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Original Research

Stabilization of Notch1 and β-catenin in response to ER- breast cancer-specific up-regulation of PSAT1 mediates distant metastasis

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ABSTRACT

Breast cancer has the highest incidence in women worldwide, with a mortality rate second only to lung cancer. Distant metastasis is the major cause of breast cancer-induced death. While upregulation of phosphoserine aminotransferase 1 (PSAT1) has been reported in several cancer types, its specific roles in breast cancer and potential involvement in distant metastasis remain unclear. In our study, PSAT1 was upregulated in metastatic breast cancer and promoted distant metastasis both in vitro and in vivo. Data obtained from transwell and wound healing, colony, sphere assays and detection of various malignant phenotypic markers showed that PSAT1 mediates distant metastasis by promoting invasion, migration, proliferation, anti-apoptosis, stemness and angiogenesis in breast cancer cells. Mechanistically, PSAT1 activated Notch and β-catenin signaling pathways, leading to enhanced distant metastasis. The clinical relevance of PSAT1 in breast cancer was additionally investigated, which revealed associations of poorer patient prognosis with high PSAT1 mRNA and protein expression. In summary, PSAT1 is a critical molecular regulator of distant metastasis that may effectively serve as a marker of poor prognosis in breast cancer.

Abbreviations

TCGA	The Cancer Genome Atlas
GEO	Gene Expression Omnibus
GSEA	Gene set enrichment analysis
qRT-PCR	Quantitative reverse transcription-polymerase chain reaction
IHC	Immunohistochemistry
Co-IP	Co-immunoprecipitation
WB	Western blot
EMT	Epithelial-mesenchymal transition
CHX	Cycloheximide.

Introduction

Breast cancer is a major threat to the physical and mental health of women worldwide. In 2021, breast cancer surpassed lung cancer in terms of incidence, with the highest number of new cases on a global scale [1,2]. Breast cancer is divided into four subtypes according to estrogen receptor (ER), progesterone receptor (PR) and human epithelial growth factor receptor 2 (Her2) status: luminal A, luminal B, Her2-enriched and triple-negative [3,4]. Among the four subtypes, luminal A and luminal B are ER-positive (ER+) while Her2-enriched and basal-like breast cancer types do not express ER. Due to the lack of targets for endocrine therapy, ER-negative (ER-) breast cancer is generally associated with poor prognosis. Distant metastasis is the major cause of breast cancer-related mortality [5]. Compared to the ER+ subtype, ER- breast cancer displays higher invasion and metastasis potential [6,7]. Bone is the most common site of breast cancer metastasis [8], followed by brain, lung, liver and other regions [9,10]. No effective therapies are currently available for distant metastatic breast cancer owing to a lack of specific targets and comprehensive treatment is usually based on the primary focus site. Thus, specific markers and efficient targets remain an urgent medical requirement to improve prognosis.

Phosphoserine aminotransferase 1 (PSAT1) is a critical enzyme in serine metabolism that acts synergistically with phosphoglycerate dehydrogenase (PHGDH), phosphoserine phosphatase (PSPH), and serine hydroxymethyltransferase (SHMT) to convert 3-phosphoglycerate (3-

Abbreviations: ER, Estrogen receptor; HE, Hematoxylin-eosin; NICD1, Notch1 intracellular domain; PSAT1, Phosphoserine aminotransferase 1; IP, Immonoprecipitation.

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PG) into L-serine [11]. Dysregulation of PSAT1 is usually associated with phosphoserine aminotransferase deficiency and Neu-Laxova Syndrome [12,13], which are correlated with abnormal serine metabolism. In recent years, PSAT1 has been identified as an oncogene. A number of studies have reported that PSAT1 is upregulated in lung, colorectal and ovarian cancer and correlated with poor prognosis [14–16]. Consistent with these findings, we observed upregulation of PSAT1 in metastatic breast cancer from several GEO datasets. However, the specific role of PSAT1 in breast cancer and its potential involvement in distant metastasis remain unclear.

PSAT1 participates in the second step of serine synthesis, which involves conversion of phosphohydroxypyruvate (3PHP) produced by PHGDH into phosphoserine under physiological conditions [17], as well as ovarian cancer growth through regulating the oxidation–reduction balance [14]. PSAT1 is reported to increase cisplatin resistance in cervical cancer cells through inducing proliferation and suppressing apoptosis via activation of the PI3K/Akt signaling pathway [18]. However, cancer metastasis is a continuous and complex process that includes epithelial-mesenchymal transition (EMT), proliferation, stemness, and angiogenesis [19], and the precise biological pathways mediating distant metastasis require further exploration.

Aberrant activation of Notch and β-catenin signaling pathways plays an important role in malignant biological processes of breast cancer, such as mediating tumor stemness to promote survival and drug resistance, ultimately enhancing distant metastasis [20,21]. In colorectal cancer, high-frequency mutations of critical molecules meditate aberrant activation of the β-catenin pathway [22] while high-frequency mutation of Notch triggers continuous activation of the pathway in leukemia [23]. However, these mutations are rare in breast cancer, which prompted us to further explore the mechanisms underlying abnormal activation of these two pathways in this cancer type. Aberrant expression of PSAT1 is clearly involved in regulation of Notch and β-catenin signaling pathways, but the specific mechanisms and potential involvement of these two signaling pathways in distant metastasis require further exploration.

Materials and methods

Tissue specimens

The breast cancer tissues and normal breast tissues were all obtained from the First Affiliated Hospital of Chongqing Medical University and Shanghai Outdo Biotech Company (China). All samples were reviewed and subjected to histological as reported previously.

Animal studies

MDA-MB-231-luc cells with PSAT1 overexpressing or PSAT1 KD and their respective control cells were injected intracardially (5×10^5) into female BALB/c-nu mice (age of 5–6 weeks, 18–20 g), metastases were viewed by bioluminescent imaging weekly. For bioluminescent imaging assay, mice were injected intraperitoneally with 150 mg/kg luciferin before imaging. After inhaling anesthetized mice with sevoflurane, images were taken and analyzed with Spectrum Living Image 4.2 software (Caliper Life Sciences). At the indicated experimental endpoints, mice were anesthetized and sacrificed, various organs (brain, lung, liver, spleen, renal and bones) were resected, sectioned, and histologically examined by H&E staining. All animal studies were approved by the SYSU Institutional Animal Care and Use Committee.

Immunohistochemistry (IHC)

Sections of BRCA tissues, adjacent normal tissues and bone metastases from metastatic mice models were obtained from the formalin-fixed, paraffin-embedded tissue blocks. A variety of antibody (Supplementary materials and methods) was used to perform immunostaining. The sections were deparaffinized in a series of xylene and hydrated in a graded series of ethanol solutions. Antigen retrieval was performed with 10mM citrate buffer solution (pH 6.0) in a pressure cooker (20psi for 10min). 3% hydrogen peroxide was used to quench endogenous peroxidase for 20 min at 37 °C and 10% normal goat serum was used to block nonspecific binding for 1 h at room temperature. Sections were then incubated with the primary antibody at 4 °C overnight. We performed chromogenic detection by using a peroxidase-conjugated secondary antibody (30min) and DAB reagents (5min). Meyer's Haematoxylin was used to counterstained tissue sections. All slides were examined by two experienced pathologists, who were blinded to all clinical data and the immunostaining was measured based on the intensity of immune staining (intensity score) and the quantity of immunoreactive cells (quantity score), as previously reported. The percent positivity was scored as 0, <1%; 1, 1%–10%; 2, 11%–50%; 3, 51%–80% and 4, >81%. The staining intensity was scored as 0, negative staining; 1, weak equivocal staining; 2, unequivocal moderate staining; 3, strong staining. And the final score was obtained by multiplying quantity score and intensity score.

