Molecular Cancer Therapeutics

BRD4 Regulates EZH2 Transcription through Upregulation of C-MYC and Represents a Novel Therapeutic Target in Bladder Cancer

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Abstract

People who develop bladder cancer frequently succumb to the intractable disease. Current treatment strategies are limited presumably due to the underlying molecular complexity and insufficient comprehension. Therefore, exploration of new therapeutic targets in bladder cancer remains necessary. Here, we identify that bromodomain-4 protein (BRD4), an important epigenome reader of bromodomain and extraterminal domain (BET) family member, is a key upstream regulator of enhancer of zeste homologue 2 (*EZH2*), and represents a novel therapeutic target in bladder cancer. We found that BRD4 was significantly overexpressed in bladder cancer cells and tissues. Inhibition of *BRD4* decreased bladder cancer cell proliferation concomitantly with the accumulation of cell apoptosis *in vitro* and suppressed tumor growth *in vivo*. We further found that suppression of *BRD4* decreased the mRNA and protein levels of *EZH2*, which was reversed by ectopic expression of *C-MYC*. In particular, individual silencing of *BRD4* using shRNA or the BET inhibitor JQ1 strikingly diminished the recruitment of C-MYC to *EZH2* promoter in bladder cancer. Briefly, our research reveals that BRD4 positively regulates *EZH2* transcription through upregulation of *C-MYC*, and is a novel promising target for pharmacologic treatment in transcriptional program intervention against this intractable disease. *Mol Cancer Ther; 15(5); 1029–42. ©2016 AACR.*

Introduction

Bladder cancer, the second most common malignancy of the urinary tract, remains a virtually intractable disease worldwide (1). Although current targeted strategies ranging from transurethral resection to systemic chemotherapy are effective in a subset of patients (2–4), the overall therapeutic efficacy is still far from satisfactory, indicating the need for innovative therapeutic strategies. Inefficiency and relapse to targeted therapies in bladder cancer has been attributed to the complicated underlying molecular mechanisms that result in functional redundancy among survival pathways. In the past several decades, a series of research efforts have been reported to identify biologic characteristics and regulatory mechanisms of these tumors, with a notable progress (2–7). However, the overall genetic regulatory networks that participate in bladder cancer pathogenesis remain incompletely understood. Therefore, exploration of new gene dysregulations and novel therapeutic targets in bladder cancer would facilitate our understanding of the potential etiology and biology.

To date, alternative treatment therapies to manipulate epigenetic regulators and control the transcriptional process that sustains tumor cell identity are emerging. As a highly conserved class of proteins, bromodomain and extraterminal domain (BET) proteins are known as important epigenome readers and exert key roles in transcriptional regulation of genes in chromatin surroundings linked to acetylated histones (8, 9). The BET family is comprised of BRD2, BRD3, BRD4, and the testis-specific protein BRDT (10), which shares the common conserved N-terminal bromodomains (BD1 and BD2), and exerts diverse roles in transcription regulation through RNA polymerase II (Pol II) (11, 12). As a well-studied member of the BET bromodomain family, BRD4 has been identified as a general transcription regulator through interactions with P-TEFb, and several histone modifiers, including the histone methyltransferase NSD3 and the demethylase protein JMJD6 (8, 13–16). Furthermore, BRD4 participates in direct regulatory interactions with a series of DNA-binding transcription factors to affect their disease-relevant functions and recruits transcriptional regulatory complexes to chromatin (17). Deregulation of BRD4 protein is increasingly found in several diseases including cancer. For example, BRD4-NUT, the BRD4 translocation fusion product, is mostly responsible for aggressive mid-line carcinomas (18, 19). In addition, BRD4 is significantly overexpressed and exerts a pro-oncogenic function in melanoma (20). In colon cancer, BRD4 was reported to frequently downregulated by aberrant promoter hypermethylation and may serve as a tumor suppressor (21). However, a role

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for BRD4 protein has yet to be reported in bladder cancer, and exploration of new regulatory pathways for BRD4 would broaden our understanding of its contribution in tumor growth.

Enhancer of zeste homologue 2 (EZH2), the catalytic subunit of Polycomb repressive complex 2 (PRC2), is significantly overexpressed in bladder cancer and plays a crucial role on tumor growth according to previous researches (22–24). Inhibition of *EZH2* induced cell-cycle arrest and cell apoptosis. Our recent work has revealed the upstream regulation of *EZH2* at the posttranscriptional level by miR-101 (23). In contrast, despite their significant relevance, the transcriptional regulatory mechanism that results in pro-oncogenic function of EZH2 in bladder cancer is still largely unknown. Thus, in this study, we sought to explore the transcriptional regulation of *EZH2* in bladder cancer.

Collectively, in our study, we assessed the expression levels and potential role of BRD4 in bladder cancer and evaluated the underlying effect of pharmacologically inhibiting BRD4 protein in bladder cancer cells *in vitro* and *in vivo*. We identify the significant effect of BRD4 inhibitor on cell proliferation, cell apoptosis, and tumor growth in bladder cancer. More importantly, our research reveals BRD4 could positively regulate *EZH2* transcription through upregulation of *C-MYC*, and is a novel promising target for pharmacologic treatment in transcriptional program intervention against this intractable disease.

Table 1. Correlation between BRD4 expression and clinicopathologic factors in bladder cancer

			BRD4 Expression		
Factors	**Group**	**Total**	**Low**	**High**	***P***
Gender	Male	39	11	28	0.533
	Female	16	6	10	
Age (years)	<55	12	4	8	1.000
	≥55	43	13	30	
Grade	LGPUC	22	14	8	0.000[a]
	HGUC	33	3	30	
Tumor stage	Ta, Tis, T1	16	7	9	0.213
	T2, T3, T4	39	10	29	
Tumor size	<3.0 cm	26	7	19	0.574
	>3.0 cm	29	10	19	
Lymph node metastasis	Absent	29	13	16	0.022[a]
	Present	26	4	22	
Distant metastasis	Absent	48	14	34	0.664
	Present	7	3	4	
Tumor multiplicity	Unifocal	30	11	19	0.386
	Multifocal	25	6	19	

[a]$P < 0.05$.

Materials and Methods

Cell lines and patient tissue specimens

The human bladder cancer cell lines EJ and T24 as well as normal human urothelial cells SV-HUC-1 were obtained from ATCC (23). Cell line identities were determined by DNA fingerprinting through AmpFlSTR Identifiler Amplification Kit (Applied Biosystems) protocols. Cells were cultured in RPMI1640 medium (Gibco) containing 10% FBS in the recommended media at 37°C supplied with 5% CO_2. Fifty-five pairs of fresh bladder cancer tissues and surrounding normal adjacent bladder tissues were selected from patients who underwent partial or radical cystectomy for urothelial carcinomas of bladder at Department of Urology of the Union Hospital of Tongji Medical College (Wuhan, China) between 2013 and 2014. Pathologic and histologic diagnoses were assessed by at least two pathologists. The specimens were classified or reclassified according to the 2004 World Health Organization Consensus Classification and Staging System. Clinicopathologic characteristics in this study are presented in Table 1. All specimens were divided into fragments and snap-frozen in liquid nitrogen immediately after surgical resection. Approval for the research was received from the Institutional Review Board of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China), and appropriate informed consent was obtained from the patients before surgery.

Plasmid construction and transfection

The shRNAs targeting *BRD4*, *C-MYC*, and *EZH2* were designed and synthesized by Genechem. Ectopic vector targeting *C-MYC* and *EZH2* were obtained from Genechem, the pcDNA4c hBrd4 full-length construct was obtained from Addgene (p14441). The sequences of shRNAs and plasmid backbones were presented in Supplementary Table S1 and Supplementary Figs. S1–S3. Twenty-four hours prior to plasmid transfection, cells were cultured in a 6-well plate (Corning) with 40%–60% confluence. Lipofectamine 2000 (Invitrogen) was used for plasmid transfection according to the manufacturer's instructions. Cells were cultured with new culture medium 4 to 6 hours after transfection. Stable cell lines were screened by the treatment with puromycin (Invitrogen).

