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Original Article

Monomethyltransferase SETD8 regulates breast cancer metabolism via stabilizing hypoxia-inducible factor 1α

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ABSTRACT

SETD8 is a methyltransferase that specifically catalyzes the monomethylation of lysine 20 on histone H4. Previous studies have demonstrated that SETD8 is associated with proper cell cycle progression, DNA damage response, and transcriptional regulation. A recent study revealed that SETD8 played an important role in epithelial–mesenchymal transition (EMT) in association with TWIST and enhanced metastatic potential of breast cancer cells. However, the contribution of SETD8 to metabolism reprogramming, one hallmark of cancer, has never been reported. In this study, we report that SETD8 was a positive regulator of anabolic metabolism. SETD8 reprograms breast cancer cell metabolism through hypoxia-inducible factor 1α (HIF1α) mediated process. Mechanistic studies indicated that SETD8 stabilized HIF1α protein level through post-transcriptional regulation. Moreover, we demonstrated that SETD8 was a HIF1α transcription target. In clinical breast cancer patient tissues, we observed a positive correlation of SETD8 with HIF1α and HIF1α target genes. Taken together, we validated SETD8 as a novel metabolic reprogramming regulator, and our mechanistic studies shed light on a novel function of SETD8 in breast cancer malignant properties maintenance.

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Introduction

Breast cancer, a malignant tumor deriving from the mammary epithelium tissue, has become the most frequent tumor among females all over the world [1,2]. Plenty of effort has been paid to explore the mechanisms of breast cancer tumorigenesis and progression to discover better treatments for those patients. Among various treatment strategies, targeting cancer cell metabolism seems rather promising [3]. Although cancer metabolism has been widely investigated, further studies are still needed to discover better drug targets to treat breast cancer patients.

To meet the soaring energy and material demand of cancer cells, a distinct cellular metabolic phenotype is required. Recently, aberrant cancer metabolism has been regarded as a hallmark of cancer [4]. One of the core pathways of regulating cancer metabolism is the rapamycin (mTOR) pathway [5,6]. Downstream of this multiple functional pathway existing a transcriptional factor, hypoxia-inducible-factor 1α (HIF1α), it is acting as a transcriptional activator modulating the expression of a series of critical glycolytic enzymes, such as glucose transporters, pyruvate kinase and lactate dehydrogenase [5,6]. Due to the expression of these enzymes, cancer cells tend to redirect glucose flux into pentose phosphate pathway and lactate production, rather than the tricarboxylic acid cycle for oxidative phosphorylation, which is known as the famous Warburg effect [7–9]. In addition to transcriptional regulation, anomalous gain-of-function of oncoproteins and loss-of-function of tumor suppressors ultimately result in HIF1α stabilization, thereby enhancing aerobic glycolysis [10,11].

In mammals, SETD8 (also known as PR-Set7, SET8, or KMT5A), a lysine methyltransferase containing a SET domain, which has been extensively investigated, is the sole methyltransferase which can monomethylate H4K20 (H4K20me1) [12,13]. Plenty of biological functions of SETD8 and H4K20me1 have been found and proved over the past decade. Using transgenic technology, researchers indicated that SETD8 is indispensable in the embryonic development and critical for proper cell cycle progression and chromatin condensation [14]. During cell mitosis, a periodic oscillation of SETD8 protein level and H4K20me1 status exists in an ubiquitin-

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dependent degradation manner, delicately synergizing other cell cycle regulators to preserve well-balanced cell proliferation [15,16]. Additionally, SETD8 has been identified as a factor for DNA damage response, as massive DNA damage could be observed after SETD8 depletion [17,18]. Interestingly, SETD8 plays a paradoxical role in transcriptional regulation, both as a transcriptional repressor and activator. For instance, H4K20me1 by SETD8 inhibits E2F target genes expression through interacting with L3MBTL1, a transcriptional repressor [19]. On the contrary, SETD8 accumulates at the promoters of Wnt3a target genes upon Wnt3a activation, stimulating target genes expression [20]. Particularly, it has been reported in breast cancer that SETD8, cooperating with TWIST, has a dual function in regulating increased N-cadherin and decreased E-cadherin expression [21]. Upon this modulation, an epithelial-to-mesenchymal (EMT) phenotype and enhanced invasive potential are imparted to breast cancer cells.