Quantitative real-time PCR

Genomic RNA were isolated from cell lines and tissues using TRIZOL Reagent (life technologies, LOT:338,111),chloroform and Isopropyl alcohol and real-time PCR (qRT-PCR) was performed using SYBR (Promega) according to the instrument manual (7500 System Software). Relative expression was calculated using the 2-△Ct method. Actin was amplified as controls for RNA integrity. The sequences of primers and reaction systems are listed in Supplementary materials and methods.

Cell culture and reagents

Breast cancer cell lines (MDA-MB231, BT-549, SK-BR-3, MCF-7, etc.), HEK293T and immortalized human mammary epithelial cell lines (MCF-10A) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) or collaborators and cultured in PRMI 1640 (Gibco) or DEME (Gibco) medium supplemented with 10% fetal bovine serum (FBS, BI) and 1% penicillin-streptomycin (Gibco) according to standard protocols. Authenticity of the cell lines was verified by short tandem repeat (STR) fingerprinting.

Construction of stable cell lines

To produce shRNA lentivirus, 293T cells were transfected with lentiviral vector and packaging plasmids (psPAX2 and PMpMD2.G). Titres were collected 48hr post-transfection. The precipitated lentivirus was resuspended in PBS and aliquoted for storage at −80 °C. For shRNA lentivirus infection, cells were incubated with shRNA lentivirus for 16hr. At 48 hrs post-infection, puromycin was added to select virally infected cells for further experiments. The sequences within genes which shRNA targeted, and DNA sequences of siRNA were listed in Supplementary materials and methods.

Western blot

Cultured cells or cells extracted from human breast cancer and patient-matched normal tissues were lysed with sample buffer (10% Glycerol, 1.5% SDS, 12.5% Tris). Western Blot (WB) was performed according to standard procedures as previously described work [24]. Primary antibodies used in WB were all listed in supplementary materials and methods. We used Restore™ Western Blot Stripping Buffer (#21,059, Thermo Fisher) to strip protein mixture on the PVDF membranes. Detection was performed by using chemiluminescence kit (Millipore, LOT: 2,101,584).

Transwell and wound-healing assays

For transwell migration assays, breast cancer cells were seeded into the upper chamber. After 24 h of incubation, non-migrated breast cancer cells were scraped off using a cotton swab, and breast cancer cells on the bottom of chamber were fixed with methanol for 10 min and stained using 0.5% crystal violet. Then 3 fields (× 200 magnification) were selected and photographed randomly using an inverted microscope (leica, Germany). Wound-healing assays were used to assess the migration ability of cells. The breast cancer cells were cultured in the six-well plates until the cell confluence reached 95%. After we performed vertical scratched in the six-well plates, serum-free medium was added in it. Afterwards, image collection and migration distance measurement were conducted at 0 h and 48h.

Sphere formation assays

Three thousand cells were seed in ultra-low adherent six-well plates (Thermo Fisher) and were cultured in sphere formation medium (DMEM/F12 serum-free medium supplemented with 2% of B27, 20 ng/ml of EGF, 20 ng/ml of b-FGF, and 4 mg/ml insulin). We added nutrient supplemented medium for the growth of spheres every 2 days for 10 days and cell spheres were photographed and counted under × 200 magnification.

Colony formation assays

The colony formation was used to detect cellular anchorage-dependent growth in vitro. The indicated cells were plated in a six well plate (MDA-MB231, 2000 cells/well; BT-549, 2000 cells/well). Surviving colonies (≥50 cells per colony) were visualized with gentian violet staining and counted.

Dual-luciferase reporter assays

Cells were seeded in quadruplicate in 48-well plates and allowed to settle for 24 hrs. 400 ng of TOP, FOP, or Notch reporter plasmids plus 8 ng of RE plasmid were transfected into the cells using the Lipofectamine 2000 reagent (Thermo Fisher). Forty-eight hours after transfection, Dual-Luciferase reporter assays were performed according to the manufacturer's instructions (Promega, Madison, WI).

Cycloheximide (CHX) chase assay

Cells were treated with 5 μM cycloheximide (MCE, LOT: HY-12,320) and harvested at indicated time points [25]. Then protein was extracted from the cells and western blot analysis was utilized to detect protein expression. Protein levels were measured with the densitometric intensity.

Immunoprecipitation

For IP experiment, Mag25K/Protein A/G (enriching biotechnology, LOT:P28005) Beads are used to adsorb specifically bound proteins. The antibodies used to incubate cell lysates are listed in Supplementary materials and methods. The Co-IP complex was analyzed by Western blot. Anti-mouse IgG was used as the second antibody to detect Co-IP complex.

Bioinformation and statistical analysis

Three data sets containing breast cancer samples from the Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO; GSE103357, GSE125989) were downloaded and were utilized to explore expression level of PSAT1.The limma package [26] was used to explore differentially expressed genes (DEGs) between primary tumor and metastatic foci from these two geo data sets (adjusted $p < 0.05$ and fold change ≥ 1). Kaplan-Meier Plotter was used to evaluate the prognostic significance of PSAT1 expression [27]. Gene set enrichment analysis was used to find potential biological changes between high and low PSAT1 expression group. GraphPad (version 8.4.0, GraphPad Prism Software, USA) and SPSS22 (version 22.0, IBM, SPSS, Chicago) were used for the statistical analyses. The chi-squared test, Student's *t*-test, Spearman correlation test, etc., were used as appropriate.

Study approval

All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Chongqing medical university. The use of breast cancer donors' samples were approved by the Institutional Research Ethics Committees of Chongqing medical university. Donors provided prior written informed consent

Results

PSAT1 is upregulated in metastatic breast cancer

To identify the critical molecules mediating breast cancer metastasis and potential therapeutic targets, differentially expressed genes were identified by analyzing GSE103357 and GSE125989 datasets in the GEO database (Fig. 1A-B). Notably, PSAT1 was one of the remarkably upregulated genes in both breast cancer cells with high metastatic potency and metastatic foci. Immunohistochemistry also indicated that PSAT1 was remarkably upregulated in breast cancer lung metastatic tissue (Fig. 1C). Interestingly, although we found that PSAT1 was downregulated in breast cancer samples in GEPIA datasets, PSAT1 was significantly upregulated in ER- breast cancer in TCGA (Fig. 1D), We further analyzed PSAT1 expression in breast cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE) (Fig. 1E). Interestingly, PSAT1 mRNA and protein levels in ER- breast cancer cell lines were markedly higher than those in normal breast cell lines while levels in ER+ breast cancer cell lines were significantly lower, as established with q-PCR and western blot analyses (Fig. 1F). Furthermore, PSAT1 expression was increased in ER- and decreased in ER+ breast cancer tissues compared with para-cancer tissues based on western blot and immunohistochemistry findings (Fig. 1G-H). As patients with ER- breast cancer were more likely to develop distant metastases than the ER+ group, we examined the hypothesis that upregulated PSAT1 in ER- breast cancer could contribute to high metastatic potential. To further establish the key regulatory genes, we employed a nude mouse model of distant metastatic breast cancer. Specifically, MDA-MB-231-luc metastatic breast cancer cells were injected into ventricles of nude mice and expression patterns of PSAT1 examined. Upon formation of brain and bone metastasis, cancer cells from the respective tissues were extracted to acquire brain-specific and bone-specific metastatic MDA-MB-231-luc cells (designated MDA-MB-231-luc-BrM and MDA-MB-231-luc-BoM, respectively) (Fig. 1I). Consistent with earlier in vitro findings, PSAT1 was upregulated in MDA-MB-231-BrM and MDA-MB-231-BoM breast cancer cells with greater metastatic potency via qRT-PCR (Fig. 1J). To further ascertain the involvement of PSAT1 in metastatic progression, the correlations between PSAT1 and metastasis-related genes or signatures from expression profiles of a cohort of breast cancer patients from The Cancer Genome Atlas (TCGA) were analyzed. As shown in Fig. 1K, expression of PSAT1 was positively correlated with CDH2, SNAI1, SNAI2 and VIM genes mediating epithelial mesenchymal transition (EMT). Taken together, our findings clearly suggest that PSAT1 participates in distant metastasis in breast cancer.

