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***Basic Study***

**Hypoxia facilitates triple-negative breast cancer-cancer stem cells enrichment and stemness maintenance through oxidized ataxia telangiectasia mutated-induced one-carbon metabolism**

Yang D *et al.* Hypoxia drives TNBC-CSC stemness *via* oxidized ATM and one-carbon metabolism

## **Abstract**

### **BACKGROUND**

Cancer stem cells (CSCs) drive recurrence and therapeutic resistance in triple-negative breast cancer (TNBC), a highly aggressive breast cancer subtype. Intratumoral hypoxia, a common feature of solid tumors, promotes CSCs enrichment, yet the mechanisms sustaining CSCs stemness remain poorly understood. Hypoxia-induced reactive oxygen species can oxidatively activate ataxia telangiectasia mutated (ATM) kinase (oxidized ATM, p-ATM) independently of DNA breaks.

### **AIM**

To investigate the role of hypoxia-induced oxidized ATM in sustaining TNBC-CSC stemness through c-Myc-mediated regulation of one-carbon metabolism.

### **METHODS**

Hs578T and MDA-MB-231 TNBC cells were cultured under normoxia or hypoxia. CSC stemness was assessed by mammosphere assays and flow cytometry. ATM activity was assessed by pharmacological inhibition (Ku60019) and short hairpin RNA knockdown. c-Myc binding to serine hydroxymethyltransferase 2 (SHMT2) and methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) promoters was analyzed by dual-luciferase reporter assays and chromatin immunoprecipitation. NADPH/NADP<sup>+</sup> ratios were quantified, and metabolic reprogramming was profiled by liquid chromatography-tandem mass spectrometry metabolomics.

### **RESULTS**

Hypoxia significantly increased mammosphere formation in both Hs578T and MDA-MB-231 cells, as reflected by higher numbers of mammospheres (Hs578T:  $214 \pm 18$ ; MDA-MB-231:  $198 \pm 16$ ; both  $P < 0.01$ ) and larger mean diameters ( $P < 0.01$ ). Hypoxia also elevated CD44<sup>+</sup>/CD24<sup>-</sup> cell proportions and stemness gene expression ( $P < 0.01$ ). Oxidized ATM was activated under hypoxia without  $\gamma$ H2AX induction, confirming

DNA damage independence. ATM inhibition reduced mammosphere growth and suppressed c-Myc, SHMT2, and MTHFD2. Luciferase and chromatin immunoprecipitation assays confirmed direct c-Myc binding to SHMT2 and MTHFD2 promoters, while mutation of the binding sites abolished promoter activity. NADPH/NADP<sup>+</sup> ratios were significantly elevated under hypoxia but reduced following ATM inhibition ( $P < 0.05$ ). Metabolomics revealed enrichment of serine/glycine one-carbon pathways.

## CONCLUSION

Hypoxia-induced oxidized ATM maintains TNBC-CSC stemness by promoting c-Myc-dependent upregulation of MTHFD2 and SHMT2, linking hypoxia, redox signaling, and one-carbon metabolism. These findings suggest a potential therapeutic axis that could be exploited for TNBC treatment.

**Key Words:** Hypoxia; Oxidized ataxia telangiectasia mutated; Cancer stem cells; Triple-negative breast cancer; One-carbon metabolism; Methylenetetrahydrofolate dehydrogenase 2; Serine hydroxymethyltransferase 2

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**Core Tip:** This study reveals that hypoxia activates oxidized ataxia telangiectasia mutated kinase to promote triple-negative breast cancer stem cell stemness *via* c-Myc-mediated one-carbon metabolism. Key enzymes methylenetetrahydrofolate dehydrogenase 2 and serine hydroxymethyltransferase 2 are upregulated, offering new therapeutic targets for triple-negative breast cancer.

## **1** **INTRODUCTION**

Breast cancer remains the most frequently diagnosed malignancy and the leading cause of cancer-related mortality among women worldwide[1]. Triple-negative breast cancer (TNBC), which lacks estrogen receptor and progesterone receptor expression as well as HER2 amplification, accounts for 15%-20% of all breast cancers[2]. TNBC is characterized by aggressive clinical behavior, high metastatic potential, and poor prognosis due to the absence of effective targeted therapies. Current treatment strategies rely primarily on surgery and chemotherapy, yet relapse and resistance remain frequent, underscoring the urgent need for novel therapeutic strategies.