RT-PCR analysis

Total RNA was isolated from tissues and cell lines with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Complementary DNA was synthesized using random primers and the reverse transcription kit PrimeScript RT reagent Kit (Takara Biomedical Technology). Quantitative real-time PCR (qPCR) analysis was determined using the SYBR Premix Ex Taq (Tli RNaseH Plus) kit (Takara Biomedical Technology). The primer set for *BRD4* was 5′-GTGGGAGGAA AGAAACAGGGACA-3′ (forward) and 5′-AGGAGGAGGATTCGGCTGAGG-3′ (reverse).The primer set for *C-MYC* was 5′-AGGGATCGCGCTGAGTATAA-3′ (forward) and 5′-TGCCTCTCGCTGGAATTACT-3′ (reverse). The primer set for *EZH2* was 5′-CCCTGACCTCTGTCTTACT-TGTGGA-3′ (forward) and 5′-ACGT CAGATGGTGCCAGCAATA-3′ (reverse). *GAPDH* was used as an internal standard with primer 5′-TCAAGAAGGTGGTGAAGCAG-3′ (forward) and 5′-CGTCAAAG GTGGAGGAGTG-3′ (reverse). The PCR amplification was performed for 40 cycles of 95°C for 5 seconds, 60°C for 30 seconds, and melting curve reaction was performed at the end. All data analyses were operated using the StepOnePlus Real-Time PCR System (Applied Biosystems). The $\Delta\Delta C_t$ method was used to assess the relative expression of different candidate genes, and the C_t values for *GAPDH* were exhibited in Supplementary Table S2.

Western blotting analysis

Tissues and cell lines were collected and lysed in RIPA (Thermo Scientific) buffer (1×) supplemented with protease inhibitor cocktail (Beyotime Institute of Biotechnology). The concentration of protein samples was detected using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Equal amounts of lysates were separated by 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1 hour in Tris-buffered saline (TBS) containing 5% nonfat milk and probed with primary antibodies (at a dilution of 1:1,000) at 4°C for 12 hours [rabbit anti-human BRD4 (Abcam, ab128874),

rabbit anti-human C-MYC (Abcam, ab39688); rabbit anti-human GAPDH (D16H11), rabbit anti-human EZH2 (D2C9) (Cell Signaling Technology Inc.)], and then incubated with the specific HRP-conjugated secondary antibody (Wuhan Boster Bioengineering Limited Company) for 2 hours before developing with the ECL kit (Beyotime Institute of Biotechnology). Data analysis was performed using ImageJ Software to evaluate the expression levels of proteins.

IHC

Immunostaining was performed on bladder cancer tissue sections that had been defined for the pathologic pattern by the pathologists. The avidin–biotin–peroxidase method was used to evaluate the location and relative expression of the target gene. The primary antibodies of BRD4, EZH2, and C-MYC were used at a dilution of 1:200, respectively. Ki-67 and TUNEL kit were used to evaluate proliferation and apoptosis of tissues. An Olympus microscope was used to collect and analyses images.

Cell viability by MTT assay

Cell viability was detected by the MTT colorimetric assay. Cells (5,000 cells/100 μL/well) were plated in 96-well microplates. After drug treatment or transfection, 20 μL MTT solutions were added to each well and incubated at 37°C in the dark for additional 4 hours. The generated formazan optical density (OD) was detected at 570 nm to determine the cell viability. All experiments were repeated at least three times.

Flow cytometry assays for the cell cycle and apoptosis

EJ and T24 cells were transfected with the corresponding plasmid vectors or treated with chemical compounds. After 72 hours, cells were harvested and stained with propidium iodide buffer (Sigma) for cell-cycle analysis. The ModFit LT software was applied to analyze the results. The Annexin V-PI and PE Annexin V apoptosis detection kits (BD Pharmingen) were used for cell apoptosis analysis according to the manufacturer's instructions.

EdU assay

EJ and T24 cells were seeded at an appropriate density in 96-well plates. Cells were transfected with corresponding plasmid vectors or treated with chemical compounds. At the indicated time points, 5-ethynyl-20-deoxyuridine assay (EdU; Cell Light EdU DNA imaging Kit, Ribo Bio) was used to assess cell proliferation viability. EdU (100 μmol/L) was added to the medium and incubated with the cells for 2 hours. Then, the images were collected and analyzed with a microscope (Olympus). Edu-stained cells were visualized with red fluorescence and Hoechst-stained cells were visualized with blue fluorescence. The rate of EdU-stained cells/Hoechst-stained cells was used to evaluate the cell proliferation activity.

Tumor xenografts and *in vivo* treatment

All procedures for the mouse experiments were approved by the Animal Care Committee of Tongji Medical College (Wuhan, China). EJ cells (1×10^6) were injected into 4-week-old nude mice suspended in 100-μL PBS to achieve tumor growth. Once the tumor volumes reached a palpable stage (100 mm^3), the animals were randomly divided into two groups ($n = 10$ per group) for JQ1 (Selleckchem) treatment trials. A stock of JQ1 in DMSO was diluted by dropwise addition of a 10% 2-hydroxypropyl-b-cyclodextrin carrier (Sigma), yielding a 5 mg/mL final solution. The animals were treated daily intraperitoneally with either freshly diluted JQ1 (50 mg/kg) or an equal volume of vehicle containing 5% DMSO. For the shRNA experiments, stable EJ cells (1×10^6) transfected with *BRD4* shRNA or negative control screened by puromycin were injected into mice ($n = 10$ per group).Tumor volume was measured in two dimensions every three days using Vernier calipers and calculated with the following equation: length $\times$ width^2 $\times$ 0.5. Three weeks later, the animals were killed and weighed simultaneously. No animal was killed or dead before the conclusion of the experiment. The tumors were excised, photographed, and weighed. Afterwards, tumor tissue sections obtained from the *in vivo* experiments were collected for immunohistochemical staining.

Luciferase reporter assays

The *EZH2* promoter reporter vector was designed and synthesized by Genechem. The reporter promoters were transiently transfected along with a *Renilla* control plasmid, concomitantly either with *BRD4* shRNA, *C-MYC* shRNA, ectopic vector, or nontargeting shRNAs. Luciferase activity was detected 24 hours after the transfection following the Dual-Luciferase Reporter Assay System (Promega). At least three independent experiments were performed, with three replicates for each condition.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed using the EZ-ChIP kit (Upstate Biotechnology). Chromatin DNA was immunoprecipitated with corresponding antibody or normal IgG, washed, and the DNA-protein cross-links were subsequently reversed. PCR primers for the *C-MYC* and *EZH2* promoter were designed with the Premier Primer 5.0 software to synthesize the corresponding regions. The primers for *C-MYC* promoter were 5′- TGCCTCTATCATTCCTCCCTAT-3′ and 5′-AAACCGCATCCTTGTCCT-3′. The primers for *EZH2* promoter were 5′-GACACGTGCTTAGAACTACGAACAG-3′ and 5′-TTTGG-CTGGCCGAGCTT-3′. The recovered DNA was assessed by PCR analysis. The relative amount of immunoprecipitated DNA was evaluated by generating a standard and normalized against the negative control.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software (SPSS Inc.) and with GraphPad Prism Version 5 (GraphPad Software). Statistically significant differences ($P < 0.05$) were determined by Student t test or ANOVA, presented as means $\pm$ SD. The χ^2 test was used to evaluate the differences in clinicopathologic features and IHC.

