

ADAMTS9-AS2 suppresses hypoxia-driven glycolytic reprogramming through EZH2-dependent regulation of ENO1 alternative splicing in bladder cancer

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ABSTRACT

Objective(s): Tumor hypoxia is a key driver of metabolic reprogramming and aggressive progression in bladder cancer; however, the upstream long non-coding RNA (lncRNA)-mediated epigenetic mechanisms that regulate these processes remain incompletely understood. This study investigated the role of ADAMTS9-AS2 in hypoxia-induced metabolic remodeling and its interaction with EZH2 and ENO1 alternative splicing.

Materials and Methods: Bladder cancer cell lines (T24 and UM-UC-3) were cultured under hypoxic or normoxic conditions. Stable knockdown and overexpression models of ADAMTS9-AS2, EZH2, and ENO1 were generated using lentiviral vectors. Functional assays included CCK-8, colony formation, wound-healing, Transwell invasion, Seahorse XF metabolic analysis, glucose uptake, lactate/ATP quantification, ROS detection, JC-1 staining, and transmission electron microscopy. Molecular interactions were assessed by RNA-FISH, RNA immunoprecipitation, biotinylated RNA pull-down, ChIP-qPCR, and RNA sequencing with alternative splicing analysis (rMATS/SUPPA2). *In vivo* effects were evaluated using subcutaneous xenograft models with bioluminescence imaging and immunohistochemistry. Rescue experiments established pathway causality.

Results: Hypoxia significantly reduced ADAMTS9-AS2 expression, which correlated with advanced clinicopathological stage and poor survival. ADAMTS9-AS2 directly interacted with nuclear EZH2 and regulated chromatin remodeling at the ENO1 locus, thereby altering ENO1 alternative splicing. Loss of ADAMTS9-AS2 promoted glycolysis, mitochondrial dysfunction, ROS accumulation, proliferation, migration, invasion, and xenograft growth. Conversely, ENO1 silencing or EZH2 signaling restoration attenuated these malignant phenotypes.

Conclusion: ADAMTS9-AS2 acts as a hypoxia-responsive tumor suppressor that restrains bladder cancer progression by regulating the EZH2/ENO1 signaling axis. These findings identify a novel lncRNA-mediated epigenetic mechanism linking hypoxia, alternative splicing, and metabolic reprogramming, highlighting ADAMTS9-AS2 and the EZH2/ENO1 pathway as promising therapeutic targets for bladder cancer.

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Introduction

One of the most prevalent urological cancers, bladder cancer is expected to cause 614,000 new cases and 221,000 deaths worldwide in 2022, with males accounting for around 75% of the burden (1). Although most patients have non-muscle-invasive conditions that may be treated endoscopically, recurrence rates are more than 50%, and up to 20–30% develop muscle-invasive bladder cancer (MIBC) (2). Despite aggressive cystectomy, perioperative chemotherapy, and new developments in immune

checkpoint inhibitors and antibody-drug conjugates, 5-year survival rates for MIBC and metastatic patients are less than 50%. The hostile tumor microenvironment (TME), where hypoxia is a key characteristic, is primarily responsible for these dismal results (3). Rapid tumor development and defective vasculature cause hypoxia, which in turn stabilizes hypoxia-inducible factor-1α (HIF-1α), thereby transcriptionally activating hundreds of genes involved in invasion, angiogenesis, and, most importantly, metabolic reprogramming (4).

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Even in the presence of oxygen, bladder cancer cells switch from oxidative phosphorylation to aerobic glycolysis under hypoxic circumstances (the Warburg effect) (5, 6). This metabolic flexibility provides lactate, ATP, and biosynthetic intermediates that acidify the extracellular environment, promoting angiogenesis, invasion, and immune evasion. Important glycolytic enzymes, such as HK2, PKM2, LDHA, and ENO1, are overexpressed in bladder cancer and are associated with poor prognosis, advanced stage, and lymph node metastases (7). Particularly important is ENO1, which catalyzes the transformation of 2-phosphoglycerate into phosphoenolpyruvate. In addition to its primary metabolic activity, ENO1 is a plasminogen receptor that facilitates cell motility and extracellular matrix breakdown (8). Importantly, alternate splicing of ENO1 results in many isoforms; the nuclear Myc-binding protein-1 (MBP-1), which binds the c-Myc promoter and exerts tumor-suppressive transcriptional repression, is produced by a shorter splice variant, whereas the full-length transcript encodes the glycolytic enzyme (9, 10). Therefore, dysregulation that favors pro-glycolytic ENO1 isoforms might intensify oncogenic signaling and metabolic addiction. This glycolytic transition is often accompanied by concurrent mitochondrial dysfunction, which contributes to tumor aggressiveness and is characterized by membrane depolarization, cristae disruption, and increased reactive oxygen species (ROS) (11).

These activities are carefully regulated by long non-coding RNAs (lncRNAs) and epigenetic regulators. Although non-canonical roles in splicing regulation are increasingly recognized, EZH2, the histone methyltransferase subunit of Polycomb repressive complex 2 (PRC2), is overexpressed in bladder cancer and primarily promotes proliferation, stemness, and invasion through H3K27me3-mediated gene silencing (12). Chromatin alterations affect spliceosome accessibility and RNA polymerase II elongation rates, which in turn affect co-transcriptional alternative splicing. Local epigenetic landscapes and splicing outcomes can be shaped by lncRNAs acting as molecular scaffolding that attract EZH2 or other chromatin modifiers to certain loci (13). The antisense lncRNA ADAMTS9-AS2, transcribed from the ADAMTS9 gene, has been shown to reduce tumor growth in bladder cancer. Its down-regulation in tumor tissues and cell lines (T24, 5637, UM-UC-3) relative to normal urothelial cells has been demonstrated in several studies. Overexpression inhibits proliferation, migration, and invasion, but knockdown has the opposite effects, partially due to miR-182-5p sponging (14, 15). Nevertheless, no prior research has examined how hypoxia regulates ADAMTS9-AS2, its nuclear activities, or its potential connections with EZH2 in regulating metabolic gene splicing.

Functionally different molecules are produced by alternative processing of the ENO1 locus. The shorter MBP-1 isoform acts as a nuclear transcriptional regulator of c-Myc and exhibits tumor-suppressive properties. In contrast, the full-length ENO1 protein is an essential glycolytic enzyme that supports tumor metabolism. Thus, changes in ENO1 transcript processing may simultaneously increase glycolysis and decrease anti-oncogenic signaling (16).

This study aimed to systematically elucidate the ADAMTS9-AS2/EZH2/ENO1 regulatory axis under hypoxic conditions in bladder cancer. The specific goals were to: (1) quantify ADAMTS9-AS2 expression in

clinical specimens, cell lines, and hypoxic cultures and correlate findings with clinicopathological parameters and patient survival; (2) confirm direct physical interaction between nuclear ADAMTS9-AS2 and EZH2 via RNA-FISH, RIP, and biotinylated RNA pull-down assays and map consequent changes in histone methylation at the ENO1 locus by ChIP-qPCR; (3) characterize hypoxia- and ADAMTS9-AS2-dependent alterations in ENO1 alternative splicing using RNA-seq, rMATS/SUPPA2 analysis, sashimi plots, and PSI quantification, validated by RT-PCR; (4) determine downstream impacts on glycolytic parameters (Seahorse ECAR/OCR, glucose uptake, lactate production, ATP levels), mitochondrial ultrastructure and membrane potential (TEM, JC-1), ROS generation, and malignant phenotypes (Cell Counting Kit-8 (CCK-8), colony formation, 5-ethynyl-2'-deoxyuridine (EdU), wound-healing, Transwell) through lentiviral knockdown/overexpression and rescue approaches; and (5) validate the axis *in vivo* using subcutaneous xenograft models in nude mice, bioluminescence imaging, tumor growth/weight analysis, H&E histology, and IHC quantification of Ki-67, EZH2, ENO1, and H3K9me3, with ENO1 silencing rescue experiments to establish causality. Collectively, this work seeks to uncover a novel hypoxia-responsive lncRNA-epigenetic-splicing-metabolic circuit that drives bladder cancer progression and to provide a foundation for precision therapeutic strategies targeting either restoration of ADAMTS9-AS2 or inhibition of EZH2/ENO1 to overcome glycolytic addiction and improve patient outcomes (Figure 1).

Materials and Methods

Cell culture and experimental design

The normal human urothelial cell line SV-HUC-1 and the human bladder cancer cell lines T24, 5637, and UM-UC-3 were obtained from verified cell banks and maintained in accordance with the suppliers' guidelines. 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin were added to the RPMI-1640 medium used to cultivate the cells. All cells were kept at 37 °C in a humidified incubator with 5% CO₂. Before further analysis, cells were placed in a tri-gas incubator with 1% O₂, 5% CO₂, and 94% N₂ for 24 to 48 hr to create a hypoxic microenvironment that resembles the physiological features of bladder cancer tissues. Control cells were grown in normoxic conditions (21% O₂). The following groups of cells were used for mechanistic investigations: control, hypoxia, hypoxia plus ADAMTS9-AS2 knockdown, hypoxia plus EZH2 overexpression, hypoxia plus ENO1 knockdown, hypoxia plus ADAMTS9-AS2 knockdown and EZH2 overexpression, and hypoxia plus EZH2 overexpression plus ENO1 knockdown. Every experiment was carried out three times.

Establishment of stable knockdown and overexpression cell lines

Using a lentiviral transduction technique, stable knockdown and overexpression cell models were created to examine the biological roles of ADAMTS9-AS2, EZH2, and ENO1. While full-length EZH2 coding sequences were cloned into pCDH expression vectors, short-hairpin RNA sequences targeting ADAMTS9-AS2 and ENO1 were developed and cloned into pLKO.1 vectors. Recombinant plasmids were co-transfected with the packaging plasmids

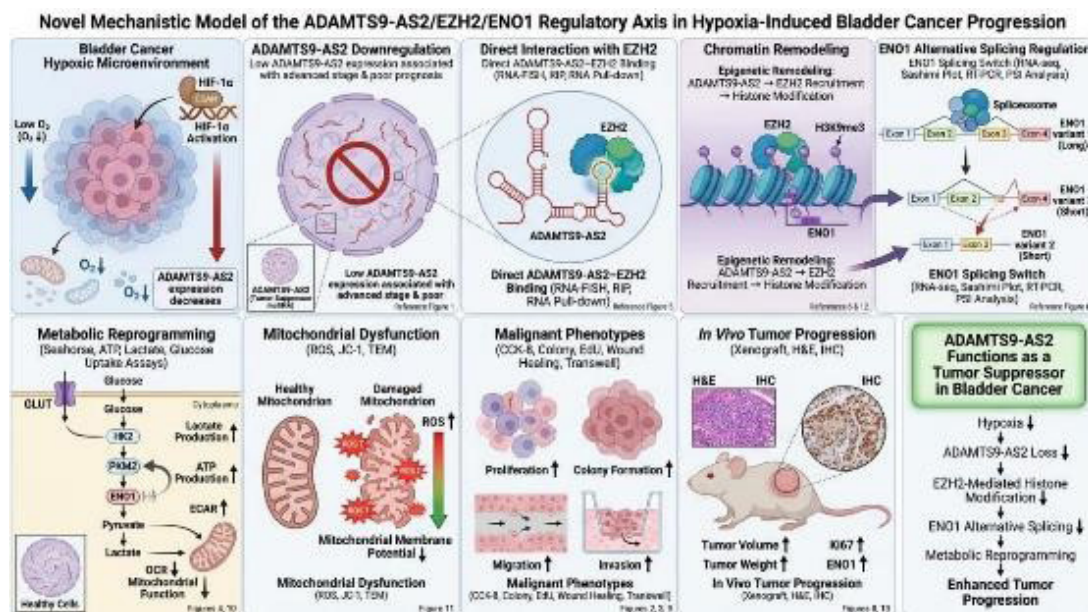


Figure 1. Novel mechanistic model of the ADAMTS9-AS2/EZH2/ENO1 regulatory axis in mouse hypoxia-induced bladder cancer progression

Schematic illustration summarizing the molecular mechanism by which ADAMTS9-AS2 suppresses bladder cancer progression under hypoxic conditions. Hypoxia within the bladder tumor microenvironment promotes HIF-1 α activation and significantly reduces ADAMTS9-AS2 expression. Decreased ADAMTS9-AS2 levels are associated with advanced tumor stage, poor clinical outcome, and enhanced malignant progression. Mechanistically, ADAMTS9-AS2 directly interacts with the epigenetic regulator EZH2 in the nucleus, as demonstrated by RNA-FISH, RNA immunoprecipitation (RIP), and RNA pull-down assays. The ADAMTS9-AS2-EZH2 complex regulates chromatin architecture at the ENO1 locus through histone methylation, resulting in altered H3K9me3 enrichment and epigenetic remodeling. Subsequently, EZH2 recruitment and chromatin modification influence ENO1 alternative splicing, thereby increasing the production of functional ENO1 splice variants. Enhanced ENO1 activity promotes metabolic reprogramming characterized by increased glucose uptake, elevated glycolytic flux, enhanced lactate production, increased ATP generation, and elevated extracellular acidification rates (ECAR), accompanied by reduced oxygen consumption rates (OCR). In parallel, ADAMTS9-AS2 deficiency induces mitochondrial dysfunction, including mitochondrial membrane depolarization, excessive ROS accumulation, mitochondrial swelling, cristae disruption, and structural fragmentation. These metabolic and mitochondrial alterations collectively promote aggressive tumor phenotypes, including enhanced cellular proliferation, clonogenic survival, migration, and invasion. In vivo, loss of ADAMTS9-AS2 accelerates xenograft growth and increases tumor burden, accompanied by elevated expression of Ki67, EZH2, ENO1, and H3K9me3. Rescue experiments further demonstrate that ENO1 functions as a critical downstream effector of the ADAMTS9-AS2/EZH2 signaling pathway, as ENO1 silencing effectively reverses the oncogenic effects induced by ADAMTS9-AS2 depletion. Overall, the study identifies a previously unrecognized ADAMTS9-AS2/EZH2/ENO1 signaling axis that links hypoxia-induced epigenetic regulation, alternative splicing, metabolic reprogramming, and bladder cancer progression. These findings suggest that restoration of ADAMTS9-AS2 expression or therapeutic targeting of the EZH2/ENO1 pathway may be promising strategies for treating bladder cancer.

