TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Su(var)3—9, Enhancer of Zeste, and Trithorax Domain-Containing 5 Facilitates Tumor Growth and Pulmonary Metastasis through Up-Regulation of AKT1 Signaling in Breast Cancer

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Several studies have confirmed the function of Su(var)3—9, Enhancer of zeste, and Trithorax (SET) domain-containing 5 (SETD5) in post-translational modifications of nonhistone proteins. Mutation of the *SETD5* gene has been implicated in the progression of many human cancers, such as breast cancer (BC), but its functional role in BC progression is still unknown. The current article investigates the clinical significance and the functional role of SETD5 in BC. Our studies show that SETD5 expression in BC was related to poor clinical outcomes, including lymph node metastasis and advanced clinical stage. SETD5 expression positively correlated with tumor-associated macrophages. SETD5 was an independent predictor of poor overall survival in BC. Furthermore, these studies show that down-regulation of SETD5 significantly decreased BC cell proliferation, metastasis, and angiogenesis, and increased apoptosis of BC cells. The mechanistic analysis showed that SETD5 contributes BC progression by interacting with AKT1 pathway. Also, *in vivo* experiments show that blocking of SETD5 expression significantly inhibited tumor growth and pulmonary metastasis of BC cells. These findings indicate that SETD5 is a potential prognosis marker and facilitates tumor progression of BC. (*Am J Pathol* 2021, 191: 180–193; <https://doi.org/10.1016/j.ajpath.2020.10.005>)

In 2018, approximately 2.1 million new breast cancer (BC) cases were diagnosed in females globally, accounting for almost one in four cancer cases in women. Current data show that BC is the most diagnosed cancer in most countries (154 of 185). Furthermore, it is the leading cause of cancer-related mortality in >100 countries.¹ In 2019, the American Cancer Society's biennial update on female BC statistics in the United States indicated that over the most recent 5-year period, BC incidence rate has increased by 0.3% per year.² Despite success of hormonal and adjuvant chemotherapeutic agents to improve BC prognosis, treating the disease is still a challenge because it is still difficult to predict an individual's response to the treatment. Currently, efforts are being made to reduce the cost of treatment through the development of targeted therapies. As such, biomarkers have been effectively applied in BC diagnosis, prognosis, predicting response to therapy, as well as

disease surveillance following treatment.³ Therefore, further elucidation of the underlying molecular mechanisms in BC progression is essential for the development of effective therapeutic agents, and the discovery of novel markers with have both prognostic and predictive value.

AKT, a serine/threonine kinase, is a signaling molecule of cell growth and differentiation.⁴ It is a well-characterized effector of phosphoinositide 3-kinase (PI3K) in PI3K/AKT/mammalian target of rapamycin signaling pathway, and its deregulation plays a crucial role in the pathogenesis of many human cancers.⁴ Three AKT genes (AKT1, AKT2, and AKT3) have been identified in the mammalian genome, and

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the principal AKT isoform is encoded by AKT1, which modulates apoptosis.⁵ AKT1 is phosphorylated *in vivo* at Ser124, Thr308, Thr450, and Ser473. Moreover, the phosphorylation of Thr308 and Ser473 is generally inducible and mostly phosphorylated after treatment of cells with extracellular stimuli, whereas Ser124 and Thr450 appear to be basally phosphorylated.⁶ Genetic and epigenetic changes in genes participating in the AKT pathway activate AKT in human cancers.⁷ Although the direct effect of methylation on AKT activation is yet to be reported, methylation of its upstream regulators in many cancers was shown to activate AKT.⁸

Su(var)3-9, Enhancer of zeste, and Trithorax (SET) domain-containing 5 (SETD5) is a member of the histone lysine methyltransferase family that is yet to be characterized and typically possesses conserved SET domain.⁹ The enzyme catalyzes monomethylation, dimethylation, or trimethylation of lysine ϵ -amine groups of proteins, specifically histones, which facilitates rearrangement of chromatin and regulation of gene expression. Epigenetic regulation appears to contribute to the initiation and progression of cancer and may precede genetic mutations. Dillon et al¹⁰ assumed that SETD5 protein participates in epigenetic regulation. *SETD5* gene mutations are associated with human neurodevelopmental disorders.^{11–14} Liu et al¹⁵ reported that mRNA levels of *SETD5* were up-regulated in BC and significantly associated with shorter survival in patients, but the functional role of SETD5 in BC progression is not fully understood.

This article investigates the clinical significance and the functional role of SETD5 in BC. Our studies show that expression of SETD5 predicts poor prognosis of BC. We hypothesize that SETD5 contributes BC progression through activation of AKT1 signaling. Of importance, our data indicate that SETD5 facilitates BC tumor growth and pulmonary metastasis and could be a potential prognosis marker.

Materials and Methods

Tissue Specimens

Tissue microarray used for immunohistochemistry (IHC) staining was purchased from Shanghai Outdo Biotech Co, Ltd (Shanghai, China). This study was conducted as per the Declaration of Helsinki principles and was approved by the Human Ethics Committee and the Research Ethics Committee of Yanbian University (Yanji, China). Tissue microarray contained 117 BC tissues that were previously fixed in formalin and then embedded in paraffin. The median follow-up duration was 112 months (range, 2 to 136 months).