PSAT1 promotes distant metastasis in vivo

To further validate PSAT1-mediated distant metastatic breast cancer, MDA-MB-231-luc cells stably overexpressing PSAT1 were generated.

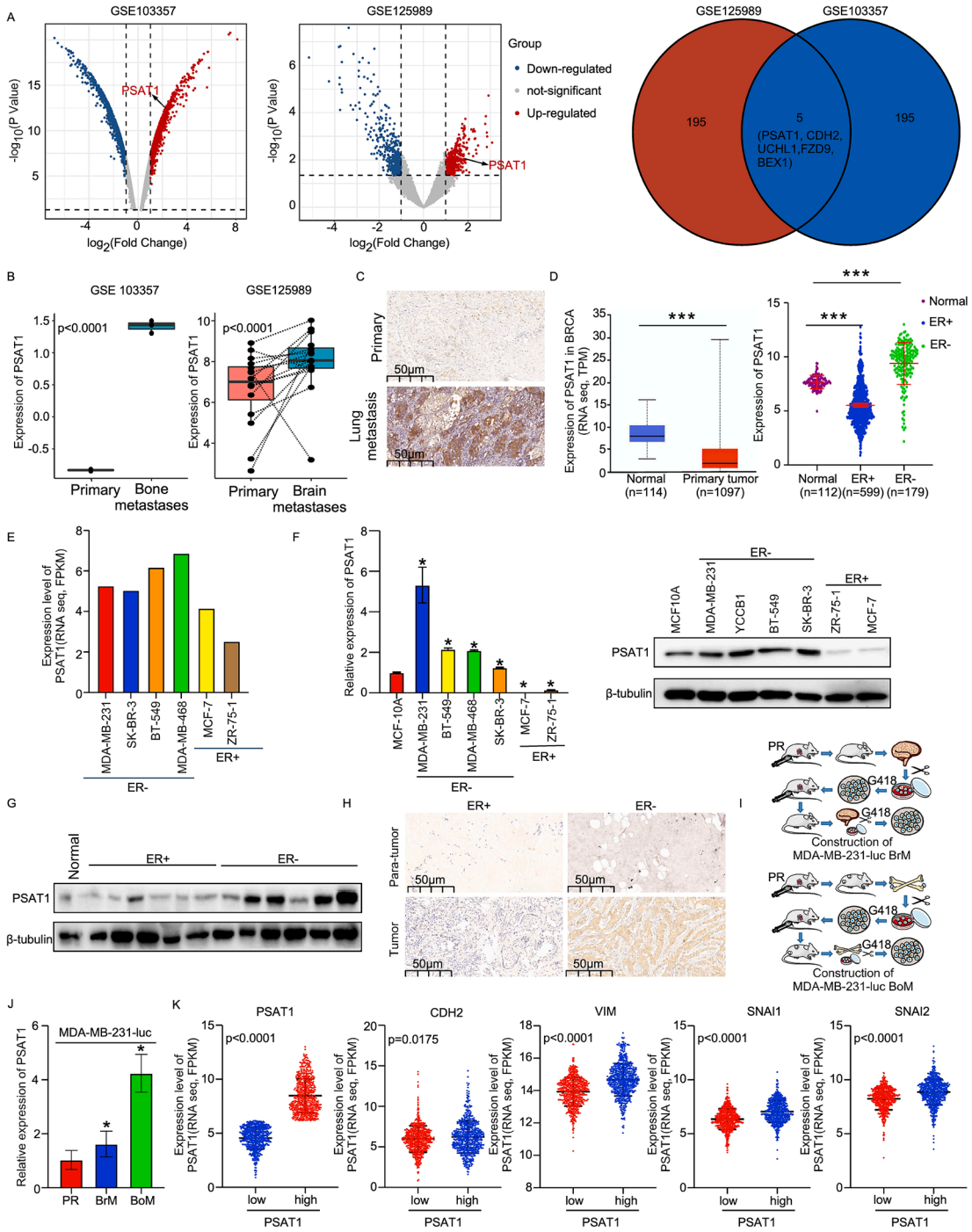

Fig. 1. PSAT1 is upregulated in metastatic breast cancer. (A) Gene difference analysis of GSE103357 (2 replicates of primary breast cancer cell line compared with 3 replicates of bone metastatic breast cancer cell lines) and GSE125989 (16 paired match from primary breast cancers and brain metastases) in GEO datasets. Volcano plots showed the top 200 differentially expressed genes in metastatic cancer cells or metastatic foci of GSE103357 and GSE125989 (left panel), respectively. $Log_2FC>1$. The upregulated genes both in the two datasets are shown in the Venn diagram (right panel). (B) Box plots of the differential expression of PSAT1 in metastatic or primary foci in GSE103357 and GSE125989. (C) Immunohistochemical analysis of expression levels of PSAT1 in primary foci of breast cancer and lung metastatic foci. Scale bar: 50μm. (D) Expression of PSAT1 in primary breast cancer and the adjacent non-tumor tissues (left panel) in GEPIA and expression of PSAT1 in normal breast sample, ER+ and ER- breast cancer samples from the TCGA database (right panel). Data are presented as mean ± SD. ANOVA was used to calculate p-values. (E) Expression of PSAT1 in breast cancer cell lines from the CCLE database. (F) qRT-PCR and western blot analyses to determine the mRNA and protein level of PSAT1 in breast cancer cell lines compared to benign primary breast epithelial cells (MCF10A), respectively. (G-H) PSAT1 protein expression in ER+ and ER- breast cancer and para-cancer tissues determined via western blot and immunohistochemistry. Scale bar: 50 μm. (I) Bone and brain metastatic nude mouse models were established via ventricle injection of MDA-MB-231-luc, bone metastatic foci and brain metastatic foci were dissected and MDA-MB-231-luc-BrM and MDA-MB-231-luc-BoM cell lines were selected. (J) PSAT1 mRNA and protein expression in MDA-MB-231-BrM (BrM) and MDA-MB-231-BoM (BoM) cell lines compared to MDA-MB-231-Parental (Parental). (K) Expression of PSAT1 and metastasis-related genes (CDH2, VIM, SNAI1 and SNAI2) levels of 890 BRCA patients in TCGA datasets grouped according to PSAT1 expression.