The contribution of SETD8 to cancer cell malignancies maintenance has been widely studied. However, its regulatory role in cancer cell metabolism, one of the hallmarks of cancer, has never been reported before. Thus, we sought to discuss the role of SETD8 in breast cancer cell metabolism and uncover the underlying mechanism. Here, for the first time we identified SETD8 as a positive regulator of anabolic glycolysis in breast cancer. With mass spectrometry technology, we identified that SETD8 interacted with proteins that regulated HIF1α protein level. This prompted us to discuss the contribution of SETD8 to HIF1α posttranslational stability. As expected, SETD8 positively regulated HIF1α protein level stability, thus reprogramming breast cancer cell metabolic profile and enhancing malignant potentials.

Materials and methods

Cells and reagents

MDA-MB-231 and MCF7 cell lines were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Human embryonic kidney 293T (HEK293T) cell line was obtained from American Type Culture Collection (ATCC). Both cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Penicillin and Streptomycin were added to the medium for preventing bacteria contamination at a final concentration of 100 U/mL and 100 mg/mL, respectively. Cells were incubated at 37 °C in a humidified CO_2 incubator. Mimetic hypoxic conditions were generated by adding cobalt chloride ($CoCl_2$, Sigma, St. Louis, Missouri) to the medium to a final concentration of 400 mM.

Plasmids

The flag-tagged coding sequences of human SETD8 were cloned into the lentiviral vector pCDH-CMV-MCS-EF1-puro (SBI, USA) to generate SETD8 expression lentiviral plasmids. In order to generate SETD8 silencing lentiviral vectors, the pLKO.1 TRC cloning vector (Addgene plasmid 10878) was used. The 21-bp targets against SETD8 were CGCAACAGAATCGCAAACTTA and CCGAGGAACAGAAGATCAAAG respectively. The DNA sequences of plasmid constructs were validated by nucleotide sequencing. pCMV-V-FLAG and pCMV-C-HA were used to generate SETD8 and HIF1α expression constructs. pEGFP-N1 plasmid was used to make EGFP fused SETD8 constructs. HRE-luciferase (Addgene plasmid 26731) was used to analyze HIF1α transcriptional activity.

Cell viability and colony formation assay

Cells were seeded onto 96-well plates at an initial density of 4500 cells/well. Cell viability was determined each day using CCK-8 (Cell Counting Kit-8; Dojindo Laboratories, Kyushu, Japan) according to the manufacturer's instructions.

Cells were seeded in triplicate onto 6-well plates at an initial density of 1000 cells/well. After 14 days, colonies were clearly visible, and the cells were fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 4 mg/ml of crystal violet (Sigma) for 1 h. Then, the cells were washed and dried for further counting and imaging. All experiments were repeated at least three times in independent experiments.

ECAR and OCR analysis

The Seahorse XF Glycolysis Stress Test Kit and Cell Mito Stress Test Kit were used to continuously monitor media acidification (ECAR) and oxygen consumption (OCR) in the Bioscience XF96 Extracellular Flux Analyzer. Cells were plated in XF96 Cell Culture Microplates (Seahorse Bioscience) at an initial cellular density of 4×10^4 cells/well the day before determination. Seahorse buffer consists of DMEM medium, phenol red, 25 mM glucose, 2 mM sodium pyruvate, and 2 mM glutamine. For ECAR measurement, 10 mM glucose, 1 μM oligomycin, and 100 mM 2-deoxy-glucose (2-DG) were automatically added to measure ECAR value. After monitoring baseline respiration, 1 μM oligomycin, 1 μM FCCP, and 1 μM rotenone were automatically injected into XF96 Cell Culture Microplates to measure the OCR. The ECAR and OCR values were calculated after normalization to cell number.

