

Original Article

INSM1 promotes breast carcinogenesis by regulating C-MYC

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Abstract: Insulinoma-associated protein-1 (INSM1), which is highly expressed in various neuroendocrine tumors, functions as a zinc finger transcription factor capable of regulating the biological behavior of tumor cells. However, its specific role in breast cancer remains unclear. This study aims to investigate the role and mechanism of INSM1 in breast cancer. A total of 158 cohorts were recruited to examine the expression of INSM1 in breast cancer tissues and their corresponding adjacent normal tissues using immunohistochemistry. Follow-up data, along with clinical and pathological information, were collected to analyze the correlation between INSM1 expression and survival outcomes in breast cancer patients. Additionally, we investigated the impact of INSM1 on breast cancer cell proliferation, migration, and aggregation. To further explore the regulatory effect of INSM1 knockdown on breast cancer tumor growth, we utilized a xenograft mouse model. The results revealed that INSM1 was significantly overexpressed in breast cancer patients and correlated with prognosis. Knockdown of INSM1 notably impaired the malignant biological effects of breast cancer cells and inhibited the growth of xenograft tumors in nude mice. Importantly, our data also suggests an interaction between INSM1 and S-phase kinase-associated protein 2 (SKP2), which in turn regulates C-MYC, thereby affecting the p-ERK pathway. Our study provides the first evidence demonstrating the contribution of INSM1 to tumor formation and growth in breast cancer. Furthermore, we found that INSM1 positively regulates C-MYC and the p-ERK pathway by interacting with SKP2 during breast cancer development. Collectively, these findings highlight INSM1 as a promising target for breast cancer treatment.

Keywords: Breast cancer, INSM1, C-MYC, SKP2, neuroendocrine

Introduction

According to the “2020 Global Cancer Statistics” report, the worldwide incidence of breast cancer has reached 2.26 million cases, surpassing lung cancer as the most prevalent cancer [1]. Breast cancer poses a significant threat to women’s health due to its high heterogeneity, morbidity, mortality, recurrence, and metastasis [2, 3]. Although treatment modalities, such as surgery, chemotherapy, radiotherapy, and molecular targeted therapy, have significantly improved the prognosis and survival of breast cancer patients, drug sensitivity varies greatly among individuals, and substantial differences in individual treatment outcomes still exist, necessitating further understanding to improve outcomes. Therefore, it is crucial for breast cancer research to identify new and reli-

able biomarkers for diagnosis and treatment, particularly by exploring underlying mechanisms.

The breast is the target organ of various endocrine hormones among which estrone and estradiol are directly related to the incidence, prognosis and treatment of breast cancer [4]. Neuroendocrine carcinoma of the breast (NEC of the breast) is a tumor with morphological features similar to neuroendocrine tumors of the gastrointestinal tract or lung. Albeit, neuroendocrine tumors originating from the gastrointestinal tract are more common than those originating from the breast. Compared to other types of invasive breast cancer, patients with neuroendocrine carcinoma of the breast exhibit more aggressive clinical stages and worse prognoses [5]. The fifth edition of the WHO clas-

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sification of breast cancer emphasizes the distinction between neuroendocrine breast cancer and breast cancer with neuroendocrine features. Classic low/intermediate neuroendocrine tumors exhibit specific morphological features and diffusely express synaptophysin or chromogranin A in immunohistochemistry. However, some breast cancers may also be positive for neuroendocrine markers without fulfilling the specific morphological criteria for neuroendocrine breast cancer [6].

Insulinoma-associated protein-1 (INSM1), a zinc-finger transcription factor, is closely associated with various neuroendocrine tumors and serves as a second-generation biomarker for neuroendocrine differentiation [7]. INSM1 shows high expression in neuroendocrine tumors (Figure S1), including medullary thyroid cancer [8], cervical cancer [9], head and neck tumors [10], lung small and large cell neuroendocrine carcinomas [11-15], pancreatic neuroendocrine tumors [16], neuroendocrine gastrointestinal tumors [17], prostate cancer [18], and skin Merkel cell carcinoma [19, 20]. Particularly in lung cancer, multiple studies have confirmed INSM1 as a crucial tumor marker for neuroendocrine lung cancer, demonstrating high sensitivity and specificity [21-24].

Neuroendocrine breast cancer does not exhibit distinct clinical features compared to non-specialized breast cancer types. In current clinical work, the detection of neuroendocrine markers is not routine (Figures S2 and S3). Endocrine treatment guidelines and norms typically follow those for non-specialized invasive breast cancer. The prognosis of breast cancer accompanied by neuroendocrine features remains controversial, but most reported studies indicate a poor response to chemotherapy and chemotherapy resistance [25-30]. Thus, the detection of neuroendocrine markers in breast cancer is necessary, and INSM1, extensively studied in numerous investigations, has demonstrated good sensitivity and specificity compared to traditional neuroendocrine factors such as chromogranin A (CgA) and synaptophysin (SYN) [31-34].

Several cases and studies suggest that INSM1 could serve as a new immunomarker for breast cancer [31, 35, 36]. However, the role and mechanism of INSM1 in breast cancer have not been extensively investigated. Only one recent

study highlighted the significance of the SCAMP1-TV2/PUM2/INSM1 pathway in regulating the biological behavior of breast cancer cells [36]. In our study, we observed overexpression of INSM1 in breast cancer, with high INSM1 expression associated with poor prognosis in breast cancer patients. To further characterize the role of INSM1 in breast cancer, we conducted both *in vitro* and *in vivo* studies. The aim of this study was to explore whether INSM1 can serve as a new biomarker and potential therapeutic target for breast cancer, with the hope of personalizing breast cancer treatment and improving patient prognosis.

Materials and methods

Human-tissue samples

All clinical sample collections were conducted in accordance with the principles of the Declaration of Helsinki. Tissue samples were obtained from 158 patients with pathologically confirmed breast cancer who underwent surgery at the Affiliated Drum Tower Hospital of Nanjing University. None of the patients had previously received anticancer therapy. The clinical stages were classified according to the guidelines of the International Union Against Cancer. The use of human subjects for this study was approved by the Ethics Committee of The Drum Tower Hospital of Nanjing University, and informed consent was obtained from all participating patients.

Immunohistochemistry

IHC was performed in tissue microarrays (TMA) as previously described [1, 2]. The TMA sections were labeled with anti-INSM1 antibodies (1:50 dilution, ab170876, Abcam, Cambridge, MA, USA) and examined under a microscope for pathological assessment.

Cell culture

The human BT-549, MCF-7, MDA-MB-231, and HS578T cell lines were purchased from ATCC (Virginia, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) (New York, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 1% L-Glutamine (Gibco, USA), 1% non-essential amino acids (Gibco, USA), and 1% penicillin-streptomycin (Gibco, USA) at 37°C with 5% CO₂.

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INSM1 down-regulation and C-MYC overexpression

Stable down-regulation of INSM1 and overexpression of C-MYC in BT-549 and MDA-MB-231 cells were achieved by using recombinant Lentivirus. Blank lentivirus was used as a vector control. Three RNA interference target sequences were designed based on the INSM1 gene to construct the target gene RNA interference lentiviral vector.