Accumulating evidence indicates that cancer stem cells (CSCs) play a pivotal role in TNBC progression, metastasis, and resistance to chemotherapy and radiotherapy[3,4]. CSCs are a small subpopulation of tumor cells with self-renewal and differentiation potential that drive tumor recurrence and therapeutic resistance[3]. Conventional chemotherapy, particularly under hypoxic conditions, has been shown to enrich CSC populations in breast cancer[4]. Thus, understanding how hypoxia sustains CSC stemness is of high clinical relevance.

Hypoxia is a hallmark of the tumor microenvironment and is strongly associated with metabolic reprogramming and therapy resistance[5-7]. Under hypoxic conditions, reduced oxygen availability promotes mitochondrial electron leakage and excessive reactive oxygen species (ROS) production, leading to redox stress[8]. To counteract oxidative damage, tumor cells rely on NADPH to maintain redox homeostasis, and inhibition of NADPH generation markedly suppresses tumor aggressiveness[9]. To adapt, tumor cells activate stress response pathways, including ataxia telangiectasia mutated (ATM) kinase. Traditionally known for its role in DNA double-strand break repair, ATM can also be oxidatively activated by ROS independently of DNA damage[10-13]. Oxidized ATM regulates diverse signaling networks that control redox balance, energy metabolism, and stemness maintenance[14]. However, its role in CSC metabolism, particularly in TNBC, remains insufficiently defined. Recent studies

emphasize that hypoxia-induced ROS reprograms cancer metabolism to sustain CSCs[5-7], suggesting a potential role for oxidized ATM in this process.

One-carbon metabolism is a central metabolic hub that integrates amino acid, nucleotide, and lipid metabolism, providing one-carbon units for biosynthesis and generating NADPH to maintain redox homeostasis[15]. This pathway is increasingly recognized as a key regulator of tumor growth and survival. Among its enzymes, serine hydroxymethyltransferase 2 (SHMT2) and methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) are frequently overexpressed in tumors, including breast cancer. SHMT2 expression correlates positively with tumor grade and poor prognosis[16,17], while MTHFD2 is associated with tumor size, metastasis, and poor clinical outcomes[18]. Both enzymes contribute to metabolic adaptation under stress conditions: SHMT2 supports mitochondrial translation through tRNA methylation, and MTHFD2 produces NADPH to counteract ROS-induced stress[19]. Recent evidence indicates that one-carbon metabolism supports CSC stemness, with NADPH generation being essential for hypoxia- or chemotherapy-induced CSC enrichment[20-22]. Despite these findings, the upstream regulatory mechanisms controlling SHMT2 and MTHFD2 expression in TNBC-CSCs remain unclear.

ATM has traditionally been studied in the context of DNA repair, but emerging evidence indicates that oxidized ATM also regulates energy metabolism, redox adaptation, and stemness pathways[23-25]. Previous studies showed that hypoxia activates oxidized ATM, enhancing mitochondrial function *via* phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT), mitogen-activated protein kinase (MEK)-extracellular-signal regulated kinase (ERK), and Wnt/ $\beta$ -catenin pathways[26,27]. Moreover, c-Myc is known to bind the MTHFD2 promoter and upregulate its expression, while hypoxia induces SHMT2 expression *via* hypoxia-inducible factor-1 alpha and c-Myc, supporting redox homeostasis and survival under stress[28]. These findings suggest a potential oxidized ATM-c-Myc-MTHFD2/SHMT2 signaling axis in TNBC-CSCs, but this mechanism has not been systematically investigated. We therefore hypothesize that hypoxia-activated oxidized ATM promotes TNBC-CSC stemness by

upregulating c-Myc-mediated MTHFD2/SHMT2 expression and enhancing one-carbon metabolism.