HIF-1 α : Hypoxia-inducible factor 1- α ; RNA-FISH: RNA fluorescence in situ hybridization; RIP: RNA immunoprecipitation; ECAR: Extracellular acidification rate; OCR: Oxygen consumption rate; ROS: Reactive oxygen species; H3K9me3: Histone H3 lysine 9 trimethylation; Ki-67: Marker of proliferation Ki-67

psPAX2 and pMD2.G into 293T cells using Lipofectamine 3000 to produce lentiviral particles. Viral supernatants were collected 48 and 72 hr after transfection, filtered through 0.45- μ m membranes, and then ultracentrifuged to concentrate them. In the presence of 8 μ g/ml polybrene, viral particles infected bladder cancer cells. Puromycin (2 μ g/ml) was used to select cells for 2 weeks after infection to achieve stable cell populations. Western blot analysis and quantitative real-time PCR were used to validate knockdown and overexpression efficiency.

Cell viability assay

The Cell Counting Kit-8 test was used to measure cell viability. In short, cells were grown under the specified experimental conditions after being seeded onto 96-well plates at a density of 3×10^3 cells per well. Ten microliters of CCK-8 reagent were added to each well at 0, 24, 48, 72, and 96 hr, and the mixture was incubated for two hours at 37 $^{\circ}$ C. A microplate reader was used to measure absorbance at 450 nm. Plotting optical density data versus incubation time produced cell growth curves.

Colony formation assay

About 500 cells were seeded into six-well plates and cultured for 10–14 days until visible colonies developed to assess long-term proliferative capacity. Every three days,

the medium was changed. Colonies were stained with 0.1% crystal violet for 30 min after being fixed with 4% paraformaldehyde for 20 min. Colony formation efficiency was computed by manually counting colonies with more than 50 cells under a light microscope.

Wound-healing and transwell assays

A wound-healing experiment was used to assess cell migration. Six-well plates were used to seed the cells, which were then allowed to achieve about 90% confluence. A linear scratch was made across the cell monolayer using a sterile 200- μ l pipette tip. Phosphate-buffered saline was used to wash away detached cells. After adding serum-free medium, an inverted microscope was used to photograph the wound closure at 0 and 24 hr. The percentage of wound closure was used to measure migration ability. Matrigel was applied to Transwell chambers with 8- μ m pore membranes for invasion tests. While complete media containing 10% fetal bovine serum was supplied to the lower chambers as a chemoattractant, cells floating in serum-free medium were implanted into the upper chambers. Cotton swabs were used to remove non-invasive cells from the top surface after a 24-hr incubation period. In five randomly selected microscopic fields, invaded cells that adhered to the lower membrane surface were fixed, stained with crystal violet, photographed, and counted.

Seahorse analysis of cellular glycolytic function

A Seahorse XF96 Extracellular Flux Analyzer was used to measure mitochondrial respiration and cellular glycolytic activity. Each cell line was seeded at its optimal density into Seahorse XF96 microplates and grown overnight. Seahorse assay medium was used instead of culture medium before analysis, and cells were cultured for 1 hr in a non-CO₂ incubator. The glycolysis stress test involved sequentially injecting glucose, oligomycin, and 2-deoxyglucose. Basal glycolysis, glycolytic capacity, and glycolytic reserve were assessed using extracellular acidification rate (ECAR). During mitochondrial stress testing, oligomycin, FCCP, and rotenone/antimycin A were injected to assess the oxygen consumption rate (OCR) simultaneously. Seahorse Wave program was used to examine the data.

Measurement of glucose uptake, lactate production, and ATP levels

The luminous glucose analog 2-NBDG was used to measure glucose uptake. After 30 min of incubation at 37 °C with 100 µM 2-NBDG, cells were washed with PBS and subjected to flow cytometry analysis. The intensity of intracellular fluorescence indicated the effectiveness of glucose absorption. A colorimetric lactate assay kit was used to measure lactate production in accordance with the manufacturer's instructions. Lactate concentrations were adjusted to total protein content in cell culture supernatants. A luciferase-based ATP measurement kit was used to quantify intracellular ATP levels. A microplate luminometer was used to measure luminescence after cell lysates were combined with luciferin-luciferase reagent.

Detection of intracellular reactive oxygen species

The fluorescent probe DCFH-DA was used to assess intracellular ROS levels. For thirty minutes, cells were treated in the dark at 37 °C with 10 µM DCFH-DA. Fluorescence intensity was measured using flow cytometry and fluorescence microscopy after PBS washing. By comparing the mean fluorescence intensity of each experimental group, the relative generation of ROS was determined.

Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde overnight at 4 °C and post-fixed with 1% osmium tetroxide for two hours to investigate changes in mitochondrial ultrastructure. After being dehydrated using a graded ethanol series, the samples were embedded in epoxy resin. An ultramicrotome was used to create ultrathin sections (70–90 nm), which were then stained with lead citrate and uranyl acetate. A

transmission electron microscope was used to analyze sections. In randomly selected fields, mitochondrial morphology, cristae structure, swelling, fragmentation, and mitochondrial number were evaluated.

RNA immunoprecipitation assay

To determine whether ADAMTS9-AS2 physically interacts with EZH2, RNA immunoprecipitation was performed. RNase inhibitor-containing RIP lysis buffer was used to lyse the cells. Magnetic beads attached to either normal rabbit IgG or anti-EZH2 antibody were used to incubate the lysates overnight. TRIzol reagent was used to extract and purify immunoprecipitated RNA. qRT-PCR was used to measure the enrichment of ADAMTS9-AS2 in immunoprecipitated complexes (Table 1).

RNA pull-down assay

In vitro synthesis of biotin-labeled ADAMTS9-AS2 transcripts was followed by incubation with cellular protein lysates. Streptavidin-coated magnetic beads were used to collect RNA-protein complexes, which were then thoroughly cleaned to remove nonspecific interactions. After bound proteins were eluted, an anti-EZH2 antibody was used for western blot analysis. Direct binding between ADAMTS9-AS2 and EZH2 was confirmed by the assay.

Chromatin immunoprecipitation assay

To determine whether EZH2-mediated histone methylation regulates ENO1 transcription and splicing, chromatin immunoprecipitation experiments were performed. After 10 min of cross-linking with 1% formaldehyde, the cells were quenched with glycine. Sonication was used to break up chromatin into 200–500 bp pieces. Antibodies against EZH2, H3K9me3, and H3K27me3 were incubated with equal volumes of chromatin for an entire night at 4 °C. Protein A/G magnetic beads were used to gather immunocomplexes. Primers targeting the ENO1 promoter and regulatory regions were used for quantitative PCR after reverse cross-linking and DNA purification. The input DNA was used to standardize the enrichment levels.

RNA sequencing and alternative splicing analysis

The TRIzol reagent was used to extract total RNA. Samples with RNA integrity scores greater than 7.0 were used for sequencing after RNA integrity was assessed using an Agilent 2100 Bioanalyzer. Oligo(dT) magnetic beads were used to concentrate polyadenylated RNA, which was then broken up into brief segments. PCR amplification, adapter ligation, end repair, and first- and second-strand

Table 1. Primer sequences used for RT-PCR validation of ENO1 alternative splicing

Gene/Target	Primer	Sequence (5'→3')	Amplicon (bp)	Purpose
ENO1 (Common)	Forward	ATGGCTGCCATCAACGATGA	168	Amplifies ENO1 transcript
ENO1 Full-length	Reverse	TGGTGATGACCTTGTCGTTGA	168	Validation of canonical ENO1 isoform
ENO1 Alternative Isoform	Reverse	CAGCTTCTTGGTGACGATGTC	142	Validation of alternatively spliced ENO1 transcript
GAPDH	Forward	GAAGGTGAAGTCTGGAGTC	145	Internal control
GAPDH	Reverse	GAAGATGGTGATGGGATTC	145	Internal control

cDNA synthesis were carried out. Paired-end reads were produced by analyzing sequencing libraries on an Illumina NovaSeq 6000 platform. Trimmomatic and FastQC were used for quality control of raw sequencing data. HISAT2 was used to align clean reads to the human reference genome. Transcript assembly and quantification were done using StringTie. DESeq2 was used to evaluate differential gene expression. rMATS and SUPPA2 software were used to identify alternative splicing events, such as exon skipping, retained introns, mutually exclusive exons, alternative 5' splice sites, and alternative 3' splice sites. To assess changes in ENO1 splicing, percent spliced-in values were computed.

Quantitative real-time PCR

TRIzol reagent was used to extract total RNA from tumor tissues and cultured cells. SYBR Green Master Mix was used in quantitative PCR experiments using an ABI 7500 real-time PCR apparatus. The $2^{-\Delta\Delta C_t}$ technique was used to determine the relative expression levels of ADAMTS9-AS2, EZH2, ENO1, and ENO1 splice variants. The endogenous normalization control was GAPDH.

RNA fluorescence *in situ* hybridization

The intracellular location of ADAMTS9-AS2 and ENO1 splice variants was investigated using RNA fluorescence *in situ* hybridization. Paraformaldehyde was used to fix the cells, followed by permeabilization with Triton X-100 and an overnight hybridization with fluorescently labeled RNA probes. Nuclei were counterstained with DAPI after thorough washing. A confocal laser-scanning microscope was used to capture images, and ImageJ was used to measure fluorescence intensity.

Western blot analysis

RIPA lysis buffer with protease and phosphatase inhibitors was used to extract total proteins. A bicinchoninic acid test was used to measure protein quantities. On 10–12% SDS-PAGE gels, equal quantities of protein were separated before being transferred to PVDF membranes. Following 1 hr of blocking with 5% non-fat milk, membranes were incubated with primary antibodies against EZH2, ENO1, H3K9me3, HK2, PKM2, LDH, HIF-1 α , and GAPDH overnight at 4 °C. Protein bands were detected using enhanced chemiluminescence reagents and quantified by densitometric analysis after being incubated with secondary antibodies coupled with horseradish peroxidase.

Xenograft mouse model

Four- to six-week-old female BALB/c nude mice were kept in a pathogen-free environment with unrestricted access to food and drink. Each mouse's right flank received a subcutaneous injection of approximately 5×10^6 bladder cancer cells suspended in 100 μ l of PBS. Digital calipers were used to measure tumor dimensions every three days, and the formula $V = (\text{length} \times \text{width}^2) / 2$ was used to compute tumor volume. At the conclusion of the trial or when the tumors reached ethical endpoints, the mice were put to death.

In vivo bioluminescence imaging and histopathological analysis

Xenograft tumors were created using bladder cancer cells tagged with luciferase. Before imaging, mice were given intraperitoneal injections of D-luciferin. An *in vivo* imaging

system was used to detect bioluminescent signals, which were then measured using specialized imaging software. Tumors and metastatic tissues were removed for histological examination after sacrifice. Tumor tissues were sectioned at a thickness of 4 μ m after being embedded in paraffin and preserved in 10% neutral-buffered formalin. Tumor architecture, necrosis, and cellular atypia were evaluated using hematoxylin and eosin staining. Collagen deposition and extracellular matrix remodeling were assessed using Masson's trichrome staining.

Immunohistochemistry

Deparaffinization, rehydration, antigen retrieval, and endogenous peroxidase blocking were applied to tissue slices embedded in paraffin. Primary antibodies against Ki-67, EZH2, ENO1, H3K9me3, and cleaved caspase-3 were incubated overnight on sections. Signals were detected using diaminobenzidine chromogen and counterstained with hematoxylin following incubation with secondary antibodies. The H-score approach was used to quantify immunoreactivity.

Statistical analysis

All experiments were independently repeated at least three times. Data are expressed as mean $\pm$ standard deviation. Statistical analyses were conducted using GraphPad Prism 9.0 and SPSS 26.0 software. Differences between two groups were analyzed using Student's t-test, whereas comparisons among multiple groups were performed using one-way analysis of variance followed by Tukey's *post hoc* test. Correlations between variables were evaluated using Pearson's correlation analysis. Survival curves were generated using the Kaplan–Meier method and compared using the log-rank test. A two-tailed *P* value less than 0.05 was considered statistically significant. Data are presented as mean $\pm$ SD from three independent biological experiments (*n*=3), unless otherwise indicated. Statistical analyses were performed using GraphPad Prism 9.0. Comparisons between two groups were analyzed using an unpaired two-tailed Student's t-test. In contrast, comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's multiple-comparison *post hoc* test, as appropriate. A value of *P*<0.05 was considered statistically significant. Statistical significance is indicated as **P*<0.05, ***P*<0.01, ****P*<0.001, and ns, not significant.