Cell proliferation and colony formation assays

Cell proliferation was quantified by counting the number of cells and plotting the proliferation curve over a five-day period. Briefly, an equal number of cells were seeded at day 0 and then counted daily at indicated time points using the celigo mechanism (Nexcelom). For the colony formation assay, cells were seeded and cultured for 14 days in medium. Colonies were fixed with a 4% paraformaldehyde solution and stained with GIEMSA dye solution. The number of colonies was counted using ImageJ software. Experiments were performed with three independent replicates.

Cell migration and invasion assays

Wound healing assay was carried out to assess cell migration according to a previously reported protocol [39, 40]. The images were scanned and analyzed using Cellomics (Thermo) at 0 hours, 24 hours, and 48 hours. For the cell invasion assays, a Transwell assay was performed using a Transwell kit (3422 Corning). Cells were resuspended in 100 μ l of serum-free medium and placed in the upper compartment of a Transwell chamber with or without matrigel. Migrating or invading cells were fixed and stained. The number of cells was determined under a light microscope by counting five random fields.

CCK-8 colony formation

The effect of INSM1 down-regulation and C-MYC overexpression on cell proliferation was determined by using the CCK-8 (Sigma, USA) colony formation assay, following a previously reported protocol [41, 42].

Flow cytometric analysis

Samples were filtered and analyzed by flow cytometry as described previously [43, 44]. All

experimental operations and steps were carried out following the instructions of the eBioscience™ Annexin V Apoptosis Detection Kits (No. 88-8007-74). Flow cytometry data was analyzed using FlowJo Software v. 10 (Tree Star, Inc., USA).

Western blotting

Whole cell lysates were analyzed by Western Blotting as described previously [45]. Briefly, lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose filter (NC) membranes. The membranes were incubated with primary antibodies that recognized INSM1 (Abcam, USA) at a 1:500 dilution or MYC (Abcam, USA) at a 1:1000 dilution. Anti-GAPDH antibody (Abcam, USA) at a 1:30000 dilution was used for standardization. Horseradish peroxidase (HRP)-coupled anti-rabbit or anti-mouse polyclonal antibodies were used as secondary antibodies at a 1:5000 dilution. Horseradish peroxidase (HRP)-coupled anti-rabbit polyclonal antibody (Abcam, USA) was used as a secondary antibody at a dilution of 1:5000. Protein bands were stained using Millipore's immobilon Western Chemiluminescent HRP Substrate kit. Band densities were evaluated using ImageJ.

Reverse transcription and quantitative real-time PCR

Total RNA was extracted from the BT549 and MDA-MB-231 cells with Trizol (Sigma, USA) and then reversed transcription into complementary DNA (cDNA) using a cDNA synthesis kit (Vazyme, China). The RNA concentration and quality were determined by measuring the 260/280 nm ratio using a Nanodrop spectrophotometer (ND-100). For mRNA expression analysis, quantitative real-time PCR (qRT-PCR) was performed using the Two-Step AceQ qPCR SYBR Green master mix (Vazyme, China). The relative gene expression levels were quantified by normalizing to GAPDH using the $2^{-\Delta\Delta Ct}$ method. GAPDH was selected as the internal reference gene for mRNA detection.

Mice

All animal experiments were approved by the Drum Tower Hospital of Nanjing University. The lenti-edited MDA-MB-231 cells were used for the in vivo study. Six-week-old BALB/C athymic

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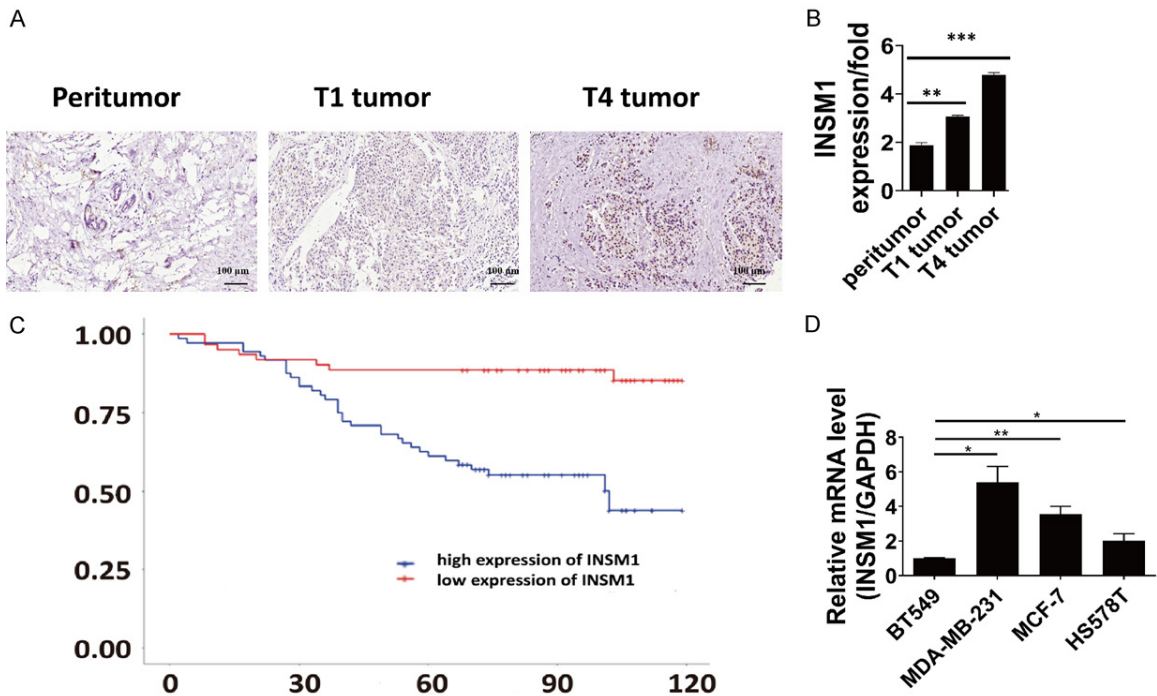


Figure 1. Clinical significance and relative expression of INSM1 in BC tissues and cells. A. Representative images of TMA stained via IHC for INSM1. Scale bar, $\times 200$. B. Statistical histograms depicting TMA staining for INSM1 (Peritumoral, $n = 19$; T1 and T4 tumors, $n = 19$). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. C. Analysis of the correlation between INSM1 expression and patient survival time, $n = 133$. D. Quantitative RT-PCR detection of INSM1 expression levels in four BC cell types, presented as mean \pm SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

nude mice were obtained from Jiangsu Jicui Yaokang Biotechnology Co. LTD. The mice were maintained in pathogen-free conditions and divided into two groups: the control group (only MDA-MB-231 cells) and the shINSM1 group (MDA-MB-231 cells infected with INSM1 stable-knockdown viruses). A total of 1.6×10^7 cells were injected into the fourth mammary fat pad of the mice. Tumor volume was measured every 7 days. Animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing University, Nanjing, China.

