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Original Research

Set7-Mediated Repression of the HIF-1α Adaptive Response Triggers Apoptosis in Hypoxic Spermatogonia

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Abstract

Background: Male infertility, often caused by oligospermia, is a major global health concern. Hypoxia is a known inducer of germ cell death, a process governed by the hypoxia-inducible factor 1α (HIF-1α). The methyltransferase Set7 represses HIF-1α activity, but its specific role in the hypoxic male germline remains unknown. **Method**: The mouse spermatogonia-derived GC-2 cell line was used. The effect of hypoxia on SET domain-containing lysine methyltransferase 7 (*Setd7*) mRNA expression was assessed by quantitative real-time polymerase chain reaction (qRT-PCR). Cells were transfected with a Set7 overexpression plasmid, and its impact was measured via luciferase reporter assays using an erythropoietin (*EPO*) promoter and qRT-PCR for HIF-1α targets (glucose transporter 1 (*Glut1*), phosphoglycerate kinase 1 (*Pgk1*), pyruvate kinase, muscle (*Pkm*)). Stable cell lines expressing wild-type Set7 or a catalytically dead mutant (Set7-H297A) were generated. Apoptosis under hypoxia was analyzed by flow cytometry and fluorescence microscopy. **Results**: Hypoxia (1% O_2) significantly suppressed *Setd7* mRNA expression while inducing HIF-1α target gene expression (*Glut1*, *Pgk1*, vascular endothelial growth factor (*Vegf*), *Pkm*). Set7 overexpression inhibited hypoxia-induced *EPO* promoter activity and blunted the hypoxic induction of *Glut1*, *Pgk1*, and *Pkm*. Although Set7 overexpression did not alter HIF-1α protein levels, it markedly increased hypoxia-induced apoptosis. This pro-apoptotic effect was significantly attenuated in cells expressing the enzymatically inactive Set7-H297A mutant. **Conclusion**: Set7 represses the essential HIF-1α-mediated adaptive response to hypoxia in spermatogonial cells. Paradoxically, this repression potentiates hypoxia-induced apoptosis in a methyltransferase-dependent manner. These findings identify Set7 as a critical molecular switch that shifts cells from cellular adaptation to death under hypoxic stress, suggesting a regulatory pathway potentially relevant to hypoxia-associated male infertility.

Keywords: histone-lysine n-methyltransferase; hypoxia-inducible factor 1; alpha subunit; hypoxia; apoptosis; spermatogonia; male infertility

1. Introduction

Infertility, affecting an estimated 12–15% of reproductive-aged couples globally, represents a critical and escalating public health challenge [1]. In approximately half of these cases, male factors are a primary contributor, underscoring the urgent need to elucidate the molecular pathologies underlying male reproductive failure [2]. Among the spectrum of male fertility disorders, oligospermia, a reduction in sperm concentration, stands as a predominant etiology [3]. Spermatogenesis is a complex, highly coordinated process reliant on precise physiological conditions, including strict oxygen homeostasis. While regulated apoptosis is essential for eliminating defective germ cells and maintaining seminiferous tubule integrity, excessive or dysregulated germ cell death is a hallmark of oligospermia and a direct cause of diminished sperm output [4]. Notably, hypoxia, a potent inducer of apoptosis in diverse cell types, including cardiomyocytes and endothelial cells [5,6], has been specifically implicated in male infertility. Experimental models demonstrate that hypoxic stress markedly increases germ cell apoptosis, with spermatocytes particularly vulnerable [7]. Clinical conditions such as varicocele, associated with testicular hypoxia, are strongly linked to oligospermia, positioning hypoxia-induced germ cell death as a pivotal mechanistic link to fertility impairment [8]. To investigate the molecular mechanisms underlying this process, we employed the GC-2 cell line, a well-established mouse model of type B spermatogonia. This cell line has been widely used to study germ cell apoptosis and hypoxic stress responses due to its retained spermatogonial characteristics, reproducible response to hypoxia, and relevance to early stages of spermatogenesis [9].

The cellular response to hypoxia is masterfully orchestrated by the hypoxia-inducible factor (HIF) family of transcription factors. HIF-1α, the oxygen-sensitive subunit, is constitutively synthesized but rapidly degraded via the ubiquitin-proteasome pathway under normoxia. Under hypoxic conditions, HIF-1α protein stabilizes, translocates to the nucleus, and dimerizes with its partner ARNT

(aryl hydrocarbon receptor nuclear translocator, also known as HIF-1β).. This complex binds to hypoxia-response elements (HREs) in target gene promoters, driving the transcription of a vast adaptive program. This program includes genes critical for angiogenesis (e.g., VEGFA, vascular endothelial growth factor A), glycolysis (e.g., SLC2A1 [solute carrier family 2 member 1], also known as GLUT1 [glucose transporter type 1]; and PGK1 [phosphoglycerate kinase 1]), and erythropoiesis (e.g., erythropoietin (*EPO*)), which collectively enhance oxygen delivery and facilitate metabolic adaptation to low oxygen [10,11]. Paradoxically, while this response promotes survival, sustained or severe hypoxia can also activate pro-apoptotic pathways via HIF-1α. In the testis, upregulated HIF-1α expression correlates with increased spermatocyte apoptosis [9], and mechanistic studies reveal HIF-1α can engage both mitochondrial and death receptor-mediated apoptotic cascades, positioning it as a central regulator of germ cell fate under hypoxic duress [12]. Thus, HIF-1α sits at a critical juncture, mediating both adaptive survival and terminal apoptosis, with the balance determining spermatogenic outcome.

The regulation of HIF-1α activity is multifaceted, extending beyond protein stability to include post-translational modifications such as methylation. SET Domain Containing 7 (*SETD7*), a protein lysine methyltransferase, catalyzes mono-methylation of histone H3 at lysine 4 (H3K4me1), a mark associated with active transcription [13]. Beyond histones, Set7 methylates numerous non-histone proteins, including p53, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), and FOXO3a (forkhead box O3a)—thereby modulating their stability, localization, and transcriptional activity, and implicating Set7 in diverse physiological and pathological processes from cancer to metabolic disease [14,15]. Crucially, Set7 has been identified as a direct negative regulator of the HIF-1α pathway. It methylates HIF-1α at lysine 32 (K32), a modification that represses HIF-1α's transcriptional activity without affecting its protein stability, thereby downregulating the expression of canonical HIF-1α target genes [16]. This established antagonistic relationship suggests Set7 could function as a molecular brake on the hypoxic response. However, the functional consequences of this interaction in the male germline remain entirely unexplored. Does Set7-mediated repression of HIF-1α confer protection by mitigating hypoxia-induced apoptosis, or does it, by crippling the essential adaptive response, inadvertently exacerbate cellular stress and death?