Simultaneously, two different short hairpin RNAs (shRNA1 and shRNA2) were utilized to silence PSAT1 in MDA-MB-231-luc (PSAT1 KD). The efficacy of ectopic expression and knockdown was validated using qPCR and western blot analyses (Fig. 2A-B). MB-231-luc cells with ectopic PSAT1 expression and the corresponding control cells were injected into the left ventricles of nude mice, followed by imaging every week. In vivo imaging revealed that overexpression of PSAT1confers potent metastatic properties to breast cancer cells, especially tend to metastasize to bone, as shown in Fig. 2C-D. HE staining experiments further prove it. (Fig. 2E). Statistical chart of metastasis in nude mice of PSAT1 and vector groups were shown in Fig. 2F, respectively.Simultaneously, MB-231-luc/PSAT1 KD and its corresponding control cells were injected into ventricles of nude mice and imaged every week. Notably, in vivo imaging disclosed lower metastatic potential in mice from the MB-

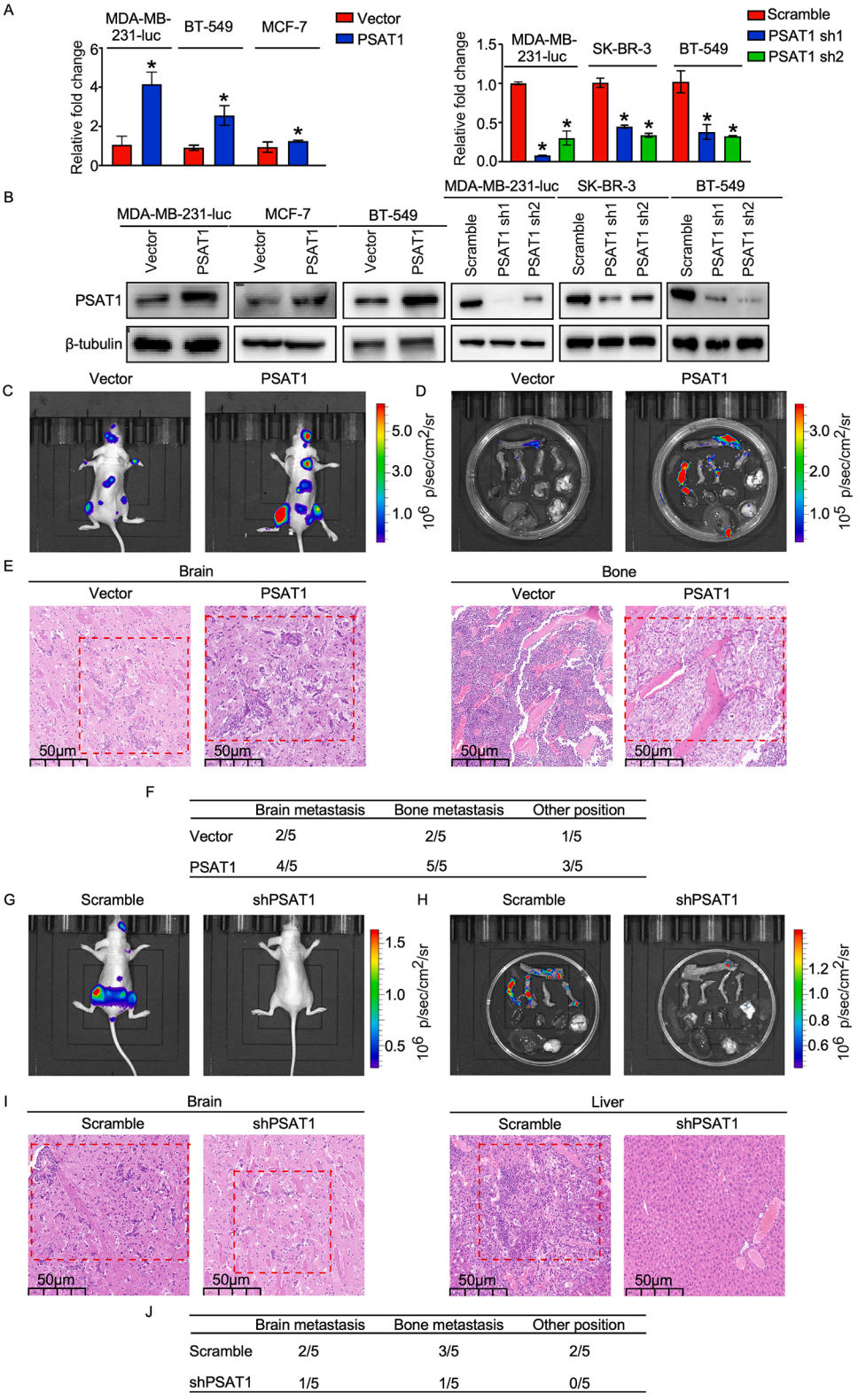

Fig. 2. PSAT1 promotes breast cancer distant metastasis in vivo. (A, B) PSAT1 stably overexpressing and knockdown (KD) breast cancer cell lines validated via qRT-PCR and western blot. (C, D) MDA-MB-231-luc-Vector and MDA-MB-231-luc-PSAT1 cells were injected into cardiac ventricles of nude mice (n = 5 per group). Representative bioluminescent images of animals and dissected organs 21 days after intraventricular injection are shown. (E) HE staining to show the distant metastasis potentials of breast cancer cells lines with or without PSAT1 ectopic expression.(F) Statistical chart of metastasis in PSAT1 and vector group of nude mice. (G, H) MDA-MB-231-luc-Scramble and MDA-MB-231-luc-KD cells were injected into cardiac ventricles of nude mice (n = 5 per group). Representative bioluminescence images of animals and dissected organs 21 days after intraventricular injection are shown. (I) HE staining of brains and livers from mice in Fig. 2g and h. (J) Statistical chart of metastasis in shPSAT1 and scramble group of nude mice.

231-luc/PSAT1 KD group relative to the control group (Fig. 2G-H). HE staining of dissected organ and bone tissues consistently revealed fewer metastatic loci in mice injected with cancer cells depleted of PSAT1 (Fig. 2I). Statistical chart of metastasis in nude mice of shPSAT1 and scramble groups were shown in Fig. 2J, respectively. These results indicate that PSAT1 plays a pivotal role in development of distant metastasis in breast cancer.

PSAT1-facilitated aggressiveness of breast cancer cells promotes early metastasis

The development of distant metastasis in breast cancer is an extremely complex biological pathway regulated by multiple biological steps including invasion and migration mediated by EMT, anti-apoptosis, stem cell tumorigenesis, and neovascularization processes that facilitate growth of metastatic foci. As shown in Fig. 2, PSAT1 promoted distant metastasis in vivo. To further establish the specific mechanisms underlying PSAT1 activity, BT-549 and MCF-7 cells stably overexpressing PSAT1 and BT-549 as well as SK-BR-3 cells with

