The Role of Polycomb Repressive Complex 2 and Effects of Lobarstin in Glioblastoma Multiforme

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The Role of Polycomb Repressive Complex 2 and Effects of Lobarstin in Glioblastoma Multiforme

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So Jin Kim

December 2013

This certifies that the dissertation of So Jin Kim is approved.

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ABBREVIATIONS

BER : Base excision repair

EED : Embryonic ectoderm development

EZH : Enhancer of zeste homolog

GBM : Glioblastoma multiforme

H3K27me3 : Trimethylation on histone H3 at lysine 27

MGMT : O^6-methylguanine-DNA methyltransferase

MMR : Mismatch repair

MPG : N-methylpurine-DNA-glycosylase

PcG : Polycomb group

PRC : Polycomb repressive complex

PTP1B : Protein tyrosine phosphatase 1B

RT-PCR : Reverse transcription-polymerase chain reaction

SUZ12 : Supporting proteins suppressor of zeste

TMZ : Temozolomide

ABSTRACT

Glioblastoma multiforme (GBM) is a grade IV primary malignant brain tumor. The elevated expression of polycomb repressive complex 2 (PRC2) proteins such as EZH2 and SUZ12 have been found in GBM and their expression levels often correlate with poor prognosis. In order to find anti-proliferative factors and to increase the potential of anti-cancer drug for glioblastoma treatment, I studied two diffetent approaches. First, I tested that PRC2 may play as master regulator in GBM. Second, I investigated the influence of co-treatment with lobarstin and TMZ on chemosesitivity of glioblastoma cells. Upon knock-down of PRC2, cell growth was attenuated and cells were accumulated at G_1 phase of the cell cycle. Among several G_1 regulators, the total and nuclear CDKN1B protein levels were drastically up-regulated by PRC2 depletion. Interestingly, the expression of SKP2, a component of the SCF^{SKP2} E3 ubiquitin ligase which is known to promote the degradation of CDKN1B protein degradation was significantly reduced under depleted expression of PRC2. Furthermore, PRC2-depletion led to down-regulation of MYC expression at both mRNA and protein levels. In agreement with the *in vitro* results, glioblastoma patients with elevated EZH2 expression showed high MYC expression but reduced CDKN1B expression. Temozolomide (TMZ) has been used as standard therapy for glioblastoma patients, however, this treatment does not improve the prognosis and survival of patients because of rapid DNA repair system. Upon transient PRC2-depletion, TMZ-induced cytotoxicity and the DNA damage were

found to are extended. Interestingly, PRC2-depletion had no effect on MGMT expression, but the expressions of the base excision repair (BER) genes such as PARP1, XRCC1, and LIG3 were significantly decreased. In agreement with the *in vitro* results, glioblastoma patients with high EZH2 expression showed increased PARP1 expression level. In order to evaluate the possibility of developing combinatorial therapy for GBM, I tested the combination effect of lobarstin and TMZ on T98G cells. Co-treatment with lobarstin and TMZ resulted in enhanced cytotoxicity for GBM cells through repressing DNA-recovery. Expressions of DNA repair genes such as MGMT, PARP1 and LIG 3 were significantly reduced in lobarstin and TMZ co-treated cells. Taken together, these results showed that the PRC2-depletion enhances inhibitory effect on GBM growth and potentiates TMZ antitumor activity. In summary, the effects of PRC2 can be considered as a potential targets for developing a novel therapeutic approaches. Additionally, lobarstin can be used as a potential combination drugs with TMZ for efficient GBM therapy.

Keywords: glioblastoma, polycomb repressive complex 2, EZH2, SUZ12, lobarstin, temozolomide, DNA repair

I. INTRODUCTION

Glioblastoma arises from the malignant transformation of astrocytes**,** is a highly aggressive brain tumor [1, 2] and is classified into four different grades (I, II, III and IV) based on the degree of malignancy [3]. Glioblastoma multiforme (GBM) is classified as grade IV and is the most aggressive form of glioblastoma accompanied by extremely poor prognosis due to the fact that the most of the GBM patients die within 2 years from the diagnosis [4, 5]. GBM has the typical characteristics of rapid, uncontrolled cell proliferation, necrosis formation, evasion of apoptosis, elevated angiogenesis and microvascular proliferation [6, 7]. Malignancy of GBM results from several genetic abnormalities such as increased levels of epidermal growth factor receptor (*EGFR*) and platelet-derived growth factor receptor (*PDGFR*), disruptions of cell cycle regulation by *INK4a-ARF* abnormalities, mutations in the *TP53* tumor suppressor gene associated with *CDK4* amplification or *RB* loss [1, 6].

