SIRT1 suppresses pituitary tumor progression by downregulating PTTG1 expression

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Abstract. Although pituitary tumors are among the most common types of brain tumor, the underlying molecular mechanism of this disease remains obscure. To this end, the role of sirtuin 1 (SIRT1) in pituitary tumors was reported. The results of reverse transcription-quantitative PCR and immunohistochemistry revealed that sirtuin 1 (SIRT1) expression was downregulated in the tumor tissues of patients with pituitary tumors. *In vitro* experiments of the present study demonstrated that SIRT1 upregulation suppressed pituitary tumor cell line growth, while SIRT1 downregulation demonstrated the opposite effect. Additionally, it was determined that the enzymatic activity of SIRT1 was required for its cellular function. A mechanistic experiment determined that SIRT1 negatively regulated pituitary tumor-transforming gene 1 (PTTG1) expression through the deacetylation of histone (H)3 lysine (K)9ac at the promoter region of PTTG1. Moreover, H3K9ac levels at the PTTG1 promoter were determined to be an essential regulatory element for PTTG1 expression. Thus, it was concluded that the SIRT1/H3K9ac/PTTG1 axis contributed to pituitary tumor formation and may represent a potential therapeutic strategy.

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Introduction

Pituitary tumors are the third most commonly diagnosed intracranial tumor after meningioma and glioma, accounting for 10-15% of all brain tumors (1). Of these, adenoma comprises most cases, with only <1% of cases developing cancer. However, due to the position and function of the pituitary gland, pituitary tumors affect patient quality of life (2). Although patients with pituitary tumors usually demonstrate a good prognosis, there is a lack of knowledge surrounding the underlying molecular mechanism of this disease.

Genetic mutations are frequently reported in patients with pituitary tumors and include mutations in GNAS complex locus, protein kinase C, PI3K, Harvey rat sarcoma virus and ubiquitin specific peptidase 8, which are known regulators of the signaling pathways that control cell proliferation, survival and motility (3). These genes are upstream of cellular signals, meaning that they produce heavily toxic effects when they are inhibited (4). The most well-known differentiated expressed gene between normal pituitary tissue and pituitary tumor tissue is pituitary tumor-transforming gene 1 (PTTG1) (5). Its expression was first determined in the GH4 rat pituitary tumor cell line, but not in the normal pituitary gland (6). A functional study indicated that PTTG1 overexpression drives pituitary adenoma formation in mice (7), while its knockout significantly inhibits pituitary tumor formation in retinoblastoma-deficient animals (8). The results of these studies demonstrate that PTTG1 is targetable in pituitary tumors.

Sirtuin 1 (SIRT1) is a protein deacetylation enzyme that removes acetyl moiety from proteins (9). The activity of this enzyme is regulated by nicotinamide adenosine dinucleotide (NAD^+), the level of which represents low energy availability in cells (10). SIRT1 is reported to be a key bridge that links metabolism with age (11). As metabolism remodeling plays a prominent role in tumor cells, SIRT1 has gained tremendous attention in cancer research. However, the function of SIRT1 in cancer is controversial due to different cancer types and the

heterogeneity of cancer tissues. To the best of our knowledge, few studies have reported the function and mechanism of SIRT1 in pituitary tumors.

The present study revealed that SIRT1 was downregulated in pituitary tumor tissues, and that SIRT1 expression inhibited tumor growth. The results also revealed that SIRT1 downregulated PTTG1 through the deacetylation of histone (H)3 lysine (K)9ac. More importantly, resveratrol, which activates SIRT1 enzymatic activity, inhibited pituitary tumor cell growth. The results of the present study concluded that targeting the SIRT1/PTTG1 axis may be a potential treatment option for patients with pituitary tumors.

Materials and methods

Clinical samples. Clinical samples of pituitary tumors tissues and paired normal tissues from patients at Shanghai Changzheng Hospital (Shanghai, China) were collected after surgery with written informed consents from all patients and in compliance with the Medical Ethics Committee of Shanghai Changzheng Hospital under the approval no. 2021SLYS5. A total of 20 patients with a median age of 38.3 years were included in the present study. Patients without any treatment prior to surgery were enrolled, and samples were obtained from January 2018 to December 2019.

Cell culture. AtT-20 (cat. no. TCM 1) and 293T (Cat. no. GNHu17) were purchased from the Cell Bank of Chinese Academy of Sciences, Shanghai, China. GT1-1 (cat. no. 1101MOU-PUMC000009) was purchased from the National Infrastructure of Cell Line Resource. AtT-20 was cultured in RPMI-1640 medium (cat. no. L210KJ; Shanghai BasalMedia Technologies Co., Ltd.) supplemented with 10% fetal bovine serum (FBS) (product code 04-001-1ACS; Wolcavi Biotech), while 293T and GT1-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) (cat. no. L110KJ; Shanghai BasalMedia Technologies Co., Ltd.) supplemented with 10% FBS. All culture medium contained penicillin and streptomycin (both were 100 U/ml; cat. no. P1400; Beijing Solarbio Science & Technology Co., Ltd.), and was conditioned at 37˚C with 5% CO_2 in an incubator (STERI-CYCLE i160; Thermo Fisher Scientific, Inc.).

Drugs and treatment. Unless otherwise stated, all drugs were obtained from MedChemExpress, and incubations were performed with full medium in the incubator for 24 h. Nicotinamide (cat. no. HY-B0150) was used at 5 mM, resveratrol (cat. no. HY-16561) was used at 10 μM, PJ34 (cat. no. HY-13688A) and DPQ (cat. no. T19849; TargetMol Chemicals, Inc.) were used at 10 μM, and JQ1 (cat. no. HY-112789) was used at 0.5 μM. Insulin (cat. no. 11038-HNAY; SinoBiological, Inc.) was used at 20 ng/ml and IGF-1 (cat. no. 50437-MNAY; SinoBiological Inc.) was used at 100 ng/ml; both growth factors were incubated with cells for 1 h in the incubator.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from pituitary tumor and adjacent normal tissues by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and quantified by NanoDrop 2000c (Thermo Fisher Scientific, Inc.). A total of 2 μg RNA was reverse transcribed according to the manufacturer's instructions using Reverse Transcription System (cat. no. A3500; Promega Corporation). For real-time PCR, 2X SYBR Premix Taq system (Yeasen Biotechnology Co., Ltd.) was used and for detection on PikoReal Real-Time PCR system (Thermo Fisher Scientific, Inc.) was used. Thermocycling conditions were as follows: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec, extension at 72˚C for 20 sec. The method of quantification used was $2^{-\Delta\Delta Cq}$ (12). Primers used to detect SIRT1 were as follows: Forward primer, 5'-GGCGGCTTGATGGTA ATCAG-3' and reverse primer, 5'-AGTCCCAAATCCAGC TCCTC-3'. Primers used to detect GAPDH were as follows: Forward primer, 5'-CCATGGGGAAGGTGAAGGTC-3' and reverse primer, 5'-GGGATCTCGCTCCTGGAAGA-3'.