Results

ADAMTS9-AS2 is down-regulated in bladder cancer and correlates with poor prognosis

To explore the potential role of ADAMTS9-AS2 in bladder cancer, we first evaluated its expression in clinical specimens and bladder cancer cell lines. The overall study workflow is presented in Figure 2A. Quantitative RT-PCR analysis demonstrated that ADAMTS9-AS2 expression was significantly decreased in bladder cancer tissues compared with matched adjacent normal tissues (Figure 2B). Consistently, bladder cancer cell lines (T24, 5637, and UM-UC-3) exhibited markedly lower ADAMTS9-AS2 expression than the normal urothelial cell line SV-HUC-1 (Figure 2C). Because hypoxia is a major characteristic of the bladder tumor microenvironment, we next examined ADAMTS9-AS2 expression under hypoxic conditions. As shown in Figure 2D, exposure to hypoxia significantly

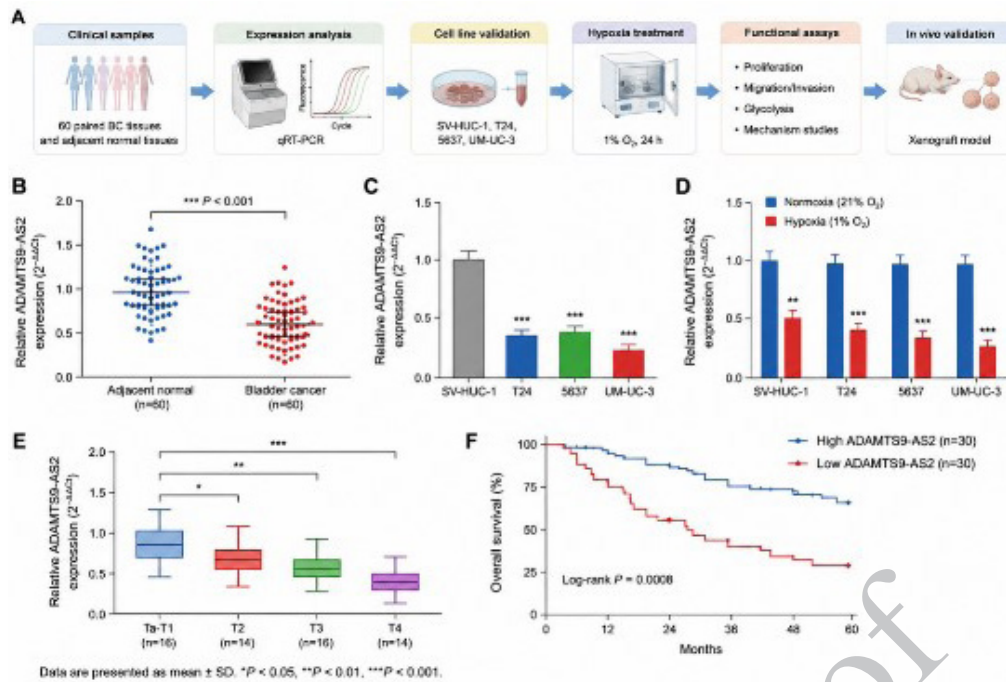


Figure 2. ADAMTS9-AS2 is down-regulated in bladder cancer and is associated with poor clinical prognosis.

(A) Schematic illustration of the overall study design. The expression pattern, biological function, molecular mechanism, and in vivo role of ADAMTS9-AS2 were systematically investigated in bladder cancer under hypoxic conditions through clinical sample analysis, cellular functional assays, mechanistic studies, and xenograft experiments. (B) Quantitative real-time PCR (qRT-PCR) analysis of ADAMTS9-AS2 expression in paired bladder cancer tissues (BC) and adjacent normal tissues (ANT). ADAMTS9-AS2 expression was significantly decreased in tumor tissues compared with corresponding normal tissues. (C) Relative expression levels of ADAMTS9-AS2 in the normal urothelial cell line SV-HUC-1 and bladder cancer cell lines T24, 5637, and UM-UC-3, as determined by qRT-PCR. ADAMTS9-AS2 expression was markedly reduced in bladder cancer cells. (D) qRT-PCR analysis of ADAMTS9-AS2 expression in T24 and UM-UC-3 cells cultured under normoxic (21% O₂) and hypoxic (1% O₂) conditions. Hypoxia significantly suppressed ADAMTS9-AS2 expression. (E) Correlation between ADAMTS9-AS2 expression and the clinicopathological stage in patients with bladder cancer. Lower ADAMTS9-AS2 expression was associated with advanced tumor stage. (F) Kaplan-Meier overall survival analysis of bladder cancer patients, stratified by ADAMTS9-AS2 expression levels. Patients with low ADAMTS9-AS2 expression had significantly poorer overall survival than those with high ADAMTS9-AS2 expression. Data are presented as mean±SD from three independent experiments. Statistical significance was determined using Student's t-test, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

qRT-PCR: Quantitative real-time polymerase chain reaction (or quantitative reverse transcription PCR); ANT: Adjacent normal tissue; BC: Bladder cancer; IHC: Immunohistochemistry (in related panels)

reduced ADAMTS9-AS2 expression in both T24 and UM-UC-3 cells. Clinical correlation analysis further revealed that low ADAMTS9-AS2 expression was associated with advanced tumor stage (Figure 2E). Kaplan-Meier survival analysis demonstrated that patients with low ADAMTS9-AS2 expression exhibited significantly poorer overall survival than those with high ADAMTS9-AS2 expression (Figure 2F). These findings suggest that ADAMTS9-AS2 may function as a tumor suppressor and that its down-regulation contributes to bladder cancer progression.

ADAMTS9-AS2 inhibits bladder cancer cell proliferation and clonogenic growth

To investigate the biological function of ADAMTS9-AS2, stable knockdown and overexpression models were established. Successful modulation of ADAMTS9-AS2 expression was confirmed by qRT-PCR (Figure 3A). CCK-8 assays revealed that hypoxia significantly promoted cellular proliferation, whereas ADAMTS9-AS2 overexpression inhibited cell growth. In contrast, ADAMTS9-AS2 knockdown markedly accelerated proliferation in both T24 and UM-UC-3 cells (Figure 3B). Similar findings were observed in colony formation assays. Representative colony images are shown in Figures 3C and 4A. Quantitative analysis demonstrated that ADAMTS9-AS2 depletion significantly increased colony numbers and colony formation efficiency, whereas ENO1 silencing suppressed

clonogenic growth (Figures 3D and 4B–4C, Table S1). To further evaluate proliferative activity, EdU incorporation assays were performed. Representative fluorescence images demonstrated increased EdU-positive nuclei following ADAMTS9-AS2 silencing (Figure 3E). Quantification confirmed a significant increase in DNA synthesis compared with control cells (Figure 3F). Collectively, these results indicate that ADAMTS9-AS2 suppresses bladder cancer cell proliferation and long-term clonogenic survival.

ADAMTS9-AS2 suppresses migration and invasion of bladder cancer cells

To determine whether ADAMTS9-AS2 influences metastatic potential, wound-healing and Transwell assays were conducted. Representative wound-healing images demonstrated that ADAMTS9-AS2 depletion significantly accelerated wound closure under hypoxic conditions (Figure 5A). Quantitative analysis revealed a marked increase in migration rates compared with controls (Figure 5B). Similarly, Transwell migration assays showed increased migration following ADAMTS9-AS2 silencing (Figure 5C), and quantitative analysis confirmed a significantly greater number of migrated cells (Figure 5D). Matrigel invasion assays further demonstrated enhanced invasive capability after ADAMTS9-AS2 depletion (Figure 5E), which was significantly reduced by ENO1 knockdown (Figure 5F). These data indicate that ADAMTS9-AS2 negatively regulates bladder cancer cell migration and invasion.

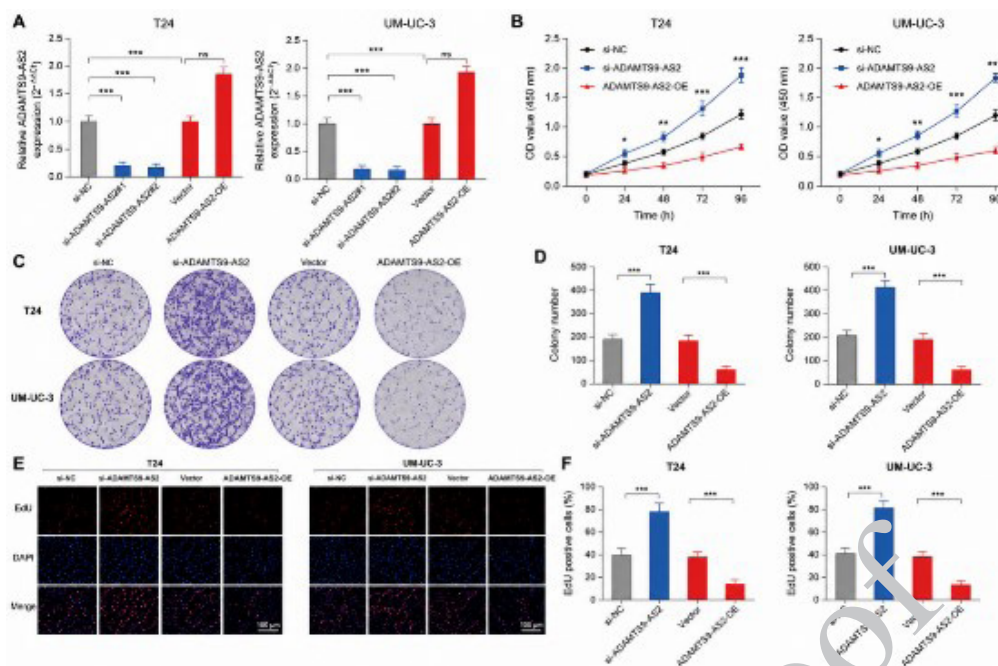


Figure 3. ADAMTS9-AS2 suppresses bladder cancer cell proliferation and clonogenic growth under hypoxic conditions

(A) Validation of ADAMTS9-AS2 knockdown and overexpression efficiency in T24 and UM-UC-3 bladder cancer cells by quantitative real-time PCR (qRT-PCR). Relative ADAMTS9-AS2 expression levels were normalized to GAPDH. (B) Cell proliferation was evaluated using the Cell Counting Kit-8 (CCK-8) assay. Growth curves of bladder cancer cells cultured under normoxic and hypoxic conditions following ADAMTS9-AS2 knockdown, EZH2 overexpression, or ENO1 silencing are shown. ADAMTS9-AS2 depletion significantly promoted cell proliferation, whereas ENO1 knockdown inhibited cell growth. (C) Representative images of colony formation assays in the indicated experimental groups. Colonies were fixed with paraformaldehyde and stained with crystal violet after 10–14 days of culture. (D) Quantitative analysis of colony numbers. ADAMTS9-AS2 knockdown significantly increased clonogenic capacity, while ENO1 depletion reduced colony formation. (E) Representative 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay images showing proliferating cells (red fluorescence) and nuclei counterstained with DAPI (blue fluorescence). (F) Quantification of EdU-positive cells. ADAMTS9-AS2 silencing significantly increased DNA synthesis activity, whereas ENO1 knockdown reduced the proportion of proliferating cells. Data are presented as mean±SD from three independent experiments. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

qRT-PCR: Quantitative real-time polymerase chain reaction; CCK-8: Cell Counting Kit-8; EdU: 5-ethynyl-2'-deoxyuridine; DAPI: 4',6-diamidino-2-phenylindole; EZH2-OE: EZH2 overexpression; si-ENO1: Small interfering RNA targeting ENO1 (ENO1 knockdown).

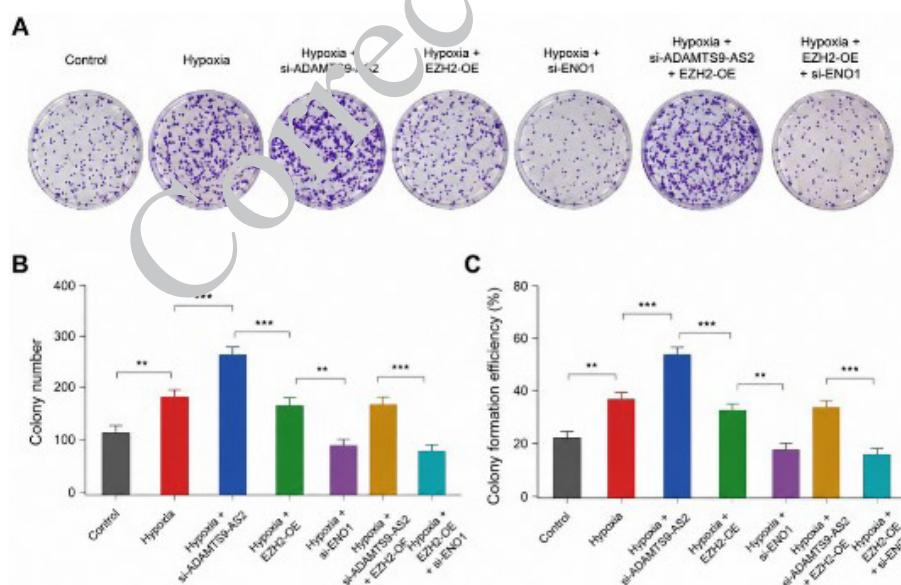


Figure 4. ADAMTS9-AS2 regulates the clonogenic survival and colony-forming capacity of bladder cancer cells

(A) Representative colony formation images of T24 and UM-UC-3 cells under different treatment conditions, including Control, Hypoxia, Hypoxia + si-ADAMTS9-AS2, Hypoxia + EZH2-OE, Hypoxia + si-ENO1, Hypoxia + si-ADAMTS9-AS2 + EZH2-OE, and Hypoxia + EZH2-OE + si-ENO1 groups. Colonies were stained with crystal violet and photographed after 10–14 days of incubation. (B) Quantitative analysis of colony numbers in each experimental group. Hypoxia significantly increased colony formation compared with the control group, while ADAMTS9-AS2 silencing further enhanced clonogenic growth. ENO1 knockdown markedly suppressed colony formation. (C) Colony formation efficiency (%) calculated as the ratio of colonies formed to the total number of seeded cells. ADAMTS9-AS2 depletion significantly increased colony formation efficiency, whereas ENO1 silencing reversed the growth-promoting effects induced by ADAMTS9-AS2 deficiency. The quantitative results correspond to the data presented in Table S1. Data are presented as mean±SD from three independent biological experiments (n=3). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison *post hoc* test unless otherwise specified. Statistical significance is indicated as * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

si-ADAMTS9-AS2: Small interfering RNA targeting ADAMTS9-AS2 (knockdown); EZH2-OE: EZH2 overexpression; si-ENO1: ENO1 knockdown (small interfering RNA); Control / Hypoxia: Experimental treatment groups