To address this fundamental question, we investigated the role of Set7 in a model of hypoxic stress using GC-2 cells, a mouse spermatogonia-derived line relevant for studying germ cell apoptosis. We hypothesized that Set7, by inhibiting HIF-1α transcriptional activity, would attenuate the hypoxic response and protect cells from hypoxia-induced apoptosis. Contrary to our hypothesis, we demonstrate that *Setd7* expression is suppressed by hypoxia, and its overexpression potently represses HIF-1α target gene activation (*Glut1*, *Pgk1*, *Pkm*). Strikingly, this is coupled with a significant enhancement of hypoxia-induced apoptosis, an effect dependent on Set7's catalytic activity. These findings reveal a novel and paradoxical role for Set7 as a potentiator of germ cell death under hypoxia, challenging the simple view of it as merely a HIF-1α inhibitor and suggesting its function is critically dependent on cellular context. This study unveils a new layer of complexity in the epigenetic regulation of hypoxic stress in spermatogenesis, with important implications for understanding the pathogenesis of oligospermia.

2. Methods and Materials

2.1 Reagents and Antibodies

Antibodies against Myc-tag (#sc-40) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, #SC-477242) were from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against HIF-1α (#36169) and Set7 (#2825) were purchased from Cell Signaling Technology (Danvers, MA, USA). An anti-β-actin antibody (#AC026) was from ABclonal (Wuhan, China). The FITC Annexin V Apoptosis Detection Kit I (#556547) was obtained from BD Pharmingen (San Diego, CA, USA).

2.2 Cell Culture and Treatments

The mouse type B spermatogonia-derived GC-2 cell line (#SCSP-5055) was obtained from the National Collection of Authenticated Cell Cultures (China). The cell line was authenticated by the supplier using short tandem repeat (STR) profiling and was confirmed to be negative for mycoplasma contamination. Cells were used within 10 passages after resuscitation and were routinely tested for mycoplasma during the study, with all tests remaining negative. The GC-2 cell line was selected for this study because it represents a well-characterized *in vitro* model of male germ cells at the spermatogonial stage. These cells are capable of undergoing apoptosis in response to hypoxic stress and have been extensively used in reproductive biology research to study spermatogenesis and germ cell death [9]. Cells were maintained in high-glucose Dulbecco's Modified Eagle Medium (DMEM; #SH30243.01, HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; #16000044, Thermo Fisher, Waltham, MA, USA) at 37 °C in a humidified incubator with 5% CO_2 (normoxic conditions). The medium was replenished every two days. For routine passage, cells at 80–90% confluence were detached using a 0.05% EDTA (ethylenediaminetetraacetic acid)-trypsin solution (#0183, ScienCell, Carlsbad, CA, USA) and reseeded at an appropriate dilution.

To chemically mimic hypoxia, cells at 70–80% confluence were treated with 200 μM cobalt chloride ($CoCl_2$; Sigma-Aldrich, USA) in complete medium for 48 hours.

For physical hypoxia, cells were placed in a modular hypoxia chamber (Billups-Rothenberg, Inc., Del Mar, CA, USA) flushed with a gas mixture of 1% O_2, 5% CO_2, and 94% N_2 for the durations specified in individual experiments.

The exposure durations were selected based on preliminary optimization experiments. The 18-hour time point was chosen to assess early transcriptional responses (qRT-PCR) and apoptotic events (flow cytometry), while the 48-hour treatment was used for evaluating sustained hypoxic effects on gene expression, allowing sufficient time for accumulation of HIF-1α target mRNAs following Set7 overexpression.

2.3 Plasmid Transfection and Luciferase Reporter Assay

The Myc-tagged empty vector (Myc-empty) and Set7 overexpression vector (Myc-Set7) were transiently transfected into GC-2 cells using the Neuroporter Transfection Kit (Sigma-Aldrich, USA) according to the manufacturer's instructions.

For the luciferase reporter assay, GC-2 cells in 24-well plates were co-transfected with the Myc-empty or Myc-Set7 plasmid, a firefly luciferase reporter plasmid driven by the erythropoietin (EPO) promoter, and a Renilla luciferase control plasmid (pRL-CMV). After 6 hours, the transfection medium was replaced, and cells were subjected to either normoxia or hypoxia (1% O_2) for 18 hours. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA). Firefly luciferase activity was normalized to Renilla luciferase activity for each sample.

2.4 RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from GC-2 cells using TRIzol reagent (Thermo Fisher, USA). First-strand cDNA was synthesized from 1 µg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher, USA). Quantitative PCR was performed using MonAmp™ SYBR Green qPCR Mix (Monad Biotech, Wuhan, Hubei, China) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was normalized to Gapdh or β-actin levels, and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences used are listed in Table 1.

2.5 Western Blot Analysis

Cells were lysed in RIPA (radioimmunoprecipitation assay) buffer (#89900, Thermo Fisher, USA) supplemented with protease inhibitors. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher, USA). Equal amounts of protein (10–30 µg) were separated by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) on 10% gels (#P0012AC, Beyotime, Shanghai, China) and transferred onto PVDF membranes (#IPVH00010, MilliporeSigma, Burlington, MA, USA). Membranes were blocked with 5% non-fat milk in TBST for 1 hour at room temperature, then incubated with primary antibodies overnight at 4 °C. After washing, membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (#AS003, ABclonal, Woburn, MA, USA) for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) substrate (Millipore, USA) and imaged with a ChemiDoc system (Bio-Rad, Hercules, CA, USA). Band intensities were quantified using ImageJ software (v.53t, National Institutes of Health, Bethesda, MD, USA).

2.6 Lentivirus Production and Generation of Stable Cell Lines

Lentiviral particles were produced by co-transfecting HEK293T cells with the packaging plasmids psPAX2 and pMD2.G, along with one of the following transfer plasmids: pHAGE (empty vector), pHAGE-Set7 (wild-type), or pHAGE-Set7-H297A (catalytically dead mutant). Viral supernatants were collected 48 hours post-transfection, filtered through a 0.45 µm membrane, and used to infect GC-2 cells in the presence of 8 µg/mL polybrene. Seventy-two hours post-infection, cells were selected with 1 µg/mL puromycin for two weeks to establish stable polyclonal populations.

2.7 Apoptosis Analysis by Flow Cytometry and Fluorescence Microscopy

For apoptosis assays, stable GC-2 cell lines were cultured under normoxia or hypoxia (1% O_2) for 18 hours. Cells were then detached, washed with cold Phosphate-buffered saline (PBS; #10010023, Thermo Fisher Scientific, Waltham, MA, USA), and stained with the FITC Annexin V Apoptosis Detection Kit I according to the manufacturer's protocol. Briefly, 1×10^5 cells were resuspended in 100 µL of 1× Binding Buffer, incubated with 5 µL of FITC Annexin V and 10 µL of propidium iodide (PI) for 15 minutes in the dark, and then analyzed immediately on a BD FACSCanto II flow cytometer. Data were processed using FlowJo software (v10.8.1, BD Biosciences, Ashland, OR, USA). For qualitative assessment, stained cells were also visualized and photographed under a fluorescence microscope (Nikon Eclipse Ti2, Nikon Corporation, Tokyo, Japan).