Immunohistochemistry. Immunostaining of SIRT1 (cat. no. 13161-1-AP; diluted 1:100) and interleukin (IL)-6 (cat. no. 21865-1-AP; diluted 1:100; both from ProteinTech Group, Inc.) was performed with paraffin-embedded pituitary tumor sections [first fixed in 4% paraformaldehyde (cat. no. P1110; Beijing Solarbio Science & Technology Co., Ltd.) for 24 h in a cold room] of 5-μm thickness. Essentially, sections were first deparaffinized at 65˚C for 30 min, and incubated with antibodies overnight in a cold room (4-8˚C), followed by incubation with an HRP-conjugated secondary antibody at room temperature for 1 h (product no. 7074; diluted 1:200; Cell Signaling Technology, Inc.). The signal was obtained with DAB Substrate Kit (product no. 8059; Cell Signaling Technology). Images were obtained at x40 and x200 magnifications with a light microscope (BX61; Olympus Corporation).

Western blotting. Western blotting was performed traditionally with slight modifications. Briefly, cells were seeded at $3x10^5$ cells/well of a 6-well plate, and were harvested 48 h later, followed by lysing in RIPA buffer (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) supplied with protease inhibitor (cat. no. B14002; Bimake.cn). Protein concentration was determined with Bradford reagent (Bio-Rad Laboratories, Inc.). Unless otherwise stated 30 μg protein samples were added per lane of 10% SDS-PAGE gel. A total of 5% BSA dissolved in TBST was used to block the PVDF membrane at room temperature for 1 h, and to dilute primary and secondary antibodies. Incubation of the primary antibodies was performed in a cold room overnight, and secondary antibody incubation at room temperature for 1.5 h. ECL (cat. no. sb-wb012; http://www.share-bio.com/) was used to detect HRP signal intensity. Chemiluminescent signals were recorded by CCD camera (Tanon system; Tanon Science & Technology Co., Ltd.). Primary antibodies used in this experiment included (unless otherwise specified all antibodies were purchased from Affinity Biosciences): anti-SIRT1 (cat. no. DF6033), anti-β-actin (cat. no. AF7018), anti-CD38 (cat. no. DF6551), anti-PTTG1 (cat. no. AF0354), pan-AKT (cat. no. AF6261), phosphorylated (p)-AKT (Thr308) (cat. no. AF3262), anti-GCN5L2 (cat. no. DF3383). The secondary antibody used was goat anti-rabbit (H + L) linked with HRP (cat. no. S0001; Affinity Biosciences).

Stable cell line construction. Stable cell lines were constructed via lentivirus-mediated gene delivery. pEF1a-MCS-IRES-GFP and pLKO.1-TRC were used to overexpress and knockdown target genes respectively. SIRT1 H363Y mutation was obtained by site-directed mutagenesis, and primers used were as follows: Forward primer, 5'-aggataattcagtgttatggttcct-3' and reverse primer, 5'-gttgcaaaggaaccataacactga-3'. To produce lentiviruses, 293T cells were transfected with the aforementioned core plasmids, along with the packaging plasmids psPAX2 and pMD2.G (cat. no. 12260 and 12259, respectively; Addgene, Inc.) at a ratio of 12:8:4 μg and Lipofectamine® 2000 transfection reagent (used according to the manufacturer's instructions; refreshing the medium 6 h post-transfection; Thermo Fisher Scientific, Inc.). Viruses were harvested at 72-h post-transfection (the medium was refreshed every 24 h). Transfection of GT1-1 and AtT20 cells was performed in 6-well plates with polybrene at a final concentration of 2.5 μg/ml for 24 h and at an MOI of 0.5. Cells were sorted for GFP signals by FACS using FACSAria (Beckman Coulter, Inc.) or selected with puromycin at 4 μg/ml for 3 days or longer. Overexpression and knockdown efficacies were demonstrated by western blot analysis. Short hairpin (sh)RNA primers used were as follows: shSIRT1-1 forward primer, 5'-CCGGAGTGA GACCAGTAGCACTAA TCTCGAGATTAGTGCTACTGG TCTCACTTTTTTG-3' and reverse primer: 5'-AATTCAAAA AAGTGAGACCAGTAGCACTAATCTCGAGATTAGTGC TACTGGTCTCACT-3'; shSIRT1-2 forward primer, 5'-CCG GCCTCGAACAATTCTTAAAGATCTCGAGATCTTTAA GAATTGTTCGAGGTTTTTG-3' and reverse primer, 5'-AAT TCAAAAACCTCGAACAATTCTTAAAGATCTCGAGAT CTTTAAGAATTGTTCGAGG-3'; shGCN5 forward primer, 5'-CCGGGCTACCTACAAAGTCAATTATCTCGAGATAA TTGACTTTGTAGGTAGCTTTTTG-3' and reverse primer, 5'-AATTCAAAAAGCTACCTACAAAGTCAATTATCTCGA GATAATTGACTTTGTAGGTAGC-3'; shBRD4-1 forward primer, 5'-CCGGTGAACCTCCCTGATTACTATACTCGA GTATAGTAATCAGGGAGGTTCATTTTTG-3' and reverse primer, 5'-AATTCAAAAATGAACCTCCCTGATTACTAT ACTCGAGTATAGTAATCAGGGAGGTTCA-3'; shBRD4-2 forward primer, 5'-CCGGGCGGCAGCTAAGTCTAGATA TCTCGAGATATCTAGACTTAGCTGCCGCTTTTTG-3' and reverse primer, 5'-AATTCAAAAAGCGGCAGCTAAGTC TAGATATCTCGAGATATCTAGACTTAGCTGCCGC-3'.