Results

BRD4 is overexpressed in bladder cancer tissues and cell lines

To evaluate the expression level of BRD4 in bladder cancer, we collected 55 pairs of primary urothelial carcinoma of bladder samples and surrounding normal bladder tissues. As shown in Fig. 1A and B, both *BRD4* mRNA and protein levels were significantly upregulated in urothelial carcinoma of bladder tissues as compared with that in corresponding normal bladder tissues ($P < 0.05$). A significant relationship was detected between high BRD4 expression and high cancer grade ($P < 0.05$) and the high lymph node metastasis ($P < 0.05$, Table 1). High levels of BRD4 were also detected in EJ and T24 cells as compared with

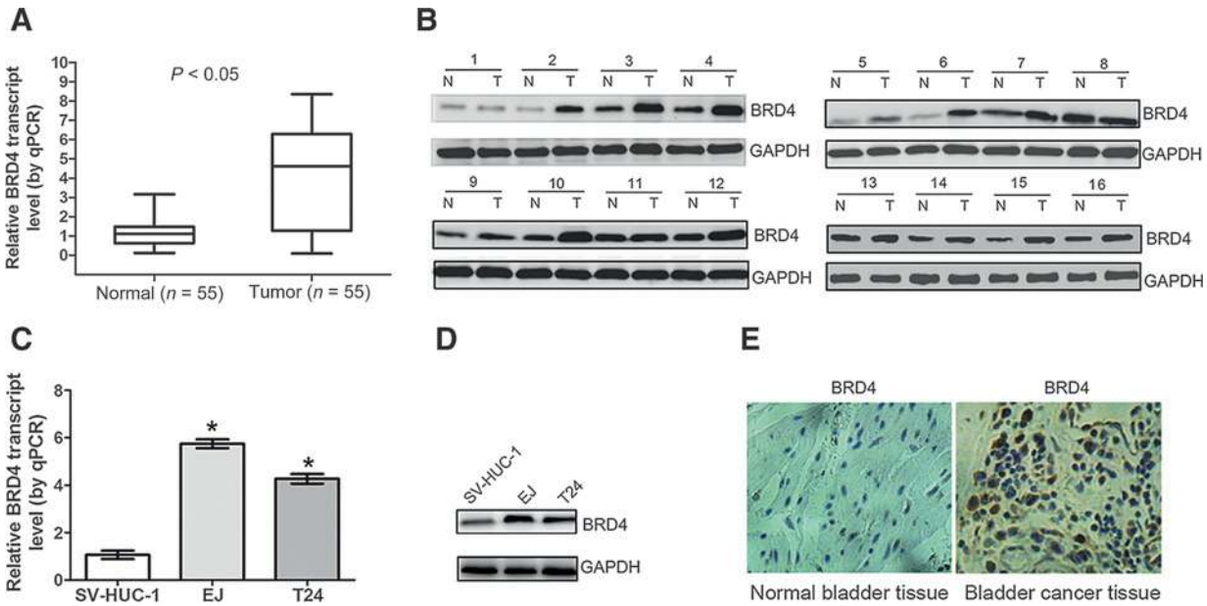

Figure 1.
BRD4 is overexpressed in bladder cancer. The expression levels of *BRD4* mRNA (A) and protein (B) in human bladder cancer samples are shown. The mRNA (C) and protein (D) levels of *BRD4* in SV-HUC-1, EJ, and T24 cells are exhibited. BRD4 expression in bladder cancer samples and surrounding normal bladder tissues were detected by IHC (400×; E). *, $P < 0.05$ compared with SV-HUC-1 cells.

SV-HUC-1 cells (Fig. 1C–D). IHC indicated that BRD4 was mainly accumulated in the cell nucleus of malignant cells, and the expression of BRD4 in urothelial carcinoma of bladder was strikingly higher than in the normal tissues (Fig. 1E). These results revealed a potential role of BRD4 protein in promoting bladder cancer progression.

Inhibition of BRD4 attenuates proliferation and induces apoptosis of bladder cancer cells

Given that BRD4 is overexpressed in bladder cancer tissues and cell lines in our study, we further explored whether inhibition of *BRD4* could affect bladder cancer cell biologic activity. Four shRNAs targeting the coding region of *BRD4* (*shBRD4*) were designed, and the pcDNA4c hBrd4 full-length construct was obtained from Addgene. As shown in Fig. 2A–B, both the mRNA and protein expression levels of *BRD4* were significantly silenced in EJ and T24 cells after transfection of shRNAs. The protein level of *BRD4* was markedly enhanced upon pcDNA4c hBrd4 full-length transfection. We ultimately selected *shBRD4-2* and *shBRD4-3* for further studies owing to the better knockdown effects. The MTT colorimetric assay exhibited that knockdown of *BRD4* or JQ1 treatment decreased cell viability in bladder cancer cells. Furthermore, JQ1 inhibited cell viability in a dose- and time-dependent manner (Fig. 2C). Meanwhile, the cell-cycle assay showed that *BRD4* knockdown or treatment with JQ1 induced cell-cycle arrest at G_0–G_1 phase in EJ and T24 cells compared with negative control (Fig. 2D). In addition, there was a further increase in cell-cycle arrest in *BRD4* knockdown cells by JQ1 treatment, but the relative increase level was lower compared with shNC cells treated by JQ1 in T24 cells (Fig. 2E). The sensitivity of the bladder cancer cells responded to JQ1 was enhanced upon *BRD4* overexpression (Fig. 2F). Consistently, the EdU assay demonstrated that suppression of *BRD4* inhibited proliferation of EJ and T24 cells (Fig. 3A–D). On the other hand, JQ1 treatment also induced considerable cell apoptosis in EJ and T24 cells (Fig. 3E–F). These observations demonstrated that inhibition of *BRD4* using shRNAs or treatment with JQ1 could suppress cell biologic activity in EJ and T24 cells, indicating the protumor function for BRD4 in bladder cancer.

Inhibition of BRD4 suppresses *EZH2* transcription in bladder cancer cells

Our previous work and several other studies have demonstrated that EZH2 is an essential factor in regulating cell biologic activity of bladder cancer. To investigate whether BRD4 influences the expression level of *EZH2*, we first detected the expression levels of *EZH2* and evaluated the potential correlation between BRD4 and EZH2 in bladder cancer tissues. As shown in Fig. 4A and B, the mRNA and protein levels of *EZH2* were upregulated in bladder cancer tissues. IHC further supported the above results (Fig. 4C). In addition, the expression of EZH2 was positively correlated with BRD4 (Figure 4D). S*hBRD4-2* and *shBRD4-3* were subsequently transfected into EJ and T24 cells. As shown in Fig. 4E and F, both *shBRD4-2* and *shBRD4-3* significantly downregulated *EZH2* mRNA and protein levels, when compared with negative control. The mRNA and protein levels of *EZH2*, but not *BRD4*, were significantly decreased upon JQ1 treatment (Fig. 4G and I). Furthermore, transfection of shBRD4 or JQ1 treatment resulted in decreased promoter activity of *EZH2* evaluated by luciferase reporter system in EJ and T24 cells (Fig. 4J and K). Meanwhile, ectopic expression of *EZH2* reversed cell-cycle arrest and cell apoptosis induced by *BRD4* inhibition (Figs. 4L and M and 5A, B, D). Knockdown of *EZH2* further strengthened cell-cycle arrest upon JQ1 treatment, but the extent of increase was smaller compared with shNC cells treated by JQ1 (Fig. 5C). These results proved