Statistical analysis

Data were presented as mean \pm SD or SEM as indicated. Statistical significance was calculated using the Student's t-test for two-group comparisons. The chi-square test was used to investigate the significance of the associations between patient variables such as age, tumor size, N classifications, ER status, PR status, ERBB2 status, and survival status. The cumulative survival time was obtained using the Kaplan-Meier method, and multiple variable

COX regression analysis was performed. Tumor volume analysis in mice was conducted using repeated measurement ANOVA with post hoc Bonferroni test. Statistical analyses were performed using the Statistical Package for Social Science 26 (26.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism (8.0, GraphPad Software Inc., San Diego, CA, USA). A p -value of ≤ 0.05 was considered statistically significant for all tests.

Results

High expression of INSM1 correlates with worse survival in breast cancer (BC) patients

To investigate INSM1 overexpression in BC patients, we performed immunohistochemistry (IHC) on a tissue microarray (TMA) containing 158 BC patient specimens and 19 normal breast tissues. Our results demonstrated that INSM1 protein was predominantly nuclear and significantly elevated in tumor tissues compared to normal tissues (**Figure 1A** and **1B**; **Table 1**). The relationship between INSM1 expression and clinicopathological variables of

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Table 1. Expression patterns in breast cancer tissues and para-carcinoma tissues revealed in immunohistochemistry analysis

INSM1 expression	Tumor tissue		Para-carcinoma tissue		p value
	Cases	Percentage	Cases	Percentage	
Low	83	52.5%	19	100.0%	0.000***
High	75	47.5%	0	-	

***P < 0.001 by t-test.

the 158 breast cancer patients is presented in **Table 1**. We observed a significant association ($P = 0.017$) between INSM1 expression and tumor size ($P = 0.041$) and T Infiltrate ($P = 0.006$) (**Figure 1A** and **1B**; **Table 2**). Furthermore, BC patients with higher INSM1 expression had a significantly lower probability of overall survival (**Figure 1C**). We conducted COX regression analysis and the results revealed that the hazard ratio (HR) was 0.212 (95% CI: 0.0968-0.4643; P -value = 0.000105), suggesting that high expression of INSM1 is an independent risk factor for poor prognosis. Additionally, we examined INSM1 expression in four cell lines (BT-549, MCF-7, MDA-MB-231, HS578T), revealing high expression of INSM1 in the latter three cell lines compared to BT-549 (**Figure 1D**). Our findings indicate that INSM1 is upregulated in BC and its expression may serve as a potential prognostic marker.

Knockdown of INSM1 inhibited the malignant phenotype of BC cells

To explore the role of INSM1 in BC, we transfected BT-549 and MDA-MB-231 cell lines with shCtrl (cells transfected with blank lentivirus) and shINSM1 (**Figure 2A** and **2B**). We evaluated the biological behaviors of the cells and observed a significant reduction in proliferation (**Figure 2C**) and colony formation (**Figure 2D**) in the shINSM1 group compared to the shCtrl group. These results suggest that INSM1 plays a vital role in cell proliferation in BC. As metastasis is a leading cause of BC-related mortality [46], we further investigated the role of INSM1 in the metastatic phenotype of BC cells. The shINSM1 group exhibited significantly decreased migration (**Figure 3A**) and invasion (**Figure 3B**) of BC cells compared to the shCtrl group, along with a significant increase in the apoptotic rate (**Figure 3C**). These findings indicate that INSM1 significantly promotes malignant characteristics of BC cells.

INSM1 depletion suppresses xenograft growth in vivo

A mouse model was established for the investigation into the role of INSM1 in BC tumor progression in vivo. The experiments consisted of a control group (NC) and an experimental group (KD) with knockdown of INSM1 with five mice per group. Mouse weight and xenograft tumor

size was measured once per week. After five weeks, the mice were sacrificed and the tumors were removed (**Figure 4A**). The tumor formation in mice with INSM1 knockdown was significantly impeded relative to the NC group (**Figure 4B**). At five weeks, the tumor weight in the NC group, 1.130 ± 0.381 g, was significantly higher than in the KD group, 0.482 ± 0.191 g ($P = 0.014 < 0.05$, **Figure 4C**). These results indicate that INSM1 knockdown significantly reduces tumor growth in vivo, indicating INSM1 acts as a prominent promoter of BC growth.

INSM1 regulates C-MYC

To gain a deeper understanding of the mechanism of INSM1 in breast cancer, we examined target genes using INACT analysis (<https://www.ebi.ac.uk/intact/home>). We identified five proteins that significantly interact with INSM1, excluding three non-human genes (**Figure 4D**). Previous studies have shown that C-MYC is a tumor suppressor gene and is involved in triple-negative breast cancer [47]. We found that C-MYC protein expression was regulated after INSM1 knockdown in BC cells (**Figure 4E**). Overexpression of MYC significantly inhibited apoptosis in BT549 and MDA-MB-231 cells (MYC+NC-SHINSM1 vs. NC(OE+KD), $P < 0.05$). INSM1 knockdown significantly promoted apoptosis (shINSM1+NC-MYC vs. NC(OE+KD), $P < 0.05$). INSM1 knockdown also significantly inhibited the apoptotic regulatory effect of MYC (shINSM1+NC-MYC vs. MYC+NC-shINSM1, $P < 0.05$) (**Figure 4F**). Additionally, MYC overexpression significantly promoted BC cell migration (MYC+NC-shINSM1 vs. NC(OE+KD), $P < 0.05$), whereas INSM1 knockdown inhibited cell migration (shINSM1+NC-MYC vs. NC(OE+KD), $P < 0.05$). INSM1 knockdown also significantly inhibited the migration regulation effect of MYC (shINSM1+NC-MYC vs. MYC+NC-shINSM1, $P < 0.01$) (**Figure 4G**). These findings demonstrate

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Table 2. Relationship between INSM1 expression and tumor characteristics in patients with breast cancer

Features	No. of patients	INSM1 expression		p value
		Low	High	
All patients	158	83	75	
Age (years)				0.130
< 55	68	40	28	
≥ 55	84	39	45	
Grade				0.642
II	74	40	34	
III	60	30	30	
AJCC stage				0.095
0	1	1	0	
1	26	16	10	
2	79	40	39	
3	36	15	21	
T Infiltrate				0.006**
0	1	1	0	
1	39	25	14	
2	87	42	45	
3	15	4	11	
4	1	0	1	
Lymphatic metastasis (N)				0.455
0	75	41	34	
1	39	18	21	
2	18	8	10	
3	13	7	6	
Tumor size				0.041*
< 3 cm	61	37	24	
≥ 3 cm	83	36	47	
ER nuclear test results				0.738
≤ 5	65	28	37	
> 5	63	29	34	
PR nuclear test results				0.396
= 0	78	33	45	
> 0	50	25	25	
HER2 testing results of membrane				0.055
≤ 6	71	37	34	
> 6	57	20	37	
KI67 nuclear test results				0.859
≤ 2.25	64	29	35	
> 2.25	64	28	36	
P53 nuclear test results				0.616
≤ 4	74	35	39	
> 4	56	24	32	
FISH data				0.503
Negative	90	43	47	
Positive	41	17	24	

**P < 0.01 by t-test; *P < 0.05 by t-test.

that C-MYC participates in the regulation of INSM1 on the biological behaviors of BC cells.