Cell Culture, Reagents, and Transfection

MCF-7, MDA-MB-231, and human umbilical vein endothelial cells (ATCC, Manassas, VA) were grown under conditions specified by the supplier. All cell lines were *Mycoplasma* free. The following reagents were purchased for *in vitro* experiments: LY294002 (MedChem Express, Shanghai, China) and

perifosine (Selleckchem, Shanghai, China). Negative control and SETD5 endoribonuclease-prepared short interfering RNA (esi-SETD5) were transfected into cells with Lipofectamine 3000 (Invitrogen, Carlsbad, CA) as per the methods described by the manufacturer. siRNA—universal negative control and esi-SETD5 were purchased from Sigma-Aldrich (St. Louis, MO). Table 1 lists the sequence of esi-SETD5.

IHC, Western Blot, Immunofluorescence, and Migration and Invasion Assays

IHC staining,¹⁶ Western blot,¹⁷ immunofluorescence,¹⁸ and migration and invasion assays¹⁸ were conducted according to previous protocols. Table 2 provides a list of antibodies used. β -Actin served as control. All experiments were repeated at least three times with similar results each time.

Semiquantitative Analysis of Immunostaining

The staining intensity and the proportion of positive cells were measured by pathologists (Y.X. and Z.Y.) with no prior knowledge of clinicopathologic results, and then staining score was assigned as follows: IHC score 1, weak staining in <50% or moderate staining in <20% of cancer cells; IHC score 2, weak staining in $\geq$ 50%, moderate staining in 20% to 50%, or strong staining in <20% of cancer cells; and IHC score 3, moderate staining in $\geq$ 50% or strong staining in $\geq$ 20% of cancer cells. After having assessed IHC score, positivity of each case was decided according to cutoff point derived from clinical study, taking clinical end points such as recurrence and patients' survival. In this study, cases with IHC score 2 and 3 were regarded as positivity for SETD5 protein expression.

Colony Formation Assay

In brief, cells were plated (200 cells/well) on 6-well plates and then maintained for 2 weeks. Next, the colonies were fixed with paraformaldehyde and stained with crystal violet (1%). Subsequently, the colonies were imaged to determine the number of colonies (>50 cells/colony). Data were graphed as means $\pm$ SD of experiments performed three times, each in triplicate.

Wound-Healing Assay

Cells were seeded in a 6-well plate and cultured for 24 hours to form confluent monolayers. A wound was generated by dragging a pipette tip through the monolayer, and plates were washed using prewarmed phosphate-buffered saline to remove cellular debris. Cell migration was monitored for 0 and 24 hours, and images were captured at each time point using a digital camera attached to an inverted phase contrast microscope. Data were graphed as means $\pm$ SD for experiments performed three times, each in triplicate.

Table 1 The Sequence of SETD5 Endoribonuclease-Prepared Short Interfering RNA

Gene	Sequence
SETD5	5'-GATGTACAGAACGCGCTTGAACAACACCTACATCTCTAGCAAGGAATTTGTGGGCAAACCTACTATTTTAGACACTATTAATAAGACTGAATTGGCCTGTAATAACACAGTTATTGGTTCCCAAAATGCAGTTACAGCTGGGAAGAGTCACTCGTGTTCAAAAACACCCGGAAGATCCTGAGGGCTGCAAGAGATTTGGCTTTGGACACTCTTATAATAGAGTATCGTGGGAAAGTCATGTTACGACAGCAATTTGAGGTCAATGGGCATTTCTTCAAAAAACCATACCCCTTTGTGCTCTTCTACTCAAAATTCATGGTGTAGAGATGTGTGTGGATGCCCGTACTTTCGGTAATGATGCTCGGTTTCATCAGAAGATCATGTACACCAATGCAGAGGTGCGACACATGATTGCAGATGGG-3'

Cell Cycle Assay

After being transfected with esi-SETD5 for 24 hours, the cells were fixed at 4°C in ethanol (70% v/v) in phosphate-buffered saline for 24 hours. After that, they were stained for 30 minutes with propidium iodide solution (10 µg/mL) comprising 5 µg/mL RNase A. Flow cytometry with fluorescence-activated cell sorting was used to examine the cell cycle progression. All experiments were repeated at least three times.

Carboxy-Fluorescein Succinimidyl Ester Staining

The BC cells (1×10^6 cells/mL) were suspended in RPMI 1640 medium, then labeled at 37°C for 20 minutes using 5 µmol/L carboxy-fluorescein succinimidyl ester (Invitrogen). To quench the labeling reaction, RPMI 1640 medium containing fetal bovine serum (10%) was added, and the mixture was incubated for 10 minutes. The carboxy-fluorescein succinimidyl ester-labeled cells were washed

Table 2 Antibodies in This Study

Antibodies against	Company	Catalog number
SETD5	NOVUS (Centennial, CO)	NBP2-38257
PI3K	Abcam (Cambridge, UK)	Ab86714
p85α	Abcam	ab86714
AKT1	Invitrogen	PA5-29169
pAKT-Ser473	Millipore (Billerica, MA)	05-1003
pAKT-Thr308	Millipore	07-1398
Snail	Abcam	ab53519
E-cadherin	Abcam	ab40772
Vimentin	Millipore	MABT121
Twist1	Abcam	Ab50887
β-Catenin	Invitrogen	TA2-62560
Bcl-2	Santa Cruz Biotechnology (Dallas, TX)	sc-7382
Bax	Santa Cruz Biotechnology	sc-493
Caspase-8	Abcam	Ab25901
Cleaved caspase-3	CST (Danvers, MA)	5 A1E
Cleaved PRAP	Abcam	Ab32064
VEGF	Millipore	ABS82
EPO	Abcam	Ab226956
CD105	Abcam	Ab170943
Ki-67	Abcam	Ab92742
D2-40	Proteintech (Wuhan, China)	1D9F3

Bax, Bcl2 associated X; Bcl-2, B-cell lymphoma 2; EPO, erythropoietin; pAKT, phosphorylated AKT; PI3K, phosphoinositide 3-kinase; PRAP, poly ADP ribose polymerase; VEGF, vascular endothelial growth factor.

twice with culture medium and then seeded onto 6-well plates. The cells were harvested at the same time. The rate of cell proliferation was examined using C6 flow cytometry. All values are means of three experiments. Statistical analysis experimental values are presented as the means $\pm$ SD for three independent experiments.

