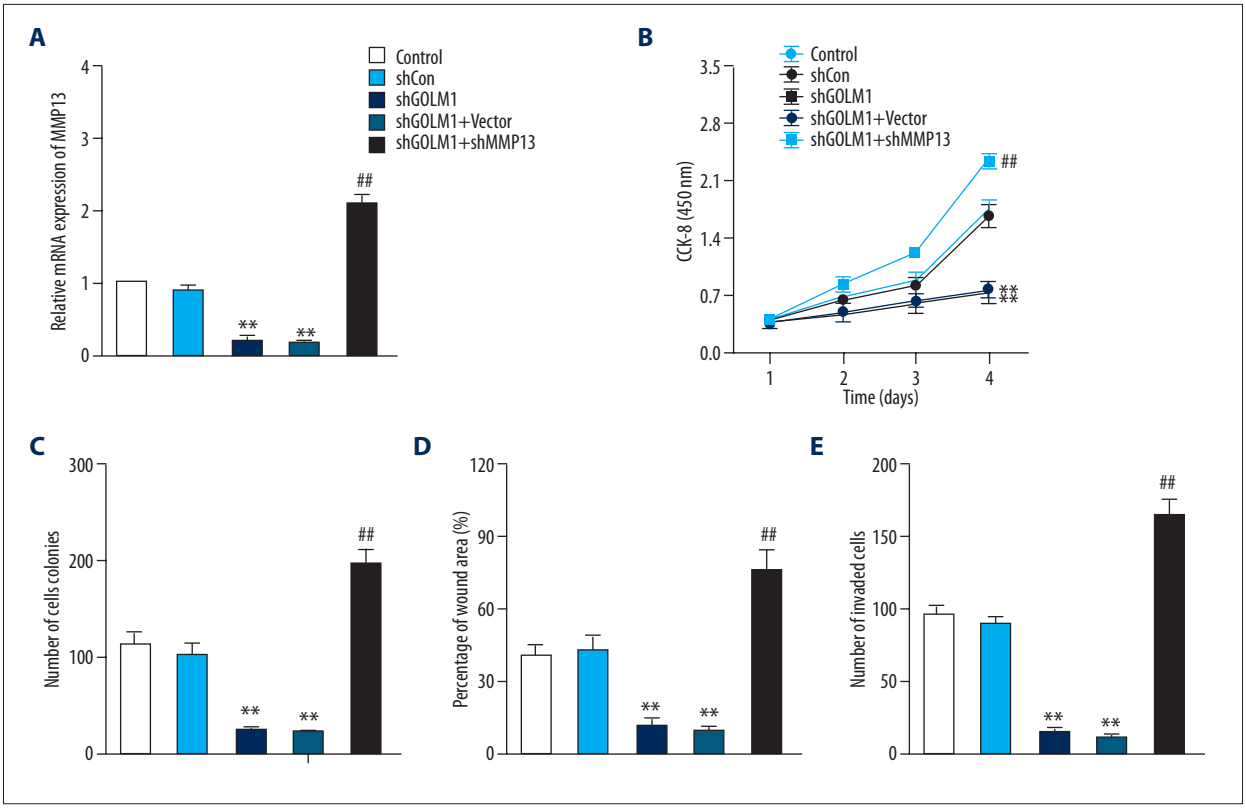

Figure 6. Upregulation of MMP13 rescued the inhibitory effects of GOLM1 underexpression. (**A**) MDA-MB-231 cells were transfected with shGOLM1 alone or cotransfected with MMP13-overexpression plasmid and shGOLM1. The level of MMP13 was determined by qRT-PCR assay. (**B**) CCK-8 assay was used to analyze MDA-MB-231 cell proliferation. (**C**) Colony formation assays of MDA-MB-231 cells. (**D**) MDA-MB-231 cells were cotransfected with MMP13-overexpression plasmid and shGOLM1, and were subjected to wound-healing assay. (**E**) Transwell invasion assay was performed after transfection of MDA-MB-231 cells with MMP13 plasmid and shGOLM1. ** P<0.01 as compared to control, ## P<0.01 as compared to shGOLM1 + Vector.

shGOLM1 and MMP13 overexpression plasmid, MDA-MB-231 cells were subjected to wound-healing assay and Transwell invasion assay. As shown in Figure 6D, 6E, the migration and invasion abilities inhibited by shGOLM1 were remarkably rescued by the overexpression of MMP13.

Discussion

Breast carcinoma is the leading cause of cancer-related death in women [21]. Recently, alternations in epigenetic modification of cancer-related genes have been identified [22]. Consistently, our study demonstrates that GOLM1 is remarkably underexpressed in breast cancer. Underexpression of GOLM1 significantly inhibits the proliferation and metastasis capacities of MDA-MB-231 cells, while overexpression of GOLM1 results in the opposite effects. Moreover, we proved that GOLM1 induces BC cell growth and metastasis by regulating MMP13.

Our results showed that GOLM1 was overexpressed in BC tissues and cell lines. Abundant evidence based on the proliferation, wound-closure, and Transwell invasion assays suggest that overexpression of GOLM1 impairs growth and metastasis of BC cells, whereas downregulation of GOLM1 resulted in the opposite results. Importantly, underexpression of GOLM1 markedly impaired BC cell tumor growth and lung metastasis *in vivo*. Furthermore, the *in vitro* experiment demonstrated that elevated expression of GOLM1 increased the level of mesenchymal marker N-cadherin and inhibited the epithelial marker E-cadherin. Thus, our results suggest that GOLM1 promotes BC cell growth, migration, and invasion and the EMT process.

To further explore whether the effects of GOLM1 on BC cells occurred entirely or partially through modulating MMP13, we upregulated the GOLM1 expression in MCF-7 with underexpression of MMP13 and performed the invasion and migration assays, showing that underexpression of GOLM1 neutralized the invasion- and migration-promoting effects of GOLM1. Then, we downregulated GOLM1 expression in MDA-MB-231 with overexpression of MMP13 and performed the migration and invasion assays. The findings indicated that upregulation

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of MMP13 rescued the inhibitory effects of GOLM1 underexpression in breast cancer cells.

In addition, we revealed that the level of MMP13 was regulated by GOLM1. Overexpression of GOLM1 increased the level of MMP13 in BC cells. Many studies have demonstrated that MMPs are associated with cancer metastasis [23]. Matrix metalloproteinases (MMPs) promote cancer cell migration, invasion, and metastasis via degradation of the extracellular matrix (ECM) around cancer cells [24]. Among MMPs, MMP9 is identified as stromelysin [25]. MMP9 degrades numerous ECM substrates, including collagens, laminins, elastin, entactin, fibronectin, and fibrin [26]. Overexpression of MMP9 has been proved to facilitate the tumor formation of BC cells in mice [27]. Furthermore, MMP9 has been proved to be upregulated in BC cells and is associated with the prognosis of patients with BC. Consistently, we demonstrate that MMP13 is overexpressed in BC tissues compared to normal tissues. Furthermore, downregulation of MMP13 by shRNA significantly suppressed BC MDA-MB-231 cell proliferation, migration, and invasion.

Conclusions

We found that GOLM1 is overexpressed in BC cells. Furthermore, overexpression of GOLM1 is significantly related to malignant phenotype of BC, including metastasis, poor prognosis, and advanced TNM stage. GOLM1 augmented BC aggressiveness via regulating the expression of MMP13. Therefore, our investigation of the vital function of GOLM1 in BC progression advances our knowledge of the underlying mechanism by which GOLM1 affects BC aggressiveness.

Conflict of interest

The authors declare no conflict of interest.

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