Am J Transl Res 2021;13(4):2331-2349
www.ajtr.org /ISSN:1943-8141/AJTR0116894

Original Article

Differentiated embryonic chondrocyte-expressed gene 1 promotes temozolomide resistance by modulating the SP1-MGMT axis in glioblastoma

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Received June 24, 2020; Accepted December 27, 2020; Epub April 15, 2021; Published April 30, 2021

Abstract: Glioblastoma multiforme (GBM) is a malignant brain tumor with a high mortality rate and poor prognosis. Temozolomide (TMZ) is a first-line drug against GBM, but resistance limits its use. We previously reported that differentiated embryonic chondrocyte (*DEC1*) expression is associated with TMZ resistance and poor prognosis in GBM; however, the underlying mechanism remains unclear. By using glioma cell lines with stably overexpressed or silenced *DEC1*, we examined the effects of *DEC1* on TMZ sensitivity using proliferation assays, Western blotting, and flow cytometry. We demonstrated that *DEC1* overexpression suppressed, whereas *DEC1* knockdown enhanced, TMZ-induced cell apoptosis in methylguanine methyltransferase (MGMT)-positive T98G and LN18 cells but not in MGMT-negative U251 cells. Mechanistically, *DEC1* positively regulated *MGMT* through specificity protein 1 (SP1). *MGMT* silencing in *DEC1*-overexpressing cells or overexpression in *DEC1*-silenced cells abrogated *DEC1*'s effects on TMZ sensitivity, and siRNA-mediated SP1 knockdown phenocopied TMZ sensitivity, which was rescued by *MGMT* overexpression. Thus, *DEC1* may control TMZ resistance via the *SP1-MGMT* axis. Immunohistochemical staining of the human glioma tissue microarray revealed that the expression levels of *DEC1* and *MGMT* were correlated. Therefore, *DEC1* expression has a predictive value for TMZ resistance and poor outcome in glioma patients, and is a novel therapeutic target in TMZ-resistant glioma.

Keywords: Temozolomide, *DEC1*, apoptosis, glioblastoma, drug resistance, *MGMT*

Introduction

Glioma is a common primary tumor in the central nervous system and classified into four pathological grades (World Health Organization [WHO] classification, grades I to IV). Glioblastoma multiforme (GBM), which belongs to WHO grade IV, constitutes more than 70% of primary intracranial tumors and has a high recurrence and mortality [1]. GBM is a highly malignant, actively proliferating, and intensively invading cancer with a very poor prognosis [2]. At present, the standard strategy for GBM is a comprehensive treatment combining advanced surgery, radiotherapy, and chemotherapy such as temozolomide (TMZ). Nonetheless, the median survival time of patients with GBM is only approximately 12-15 months [3]. Drug resistance represents a key challenge in clinical practice and limits the therapeutic application of TMZ. Therefore, understanding the mechanisms underlying this drug resistance and identification of novel therapeutic targets and strategies are of great importance.

Temozolomide, as the first-line chemotherapeutic agent in glioma, exerts its anticancer effects by methylating the O^6 position of guanine (O^6-G) in the deoxyribonucleic acid (DNA) molecule. O^6-methylguanine (O^6-MeG) mispairs with thymine during DNA replication, which evokes the DNA mismatch repair system to recognize this mismatch region and excise the thymine. Persistent O^6-MeG caused by TMZ in the template strand and the following mispair with thymine triggers futile repair cycles, ultimately

leading to DNA strand breaks and, thus, cell death [4]. O^6-methylguanine-DNA methyltransferase (MGMT), a suicide DNA repair enzyme, is able to directly reverse O^6-MeG lesions by transferring these groups to its active center and thus becoming inactivated [5]. Therefore, MGMT is a key player to mediate the resistance to TMZ, and MGMT levels are regarded as an important biomarker to evaluate the responsiveness to TMZ [5]. It has been well known that the epigenetic regulation of MGMT plays an important role in glioma, with approximately 20% of primary GBMs lacking MGMT activity and approximately 40% of the gliomas displaying hypermethylation in the *MGMT* promoter region [6, 7]. Moreover, several transcription factors, such as secreted protein 1 (SP1), activator protein 1 (AP1), CCAAT-enhancer-binding proteins (CEBP), hypoxia inducible factor-1α (HIF-1α), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), bind to the *MGMT* promoter to induce or suppress *MGMT* expression [4]. Previous studies have reported that the MGMT level is primarily determined by the transcription factor *SP1* and upregulated by glucocorticoids [8], whereas *p53* can negatively regulate *MGMT* expression via interaction with and sequestration of SP1 to the cognate elements in the *MGMT* promoter [9]. However, the regulation of *MGMT* and its relevance are far from being clarified.

Differentiated embryonic chondrocyte-expressed gene 1 (*DEC1*), also known as split- and hairy-related protein-2 (*SHARP-2*), basic helix-loop-helix binding protein 2 (*BHLHB2*), or stimulated with retinoic acid 13 (*Stra13*), belongs to a subfamily of the basic helix-loop-helix family, which also contains *DEC2* (*BHLHE41/SHARP-1*) [10]. The *DEC1* gene is located at chromosome 9q33.1. The human DEC1 protein consists of 412 amino acid residues with basic helix-loop-helix (bHLH) and Orange domains. As a transcription factor, DEC1 either represses gene transcription by directly binding to E-box motifs of its target genes such as *DEC2* [11], *CCND1* [12], and *LKB1* [13], or activates gene transcription by binding to the *SP1* site of its target genes, such as *SURVIVIN* [14]. DEC1 can also act as a cofactor to inhibit the SP1-mediated claudin-1 (*CLDN1*) transactivation [15]. In function, *DEC1* is an essential regulator of circadian rhythms [16]. In addition, *DEC1* has an important role in various cellular processes such as cell growth [10], cell cycles [17], cell metabolism [18], cell differentiation, and apoptosis [19]. The aberrant expression of *DEC1* is involved in the pathologies of various disorders [20]. Particularly, *DEC1* is closely associated with cancer but its functions are controversial in different types of cancer. It was reported that DEC1 suppresses cell proliferation, migration, and invasion in breast and oral cancer [21], and its expression is correlated with clinicopathological parameters [22]. However, in thyroid cancer, *DEC1* is significantly overexpressed in all major histologic types and promotes cell growth and invasiveness via an interplay with *NOTCH1* [17]. We previously reported that *DEC1* expression is increased in glioma in comparison to normal tissues, and its expression is correlated with malignancy grade, prognosis, as well as TMZ resistance [3]. Furthermore, *DEC1* was reported to promote GBM tumor cell proliferation, migration, and invasion [23]. However, to the best of our knowledge, far less is known about whether, and how, *DEC1* is involved in TMZ resistance.

Since MGMT is the most important factor in the response of glioma cells to TMZ as mentioned above, we speculated that *DEC1* may regulate cellular sensitivity to TMZ via MGMT. Therefore, the present study intended to explore the role of *DEC1* in the response to TMZ and the interactive relationships between *DEC1* and *MGMT*. Our results showed that *DEC1* can positively regulate the expression of *MGMT* through SP1, and this axis can promote therapeutic resistance in response to TMZ.

Material and methods

Patients' samples

The tissue microarrays were preloaded with brain tissue samples from five healthy controls and 35 patients with GBM, containing 80 points (10 for normal brains and 70 for GBM); they were commercially obtained from Xi'an Alenabio Technology Co. Ltd. (GI805; Xi'an, China). The experiments were approved by the Research Ethics Committee and the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/), revised in 2013.

The Cancer Genome Atlas (TCGA)

DEC1 gene expression profile for GBM patients was obtained from the The Cancer Genome Atlas (TCGA) data portal (https://tcga-data.nci.nih.gov/tcga/), which contains 249, 265 and 153 samples of GII, GIII and GIV, respectively.

Reagents and plasmids

Dimethyl sulfoxide (DMSO; cat no. DH105-2) was purchased from Xi'an Kehao Bioengineering Co., Ltd. (Xi'an, China). Temozolomide (cat no. 85622-93-1) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in DMSO to obtain a 100-mM stock concentration. Puromycin (cat no. HY-CL13900) was purchased from MCE Bioengineering Co., Ltd. (Shanghai, China) and dissolved in phosphate-buffered saline (PBS) at a 1 mg/ml concentration. Hygromycin B (cat no. HY-B0490) was purchased from MCE Bioengineering Co., Ltd. (Shanghai, China) and dissolved in phosphate-buffered saline (PBS) at a 500 mg/ml concentration. Geneticin (cat no. DH138-2) was purchased from Xi'an Kehao Bioengineering Co., Ltd. and was dissolved in PBS to obtain a stock solution of 20 mg/ml.

Lentivirus plasmids containing full-length *DEC1* (EX-DEC1-pEZ-Lv105) or *MGMT* (EX-MGMT-pEZ-Lv151) or their corresponding empty cherry plasmids (i.e., EX-pEZ-Lv105 and EX-pEZ-Lv151) were commercially purchased (Guangzhou Fulengen Co., Ltd., Guangdong, China). The following lentivirus plasmids were purchased from Guangzhou FulenGen Co., Ltd.: short hairpin *DEC1* and its empty scrambled plasmid (CSHCTR001-LVRU6P), as well as short hairpin *MGMT* and its empty scrambled plasmid (CSHCTR001-LVRU6GH). The full-length *MGMT* promoter-reporter construct (pGL3.0-pMGMT-Luc vector, forward: 5'-AATATCAGTC-GGCACTCTGATTCCA-3'. reverse: 5'-CTCTTGC-TTTTCTCAGGTCC-3') and its empty cherry vector were purchased from BokeGen Co., Ltd. (Xi'an, China).