MTT assay. MTT assays were performed to determine the cell proliferation rate. GT1-1 cells were dissociated from a 10-cm plate, AtT-20 cells were pelleted down, and the cell concentration was determined by autonomous cell counter. Cells ($1x10^3$) were added into each well of a 96-well plate. After 24 h of seeding in the incubator, MTT was added into the 96-well plate for 4 h, followed by aspiration to remove the media. DMSO was then used to dissolve cellular debris and the OD570 was measured and recorded. These steps were repeated for the next 4 days. All data was combined and analyzed using GraphPad Prism 6.0 software (GraphPad Software, Inc.).

Cell viability. Cell viability was determined by Cell Counting Kit-8 (CCK-8) assay. Cells were seeded at $1x10^4$ cells/well of a 96-well plate. After 24 h of seeding, drugs indicated in the specific assays were added. After another 24 h, 20 μl CCK-8 was added, and incubated for 3 h. Following incubation, the absorbance at 450 nm was determined using a microplate reader (PerkinElmer Enspire; PerkinElmer, Inc.). Data was processed using GraphPad Prism 6.0 software.

Invasion assay. The invasion assay was performed using a Transwell chamber (8 μM pore size; product no. 3422; Corning, Inc.). A total of $1x10^6$ cells resuspended in 200 μl DMEM or RPMI-1640 medium were seeded into each Transwell with 50 μl Matrigel (precoated at 37˚C for 30 min and diluted 1:2) while 700 μl full medium was added to the lower compartment. Following incubation for 48 h in the incubator, cells on the outer membrane were stained at room temperature with 0.05% crystal violet containing 20% methanol for 10 min. Images were obtained with a light microscope (RVL-100-G; ECHO).

Chromatin immunoprecipitation (ChIP). ChIP-qPCR assays were used to determine the binding of SIRT1 and H3K9ac to PTTG1 promoter. Cells were first cross-linked with 1% formaldehyde, followed by scraping into ice-cold PBS with protease inhibitors. Then cells were resuspended in lysis buffer (20 mM HEPES, pH=7.9, 420 mM NaCl, 0.2 mM EDTA, 0.5% NP-40, 25% glycerol and 1.5 mM $MgCl_2$), followed by several brief periods of sonication. One-third of the cell extract was kept as the input sample, and two-thirds of the cell extract was used as the substrate for immunoprecipitation with anti-SIRT1 and anti-H3K9ac antibodies. A total of 5 M NaCl was used to reverse the cross-linking after which the eluted DNA was extracted for PCR analysis. The primer sequences for the PTTG1 promoter are listed as follows: forward primer, 5'-ttc gtgaaagagtaatatgg3' and reverse primer, 5'-atcctcaatacttcaggc taag-3'.

Statistical analysis. All data analyzed is presented as the mean ± standard deviation (SD), and was performed using GraphPad Prism 6. Paired Student's t-test was used to compare variants in two groups. For each Student's t-test, each group contained more than 3 replicates. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SIRT1 expression reduces pituitary tumor cell growth and invasion. The expression pattern of SIRT1 was assessed in 20 pairs of clinical pituitary tumors by RT-qPCR. The results demonstrated that SIRT1 expression was lower in tumor tissue compared with paired normal tissue in 15 (75% of total samples examined) patients (Fig. 1A). Consistently, immunohistochemical staining of SIRT1 also revealed that SIRT1 expression was decreased in tumors compared with normal tissue (Fig. 1B). To determine the function of SIRT1 in pituitary tumors, the endogenous expression of SIRT1 was knocked down in AtT20 cells (a mouse pituitary tumor cell line) (Fig. 1C). Subsequently, cellular growth rate and invasion were assessed. The results revealed that the growth rate of tumor cells was accelerated, while AtT20 cell invasion was also increased, as a result of SIRT1 knockdown (KD) (Fig. 1C and D). The aforementioned results were also observed in the GT1-1 cell line (Fig. 1E and F). Furthermore,