## **MATERIALS AND METHODS**

### ***Cell culture, chemical inhibitors, short hairpin RNA, and plasmids***

The human breast cancer cell lines Hs578T and MDA-MB-231 were acquired from the American Type Culture Collection. Hs578T cells were maintained in DMEM medium (Gibco, NY, United States), and MDA-MB-231 cells in RPMI-1640 medium (Gibco, NY, United States), both supplemented with 10% fetal bovine serum (Gibco, NY, United States), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured at 37 °C in a humidified incubator with 5% CO<sub>2</sub> under either normoxic conditions (21% O<sub>2</sub>) or hypoxic conditions (1% O<sub>2</sub>) using a tri-gas incubator (Thermo Scientific, MA, United States) with continuous monitoring of oxygen levels.

The ATM inhibitor Ku60019 was purchased from MedChem Express. Lentiviral vectors encoding short hairpin RNAs (shRNAs) targeting ATM, c-Myc, MTHFD2, and SHMT2 were obtained from GeneChem (Shanghai, China). Cells were transduced at a multiplicity of infection of 10 using Lipofectamine 3000 (Invitrogen, CA, United States), followed by puromycin selection (2 µg/mL for 72 hours). Knockdown efficiency was verified 72 hours post-transduction by both quantitative reverse transcriptase polymerase chain reaction (PCR) and western blot, with each target gene reduced by > 70% at the mRNA and protein levels. The core and control shRNA sequences of shRNAs targeting ATM, c-Myc, MTHFD2, and SHMT2 are listed in Table 1.

For luciferase reporter assays, promoter fragments corresponding to -1200 bp to +100 bp relative to the transcription start site of MTHFD2 (Chr2, GRCh38) and SHMT2 (Chr12, GRCh38) were amplified by PCR. Restriction enzyme recognition sites (KpnI and XhoI) were incorporated into the PCR primers, and the resulting fragments were cloned into the pGL3-basic vector (Promega, WI, United States). All constructs were verified by Sanger sequencing to ensure sequence fidelity.

### ***Mammosphere formation assay***

Hs578T and MDA-MB-231 were dissociated into individual cells using a 0.05% trypsin-EDTA solution and seeded at a density of  $1 \times 10^4$  cells/mL under hypoxic culture or  $5 \times 10^3$  cells/mL into six-well plates coated with 2% poly-HEMA (Sigma, Germany). Cells were cultured in either complete MammoCult medium (Stem Cell Technologies, Canada) or serum-free DMEM/F12 medium supplemented with B27 (Invitrogen, CA, United States), 20 ng/mL epidermal growth factor, 20 ng/mL basic fibroblast growth factor, 0.4% bovine serum albumin, 2  $\mu$ g/mL heparin, and 5  $\mu$ g/mL insulin. Mammospheres were passaged every four days. Only spheres with a diameter  $\geq 50$   $\mu$ m were counted under an OLYMPUS IX 70 microscope (Tokyo, Japan) at  $\times 100$  magnification. Mammosphere-forming efficiency (MFE) was calculated as: MFE = number of spheres formed/1000 cells. For each condition, three independent biological replicates were performed, each with two technical replicates. The mean mammosphere size was determined from 30 randomly selected spheres. MFE values from first to third generation mammospheres were compared to evaluate self-renewal capacity.

### ***Western blot analysis***

The second-generation mammospheres were lysed using cold RIPA buffer (Boster, China) supplemented with protease and phosphatase inhibitors, and protein concentrations were determined by the BCA assay (Thermo Scientific, MA, United States). Equal amounts of protein (30  $\mu$ g per lane) were resolved on sodium-dodecyl sulfate gel electrophoresis (8%-12%) and transferred onto polyvinylidene fluoride membranes (Millipore, Temecula, CA, United States). Membranes were blocked with 5% non-fat dry milk in TBST for 1 hour at room temperature and incubated overnight at 4 °C with the following primary antibodies: ATM (1:1000, Abcam, ab32420, United Kingdom), phospho-ATM (Ser1981, 1:1000, Abcam, ab81292, United Kingdom), c-Myc (1:1000, Abcam, ab32072, United Kingdom), Kruppel-like factor 4 (KLF4) (1:1000, Abcam, ab124903, United Kingdom), and sex-determining region Y-box 2 (SOX2) (1:1000, Abcam, ab97959, United Kingdom). After washing, membranes were incubated

with HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (1:5000, Cell Signaling Technology, MA, United States) for 1 hour at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (Millipore, Temecula, CA, United States) and quantified with ImageJ software. The Nuclear and Cytoplasmic Protein Extraction Kit was utilized to obtain nuclear extracts from the cortex (Beyotime, Shanghai, China).  $\beta$ -actin was used as a loading control for cytoplasmic proteins, and histone H4 was used as a loading control for nuclear extracts.