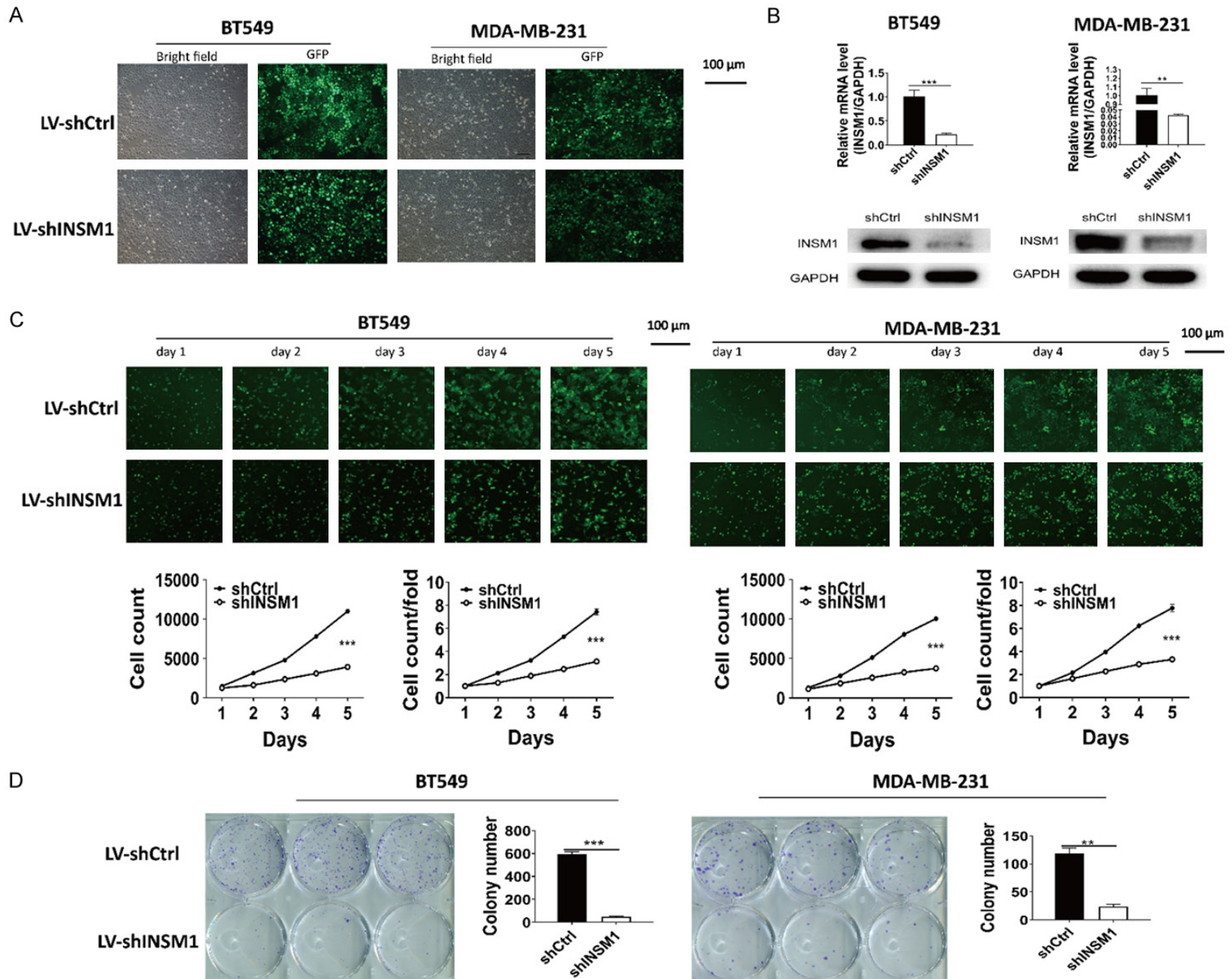
INSM1 interacts with SKP2 to regulate C-MYC

Previous studies have suggested that MYC can block p21 transcription or induce S-phase kinase-associated protein 2 (SKP2) [49, 50]. Therefore, we investigated the relationship between INSM1 and SKP2 on C-MYC regulation. We successfully established BC cells infected with SKP2 overexpressed lentivirus. Compared to the control group, down-regulation of INSM1 and overexpression of SKP2 upregulated C-MYC (**Figure 5A** and **5B**). Moreover, the protein stability of C-MYC significantly decreased following CHX treatment after INSM1 knockdown and SKP2 overexpression (**Figure 5C** and **5D**). To determine the correlation between SKP2 and INSM1, we performed Co-IP and found that INSM1 interacts with SKP2 (**Figure 5F**). Results showed that the ubiquitination level of C-MYC significantly increased after INSM1 knockdown (**Figure 5E**), demonstrating that INSM1 interacts with SKP2 to regulate C-MYC.

INSM1 affects p-ERK phosphorylation

Research has shown that INSM1 plays a key role in NE lung cancer through Shh signaling, which crosstalks with the PI3K/AKT and MEK/ERK pathways to enhance N-MYC stability [51]. To gain a bet-

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Figure 2. INSM1 knockdown restricts BC cell malignant phenotypes. A. Immunofluorescence images of BC cells post-lentiviral infection. B. Cells transfected with an empty vector serve as a negative control. Both RT-qPCR and Western blot analyses were performed to determine mRNA and protein levels respectively in BC cells following INSM1 gene downregulation or negative control. C. Cell proliferation assay predicts potential of INSM1 to promote BC cell proliferation. D. Colony formation assay exploring INSM1's impact on BC cell colony formation capabilities.