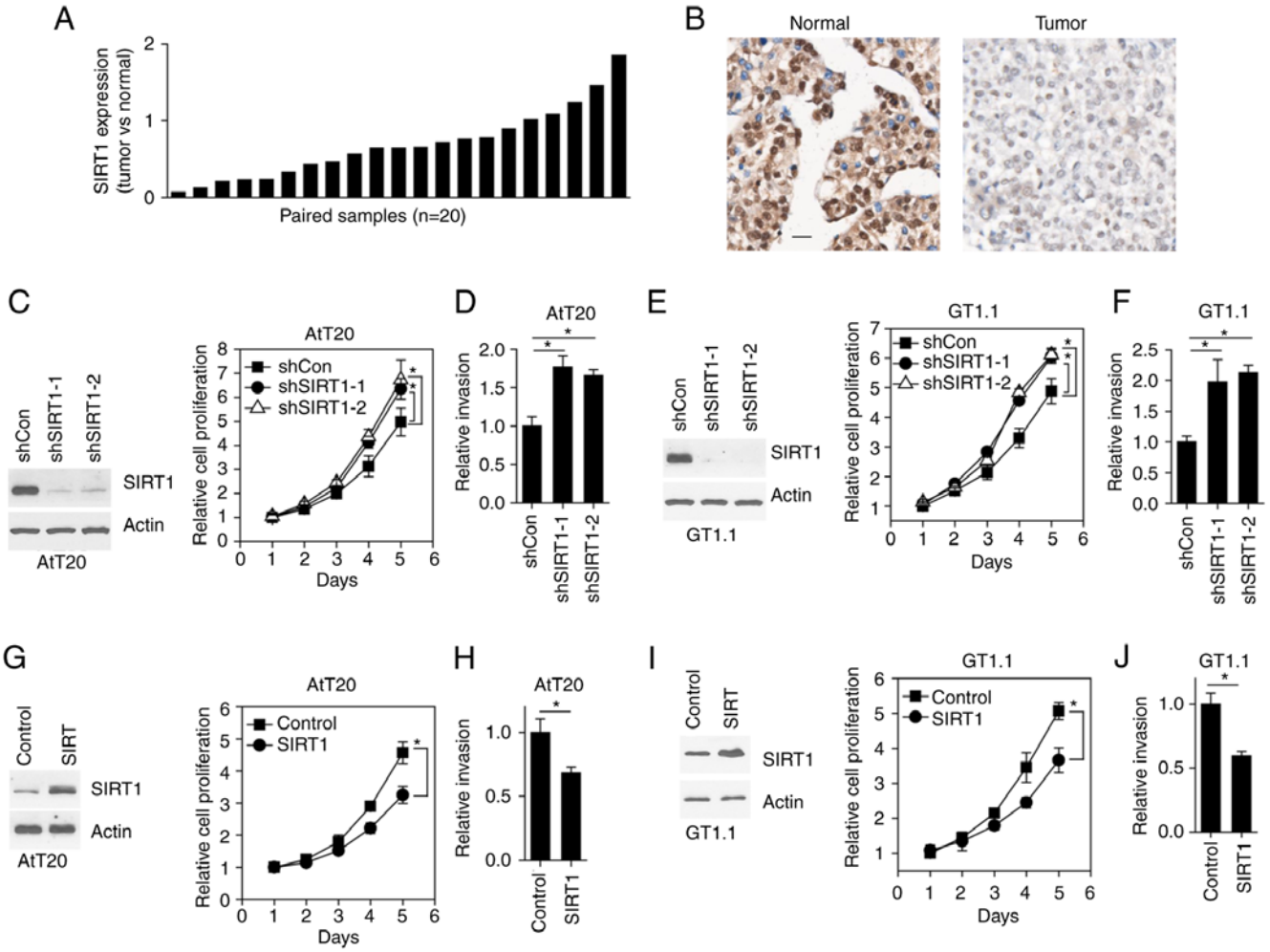

Figure 1. SIRT1 expression decreases pituitary tumor cell growth and invasion. (A) Reverse transcription-quantitative PCR results revealing SIRT1 expression in human pituitary tumor tissues. (B) Immunohistochemical staining of SIRT1 in human pituitary sections. Scale bar, 50 μm. (C) Western blotting results revealing the protein expression in cells (left panel). Line plot of the cell proliferation rate (right panel). *P<0.05, determined by paired Student's t-test. (D) Bar plot revealing the number of invasive cells. *P<0.05, determined by paired Student's t-test. (E) Western blotting results revealing the protein expression in cells (left panel). Line plot of the cell proliferation rate (right panel). *P<0.05, determined by paired Student's t-test. (F) Bar plot showing the number of invasive cells. *P<0.05, determined by paired Student's t-test. (G) Western blotting results revealing the protein expression in cells (left panel). Line plot of the cell proliferation rate (right panel). *P<0.05, determined by paired Student's t-test. (H) Bar plot revealing the number of invasive cells. *P<0.05, determined by paired Student's t-test. (I) Western blotting results revealing the protein expression in cells (left panel). Line plot of the cell proliferation rate (right panel). *P<0.05, determined by paired Student's t-test. (J) Bar plot revealing the number of invasive cells. *P<0.05, determined by paired Student's t-test. SIRT1, sirtuin 1.

SIRT1 was exogenously expressed in both AtT20 and GT1-1 cells (Fig. 1G and I). To this end, SIRT1 overexpression in both cell lines inhibited cell growth and invasion (Fig. 1G-J). Collectively, SIRT1 was downregulated in pituitary tumors and its expression reduced pituitary tumor cell growth and invasion.

SIRT1 activity regulates pituitary tumor cell growth and invasion. Due to the fact that resveratrol is an activator of SIRT1 (10), the present study aimed to determine whether resveratrol mimicked the tumor suppressive effect of SIRT1 overexpression in pituitary tumor cells. The results revealed that resveratrol induced AtT20 and GT1-1 cell death in a concentration-dependent manner (Fig. 2A). The invasion of AtT20 and GT1-1 cells was also inhibited (Fig. 2B). While these results indicated that the promotion of SIRT1 activity inhibited pituitary tumor cell growth and invasion, whether SIRT1 inhibition could increase tumor cell growth and invasion required further elucidation. As nicotinamide is a known antagonist of SIRT1 (10), the present study treated pituitary cells with nicotinamide. The results demonstrated that nicotinamide promoted cell growth (Fig. 2C) and invasion (Fig. 2D).

It has been demonstrated that a mutation of histidine to tyrosine in the SIRT1 protein at position 363 abrogates the enzymatic activity of SIRT1 (13). To further verify that SIRT1 activity regulated pituitary tumor cell growth and invasion, wild-type (WT) SIRT1 and H363Y mutant SIRT1 was expressed in AtT20 cells (Fig. 2E). While WT SIRT1 inhibited cell growth, H363Y mutant SIRT1 exerted no effect (Fig. 2F). Further treatment with resveratrol induced the robust inhibition of cell growth in AtT20 cells expressing WT SIRT1; however, this effect was not observed in H363Y SIRT1-expressed cells (Fig. 2G). The endogenous activity of SIRT1 is mainly regulated by NAD^+ levels, indicating that upregulating cellular NAD^+ levels may promote SIRT1