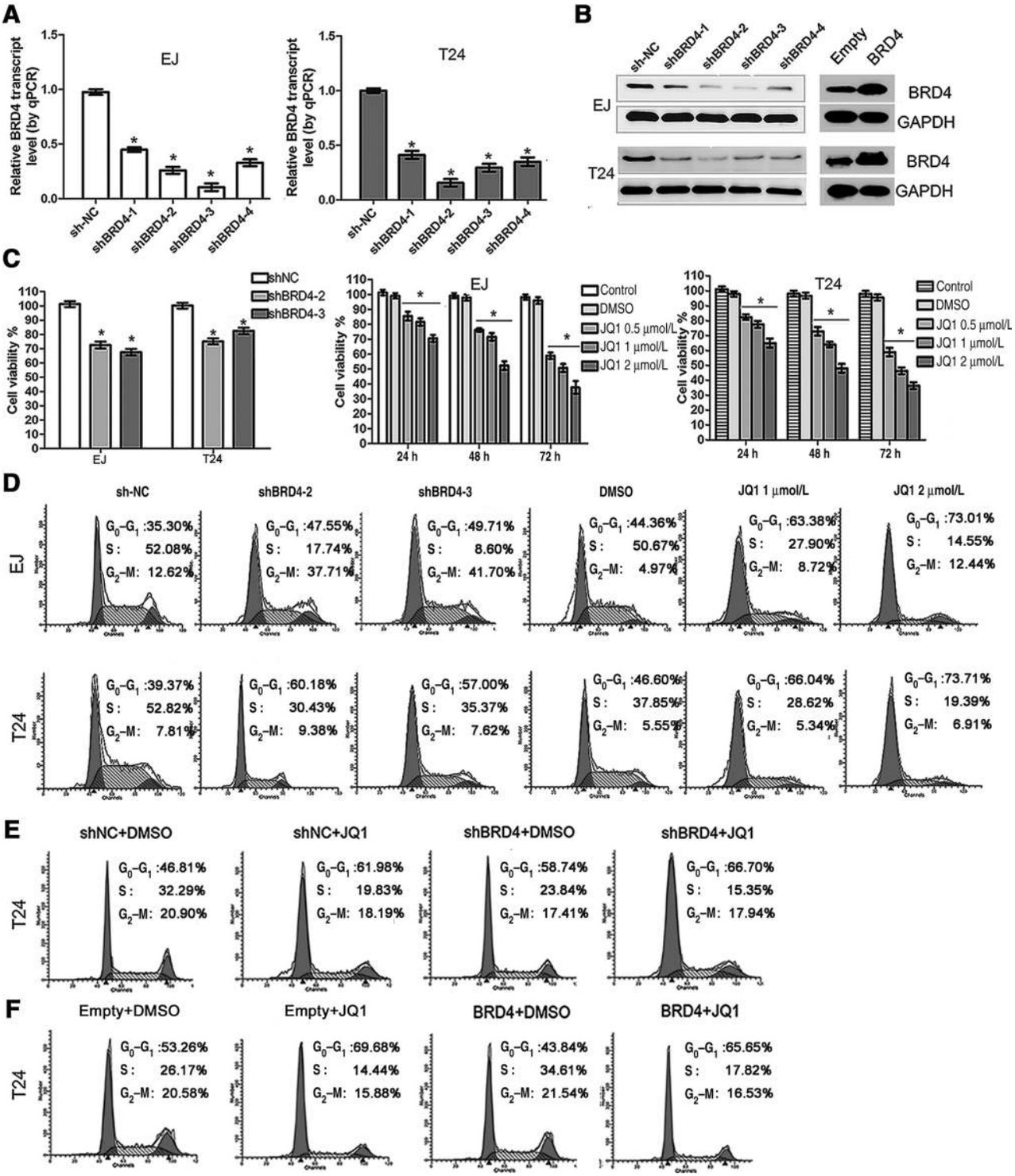

Figure 2.

Inhibition of BRD4 suppresses cell viability and induces cell-cycle arrest in bladder cancer. The expression levels of *BRD4* mRNA (A) and protein (B) after transfected with four *BRD4* shRNAs or pcDNA4c hBrd4 full-length construct in EJ and T24 cells were evaluated. Forty-eight hours after *shBRD4* transfection and 24, 48, and 72 hours after JQ1 (0.5 μmol/L, 1 μmol/L, 2 μmol/L) treatment, MTT assays were performed to evaluate the cell viability (C). Flow cytometry showed that *BRD4* shRNAs and JQ1 resulted in G_0–G_1 arrest in both EJ and T24 cells (D). The cell-cycle distributions in *BRD4* knockdown or overexpression cells upon JQ1 treatment are exhibited (E and F). *, $P < 0.05$ compared with respective control.

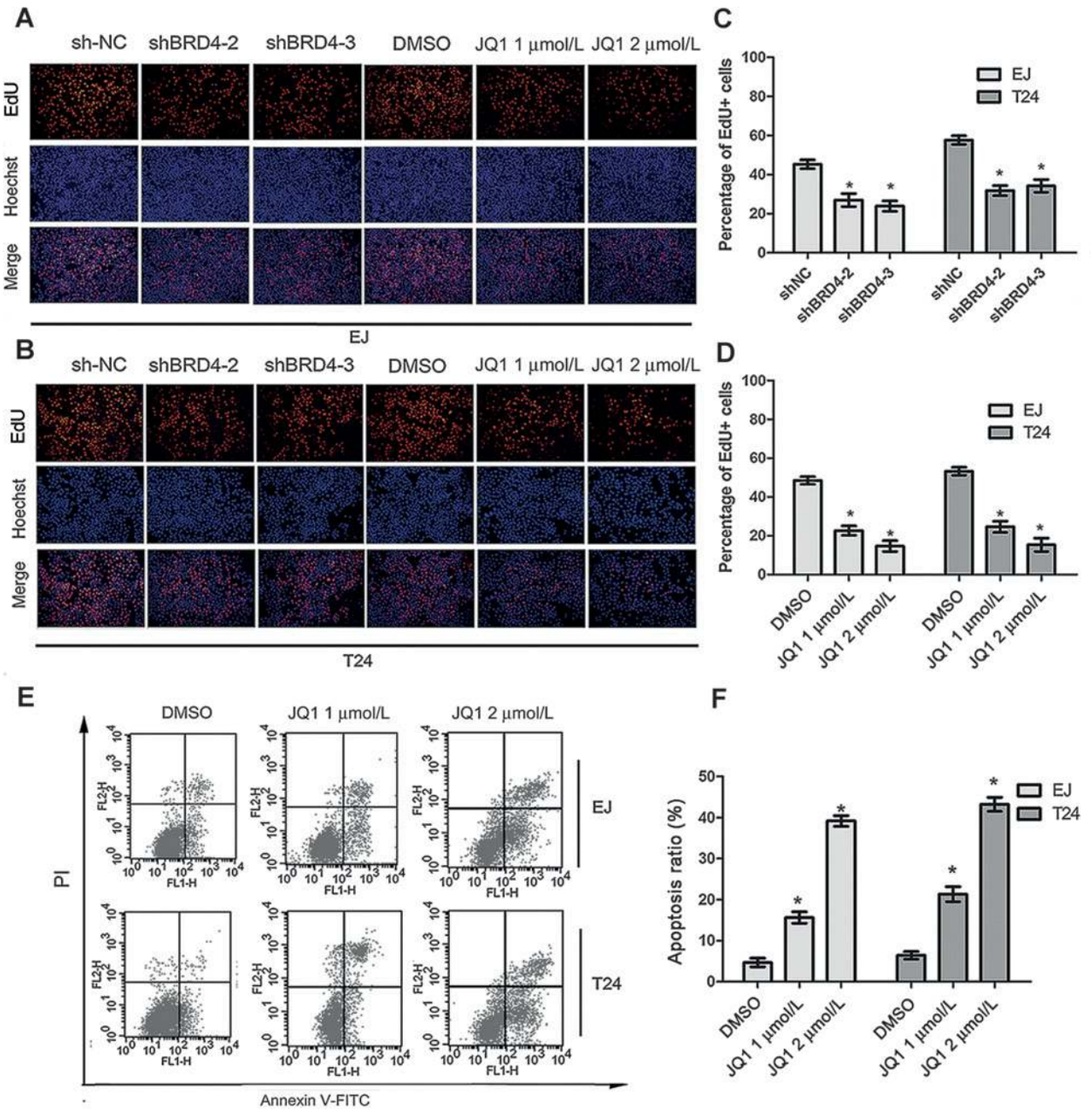

Figure 3.
Inhibition of BRD4 suppresses cell proliferation and promotes cell apoptosis in bladder cancer. EdU assay shows that *BRD4* shRNAs and JQ1 inhibited proliferation of EJ and T24 cells (A–D). Annexin V and PI staining flow cytometry assay show that JQ1 treatment increased the rate of apoptosis in EJ and T24 cells (E and F). *, $P < 0.05$ compared with respective control.

that BRD4 could positively regulate *EZH2* transcription in bladder cancer.