Cell culture, cell transfection, and lentivirus infection

Human GBM cell lines (T98G, LN18, and U251), normal human glial cells (HEB) and human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA, USA) were maintained in Dulbecco's Modified Eagle's Medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in a humidified incubator containing 5% carbon dioxide (CO_2) at 37°C. In addition, 100 μg/mL streptomycin and 100 U/mL penicillin were routinely added to the medium. For lentiviral packaging, HEK293T cells were seeded into 10-cm plates, and when the density reached 80%, the Lenti-Pac HIV Expression Packaging Kit (Genecoponia, Inc., Rockville, MD, USA) was applied. The recombinant expression plasmid or empty cherry plasmid were transfected into HEK293T cells together with the packing plasmid mix (Genecoponia, Inc., Rockville, MD, USA) using lipofectamine 2000 (Thermo Fisher Scientific, Inc.). The supernatants which contain virus were harvested after 48 hours. Glioma cells were then infected with the individual recombinant virus. Stable T98G, LN18, and U251 cells with overexpressed/silenced *DEC1* and their corresponding control cells expressing Cherry were generated using selection with 0.8 mM (for T98G and LN18) or 0.4 mM (for U251) puromycin (Invitrogen; Thermo Fisher Scientific, Inc.). Stable T98G and LN18 cells overexpressing *MGMT* or Cherry were generated by lentivirus infection and selection with 400 or 600 mM G418. Stable T98G and LN18 cells with suppressed *MGMT* or negative control were generated by lentivirus infection and selection with 600 mM hygromycin B in T98G or LN18 cells, respectively. For transient transfection, cells were seeded into six-well plates and transfected with individual plasmids or siRNAs using lipofectamine 2000 (Thermo Fisher Scientific, Inc.), based on the manufacturer's instructions.

Quantitative real-time PCR analysis

Total RNA was extracted from U251, T98G, or LN18 cells using RNAiso Plus (Takara Bio, Inc., Kusatsu, Japan). The One-Step SYBR PrimeScript RT-PCR Kit (Takara Bio, Inc., Kusatsu, Japan) was used for qRT-PCR detection of *DEC1, MGMT*, or *β-actin*. The primers were as follows: *DEC1* forward: 5'-ACTTACCTTGAAG-CATGTGAAAGCA-3', reverse: 5'-CATGTCTGGA-AACCTGAGCAGAA-3'; *β-actin* forward: 5'-TGA-CGTGGACATCCGCAAAG-3', reverse: 5'-CTGG-AAGGTGGACAGCGAGG-3'; *MGMT* forward: 5'-

GCAATTAGCAGCCCTGGCA-3', reverse: 5'-CACTCTGTGGCACGGGAT-3'. The qRT-PCR was conducted using a Bio-Rad C1000 thermal cycler with the following conditions: 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds and 55°C for 30 seconds. The expression levels were calculated relative to the β-actin levels as the endogenous control. Relative expression was calculated as $2^{(Ct\ test\ gene\ -\ Ct\ ACTB)}$.

Luciferase reporter assay

HEK293T cells, at 60% confluence, were seeded into six-well plates and then transfected with *DEC1*-encoding plasmid (EX-DEC1-pEZ-Lv105) or its corresponding empty cherry plasmid (EX-pEZ-Lv105), together with the control luciferase vector pGL3.0-Luc or the luciferase reporter pGL3.0-pMGMT-Luc which were commercially purchased (Guangzhou Fulengen Co., Ltd., Guangdong, China). The cells were harvested 48 hours after transfection. The Luc activity was determined, based on the manufacturer's instructions (Promega, Madison, WI, USA).

RNA interference

Chemically synthesized siRNA duplexes were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). Negative control siRNA sequence: 5'-UUCUCCGAACGUGUCACGUTT-3'; si*SP1*#1: 5'-GCCGUUGGCUAUAGCAAAUTT-3'; si-*SP1*#2: 5'-CCAGCAACAUGGGAAUUAUTT-3'; si-*SP1*#3: 5'-GUGCAAACCAACAGAUUAUTT-3'. siRNA transfections were conducted with lipofectamine 2000, based on the manufacturer's instructions.

Cell proliferation assay

T98G, LN18, and U251 cells were seeded at a density of 1,000 cells/well in 96-well plates in septuplets. Cell proliferation was estimated using the CCK8 (Apexbio, Co., Ltd., Boston, MA, USA; cat no. k1018). To each well, 10 μL of CCK-8 solution were added, and the cells were incubated for 3 hours at 37°C. The absorbance at 490 nm was measured using a microplate reader (SH-9000; Hitachi, Tokyo, Japan).

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (50 nM Tris-HCl, 150 nM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate [SDS], 0.5% deoxycholate, and 1 mM phenylmethanesulfonyl fluoride) for 30 minutes on ice. A total of 30 μg protein was separated by gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (4%-12% polyacrylamide). Proteins were then transferred to nitrocellulose membranes. The membranes were blocked, immunoblotted with the indicated primary antibodies, and subsequently incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies. The signals were detected with an enhanced chemiluminescence system (Tanon 5500; Tanon Science and Technology, Co., Ltd., Shanghai, China). The following commercial antibodies were purchased: DEC1 (dilution 1:5,000; rabbit monoclonal; cat no. ab70723; Abcam, Cambridge, UK), MGMT (dilution 1:1,000; mouse monoclonal; cat no. ab69629; Abcam, Cambridge, UK), Bcl-2 (dilution 1:1,000; rabbit monoclonal; cat no. 2876; CST, Boston, MA, USA), SP1 (dilution 1:1,000; rabbit monoclonal; cat no. 5931; CST, Boston, MA, USA), GAPDH (dilution 1:5,000; rabbit monoclonal; Zhuangzhibio; Xi'an, China). Horseradish-peroxidase-conjugated secondary antibodies (cat no. abs921; absin, Shanghai, China).

Apoptosis assay

V-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (Cat 55647; BD, Franklin Lakes, NJ, USA) was used. Cells were treated with TMZ or DMSO. A total of 10^5 cells were collected and resuspended in binding buffer. The FITC-Annexin V and PI (each 5 μL) were then added to the cell suspension and incubated for 15 minutes at room temperature in the dark. Stained cells were analyzed using a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA). Annexin V+/PI- (i.e., early apoptotic cells) and Annexin V+/PI+ (i.e., late apoptotic cells) were calculated as total apoptotic cells (from triplicates and using Student's t-test).

Immunohistochemistry

Immunohistochemistry was conducted, based on standard procedures. Primary antibodies directed against DEC1 (1:250 dilution; cat no. ab90594; Abcam, Cambridge, UK), or MGMT (1:100 dilution; cat no. ab69629; Abcam, Cambridge, UK) were used with the ABC Kit and

DAB (both obtained from Vector Resources, Inc., Torrance, CA USA) for staining. The results were obtained using a digital slide scanner (3DHISTECH, Ltd., Budapest, Hungary). The immunoreactive score (IRS) was applied. The deliberate score standard was as follows (presented as the percentage of cells with positive staining and score number): < 5%, 0; 5%-25%, 1; 26%-50%, 2; 51%-75%, 3; >75%, 4. The color score standard used the staining intensity, as follow: colorless, 0; mild, 1; moderate, 2; and strong, 3.

Colony formation assay

T98G, LN18, and U251 cells were seeded into six-well plates at a density of 300 cells/well in triplicate. The plates were incubated at 37°C with 5% CO_2 for 3 weeks. The plates were then washed with PBS, the developed cell colonies were fixed with 4% formaldehyde, and the cells were stained with Giemsa for 20-30 minutes. The number of colonies was directly reported.

Statistical analysis

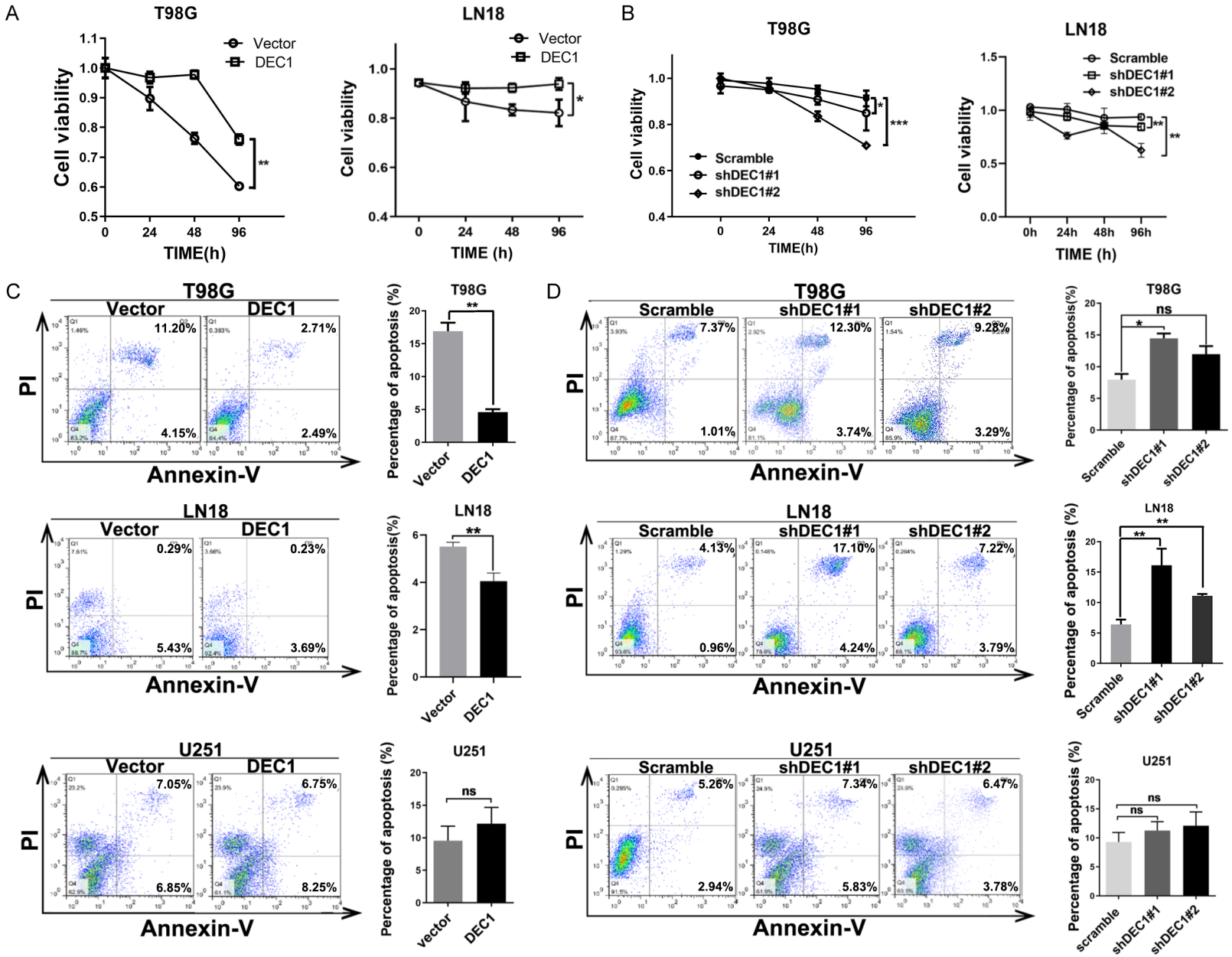
All statistical analyses were conducted using SPSS 13.0 package (SPSS Inc., Chicago, IL, USA). Quantitative data are presented as the mean ± the standard deviation of at least three independent experiments. For comparison of two-groups datasets, 2-sided, unpaired t-test was applied. For comparison of the clinical relevance of DEC1 and MGMT expression, 2-sided, unpaired t-test and chi-square test was applied. Statistical significance was defined as P < 0.05. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$, and ****; $P < 0.0001$.