2.8 Statistical Analysis

All experiments were performed in at least three independent biological replicates. Data are presented as mean ± standard deviation (SD). Statistical significance between two groups was determined using an unpaired two-tailed Student's *t*-test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. A p-value of <0.05 was

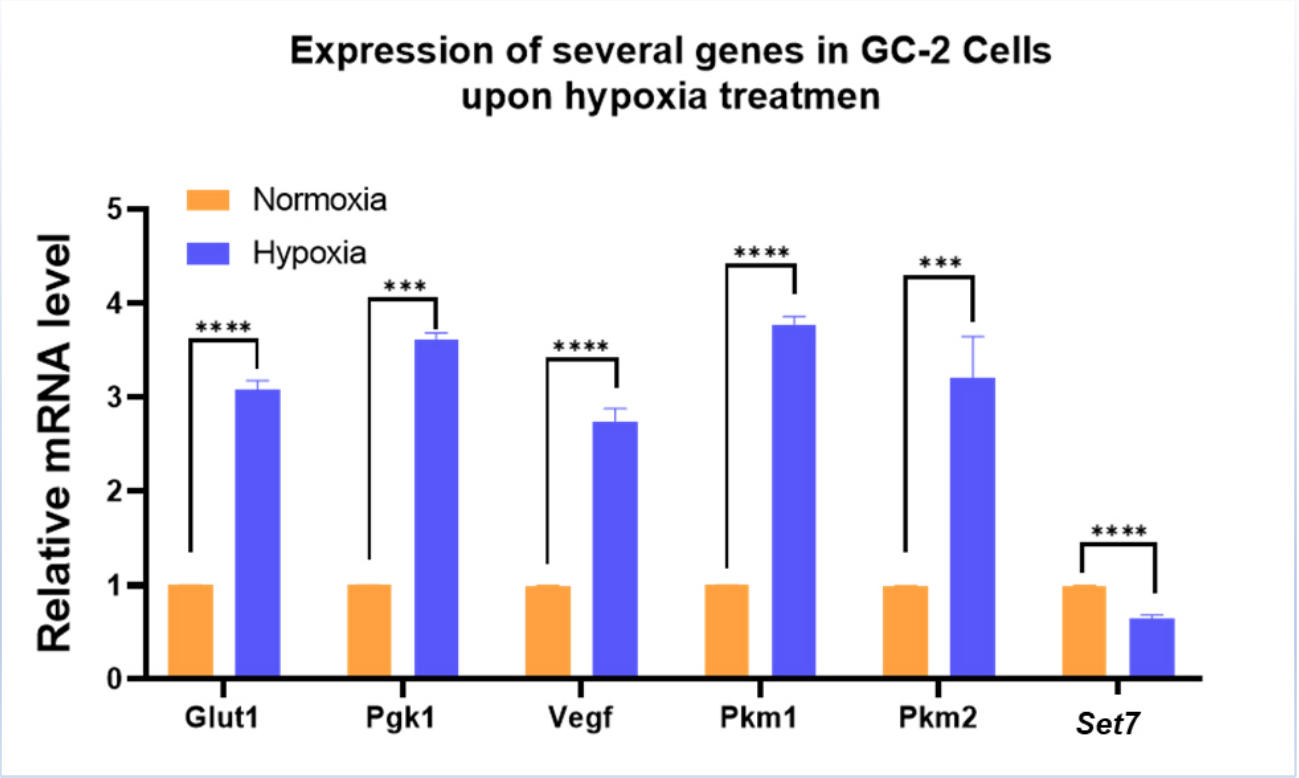

Fig. 1. Hypoxia suppresses *Setd7* expression while inducing hypoxia-inducible factor 1α (HIF-1α) target genes in GC-2 cells. GC-2 cells were exposed to normoxia (N) or hypoxia (H, 1% O_2) for 18 hours. mRNA levels of the indicated genes were quantified by qRT-PCR. Gene expression was normalized to Gapdh and is presented relative to the normoxia control. Data are shown as mean ± SD (n = 3). ***$p < 0.001$, ****$p < 0.0001$ vs. normoxia group (unpaired Student's *t*-test).

Table 1. Primer sequences for quantitative real-time PCR.

Gene	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
Glut1	GGAGAGGTGTCACCTACAGC	AAGGATGCCAACGACGATTC
Pgk1	GGAGAGTCCAGAGCGACCCT	GCAACTTTAGCGCCTCCCA
Vegf	TGACAAGCCAAGGCGGTGAG	CTCCTCTTCCTTCATGTCAG
Pkm	TTGTGCGAGCCTCCAGTC	ACTCCGTGAGAACTATCAAAGC
Setd7	TTCACTCCAAACTGCATCTACGA	GGGTGCGGATGCATTTG
Gapdh	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG
β-actin	TGGAATCCTGTGGCATCCATGAAAC	TAAAACGCAGCTCAGTAACAGTCCG

Note: *Glut1*, glucose transporter type 1 (SLC2A1); *Pgk1*, phosphoglycerate kinase 1; *Vegf*, vascular endothelial growth factor A; *Pkm*, pyruvate kinase M1/2; *Setd7*, SET domain containing 7 (SET7/9); *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase; *β-actin*, beta-actin.

considered statistically significant. All analyses and graph generation were performed using GraphPad Prism software (v8.0, GraphPad Software, Boston, MA, USA).

3. Results

3.1 Hypoxia Downregulates Setd7 Expression in GC-2 Cells

We first assessed the transcriptional response of GC-2 cells to hypoxia. Cells were subjected to normoxia (21% O_2) or hypoxia (1% O_2) for 18 hours, and mRNA levels were quantified by qRT-PCR using gene-specific primers (Table 1). As expected, hypoxia triggered a significant induction of canonical HIF-1α target genes involved in glycolysis and angiogenesis, including *Glut1*, *Pgk1*, *Vegf*, and *Pkm*. In contrast, the mRNA level of *Setd7* (encoding Set7 protein) was markedly suppressed under the same hypoxic conditions (Fig. 1). This reciprocal expression pattern suggests that *Setd7* may be transcriptionally regulated as part of the cellular adaptation to hypoxia.