BRD4 regulates transcriptional expression of *EZH2* through *C-MYC* in bladder cancer *in vitro*

Next, we sought to elucidate the underlying mechanism of EZH2 regulated by BRD4 at transcriptional level. We found that C-MYC was also upregulated in bladder cancers (Fig. 6A and C), and C-MYC expression was positively correlated with BRD4 (Fig. 6D). The expression levels of *C-MYC* mRNA and protein were decreased in EJ and T24 cells upon transfection of *shBRD4* or treatment with JQ1, as evaluated by RT-PCR and Western blot analysis (Fig. 6E and G). Enforced expression of *C-MYC* partly reversed cell apoptosis induced by JQ1, and the efficiency is similar to *EZH2* overexpression (Fig. 6H and I). Meanwhile, knockdown of *C-MYC* with shRNA significantly decreased the levels of *EZH2* mRNA and protein, and ectopic expression of *C-MYC* upregulated *EZH2* expression in EJ and T24 cells

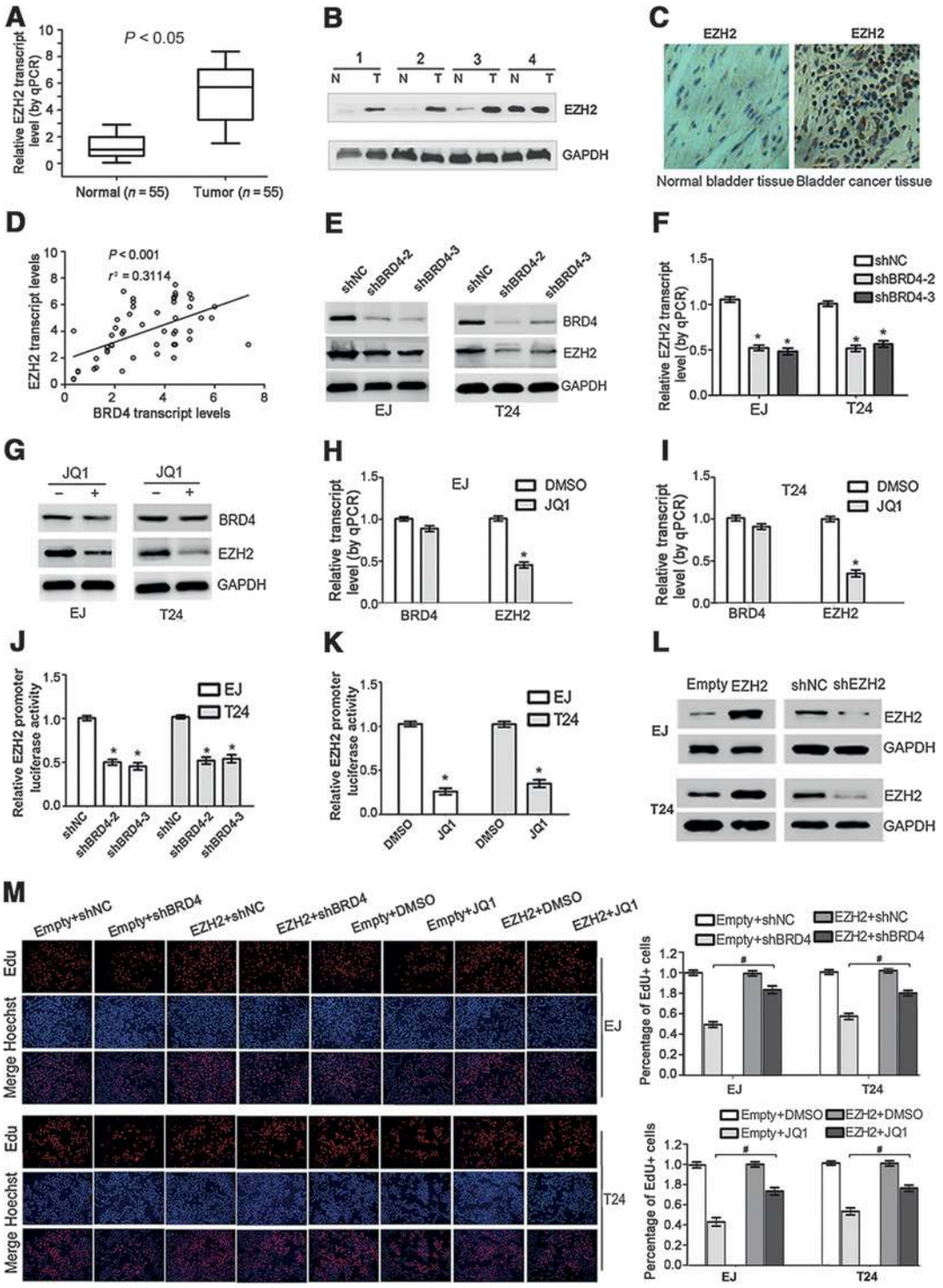

Figure 4.

Inhibition of BRD4 results in downregulation of EZH2 expression and ectopic expression of EZH2 reverses cell proliferation suppression induced by BRD4 knockdown or JQ1 treatment. The mRNA level of *EZH2* in normal bladder (N) and paired cancerous (T) tissues was detected by qPCR (A), and the protein level was evaluated by Western blotting (B) and IHC (C), respectively. The linear regression between the expression of *BRD4* mRNA and EZH2 was analyzed (D). Knockdown of *BRD4* decreased the expression levels of *EZH2* protein (E) and mRNA (F) in EJ and T24 cells. JQ1 (1 μmol/L) treatment decreased *EZH2* protein (G) and mRNA (H and I) expression but not *BRD4* protein (G) and mRNA (H and I) expression. *BRD4* shRNAs and JQ1 (1 μmol/L) repressed *EZH2* promoter activity in EJ and T24 cells (J and K). The protein levels of *EZH2* were significantly changed upon *EZH2* knockdown or overexpression (L). Ectopic expression of *EZH2* reversed *BRD4* shRNAs and JQ1 (1 μmol/L) induced cell proliferation suppression (M). *, $P < 0.05$ compared with respective control; #, $P < 0.05$ compared with empty vector group.

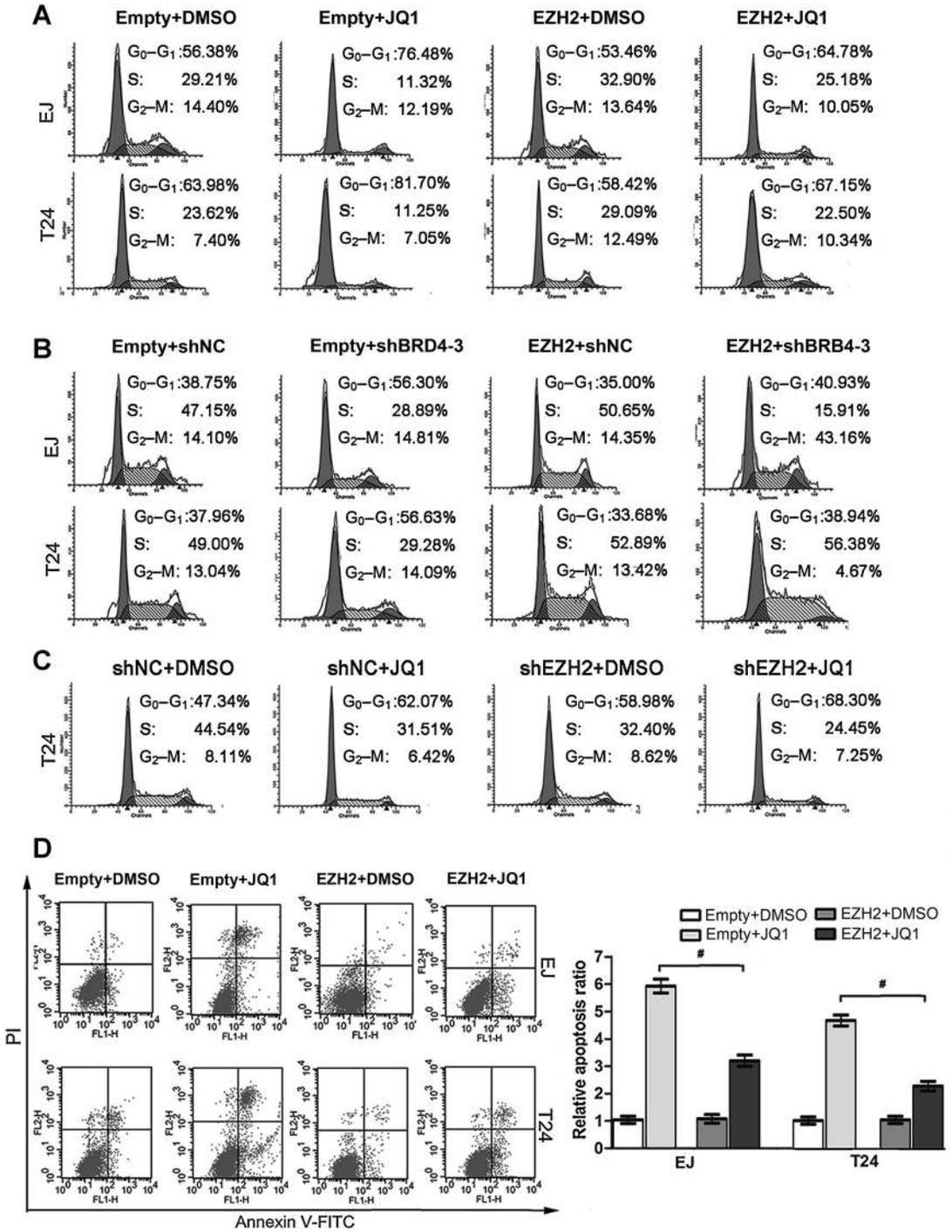

Figure 5.