Results

DEC1 promotes TMZ resistance in MGMT-positive GBM cells

Our previous study showed that *DEC1* expression is positively correlated with the malignancy grade and the TMZ resistance of GBM [3]. Therefore, we speculated that *DEC1* is involved in TMZ resistance by regulating key molecules that determine TMZ sensitivity. To test this hypothesis, we chose three GBM cell lines: T98G, LN18, and U251. Among these cell lines, U251 is MGMT-deficient and TMZ-sensitive, whereas T98G and LN18 express high levels of MGMT, rendering them resistant to TMZ [24]. Consistent with the literature, our Western blot analysis showed that T98G and LN18, but not U251, expressed high levels of MGMT (Figure S1A). Moreover, the cell counting kit-8 (CCK-8) assay, used to test the sensitivity of cells to TMZ, demonstrated that TMZ significantly inhibited cell proliferation in U251, but T98G and LN18 exhibited much more resistance to TMZ (Figure S1B). Therefore, a TMZ concentration of 600 μM in LN18 and T98G and 200 μM in U251 were applied in the subsequent experiments, according to our result and the recent study [43]. We then established stable T98G, LN18, and U251 cell lines by individually infecting cells with *DEC1* or *shDEC1* recombinant lentivirus to up- or downregulate the expression of *DEC1*. The success of stable infection in these cell lines was confirmed by Western blotting analysis and by real-time polymerase chain reaction (PCR) assay (Figure S1C and S1D). The CCK-8 and the colony formation assays demonstrated that upregulation of *DEC1* significantly promoted cell growth, colony-forming ability and cell migration, while downregulation of *DEC1* had the opposite effect (Figure S1E-J). These results indicated that *DEC1* has an important role in the proliferation and migration of the chosen GBM cell lines.

To examine whether *DEC1* expression affects the sensitivity of glioma cells to TMZ, we conducted a CCK-8 assay in these cells upon treatment with TMZ. As shown in **Figure 1A** and **1B**, T98G and LN18 cells stably overexpressing *DEC1* were more resistant to TMZ, compared to the vector cells, whereas *DEC1* knockdown significantly reverted TMZ resistance in these cells. Next, we conducted propidium iodide (PI)-annexin V double staining and flow cytometry analysis to examine apoptosis in these cells after treatment with TMZ. Our results demonstrated that *DEC1* overexpression was able to decrease apoptosis in T98G cells (4.60% ± 0.45% vs 16.89% ± 1.29%, $P < 0.01$) and LN18 cells (4.06% ± 0.19% vs 5.58% ± 0.09%, $P < 0.01$; **Figure 1C**). By contrast, *DEC1* knockdown increased apoptosis in T98G cells (14.49% ± 0.78% and 11.40% ± 0.94% vs 7.96 ± 0.88%, $P < 0.05$, and $P = 0.15$, respectively) and in LN18 cells (16.12% ± 2.72% vs 6.41% ± 0.81%, $P < 0.05$, and 11.10% ± 0.36% vs 6.41% ± 0.81%, $P < 0.01$, respectively; **Figure 1D**). In a similar manner, no significant difference existed in TMZ-induced cell apoptosis between U251-


A
T98G
Vector
DEC1
Cell viability
TIME(h)
LN18
Vector
DEC1
Cell viability
TIME (h)
B
T98G
Scramble
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shDEC1#2
Cell viability
TIME(h)
LN18
Scramble
shDEC1#1
shDEC1#2
Cell viability
TIME (h)
C
T98G
Vector
DEC1
11.20%
2.71%
4.15%
2.49%
PI
Annexin-V
Percentage of apoptosis (%)
LN18
Vector
DEC1
0.29%
0.23%
5.43%
3.69%
U251
Vector
DEC1
7.05%
6.75%
6.85%
8.25%
ns
D
T98G
Scramble
shDEC1#1
shDEC1#2
7.37%
12.30%
9.28%
1.01%
3.74%
3.29%
Percentage of apoptosis(%)
LN18
Scramble
shDEC1#1
shDEC1#2
4.13%
17.10%
7.22%
0.96%
4.24%
3.79%
U251
Scramble
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shDEC1#2
5.26%
7.34%
6.47%
2.94%
5.83%
3.78%

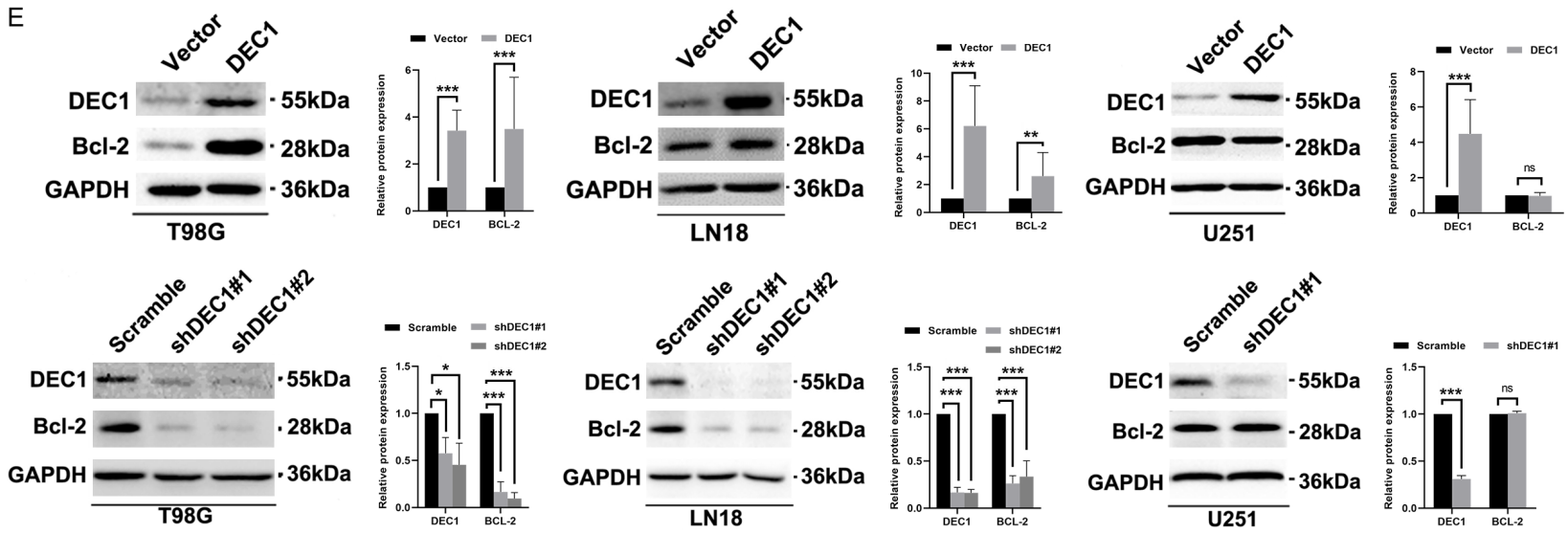


Figure 1. *DEC1* promotes TMZ resistance in MGMT-positive GBM cells. Stable GBM cell lines (T98G, LN18, and U251) were established by an infection with *DEC1*- or *shDEC1*-recombinant lentivirus and selection with puromycin. (A and B) LN18 and T98G were treated with 600 μM TMZ at different time points. cell survival was evaluated with CCK-8 assays (n = 3). (C and D) Cells were treated with TMZ (600 μM for LN18 and T98G, and 200 μM for U251) for 48 hours; cell apoptosis was evaluated using flow cytometry analysis. Representative images are shown (n = 3). (E) Cells were treated with 600 μM TMZ for 48 hours; the expression level of DEC1 and Bcl-2 were evaluated using Western blot (n = 3). In (A-D), data are presented as the mean ± SD. Unpaired *t* test for two-groups datasets. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$. CCK-8, cell counting kit 8; DEC1, differentiated embryonic chondrocyte; GBM, glioblastoma multiforme; MGMT, methylguanine methyltransferase; RT-qPCR, quantitative reverse transcription polymerase chain reaction; SD, standard deviation; shDEC1, short hairpin differentiated embryonic chondrocyte; specificity protein 1; TMZ, temozolomide.

DEC1 cells and U251-vector cells (12.16% ± 2.51% vs 9.55% ± 2.23%, $P = 0.40$), as well as between U251-scramble cells and U251-*shDEC1*#1 and U251-*shDEC1*#2 cells (11.26% ± 1.53% and 12.08% ± 2.38% vs 9.30% ± 1.63%, $P = 0.40$ and $P = 0.40$, respectively; **Figure 1C** and **1D**). These data suggested that *DEC1* regulated TMZ resistance in an MGMT-dependent manner. To further confirm these results, Bcl-2 level, which has been widely used as an anti-apoptosis indicator, was assessed by using Western blotting and exhibited consistent alterations (**Figure 1E**).