Hoechst 33258 Staining

Hoechst 33258 staining was conducted using Hoechst Staining Kit (Beyotime Biotechnology, Shanghai, China) to detect the nuclei morphology, as per the methods described by the manufacturer. The experiments were biologically repeated for three times, and data are shown as means $\pm$ SD.

Co-IP Data

The Thermo Scientific Pierce co-IP kit (Thermo Scientific, Rockford, IL) was used to perform co-immunoprecipitation (co-IP) following the instructions provided by the manufacturer. Results shown are representative of experiments performed at least three times with similar results each time.

Tube Formation Assay

Briefly, cold Matrigel (Corning Inc., Corning, NY) was used to coat the 96-well plate. Next, the plates were incubated at 37°C for 4 hours, and then human umbilical vein endothelial cells (2×10^4 cells/well) in 150 µL conditioned medium, diluted at 2:1 in RPMI 1640 medium without fetal bovine serum, were seeded to Matrigel-coated wells and incubated for 4 to 6 hours at 37°C. A microscope was used to observe the formation of capillary-like structures. Image J software version 1.52 (NIH, Bethesda, MD; <http://imagej.en.softonic.com>, last accessed October, 2019) was used to assess total tube length and branching points formed by endothelial cells per image field. Here and later, data were graphed as means $\pm$ SD for experiments performed three times, each in triplicate.

Vasculogenic Mimicry Assay

A total of 3×10^4 cells in RPMI 1640 medium without fetal bovine serum were seeded onto 96-well plates precoated with Matrigel. Tube formation was assessed under a phase-contrast microscope for 4 to 6 hours after seeding.

Figure 1 SETD5 expression is related to poor prognosis in breast cancer (BC). Immunohistochemistry on tissue microarrays for SETD5 to investigate SETD5 expression in human BC. **A–C:** SETD5 expressed in ductal carcinoma *in situ* tissues (**A**) and invasive ductal carcinoma of breast (**B** and **C**). **D:** Immunostaining of SETD5 (red reaction product) in the cancer cells and D2-40 (brown reaction product) around the cancer cells in the lymphatic invasion of breast ductal carcinoma (**blue arrows**). **E:** SETD5 protein expression detected in normal tissues and BC tissues using the online web portal UALCAN (<http://ualcan.path.uab.edu>, last accessed July, 2020) based on Clinical Proteomic Tumor Analysis Consortium. **F:** Kaplan-Meier analysis employed to study the relationship between SETD5 expression and overall survival (OS) in patients with BC. OS outcomes of the SETD5-positive group of patients (**solid red line**) versus the SETD5-negative group of patients (**solid blue line**). The OS time in months is displayed on the x axis, and the probability of OS is displayed on the y axis. $n = 18$ normal tissues (**E**); $n = 125$ BC tissues (**E**). *** $P < 0.001$ versus controls (log-rank test). Scale bars = 100 μm (**A–D**). Original magnification, $\times 200$ (**A–D**).

GSEA Data

The raw data can be accessed in the National Center for Biotechnology Information Gene Expression Omnibus site (<https://www.ncbi.nlm.nih.gov/geo>; accession number GSE29431, last accessed May, 2019). The database was analyzed using the Gene Set Enrichment Analysis (GSEA). GSEA version 2.2.4 software (<http://www.broadinstitute.org/gsea>, last accessed May, 2019) was used to explore the relationship between SETD5 expression and PI3K/AKT signaling. The GSEA includes three key statistics: false discovery rate, nominal P value, and normalized enrichment score.

In Vivo Experiments

All animal experiments were conducted as per the recommendations of the *Guide for the Care and Use of Laboratory Animals*¹⁹ of the NIH and were approved by the Yanbian University Animal Care and Use Committee. A total of 100 μL of cell suspension MDA-MB-231 cells (1×10^7) in phosphate-buffered saline comprising 50% Matrigel was inoculated (subcutaneously) into the right or

left flank of 5-week-old BALB/c nude female mice (Vital Rivers, Beijing, China) to generate a tumor model. Tumors were harvested at 4 weeks for cells after inoculation. To detect lung metastasis, MDA-MB-231 cells (1×10^6) in saline were injected into the tail vein of nude mice. The mice were sacrificed at 4 weeks following injection. The nodules were counted after harvesting the lungs. For histologic examination, hematoxylin and eosin staining was conducted.