Ectopic expression of EZH2 reverses cell-cycle arrest and cell apoptosis induced by BRD4 inhibition. Ectopic expression of *EZH2* reversed *BRD4* inhibition induced cell-cycle arrest (A and B). Knockdown of *EZH2* further strengthened cell-cycle arrest upon JQ1 treatment, but the extent of increase was smaller compared with shNC cells treated by JQ1 (C). Cell apoptosis induced by JQ1 (1 μmol/L) was reversed by ectopic expression of *EZH2* in EJ and T24 cells (D). #, $P < 0.05$ compared with empty vector group.

(Fig. 6J and L). Importantly, ectopic expression of *C-MYC* efficiently reversed the suppression of *EZH2* protein and mRNA levels induced by JQ1 and *BRD4* shRNA (Fig. 7A and D). Furthermore, as shown in Fig. 7E and H, ectopic expression of *C-MYC* also reversed the attenuation of *EZH2* promoter activity upon *BRD4* inhibition. These results demonstrated that BRD4 positively regulated *EZH2* transcription through upregulation of *C-MYC* in bladder cancer cells.

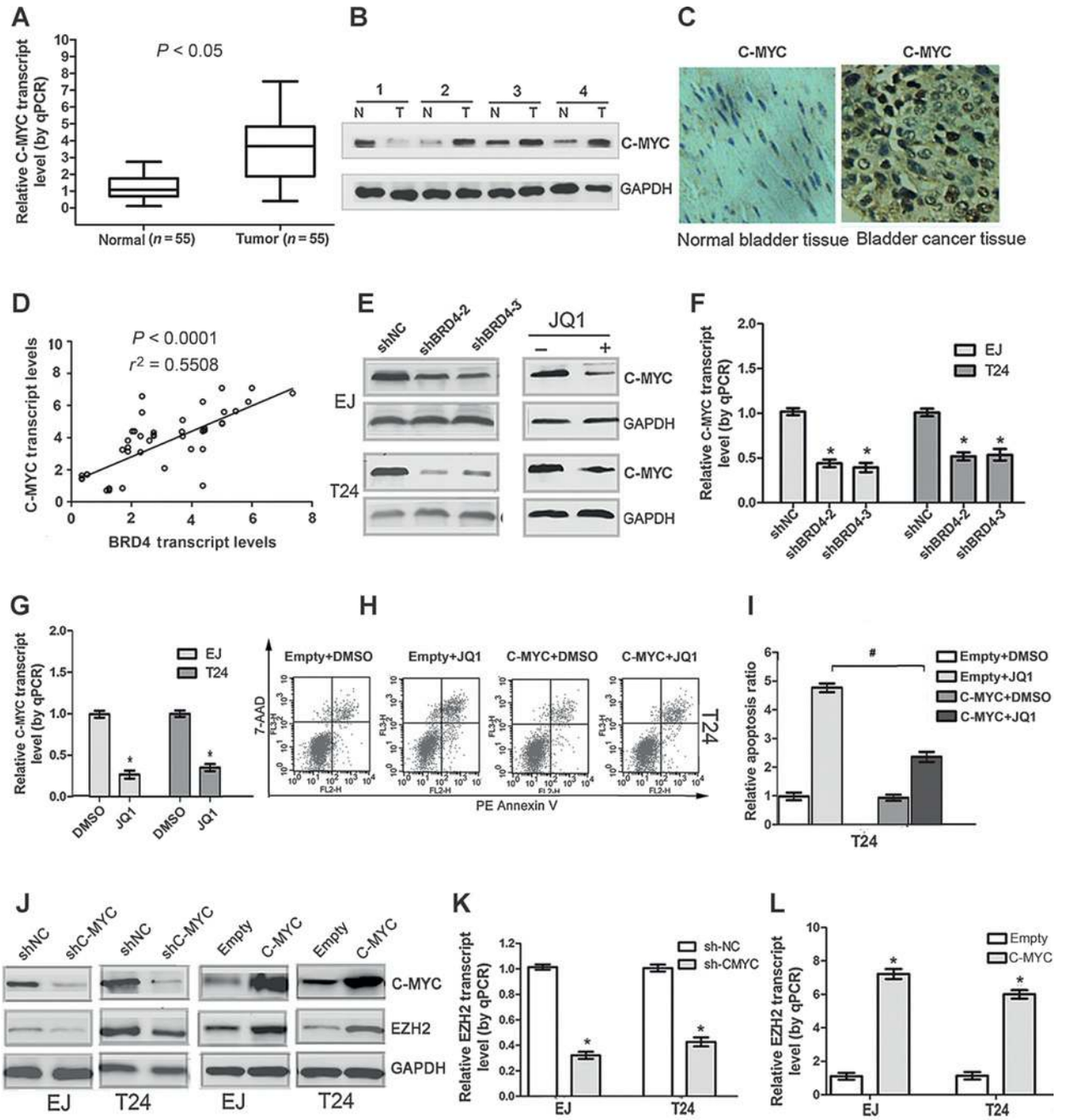

Figure 6.
Inhibition of BRD4 suppresses C-MYC expression and C-MYC enhances EZH2 expression in bladder cancer cells. The mRNA level of *C-MYC* in normal bladder (N) and paired cancerous (T) tissues was detected by qPCR (A). Meanwhile, the protein level of *C-MYC* was evaluated by Western blotting (B) and IHC (C) respectively. The linear regression between the expression of BRD4 mRNA and C-MYC was analyzed (D). The effects of *shBRD4* and JQ1 (1 μmol/L) on *C-MYC* expression were confirmed through Western blotting (E) and RT-PCR (F and G) respectively. Enforced expression of C-*MYC* partly reversed cell apoptosis induced by JQ1 treatment (H and I). Then, the effects of *shC-MYC* and ectopic expression *C-MYC* on *EZH2* expression were evaluated (J–L). *, $P < 0.05$ compared with respective control; #, $P < 0.05$ compared with empty vector group.

Inhibition of BRD4 attenuates the recruitment of C-MYC to *EZH2* promoter in bladder cancer

It has been reported that C-MYC could bind directly to *EZH2* promoter to promote its transcription. To further explore the role of C-MYC in mediating transcriptional regulation of *EZH2* by BRD4, ChIP experiments were performed subsequently. We found that BRD4 was enriched in the *C-MYC* promoter region, and there was no obvious recruitment of BRD4 to detect *EZH2*