DEC1 positively regulates SP1 and MGMT

It is a consensus that MGMT is a critical molecule in the development of resistance to TMZ [26]. High levels of MGMT are associated with TMZ resistance and poor prognosis of patients with GBM [27]. On account that *DEC1* modulates TMZ sensitivity in *MGMT*-positive but not *MGMT*-negative cell lines, we tested whether *DEC1* is able to regulate *MGMT* expression. As shown in **Figure 2A**, Western blot results demonstrated that MGMT could be upregulated by *DEC1* overexpression and downregulated by *DEC1* knockdown. Moreover, real-time PCR assays revealed similar results (**Figure 2B** and **2C**), which indicated that DEC1 enhances *MGMT* at the transcription level. To further confirm this finding, pGL3.0-pMGMT-Luc, a luciferase reporter plasmid containing the promoter of *MGMT*, or the control luciferase vector pGL3.0-Luc was transfected into 293T cells together with the *DEC1*-overexpression vector or an empty vector to evaluate luciferase activity. As shown in **Figure 2D**, DEC1 significantly enhanced the fluorescence signal, compared to the control group. This indicated that *DEC1* positively regulates *MGMT* at the transcriptional level.

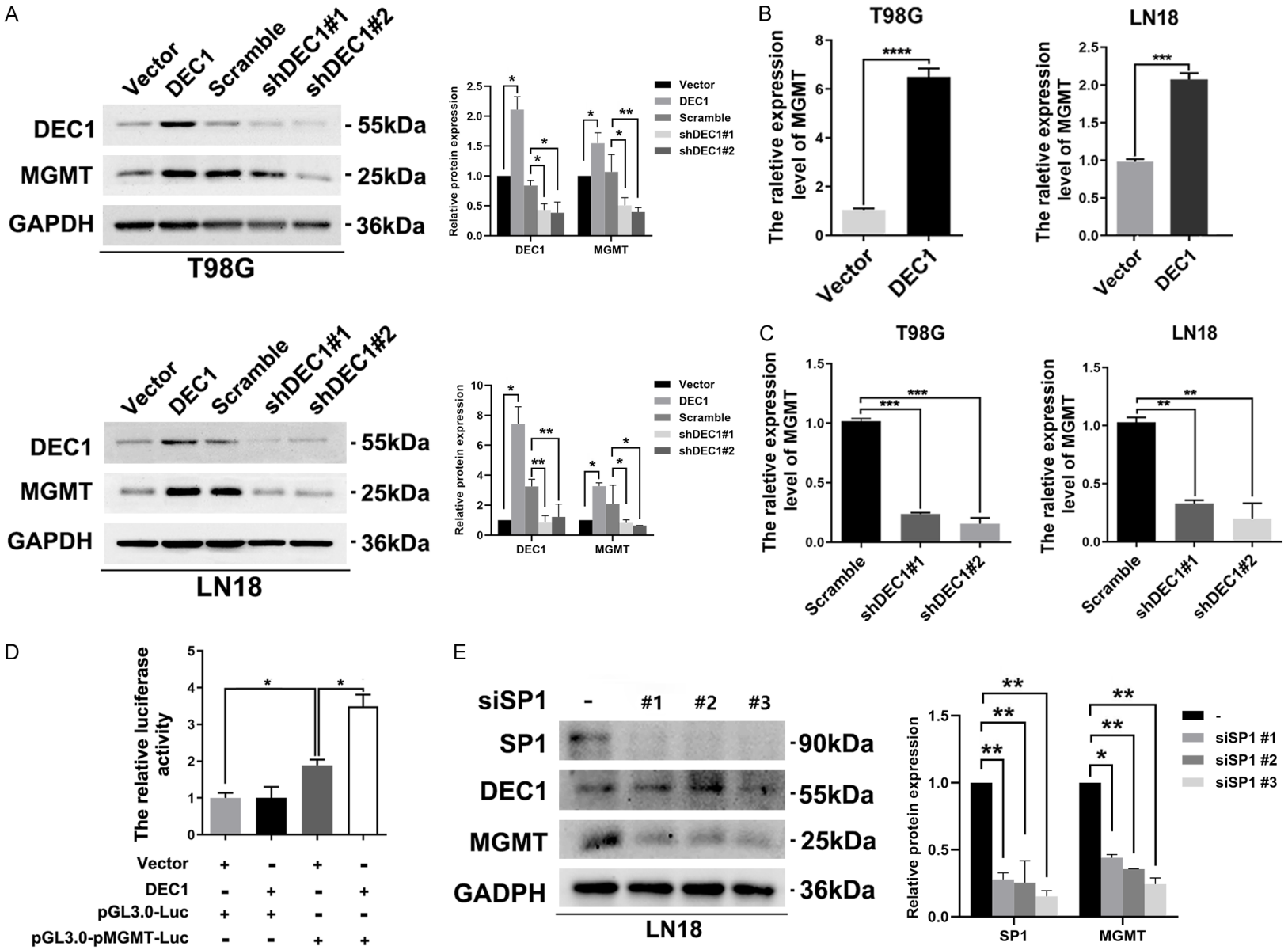
We were also interested in determining whether DEC1 acted directly on the *MGMT* promoter or whether its activity relied on other transcription factors. It has been reported that the *MGMT* promoter contains binding sites for a series of transcription factors such as *SP1* and *AP1* [9]. Interestingly, Zheng et al. demonstrated that DEC1 suppresses the transcription of *CLDN1* by interaction with SP1 but not by binding to the E-box motifs in the *CLDN1* promoters, which also contain *SP1* binding sites [15]. Similar to *CLDN1*, the *MGMT* promoter contains SP1 binding sites and E-box motifs; therefore, we wondered whether *DEC1* regulates *MGMT* transcription via SP1.

We first examined SP1 levels in *DEC1*-silencing cells. Strikingly, we found that *SP1* was positively regulated by *DEC1* in LN18, T98G, and U251 cells (**Figure 2F**). To address whether *DEC1* regulates *MGMT* transcription via SP1, we silenced *SP1* expression using siRNAs in T98G and LN18 (**Figure 2E**) to examine the expression of *MGMT* and *DEC1*. As shown in **Figure 2E**, the silencing of *SP1* led to a dramatic decrease in *MGMT* expression but had no effect on DEC1 levels. Thus, *SP1* is a downstream target of *DEC1* and an upstream regulator of *MGMT*.

We then knocked down SP1 with siRNAs in *DEC1*-overexpressing LN18 cells to observe its effects on DEC1-dependent MGMT alterations. The results showed that the regulation of *MGMT* by *DEC1* could be abrogated by *SP1* (**Figure 2G**). Together, these data indicated that *SP1* is an indispensable factor for *MGMT* regulation by *DEC1*.

The SP1-MGMT axis is essential for DEC1-mediated TMZ resistance

We have already provided evidence that DEC1 can promote TMZ resistance and upregulate MGMT expression in GBM cell lines. We next examined whether *DEC1* exerts its role in the cellular response to TMZ via *MGMT*. For this purpose, we further knocked down *MGMT* in *DEC1*-overexpressing cells or overexpressed *MGMT* in stable *DEC1*-silenced T98G and LN18 cells. The expression levels of MGMT were confirmed by Western blot analysis (**Figure 3A**). We then treated these cells with TMZ and determined cell survival via CCK-8 assay and cell apoptosis via flow cytometry assay. As shown in **Figure 3B-D**, cell survival was significantly increased while apoptosis decreased after *DEC1* overexpression, and this was reversed when *MGMT* was simultaneously downregulated. Similarly, *DEC1*-silenced T98G and LN18 cells exhibited significantly lower viability but higher apoptosis rates than their scrambled control group, but this effect could be rescued by forced *MGMT* upregulation. Western blot analyses of *Bcl-2* also corroborated these results (**Figure 3A**). These data indicated that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MGMT
LN18
B
T98G
LN18
The raletive expression level of MGMT
C
Scramble
shDEC1#1
shDEC1#2
D
The relative luciferase activity
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siSP1 #3