Statistical Analysis

Data analysis was performed using SPSS version 25.0 (SPSS, Inc., Chicago, IL) and GraphPad Prism version 5.01 (GraphPad Inc., San Diego, CA). Correlation analysis was performed using Spearman correlation analysis. Overall survival (OS) was calculated using the Kaplan-Meier method followed by the log-rank test (for comparing the survival curves). Survival time was the period from the date of surgery to the date a patient died or the date of the last follow-up. The Cox proportional hazards model was used for multivariate analysis. Clinicopathologic factors, which were statistically significant in univariate analysis, were

Table 3 Comparison of Clinicopathologic Characteristics According to the SETD5 Expression in Breast Cancer Tissues

Variable	<i>n</i>	SETD5 (-), <i>n</i> (%)	SETD5 (+), <i>n</i> (%)	χ^2	R	<i>P</i> value
Age, years				5.922	0.237	0.015
≤50	82	25 (30.5)	57 (69.5)			
>50	35	3 (8.6)	32 (91.4)			
Grade				0.649	0.012	0.723
Well	31	9 (29.0)	22 (71.0)			
Moderately	43	9 (20.9)	34 (79.1)			
Poorly	43	10 (23.3)	33 (76.7)			
pT stage				5.623	0.226	0.131
T1	10	3 (30.0)	7 (70.0)			
T2	50	15 (30.0)	35 (70.0)			
T3	48	10 (20.8)	38 (79.2)			
T4	9	0 (0.0)	9 (100.0)			
Lymph node metastasis				15.786	0.388	<0.001
Negative	66	25 (37.9)	41 (62.1)			
Positive	51	3 (5.9)	48 (94.1)			
Clinical stage				12.755	0.307	0.013
I	46	18 (39.1)	28 (60.9)			
II	35	8 (22.9)	27 (77.1)			
III	29	2 (6.9)	27 (93.1)			
IV	7	0 (0.0)	7 (100.0)			
ER				1.885	0.127	0.170
Negative	62	18 (29.0)	44 (71.0)			
Positive	55	10 (18.2)	45 (81.8)			
PR				1.270	0.104	0.260
Negative	56	16 (28.6)	40 (71.4)			
Positive	61	12 (19.7)	49 (80.3)			
HER2				1.631	0.118	0.202
Negative	41	7 (17.1)	34 (82.9)			
Positive	76	21 (27.6)	55 (72.4)			
P53				0.423	0.060	0.516
Negative	81	18 (22.2)	63 (77.8)			
Positive	36	10 (27.8)	26 (72.2)			

ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor; pT, pathologic tumor.

included as covariables in multivariate analysis. Hazard ratio and 95% CIs were assessed for each factor. Two-group comparisons were analyzed using *t*-test (two tailed). $P < 0.05$ was considered statistically significant.

Results

Expression of SETD5 Is Related to Unfavorable Clinicopathologic Characteristics and Poor Disease Outcomes in BC

Genetic mutation in the gene (*SETD5*) that codes for histone lysine methyltransferases has been shown to be significantly related to the progression of BC and OS of patients.¹⁵ As a methyltransferase, SETD5 controls histone acetylation, mediates transcription, and functions in cell cycle progression and embryo development in mammals.²⁰ To fully explore the clinical significance of SETD5 in the context of BC, tissue microarray analysis was performed on 117 primary human BC specimens which were subsequently stained for SETD5 expression using IHC (Figure 1, A–D). The current results

indicate that 89 BC cases (76%) were positive for SETD5 staining (Table 3). SETD5 had a low expression in ductal carcinoma *in situ* tissues (pT0) (Figure 1A), and medium expression in invasive ductal carcinoma of breast (pT1 and pT3) (Figure 1, B and C). The high expression of SETD5 cancer cells and D2-40–positive lymph vessels was observed in the lymphatic invasion of breast ductal carcinoma samples (Figure 1D). Among 51 cases of patients with lymph node metastasis, 48 (94%) were positive for SETD5 staining, which was significantly higher compared with those for patients without lymph node metastasis (62.1%) ($P < 0.001$) (Table 3). The prevalence of SETD5 expression gradually increased with disease progression. In patients with BC with early clinical stage disease, 60.9% exhibited positive staining for SETD5, whereas in patients with more advanced stage II, stage III, and stage IV clinical disease, the prevalence was 77.1%, 93.1%, and 100%, respectively ($P < 0.001$) (Table 3). The association between SETD5 protein expression and clinical pathologic diagnosis from Clinical Proteomic Tumor Analysis Consortium database in BC tissues was also analyzed. The results obtained from the online tool

Table 4 Univariate and Multivariate Analysis for Prognostic Variables of Overall Survival in Breast Cancer Patients Using Cox Proportional-Hazards Regression

Characteristic	Univariate analyses			Multivariate analyses		
	HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value
Age, years			0.097			0.088
<50	1.00			1.00		
≥50	1.493	0.931–2.397		1.627	0.931–2.842	
pT stage			0.001			0.009
T1	1.00			1.00		
T2	3.173	0.885–11.378		2.137	0.425–10.754	
T3	6.511	2.052–20.665		5.859	1.361–25.221	
T4	8.307	2.315–29.817		7.141	1.422–35.858	
Lymph node metastasis			0.002			0.164
Negative	1.00			1.00		
Positive	2.130	1.326–3.422		1.512	0.845–2.706	
Distant metastasis			0.012			0.110
Negative	1.00			1.00		
Positive	1.738	1.128–2.679		1.498	0.912–2.460	
SETD5			0.010			0.036
Negative	1.00			1.00		
Positive	1.830	1.153–2.904		1.687	1.034–2.754	

HR, hazard ratio; pT, pathologic tumor.