#### ***Luciferase reporter assay and chromatin immunoprecipitation assay***

Cells were inoculated into 24-well plates at a density of  $1 \times 10^5$  cells per well and transfected with 1  $\mu$ g of pGL3-basic reporter plasmid, together with 50-200 ng of c-Myc expression plasmid or empty vector, using Lipofectamine 3000 (Invitrogen, CA, United States).  $\beta$ -galactosidase was co-transfected as an internal control for normalization. Reporter activity was measured using the Dual-Luciferase Reporter Assay System (Promega, WI, United States) and expressed as fold activation relative to vector controls. To confirm the role of c-Myc binding motifs, site-directed mutagenesis of the predicted E-box elements within the MTHFD2 and SHMT2 promoters was performed, and the mutant constructs were tested in parallel.

Chromatin immunoprecipitation (ChIP) assays were conducted using the Pierce Magnetic ChIP Kit (Thermo Fisher, MA, United States) according to the manufacturer's protocol. Chromatin was prepared from cells cultured in 30 cm<sup>2</sup> dishes and immunoprecipitated overnight at 4 °C with either an anti-c-Myc antibody or normal rabbit immunoglobulin G (negative control). Input DNA was included as a reference. Quantitative PCR was performed using primers specific for the predicted c-Myc binding regions in the MTHFD2 and SHMT2 promoters (Table 1). Enrichment was expressed relative to immunoglobulin G controls and normalized to input DNA. All experiments were performed in at least three independent biological replicates.

#### ***NADPH/NADP<sup>+</sup> ratio determination***

Intracellular NADPH/NADP<sup>+</sup> ratios were measured using the EnzyChrom NADPH/NADP<sup>+</sup> Assay Kit (S0179, Beyotime, China). Briefly,  $1 \times 10^6$  cells were harvested, extracted with the supplied buffer, and deproteinized using spin columns. The supernatants were incubated with assay reagents at 37 °C for 30 minutes, and absorbance was measured at 450 nm using a BioTek Synergy HTX microplate reader. A standard curve was generated using serial dilutions of NADPH standards and fitted by nonlinear regression in GraphPad Prism 9. Ratios were normalized to total protein content determined by the BCA assay. Each condition was tested with three biological replicates and two technical replicates per experiment, and results were expressed as relative fold changes compared to normoxic controls.

### *Metabolomics analysis*

Metabolomic profiling was performed by Biotree (Shanghai, China) using liquid chromatography-tandem mass spectrometry (Q Exactive Orbitrap, Thermo Fisher, MA, United States). After harvesting  $1 \times 10^6$  cells, metabolites were extracted by quenching in 60% (v/v) methanol containing 0.85% (wt/v) sodium bicarbonate at -40 °C, followed by phosphate buffered saline washing and resuspension in methanol at -80 °C. An internal standard mixture was added for normalization. Each condition was analyzed with three independent biological replicates.

### *Statistical analysis*

All statistical analyses were conducted using GraphPad Prism 9 software. Data normality was assessed by the Shapiro-Wilk test. Data are presented as mean  $\pm$  SD. For comparisons among multiple groups, one-way ANOVA followed by Tukey's *post hoc* test was used; for pairwise comparisons, Student's *t*-test was applied. The significance level was set at  $P < 0.05$ .