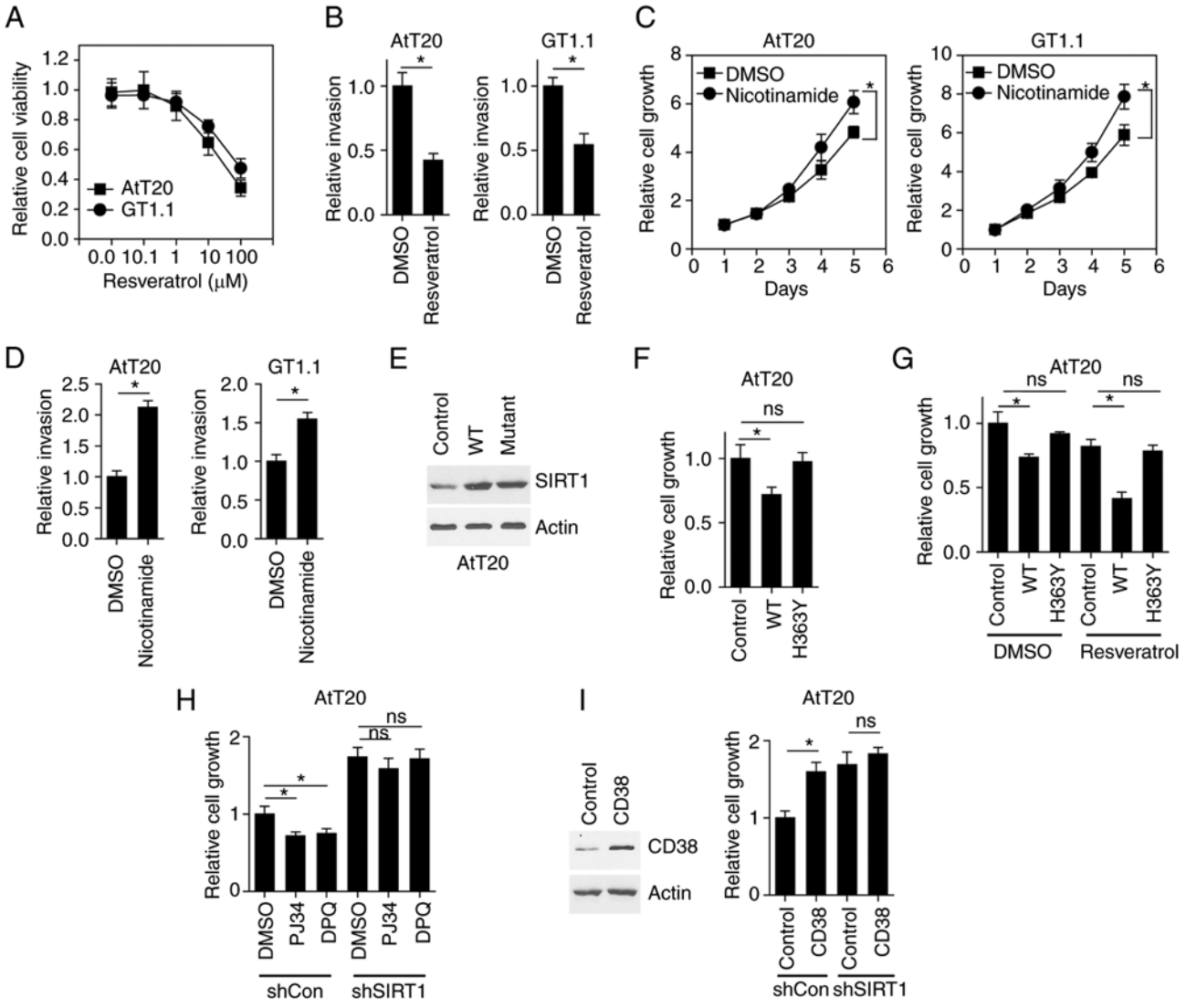

Figure 2. SIRT1 activity regulates pituitary tumor cell growth and invasion. (A) Line plot revealing the cell viability. (B) Bar plot demonstrating the relative number of invasive cells. *P<0.05, determined by paired Student's t-test. (C) Line plot revealing the cell growth rate. *P<0.05, determined by paired Student's t-test. (D) Bar plot demonstrating the relative number of invasive cells. *P<0.05, determined by paired Student's t-test. (E) Western blotting results revealing the protein expression in cells. (F-H) Bar plot revealing the cell growth. *P<0.05, determined by paired Student's t-test. (I) Western blotting results revealing the protein expression in cells (left panel). Line plot of the cell growth (right panel). *P<0.05, determined by paired Student's t-test. SIRT1, sirtuin 1; ns, not significant.

activity. PJ34 and DPQ are two chemical compounds that are known to inhibit PARP function and upregulate cellular NAD^+ levels (14). In the present study, treatment of control AtT20 cells with PJ34 and DPQ inhibited cell growth, while SIRT1 KD abolished this effect (Fig. 2H). To decrease cellular NAD^+ levels, CD38 was overexpressed, as it is an NADase enzyme. In AtT20 cells, the cell growth assay revealed that CD38 overexpression promoted cell growth, while SIRT1 KD inhibited this effect (Fig. 2I). Thus, the enzymatic activity of SIRT1 regulated pituitary tumor cell growth and invasion.

SIRT1 regulates PTTG1 expression. PTTG1 is an oncogene that is expressed in pituitary tumors but not in normal pituitary glands. As SIRT1 is mainly localized to the nucleus (10), the present study aimed to determine whether PTTG1 expression was regulated by SIRT1. The results demonstrated that PTTG1 mRNA and protein levels were increased upon SIRT1 inhibition (Fig. 3A). Furthermore, SIRT1 overexpression in AtT20 cells led to decreased levels of PTTG1 mRNA and protein expression (Fig. 3B). Moreover, SIRT1 enzymatic activation by resveratrol decreased PTTG1 expression, while its inhibition by nicotinamide increased PTTG1 expression (Fig. 3C and D). This trend was also observed in clinical pituitary tumor tissues (Fig. 3E). As insulin and insulin-like growth factor 1 (IGF-1) activate PTTG1 mRNA transcription (15), the present study aimed to elucidate whether SIRT1 was involved in this process. As presented in Fig. 3F, insulin and IGF-1 treatment increased PTTG1 expression, while resveratrol attenuated PTTG1 expression. The aforementioned results demonstrated that SIRT1 regulated PTTG1 expression.

SIRT1 deacetylates the H3K9 to regulate PTTG1 expression. To gain insight into the mechanism underlying PTTG1 regulation by SIRT1, the present study hypothesized that SIRT1 may deacetylate H3K9 to suppress PTTG1 transcription. The results demonstrated that SIRT1 was localized to the