UALCAN showed that SETD5 protein expression significantly increased in BC tissues relative to that in the normal tissues ($P < 0.001$) (Figure 1E). Kaplan-Meier analysis showed that SETD5 expression in BC specimens was significantly associated with poor OS of patients ($P = 0.009$) (Figure 1F). Univariate Cox regression analysis identified pathologic tumor stage ($P = 0.001$), lymph node metastasis ($P = 0.002$), distant metastasis ($P = 0.012$), and SETD5 expression ($P = 0.010$) as significant prognostic factors of poor OS for patients with BC (Table 4). Multivariate Cox regression analysis revealed that pathologic tumor stage ($P = 0.009$) and SETD5 expression ($P = 0.036$) were independent prognostic factors for poor OS (Table 4). These findings imply that SETD5 expression may be a potential biomarker for poor prognosis of BC.

SETD5 Expression Positively Correlates with TAMs in BC

Properties of tumor-associated macrophages (TAMs) that help in the progression of the tumor, their main functional peculiarities related to the suppression of immune reaction, extracellular matrix remodeling, and angiogenesis stimulation remain to be the most important factors for tumor development.²¹ SETD5 had high expression in adjacent macrophage population of BC, implying TAM population infiltration along with high SETD5 expression in areas around the cancer cells (Figure 2A). SETD5 expression positively correlated with TAM makers, such as CD68 ($P < 0.001$), CD204 ($P < 0.001$), CD206 ($P < 0.001$), T-cell immunoglobulin mucin family member 3 ($P < 0.001$), and CD163 ($P = 0.0048$) in BC samples by Gene Expression Profiling Interactive Analysis through The

Cancer Genome Atlas database (Figure 2, B–F). Thus, TAMs with high SETD5 expression play an important role in progression of BC. However, further studies are required to determine a specific relationship between SETD5 expression and TAM infiltration in BC.

Inhibition of SETD5 Expression Suppresses BC Cell Proliferation

SETD5 was not related to progesterone receptor, estrogen receptor, or human epidermal growth factor receptor 2 (Table 3). Thus, aggressive triple-negative MDA-MB-231 and nonaggressive estrogen receptor α -positive MCF-7 cell lines were used as a contrast in BC. To determine how SETD5 affects BC progression, the impact of SETD5 on cell apoptosis and proliferation was examined. First, SETD5 expression in MDA-MB-231 and MCF-7 cells was inhibited by transfecting them with SETD5-targeted endoribonuclease-prepared short interfering RNA (Figure 3A). Hoechst 33258 staining revealed significant increases in the morphologic hallmarks of apoptotic cell death in SETD5 knockdown cells relative to the control cells (Figure 3B). These observations were confirmed through the Western blot assay. The results of the current study indicates that inhibition of SETD5 expression significantly enhances the expression of apoptotic markers in BC cells, including caspase-8, cleaved poly ADP ribose polymerase, cleaved caspase-3, and Bcl2 associated X/B-cell lymphoma 2 (Figure 3C). In addition, carboxy-fluorescein succinimidyl ester assays (Figure 3D) and colony formation assays (Figure 3E) indicated that down-regulation of SETD5 significantly inhibited cell clonogenicity ($P < 0.001$) and

Figure 2 SETD5 expression positively correlates with tumor-associated macrophages (TAMs) in breast cancer (BC). **A:** Immunohistochemistry on tissue microarrays for SETD5 to investigate SETD5 expression in adjacent macrophage of breast ductal carcinoma cells (**blue arrows**). **B–F:** The correlation between SETD5 and mRNA levels of TAM makers CD68 (**B**), CD204 (**C**), CD206 (**D**), T-cell immunoglobulin mucin family member 3 (TIM-3; **E**), and CD163 (**F**) was analyzed in BC tissues by Gene Expression Profiling Interactive Analysis (<http://gepia.cancer-pku.cn>, last accessed July 20, 2020). Scale bar = 100 μ m (**A**). Original magnification, $\times 200$ (**A**). TPM, transcript per million.

proliferation ($P < 0.001$). Flow cytometry analyses showed that knockdown of SETD5 expression primarily decreased the subpopulations in MDA-MB-231 and MCF-7 cells in S phase (Figure 3F). Collectively, these results indicate that SETD5 contributed to BC progression.

SETD5 Expression Is Present in Conjunction with EMT-Like Changes in BC

Tumor budding is a phrase used to describe a thin anaplastic cell cord, undifferentiated tumor cells, and individual free cells at the invasion front of tumors and is recognized as being composed of neoplastic epithelium or cancer cell aggregates (up to five cells).²² SETD5 expression was diffuse yet strong in the tumor budding of BC specimens (Supplemental Figure S1). Tumor budding is closely related to epithelial-mesenchymal transition (EMT), and the cells exhibit both invasive and migratory characteristics.^{23,24} EMT is an important biological process that involves the release of epithelial cells from the surrounding tissue, which then gain the features of mesenchymal cells, such as distinctive, motile, spindle-shaped cells with end-to-end polarity. Therefore, the effects of inhibiting SETD5 expression on EMT were assessed. Frequently used

molecular indicators of EMT include up-regulation of vimentin and N-cadherin and increased production of transcription factors that inhibit E-cadherin and β -catenin production, including Slug, Snail, Twist, and zinc finger E-box binding homeobox 1.^{25,26} These studies showed that inhibiting SETD5 expression significantly decreased the levels of Snail, Twist1, and vimentin proteins and significantly increased the levels of E-cadherin and β -catenin proteins in MCF-7 and MDA-MB-231 cells (Figure 4A). Immunofluorescence staining further confirmed that esi-SETD5 up-regulated E-cadherin, while down-regulating vimentin (Figure 4B). EMT plays an essential role during the initial stages of metastasis.²⁵ To investigate the function of SETD5 in metastasis of BC cells, transwell and wound-healing assays were performed. Data from these assays indicate that SETD5 silencing markedly suppressed the invasive and proliferative abilities of BC cells (Figure 4C and Supplemental Figure S2). Collectively, these findings indicate that SETD5 facilitates the migration and invasion of cells during BC progression via EMT.