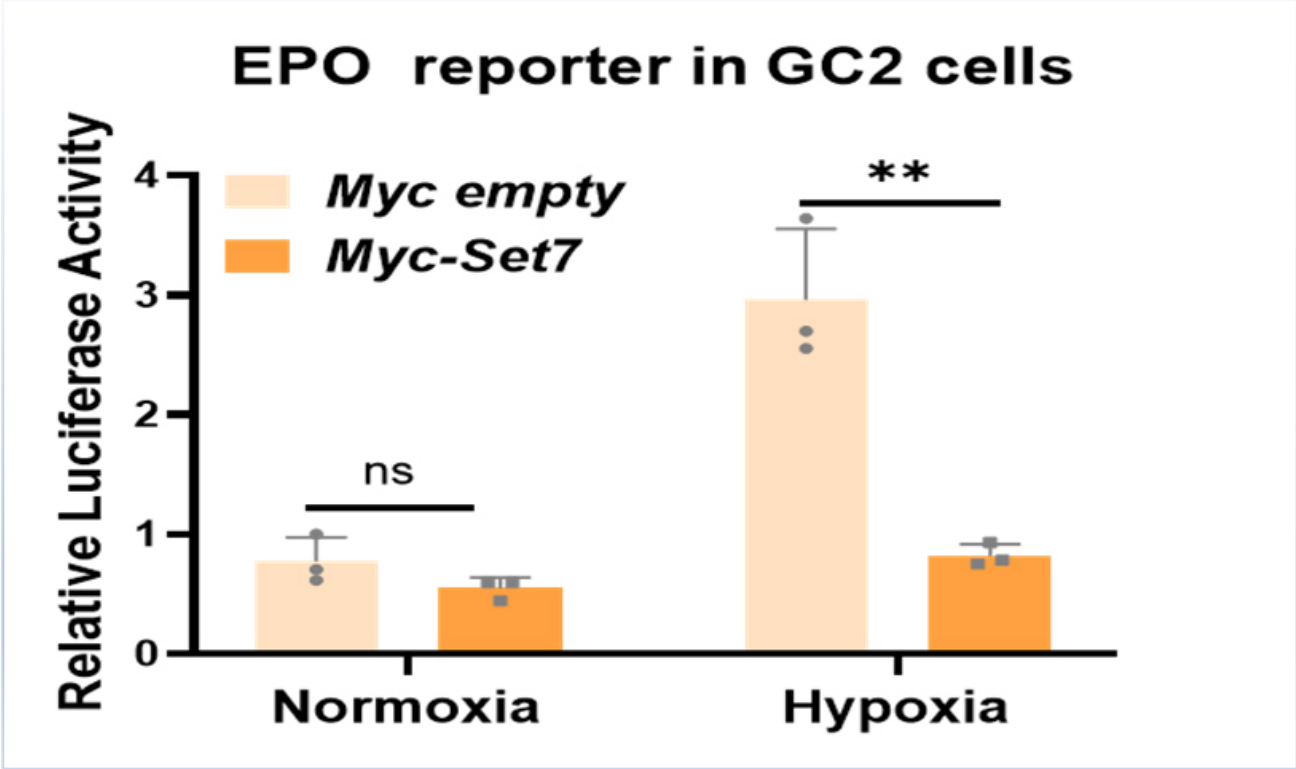

Fig. 2. Set7 inhibits HIF-1α-dependent transcriptional activation. GC-2 cells were co-transfected with an erythropoietin (EPO) promoter-luciferase reporter (constructed in-house using pGL3-Basic vector, Promega Corporation, Madison, WI, USA), a Renilla luciferase control plasmid, and either Myc-Set7 or Myc-empty vector. After transfection, cells were maintained under normoxia or hypoxia (1% O_2) for 18 hours. Firefly luciferase activity was normalized to Renilla luciferase activity. Data are presented as mean ± SD (n = 4). ns, not significant; $^{**}p < 0.01$ (one-way ANOVA with Tukey's post-hoc test using GraphPad Prism version 8.0, GraphPad Software, Boston, MA, USA). ANOVA, analysis of variance.

3.2 Set7 Negatively Regulates HIF-1α-Dependent Transcription

To determine if Set7 functionally represses the HIF-1α pathway, we performed a luciferase reporter assay. GC-2 cells were co-transfected with an EPO promoter-driven firefly luciferase reporter, a Renilla control plasmid, and either a Myc-tagged Set7 overexpression vector (Myc-Set7) or an empty vector control (Myc-empty). Under normoxic conditions, Set7 overexpression did not alter EPO promoter activity. However, under hypoxic stress, Set7 overexpression resulted in a significant reduction in hypoxia-induced luciferase activity compared with the empty vector control (Fig. 2). This result demonstrates that Set7 inhibits HIF-1α-mediated transcription.

3.3 Set7 Overexpression Represses the Expression of Hypoxia-Inducible Genes

We next examined the effect of Set7 on endogenous HIF-1α target gene expression using two independent hypoxia models. First, treatment with the chemical hypoxia mimetic $CoCl_2$ (200 µM, 48 h) robustly induced *Glut1* mRNA. This induction was significantly attenuated in cells overexpressing Set7 (Fig. 3A). We then validated this finding under physiological hypoxia (1% O_2, 48 h). As summarized in Table 2, qRT-PCR analysis revealed that hypoxia strongly induced the expression of Glut1, Pkm, and Pgk1 in control cells. Strikingly, this hypoxic induction was significantly blunted in cells overexpressing Set7 (Fig. 3B–D; Table 2). Set7 overexpression had no significant effect on the basal expression of these genes under normoxia. Successful overexpression of Set7 in transfected GC-2 cells was confirmed by qRT-PCR for *Setd7* mRNA (data not shown) and by the functional repression of HIF-1α target genes presented below.

We confirmed that the repressive effect of Set7 on HIF-1α target genes was not due to altered HIF-1α protein stability. Western blot analysis showed that Set7 overexpression did not affect HIF-1α protein levels under either normoxic or hypoxic conditions (Fig. 4).

3.4 Validation of Set7 Overexpression in GC-2 Cells

To confirm successful overexpression of Set7 in our experimental systems, we performed qRT-PCR analysis of *Setd7* mRNA levels. Transient transfection of Myc-Set7 resulted in an approximately 8-fold increase in *Setd7* mRNA compared to Myc-empty control (Fig. 5A). Similarly, sta-