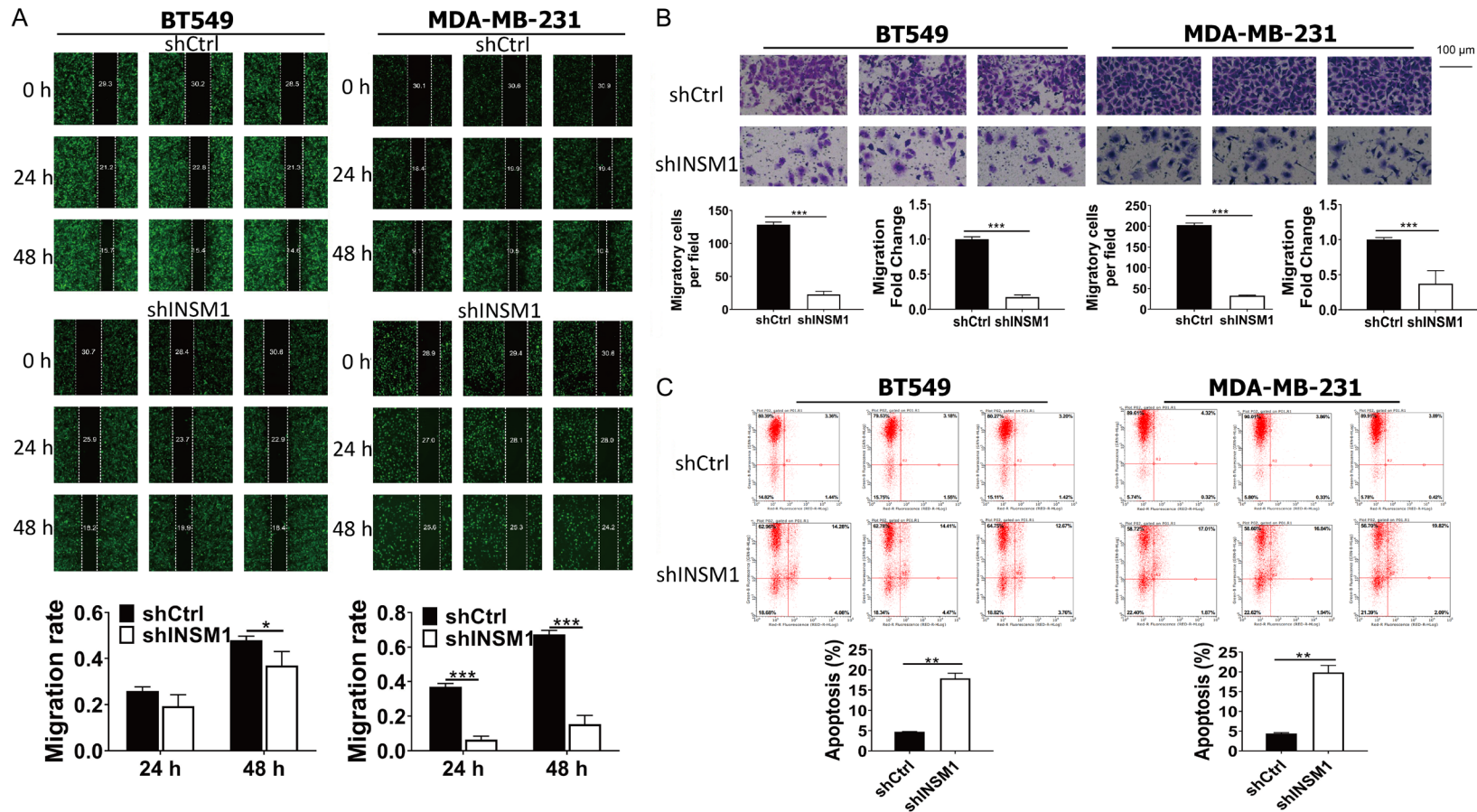
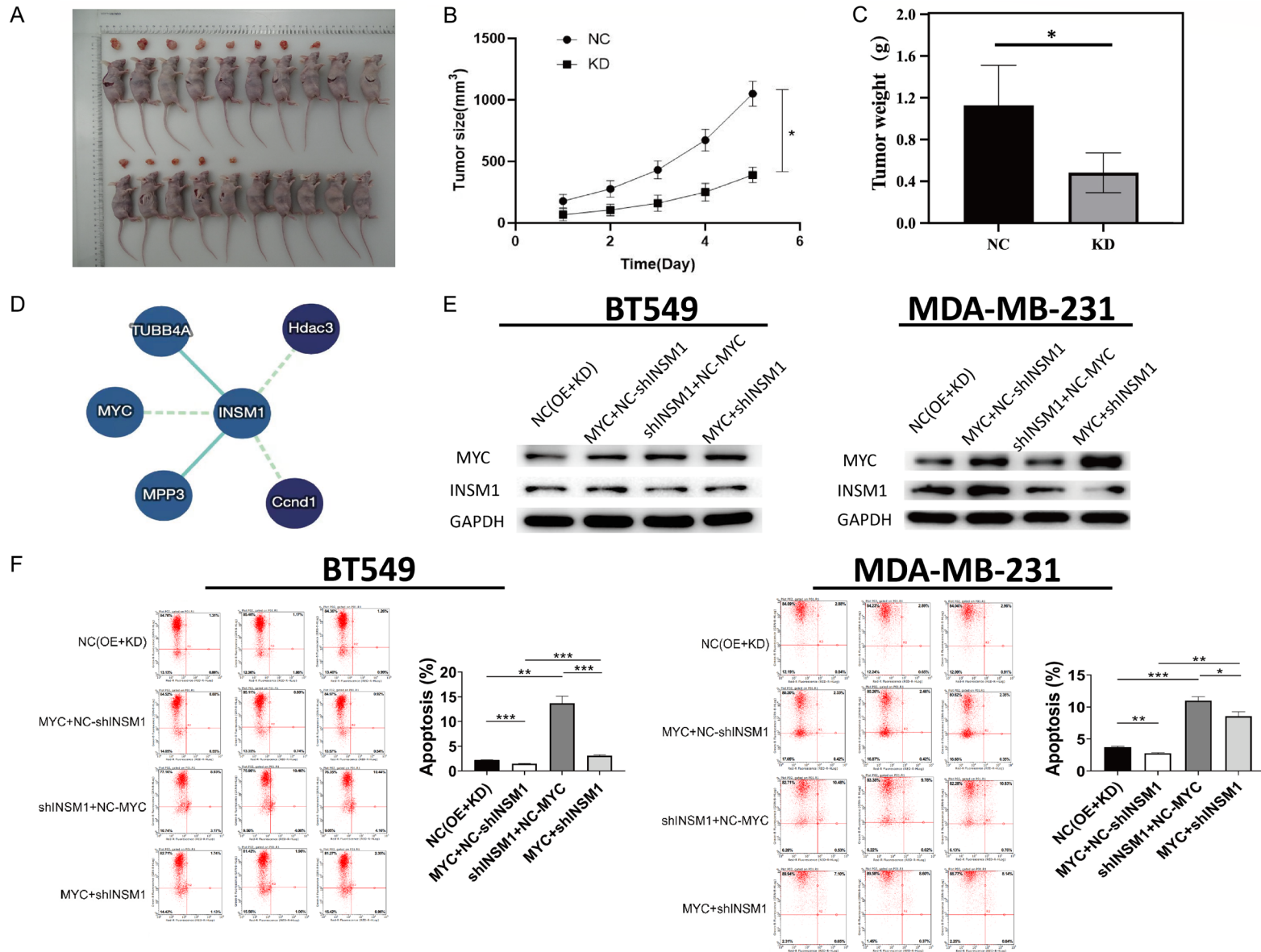