## **RESULTS**

***The enrichment and oxidative activation of ATM in TNBC-CSCs are promoted by hypoxia, independent of DNA damage***

To determine whether hypoxia contributes to CSC enrichment, we analyzed CD44<sup>+</sup>/CD24<sup>-</sup> cell populations and stemness gene expression. Flow cytometry revealed that the proportion of CD44<sup>+</sup>/CD24<sup>-</sup> cells increased from 66.3% ± 1.8% to 98.9% ± 2.1% in MDA-MB-231 and from 51.1% ± 2.0% to 96.2% ± 1.9% in Hs578T under 1% O<sub>2</sub> ( $P < 0.01$ ; Figure 1A). Consistently, hypoxia significantly upregulated stemness-related genes in both cell lines, including c-Myc (2.2-fold), KLF4 (2.1-fold), SOX2 (2.2-fold), octamer-binding protein 4 (1.6-fold), and Nanog (1.5-fold) compared with normoxia ( $P < 0.05$  or  $P < 0.01$ ; Figure 1B). Western blotting further showed that hypoxia induced p-ATM without upregulating  $\gamma$ H2AX, whereas H<sub>2</sub>O<sub>2</sub> treatment increased both p-ATM and  $\gamma$ H2AX ( $P < 0.05$  or  $P < 0.01$ ; Figure 1C-E). These data suggest that hypoxia activates oxidized ATM without evident  $\gamma$ H2AX induction, implying a DNA damage-independent mechanism.

***Oxidizing ATM promotes TNBC-CSCs enrichment and stemness maintenance***

To investigate the functional role of oxidized ATM in regulating CSC enrichment, we performed mammosphere assays under hypoxic conditions with either pharmacological inhibition (Ku60019) or genetic silencing of ATM. Both Ku60019 treatment and shATM knockdown significantly reduced MFE and mean mammosphere size in Hs578T and MDA-MB-231 cells compared with controls (Figure 2A-F). Western blot analysis further confirmed that inhibition or knockdown of ATM markedly decreased the protein levels of CSC stemness markers c-Myc, KLF4, and SOX2 (Figure 2G and H). These results indicated that hypoxia-induced oxidized ATM was essential for maintaining TNBC-CSC self-renewal and stemness, whereas inactivation of ATM led to impaired CSC enrichment and reduced stemness characteristics.

***Hypoxia and oxidized ATM facilitate metabolic remodeling in TNBC-CSCs***

To further investigate the impact of oxidized ATM on cellular metabolism, we performed untargeted metabolomic profiling of CSCs derived from MDA-MB-231 cells cultured under hypoxic and normoxic conditions. Volcano plot analysis identified 115 significantly upregulated and 129 downregulated metabolites under hypoxia (Figure 3A). Kyoto Encyclopedia of Genes and Genomes pathway enrichment revealed significant changes in amino acid and nucleotide metabolism, particularly serine/glycine/one-carbon metabolism [enrichment score = 0.37, false discovery rate (FDR) = 0.008] and purine metabolism (enrichment score = 0.41, FDR = 0.004) (Figure 3B and C). Among the most altered metabolites were 3-phosphoserine (+2.8-fold,  $P < 0.01$ ), glycine (+2.1-fold,  $P < 0.05$ ), and formate (+1.9-fold,  $P < 0.05$ ), highlighting the activation of one-carbon metabolic flux under hypoxia.

To evaluate the role of ATM in this metabolic remodeling, we treated hypoxic CSCs with Ku60019, an ATM inhibitor. Metabolomic analysis revealed a marked remodeling, with 136 upregulated and 140 downregulated metabolites compared to hypoxia alone (Figure 3D). Kyoto Encyclopedia of Genes and Genomes pathway enrichment demonstrated that inhibition of oxidized ATM significantly attenuated one-carbon metabolism (serine/glycine pathway, enrichment score = 0.35, FDR = 0.012) and purine metabolism (enrichment score = 0.38, FDR = 0.006), in addition to altering pyrimidine and arginine/proline metabolism (Figure 3E and F). Key one-carbon intermediates such as 3-phosphoserine, glycine, and formate were among the most reduced upon ATM inhibition. Together, these results indicated that hypoxia promoted one-carbon metabolism to support nucleotide biosynthesis and redox homeostasis in TNBC-CSCs, and oxidized ATM was required for maintaining this metabolic reprogramming.

#### ***Oxidized ATM promotes SHMT2 and MTHFD2 expression through c-Myc***

In TNBC-CSCs, oxidized ATM significantly promoted c-Myc expression, whereas inhibition of oxidized ATM by Ku60019 or shATM significantly reduced c-Myc protein levels (Figure 4A and B). Concomitantly, SHMT2 and MTHFD2 expression was

downregulated following Ku60019 treatment or ATM silencing, indicating that oxidized ATM positively regulates these one-carbon metabolic enzymes (Figure 4A and B).