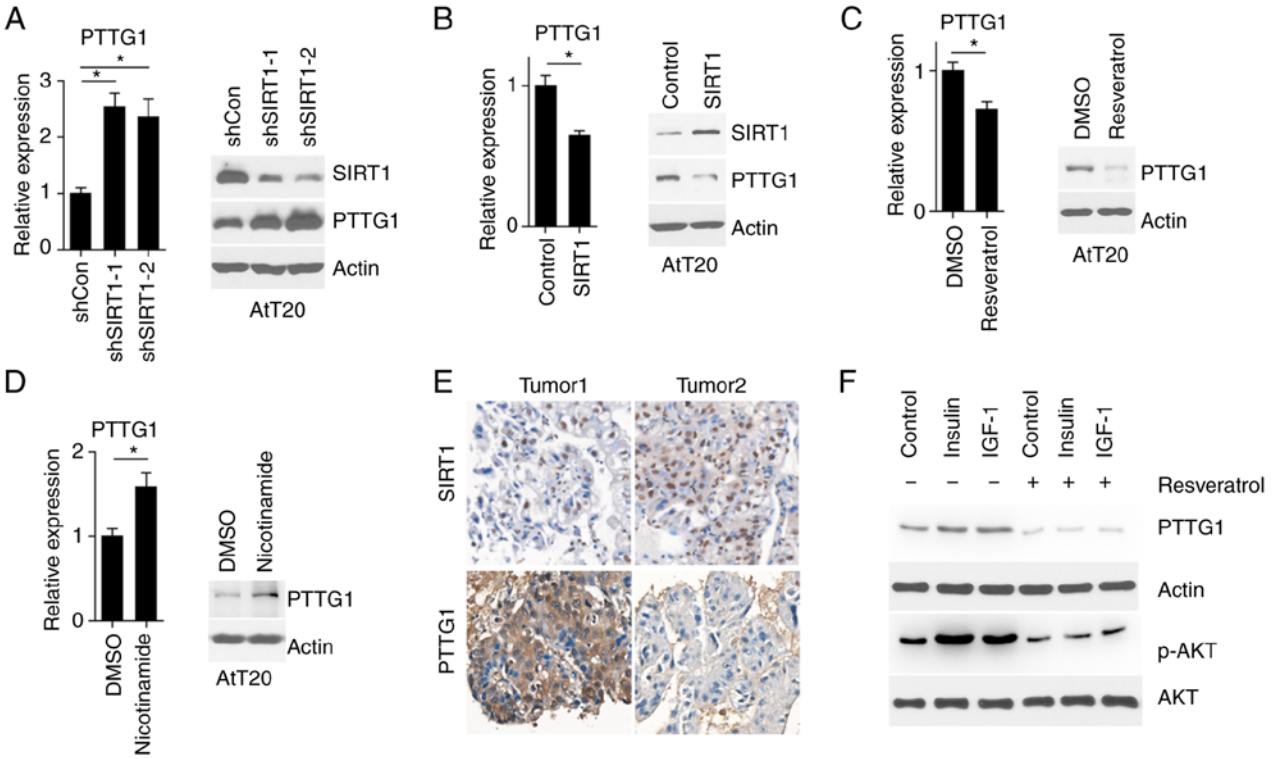

Figure 3. SIRT1 regulates PTTG1 expression. (A) Bar plot revealing the relative mRNA expression (left panel). *P<0.05, determined by paired Student's t-test. Western blotting results revealing the protein expression in cells (right panel). (B-D) Bar plots revealing the relative mRNA expression (left panels). *P<0.05, determined by paired Student's t-test. Western blotting results revealing the protein expression in cells (right panels). (E) Representative images revealing the immunohistochemical staining results. Magnification, x100. (F) Western blotting results revealing the protein expression in cells. SIRT1, sirtuin 1; PTTG1, pituitary tumor-transforming gene 1.

PTTG1 promoter region, and its inhibition enriched H3K9ac at the PTTG1 promoter (Fig. 4A). Furthermore, resveratrol treatment, which activated SIRT1, decreased H3K9ac levels at the PTTG1 promoter (Fig. 4B). To confirm that H3K9ac levels regulated PTTG1 expression, the expression of GCN5 was knocked down, as GCN5 is known to modify H3K9 with acetyl moiety (16). The results of the chromatin immunoprecipitation assay revealed that H3K9ac levels were decreased at the PTTG1 promoter (Fig. 4C). Furthermore, GCN5 inhibition resulted in the downregulation of PTTG1 mRNA (Fig. 4D). As GCN5 inhibition decreased H3K9ac levels at the PTTG1 promoter, the present study aimed to determine whether SIRT1 regulated PTTG1 expression independently of H3K9ac. As presented in Fig. 4E, while SIRT1 KD upregulated PTTG1, this regulation did not occur in GCN5 KD cells. The results suggested that SIRT1 deacetylated the H3K9 to regulate PTTG1 expression.

H3K9ac reader bromodomain containing 4 (BRD4) is required for PTTG1 expression. To corroborate that H3K9ac mediated the regulation of PTTG1 expression by SIRT1, the expression of BRD4 was knocked down. BRD4 is an H3K9ac reader that when coupled to this, causes downstream RNA synthesis (17). The results revealed that BRD4 KD reduced PTTG1 expression (Fig. 5A and B). JQ1 has been reported to be an inhibitor of the BRD protein family (18), thus indicating that JQ1 treatment may mimic the effect of BRD4 KD. As presented in Fig. 5C and D, JQ1 treatment led to a decreased expression of PTTG1 in a concentration-dependent manner. Furthermore, treatment of JQ1 abolished the PTTG1 expression that was induced by SIRT1 KD (Fig. 5E). The aforementioned results revealed that PTTG1 expression was regulated by H3K9ac, and that this modification could be divided into two aspects: Processes determining the abundance of H3K9ac and cellular ability to decode H3K9ac. While SIRT1 reduced the abundance of H3K9ac, BRD4 KD attenuated the ability of cells to decode the H3K9ac signal. To assess whether SIRT1 activation and BRD4 inhibition would synergically inhibit PTTG1 expression, AtT20 and GT1-1 cells were treated with resveratrol and JQ1. It was demonstrated that resveratrol treatment alone inhibited cell growth, and that the concurrent treatment of resveratrol and JQ1 significantly reduced cell growth (Fig. 5F and G). The results suggested that H3K9ac mediated PTTG1 expression via SIRT1.

Discussion

Gene regulation has been studied to a lesser degree in pituitary tumors when compared with other tumor types. Although SIRT1 dysregulation has been reported in numerous types of cancer, to the best of our knowledge, no study has described the role of SIRT1 in pituitary tumors. While SIRT1 was reported to be upregulated and a predictor of poor survival in lung (19), breast (20), gastric (21), colon (22) and prostate cancer (23), along with glioma (24) and sarcoma (25), its expression was confirmed to be downregulated in head and neck cancer (26). The results of the present study revealed that SIRT1 was downregulated in pituitary tumors, and that activation of its