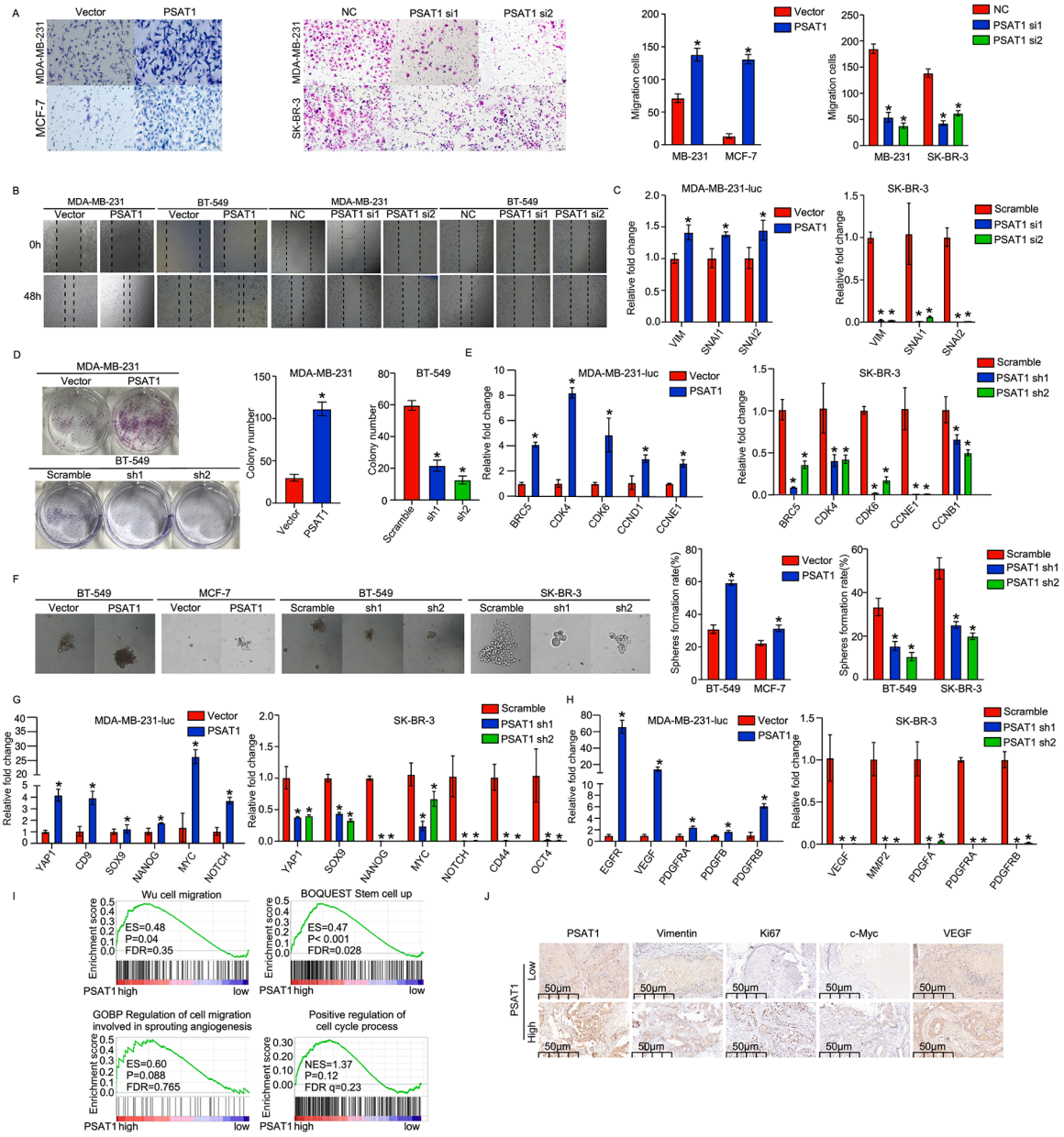

Fig. 3. PSAT1-facilitated aggressiveness of breast cancer cells mediates early metastasis. (A) Transwell assay to evaluate the migration ability of breast cancer cells with PSAT1 overexpression or depletion. Scale bar: 50 μm. (B) Wound healing assay performed to assess the migration abilities of PSAT1-overexpressing and PSAT1 KD breast cancer cells. Scale bar: 50 μm. (C) qRT-PCR analysis of changes in EMT markers in PSAT1-overexpressing and PSAT1 KD cells. (D) Colony forming ability of PSAT1-overexpressing and knockdown cells. (E) qRT-PCR analysis of changes in proliferation and anti-apoptosis markers of breast cancer cells with PSAT1 overexpression or knockdown. (F) Sphere assays to assess stemness of breast cancer cells with overexpression and knockdown of PSAT1. Scale bar: 50 μm. (G) qRT-PCR analysis of changes in stemness markers of breast cancer cells with PSAT1 overexpression or knockdown. (H) qRT-PCR analysis of changes in angiogenesis markers in PSAT1-overexpressing and knockdown breast cancer cells. (I) GSEA for exploring correlations of PSAT1 with markers of EMT, proliferation, anti-apoptosis, stemness, and angiogenesis based on expression profiles of breast cancer patients in TCGA. (J) Immunohistochemical analysis of expression levels of EMT, proliferation, anti-apoptosis, stemness and angiogenesis markers in primary breast cancer tissues with high expression of PSAT1 and low expression of PSAT1.Scale bar: 50 μm.

silencing of PSAT1 were successfully constructed (Fig. 2A-B). In transwell and wound healing assays, overexpression of PSAT1 promoted the invasion and migration abilities of breast cancer cells. Conversely, decreased invasive and migratory capacity was observed in breast cancer cells depleted of PSAT1. EMT markers were upregulated in PSAT1-overexpressing but downregulated in PSAT1 KD breast cancer cells, as determined via qRT-PCR (Fig. 3A-C). In the colony formation assay, upregulation of PSAT1 induced an increase in the number of colonies, which was decreased upon downregulation of PSAT1. qRT-PCR findings disclosed higher expression of proliferation- and anti-apoptosis-related genes in PSAT1-overexpressing cells, with the opposite trend in PSAT1 KD groups (Fig. 3D-E). In the sphere assay, overexpression of PSAT1 markedly promoted tumor sphere formation in breast cancer cells while its silencing prevented breast cancer cells from forming tumor spheres. Simultaneously, markers of stemness were upregulated in PSAT1-overexpressing cells, supporting the theory that PSAT1 regulates cancer stem cell properties (Fig. 3F-G). At the same time, the angiogenesis markers were also obviously upregulated in PSAT1 overexpressing cells (Fig. 3H). Our results collectively indicate that PSAT1 promotes the processes of EMT, proliferation, anti-apoptosis, stemness and angiogenesis. Next, we focused on the clinical significance of PSAT1 in regulating the malignant biological process. GSEA was performed on the expression profiles of breast cancer patients in TCGA to explore potential correlations of PSAT1 with markers of EMT, proliferation, anti-apoptosis, stemness and angiogenesis (Fig. 3I). Our data showed associations of PSAT1 with EMT, proliferation, anti-apoptosis, stemness and angiogenesis of cancer cells in breast cancer patients. Next, we ascertained whether PSAT1-mediated metastasis of breast cancer involves regulatory effects on EMT, proliferation, stemness, anti-apoptosis and angiogenesis. In primary breast cancer tissues, markers of EMT, proliferation, anti-apoptosis, stemness and angiogenesis were upregulated in tissues with high expression of PSAT1(Fig. 3J). Data from our in vitro experiments along with changes in expression patterns of the above markers collectively suggest that PSAT1 promotes metastasis through regulation of EMT, proliferation, anti-apoptosis, stemness and angiogenesis.

PSAT1 activates wnt/β-catenin and notch signaling pathways by stabilizing the respective proteins

We further explored the mechanisms underlying PSAT1-induced aggressiveness of breast cancer. To this end, expression patterns of downstream genes of several cancer-related signaling pathways (Notch, Wnt/β-catenin, NF-κB, HIF-1, TGF-β and MAPK) were examined in breast cancer cells with PSAT1 overexpression or depletion via qRT-PCR. Consistently, the majority of markers of β-catenin and Notch pathways were upregulated in PSAT1-overexpressing breast cancer cells and conversely downregulated in PSAT1 KD cells (Fig. 4A and supplementary Fig. A-B). In the luciferase reporter assay, overexpression of PSAT1 significantly activated transcription of β-catenin and Notch while silencing of PSAT1 exerted the opposite effects (Fig. 4B-C). To validate whether PSAT1 regulates β-catenin and Notch signaling in breast cancer patients, GSEA analysis was conducted (Fig. 4D). The results revealed association of high PSAT1 expression with enriched Notch and Wnt/β-catenin signaling. The mechanisms by which PSAT1 regulates β-catenin and Notch signaling pathways were further explored. Overexpression of PSAT1 clearly increased Notch1 and β-catenin protein levels while its silencing led to decreased expression of Notch1 and β-catenin (Fig. 4E). Following co-immunoprecipitation (Co-IP) experiments using 293T cells, we found that PSAT1 could combined with NUMB and interferes with binding of the NUMB to Notch1 and β-catenin, leading to reduced ubiquitination of the two proteins (Fig. 4F). Simultaneously, cells were further treated with cycloheximide (CHX) to inhibit protein biosynthesis and protein samples acquired at 0, 1, 2, 4, 8, and 24 h. Overexpression of PSAT1 induced a significant extension in the half-lives of NICD1 and β-catenin while silencing of PSAT1 led to shorter half-lives (Fig. 4G-H). These results support the theory that the ability of PSAT1 to promote breast cancer metastasis is potentially related to regulatory effects on Notch and Wnt/β-catenin signaling pathways. In conclusion, PSAT1 inhibits the ubiquitination-mediated degradation of β-catenin and Notch1, leading to activation of the respective signaling pathways.