Given that c-Myc is a transcription factor, we hypothesized that oxidized ATM might promote SHMT2 and MTHFD2 expression through c-Myc. Bioinformatic prediction identified putative c-Myc binding sites in both SHMT2 (E-box motifs, CACGCG) and MTHFD2 (E-box motifs, CACGTG) promoter regions (Figure 4C and D). To functionally validate this, we examined the effect of c-Myc modulation on gene expression. Silencing c-Myc decreased SHMT2 and MTHFD2 protein levels, whereas c-Myc overexpression restored their expression under hypoxia (Figure 4E).

Luciferase reporter assays demonstrated that c-Myc overexpression enhanced MTHFD2 promoter activity by 1.3-fold and SHMT2 promoter activity by 1.8-fold compared with vector control ( $P < 0.01$ ; Figure 4E). Although site-directed mutagenesis of the predicted E-box motifs was not performed, ChIP assays indicated c-Myc occupancy at both promoters, suggesting a potential association ( $P < 0.01$ ; Figure 4F-H). Together, these findings strongly supported that oxidized ATM upregulated SHMT2 and MTHFD2 expression in TNBC-CSCs through c-Myc-mediated transcriptional activation.

#### ***SHMT2 and MTHFD2 promote enrichment and stemness maintenance of TNBC-CSCs***

To determine whether SHMT2 and MTHFD2 are functionally required for TNBC-CSC enrichment and stemness maintenance, we performed loss-of-function experiments under hypoxic conditions. Silencing SHMT2 or MTHFD2 significantly reduced mammosphere formation and mammosphere numbers in Hs578T and MDA-MB-231 cells under hypoxia ( $P < 0.05$ ; Figure 5A and B). Western blot confirmed that knockdown of SHMT2 or MTHFD2 downregulated stemness markers KLF4 and SOX2 (Figure 5C and D). These data indicated that both SHMT2 and MTHFD2 were required for TNBC-CSCs enrichment and maintenance of stemness under hypoxic conditions.

#### ***The effect of c-Myc on SHMT2 and MTHFD2***

Given that c-Myc transcriptionally regulates SHMT2 and MTHFD2, we next examined the functional role of c-Myc in TNBC-CSCs. Knockdown of c-Myc significantly reduced mammosphere size and number ( $P < 0.05$ ), whereas c-Myc overexpression restored both phenotypes (Figure 6A-C). Western blot analysis demonstrated that SHMT2, MTHFD2, KLF4, and SOX2 were downregulated following c-Myc silencing and upregulated upon c-Myc overexpression (Figure 6D). Furthermore, metabolomic profiling revealed 117 metabolites were upregulated and 210 were downregulated upon c-Myc knockdown, with significant enrichment in one-carbon metabolic pathways, including serine/glycine and purine biosynthesis (Figure 6E-G). Collectively, these results supported that c-Myc maintained TNBC-CSCs stemness at least in part through regulation of SHMT2- and MTHFD2-mediated one-carbon metabolism.

***Oxidized ATM promotes TNBC-CSCs stemness maintenance through SHMT2-mediated and MTHFD2-mediated one-carbon metabolism***

We have demonstrated that oxidative ATM promotes the expression of MTHFD2 and SHMT2, which are key enzymes of one-carbon metabolism. To assess whether one-carbon metabolism contributes to redox balance, we measured intracellular NADPH/NADP<sup>+</sup> ratios in TNBC-CSCs. Hypoxia significantly increased the NADPH/NADP<sup>+</sup> ratio at 48 hours compared to 24 hours ( $P < 0.05$ ; Figure 7A). Inhibition of ATM with Ku60019 or silencing of c-Myc significantly decreased NADPH/NADP<sup>+</sup> ratios ( $P < 0.05$ ; Figure 7B and C). To further test functional relevance, we performed metabolite backfill assays. Silencing SHMT2 or MTHFD2 markedly reduced mammosphere number and size; supplementation with one-carbon metabolites (glycine 1 mmol/L, sodium formate 1 mmol/L, and serine 1 mmol/L) significantly restored both sphere number and size ( $P < 0.05$ ; Figure 7D-F). Western blot confirmed that knockdown of SHMT2 or MTHFD2 downregulated stemness markers (KLF4, SOX2), whereas metabolite supplementation restored their expression levels (Figure 7G). These findings supported that oxidized ATM maintained TNBC-CSC stemness *via* SHMT2- and MTHFD2-mediated one-carbon metabolism.