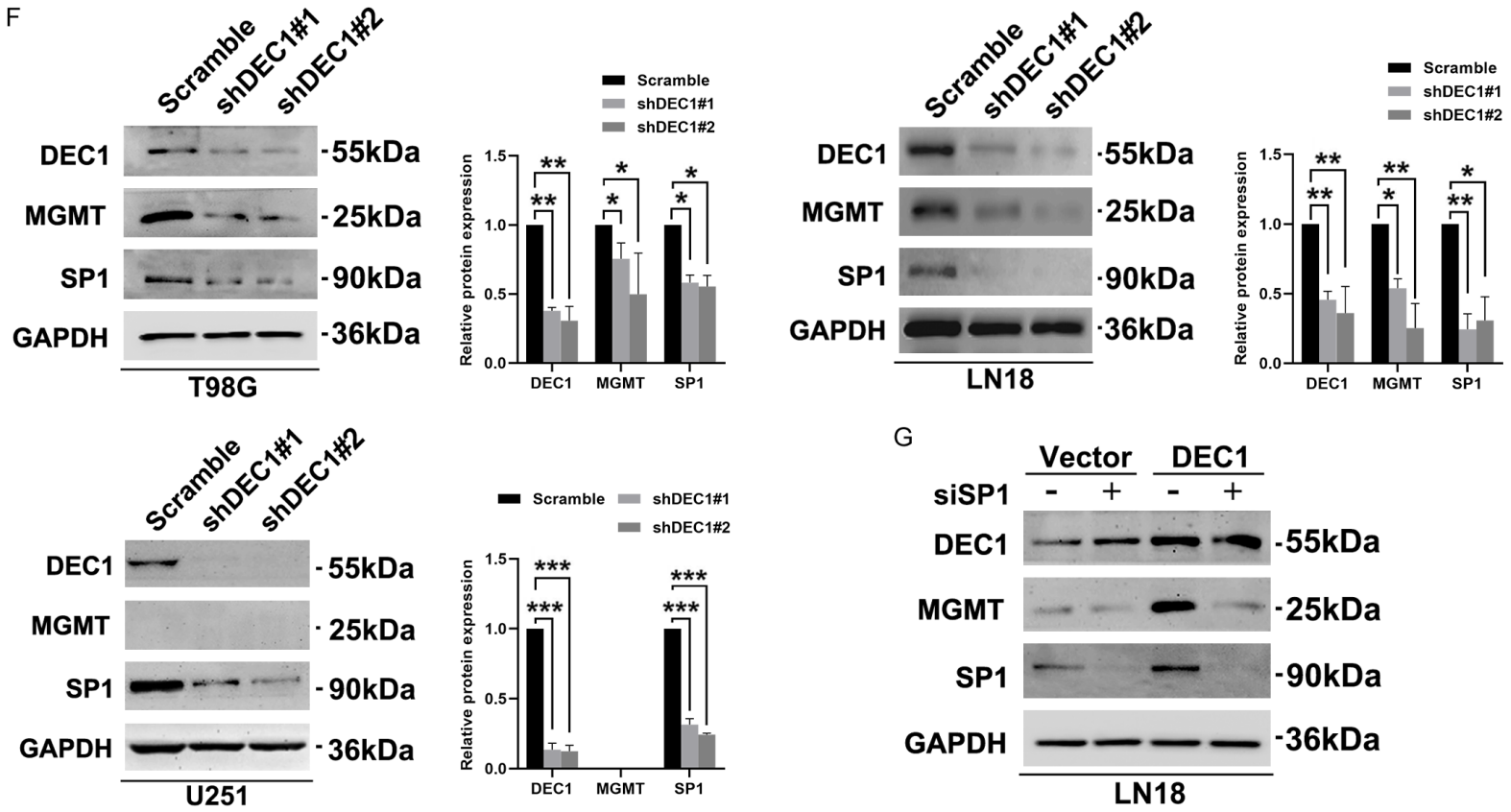
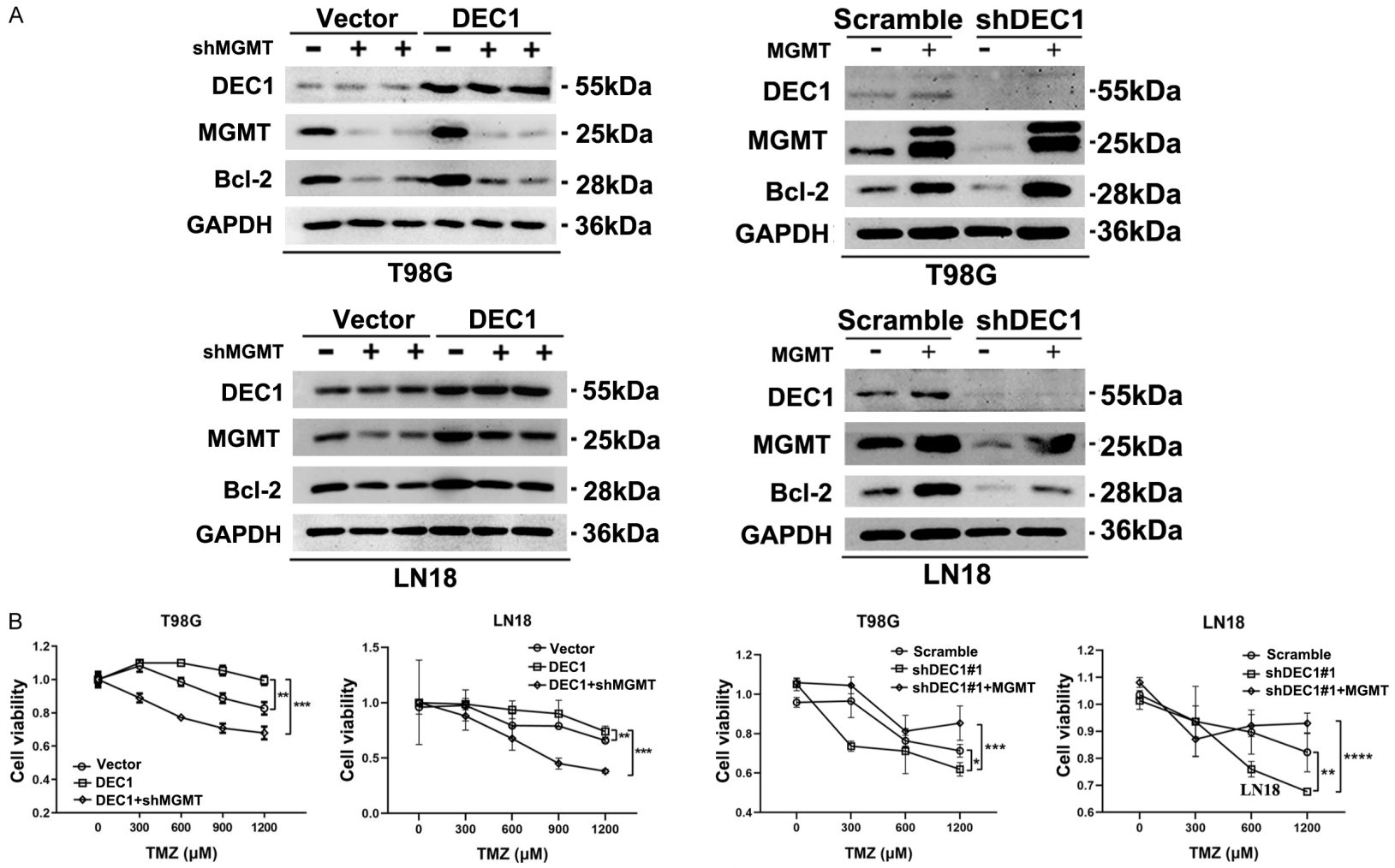


Figure 2. *DEC1* positively regulates *SP1* and *MGMT*. (A) The expression level of DEC1, MGMT and GAPDH in stable GBM cells was detected with Western blot analysis (n = 3). (B and C) The transcriptional levels of MGMT was evaluated using RT-qPCR (n = 3). (D) The 293T cells were transiently co-transfected with the *DEC1*-encoding construct or empty vector, and the control luciferase vector pGL3.0-Luc or the luciferase reporter pGL3.0-pMGMT-Luc. Luciferase activity was evaluated 48 hours after transfection and shown as the ratio of promoter-reporter activity to control vector luciferase activity (n = 3). (E) The LN18 cells were transfected with siNC, siSP1#1, siSP1#2 or siSP1#3. The expression levels of SP1, DEC1, and MGMT were evaluated with Western blot (n = 3). (F) In stable DEC1 GBM cell lines (i.e., T98G, LN18, and U251), the expression level of SP1 along with DEC1 and MGMT were evaluated with Western blot (n = 3). (G) Stable *DEC1*-overexpressing LN18 was transiently transfected with si*SP1*#1 or siNC. The expression levels of MGMT along with SP1 and DEC1 were evaluated with Western blot. In (B-D), the data are presented as the mean ± SD. Unpaired t test for two-groups datasets. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$.


A
Vector
DEC1
shMGMT
DEC1
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GAPDH
55kDa
25kDa
28kDa
36kDa
T98G
Scramble
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MGMT
LN18
B
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Cell viability
TMZ (μM)
Vector
DEC1
DEC1+shMGMT
Scramble
shDEC1#1
shDEC1#1+MGMT