Figure 3. INSM1 expression's effect on BC cell migration, invasion, and apoptosis. A. Wound healing assay assessing BC cell migration. B. Transwell assay examining alterations to the invasion phenotype of cells with varying INSM1 expression levels. C. Flow cytometric analysis investigating INSM1 influence on BC cell apoptosis. Data are presented as mean \pm SD from a minimum of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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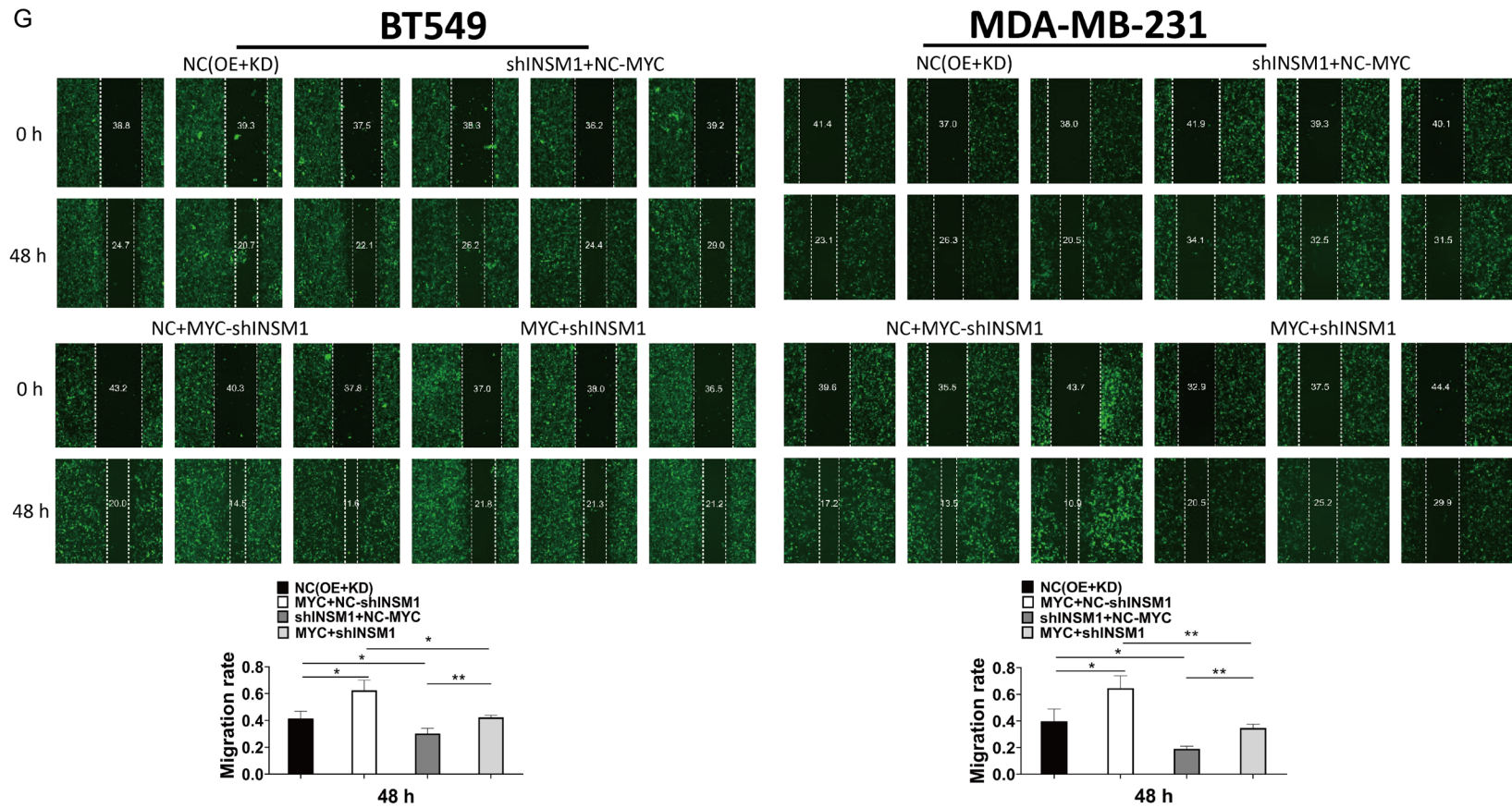


Figure 4. INSM1 inhibits BC tumor growth in vitro and regulates C-MYC. A. Tumor images from INSM1 knockout and control groups. B. Tumor sizes assessed via repeated measures ANOVA, followed by Bonferroni's post hoc test. C. Tumor weights were measured post-surgical dissection, n = 10 for NC and KD groups. D. INACT analysis identified five proteins significantly interacting with INSM1. E. Western blot analysis of INSM1 and C-MYC expression in stably transfected BC cells. F. Flow cytometry examines how INSM1 regulation of C-MYC influences BC cell diagnosis. G. Wound healing assay to determine the effect of downregulated INSM1 on C-MYC's promotion of cell migration.

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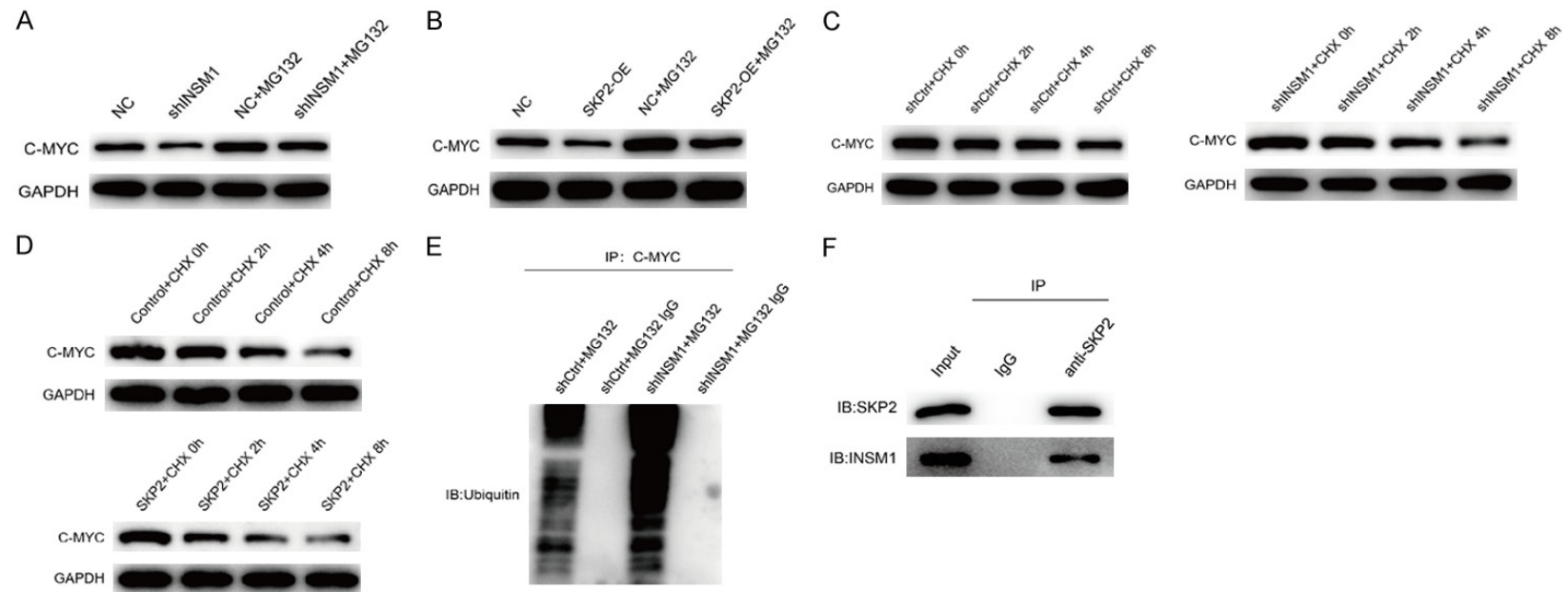


Figure 5. INSM1 interacts with SKP2 to regulate C-MYC. A. Western blot analysis of INSM1 and C-MYC in BC cells with or without MG132. B. Western blot analysis of SKP2 and C-MYC in BC cells with or without MG132. C. Western blot analysis of INSM1 and C-MYC in BC cells with or without CHX. D. Western blot analysis of SKP2 and C-MYC in BC cells with or without CHX. E. Co-IP explores the potential interaction between SKP2 and INSM1. F. Effects of INSM1 knockdown on C-MYC protein stability through determination of ubiquitination patterns.