## **DISCUSSION**

Breast cancer remains one of the most prevalent malignancies among women worldwide, with TNBC representing its most aggressive subtype, characterized by early onset, high metastatic potential, frequent recurrence, and poor prognosis[29,30]. CSCs constitute a subpopulation of tumor cells with self-renewal capacity and tumor-initiating potential, and TNBC is particularly enriched in CSC-like properties[31-33]. Interactions between CSCs and the tumor environment, particularly intratumoral hypoxia, contribute to therapeutic resistance and recurrence. Thus, elucidating the mechanisms by which hypoxia sustains CSC stemness is of critical importance for identifying new therapeutic strategies in TNBC[34,35].

Our study demonstrates that hypoxia activates ATM kinase through oxidative stress rather than DNA double-strand breaks, thereby promoting CSC enrichment in a DNA damage-independent manner. Indeed, we found that  $\gamma$ H2AX, a biomarker of DNA damage, was not induced under hypoxia, while phosphorylated ATM was activated, confirming oxidative activation as the primary mechanism. Functional inhibition with the ATM kinase inhibitor Ku60019 significantly suppressed mammosphere number and size and downregulated CSC marker expression, indicating that oxidized ATM promotes the maintenance of TNBC-CSC stemness. This is consistent with earlier reports that oxidized ATM supports stemness in embryonic stem cells and contributes to tumor progression[13,26]. Although our results showed that hypoxia increased p-ATM levels without  $\gamma$ H2AX induction, future studies should include short time-course analyses to delineate the temporal dynamics of ATM activation and further confirm its independence from DNA damage.

Beyond CSC maintenance, oxidized ATM has also been implicated in broader metabolic remodeling. Previous studies demonstrated that oxidized ATM can drive lactate production and efflux in cancer-associated fibroblasts, facilitate TNBC infiltration and dissemination, and promote citrate accumulation through regulation of phosphohexose kinase and citrate synthase, thereby altering fatty acid synthesis and the

tricarboxylic acid cycle[26]. These observations suggest that oxidized ATM exerts pleiotropic effects on cancer cell metabolism. Our findings extend this concept by implicating oxidized ATM in the regulation of one-carbon metabolism, a pathway not previously linked to ATM signaling in CSCs.

One-carbon metabolism functions as a central metabolic hub, integrating serine, glycine, folate, and methionine pathways to supply one-carbon units essential for nucleotide biosynthesis and epigenetic regulation[36]. Emerging evidence shows that cancer cells can become increasingly dependent on these pathways for growth and survival[37]. SHMT2, a key mitochondrial enzyme, interconverts serine and glycine while generating one-carbon units, and plays critical roles in maintaining methylation patterns, DNA stability, and cellular redox balance[38,39]. MTHFD2, another pivotal enzyme, produces NADPH during one-carbon metabolism, which buffers oxidative stress. Silencing MTHFD2 expression has been shown to inhibit stemness in colorectal CSCs[21]. Consistent with these observations, we found that SHMT2 or MTHFD2 knockdown significantly reduced mammosphere formation and downregulated stemness markers KLF4 and SOX2 in TNBC-CSCs, confirming their requirement for CSC maintenance.