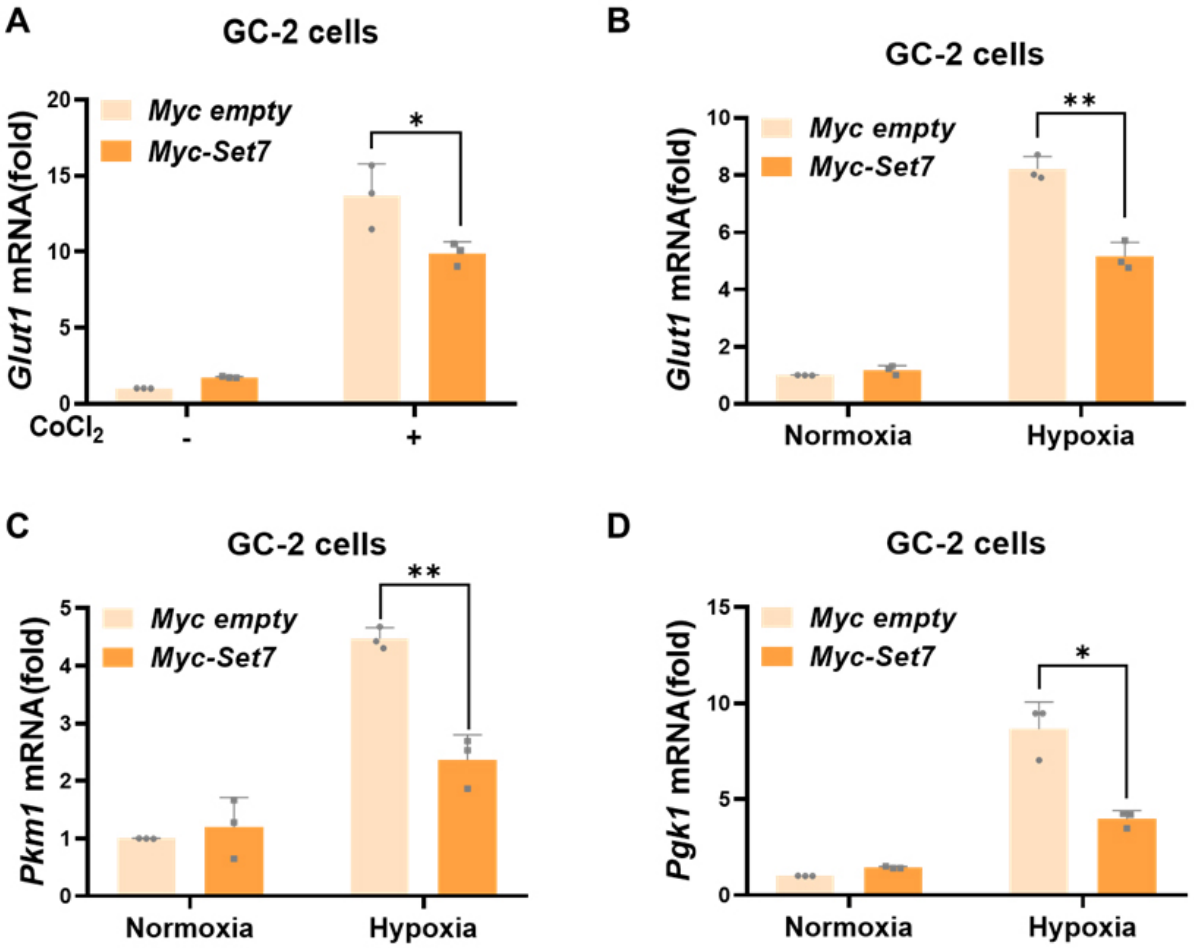

Fig. 3. Set7 represses the hypoxic induction of HIF-1α target genes. (A) GC-2 cells transfected with Myc-Set7 or Myc-empty vector were treated with or without 200 μM $CoCl_2$ for 48 hours. *Glut1* mRNA levels were analyzed by qRT-PCR. (B–D) GC-2 cells transfected as in (A) were exposed to normoxia or hypoxia (1% O_2) for 48 hours. mRNA levels of *Glut1* (B), *Pkm* (C), and *Pgk1* (D) were quantified. All qRT-PCR data are normalized to β-actin (or Gapdh, with similar results) and presented as mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ (one-way ANOVA with Tukey's post-hoc test).

Table 2. Set7 overexpression attenuates the hypoxic induction of glycolytic genes.

Gene	Fold Change (Hypoxia/Normoxia)	
	Myc-empty	Myc-Set7
Glut1	4.8 ± 0.5	1.9 ± 0.3*
Pkm	3.2 ± 0.4	1.5 ± 0.2*
Pgk1	5.1 ± 0.6	2.2 ± 0.4*

Note: GC-2 cells transfected with Myc-empty or Myc-Set7 were exposed to normoxia or hypoxia (1% O_2) for 48 hours. mRNA levels were quantified by qRT-PCR. Data represent the mean fold induction (Hypoxia/Normoxia) ± SD from three independent experiments. *$p < 0.05$ vs. Myc-empty group under hypoxia (unpaired Student's *t*-test).

ble lentiviral expression of pHAGE-Set7 and pHAGE-Set7-H297A yielded comparable levels of *Setd7* mRNA, with both showing approximately 8-fold increases relative to the pHAGE control (Fig. 5B). These data confirm that both wild-type and catalytically dead Set7 were successfully overexpressed in GC-2 cells.

3.5 Set7 Promotes Hypoxia-Induced Apoptosis in a Methyltransferase-Dependent Manner

Given that Set7 suppresses the canonical adaptive response to hypoxia, we investigated its functional consequence on cell survival. We generated GC-2 cells stably expressing either wild-type Set7 (pHAGE-Set7), a catalytically dead mutant (Set7-H297A; pHAGE-Set7-H297A), or an empty vector control (pHAGE).

Under normoxia, apoptosis rates were low and indistinguishable among the three cell lines. Hypoxia (1% O_2, 18 h) induced apoptosis in all groups. However, cells overexpressing wild-type Set7 exhibited a significantly higher apoptotic rate compared to control cells. Notably, this