ATP production analysis

The ENLITEN ATP Assay System (Promega, FF2000) was used according to the manufacturer's instructions. Cells were seeded onto 24-well plates at an initial density of 3×10^4 cells/well the day before determination. Cells were harvested by digesting with trypsin-EDTA (Gibco) and then resuspended in PBS. ATP was extracted by adding 5% trichloroacetic acid (TCA) and then TCA was diluted to a final concentration of 0.1% with Tris-acetate buffer (pH 7.75). The luminescence of diluted sample (40 μl) mixed with an equal volume of rL/L Reagent (Promega, FF2000) was measured. Standard ATP regression curve was created using ATP standard solution supplied in the kit. Then relative ATP concentration was determined and normalized to that of the control group.

Western blot

Western blotting was performed according to the previously published paper [22]. Antibodies used in this study are listed in Supplementary Table S1. β-actin was used as a loading control.

Quantitative real-time PCR

RNA was isolated using Trizol method according to manufacturer instructions (Invitrogen, USA). Complementary DNA was synthesized by reverse transcription using a TaKaRa PrimeScript RT reagent kit and then subjected to quantitative real-time PCR using ABI 7900HT Real-Time PCR system (Applied Biosystems, USA). Each reaction was performed in triplicate. Primer sequences are listed in Supplementary Table S2.

Co-immunoprecipitation (CoIP) and immunoblotting

Whole-cell protein lysates for immunoprecipitation (IP) were extracted in IP-lysis buffer [20 mM pH 8.0 Tris·Cl, 180 mM NaCl, 1.0% Nonidet P-40, 10% glycerol, 0.4% EDTA, 1 × phosphatase inhibitors (Thermo) and 1 × protease inhibitors (Thermo)] at 4 °C. Supernatants were clarified by centrifugation at the speed of 12000 rpm for 10 min at 4 °C. For endogenous IP, supernatants were incubated with the corresponding antibodies as indicated and 50 μL of protein A/G Dynabeads (Thermo Fisher Scientific) at 4 °C overnight. For exogenous IP, supernatants were incubated with anti-flag beads (Sigma, USA) at 4 °C overnight. After the conjugation, beads were washed with pre-cooled IP-lysis buffer five times. After that, input and immunoprecipitates were subjected to Western blotting.

Silver staining and mass spectrometry analysis

Whole-cell protein lysates were extracted 48 h after transfected with flag-tagged SETD8. Exogenous IP was performed as described above. Differently, beads were washed with flag peptide, and then supernatant mixed with loading buffer was subjected to SDS-page electrophoresis. Silver staining was performed using the Fast Silver Stain Kit (Beyotime Biotechnology) according to the manufacturer's instructions. Stained gel bands were retrieved and analyzed by mass spectrometry.

Confocal microscopic analysis

Cells were transfected with indicated plasmids after seeded onto glass coverlips. After 48 h, immunofluorescence staining assay was carried out as previously described [21]. Primary HIF1α antibody was purchased from Abcam (ab51608), respectively. Secondary goat anti-rabbit Alexa Fluor 594 antibody was purchased from Invitrogen. Images were visualized and recorded with Leica TCS SP5 confocal microscope.

Dual-luciferase reporter assay

Cells were seeded onto 96-well plates at an initial density of 2×10^4 cells/well. Totally, 2 μg plasmids were transfected into each well. Cells were transfected with 0.5 μg of promoter-luciferase plasmid pGL2-HRE and 0.5/1.0 μg pGL2-Basic plasmid along with 1 μg pCMV-c-flag or 1 μg pCMV-SETD8-flag. To normalize transfection efficiency, 60 ng Renilla luciferase plasmid was transfected into each well simultaneously. Forty-eight hours after transfection, luciferase activity was determined using the Dual-Luciferase Assay System (Promega). Three independent experiments were performed.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed according to manufacturer's instructions using the EZ-ChIP kitfrom Millipore. Proteins and chromatins were immunoprecipitated using antibody against HIF1α (Abcam, ab51608). Primers to detect SETD8 promoter

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