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ter understanding of the possible mechanism of INSM1 action in BC, we investigated the relationship between INSM1 and phosphorylation in the ERK pathway. We assessed BC cell proliferation and diagnosis using CCK-8, colony formation, and flow cytometric analysis, and explored the expression levels of ERK and p-ERK proteins through Western blot analysis (Figure 6A-C). Our data revealed that INSM1 infection significantly influenced p-ERK phosphorylation and its expression in BC cells (BT549 and MDA-MB-231) after lentivirus infection.

Discussion

Breast cancer is a relatively common tumor. In recent years, advancements in surgical techniques, chemotherapy, radiotherapy, molecular targeted therapy, and immunotherapy have significantly reduced the mortality rate [14, 52]. However, breast cancer remains one of the leading causes of cancer-related deaths in women. Patients with similar pathological types, surgical methods, endocrine treatments, and targeted drug therapies exhibit varying prognosis and treatment outcomes [53]. Therefore, targeted research is of particular importance for the future diagnosis and treatment of breast cancer.

This study concludes that high expression of INSM1 is significantly correlated with advanced stage and tumor growth in breast cancer patients. Additionally, overexpression of INSM1 is associated with shorter survival time in BC patients, indicating its significant role in promoting malignant behaviors in BC cells. These findings suggest that INSM1 may play an oncogenic role and contribute to the occurrence and development of breast cancer. Moreover, INSM1 has the potential to serve as a molecular target for the diagnosis and treatment of breast cancer. Several studies in breast cancer have demonstrated that compared to traditional neuroendocrine markers such as CgA, CD56, and SYN [32, 34, 35], INSM1 exhibits lower sensitivity but higher specificity. Similar trends have been observed in neuroendocrine lung cancer, where INSM1 is recognized as a pathological indicator for small cell lung cancer [13, 21, 24, 54]. Based on the immunohistochemical and correlation analysis results of this study, it is suggested that INSM1 should be

included in the immunohistochemical pathological detection of breast cancer.

Zhang et al. [55] discovered that compared to other types of breast cancer, neuroendocrine breast cancer exhibited a larger tumor diameter, which aligns with our findings. However, we did not observe a significant difference between neuroendocrine breast cancer and lymph node metastasis while Zhang et al. [55] demonstrated a lower incidence of lymph node metastasis in neuroendocrine breast cancer compared to other types of breast cancer. Some studies have indicated a significant correlation between neuroendocrine differentiation and estrogen positivity in invasive breast cancer [56]. Kawasaki et al. [35] identified a significant positive correlation between INSM1 and estrogen receptor expression. INSM1 also exhibited a negative correlation with Human Epidermal Growth Factor Receptor-2 (HER-2) and high molecular weight cytokeratin. INSM1 can be used to classify invasive breast cancer into different prognostic subtypes based on neuroendocrine characteristics. Moreover, it was suggested that patients with high INSM1 expression had a better prognosis. However, our study did not reveal a significant correlation with ER, PR, and HER-2, and we believed that patients with high INSM1 expression had a worse prognosis. The differences observed may be attributed to variations in inclusion criteria, ethnic backgrounds, and interpretations of neuroendocrine characteristics among breast cancer patients. It should be noted that not all the fundamental clinical information of breast cancer patients was completely obtained in our study, and the lack of access to certain data may have resulted in the exclusion of corresponding patients. Additionally, there may be some statistical bias in the telephone and outpatient follow-up information, which cannot be entirely objective. Therefore, larger-scale, multi-center studies are necessary to assess the impact of INSM1 expression on the prognosis of breast cancer patients.

The MYC genes consist of three types: N-MYC, C-MYC, and L-MYC. Among these, C-MYC gene expression disorder is estimated to occur in up to 70% of human cancers [57]. C-MYC is one of the most extensively studied oncogenes and is closely associated with cancer growth, progression, and maintenance. The regulation of

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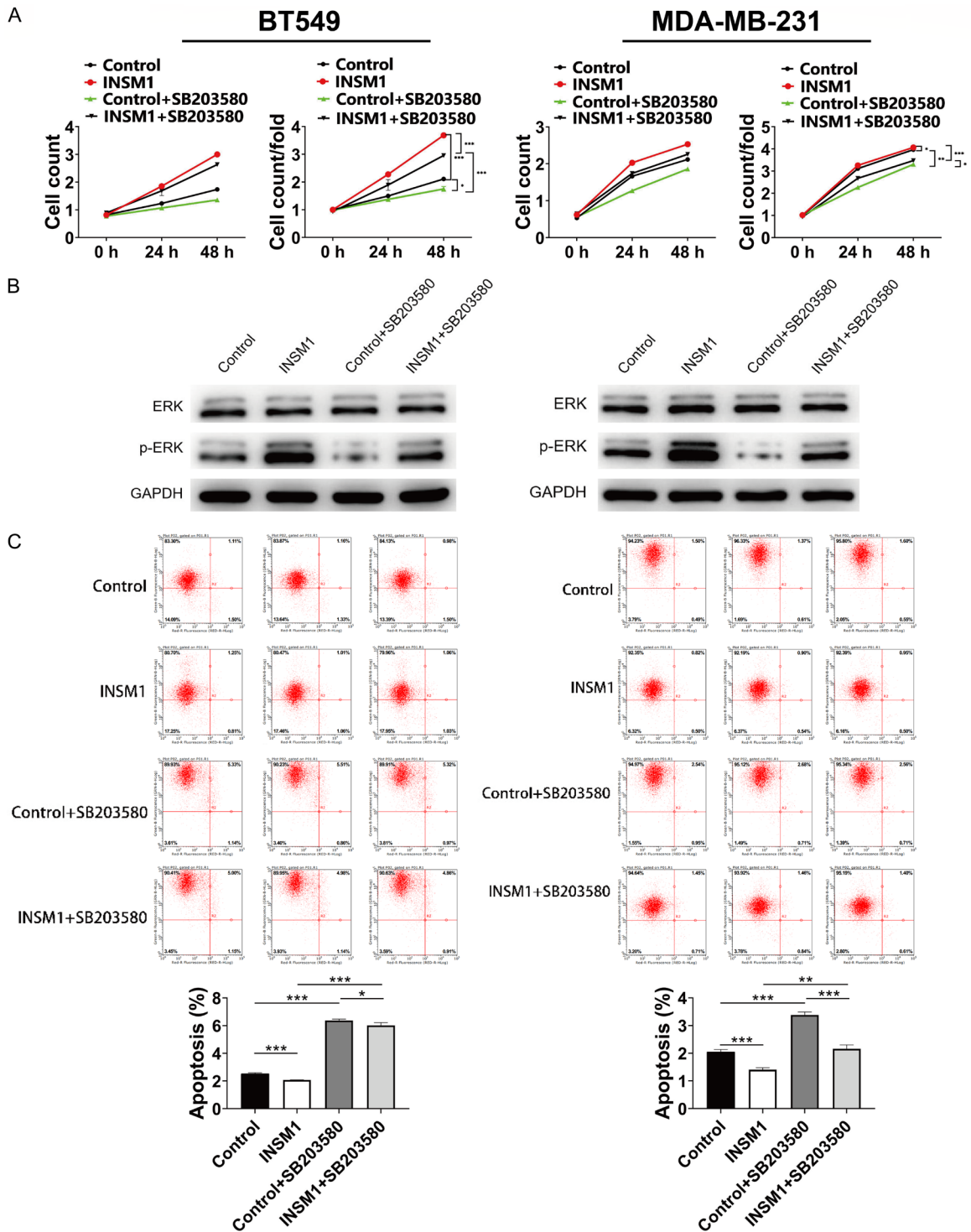