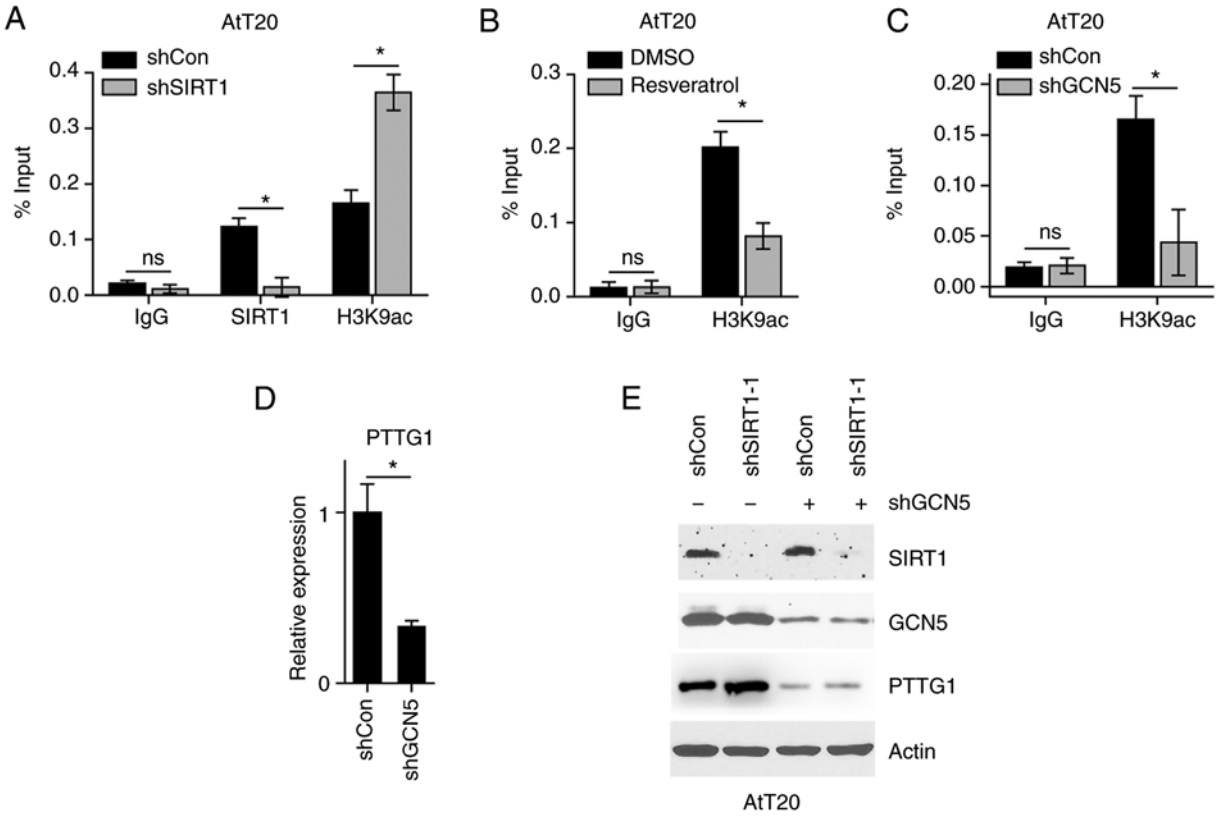

Figure 4. SIRT1 deacetylates the histone H3K9 to regulate PTTG1 expression. (A-C) Bar plots showing the quantity of enriched PTTG1 promoter region. *P<0.05, determined by paired Student's t-test. (D) Bar plot revealing the relative mRNA expression. *P<0.05, determined by paired Student's t-test. (E) Western blotting results revealing the protein expression in cells. SIRT1, sirtuin 1; PTTG1, pituitary tumor-transforming gene 1; ns, not significant.

expression or enzymatic activity inhibited pituitary tumor cell growth. Since SIRT1 could be activated by resveratrol, which is a small chemical, it may represent a potential treatment strategy for patients with pituitary tumors.

The present study also revealed that the mRNA expression of PTTG1 was regulated by SIRT1 in pituitary tumors. Reported mechanisms underlying the role of SIRT1 in other malignancies have included: i) Direct deacetylation and inactivation of tumor suppressor genes, such as tumor protein 53 (27) and retinoblastoma (28); ii) activation of oncogenic transcription factors such as c-Myc (29), beta-catenin (30) and hypoxia-inducible factor 1α (31). Moreover, SIRT1 is classified as a H deacetylase, which indicates its role in the modification of Hs, its role in the regulation of chromatin structure and gene expression (32). H substrates of SIRT1 are mainly comprised of H3 and H4, with strong deacetylation activities for H3K9ac and H4K16ac, and minor deacetylation activities for H3K14ac, H4K8ac and H4K12ac (33). H3K9ac is the classical substrate of SIRT1, and it has been well established that H3K9ac activates gene transcription at promoter regions (34). The results of the present study revealed that SIRT1 expression inhibited PTTG1 expression, but that this was dependent on its enzymatic activity. SIRT1 was additionally determined to bind to the promoter of PTTG1, deacetylate H3K9ac and thus attenuate PTTG1 mRNA expression. The function and mechanism of PTTG1 protein in the development and progression of different cancer types had gained tremendous interest, and a number of studies have demonstrated that PTTG1 serves as an oncogene (35). PTTG1 acts as a modulator of the sister chromatid separation process (36). Its upregulation leads to genetic instability, including aneuploidy, and thus contributes to tumor cell evolution. Functional studies have demonstrated that PTTG1 is an initiator of tumorigenesis as well as a promoter of tumor progression. Nevertheless, regulation of PTTG1 mRNA expression has been less frequently reported. Although the SIRT1/H3K9ac/gene transcription pathway is well accepted, the specific regulation of PTTG1 by SIRT1 via H3K9ac deacetylation has not yet been reported to the best of our knowledge.