SETD5 Induces Tumor Angiogenesis

The clinical failure of anti-angiogenic drugs in the treatment of BC may be attributed to the fact that many factors

Figure 3 SETD5 positively regulates breast cancer (BC) cell proliferation. **A:** Effects of silencing SETD5 in MDA-MB-231 and MCF-7 cells evaluated by Western blot analysis. β -Actin served as control. **B:** Apoptosis-associated morphologic changes of BC cells analyzed using Hoechst 33258 fluorescent staining. **C:** Western blot assay showing the expression of apoptosis-related proteins in BC cells in the control (Con) group versus the SETD5 endoribonuclease-prepared short interfering RNA (esi-SETD5) group. **D** and **E:** Cell proliferation in the SETD5-knockdown group versus the control group based on carboxy-fluorescein succinimidyl ester (CFSE) staining (**D**) and colony formation assay (**E**). **F:** Representative histograms of the cell cycle distribution in two BC cell lines transfected with esi-SETD5 versus the control cells based on flow cytometry. *** $P < 0.001$ versus controls. Scale bars = 100 μ m (**B**). Original magnification, $\times 200$ (**B**). Bax, Bcl2 associated X; Bcl-2, B-cell lymphoma 2; FITC, fluorescein isothiocyanate; PARP, poly ADP ribose polymerase.

involved in metastasis and angiogenesis are still unidentified. To determine the effects of SETD5 on angiogenesis, costaining for SETD5 and microvessel density marker CD105²⁷ was performed via IHC. The results revealed that CD105 was highly expressed in new capillary blood vessels near the cancer cells in the host (Supplemental Figure S3). Microvessel density was significantly higher in SETD5-positive tissues of patients with BC (36.64 ± 15.395) compared with that in SETD5-negative BC patient tissues (30 ± 13.172 ; $P = 0.039$) (Figure 5A). The process of angiogenesis is mediated by several classic factors, including vascular endothelial growth factor (VEGF), and other endogenous nonclassic peptides, such as erythropoietin (EPO).²⁸ Inhibition of SETD5 expression reduced the protein levels of VEGF and EPO (Figure 5B). Tube formation assays (Figure 5C) and vascular mimicry assays (Figure 5D) showed that SETD5 knockdown significantly inhibited tube formation, which was quantified as total branching length and intersection number, and confirmed the proangiogenic activity of SETD5.

SETD5 Enhances BC Tumorigenesis through the AKT1 Signaling Pathway

Many studies have shown that critical epigenetic modifiers are regulated by the PI3K/AKT signaling pathway and are involved in the oncogenicity of the PI3K cascade in cancers.²⁹ To identify the potential mechanism of SETD5 in BC, gene expression signatures of SETD5 high-expressing BC were investigated using GSEA and a public data set. Herein, 27 SETD5 high-expressing and 27 SETD5 low-expressing BC groups were chosen from a sample pooled cohort of 54 patients with BC in the Gene Expression Omnibus database. GSEA showed that SETD5 expression levels were closely related to the PI3K/AKT signaling pathway (normalized enrichment score = 1.53; false discovery rate $q = 0.19$) (Figure 6A). Tissue microarray analysis of BC tissues revealed a positive correlation between SETD5 expression and the expression of genes linked to PI3K/AKT signaling pathway activation, including p85 α ($P = 0.004$) and phosphorylated AKT (pAKT)—Ser473

Figure 4 SETD5 positively modulates epithelial-mesenchymal transition (EMT) in breast cancer (BC). **A:** Western blot analysis of EMT marker expression in BC cells and cells transfected with SETD5 endoribonuclease-prepared short interfering RNA (esi-SETD5). β -Actin was used as the loading control (Con). **B:** The expression of E-cadherin (E-cad) and vimentin was determined by immunofluorescence. **C:** MDA-MB-231 and MCF-7 cell migration and invasion ability analyzed using transwell and Matrigel invasion assays after being transfected with esi-SETD5. The invading cells were stained and counted. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus controls. Scale bars = 100 μ m (**B** and **C**). Original magnification, $\times 200$ (**B** and **C**).