Figure 6. INSM1 influences p-ERK phosphorylation. A. CCK-8 assay investigates changes in cell proliferation when INSM1 is downregulated in BC cells, with or without the p38 MAPK inhibitor SB203580. B. Western blot identifies changes in ERK and p-ERK protein levels in cells with downregulated INSM1, with or without SB203580. C. Flow cytometry examines the impact of INSM1-mediated p-ERK phosphorylation on BC cell diagnosis. Data are presented as mean \pm SD from a minimum of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

C-MYC protein is involved in various cellular processes, including growth, cell cycle, differ-

entiation, apoptosis, angiogenesis, metabolism, DNA repair, protein translation, immune

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response, and stem cell formation [58-60]. Research suggests that the interaction between MYC and the zinc finger protein MIZ1 contributes to the downregulation of certain genes [61]. Chen et al. [62] demonstrated that INSM1 increases the level of MYC and enhances its stability by activating the PI3K/AKT/GSK3 β signaling pathway in neuroblasts. The positive feedback loop between MYC and INSM1 stimulates the proliferation of neuroendocrine cells. Additionally, INSM1 expression enhances the invasiveness and tumorigenicity of neuroendocrine cells [48]. Studies on neuroendocrine lung cancer have shown that INSM1 activation promotes MYC stabilization in neuroendocrine lung cancer cells, and INSM1 is a necessary signaling pathway for MYC carboxylation, suggesting a positive regulation of MYC by INSM1 [62-64]. Chen et al. [62] demonstrated that INSM1 increases the level of MYC and enhances its stability by activating the PI3K/AKT and MEK/ERK 1/2 signaling pathways in neuroblasts. In our study, we observed that knocking down INSM1 in breast cancer cells led to a downregulation of C-MYC protein levels and inhibited the regulatory effect of C-MYC on breast cancer cells. Furthermore, we discovered that INSM1 works in conjunction with SKP2 to influence and regulate C-MYC, which aligns with the research conducted by Kim et al. [65], where SKP2 was found to regulate MYC protein stability and activity.

Although current studies focusing on targeting in breast cancer generally consider direct targeting of transcription factors as challenging for achieving effective tumor suppression, research in lung cancer has demonstrated the effectiveness of targeting INSM1-related signaling pathways in the presence of INSM1 expression [66], which aligns with the findings of this study. Therefore, targeting multiple signaling pathways associated with the activation of the INSM1/MYC transcription factor may be an important approach to delaying or blocking the growth of breast cancer. This study suggests that INSM1 could serve as a novel therapeutic target for breast tumors. MYC plays a significant role in promoting the survival and migration of various tumors through diverse pathways [67, 68]. Numerous studies have also explored MYC-targeted therapies to achieve the inhibition of different types of tumors, although direct targeting of MYC has been con-

sidered challenging [69-71]. This study introduces a new concept for the targeted regulation of MYC and the ERK pathway: by selectively modulating INSM1, it affects the stability of the SKP2/C-MYC/ERK pathway and thereby achieves therapeutic effect in breast cancer treatment.

Conclusions

Our study indicates that INSM1 is highly expressed in breast cancer. INSM1 participates in promoting the development of breast cancer by interacting with SKP2 to regulate MYC and the phosphorylation of ERK pathway. Finally, low INSM1 expression is correlated with less aggressive clinicopathological staging and favorable survival outcomes.

Disclosure of conflict of interest

None.

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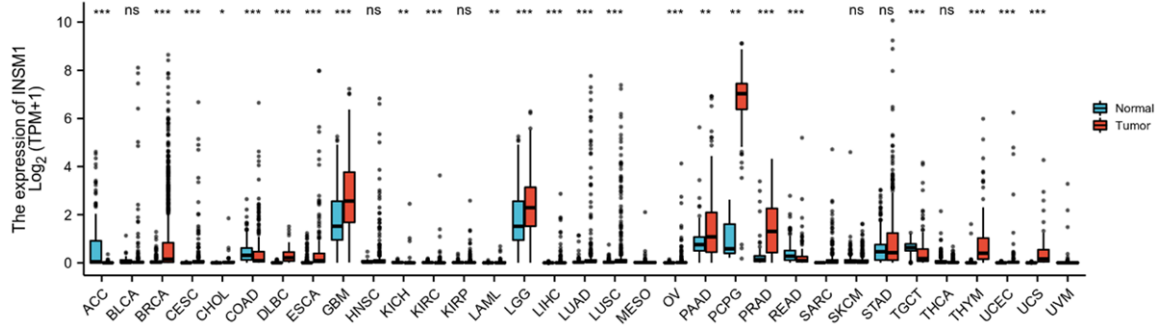


Figure S1. INSM1 is highly expressed in a variety of cancer tissues.

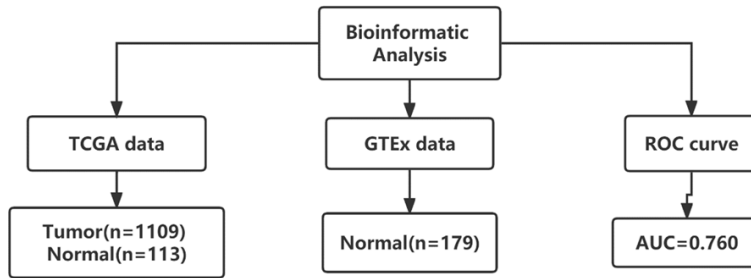


Figure S2. Flow chart of bioinformatic analysis.

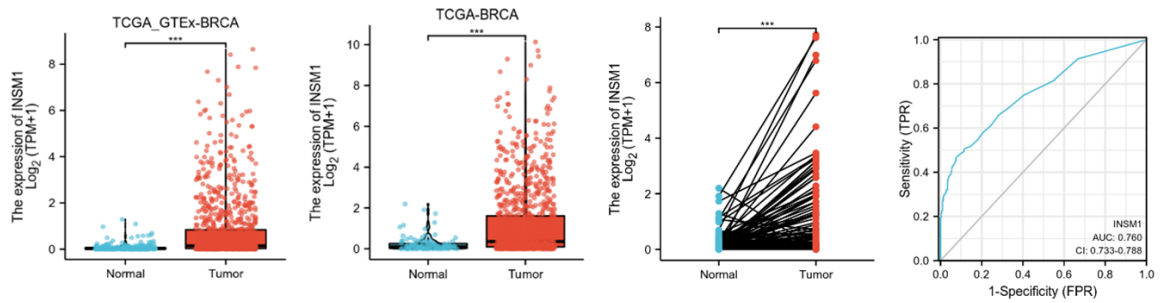


Figure S3. Figures of bioinformatic analysis. Abbreviations: TCGA: The Cancer Genome Atlas project; BRCA: breast cancer.