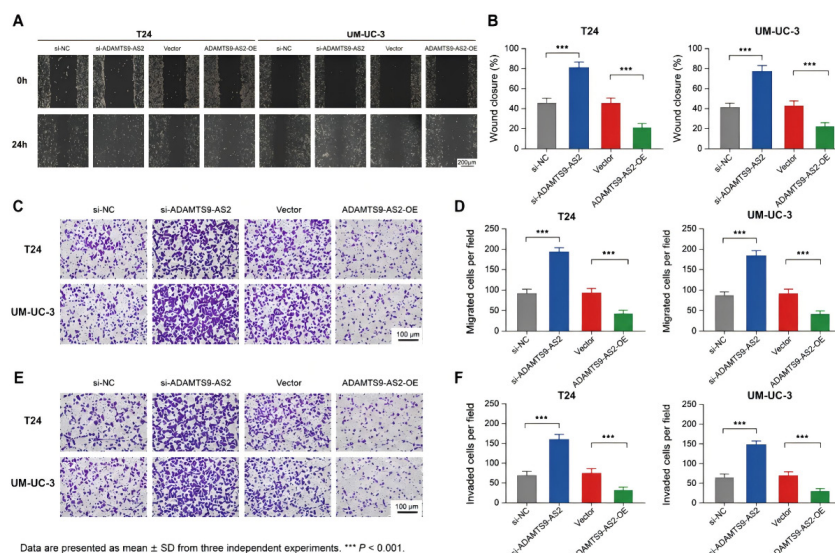


Figure 5. ADAMTS9-AS2 inhibits migration and invasion of bladder cancer cells under hypoxic conditions

(A) Representative wound-healing assay images showing the migratory ability of T24 and UM-UC-3 cells under different treatment conditions. Images were captured at 0 h and 24 h after scratch generation. Cells were treated with hypoxia, si-ADAMTS9-AS2, EZH2-OE, si-ENO1, or the indicated combination treatments. ADAMTS9-AS2 depletion accelerated wound closure, whereas ENO1 silencing markedly inhibited cell migration. (B) Quantitative analysis of wound closure percentage. Migration rates were calculated as the percentage reduction in wound area relative to the initial scratch area. ADAMTS9-AS2 knockdown significantly increased wound closure, while ENO1 depletion reversed the pro-migratory effects induced by ADAMTS9-AS2 deficiency. (C) Representative Transwell migration assay images. Migrated cells on the lower surface of the membrane were fixed, stained with crystal violet, and photographed under a light microscope. An increased number of migrated cells was observed following ADAMTS9-AS2 silencing. (D) Quantification of migrated cells per microscopic field. Hypoxia significantly promoted cell migration compared with the control group, and ADAMTS9-AS2 depletion further enhanced migratory capacity. In contrast, ENO1 knockdown significantly reduced the number of cells that migrated. (E) Representative Matrigel-coated Transwell invasion assay images. Invading cells that penetrated the extracellular matrix barrier were stained with crystal violet and imaged. ADAMTS9-AS2 knockdown markedly increased the number of invasive cells, whereas ENO1 silencing suppressed invasion. (F) Quantitative analysis of invasive cells per field. The invasion rate was significantly increased following ADAMTS9-AS2 depletion and was partially reversed by EZH2 overexpression. Silencing ENO1 effectively inhibited the invasive phenotype, indicating that ENO1 functions as a critical downstream mediator of ADAMTS9-AS2-regulated metastatic behavior. The quantitative invasion data correspond to the results presented in Table S2. Data are presented as mean ± SD from three independent biological experiments (n=3). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. A value of $P < 0.05$ was considered statistically significant. Statistical significance is indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars = 100 μ m.

si-ADAMTS9-AS2: ADAMTS9-AS2 knockdown; EZH2-OE: EZH2 overexpression; si-ENO1: ENO1 knockdown; Matrigel: Basement membrane matrix (for invasion assay)

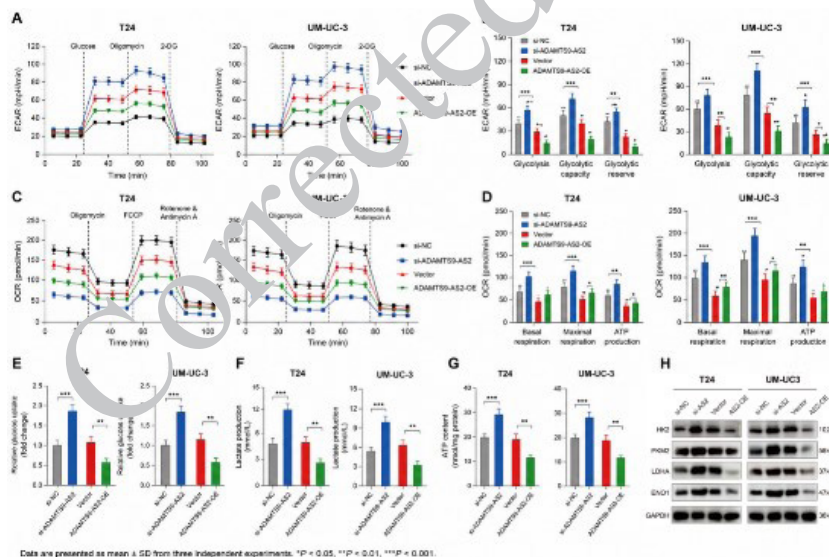


Figure 6. ADAMTS9-AS2 suppresses glycolytic metabolism in bladder cancer cells by regulating ENO1

(A) Representative Seahorse XF ECAR profiles of T24 and UM-UC-3 cells under the indicated treatment conditions. Sequential injections of glucose, oligomycin, and 2-deoxy-D-glucose (2-DG) were performed to evaluate glycolytic activity. ADAMTS9-AS2 knockdown significantly enhanced ECAR, whereas ENO1 silencing reduced glycolytic flux. (B) Quantification of glycolytic capacity derived from ECAR measurements. Hypoxia significantly increased glycolytic activity compared with normoxic controls, and ADAMTS9-AS2 depletion further elevated glycolytic capacity. In contrast, ENO1 knockdown markedly suppressed glycolytic metabolism. (C) Representative Seahorse XF OCR profiles showing mitochondrial respiratory function in the indicated groups. Sequential injections of oligomycin, FCCP, and rotenone/antimycin A were used to assess mitochondrial respiration. (D) Quantitative analysis of basal respiration determined from OCR measurements. ADAMTS9-AS2 silencing significantly reduced mitochondrial respiratory activity, whereas ENO1 depletion restored oxidative phosphorylation capacity. (E) Quantification of glucose uptake determined using fluorescent glucose analog assays. Hypoxia promoted glucose uptake, and ADAMTS9-AS2 knockdown further increased glucose consumption compared with control cells. (F) Quantification of lactate production in cell culture supernatants. Lactate secretion was significantly elevated following ADAMTS9-AS2 depletion, indicating enhanced aerobic glycolysis. (G) Measurement of intracellular ATP levels. ADAMTS9-AS2 knockdown significantly increased ATP production, whereas ENO1 silencing reduced cellular ATP generation. (H) Representative western blot analysis of glycolysis-associated proteins, including HK2, PKM2, LDHA, and ENO1, in the indicated treatment groups. GAPDH was used as a loading control. ADAMTS9-AS2 depletion increased glycolytic enzyme expression, whereas ENO1 knockdown attenuated activation of the glycolytic pathway. The quantitative glucose uptake and lactate production data correspond to Table S4, whereas ATP levels correspond to Table S3. ECAR and OCR data are presented in Figures 6B and 6D, respectively. Data are presented as mean ± SD from three independent experiments. Statistical significance was analyzed using one-way ANOVA followed by Tukey's *post hoc* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

ECAR: Extracellular acidification rate; OCR: Oxygen consumption rate; 2-DG: 2-deoxy-D-glucose; HK2: Hexokinase 2; PKM2: Pyruvate kinase M2; LDHA: Lactate dehydrogenase A; Seahorse XF: Seahorse Extracellular Flux Analyzer

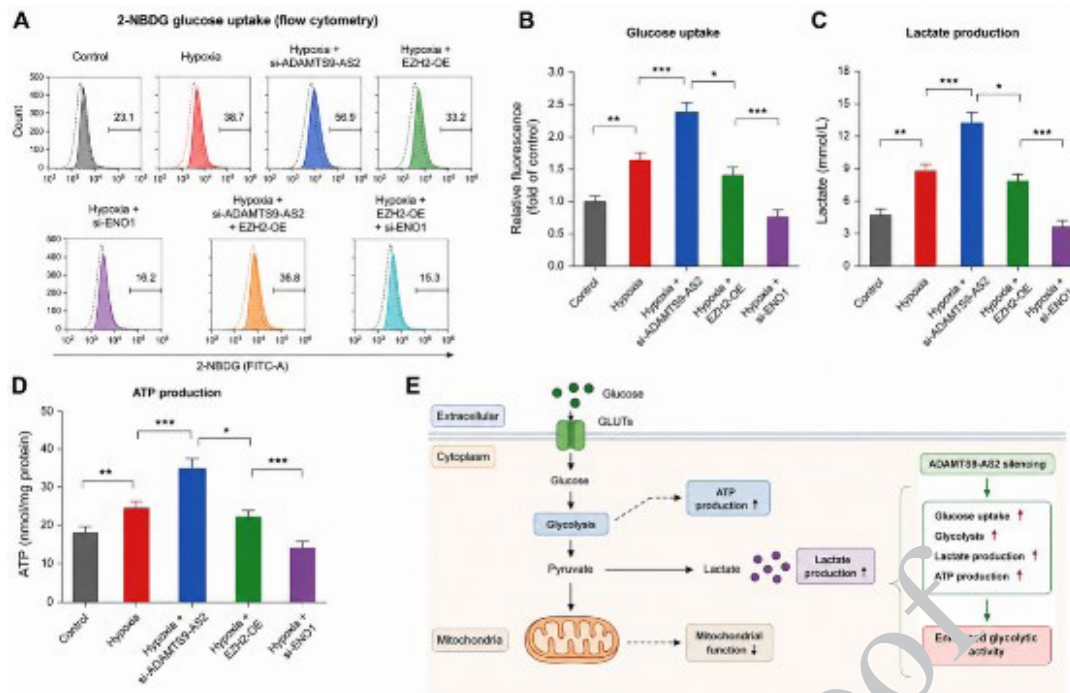


Figure 7. ADAMTS9-AS2 regulates glycolytic activity and cellular energy metabolism in bladder cancer cells.

(A) Representative flow cytometric histograms of 2-NBDG uptake in bladder cancer cells under different treatment conditions. Increased fluorescence intensity indicates enhanced glucose uptake. ADAMTS9-AS2 depletion significantly increased glucose uptake, whereas ENO1 silencing reduced glucose consumption. (B) Quantitative analysis of relative glucose uptake. Fluorescence intensity was normalized to the control group. Hypoxia-induced glucose uptake was further enhanced by ADAMTS9-AS2 knockdown and significantly suppressed by ENO1 depletion. (C) Quantification of extracellular lactate production. Lactate concentrations were measured in conditioned culture medium using a colorimetric lactate assay kit. ADAMTS9-AS2 silencing significantly increased lactate secretion, indicating enhanced glycolytic flux. (D) Quantification of intracellular ATP levels. ATP concentrations were determined using a luciferase-based ATP detection assay. ADAMTS9-AS2 depletion increased ATP production, whereas ENO1 knockdown reduced ATP generation and cellular metabolic activity. (E) Schematic illustration of the proposed metabolic regulatory pathway. Under hypoxic conditions, reduced ADAMTS9-AS2 expression promotes EZH2-dependent regulation of ENO1, resulting in enhanced glycolytic metabolism characterized by increased glucose uptake, elevated lactate production, increased ATP generation, and metabolic reprogramming that supports bladder cancer progression. The quantitative glucose uptake and lactate production results correspond to Table S4, whereas ATP levels correspond to Table S3. Data are expressed as mean $\pm$ SD from three independent biological replicates. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as the hypoxia group. 2-NBDG: 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose (fluorescent glucose analog); ECAR: Extracellular acidification rate; ATP: Adenosine triphosphate; Flow cytometry: Fluorescence-activated cell sorting/analysis.

ADAMTS9-AS2 inhibits glycolytic reprogramming in bladder cancer cells

To investigate whether ADAMTS9-AS2 regulates cellular metabolism, Seahorse XF analysis was performed. As shown in Figure 6A, hypoxia significantly increased extracellular acidification rates (ECAR), indicating enhanced glycolysis. ADAMTS9-AS2 knockdown further increased glycolytic activity, whereas ENO1 depletion significantly suppressed ECAR values. Quantification of glycolytic capacity is presented in Figure 6B. In contrast, oxygen consumption rate (OCR) analysis demonstrated reduced mitochondrial respiration following ADAMTS9-AS2 depletion (Figure 6C), while basal respiratory capacity was significantly restored after ENO1 knockdown (Figure 6D). To further validate these observations, glucose uptake, lactate secretion, and ATP production were measured. Flow cytometric analysis of 2-NBDG uptake demonstrated significantly enhanced glucose uptake following ADAMTS9-AS2 silencing (Figure 7A), and quantitative results are shown in Figure 7B and Table S4. Lactate production was also markedly increased (Figure 7C), whereas ATP levels were significantly elevated (Figure 7D, Table S3). Western blot analysis further revealed increased expression of glycolytic enzymes HK2, PKM2, LDHA, and ENO1 after ADAMTS9-AS2 depletion (Figure 6H). The overall metabolic regulatory pathway is summarized in Figure 7E. Together, these findings demonstrate that ADAMTS9-AS2 suppresses glycolytic

metabolism in bladder cancer cells.