Activation of β-catenin and notch signaling mediates PSAT1-induced aggressiveness of breast cancer cells

In view of the critical roles of Notch and β-catenin signaling in regulation of multiple malignant processes (including proliferation, EMT, stemness and angiogenesis), we speculated that PSAT1 promotes malignant phenotypes via these pathways. To examine this hypothesis, PSAT1 KD cancer cells overexpressing NICD1 and β-catenin were generated (Fig. 5A). Interestingly, we found that markers of EMT, proliferation, stemness and angiogenesis were significantly upregulated in PSAT1 KD cells with overexpression of NICD1 and β-catenin (Fig. 5B). The sphere formation ability of PSAT1 KD cancer cells was remarkably increased under conditions of overexpression of NICD1 and β-catenin (Fig. 5C). At the same time, the invasion ability of cells overexpressing NICD1 and β-catenin was also enforced via transwell assay (Fig. 5D). Based on the collective results, we proposed that activation of β-catenin and Notch signaling pathways could enforce the ability of breast cancer cells in promoting breast cancer distant metastasis when silenced PSAT1. Additionally, the above results further identified that PSAT1 mediates distant metastatic breast cancer via β-catenin and Notch signaling pathways.

Aberrant upregulated PSAT1 is a potential biomarker of early metastasis in breast cancer

To further explore the clinical relevance of PSAT1 in breast cancer metastasis, Kaplan-Meier Plotter was applied for analysis of the correlation between PSAT1 expression and survival. Patients expressing higher PSAT1 tended to have shorter overall survival (OS) and distant metastasis-free survival (DMFS) than those with lower PSAT1 expression (Fig. 6A-B). Besides, in different grades of breast cancer, patients with high expression of PSAT1 had shorter DMFS (Fig. 6C). Moreover, in both ER+ and ER- breast cancer groups, patients with high expression of PSAT1 also had shorter DMFS, suggesting that PSAT1 also participates in ER+ breast cancer metastasis despite relatively lower levels of expression (Fig. 6D). Kaplan-Meier Plotter data showed a correlation between PSAT1 mRNA expression and prognosis. Accordingly, tissue chip immunohistochemistry was conducted to explore the correlation between PSAT1 protein expression and prognosis (Fig. 6E). In view of the marked upregulation of PSAT1 in ER- breast cancer, we divided patients into two groups according to ER status. ER- breast cancer patients with high expression of PSAT1 tended to have significantly shorter survival than those with low expression of PSAT1. While PSAT1 expression was generally lower in ER+ patients, shorter OS was reported in patients with relatively high expression of PSAT1 (Fig. 6F). The above results clearly indicate that PSAT1 acts as an important mediator of distant metastasis in patients with both ER- negative and ER-positive breast cancer. In view of the lack of clinical biomarkers for early diagnosis of metastatic breast cancer, our findings that PSAT1 is associated with poorer survival prognosis in advanced disease and its high expression signifies earlier distant metastasis in early disease support the utility of PSAT1 as a potential marker of poor prognosis for breast cancer patients.

Discussion

Phosphoserine aminotransferase 1 (PSAT1) is one of the three key enzymes contributing to serine synthesis. In the physiological state, PSAT1 participates in the second step of serine synthesis, which involves conversion of phosphohydroxypyruvate (3PHP) catalyzed by

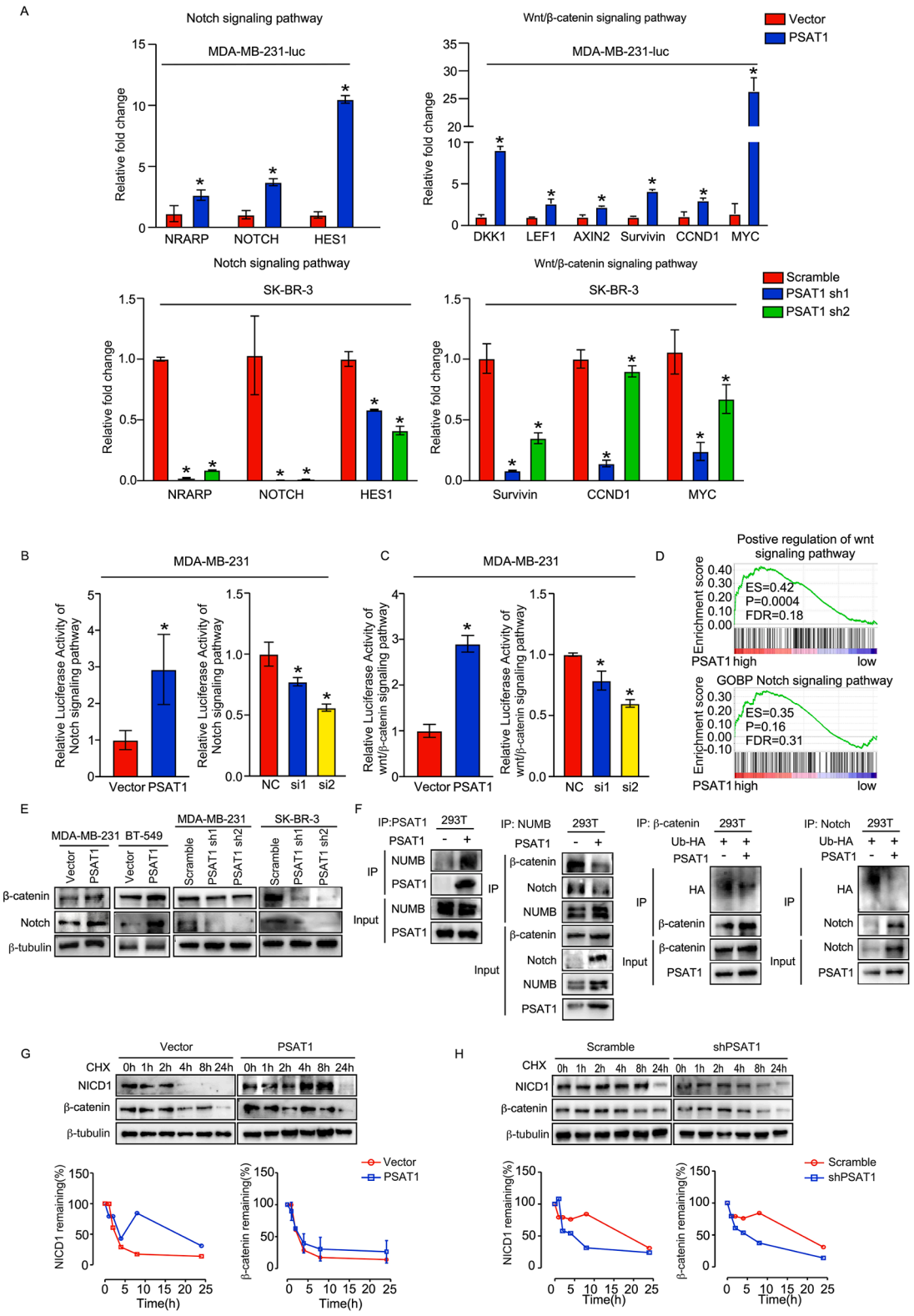

Fig. 4. PSAT1 activates β-catenin and Notch signaling pathways by stabilizing β-catenin and Notch 1 proteins. (A) qRT-PCR analysis of expression level of markers of changes in of Notch, Wnt/β-catenin in breast cancer cells with PSAT1-overexpression or depletion. (B, C) Luciferase reporter assay evaluating transcription of β-catenin and Notch under conditions of overexpression and knockdown of PSAT1. (D) GSEA for exploring the correlations between PSAT1 and markers of β-catenin and Notch1 based on expression profiles of breast cancer patients in TCGA database. (E) WB to assess the protein level of β-catenin and Notch1 in breast cancer cells with PSAT1-overexpression or depletion. (F) Co-immunoprecipitation was conducted to establish the molecules interacting with PSAT1. (G, H) CHX intervention experiments performed to determine the half-lives of NICD1 and β-catenin under conditions of overexpression and silencing of PSAT1.