($P = 0.027$) (Supplemental Figure S4, A–D, and Supplemental Table S1), which confirmed the GSEA results. Furthermore, when MCF-7 and MDA-MB-231 cells were transfected with esi-SETD5 or treated with inhibitors for PI3K (LY294002, 10 μ mol/L) or AKT1 (perifosine, 10 μ mol/L), the upstream and downstream relationships among PI3K, AKT1, and SETD5 were confirmed. LY294002 and perifosine decreased AKT1 phosphorylation at pAKT-Thr308 and pAKT-Ser473, respectively. The expression of SETD5 was not significantly down-regulated in the cells when treated with LY294002 or perifosine. However, silencing of SETD5 had a marked effect on AKT1 phosphorylation at pAKT-Thr308 and pAKT-Ser473 (Figure 6B). SETD5 was clearly pulled down by an anti-AKT1 antibody in BC cells during co-IP, which indicated close binding between SETD5 and AKT1 (Figure 6C). Moreover, immunofluorescence was used to evaluate the correlation between SETD5 expression and AKT1. The results showed that SETD5 was colocalized with AKT1 phosphorylation at pAKT-Thr308 and pAKT-Ser473 in BC patient tissues (Figure 6D). The positive relationship between the expression of SETD5 and AKT1 mRNA levels was further analyzed by Spearman correlation analysis of Gene Expression Profiling Interactive Analysis ($R = 0.25$; $P < 0.001$) (Supplemental Figure S4E). This points towards SETD5 working as an upstream signal of AKT1 and

regulating AKT1 phosphorylation in BC cells as a possible mechanism.

Experiments *in Vivo*

To further confirm whether SETD5 affects the proliferation of tumor cells, SETD5-depleted MDA-MB-231 cells were injected (subcutaneously) into the right flank of 5-week-old female BALB/c nude mice. Inhibition of SETD5 expression significantly inhibited tumor growth (Figure 7A). Inhibition of SETD5 significantly reduced the scores of the cancer cells stained by Ki-67 (a typical biomarker of cell proliferation), vimentin, VEGF, and EPO, and increased the expression of cleaved caspase-3 and E-cadherin in xenograft tumor tissues, as indicated by IHC (Figure 7, B and C). Collectively, these observations suggest that SETD5 can induce the growth of BC tumors, supporting the hypothesis that SETD5-targeted endoribonuclease-prepared short interfering RNA may function as a suppressor of BC tumors.

In vivo pulmonary metastasis experiments were subsequently performed to further confirm the role of SETD5 in BC cell micrometastases. Metastatic nodules of tumor xenografts were counted in lung sections of the mice 4 weeks after tail vein injection. Inhibiting the expression of SETD5 was found to markedly reduce the numbers of metastatic lung nodules (Figure 7D). Histologic analysis confirmed the

Figure 5 Proangiogenic role of SETD5 in breast cancer (BC). **A:** Association between SETD5 and CD105 in BC tissues. **B:** Western blot analysis of vascular endothelial growth factor (VEGF) and erythropoietin (EPO) protein expression in normal MCF-7 and MDA-MB-231 cells and cells transfected with SETD5 endoribonuclease-prepared short interfering RNA (esi-SETD5). **C** and **D:** Tube formation assays (**C**) and vasculogenic mimicry assays (**D**) performed to analyze the effect of esi-SETD5 on tumor angiogenesis. Quantitation of total branching length of tubes and numbers of intersections performed using ImageJ software version 1.52 with the angiogenesis plugin. * $P < 0.05$, *** $P < 0.001$ versus controls (Con). Scale bars = 100 μ m (**C** and **D**). HUVEC, human umbilical vein endothelial cell; MVD, microvessel density.

presence of BC metastases in the lungs of the mice, as demonstrated by the photomicrographs of hematoxylin and eosin–stained specimens (Figure 7D). Collectively, these data clearly demonstrate a vital role of SETD5 in the occurrence of pulmonary metastasis in BC.

Discussion

SETD5 has H3K36 methyltransferase activity, which is crucial in the elongation and processing of RNA, as well as correct gene transcription.³⁰ Alteration in promoter-proximal pausing of the polymerase usually impairs this process, thus leading to

several pathologic disorders, such as developmental syndromes and cancer.^{31,32} Although the up-regulation of SETD5 in BC has been previously reported,¹⁵ the fact that SETD5 is related to poor disease outcomes in patients with BC and the mechanisms underlying its involvement in tumor progression have not been elucidated. The current study involves a comprehensive investigation of SETD5 with respect to tumor progression, which eventually results in a poor prognosis for patients with BC. First, the expression of SETD5 was shown to be increased in BC tissues and be positively associated with lymph nodes metastasis, advanced clinical stages, and patient OS. Additionally, SETD5 was determined to be an independent prognostic factor for patients with BC. Second, SETD5 was shown to be

Figure 6 SETD5 interacts with AKT1 in breast cancer (BC). **A:** Gene Set Enrichment Analysis results for phosphoinositide 3-kinase (PI3K)/AKT in SETD5 high-expressing BC. **B:** Western blot analysis of PI3K, p85α, AKT1, phosphorylated AKT (pAKT)—Thr308, pAKT-Ser473, and SETD5 in MDA-MB-231 and MCF-7 cells after treatment with the PI3K inhibitor LY294002 (LY) or AKT inhibitor perifosine (Per) or transfection with SETD5 endoribonuclease-prepared short interfering RNA (esi-SETD5). **C:** SETD5 and AKT1 protein interactions in MDA-MB-231 and MCF7 cells were analyzed by co-immunoprecipitation (IP). **D:** Immunofluorescence analysis of the colocalization of SETD5 and AKT1 phosphorylation at pAKT-Thr308 and pAKT-Ser473 in BC tissues. Scale bars = 100 μm (D). Original magnification, ×200 (D). FDR, false discovery rate; IB, immunoblot; NES, normalized enrichment score.

an upstream signal of AKT1 in BC cells. Third, inhibition of SETD5 suppressed the tumor growth and metastasis of BC cells *in vivo* and *in vitro*. Collectively, these data demonstrated that SETD5 promotes tumor progression via AKT1 signaling, ultimately leading to a poor prognosis for patients with BC.