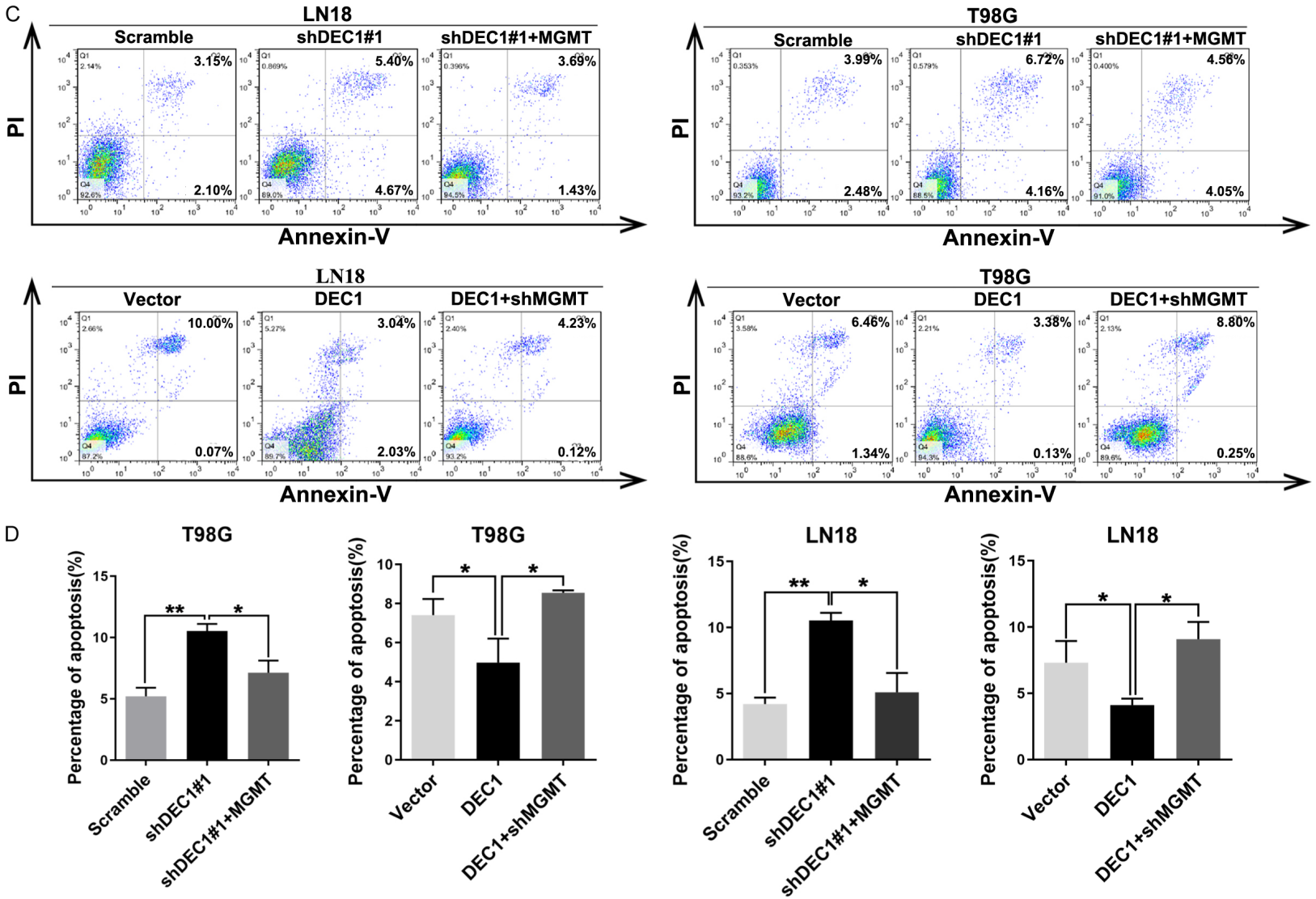

Figure 3. The SP1-MGMT axis is essential for DEC1-mediated TMZ resistance. After being individually infected with *DEC1* or *shDEC1* recombinant lentivirus, the T98G and LN18 cells were further infected with *MGMT*, *shMGMT* or their individual empty control recombinant lentivirus and selected with hygromycin and G418 to establish stable cell lines. (A) Cells were treated with TMZ (600 μM) for 48 hours. The expression level of MGMT, DEC1, and Bcl-2 were determined by Western blot. (B) Cells indicated were treated with different concentration of TMZ for 48 hours, and cell survival was evaluated by CCK-8 assay (n = 3). (C and D) Cells were treated with 600 μM TMZ for 48 hours and cell apoptosis was evaluated by flow cytometry analysis (n = 3). Representative images are shown in (C). In (B and D), data are presented as the mean ± the SD. Unpaired t test for two-groups datasets. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$, ****; $P < 0.0001$.

MGMT is an indispensable factor that mediates the effect of *DEC1* on TMZ resistance.

Secreted protein 1 (SP1) is indispensable for *MGMT* regulation by *DEC1* (see **Figure 2G**); therefore, we then investigated whether *SP1* has an important role in *MGMT* expression and resistance to TMZ. To test this, we knocked down SP1 via siRNA in T98G and LN18 cells and observed the effects on TMZ sensitivity by performing CCK-8 assays, apoptosis analyses, and Western blots. When *SP1* was repressed, *MGMT* expression was also markedly downregulated (**Figure 4A**), and more cells underwent apoptosis (**Figure 4A-C**). More importantly, the effects of *SP1* knockdown on cell survival and apoptosis was entirely abrogated if MGMT was forcedly overexpressed in the cells at the same time (**Figure 4A-C**). These results collectively indicated that the *SP1-MGMT* axis is essential for *DEC1*-mediated TMZ resistance.

DEC1 is overexpressed in GBM and is correlated with the expression level of MGMT

Based on the findings of our previous study [3], high expression levels of *DEC1* are associated with poor prognosis and chemotherapeutic resistance to TMZ. In this study, we examined the discrepancy between endogenous *DEC1* expression levels in GBM (T98G, LN18, and U251) and normal cerebral (i.e., HEB) cells by Western blot, and found that *DEC1* was overexpressed in GBM cell lines (**Figure 5A**). We also analyzed the glioma database provided by the Cancer Genome Atlas (GII: 249 samples, GIII: 265 samples, GIV: 153 samples). Student's t-test analysis confirmed that the expression level of DEC1 in glioblastoma was significantly higher than those in GII and GIII giloma (**Figure 5B**, GIV vs GII, G vs GIII, $P < 0.0001$). To further explore the clinical relevance of *DEC1* and *MGMT* expression, immunohistochemical (IHC) staining of glioblastoma TMA, containing 35 patients and 5 normal tissue samples, was used to determine the expression levels of DEC1 and MGMT. The result showed that the expression levels of both DEC1 and MGMT were significantly higher in glioblastoma tissues than in normal brain tissues (**Figure 5C** and **5D**, $P < 0.01$); moreover, *DEC1* expression levels were evaluated by IHC, and patients were stratified as low *DEC1* expression (IRS < 6) or high *DEC1* expression (IRS ≥ 6). According to the IRS of MGMT, we found that percentage of high *MGMT* expression (IRS ≥ 6) in high *DEC*1 expression group is much higher than those in low *DEC1* expression group (**Figure 5E** and **5F**, $P < 0.05$). These findings are in accordance with our cytological results, indicating that the expression of *DEC1* is positively associated with that of *MGMT*. In summary, *DEC1* is upregulated in GBM tissue, compared to normal human tissue, and its expression is correlated with the GBM malignancy grade, and the expression of *MGMT*.

Discussion

Our previous study showed that *DEC1* is positively associated with grade, prognosis, and TMZ resistance of glioma [3], but the molecular mechanisms for these effects remained unclear. Our study demonstrated that forced expression of *DEC1* suppressed TMZ-induced cell apoptosis, whereas the knockdown of *DEC1* had the opposite effect in MGMT-positive glioma cell lines, which indicated that *DEC1* promoted TMZ resistance in an MGMT-dependent manner. Regarding the mechanism, we confirmed that *DEC1* positively regulated *MGMT* at the transcriptional level. We further showed that *SP1* was an essential factor for the regulation of *MGMT* by *DEC1*. Moreover, *DEC1*-dependent TMZ resistance can be blocked by MGMT manipulation. These data illustrated that *DEC1* is involved in glioma cell sensitivity to TMZ by regulating the *SP1-MGMT* axis. In the samples of TCGA, *DEC1*'s expression level increased in GBM compared to gradeII or gradeIII samples, which indicated that DEC1 was positively associated with the glioma grades. Finally, immunohistochemistry confirmed that the expression of *DEC1* and *MGMT* was increased in GBM tissues and the expression level of *DEC1* was positively associated with those of *MGMT*. Our data demonstrated for the first time that *DEC1*, as an important upstream regulator of *MGMT*, controlled TMZ sensitivity. This finding suggested that it is a novel potential target for glioma treatment.

The expression of *DEC1* is differentially upregulated or downregulated among various cancers and is associated with tumor grade, malignant characteristics, and patient prognosis in various cancers [10, 21, 30-32]. However, the evidence is inconsistent and even contradictory

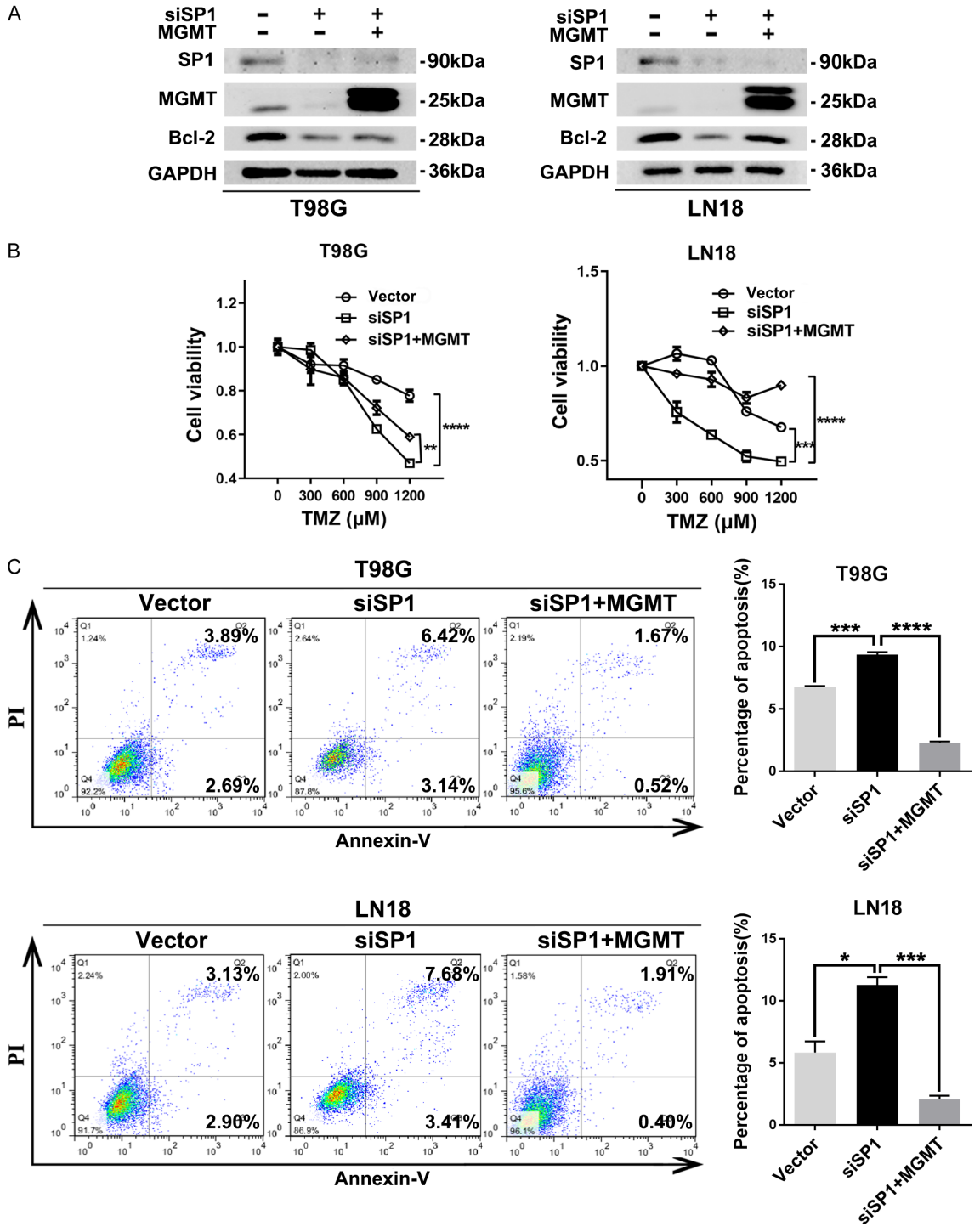

Figure 4. SP1 regulates TMZ sensitivity via MGMT. The T98G and LN18 cells infected with *MGMT* or empty Cherry recombinant lentivirus were further transiently transfected with si*SP1*#NC or si*SP1*#1. A. Cells were treated with TMZ at 600 μM for 48 hours. The expression level of SP1, MGMT, DEC1, and Bcl-2 were analyzed using Western blot. B. Cells were treated with different concentrations of TMZ for 48 hours and cell survival were evaluated using CCK-8 assays (n = 3). C. Cell apoptosis was evaluated by using flow cytometry analysis (n = 3). Data are presented as the mean ± the SD. Unpaired *t* test for two-groups datasets. *; $P < 0.05$, **; $P < 0.01$, ***; $P < 0.001$, ****; $P < 0.0001$.