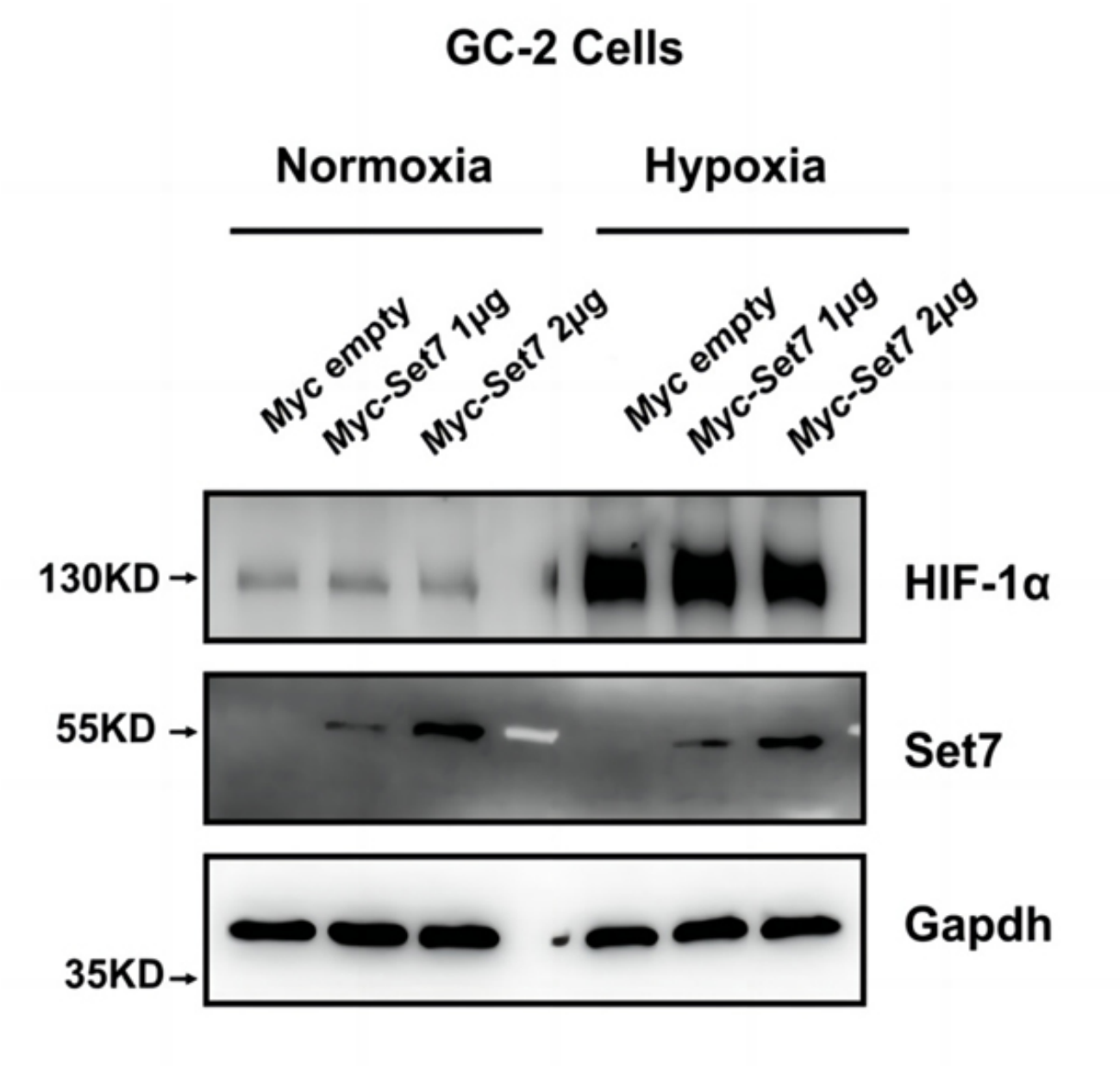

Fig. 4. Set7 overexpression does not affect HIF-1α protein stability. GC-2 cells stably expressing Myc-Set7 or containing the empty vector (pHAGE) were cultured under normoxia or hypoxia (1% O_2) for 18 hours. Whole-cell lysates were analyzed by Western blotting using antibodies against HIF-1α and GAPDH (loading control). A representative blot from three independent experiments is shown.

pro-apoptotic effect was substantially attenuated in cells expressing the enzymatically inactive Set7-H297A mutant (Fig. 6A). The quantitative apoptosis data from flow cytometry are summarized in Table 3.

Fluorescence microscopy analysis of Annexin V-FITC/PI-stained cells corroborated the flow cytometry results. Under hypoxia, a pronounced increase in Annexin V-FITC signal (green fluorescence, indicating early apoptosis) was observed in the pHAGE-Set7 group compared to the pHAGE control, while the signal in the pHAGE-Set7-H297A group was intermediate (Fig. 6B).

4. Discussion

This study elucidates a novel and paradoxical role for the protein methyltransferase Set7 in the hypoxic stress response of mouse spermatogonial cells. We demonstrate that hypoxia transcriptionally represses *Setd7* expression while simultaneously activating the canonical HIF-1α pathway. Functionally, Set7 acts as a potent repressor of HIF-1α-mediated transcription and significantly blunts the induction of key adaptive genes under hypoxic conditions. Contrary to the expectation that inhibiting a stress-response pathway would be protective, Set7 overexpression markedly exacerbates hypoxia-induced apoptosis in GC-2

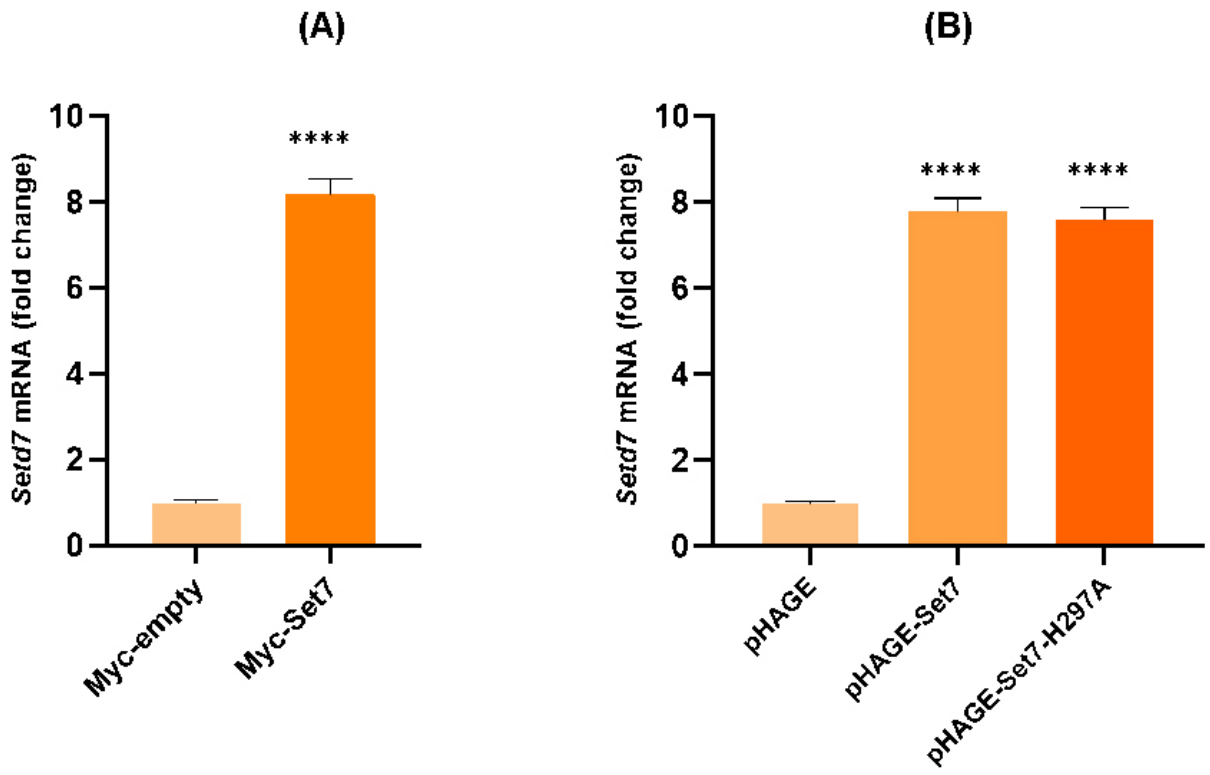

Fig. 5. Validation of Set7 overexpression in GC-2 cells. (A) GC-2 cells were transiently transfected with Myc-empty (control) or Myc-Set7 for 24 hours. *Setd7* mRNA levels were quantified by qRT-PCR and normalized to *Gapdh*. Data are presented relative to the Myc-empty control (set to 1.0). (B) GC-2 cells stably expressing pHAGE (empty vector control), pHAGE-Set7 (wild-type), or pHAGE-Set7-H297A (catalytically dead) were established by lentiviral infection. *Setd7* mRNA levels were quantified by qRT-PCR and normalized to *Gapdh*. Data are presented relative to the pHAGE control (set to 1.0). All data are shown as mean ± SD (n = 3). ****$p <$ 0.0001 vs. respective control (one-way ANOVA with Tukey's post-hoc test).