ADAMTS9-AS2 regulates mitochondrial function and oxidative stress

Because glycolytic activation is often accompanied by mitochondrial dysfunction, ROS generation and mitochondrial membrane potential were assessed. Fluorescence microscopy revealed significantly elevated intracellular ROS levels in ADAMTS9-AS2-deficient cells (Figure 8A), which were confirmed by quantitative analysis (Figure 8B, Table S5). JC-1 staining demonstrated a substantial reduction in mitochondrial membrane potential following ADAMTS9-AS2 knockdown (Figure 8C), and quantification of the red/green fluorescence ratio further confirmed mitochondrial depolarization (Figure 8D). Transmission electron microscopy revealed severe mitochondrial swelling, cristae disruption, and fragmentation in ADAMTS9-AS2-silenced cells (Figure 8E). Quantitative analysis of mitochondrial morphology revealed significantly more structural abnormalities than in controls (Figure 8F). These findings indicate that ADAMTS9-AS2 plays a critical role in maintaining mitochondrial integrity and redox homeostasis.

ADAMTS9-AS2 directly interacts with EZH2

To elucidate the molecular mechanism underlying ADAMTS9-AS2 function, we investigated its interaction

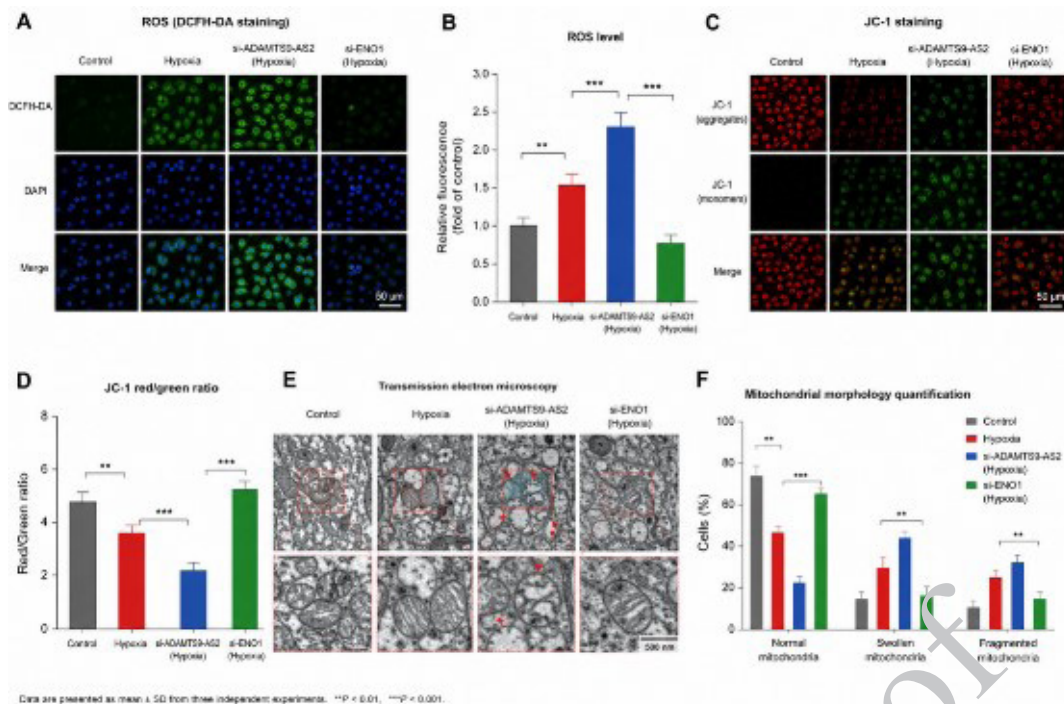


Figure 8. ADAMTS9-AS2 regulates mitochondrial function and oxidative stress in bladder cancer cells

(A) Representative fluorescence microscopy images of intracellular ROS detected using the DCFH-DA fluorescent probe in 24 h HIF-1 α UCA-1 cells under the indicated treatment conditions. Increased green fluorescence intensity indicates elevated ROS production. ADAMTS9-AS2 knockdown significantly enhanced oxidative stress, whereas ENO1 silencing reduced ROS accumulation. Scale bars=100 μ m. (B) Quantitative analysis of intracellular ROS levels. Fluorescence intensity was normalized to the control group. Hypoxia significantly increased ROS generation, and depletion of ADAMTS9-AS2 further exacerbated oxidative stress. In contrast, ENO1 knockdown markedly suppressed ROS production. (C) Representative JC-1 staining images showing mitochondrial membrane potential ($\Delta\Psi$ m) in bladder cancer cells. Red fluorescence indicates polarized mitochondria with intact membrane potential, whereas green fluorescence indicates depolarized mitochondria. ADAMTS9-AS2 knockdown increased mitochondrial depolarization, while ENO1 silencing restored mitochondrial function. Scale bars=50 μ m. (D) Quantification of mitochondrial membrane potential expressed as the JC-1 red/green fluorescence ratio. ADAMTS9-AS2 depletion significantly reduced mitochondrial membrane potential compared with control cells, whereas ENO1 knockdown increased the JC-1 ratio, indicating improved mitochondrial integrity. (E) Representative transmission electron microscopy (TEM) images of mitochondrial ultrastructure in Control, Hypoxia, si-ADAMTS9-AS2, and si-ENO1 groups. Control cells displayed normal mitochondrial morphology with intact cristae and regular membrane structure. Hypoxia induced moderate mitochondrial swelling and cristae disruption. ADAMTS9-AS2 knockdown led to severe mitochondrial damage, characterized by swelling, fragmented cristae, vacuolization, and loss of structural integrity. In contrast, ENO1 silencing preserved mitochondrial morphology and reduced ultrastructural abnormalities. Scale bars=500 nm. (F) Quantitative analysis of mitochondrial morphology. Mitochondrial damage scores were calculated based on cristae disruption, swelling, fragmentation, and vacuole formation. ADAMTS9-AS2-deficient cells exhibited significantly increased mitochondrial abnormalities, whereas ENO1 knockdown markedly improved mitochondrial structural integrity. Data are presented as mean $\pm$ SD from three independent biological experiments (n=3). Statistical significance is indicated as * P <0.05, ** P <0.01, *** P <0.001. These results demonstrate that ADAMTS9-AS2 maintains mitochondrial homeostasis and suppresses oxidative stress in bladder cancer cells. Loss of ADAMTS9-AS2 promotes mitochondrial dysfunction and ROS accumulation, whereas ENO1 inhibition partially restores mitochondrial integrity and cellular redox balance.

ROS: Reactive oxygen species; DCFH-DA: 2',7'-dichlorofluorescein diacetate (fluorescent ROS probe); JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (mitochondrial membrane potential dye); TEM: Transmission electron microscopy $\Delta\Psi$ m: Mitochondrial membrane potential

with EZH2. Bioinformatic prediction identified several potential EZH2-binding motifs within the ADAMTS9-AS2 transcript (Figure 9A). RNA-FISH analysis demonstrated that ADAMTS9-AS2 was predominantly localized in the nucleus (Figure 9B), suggesting a potential role in epigenetic regulation. RIP assays demonstrated significant enrichment of ADAMTS9-AS2 in EZH2 immunoprecipitates compared with IgG controls (Figure 9C), and quantitative analysis confirmed approximately 8-fold enrichment (Figure 9D, Table S6). RNA pull-down assays further validated the direct interaction between ADAMTS9-AS2 and EZH2 (Figure 9E), while western blotting confirmed specific enrichment of EZH2 in the pull-down complexes (Figure 9F). A schematic representation of the ADAMTS9-AS2–EZH2 interaction is shown in Figure 9G. These findings indicate that ADAMTS9-AS2 directly associates with EZH2 and may function as an epigenetic scaffold.

ADAMTS9-AS2/EZH2 regulates ENO1 alternative splicing through histone modification

RNA sequencing was performed to identify downstream

targets regulated by ADAMTS9-AS2. Differential expression analysis identified extensive transcriptomic alterations (Figure 10A), while heatmap analysis revealed significant changes in alternative splicing events (Figure 10B). Among the affected genes, ENO1 exhibited the most prominent splicing alterations. Sashimi plot analysis demonstrated altered exon usage following ADAMTS9-AS2 depletion (Figure 10C). RT-PCR validation confirmed significant changes in ENO1 splice variant abundance (Figure 10D), while PSI analysis further quantified these splicing alterations (Figure 10E, Table S7). ChIP-qPCR analysis demonstrated significantly increased EZH2 occupancy at ENO1 regulatory regions (Figure 10F, Table S8). Furthermore, H3K9me3 enrichment was markedly elevated following ADAMTS9-AS2 depletion (Figure 10G), suggesting chromatin remodeling at the ENO1 locus. The proposed epigenetic mechanism is summarized in Figure 10H and further illustrated in Figure 11E, whereby ADAMTS9-AS2 recruits EZH2 to regulate H3K9me3 deposition and ENO1 alternative splicing. ChIP-qPCR demonstrated a significant increase in EZH2 occupancy

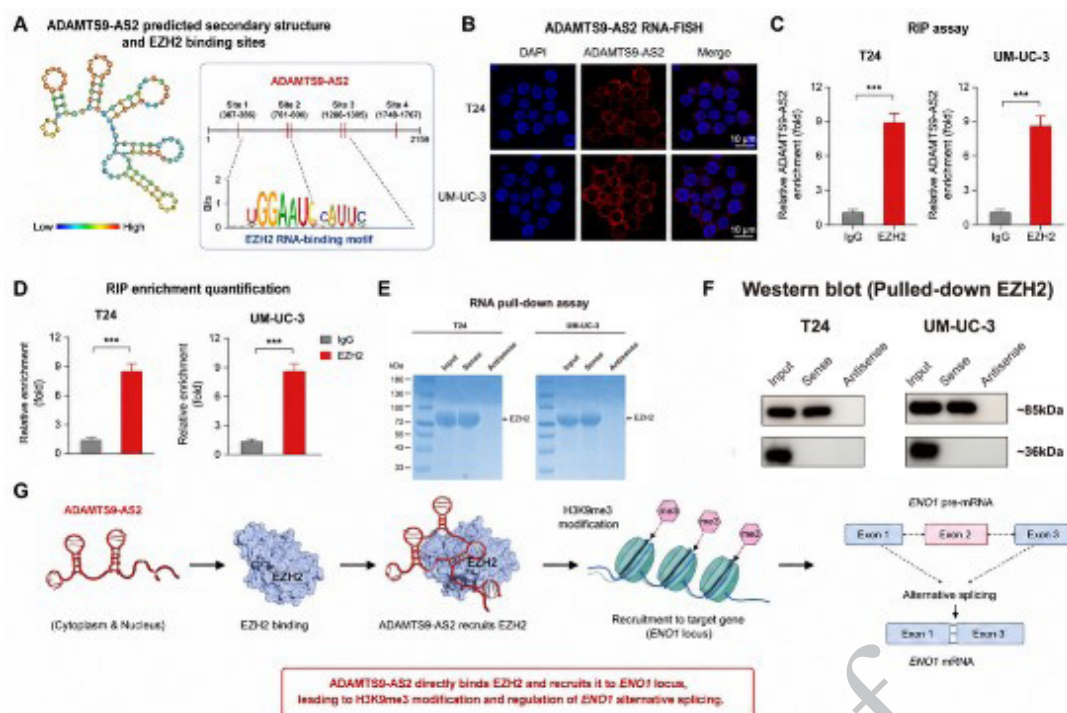


Figure 9. ADAMTS9-AS2 directly interacts with EZH2 and functions as a nuclear epigenetic regulator in bladder cancer cells

(A) Bioinformatic prediction of the secondary structure of ADAMTS9-AS2 and identification of potential EZH2-binding regions. Putative interaction motifs within the ADAMTS9-AS2 transcript are highlighted, suggesting direct association with the EZH2 protein. (B) Representative RNA fluorescence in situ hybridization (RNA-FISH) images showing the subcellular localization of ADAMTS9-AS2 in T24 and UM-UC-3 cells. ADAMTS9-AS2 signals (red) were predominantly localized in the nucleus, while nuclei were counterstained with DAPI (blue). Scale bars=20 μ m. (C) RNA immunoprecipitation (RIP) assay demonstrating enrichment of ADAMTS9-AS2 in EZH2 immunoprecipitates compared with IgG controls. Total RNA was extracted from immunoprecipitated complexes and analyzed by qRT-PCR. (D) Quantitative analysis of RIP enrichment. ADAMTS9-AS2 was significantly enriched in the EZH2-RIP fraction, showing approximately an 8-fold increase over the IgG control group, indicating a specific interaction between ADAMTS9-AS2 and EZH2. (E) RNA pull-down assay using biotin-labeled ADAMTS9-AS2 transcripts. Protein complexes associated with ADAMTS9-AS2 were isolated using streptavidin magnetic beads and subsequently analyzed. (F) Western blot analysis of proteins isolated from RNA pull-down assays. EZH2 protein was specifically detected in complexes precipitated by biotin-labeled ADAMTS9-AS2 but not by antisense or negative control probes, confirming direct physical binding between ADAMTS9-AS2 and EZH2. (G) Proposed model illustrating the interaction between ADAMTS9-AS2 and EZH2. Nuclear ADAMTS9-AS2 functions as a molecular scaffold that recruits EZH2 to chromatin-associated regulatory regions, thereby modulating histone methylation and downstream gene regulation, including ENO1 alternative splicing and metabolic reprogramming. The RIP quantitative results correspond to the data presented in Table S6. Data are presented as mean $\pm$ SD from three independent biological experiments (n=3). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. Statistical significance is indicated as * P <0.05, ** P <0.01, *** P <0.001. These findings demonstrate that ADAMTS9-AS2 directly binds EZH2 and suggest that the ADAMTS9-AS2–EZH2 complex functions as an important epigenetic regulatory unit that controls alternative splicing and glycolytic metabolism in bladder cancer cells.

at the ENO1 regulatory region following ADAMTS9-AS2 depletion. Consistent with canonical PRC2 activity, H3K27me3 enrichment was significantly elevated at the same genomic regions. Increased H3K9me3 enrichment was also observed, suggesting coordinated chromatin remodeling rather than direct H3K9 methylation by EZH2.