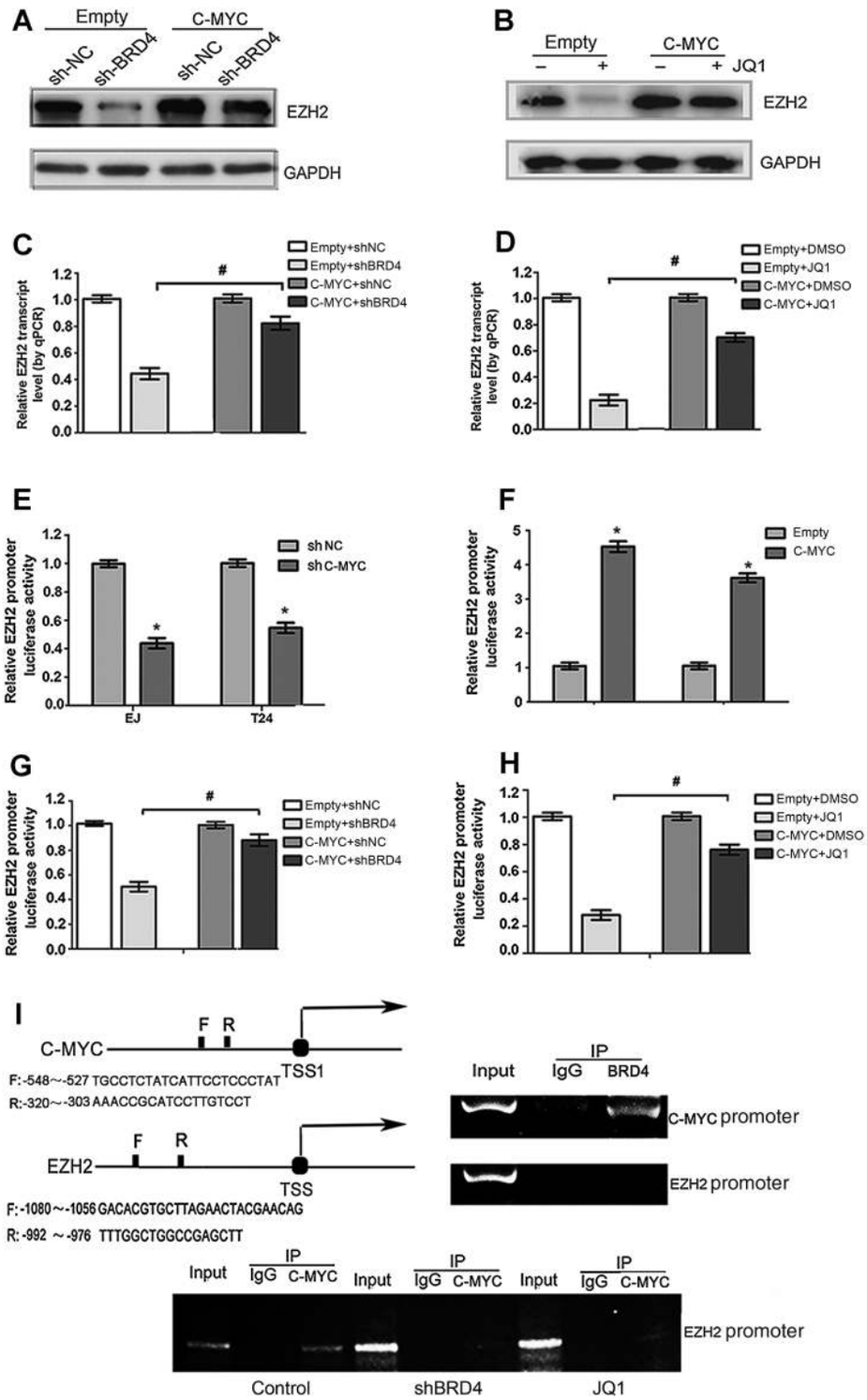

Figure 7.
BRD4 regulates EZH2 transcription through upregulating C-MYC expression in bladder cancer cells. Ectopic expression of *C-MYC* reversed the suppression of *EZH2* protein (A and B) and mRNA (C and D) levels induced by JQ1 and *BRD4* shRNA. Knockdown of *C-MYC* suppressed *EZH2* promoter activity (E), while ectopic expression of *C-MYC* enhanced *EZH2* promoter activity (F). Ectopic expression of *C-MYC* reversed the attenuation of *EZH2* promoter activity induced by *shBRD4* and JQ1 (1 μmol/L; G and H). The location of primers for ChIP experiments is shown (I). ChIP experiments demonstrated that BRD4 could be recruited to *C-MYC* promoter, and there was no obvious enrichment of BRD4 to detected *EZH2* promoter region (I). *, $P < 0.05$ compared with respective control; #, $P < 0.05$ compared with empty vector group.

promoter region in bladder cancer cells (Fig. 7I). ChIP experiments also showed that transfection of *BRD4* shRNA or JQ1 treatment resulted in considerable loss of C-MYC recruitment to *EZH2* promoter region (Fig. 7I). These data suggest that BRD4 could probably interact with *C-MYC* promoter to promote its expression, and subsequently enhance the direct binding of C-MYC to *EZH2* promoter, which result in the upregulation of *EZH2* transcription.

Suppression of BRD4 impairs bladder cancer tumor growth *in vivo*

To evaluate the antitumor potential of *BRD4* inhibition *in vivo*, we detected the effects of *BRD4* shRNA and JQ1 in a xenograft mouse model. JQ1-treated mice displayed a considerable reduction in tumor weight and tumor volume at the end of the experiment ($P < 0.05$, Fig. 8A, C, and E). Stable EJ cells transfected with control or *shBRD4* constructs were injected into the flanks of mice for shRNA experiments. S*hBRD4*-injected mice showed a reduction in tumor weight and tumor volume at the end compared with their control groups ($P < 0.05$, Fig. 8A, B, and D). Moreover, inhibition of *BRD4* suppressed proliferation and promoted apoptosis in tumors (Fig. 8F). Meanwhile, we did not find any distal and lymph node metastatic in control or *BRD4* inhibition groups. Histologic analysis of tumors suggested positive correlations between BRD4 and C-MYC as well as EZH2

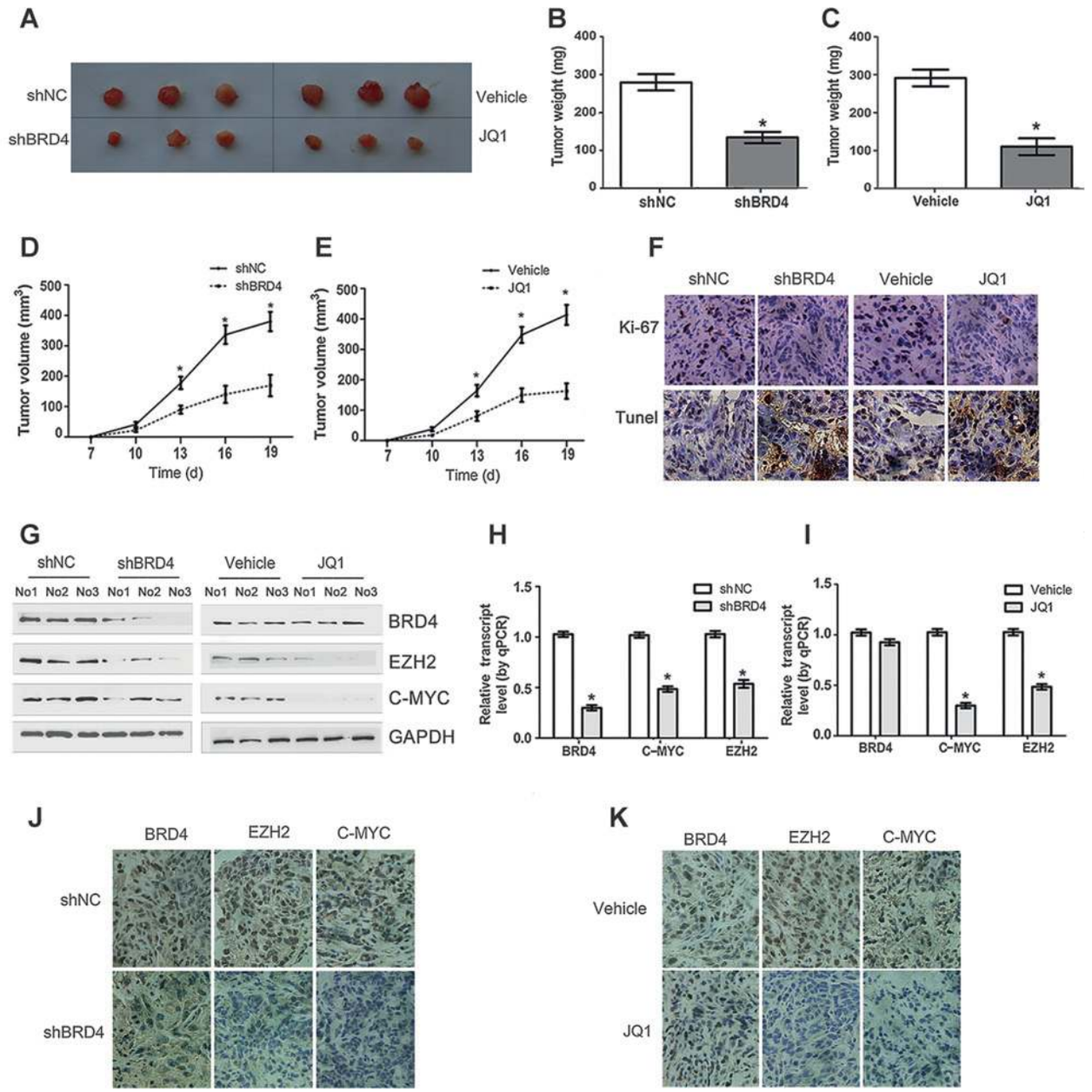

Figure 8.