Table 3. Catalytic activity of Set7 is required for its pro-apoptotic function under hypoxia.

Cell Line	Apoptosis Rate (%)	
	Normoxia	Hypoxia
pHAGE (Control)	4.2 ± 0.8	18.5 ± 2.1
pHAGE-Set7	4.5 ± 1.1	42.3 ± 3.4****
pHAGE-Set7-H297A	4.8 ± 0.9	24.7 ± 2.2***

Note: Stable GC-2 cell lines were cultured under normoxia or hypoxia (1% O_2) for 18 hours. Apoptosis was assessed by Annexin V-FITC/PI staining and flow cytometry. Data represent the mean percentage of apoptotic cells (Annexin V+) ± SD from four independent experiments. ***$p < 0.001$, ****$p < 0.0001$ vs. pHAGE control under hypoxia; (one-way ANOVA with Tukey's post-hoc test).

cells. Critically, this pro-apoptotic function is largely dependent on Set7's methyltransferase activity. These findings position Set7 not merely as a feedback inhibitor of HIF-1α but as a decisive regulator that tips the cellular balance from adaptation towards apoptosis under hypoxic duress.

Hypoxia is a well-documented inducer of germ cell apoptosis and a key pathophysiological factor in oligospermia, a predominant cause of male infertility [3,7]. The cellular response to oxygen deprivation is orchestrated by HIF-1α, which coordinates a transcriptional program to enhance oxygen delivery and rewire metabolism towards glycolysis for survival. Our data confirm the robust activation of this program in GC-2 cells under hypoxia. However, this adaptive response has a paradoxical dimension; under severe or prolonged hypoxia, HIF-1α can also activate pro-apoptotic pathways [9,12]. Our findings reveal that Set7 intersects with this critical juncture. While Set7 is established as a negative regulator of HIF-1α transcriptional activity through lysine methylation [16,17,18], its functional outcome in germ cell fate was unknown.

We observed a significant downregulation of *Setd7* mRNA under hypoxia, which aligns with its role as a repressor of the hypoxic response. This suggests the existence of a regulatory loop where hypoxia actively suppresses its own inhibitor, thereby amplifying the HIF-1α signal, a potential mechanism to ensure a robust initial adaptive response. Consistent with its known biochemical function as

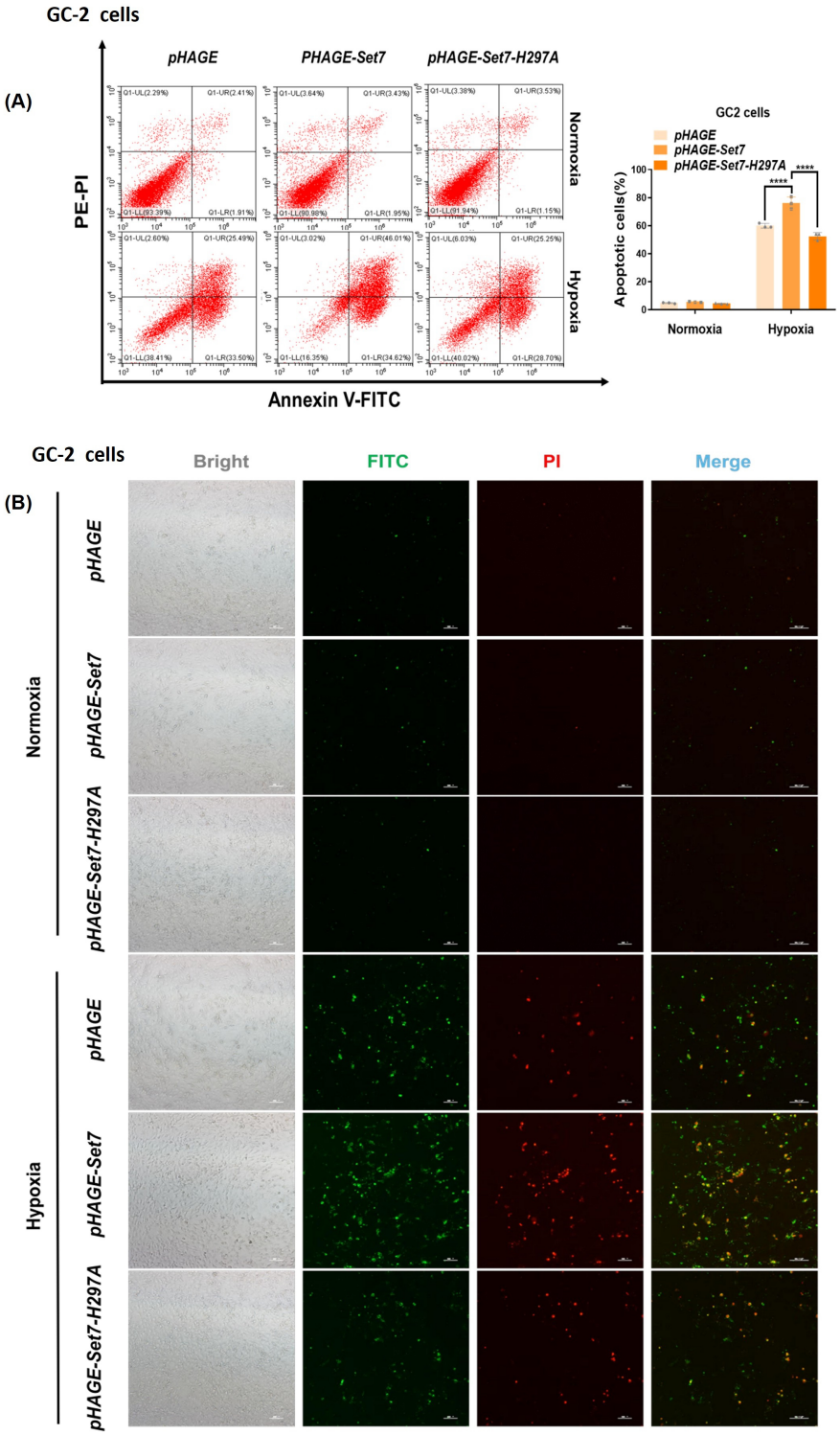

Fig. 6. Set7 potentiates hypoxia-induced apoptosis in a methyltransferase-dependent manner. (A) Stable GC-2 cell lines (pHAGE, pHAGE-Set7, pHAGE-Set7-H297A) were cultured under normoxia or hypoxia (1% O_2) for 18 hours and analyzed by flow cytometry following Annexin V-FITC/PI staining. Representative dot plots are shown (left). The right panel shows the quantitative analysis of the total apoptotic cell population (Annexin V+). Data are mean ± SD (n = 4). (B) Fluorescence microscopy images of the same cell lines and treatments stained with Annexin V-FITC (green) and PI (red). Representative images from three independent experiments are shown. Original magnification: 200×; scale bar: 50 μm. Statistical significance: ****$p < 0.0001$ (one-way ANOVA with Tukey's post-hoc test).