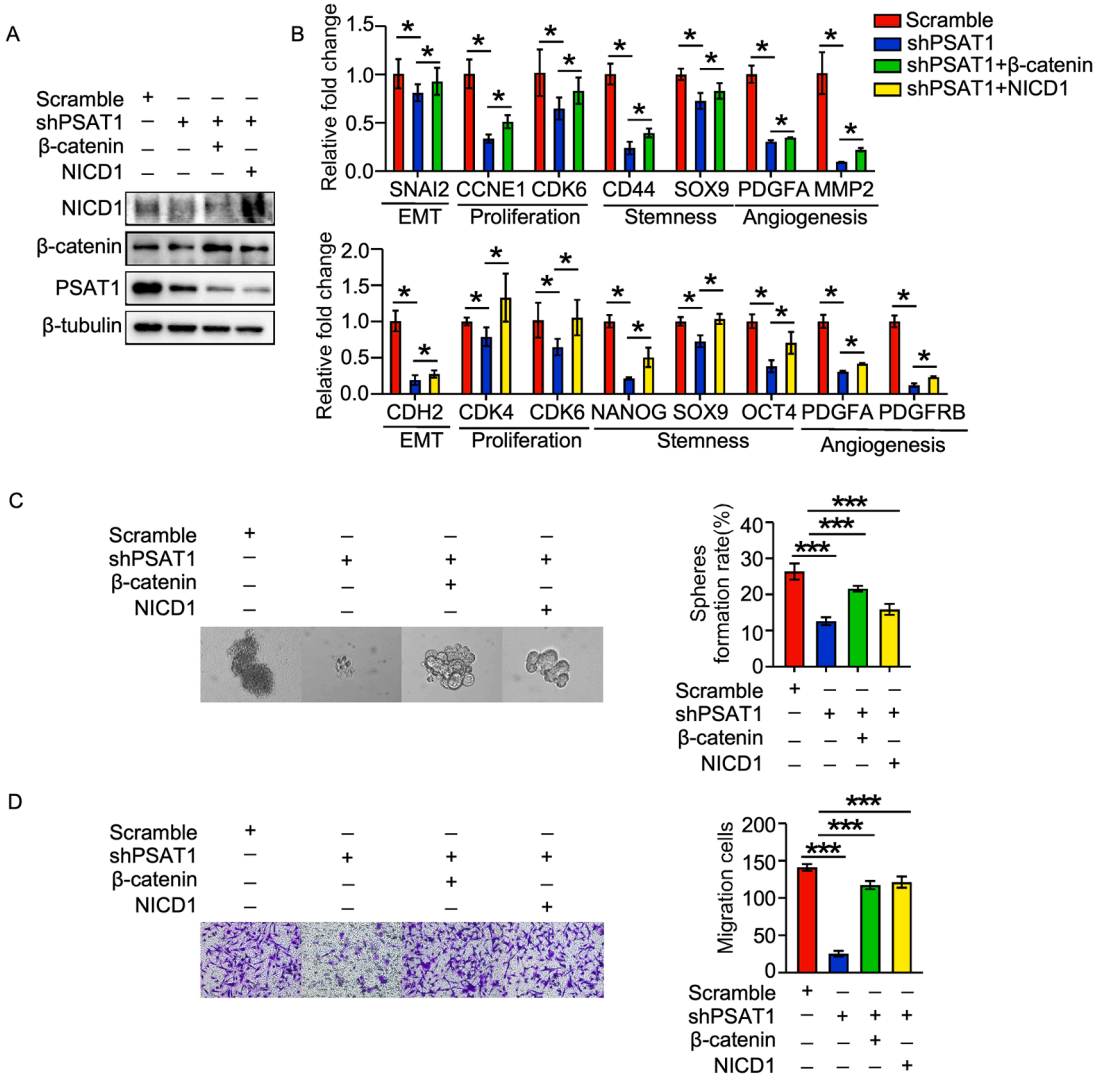

Fig. 5. Activation of β-catenin and Notch signaling mediates PSAT-induced aggressiveness of breast cancer cells and β-catenin and Notch serve as crucial downstream factors promoting metastasis. (A) Western blot experiments validating the efficiency of NICD1 and β-catenin overexpression. (B) qRT-PCR experimets validating the changes of markers of EMT, proliferation, stemeness and angiogenesis in cancer cells overexpressing NICD1 and β-catenin. (C) Sphere assay of stemness of breast cancer cells under conditions of overexpression of NICD1 and β-catenin. (D) Transwell assay for evaluating the migration ability of cancer cells overexpressing NICD1 and β-catenin.

phosphoglycerate dehydrogenase (PHGDH) into phosphoserine [28]. However, aberrant expression of PSAT1 in tumors potentially contributes to tumor progression. For example, PSAT1 is reported to promote tumor cell proliferation by activating the GSK3β/β-catenin/cyclin D1 pathway [15,29]. Moreover, PSAT1 inhibits cell death, thereby promoting chemoresistance in colorectal cancer patients [30]. In the current study, PSAT1 was upregulated in metastatic breast cancer samples from multiple data sets. We further focused on whether PSAT1 acts as a mediator of distant metastatic breast cancer. While previous studies have shown that PSAT1 promotes distant metastasis of lung cancer, the specific roles and underlying mechanisms of action of PSAT1 in breast cancer metastasis have not been established to date [31]. Our experiments revealed high PSAT1 expression in ER- breast cancer samples, which were more prone to distant metastasis. Additionally, ectopic expression of PSAT1 promoted distant metastasis, which was inhibited upon silencing PSAT1 in vivo. Our in vitro experiments consistently demonstrated that PSAT1 promotes the invasion and migration ability of breast cancer cells, supporting a novel role of PSAT1 in the pathology of breast cancer. Furthermore, PSAT1 promoted distant metastasis of breast cancer to the bone in particular. However, the detailed mechanisms underlying PSAT1-mediated metastasis require further elucidation.