Currently, standard treatment for GBM consists of surgical resection, radiotherapy, concomitant and adjuvant with temozolomide (TMZ). However, the outcome are extremely poor [7] and these treatments result in several side effects that diminishes the quality of life of the patients. Among several chemotherapeutics, TMZ is the most widely used for the treatment of GBM and induces the cell death by generating a spectrum of methyl-adducts on DNA [8, 9]. The major site of TMZ-induced DNA methyl-adducts are N^7-methylguanine (>70%), N^3-methyladenine (9.2%), and O^6-methylguanine (5%) [10-12]. The cytotoxicity of TMZ is mainly dependent on the

efficiency of DNA repair system. Improper DNA repair system results in DNA nick formation followed by cell apoptosis and cell death [13]. Although O^6-methylguanine adduct is about 5%, which is consider as most toxic lesion and is directly repaired by O^6-methylguanine methyltransferase (MGMT) and mismatch repair (MMR) [14].

Another important mechanism involved in resistance to TMZ therapy is a base excision repair (BER), which repairs N^7-methylguanine and N^3-methyladenine DNA adducts. BER is initiated by N-methyl-purine DNA glycosylase (MPG) which creates apurinic/apyrimidinic (AP) site through the N-glycosidic bond cleavage of deoxy-guanosine residues [15]. Next, AP endonuclease 1 (APE1) recognizes and cleaves an AP site to yield a 3′-hydroxyl group adjacent to a 5′-deoxyribose phosphate [15]. Poly (ADP-ribose) polymerase 1 (PARP1) acts as a DNA nick-sensor, binds to DNA damage sites, and catalyzes poly **(**ADP-ribosylation**)** the chromatin-associated protein including itself [16, 17]. X-ray repair cross-complementing protein 1 (XRCC1) has a tendency to interact with PARP1. Thus, XRCC1 acts as a scaffolding protein by directly binding to PARP1 and activates other BER components such as DNA polymerase beta (POLß) and DNA ligase III (LIG3) [18] which are involved in the DNA repair mechanism. In spite of N^7-methylguanine and N^3-methyladenine DNA adducts comprise most of the TMZ-induced DNA lesions, toxicity is relatively less due to rapid repair of these adducts by BER [9]. Many agents, including the MGMT inhibitor [19], PARP1 inhibitor [20], ribonucleotide reductase inhibitors [21], anti-epileptic drugs [22, 23], resveratrol

[24], rapamycin analogs [25] and cold atmospheric plasma [26] have been reported to enhance the sensitivity of TMZ [27]. However, further research remains until the usage of these agents at the clinical level.

The epigenetics is defined as a regulation of the gene expression by modifying the conformation of chromatin or DNA, that is not due to changes in the nucleotide sequence of the gene, but the changes persists for generation [28-30]. Epigenetic gene regulation is essential for the silencing of a multitude of cellular gene loci including embryonic development, maintenance of cell fates and X-chromosome inactivation [31-35], polycomb group (PcG) and trithorax group (trxG) proteins are considered as critical regulators [36]. TrxG gene expression remains in active state which is characterized by the methylation of histone H3 at Lys 4 (H3K4). On the other hand, PcG shows repressive function and counteracts this gene inactivation by tri-methylation of histone H3 at Lys27 (H3K27me3) [37]. PcG proteins were discovered first in the fruit fly *Drosophila melanogaster* development as repressors of Homeobox (*HOX*) genes, which are necessary for determining the body plan and the formation of body segments [38]. In mammalian cells, PcG proteins form large multimeric complexes that silence the target genes as well as *HOX* genes through chromatin modification such as acetylation and methylation [38, 39]. Recently, several studies have reported that the dysregulation of PcG proteins have been found in various cancer types including glioblastoma and strongly associate with a poor prognosis or metastasis [33, 40].

PcG proteins are classified into two distinct multi-protein complexes, the

polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) based on their roles for gene suppression [32]. PRC1 consists of BMI1/RING1/Mel-18 proteins which is required for the maintenance of silenced state through ubiquitination of histone H2A at lysine 119 (H2AK119ub), and participates in compaction of polynucleosomes [35, 41, 42]. These proteins play an important role in the regulation of differentiation and the maintenance of stem cell populations [31, 43]. PRC2 contains the PcG proteins such as enhancer of zeste homolog 2 (EZH2), supporting proteins suppressor of zeste 12 (SUZ12) and embryonic ectoderm development (EED) [32] and is involved in the initiation of silencing through H3K27me3 [32, 44]. PRC2 catalyzes the H3K27me3 whereas PRC1 dynamically recruits the modified histones, which then mediates the ubiquitination of H2AK119 resulting in the repression of targeted genes. PRC2 leads to a more condensed chromatin and cooperates with histone deacetylases (HDAC), DNA methyltransferase (DNMT), co-repressor, etc [45, 46].