Rescue experiments confirm the ADAMTS9-AS2/EZH2/ENO1 regulatory axis

To validate the functional relationship among ADAMTS9-AS2, EZH2, and ENO1, rescue experiments were performed as shown in Figure 12A. ADAMTS9-AS2 knockdown significantly increased proliferation (Figure 12B), colony formation (Figure 12C), migration (Figure 12D), invasion (Figure 12E), and glycolytic activity (Figure 12F). Interestingly, EZH2 overexpression partially attenuated these phenotypic changes, whereas ENO1 silencing produced a more pronounced inhibitory effect. Western blot analysis confirmed corresponding changes in EZH2, ENO1, HK2, PKM2, and LDHA expression (Figure 12G). Quantitative analysis is summarized in Figure 12H. These findings demonstrate that ENO1 acts as a critical downstream effector of the ADAMTS9-AS2/EZH2

signaling axis.

ADAMTS9-AS2 suppresses tumor growth in vivo

To verify the *in vitro* findings, xenograft models were established (Figure 13A). Representative tumors are shown in Figure 13B. Tumors derived from ADAMTS9-AS2-deficient cells grew significantly faster than control tumors (Figure 13C) and exhibited increased tumor weight at sacrifice (Figure 13D, Table S9). Bioluminescence imaging further confirmed enhanced tumor burden following ADAMTS9-AS2 depletion (Figure 13E). Histological examination with HE staining revealed increased tumor aggressiveness (Figures 13F and 14A). Immunohistochemical analyses revealed elevated expression of Ki67, EZH2, ENO1, and H3K9me3 in tumors derived from ADAMTS9-AS2-silenced cells (Figures 14B–14E). Quantification of H-scores confirmed significant increases in proliferative and epigenetic markers (Figures 14F–14I, Table S10). Taken together, these *in vivo* findings strongly support the tumor-suppressive role of ADAMTS9-AS2 and validate the ADAMTS9-AS2/EZH2/ENO1 signaling pathway in bladder cancer progression. Figure 15 shows the results of the study.

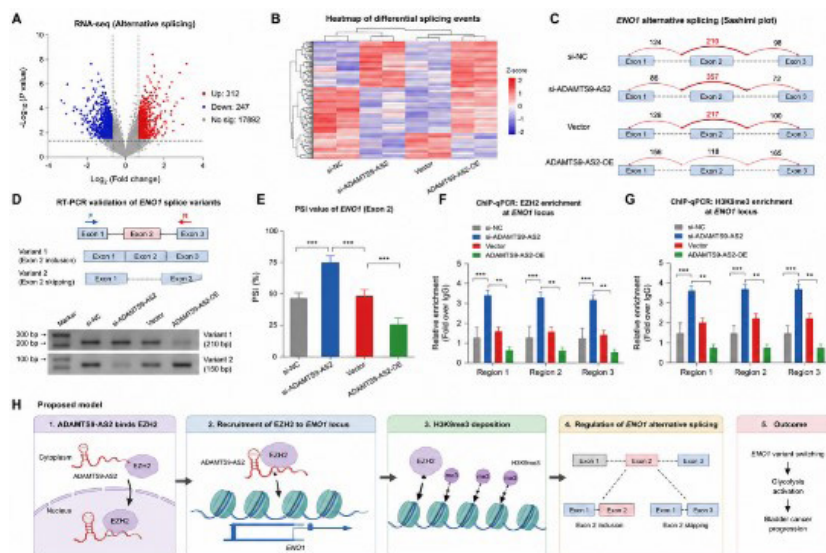


Figure 10. ADAMTS9-AS2/EZH2 regulates ENO1 alternative splicing through epigenetic modification in bladder cancer cells

(A) RNA sequencing (RNA-seq) volcano plot showing differentially expressed and alternatively spliced genes between control and ADAMTS9-AS2 deficient bladder cancer cells. Significantly up-regulated genes are shown in red, significantly down-regulated genes are shown in blue, and non-significant genes are shown in gray. ENO1 was identified as one of the major downstream targets affected by ADAMTS9-AS2 depletion. (B) Heatmap of differentially spliced genes identified by RNA-seq analysis. Hierarchical clustering demonstrates distinct alternative splicing patterns among experimental groups, indicating extensive transcriptomic remodeling following ADAMTS9-AS2 depletion. (C) Sashimi plot illustrating alternative splicing events within the ENO1 locus. Read coverage and splice junction counts reveal altered exon inclusion and exon-skipping events in ADAMTS9-AS2-deficient cells compared with controls. (D) RT-PCR validation of ENO1 splice variants. Agarose gel electrophoresis demonstrates differential abundance of ENO1 transcript isoforms among experimental groups. GAPDH was used as an internal control. (E) Quantification of percent spliced-in (PSI) values for the major ENO1 splice variant. ADAMTS9-AS2 knockdown significantly increased exon inclusion events, whereas EZH2 overexpression and ENO1 silencing altered splice-site selection. Quantitative data correspond to Table S7. (F) Chromatin immunoprecipitation (ChIP)-qPCR analysis of EZH2 occupancy at regulatory regions of the ENO1 gene. ADAMTS9-AS2 depletion significantly increased EZH2 recruitment to the ENO1 locus, indicating enhanced chromatin association. (G) ChIP-qPCR analysis of H3K9me3 enrichment at the ENO1 promoter and adjacent regulatory regions. Increased H3K9me3 deposition was observed following ADAMTS9-AS2 knockdown, suggesting epigenetic regulation of ENO1 transcription and splicing. (H) Proposed mechanistic model illustrating the role of the ADAMTS9-AS2/EZH2 complex in regulating ENO1 alternative splicing. Nuclear ADAMTS9-AS2 interacts with EZH2 and modulates chromatin architecture through histone methylation, thereby influencing ENO1 splice-site selection and downstream metabolic reprogramming. The PSI values correspond to the quantitative results presented in Table S7. Data are presented as mean $\pm$ SD from three independent biological experiments (n=3). RNA sequencing was performed using three independent biological replicates per group. Differential alternative splicing events identified by rMATS and SUPPA2 were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR), with FDR<0.05 considered statistically significant. ChIP-qPCR validation experiments were analyzed using one-way ANOVA followed by Tukey's multiple-comparison post hoc test. Statistical significance is indicated as * P <0.05, ** P <0.01, *** P <0.001. These findings demonstrate that ADAMTS9-AS2 regulates ENO1 alternative splicing through an EZH2-dependent epigenetic mechanism, linking lncRNA-mediated chromatin regulation to metabolic adaptation in bladder cancer cells. RNA-seq: RNA sequencing; ChIP-qPCR: Chromatin immunoprecipitation followed by quantitative PCR; PSI: Percent spliced-in (measure of alternative splicing); rMATS / SUPPA2: Bioinformatics tools for alternative splicing analysis (replicate Multivariate Analysis of Transcriptome Splicing / SUPPA2); Sashimi plot: Visualization of alternative splicing events

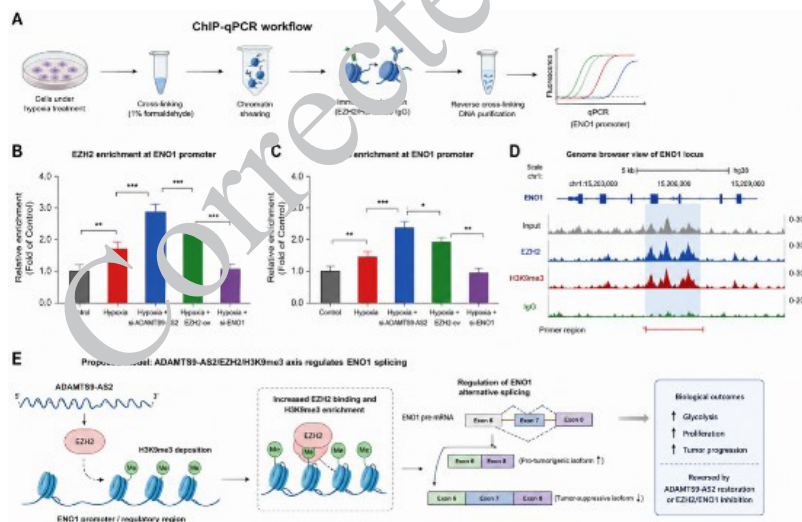


Figure 11. EZH2-mediated histone modification regulates ENO1 transcriptional and splicing activity downstream of ADAMTS9-AS2

(A) Schematic representation of the ChIP-qPCR experimental workflow. Chromatin from bladder cancer cells was cross-linked, fragmented, immunoprecipitated using antibodies against EZH2 or H3K9me3, and analyzed by quantitative PCR using primers targeting ENO1 regulatory regions. (B) Quantitative ChIP-qPCR analysis of EZH2 enrichment at the ENO1 promoter. Hypoxia and ADAMTS9-AS2 depletion significantly increased EZH2 occupancy, whereas ENO1 silencing had minimal effects on EZH2 recruitment. Quantitative results correspond to Table S8. (C) Quantitative ChIP-qPCR analysis of H3K9me3 enrichment at the ENO1 locus. Increased histone methylation was observed following ADAMTS9-AS2 knockdown, suggesting enhanced epigenetic remodeling and transcriptional regulation of ENO1. (D) Genome browser visualization of the ENO1 genomic region. Peaks representing EZH2 binding and H3K9me3 occupancy are shown across promoter and exon regions associated with alternative splicing events. Differential chromatin enrichment patterns among experimental groups are highlighted. (E) Integrated mechanistic model summarizing the ADAMTS9-AS2/EZH2/ENO1 regulatory axis. Under hypoxic conditions, reduced ADAMTS9-AS2 expression is associated with altered EZH2 occupancy and increased H3K9me3 enrichment at the ENO1 locus. These epigenetic alterations influence ENO1 alternative splicing and transcriptional activity, leading to enhanced glycolytic metabolism, increased cellular proliferation, migration, invasion, and bladder cancer progression. The quantitative ChIP-qPCR results correspond to the data presented in Table S8. Data are presented as mean $\pm$ SD from three independent biological experiments (n=3). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison post hoc test. Statistical significance is indicated as * P <0.05, ** P <0.01, *** P <0.001. These results provide mechanistic evidence that ADAMTS9-AS2 functions as an epigenetic regulator by modulating EZH2-mediated histone methylation at the ENO1 locus, thereby controlling alternative splicing and metabolic reprogramming in bladder cancer cells. ChIP-qPCR: Chromatin immunoprecipitation quantitative PCR; H3K9me3: Histone H3 lysine 9 trimethylation; H3K27me3: Histone H3 lysine 27 trimethylation (mentioned in methods context)

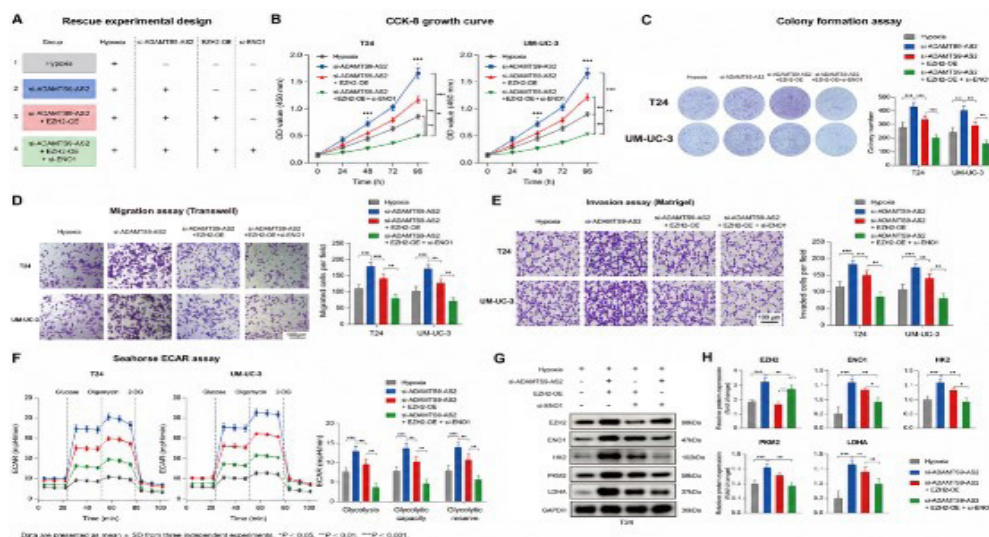


Figure 12. Rescue experiments validate the ADAMTS9-AS2/EZH2/ENO1 signaling axis in bladder cancer cells