As a key enzyme in L-serine synthesis in the normal physiological state [32], PSAT1 utilizes the PHGDH product 3PHP to convert glutamate to α-ketoglutarate, which acts as an anaplerotic intermediate that refuels the TCA cycle and maintains cancer metabolism [33,34]. However, it is unclear whether the regulatory effects of PSAT1 on breast cancer metastasis depend on the function of serine synthetase. Cancer metastasis is a complex and continuous biological process involving several key steps [35]. The tumor cells that initiate formation of the metastatic colony need to break away from the primary tumor, invade the local host tissue matrix, penetrate local lymphatics and blood vessels, survive in the circulation, remain in the capillaries or venules of other organs, penetrate the corresponding parenchyma, and adapt to the new colonized environment or subvert the local microenvironment to adapt to their own needs, and divide to form new tumors [36]. Therefore, EMT, proliferation, anti-apoptosis, angiogenesis and stemness of cancer cells are all indispensable for metastasis. Our in vitro wound healing and transwell assays showed that overexpression of PSAT1 could facilitate invasion and migration of cancer cells. Data from the colony

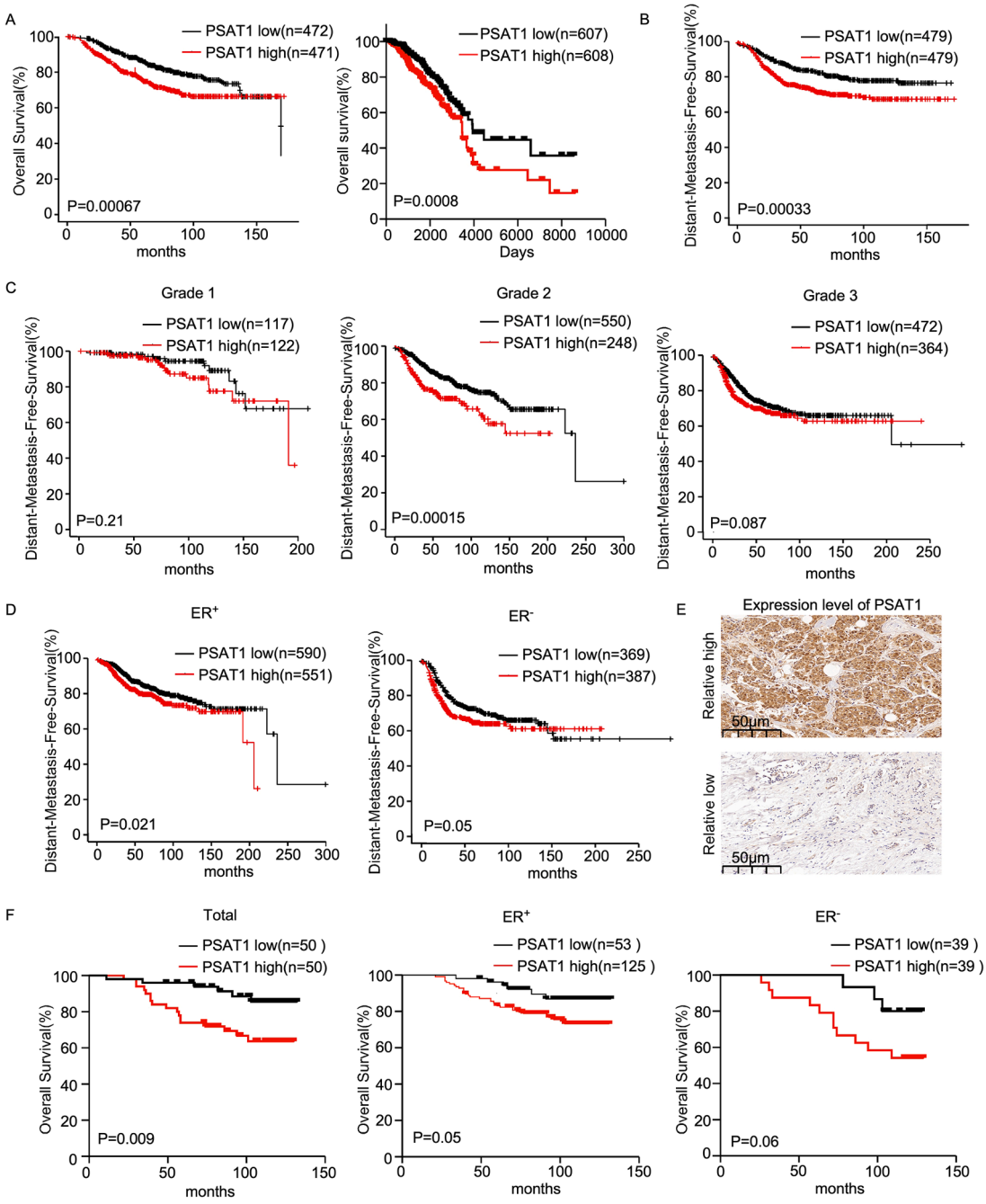

Fig. 6. Aberrantly upregulated PSAT1 is a potential biomarker for indicating early metastasis in breast cancer. (A, B) Kaplan-Meier Plotter analysis of the correlations between PSAT1 and OS and DMFS in breast cancer patients. (C) Kaplan-Meier Plotter analysis of the correlations between PSAT1 and DMFS in patients with grade 1 (left), grade 2 (middle) and grade 3 (right) breast cancer. (D) Kaplan-Meier Plotter analysis of correlations between PSAT1 and DMFS in ER+ (left) and ER- (right) breast cancer patients. (E) Immunohistochemical analysis of tissue chips to evaluate protein expression of PSAT1. Scale bar: 50 μm. (F) OS curve of total (left), ER+ (middle) and ER-(right) breast cancer tissue were generated according to immunohistochemical scores. P values were calculated based on the log-rank test.

formation assay revealed that overexpression of PSAT1 enhanced the proliferative and anti-apoptosis properties of breast cancer cells. Furthermore, sphere assays and qRT-PCR detection of angiogenesis markers confirmed increased stemness and angiogenesis abilities of PSAT1-overexpressing cancer cells. Additionally, PSAT1 mediated EMT, proliferation and anti-apoptosis, stemness and angiogenesis based on data obtained from patient expression profiles in TCGA. In view of the collective findings, we conclude that PSAT1 promotes the malignant phenotypes of EMT, proliferation, anti-apoptosis, stemness and angiogenesis in breast cancer cells, ultimately facilitating distant metastasis.

PSAT1 is reported to promote breast cancer cell proliferation by activating the GSK3β/β-catenin/cyclin D1 signaling pathway [29] in addition to inhibiting cancer cell apoptosis, promoting cell growth and cisplatin resistance through stimulation of the PI3K/AKT signaling pathway [18]. ATF4 has been identified as a downstream factor of PSAT1 in the mediation of cancer cell proliferation [29,37]. However, the precise mechanisms underlying PSAT1-mediated distant metastatic breast cancer have not been elucidated until now. Data from the current study showed association of PSAT1 with increased expression of Notch and β-catenin at the protein level. CHX intervention experiments disclosed that PSAT1 could extend the half-lives of Notch1 intracellular domain (NICD1) and β-catenin and Co-IP experiments additionally

revealed that PSAT1 interacted with NUMB. Thus, we have uncovered a novel pathway whereby PSAT1 activates Notch and β-catenin pathways via inhibiting combination of NUMB with Notch1 and β-catenin, which mediates distant metastasis.

PSAT1 has been identified as a poor prognostic marker in ovarian and colorectal cancer types [38,39]. In view of the finding that PSAT1 promotes distant metastasis of breast cancer both in vivo and in vitro, we further investigated its clinical significance in breast cancer patients. Kaplan-Meier analysis and tissue chip immunohistochemistry showed that patients with high PSAT1 expression (both mRNA and protein) have shorter OS and DMFS. Additionally, patient groups with ER+ and ER- breast cancer were analyzed. Surprisingly, within both patient groups, those with high PSAT1 expression had shorter DMFS than those with low PSAT1 expression, supporting the theory that PSAT1 is a crucial mediator of distant metastasis. Interestingly, in patients with breast cancer diagnosed at an early stage, higher expression of PSAT1 was associated with shorter DMFS. In summary, PSAT1 is a critical molecular regulator that may effectively serve as a potential biomarker of early metastasis in breast cancer patients.

Supplementary material

PSAT1 supplementary information-final.docx
Supplementary figure.jpg

Author contributions

SZ carried out experiments, analyzed data and wrote a draft of the paper with the help of XW; LL and GR conceived the ideas, designed and discussed experiments, supervised progress and extensively edited and communicated regarding the manuscript. All authors were involved in writing the paper and had fiinal approval of the submitted and published versions.

Declaration of Competing Interest

All authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2022.101399.

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