a methyltransferase [14,19], enforced Set7 expression effectively silenced HIF-1α-driven transcription. This repression occurred without altering HIF-1α protein stability, consistent with the notion that Set7 modulates transcriptional activity rather than protein stability, likely through the previously reported K32 methylation [16].

The central and unexpected finding is that Set7-mediated repression of the hypoxic adaptive program sensitizes GC-2 cells to apoptosis. This presents an intriguing paradox: inhibiting a pathway often associated with promoting cell survival under stress leads to increased cell death. We propose a mechanistic model to resolve this. In spermatogonial cells, the HIF-1α-mediated shift to glycolysis may be essential for maintaining energy homeostasis and viability during hypoxia [20,21]. Genes like *Glut1* and *Pgk1* are critical for survival. By dampening this essential metabolic adaptation, Set7 may precipitate a bioenergetic crisis, pushing cells past a viability threshold. This is supported by our data showing that the pro-apoptotic effect is significantly dependent on Set7's enzymatic activity. The partial effect of the Set7-H297A mutant suggests that while the catalytic activity is predominant, protein-protein interactions may also contribute minimally.

Furthermore, it is plausible that Set7 exerts its pro-apoptotic effect through additional targets beyond HIF-1α. Set7 methylates numerous non-histone proteins involved in stress and apoptosis signaling [14,15]. Under hypoxic stress, Set7 may methylate and alter the activity of such pro-apoptotic factors, creating a dominant death signal that overrides or works in concert with its inhibition of the HIF-1α pathway. While our functional data support this model, direct biochemical confirmation of specific methylation events in spermatogonial cells under hypoxia remains an important goal for future studies.

This dual-target hypothesis warrants future investigation. Our findings have important implications for understanding the etiology of male infertility. Conditions such as varicocele induce testicular hypoxia [8], and the Set7-mediated pathway identified here could be a key amplifier of hypoxic damage in the male germline, contributing to oligospermia [3].

5. Limitations and Future Directions

This study has several limitations. First, it is based on an *in vitro* model using a mouse spermatogonial cell line. While GC-2 cells are a validated model [9], the complexity of the testicular niche is not captured. Second, we have established a correlative link; future work using metabolomic approaches is needed to directly prove the bioenergetic crisis model. Third, the global landscape of Set7 methylation targets in hypoxic germ cells remains unknown. Fourth, while we demonstrate transcriptional suppression of *Setd7* under hypoxia, confirmation at the protein level was not performed in the current study. Protein-level validation would further strengthen the link between reduced mRNA and functional outcomes, and represents an important direction for future investigation. Fifth, while functional assays confirmed the activity of overexpressed Set7 constructs, direct visualization of Set7 protein levels by Western blot was not included in the current study. Future work should include protein-level validation of overexpression efficiency in this system. Sixth, while the use of the catalytically dead Set7-H297A mutant serves as a genetic rescue demonstrating the methyltransferase-dependent nature of the observed apoptosis, additional rescue strategies, such as co-expression of constitutively active HIF-1α or key target genes like *Glut1*—would further delineate the specific downstream mediators of this pathway. Given that Set7 likely has multiple targets beyond HIF-1α, such experiments represent an important direction for future mechanistic dissection. Seventh, while our functional data, particularly the repression of HIF-1α transcriptional activity without affecting its protein stability (Fig. 4) and the attenuated pro-apoptotic effect of the catalytically dead Set7-H297A mutant (Fig. 6), strongly support a methyltransferase-dependent mechanism consistent with previous reports [16], we acknowledge that direct biochemical evidence of Set7-HIF-1α interaction and methylation in GC-2 cells is not provided in the current study. Eighth, while representative Western blot images from three independent experiments are shown (Fig. 4), quantitative densitometry analysis was not performed. Visual inspection of all three independent replicates consistently showed that Set7 overexpression did not alter HIF-1α protein levels under either normoxic or hypoxic conditions. Future studies should include densitometric quantification to further validate this finding.

To address these, future studies should: (1) validate these findings *in vivo* using a testis-specific *Setd7* knockout mouse model subjected to hypoxic challenge; (2) perform chromatin immunoprecipitation sequencing (ChIP-seq) and mass spectrometry to identify genome-wide targets of Set7 under hypoxia; (3) investigate whether pharmacological inhibition of Set7 can protect germ cells from hypoxia-induced apoptosis; (4) perform Western blot analysis to confirm Set7 protein expression under hypoxic conditions in spermatogonial cells; (5) perform targeted rescue experiments, such as co-expression of constitutively active HIF-1α or its downstream targets, to identify the specific mediators of Set7's pro-apoptotic effect; and (6) employ co-immunoprecipitation and *in vitro* methylation assays in spermatogonial cells to directly confirm the Set7-HIF-1α interaction and validate K32 as the critical methylation site under hypoxic conditions; and (7) include densitometric quantification of Western blot replicates to better assess experimental variability.

6. Conclusion

This study reveals a novel and pivotal function for the methyltransferase Set7 in the cellular response to hypoxia

within the male germline. We demonstrate that hypoxia suppresses *Setd7* expression while Set7 protein acts to potently repress the HIF-1α-mediated transcriptional adaptation essential for cell survival. Paradoxically, this repression shifts the cellular fate from adaptation to apoptosis, an effect critically dependent on Set7's catalytic activity. These findings establish a key regulatory mechanism where Set7 serves as a decisive molecular switch, sensitizing spermatogonial cells to hypoxic damage. This newly defined Set7-HIF-1α-apoptosis regulatory pathway provides a candidate molecular insight into the pathogenesis of conditions like varicocele, though *in vivo* validation will be necessary to confirm its physiological relevance. Positioning Set7 as a potential therapeutic target for hypoxia-induced male infertility will require further investigation.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

YS: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. ZC: Validation, Resources, Conceptualization. JS: Writing – review & editing, Resources, Project administration. JZ: Writing – review & editing, Supervision, Resources, Methodology, Project administration, Funding acquisition, Conceptualization. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

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