(A) Schematic illustration of the rescue experimental design. Bladder cancer cells were divided into four treatment groups: Hypoxia, Hypoxia+si-ADAMTS9-AS2+EZH2 overexpression (EZH2-OE), and Hypoxia+si-ADAMTS9-AS2+EZH2-OE+si-ENO1. Functional and molecular analyses were subsequently performed to evaluate the regulatory relationship among ADAMTS9-AS2, EZH2, and ENO1. (B) CCK-8 proliferation assay showing relative cell viability in the indicated groups. ADAMTS9-AS2 knockdown significantly enhanced cellular proliferation under hypoxic conditions. EZH2 overexpression partially attenuated the proliferative phenotype, whereas subsequent ENO1 silencing markedly inhibited cell growth. (C) Representative colony formation assay images and quantitative analysis of clonogenic survival. ADAMTS9-AS2 depletion promoted colony formation, while EZH2 overexpression was associated with reduced colony growth. ENO1 knockdown further suppressed colony formation and reduced clonogenic capacity. (D) Quantification of wound-healing migration assays. ADAMTS9-AS2 silencing significantly increased wound closure rates compared with the hypoxia group. The enhanced migratory ability was partially reversed by EZH2 overexpression and substantially inhibited following ENO1 depletion. (E) Quantitative analysis of Matrigel invasion assays. Increased invasive capacity induced by ADAMTS9-AS2 deficiency was reduced by EZH2 restoration and almost completely abolished by ENO1 knockdown, demonstrating that ENO1 mediates the pro-invasive phenotype. (F) Seahorse XF extracellular acidification rate (ECAR) analysis. ADAMTS9-AS2 knockdown significantly enhanced glycolytic activity, whereas EZH2 overexpression reduced ECAR values. Silencing ENO1 further suppressed glycolysis and reduced metabolic homeostasis. (G) Representative western blot analysis of ADAMTS9-AS2/EZH2/ENO1 pathway-associated proteins. Protein expression levels of EZH2, ENO1, HK2, PKM2, and LDHA were examined in the indicated groups. ADAMTS9-AS2 depletion increased ENO1 and glycolysis-associated proteins, whereas ENO1 silencing attenuated activation of the glycolytic pathway. GAPDH served as the loading control. (H) Integrated quantitative summary of proliferation, migration, invasion, glycolytic activity, and protein expression changes among rescue groups. Relative values were normalized to the hypoxia group to facilitate comparison of biological responses. Data are presented as mean±SD from three independent biological experiments (n=3). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. Statistical significance is indicated as **P*<0.05, ***P*<0.01, ****P*<0.001. These rescue experiments demonstrate that ENO1 functions as a critical downstream effector of the ADAMTS9-AS2/EZH2 regulatory pathway. EZH2 overexpression partially modulated the phenotypic changes induced by ADAMTS9-AS2 depletion, whereas ENO1 silencing markedly attenuated the enhanced proliferation, migration, invasion, and glycolytic metabolism. These findings support the functional involvement of the ADAMTS9-AS2/EZH2/ENO1 regulatory pathway in bladder cancer.

si-ADAMTS9-AS2: ADAMTS9-AS2 knockdown; EZH2-OE: EZH2 overexpression; si-ENO1: ENO1 knockdown; ECAR: Extracellular acidification rate; Western blot: Protein expression analysis by immunoblotting

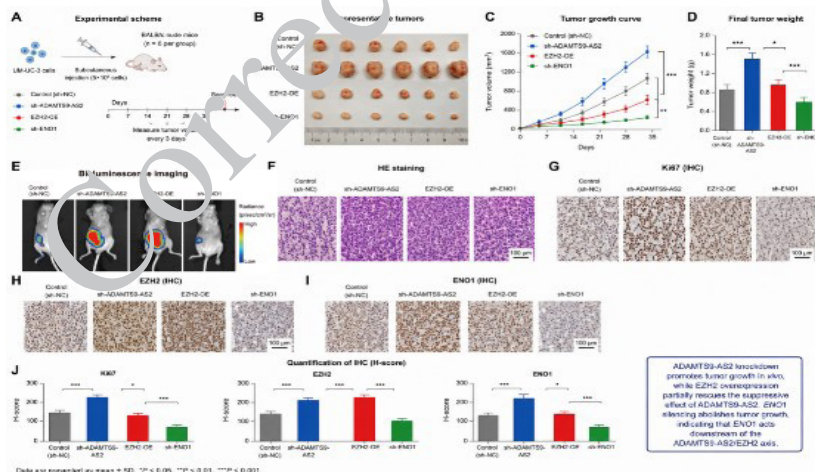


Figure 13. ADAMTS9-AS2 suppresses bladder cancer tumor growth *in vivo* by regulating the EZH2/ENO1 signaling pathway

(A) Schematic illustration of the xenograft experimental design. Stable bladder cancer cells with ADAMTS9-AS2 knockdown, EZH2 overexpression, or ENO1 silencing were subcutaneously injected into BALB/c nude mice. Tumor growth was monitored periodically, and tumors were harvested at the end of the experimental period for molecular and histological analyses. (B) Representative images of xenograft tumors excised from mice in the indicated experimental groups. Tumors derived from ADAMTS9-AS2-silenced cells were visibly larger than those from the control group, whereas ENO1 depletion markedly reduced tumor size. (C) Tumor growth curves showing changes in tumor volume over time. ADAMTS9-AS2 knockdown significantly accelerated tumor growth throughout the experimental period, whereas EZH2 overexpression partially suppressed tumor progression. ENO1 silencing markedly inhibited tumor expansion. (D) Quantitative analysis of final tumor weight at the endpoint of the experiment. Tumors from the si-ADAMTS9-AS2 group exhibited significantly greater mass than controls, whereas ENO1 knockdown resulted in the lowest tumor weights. Quantitative results correspond to Table S9. (E) Representative bioluminescence imaging of xenograft-bearing mice. Increased luminescence intensity was observed in the ADAMTS9-AS2-deficient group, indicating enhanced tumor burden and metabolic activity. Reduced bioluminescence signals were detected following ENO1 silencing. (F) Quantification of total bioluminescence intensity in each treatment group. Relative photon flux was calculated to evaluate *in vivo* tumor progression and metabolic activity. (G) Schematic summary illustrating the role of ADAMTS9-AS2 in suppressing bladder cancer growth through modulation of the EZH2/ENO1 signaling pathway under hypoxic conditions. The quantitative tumor growth and tumor weight data correspond to the results presented in Table S9. Data are presented as mean±SD (n=6 mice per group). Tumor growth curves and quantitative analyses were evaluated using one-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. A value of *P*<0.05 was considered statistically significant. Statistical significance is indicated as **P*<0.05, ***P*<0.01, ****P*<0.001. These results demonstrate that ADAMTS9-AS2 functions as a potent tumor suppressor *in vivo* and that activation of the EZH2/ENO1 pathway contributes significantly to bladder cancer progression.

IHC: Immunohistochemistry; Ki-67: Proliferation marker; Ki-67; H3K9me3: Histone H3 lysine 9 trimethylation; Bioluminescence imaging: *In vivo* luciferase-based tumor imaging; H&E: Hematoxylin and eosin (histological staining)

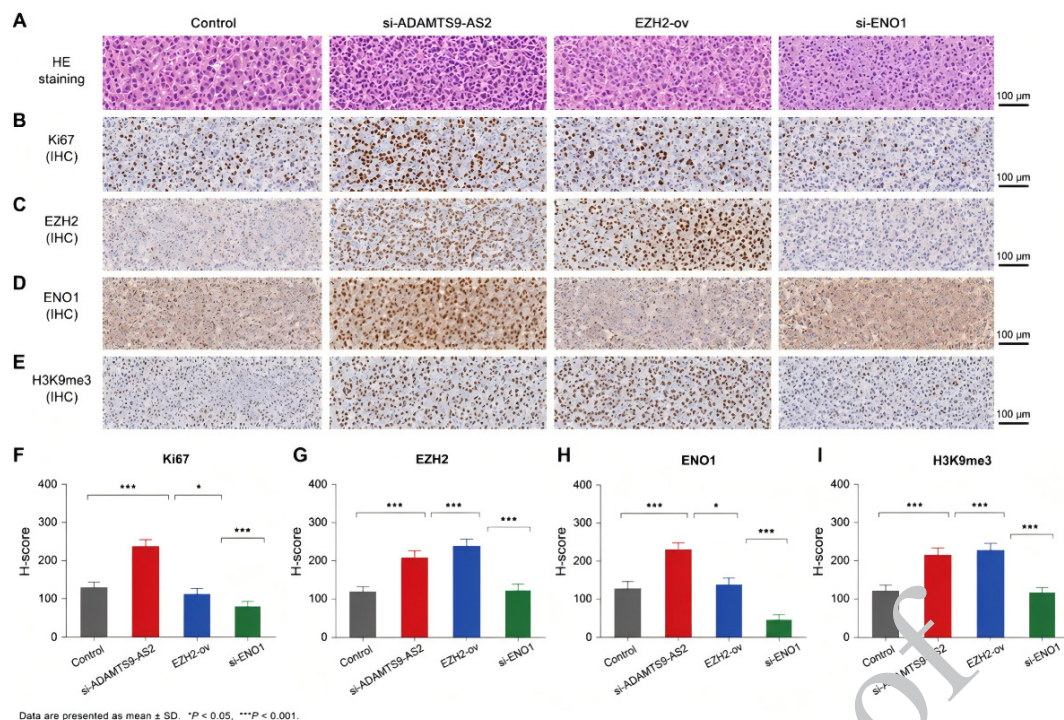


Figure 14. Histological and immunohistochemical validation of the ADAMTS9-AS2/EZH2/ENO1 signaling axis in bladder cancer xenograft tumor

(A) Representative hematoxylin and eosin (H&E) staining of xenograft tumor sections from the indicated experimental groups. Tumors derived from ADAMTS9-AS2-silenced cells exhibited increased cellular density, enlarged nuclei, nuclear pleomorphism, and aggressive histopathological features compared with control tumors. ENO1 knockdown alleviated these malignant characteristics. Scale bars=100 μ m. (B) Representative immunohistochemical (IHC) staining of Ki67 in xenograft tumor tissues. An increase in Ki67-positive nuclei was observed in tumors depleted of ADAMTS9-AS2, indicating increased proliferative activity. ENO1 silencing significantly reduced Ki67 expression. Scale bars=50 μ m. (C) Representative IHC staining of EZH2. Elevated EZH2 expression was detected in ADAMTS9-AS2-deficient tumors and EZH2-overexpressing tumors, confirming activation of the epigenetic regulatory pathway. Scale bars=50 μ m. (D) Representative IHC staining of ENO1. Strong ENO1 immunoreactivity was observed in the si-ADAMTS9-AS2 group, whereas ENO1 knockdown markedly reduced staining intensity. Scale bars=50 μ m. (E) Representative IHC staining of H3K9me3 in xenograft tumor sections. Increased H3K9me3 deposition was detected in ADAMTS9-AS2-deficient tumors, supporting enhanced EZH2-mediated epigenetic modification. Scale bars=50 μ m. (F) Quantification of Ki67 immunohistochemical H-scores. ADAMTS9-AS2 knockdown significantly increased proliferative activity, while ENO1 depletion reduced Ki67 expression. (G) Quantification of EZH2 H-scores. Elevated EZH2 expression was observed in the ADAMTS9-AS2-deficient and EZH2-overexpressing groups. (H) Quantification of ENO1 H-scores. ENO1 expression was significantly increased following ADAMTS9-AS2 depletion and markedly reduced after ENO1 silencing. (I) Quantification of H3K9me3 H-scores. Enhanced histone methylation was observed in tumors with reduced ADAMTS9-AS2 expression, supporting activation of the epigenetic regulatory mechanism. Quantitative immunohistochemical analyses are presented as mean $\pm$ SD from at least five randomly selected microscopic fields per tumor section (n=6 mice per group). Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple-comparison *post hoc* test. Statistical significance is indicated as * P <0.05, ** P <0.01, *** P <0.001. These histological and immunohistochemical findings provide *in vivo* validation of the proposed ADAMTS9-AS2/EZH2/ENO1 signaling pathway and demonstrate that loss of ADAMTS9-AS2 promotes tumor proliferation, metabolic reprogramming, and epigenetic activation in bladder cancer.

H&E: Hematoxylin and eosin staining; IHC: Immunohistochemistry; Ki-67: Proliferation marker; EZH2: Enhancer of zeste homolog 2; ENO1: Enolase 1; H3K9me3: Histone H3 lysine 9 trimethylation; H-score: Histological scoring method for IHC quantification

Discussion

In this study, we identified a novel hypoxia-responsive regulatory axis involving the long non-coding RNA ADAMTS9-AS2, the epigenetic modifier EZH2, and the glycolytic enzyme ENO1 in bladder cancer. Our findings demonstrate that down-regulation of ADAMTS9-AS2 under hypoxic conditions promotes EZH2-mediated epigenetic remodeling at the ENO1 locus, leading to altered alternative splicing that favors pro-oncogenic ENO1 isoforms. This cascade enhances aerobic glycolysis, induces mitochondrial dysfunction, and drives aggressive tumor phenotypes in both *in vitro* and *in vivo* settings. These results significantly extend previous observations that ADAMTS9-AS2 functions as a tumor suppressor and provide the first mechanistic link between this lncRNA, epigenetic control of splicing, and metabolic reprogramming in the hypoxic tumor microenvironment. Expression analyses confirmed that ADAMTS9-AS2 is markedly down-regulated in bladder cancer tissues and cell lines, consistent with earlier reports.

EZH2 is the catalytic subunit of PRC2 and primarily mediates H3K27 trimethylation rather than H3K9 trimethylation. Therefore, the increased H3K9me3 observed in our study should be interpreted as an associated chromatin modification occurring alongside

enhanced EZH2 recruitment and H3K27me3 deposition (17). Previous studies have demonstrated functional cooperation between PRC2 and H3K9 methyltransferases, including SUV39H1 and SETDB1, during establishment of transcriptionally repressive chromatin domains. Our findings support a model in which ADAMTS9-AS2 deficiency promotes EZH2 recruitment to the ENO1 locus, leading to canonical H3K27me3 deposition and coordinated H3K9me3 enrichment through additional chromatin regulatory mechanisms. Future studies are required to identify the methyltransferase directly responsible for H3K9 trimethylation at this locus (18).