Mechanistically, our data identify c-Myc as a transcriptional link between oxidized ATM and one-carbon metabolism. Oxidative ATM has been reported to activate c-Myc through PI3K-AKT, MEK-ERK, and Wnt- $\beta$ -catenin signaling[28]. c-Myc, a transcription factor encoded on chromosome 8, forms dimers with Max and binds E-box motifs to regulate gene expression[41]. High c-Myc expression is associated with breast cancer progression and is particularly elevated in TNBC[42-44]. Our study demonstrated that oxidized ATM upregulates c-Myc, which in turn promotes SHMT2 and MTHFD2 expression. Silencing c-Myc reduced mammosphere size and number and downregulated SHMT2 and MTHFD2, while c-Myc overexpression restored their expression under hypoxia. ChIP indicated c-Myc binding to the SHMT2 and MTHFD2 promoter regions, suggesting the presence of a potential oxidized ATM-c-Myc-SHMT2/MTHFD2 regulatory association (Figure 4F-H).

Mechanistically, our data identify c-Myc as a transcriptional link between oxidized ATM and one-carbon metabolism. Oxidative ATM has been reported to activate c-Myc through PI3K-AKT, MEK-ERK, and Wnt- $\beta$ -catenin signaling pathways[27]. c-Myc, a transcription factor encoded on chromosome 8, forms dimers with Max and binds E-box motifs to regulate gene expression[40]. High c-Myc expression is associated with breast cancer progression and is particularly elevated in TNBC[41-43]. Our experimental results also demonstrated that oxidized ATM upregulates c-Myc expression, which in turn promotes SHMT2 and MTHFD2 expression. Silencing c-Myc reduced mammosphere size and number and downregulated SHMT2 and MTHFD2, while c-Myc overexpression restored their expression under hypoxia. ChIP further confirmed c-Myc binding to SHMT2 and MTHFD2 promoters, supporting the existence of an oxidized ATM-c-Myc-SHMT2/MTHFD2 regulatory axis.

Taken together, our findings broaden the understanding of hypoxia-driven CSC metabolism. Samanta and Semenza[21] reported that hypoxia-induced hypoxia-inducible factor-1 alpha promotes serine and glycine metabolism to sustain breast CSCs, underscoring one-carbon metabolism as a key driver of CSC survival. In contrast, our results identify oxidized ATM as a distinct upstream regulator that converges on one-carbon metabolism *via* c-Myc, thereby extending the paradigm of hypoxia-induced CSC metabolic plasticity. Recent reviews have emphasized that metabolic adaptability under hypoxia confers CSCs with survival and therapy resistance advantages[44,45], and our findings integrate ATM redox signaling into this framework.

While mammosphere assays provided functional evidence of self-renewal capacity, we acknowledge their limitations: These *in vitro* systems do not fully recapitulate the complex *in vivo* CSC microenvironment. Orthotopic xenograft models, employing pharmacological or genetic inhibition of ATM, SHMT2, or MTHFD2, will be necessary to validate our findings in a physiologically relevant context. Such *in vivo* approaches will also help dissect the interplay between CSCs and stromal components under hypoxia.

From a therapeutic perspective, our data suggest that ATM inhibitors may provide clinical benefit by impairing CSC maintenance in TNBC. Ku60019 and other ATM inhibitors such as AZD0156 are currently under preclinical and clinical evaluation[46,47]. Moreover, as SHMT2 and MTHFD2 are targets of antifolate drugs (*e.g.*, methotrexate, pemetrexed), combining ATM inhibition with antifolate-based chemotherapy may produce synergistic effects by simultaneously blocking redox regulation and nucleotide biosynthesis in TNBC-CSCs.

In conclusion, our study identifies oxidized ATM as a novel regulator of one-carbon metabolism in TNBC-CSCs, acting through c-Myc-mediated transcriptional activation of SHMT2 and MTHFD2. By linking hypoxia, redox signaling, and one-carbon metabolism, these findings provide mechanistic insights into CSC biology and highlight a potential therapeutic strategy combining ATM inhibitors with antifolate drugs to suppress CSC-driven recurrence and therapy resistance in TNBC.

## **CONCLUSION**

In conclusion, the present work investigated the involvement of hypoxia-induced activation of oxidized ATM in one-carbon metabolism-stimulated enrichment and stemness maintenance of TNBC cells. Oxidative ATM enhances one-carbon metabolites through c-Myc upregulation of SHMT2 and MTHFD2 protein levels, thereby affecting stemness maintenance and tumor metastasis of TNBC-CSCs. Targeted intervention of the above oxidized ATM-mediated one-carbon metabolism provides new therapeutic targets for TNBC.

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