regarding whether DEC1 functions as a tumor promoter or suppressor. For example, *DEC1* was shown to inhibit cell proliferation of breast cancer [33], colon cancer [34], and oral cancer

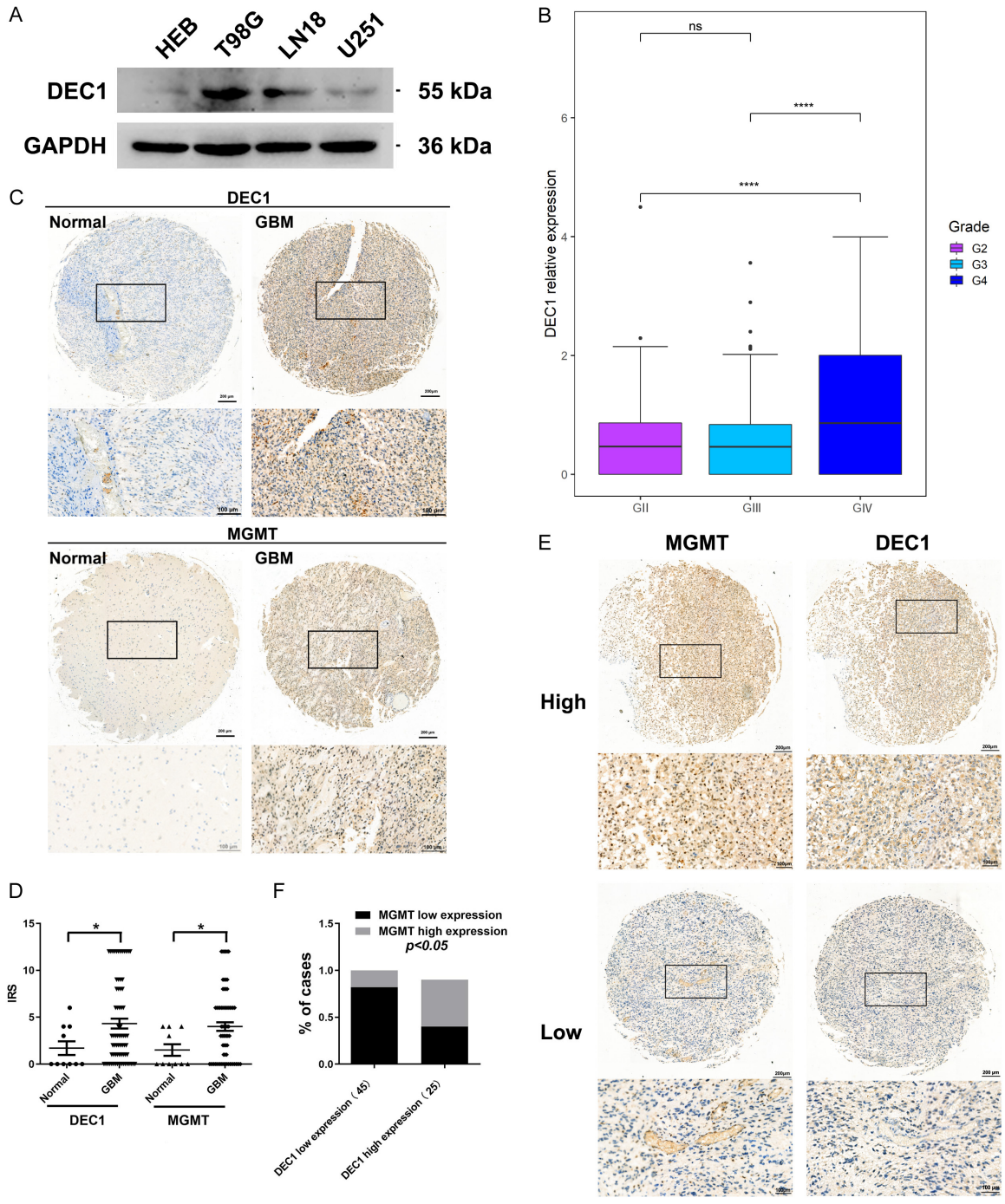

Figure 5. The expression of *DEC1* is upregulated in GBM and correlated with the expression of *MGMT*. A. The expression level of DEC1 was analyzed using Western blot in normal human brain cell line HEB and GBM cell lines (T98G, LN18, and U251). B. The correlation between the expression level of DEC1 and the glioma grade was analyzed, based on the database information provided by the Cancer Genome Atlas (TCGA). C and D. The expression level of DEC1 and MGMT were analyzed by immunohistochemical staining of glioma tissue microarray, which contains 35 GBM tissues and 5 normal brain tissues. E. Representative immunohistochemistry images with high and low levels of DEC1 and MGMT are shown. F. The IRS scores of DEC1 and that of MGMT (low expression group: IRS < 6 or high expression group: IRS ≥ 6) were counted and the percentage of high/low MGMT in DEC1 high/low were shown. $P < 0.05$, chi-square test was applied.

cells, probably by regulating cyclin D1 [22] or cyclin E [33]. By contrast, DEC1 promotes cell growth in thyroid cancer [17] and gastric cancer [19] by controlling cell cycle-related genes.

Moreover, *DEC1* facilitates epithelial-mesenchymal transition processes and invasiveness in breast [21], thyroid [17], pancreatic [37], and liver cancers but inhibits migration and invasion in oral cancer cells [10]. Thus, DEC1 may act as a tumor promoter or suppressor, depending on different tumor tissue contexts. Our previous study showed for the first time that in glioma, the expression of *DEC1* is upregulated and positively associated with the tumor grade. Our current study further demonstrated that *DEC1* can antagonize TMZ-induced cell apoptosis, thereby promoting TMZ resistance. Of note, *DEC1* was reported to be upregulated in several types of cancers and to protect against serum-starvation-induced apoptosis by transcriptionally activating *SURVIVIN*, an anti-apoptotic protein [14], or antagonize DNA damage-induced cell death by interacting with *p53* and weakening its induction of *MIC-1* [38]. Similarly, *DEC1* inhibited apoptotic effects in mouse mammary carcinoma cell lines induced by a TGF-βR kinase inhibitor [39]. Interestingly, there is also contradictory evidence showing that *DEC1* exhibits pro-apoptotic effects in human esophageal [40] and breast [41] cancer cells after treatment with chemotherapeutic agents such as cisplatin or paclitaxel [40, 41]. As such, the exact role and mechanism of *DEC1* may be highly context-dependent in different types of cancer in response to various stimuli, deserving further investigations.

The novel mechanistic finding of our study is that *DEC1* regulates the *SP1-MGMT* axis in glioma cells. It is well-established that *MGMT* is a key regulator of TMZ sensitivity in glioma cells, and epigenetic silencing, via methylation of its promoter, contributes so much to its expression level that the MGMT methylation status is used as a predictive marker for therapy [13]. Recently, accumulating studies have revealed that *MGMT* is subjected to regulation by a variety of transcription factors, such as AP1 [42], SP1 [8], and NF-κB, which play an important role in the resistance of glioma to TMZ [24, 43].

In particular, many *SP1* sites exist within the *MGMT* promoter so the basal level of *MGMT* expression is primarily determined by the SP1 level [8]. Consistent with this finding, we showed that *SP1* knockdown significantly reduced the *MGMT* expression level and enhanced TMZ-induced apoptosis in glioma cells. Of note, the study by Kitange et al. [28] also demonstrated that increased SP1 recruitment within the *MGMT* promoter is involved in *MGMT* upregulation and acquired TMZ resistance in GBM. Considering that *SP1* is upregulated in GBM and promotes glioma cell proliferation [35], whereas its inhibition leads to decreased cell migration and invasion in glioma and other cancers [35, 36], our study is in support of other publications and strengthens the notion that *SP1* might be a potential therapeutic target in glioma.

In addition, we showed that *DEC1* regulates TMZ sensitivity via the *SP1-MGMT* axis because the effects of *DEC1* on TMZ-induced cell apoptosis rely on the presence of *MGMT* and can be completely abolished by the manipulation of *MGMT* expression. As a transcription factor, DEC1 can either activate or repress its target genes [25]. Interestingly, DEC1 can not only directly bind to the *SP1* sites of its target genes to activate gene transcription such as *SURVIVIN* [14] but also interact with SP1 and prevent its binding to the promoter to suppress *CLDN1* transcription [15]. Our current study showed that *DEC1* can also regulate SP1 levels, albeit the detailed mechanism still needs to be clarified. Together, it seems an intimate interplay exists between SP1 and DEC1 in the regulation of gene transcription, wherein DEC1 can function by occupying *SP1* sites, sequestering SP1 binding, or regulating *SP1* content.

We provided robust evidence that *DEC1* regulates TMZ sensitivity via the *SP1-MGMT* axis and that its expression is upregulated in GBM and correlated with the expression of *MGMT*; however, we have not clarified how *DEC1* regulates *SP1*. It is still needed to address whether *DEC1* regulates *SP1* transcriptionally, post-transcriptionally, or epigenetically, and to evaluate its clinical relevance. In addition, a major limitation of this study was that glioma cell lines, but not patient-derived primary glioma cells or an orthotopic glioblastoma mouse model, were utilized to identify the role of *DEC1* in glioma, which is worth an in-depth investigation in the future.