We further showed that hypoxia suppresses ADAMTS9-AS2. This links the lncRNA directly to the HIF-1 α -driven program that promotes glycolytic adaptation. The inverse correlation between ADAMTS9-AS2 levels and tumor stage, together with poorer survival in low-expression cases, supports its potential as a prognostic biomarker. While prior work emphasized cytoplasmic miRNA sponging, our data highlight a nuclear role through direct EZH2 binding, suggesting context-dependent compartmental functions. ADAMTS9-AS2 knockdown promoted proliferation, clonogenicity, migration, and invasion, while driving a robust shift toward aerobic glycolysis, increasing ECAR,

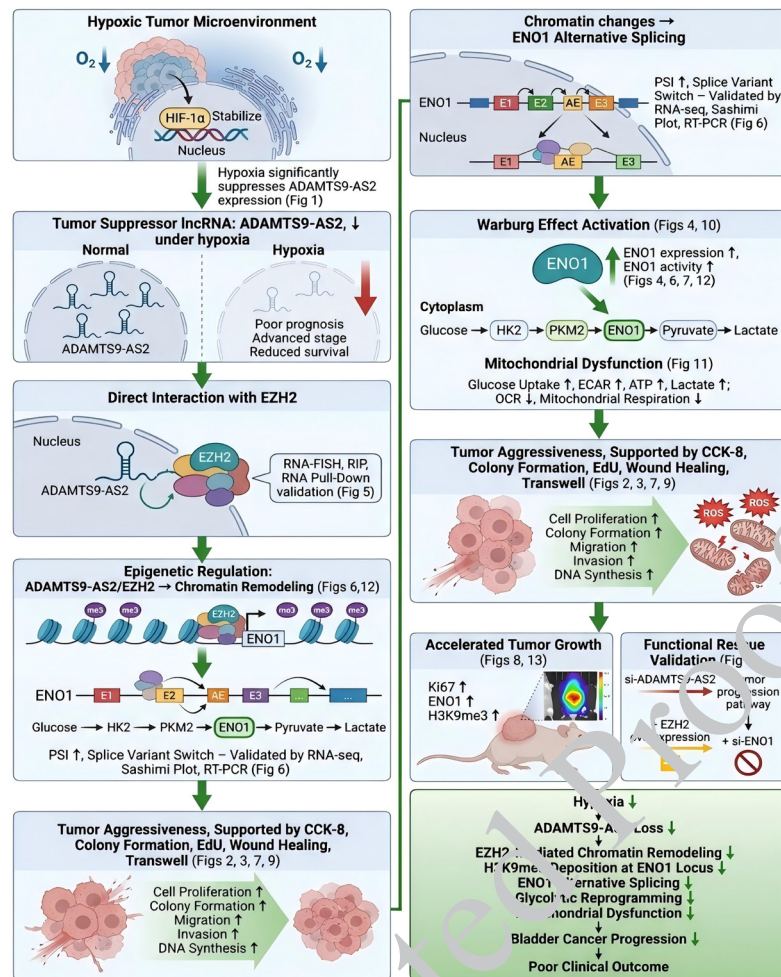


Figure 15. Proposed mechanism underlying ADAMTS9-AS2-mediated suppression of bladder cancer progression under hypoxic conditions

Schematic diagram illustrating the molecular mechanism by which ADAMTS9-AS2 regulates bladder cancer progression through epigenetic and metabolic pathways under hypoxic conditions. Hypoxia within the bladder tumor microenvironment induces HIF-1 α stabilization and significantly suppresses ADAMTS9-AS2 expression. Reduced ADAMTS9-AS2 levels are associated with advanced tumor stage, unfavorable clinical characteristics, and poor overall survival. As a tumor-suppressive long non-coding RNA, ADAMTS9-AS2 directly interacts with the epigenetic regulator EZH2 in the nucleus, as demonstrated by RNA-FISH, RNA immunoprecipitation (RIP), and RNA pull-down analyses. Following formation of the ADAMTS9-AS2-EZH2 complex, EZH2 is recruited to regulatory regions of the ENO1 gene, resulting in chromatin remodeling and increased histone methylation, including H3K9me3 deposition. These epigenetic modifications alter ENO1 transcript processing and promote alternative splicing events, leading to increased production of functionally active ENO1 splice variants. Consequently, ENO1 expression and enzymatic activity are enhanced, establishing ENO1 as a key downstream effector of the ADAMTS9-AS2/EZH2 signaling pathway. Activation of ENO1 promotes glycolytic reprogramming characterized by increased glucose uptake, enhanced extracellular acidification rate (ECAR), elevated ATP production, increased lactate secretion, and reduced oxidative phosphorylation. Simultaneously, dysregulated cellular metabolism contributes to mitochondrial dysfunction, including mitochondrial membrane depolarization, excessive ROS accumulation, mitochondrial swelling, cristae disruption, and structural fragmentation. These metabolic alterations collectively promote malignant phenotypes, including increased cellular proliferation, clonogenic survival, DNA synthesis, migration, and invasion. In vivo studies further demonstrate that loss of ADAMTS9-AS2 accelerates xenograft tumor growth and increases tumor burden, accompanied by elevated expression of Ki67, ENO1, and H3K9me3. Functional perturbation experiments support the involvement of ENO1 in the ADAMTS9-AS2/EZH2 regulatory axis, as EZH2 overexpression partially attenuates the effects of ADAMTS9-AS2 depletion, whereas ENO1 silencing markedly suppresses the enhanced proliferation, metastatic behavior, glycolytic activation, and tumor progression observed following ADAMTS9-AS2 depletion. Collectively, these findings identify a novel hypoxia-responsive ADAMTS9-AS2/EZH2/ENO1 signaling axis that links epigenetic regulation, alternative splicing, metabolic reprogramming, mitochondrial dysfunction, and bladder cancer progression. The proposed mechanism suggests that restoration of ADAMTS9-AS2 expression or therapeutic targeting of EZH2- and ENO1-dependent pathways may represent promising strategies for bladder cancer treatment and precision oncology.

HIF-1 α : Hypoxia-inducible factor 1-alpha; H3K9me3: Histone H3 lysine 9 trimethylation; ECAR: Extracellular acidification rate; OCR: Oxygen consumption rate; ROS: Reactive oxygen species; Ki-67: Proliferation marker Ki-67

glucose uptake, lactate production, and ATP production, and reducing oxidative phosphorylation. Elevated glycolytic enzyme levels and profound mitochondrial impairment, including membrane depolarization, cristae disruption, and ROS accumulation, accompanied these metabolic alterations. ENO1 silencing largely reversed these phenotypes, aligning with evidence that ENO1 promotes bladder cancer progression via both glycolytic and non-glycolytic mechanisms and that its alternative splicing generates isoforms with opposing tumorigenic roles. Mechanistically, nuclear ADAMTS9-AS2 directly interacts with EZH2, modulating chromatin at the ENO1 locus.

ADAMTS9-AS2 depletion was associated with increased EZH2 occupancy and H3K9me3 enrichment at the ENO1 locus, correlating with shifts in ENO1 splicing detected by RNA-seq, PSI analysis, and RT-PCR. These findings are consistent with the concept that histone modifications influence co-transcriptional splicing by affecting RNA polymerase II elongation and spliceosome recruitment. ADAMTS9-AS2 thus appears to act as an epigenetic scaffold restraining EZH2 at metabolic loci; its hypoxia-induced loss derepresses this control, favoring oncogenic ENO1 variants. Rescue experiments and xenograft models provided causal validation. EZH2 overexpression partially

attenuated the phenotypic effects of ADAMTS9-AS2 knockdown, while ENO1 silencing robustly suppressed them. *In vivo*, ADAMTS9-AS2-deficient tumors exhibited faster growth, a higher bioluminescent burden, and elevated levels of Ki-67, EZH2, ENO1, and H3K9me3. These results confirm ENO1 as a key effector and highlight the therapeutic potential of targeting the axis. The identification of this circuit has several implications. It integrates lncRNA-mediated epigenetic regulation with splicing and metabolic reprogramming in bladder cancer, where hypoxia is common and therapeutically challenging. The splicing-dependent activation of ENO1 expands understanding of how non-coding RNAs control isoform usage. Notably, the tumor-suppressive role of ADAMTS9-AS2 in bladder cancer contrasts with its oncogenic activity reported in other malignancies, underscoring the tissue-specific functions of lncRNAs. Therapeutically, restoring ADAMTS9-AS2 expression or inhibiting EZH2 or ENO1 could disrupt hypoxia-driven progression. ADAMTS9-AS2 levels or ENO1 splice ratios may also serve as biomarkers for stratification or treatment monitoring. Our alternative splicing analyses suggest that hypoxia-induced ADAMTS9-AS2 deficiency favors production of glycolytically active ENO1 transcripts while reducing the relative abundance of transcript variants associated with MBP-1 generation. Given the opposing biological functions of these isoforms, this shift provides a plausible explanation for the enhanced glycolytic metabolism and malignant phenotypes observed in bladder cancer cells. Although direct characterization of MBP-1 protein abundance was beyond the scope of the present study, future studies that combine isoform-specific antibodies, targeted proteomics, and CRISPR-mediated splice-site editing will further define the functional contributions of individual ENO1 isoforms (19).

From a translational perspective, pharmacological inhibition of EZH2 represents a particularly attractive therapeutic strategy. Tazemetostat, an FDA-approved selective EZH2 inhibitor, has demonstrated clinical efficacy in several malignancies and may warrant investigation in bladder cancers exhibiting activation of the ADAMTS9-AS2/EZH2 signaling axis. Although clinically approved ENO1 inhibitors are currently unavailable, multiple experimental approaches including monoclonal antibodies, peptide inhibitors, RNA interference, and small-molecule compounds targeting ENO1 have demonstrated encouraging anti-tumor activity in preclinical studies (20). Future investigations should evaluate whether restoration of ADAMTS9-AS2 expression enhances sensitivity to EZH2 inhibition or whether combined targeting of the ADAMTS9-AS2/EZH2/ENO1 pathway provides synergistic therapeutic benefit under hypoxic conditions (21).

Previous studies have consistently described ADAMTS9-AS2 as a tumor suppressor in several malignancies, including breast cancer, gastric cancer, colorectal cancer, hepatocellular carcinoma, glioma, ovarian cancer, and cervical cancer, where it predominantly functions by regulating miRNA networks or transcriptional signaling pathways (22). In contrast, the present study identifies a previously unrecognized hypoxia-responsive nuclear mechanism in bladder cancer in which ADAMTS9-AS2 directly interacts with EZH2 to regulate chromatin remodeling, ENO1 alternative splicing, and metabolic reprogramming. These observations highlight the tissue-specific functional diversity of ADAMTS9-AS2 while

extending its biological role beyond canonical competing endogenous RNA mechanisms (23).

Limitations include reliance on established cell lines and subcutaneous xenografts, which may not fully recapitulate the native bladder microenvironment or metastasis. Orthotopic models and patient-derived systems would strengthen translational relevance. While focused on H3K9me3 at ENO1, broader epigenomic profiling could identify additional regulated loci. Although the present study demonstrated significant alterations in ENO1 transcript processing using RNA sequencing, rMATS, SUPPA2, PSI analysis, and RT-PCR validation, we did not directly quantify individual ENO1 protein isoforms or selectively manipulate the canonical ENO1 and MBP-1 isoforms. Therefore, the functional contribution of each splice variant was inferred from transcript-level analyses and previously published studies describing their biological activities. Future investigations employing isoform-specific antibodies, CRISPR/Cas9-mediated splice-site editing, or isoform-specific overexpression and knockdown strategies will be important to establish the precise contribution of each ENO1 isoform to metabolic reprogramming and bladder cancer progression. The precise steps linking histone changes to ENO1 splice-site selection require further study. CRISPR-based approaches will help confirm specificity in future work. In conclusion, this work establishes the ADAMTS9-AS2/EZH2/ENO1 axis as a critical mediator of hypoxia-driven metabolic reprogramming and progression in bladder cancer. By connecting lncRNA biology, chromatin regulation, and tumor metabolism, these findings open new avenues for mechanistic understanding and targeted therapeutic development against this disease.

Conclusion

This study demonstrates that ADAMTS9-AS2 is a critical hypoxia-responsive tumor suppressor that coordinates epigenetic regulation, alternative splicing, and metabolic reprogramming in bladder cancer. We show that hypoxia markedly suppresses ADAMTS9-AS2 expression, which correlates with advanced disease stage and unfavorable patient prognosis. Mechanistically, ADAMTS9-AS2 directly interacts with EZH2 in the nucleus to regulate chromatin remodeling at the ENO1 locus, thereby modulating ENO1 alternative splicing and promoting a metabolic shift toward aerobic glycolysis. Loss of ADAMTS9-AS2 enhances glucose metabolism, mitochondrial dysfunction, oxidative stress, cellular proliferation, migration, invasion, and tumor growth. In contrast, inhibition of ENO1 effectively reverses these oncogenic phenotypes, confirming its role as a key downstream effector. Collectively, these findings establish the ADAMTS9-AS2/EZH2/ENO1 axis as a previously unrecognized molecular pathway linking hypoxia-induced epigenetic remodeling with alternative splicing and metabolic adaptation during bladder cancer progression. This work expands current understanding of lncRNA-mediated regulation of tumor metabolism and provides mechanistic evidence supporting the therapeutic potential of restoring ADAMTS9-AS2 expression or targeting EZH2- and ENO1-dependent signaling. Future studies using larger clinical cohorts and preclinical therapeutic models will be valuable for validating the clinical applicability of this pathway and for facilitating the development of precision therapies for patients with bladder cancer.

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Ethics Approval and Consent to Participate

This study was approved by the Biological and Medical Ethics Committee of Hubei Polytechnic University (Approval No. Hbpu2024015). All procedures involving experimental materials were conducted in accordance with the ethical standards of the institutional research committee and with the Declaration of Helsinki.

Availability of Data and Materials

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Authors' Contributions

G L and X W contributed equally to this work and share first authorship. They participated in study design, data acquisition, analysis, and manuscript preparation. D G and T Z contributed to project supervision, interpretation of results, and critical revision of the manuscript. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare that they have no competing interests.

Declaration

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