Suppression of BRD4 impairs tumor growth in bladder cancer *in vivo*. Tumors collected from mice are exhibited (A). Tumor weight of mouse was measured after *BRD4* shRNA (B) and JQ1 (C) treatment. Tumor volume curve of mouse upon *BRD4* shRNA (D) and JQ1 (E) treatment was analyzed. Immunohistochemical staining of Ki-67 was used to assess proliferation, and apoptosis was detected using TUNEL kit (F). The mRNA and protein levels of *BRD4, EZH2* and *C-MYC* in tumors (G–I) were assessed. Immunohistochemical analyses of BRD4, EZH2, and C-MYC were obtained from tumors (400×; J and K). *, $P < 0.05$ compared with respective control.

expression in both control and *BRD4* inhibition cohorts (Fig. 8G and K), which was consistent with the data obtained from clinical specimens. These results further verified the role of BRD4 in promoting tumor growth and provided more evidence for therapeutic strategy targeting *BRD4* in bladder cancer treatment.

Discussion

Current treatment strategies for bladder cancer present limited efficacy in delaying tumor progression and recurrence. Approximately 50% to 70% of patients with superficial bladder cancer will recur after administration of transurethral resection followed with or without intravesical chemotherapy (25), and a considerable proportion of carcinoma *in situ* probably develop invasive bladder cancer within several years (26). To date, the significant impact of gene dysregulation on tumorigenesis and progression in bladder cancer are gradually disclosed and are currently the focus subject of investigation, and alternative therapeutic strategies targeting gene aberration are necessary to be explored to improve the survival of patients with bladder cancer.

As well-known conserved epigenome readers, the BET proteins link to chromatin remodeling, and function as transcription regulators in a context-dependent manner (8). The important BET family member BRD4 has been demonstrated to possess significant effect on cell biologic activity. Recently, several selective small-molecule chemical compounds that target the acetyl-lysine binding of BET proteins have been developed to exhibit the antitumor effects in a variety of malignancies (27, 28). JQ1, a first-in-class small-molecule inhibitor of BRD4, engages to competitively bind to the acetyl-lysine recognition area of BRD4, displaces BRD4 from acetylated chromatin, and represses transcription of targeted genes in the influenced chromatin region. The specific BET inhibitors have demonstrated apparent efficacy in blocking tumor progression in a range of cancer models including castration-resistant prostate cancer (29), acute myeloid leukemia (30), melanoma (20), multiple myeloma (31), and lung carcinoma (32–34). Nevertheless, the role of BRD4 in bladder cancer progression and the therapeutic effect of BRD4 inhibitor in treatment of bladder cancer are still unknown. Here, we found that JQ1 treatment and shRNA-mediated *BRD4* knockdown induced cell-cycle arrest, cell apoptosis, and suppressed tumor growth in bladder cancer both *in vitro* and *in vivo*. Although knockdown of BRD4 could result in variable effects on cell-cycle progression in EJ cells, the general cell-cycle arrest effects of *BRD4* shRNAs were consistent with JQ1 treatment, and the variability is presumably related to cell status. These results suggest that BRD4 probably exert important roles in the growth of bladder cancer cells and JQ1 may be an innovative therapeutic approach for treatment of bladder cancer, which has not been reported before. In addition, recent study has identified BRD4 as a predictor of survival for bladder cancer. These data further support our findings that BRD4 is a critical regulator in bladder cancer progression (35). It has been demonstrated that a number of therapeutic strategies enhanced chemotherapy in human bladder cancer cells (36, 37). Whether treatment with JQ1 combined with other chemotherapeutics simultaneously exert the synergistic effect in bladder cancer therapy needs to be answered in the future.

Our previous study has demonstrated EZH2 is a key regulator and exerts an important role in bladder cancer growth, and miR-101 could target *EZH2* 3′-untranslated region (UTR) to inhibit *EZH2* expression at a posttranscriptional level (23). Recently, a series of reports identified miR-26b and/or miR26a as potential miRNAs that could regulate *EZH2* expression in prostate cancer, nasopharyngeal carcinoma, or lymphoma (38–40). In this study, we identified EZH2 as a downstream effecter of BRD4, and BRD4 could positively regulate *EZH2* expression at transcription level. The E2Fs transcription factors have been reported to manipulate *EZH2* transcription in several malignancies including bladder cancer (41). However, knockdown of *BRD4* failed to affect E2Fs expression according to previous researches (42). C-MYC is a transcription factor which controls the expression of genes involved in cancer cell-cycle progression. Recently, C-MYC has been demonstrated to effectively regulate *EZH2* expression through transcriptional approach in leukemia and prostate cancer (39, 43). In this study, we found that BRD4 positively regulated *EZH2* transcription through upregulation of *C-MYC* in bladder cancer cells. Moreover, BRD4 could interact with *C-MYC* promoter and BRD4 inhibition attenuated the recruitment of C-MYC to *EZH2* promoter. The interaction of BRD4 and *C-MYC* promoter is supported by recent studies that BRD4 could directly regulate transcriptional expression of *C-MYC* in a subset of cancer types (30, 31, 44). On the other hand, previous studies have also suggested a potential enrichment of BRD4 at the *EZH2* enhancer or promoter (14, 45). However, our experiments showed that there was no obvious recruitment of BRD4 to detected *EZH2* promoter region. The differences are presumably caused by following reasons. First, the enrichment area of BRD4 is probably beyond our detected *EZH2* promoter regions, which has led to negative results. Second, the binding capacity of BRD4 to *EZH2* promoter region is possibly vulnerable, which resulted in variable results. Taken together, our study reveals that the BRD4/C-MYC/EZH2 axis plays a vital role in the regulation of bladder cancer cell viability, which extends the knowledge about the upstream regulation of *EZH2* at the transcriptional level and facilitates our understanding of the potential etiology and biology of bladder cancer progression. In addition, knockdown of *EZH2* further strengthened cell-cycle arrest upon JQ1 treatment, according to the *in vitro* experiments. Thus, treatment with JQ1 together with EZH2 inhibitors may lead to an enhanced therapeutically effect *in vivo*.

It has been previously reported that BRD4 could be recruited to specific gene transcriptional sites through interactions with several sequence-specific transcription factors (17, 46). Besides, BRD4 may exert roles to help stabilize its interacting proteins on or off chromatin, and indirectly manipulate gene transcription by modulating protein stability according to previous studies (47). Although our study and recent reports demonstrate that the recruitment of BRD4 to the promoter region plays a crucial function in the regulation of *C-MYC* transcription, high expression of C-MYC in bladder cancer might also reflect the activity of transcription factors that manipulate upstream of *MYC* family members and that depend on BRD4 interactions to activate *MYC* family transcription. Moreover, direct protein–protein association between BRD4 and C-MYC/MAX complex, which has been found in recent studies (17), probably also manipulates C-MYC–regulated gene transcription and potentially modulates C-MYC protein stability. These intriguing possibilities concerning the functional implication of protein–protein interaction still need to be experimentally verified.

In summary, we identify BRD4 as an essential factor in regulating proliferation and apoptosis of bladder cancer cells, and provide the possibility of applying BET inhibitors for treatment of bladder cancer. Besides, our study reveals a novel regulatory

pathway that BRD4 could positively regulate *EZH2* transcription through upregulation of *C-MYC*, which extends the knowledge about the molecular mechanism underlying bladder cancer progression.

Disclosure of Potential Conflicts of Interest

The authors disclose no potential conflicts of interest.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Wu, D. Liu, W. Xiang, L. Wang, G. Luo, Y. Li, G. Jiang
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