In conclusion, and to the best of our knowledge, our study, for the first time provided evidence that *DEC1* promotes the chemotherapeutic resistance of glioma cells to TMZ via positively

regulating the *SP1-MGMT* axis. Our study also explained the mechanism of TMZ resistance in GBM treatment. We anticipate that our findings may provide new therapeutic and prophylactic target molecules to improve prognosis for patients.

Acknowledgements

This work was supported by the Natural Science Foundation of China (grant no.81572469), and Technology Breakthrough Project of Shaanxi Province (2016SF-094). All research activity was monitored with regard to study design, collection of data and its analysis. The final version of the manuscript was revised by Elsevier Language Editing Services.

Disclosure of conflict of interest

None.

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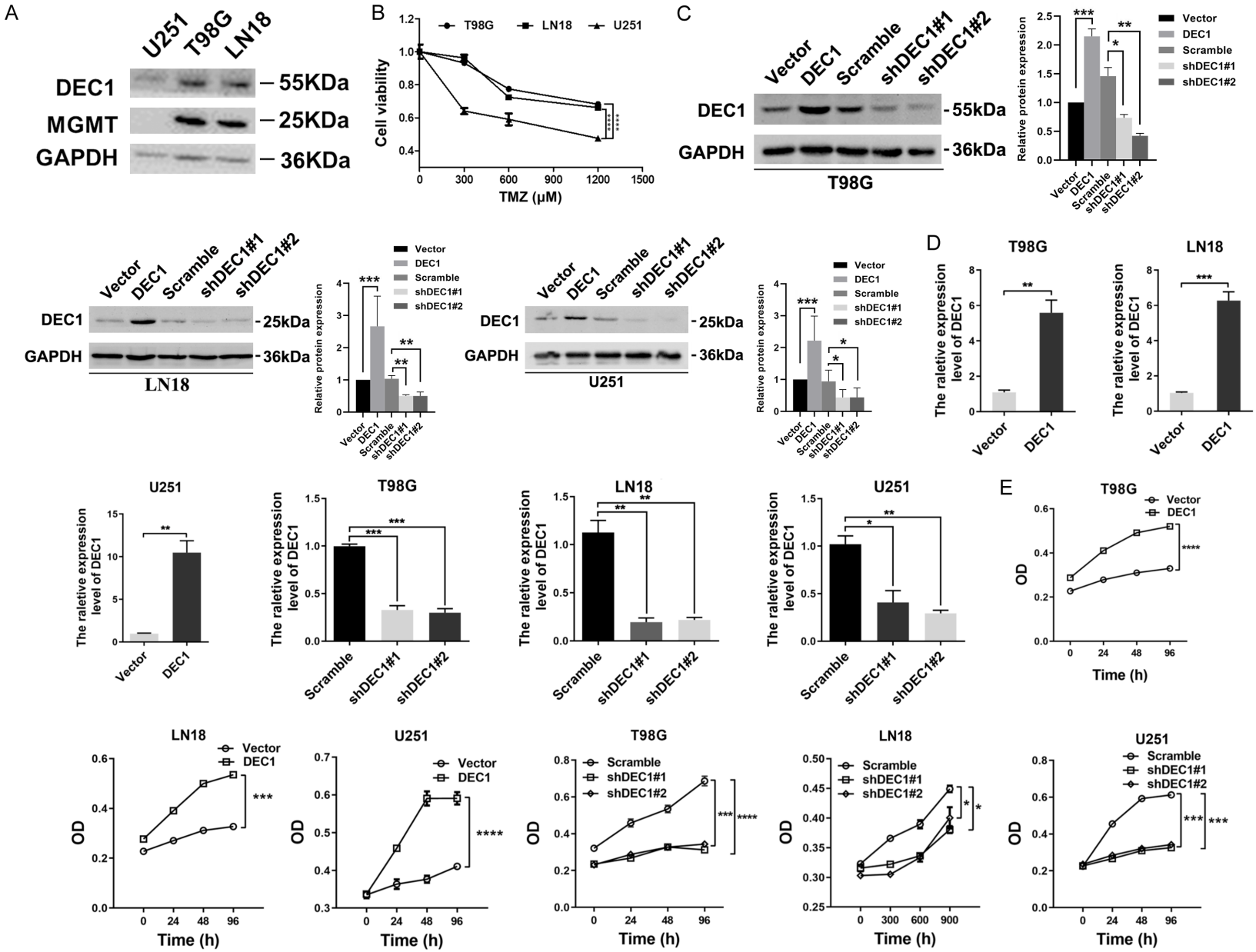
A
U251
T98G
LN18
DEC1
MGMT
GAPDH
55KDa
25KDa
36KDa
B
T98G
LN18
U251
Cell viability
TMZ (μM)
C
Vector
DEC1
Scramble
shDEC1#1
shDEC1#2
DEC1
GAPDH
55kDa
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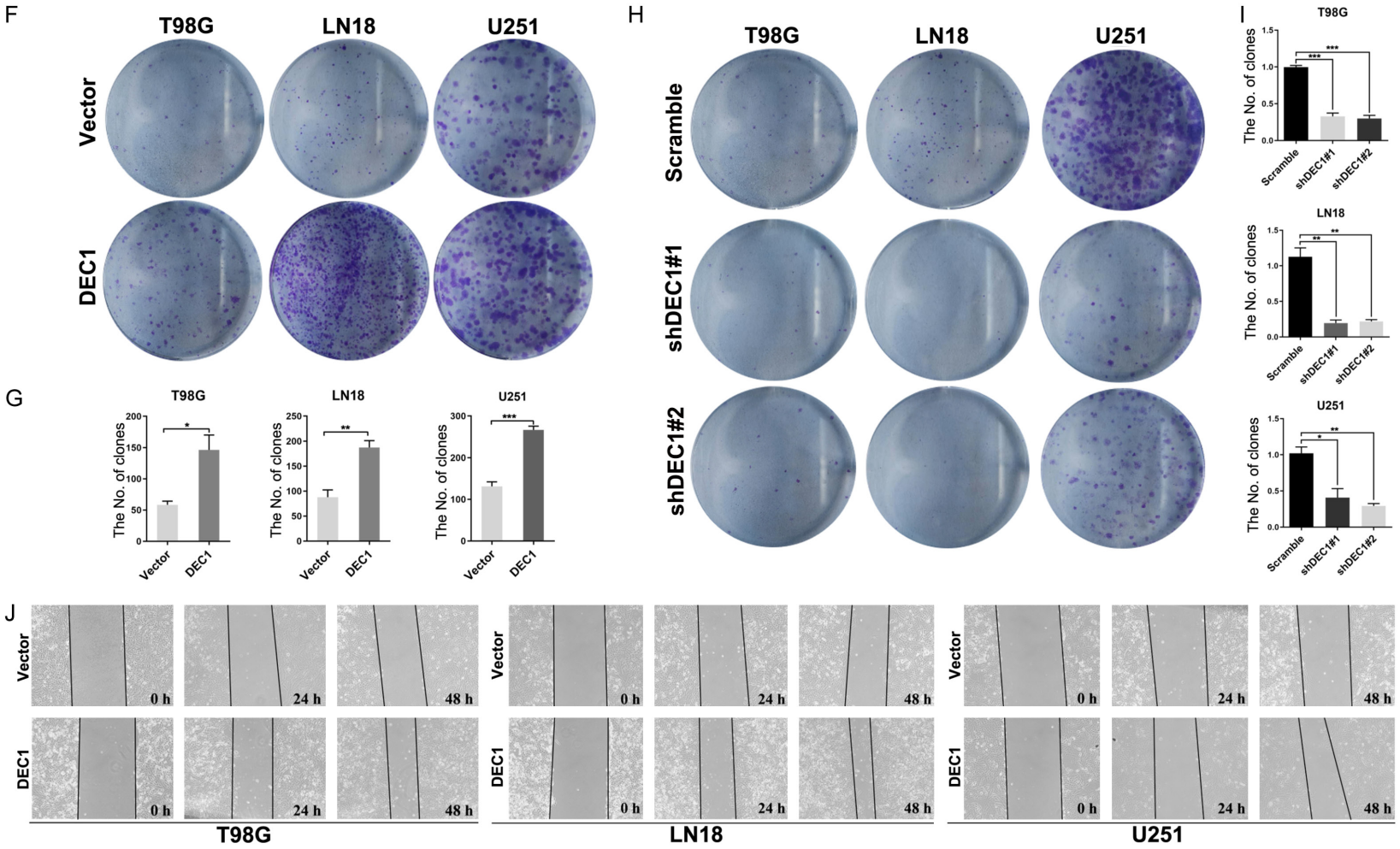

Figure S1. (A) The expression level of DEC1 in GBM cell lines (i.e., T98G, LN18 and U251) and MGMT was analyzed with western blot. (B) The GBM cell lines T98G, LN18 and U251 were treated at different concentrations of TMZ for 48 hours. The cell viability, based on TMZ half-maximal inhibitory concentration (IC50) doses, was evaluated by using CCK-8 (n = 3, ****P < 0.0001, the U251 group versus the LN18 group, the U251 group versus the T98G group). (C and D) The stable DEC1 GBM cell lines (i.e., T98G, LN18, and U251) were confirmed with western blot analysis (C) and RT-qPCR (D) (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001 versus the control group). (E) The viability of stable DEC1 GBM cell lines were analyzed at different time points by using CCK-8 assay (n = 3, *P < 0.05, ***P < 0.001, ****P < 0.0001 versus the control group). (F-I) Stable DEC1 GBM cell lines were seeded into 6-well plates and cultured for 14 days to examine colony-forming ability. (J) Migration of stable DEC1 GBM cell lines were evaluated by wound healing assay (n = 3, *P < 0.05, **P < 0.01, ***P < 0.001 versus the control group). The data are presented as the mean ± the SD. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.