The expression of *SETD7* of the *SETD* methyltransferase family of genes is positively associated with metastasis, recurrence, and poor prognosis of hepatocellular carcinoma.³³ Consistent with the report by Chen et al,³³ the current study found that SETD5 had high expression in BC tissues and positively correlated with patient age, lymph node metastasis, and clinical stage. Survival analyses showed that expression of SETD5 was significantly related to a poorer prognosis. Cox regression analysis indicated that the expression of SETD5 was an independent prognostic factor for patients with BC. While the collective data support the potential value of SETD5 as a novel prognostic marker for BC, the role of SETD5 in BC tumorigenesis is not fully understood.

Studies have shown that embryonic stem cells lacking SETD5 expression show high apoptosis rate, poor proliferation, defective cell cycle progression, weakened ability to

differentiate to cardiomyocytes, and poor gene expression.^{20,34}

In the current study, SETD5-deficient BC cells were found to induce tumor cell apoptosis and to inhibit tumor cell proliferation *in vitro*. A xenograft mouse model further confirmed that knockdown of SETD5 inhibited tumor growth. Also, *in vivo* experiments further confirmed the biological function of SETD5 regarding cancer cell apoptosis and proliferation. Thus, it is conceivable that SETD5 expression decreases apoptosis and promotes proliferation of BC cells.

Yu et al³⁵ have shown that SETD5 may promote the invasive and migration abilities of non-small-cell lung cancer by promoting Snail expression, while suppressing zonula occludens protein 1 expression. It will be interesting to determine whether SETD5 regulates the expression of molecules related to the EMT in BC. Consistent with this, the current study show that SETD5 knockdown influenced the expression of EMT-related genes in both BC cells and xenograft tumor tissues. The most important clinical significance of the EMT process is its influence on BC cell invasion and metastasis. Inhibiting the expression of SETD5 was found to lead to decreased cell migration and invasion

Figure 7 The anticancer growth effect of SETD5 endoribonuclease-prepared short interfering RNA (esi-SETD5) *in vivo*. **A:** Mice with tumors formed by subcutaneously injected normal MDA-MB-231 cells (black circles) and MDA-MB-231 cells transfected with esi-SETD5 (black squares). Tumor volumes and tumor weights of each group at the indicated time after injection are shown. **B** and **C:** Immunohistochemistry (IHC) staining and scores of SETD5, Ki-67, cleaved caspase-3, E-cadherin (E-cad), vimentin, vascular endothelial growth factor (VEGF), and erythropoietin (EPO) in xenograft tumors. **D:** MDA-MB-231 cells and MDA-MB-231 cells transfected with esi-SETD5 were injected via tail-vein injection into the mice. Representative micrographs of hematoxylin and eosin-stained metastatic nodules. Metastatic nodules (blue arrows) in the lungs were counted. *** $P < 0.001$ versus controls (Con). Scale bars = 100 μ m (**B** and **D**). Original magnification: $\times 200$ (**B**); $\times 100$ (**D**).

in vitro. More important, SETD5 knockdown inhibited the metastasis of BC cells to the lungs *in vivo*. Taken together, it is conceivable that SETD5 expression may lead to EMT program and promote lung micrometastases of BC cells.

Tumor angiogenesis refers to the formation of new blood vessels to serve the tumor tissues and is used as a therapeutic target in cancer treatment. Notably, the process is vital in the proliferation and metastasis of cancer cells.^{36,37} Here we used a series of experiments to explore the role of SETD5 in tumor angiogenesis, which has never been reported before. In the current study, IHC of specimens from patients with BC revealed that SETD5, along with CD105, was markedly up-regulated in cancer tissues in comparison to the adjacent tissues. Additional studies showed that SETD5 influenced total branching length and the intersection number of tumor

blood vessels and promoted angiogenesis and VEGF and EPO expression in BC cells. In addition, silencing SETD5 was demonstrated to down-regulate the expression of VEGF and EPO in BC xenograft tumor tissues. These findings may have significant implications for treating BC angiogenesis via targeting SETD5, which, in turn, may further suppress tumor proliferation, invasion, and metastasis. However, the molecular mechanisms by which SETD5 affects angiogenesis and promotes BC cell proliferation, invasion, and metastasis require further investigation.

Development of anticancer drug resistance has been associated with the activation of the PI3K/AKT pathway. AKT activation leads to the phosphorylation of many substrates, as well as downstream effectors, including members of the caspase family and cell cycle protein family, which

facilitate cell survival, proliferation, metastasis, and chemoresistance.^{38–40} Thus, it is logical to investigate the association between SETD5 and AKT in BC. (Figure 6), GSEA analysis, IHC staining, and co-IP assays clearly indicated that SETD5 expression was closely related to AKT1 signaling in BC. Furthermore, silencing of SETD5 resulted in decreased AKT1 phosphorylation at pAKT-Thr308 and pAKT-Ser473. These observations suggest that SETD5 signaling functions as an upstream signal of AKT1 and is crucial for the targeted activation of AKT1. The current findings broaden understanding of SETD5-regulated BC progression and demonstrate that this regulation was primarily through the AKT1 pathway.

In conclusion, SETD5 may act as a potential prognostic biomarker for patients with BC. The current study reports a novel role for SETD5 in promoting tumor progression by activating the AKT1 pathway in BC cells, promoting BC cell growth and motility *in vitro* and facilitating tumor growth and metastasis *in vivo*.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2020.10.005>.

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