Three elements are central to H3K9 acetylation modifications: Writers, erasers and readers (37). The present study identified three important players that regulate H3K9ac at the promoter region of PTTG1: GCN5 (the writer), SIRT1 (the eraser) and BRD4 (the reader). GCN5 belongs to the H acetyltransferase (HAT) family, which consists of five subfamilies: HAT1, GCN5/P300/CBP-associated factor, MYST (full name MOZ, Ybf2/Sas3, Sas2 and Tip60), P300/CBP and Rtt109 (37). Among these HATs, GCN5 has been demonstrated to acetylate K9 and 14 of H3, and regulate diverse cellular processes such as cell cycle progression, metabolism and spermiogenesis (38). GCN5 was also reported to be involved in cancer progression. For instance, GCN5 acetylated c-Myc at K323 to stabilize this oncoprotein (39). In non-small cell lung cancer, GCN5 was localized to the promoter regions of E2F transcription factor 1, cyclin D1 and cyclin E1 to promote their gene transcription (40). Consistent with this model, the present study revealed that GCN5 upregulated H3K9ac levels at the promoter region of

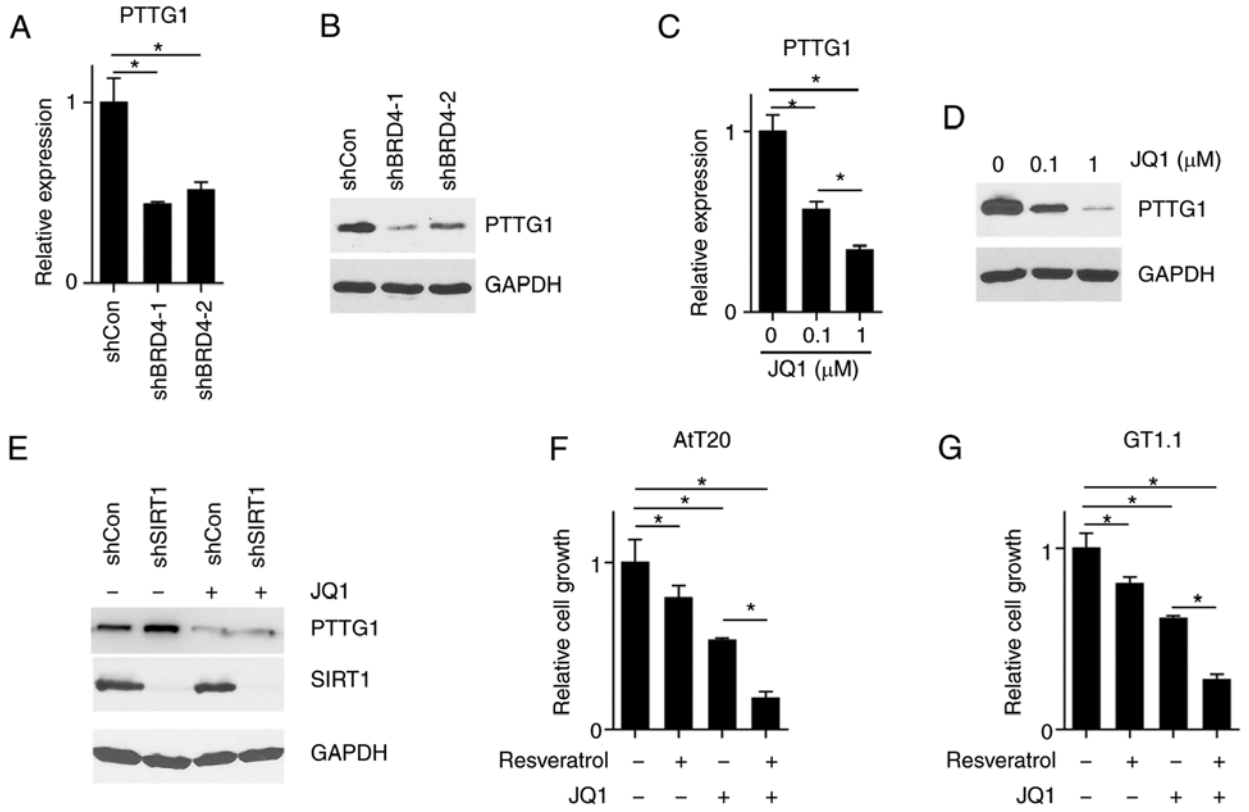

Figure 5. H3K9ac reader BRD4 is required for PTTG1 expression. (A) Bar plot revealing the relative mRNA expression. *P<0.05, determined by paired Student's t-test. (B) Western blotting results revealing the protein expression in cells. (C) Bar plot revealing the relative mRNA expression. *P<0.05, determined by paired Student's t-test. (D and E) Western blotting results revealing the protein expression in cells. (F and G) Bar plots revealing the cell growth. *P<0.05, determined by paired Student's t-test. BRD4, bromodomain containing 4; PTTG1, pituitary tumor-transforming gene 1; SIRT1, sirtuin 1.

PTTG1, which in turn promoted PTTG1 expression. However, it should be noted that other HAT proteins may also regulate this process, and that further study is required to elucidate these. BRD4 is the most studied member of the bromodomain and extraterminal family. It contains two bromodomains that recognizes acetylated K residues (41). Reported substrates of BRD4 include H3K9ac, H3K14ac and H3K27ac, as well as acetylated Ks in histone 4 (42). When BRD4 binds to its substrate Hs, it recruits transcription machinery to gene promoters or enhancers to regulate gene expression (42). The present study revealed that PTTG1 upregulation, occurring through SIRT1 inhibition, was reversed by BRD4 inhibition. Since it was demonstrated that SIRT1 inhibition upregulated H3K9ac at the PTTG1 promoter region, the present results indicated that H3K9ac was read by BRD4 at PTTG1 promoter regions. Furthermore, a synergistic effect was observed when combining resveratrol and JQ1 to inhibit pituitary cells. This observation was likely due to the fact that resveratrol decreases H3K9ac levels at the PTTG1 promoter region, but does not inhibit them, thus interrupting the readout of H3K9ac, and therefore inhibiting the binding efficacy of BRD4 to H3K9ac, further downregulating the expression of PTTG1. The present study not only validated H3K9ac as an essential regulatory element of PTTG1 expression, but also identified the writer, the eraser and reader of H3K9ac at the PTTG1 promoter. The results also determined that the combination of resveratrol and JQ1 inhibited the progression of pituitary tumor cells, indicating that they may serve as a potential treatment for patients with pituitary tumors. However, there are certain limitations in the present study. First, pituitary tumor subtypes were not addressed in this study since the clinical samples collected were limited and second, most of the functional experiments were performed using two cell lines which render the conclusions preliminary.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XD conceived the study. JH and FZ performed most of the experiments. GH performed RT-PCR and immunohistochemical assays. YP performed certain drug treatment assays. WS, LJ, PW provided essential advice and contributed to the

design of this study. JQ analyzed the data and contributed to the interpretation of all the experimental results. XD, JH and FZ confirm the authenticity of all the raw data. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All experimental protocols were in compliance (approval no. 2021SLYS5) with the Medical Ethics Committee of Shanghai Changzheng Hospital (Shanghai, China) and written informed consents were obtained from all the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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