EZH2 contains the catalytic SET domain and transfers a methyl group from S-adenosylmethionine (SAM) when complexed with SUZ12 and EED [35, 47-51]. SUZ12 is required for the stabilization of EZH2 along with EED [31, 52, 53]. Several studies showed that EZH2 regulates the silencing of tumor suppressor genes (*e.g.*, $p16^{INK4A}$, ADRB2, DAB2IP, Kruppel-like foator) [54-58], the regulations of cell cycle (*e.g.*, EGFR2, p53, Wnt/b-catenin, Ras), invasion (*e.g.*, NF-kB, ß-adregenic signaling, E-cadherin), differentiation (*e.g.*, BMP, Notch signaling) and angiogenesis (*e.g.*, VEGF) [59-62]. In glioblastoma, EZH expression level increases

along with tumor grade and is also known to play a key role in stem cells maintenance [63, 64]. Down-regulation of EZH2 by RNA interference induces cell cycle arrest at G_0/G_1 phase and apoptosis in U87 human glioma cells [65]. However, many aspects of molecular mechanisms associated with EZH2 and glioblastoma cell proliferation are still unclear. Recent studies have shown that SUZ12 expression level is also elevated in several human cancers, signifying its importance in cell proliferation, invasion and blocking apoptosis [52, 66]. Although, SUZ12 plays a role in tumorigeneisis, its molecular mechanisms in the progression of tumorigenesis are not yet clearly understood. Based on fact that the SUZ12 knockdown strongly impaired the EZH2 activity as well as expressions [52, 67, 68], I determined the substantial impact of PRC2 silencing in human glioblastoma. In order to investigate the tumorigenesis and chemoresistance by targeting PRC2 in glioblastoma, I generated PRC2-knockdown cell lines by using lenti-viral shSUZ12 system in T98G Cells.

From GBM genome analysis, the deletion of *CDKN2A/CDKN2B* locus and the amplifications of *EGFR*, *PDGFRA*, *CDK4* genes were reported to be involved in cell proliferation and the deficient frequency is as high as 46.4%, especially for *CDKN2A/CDKN2B* [69]. *CDKN2A/CDKN2B* loci is a target for PcG which is involved in cell senescence, regulation of the cell cycle, and maintenance of stemness [70-77]. If PcG delays the cell cycle process in glioblastoma, then there could be a possibility of another CDK inhibitor function other than *CDKN2A/CDKN2B*. The cyclin-dependent kinase inhibitor (CDKN1B) is a central

regulator of the cell cycle [78] leading to an arrest in the G_1-phase of the cell cycle by inhibiting the activity of Cyclin-CDK2 [79]. CDKN1B frequently acts as a potent tumor suppressor in many aggressive human cancers, and its inhibition is associated with poor prognosis in many cancers [80, 81]. Degradation of CDKN1B is regulated by two different mechanism, one is by ubiquitin-mediated degradation and another is by proteolytic processing by 26S proteasome [82, 83]. In case of ubiquitin-mediated degradation, CDKN1B protein level is reduced due to the rapid protein degradation by ubiquitin protein ligase complex called SCF (SKP1-cullin-F-box) [84, 85]. The S-phase kinase protein, SKP*2* is overexpressed in human cancers [86]. Down-regulation of SKP2 leads to G_1 arrest and growth inhibition [87]. SKP2 is a component of SCF complex, which is required for the ubiquitin-mediated degradation of several cell cycle regulators, such as the cell cycle inhibitor CDKN1B and Cyclin D1[78, 88]. SKP2 recruits CDKN1B to the SCF complex and induces polyubiquitination and promotes for CDKN1B degradation through the 26S proteasome [78, 84, 89-91]. MYC, a proto-oncogenic transcription factor promotes cell proliferation and plays a central role in the genesis of many human cancers [92-95]. In human leukemia cells, SKP2 is known as a direct target to MYC and consequently leads to MYC-SKP2-CDKN1B axis [90, 96]. However, the existence of the MYC-SKP2-CDKN1B axis and its regulatory mechanisms in glioblastoma remains to be investigated.

Several lichen extracts have been used as the traditional folk medicine for the disease treatments and recent studies have suggested that lichen metabolites contain

many effective biological activities such as antibiotic, anti-mycobacterial, anti-viral, analgesic, and anti-pyretic [97, 98]. Antarctic lichen produce a great variety of secondary metabolites and most of them are unique because they have protective mechanisms to avoid DNA damage under extreme environment including high UV radiation and low temperature [99-101]. Therefore, the possibility of using lichen metabolites as potential and novel medical drugs has become a promising approach [97]. Recently several metabolites from the Antarctic lichen *Stereocaulon alpinum* have been isolated [97, 102, 103] and proved to have several biological activities such as antibacterial and antioxidant activity as well as protein tyrosine phosphatase 1B (PTP1B) inhibitory activity which acts as a negative regulator for insulin signaling [101]. Among several metabolites, lobarstin is known to play an important role as PTP1B inhibitor. However, its exact function is not yet clearly understood. Therefore, further studies are required to elucidate the molecular mechanism and its regulatory functions. My main aim of the present study is to develop effective anticancer drug for the difficult-to-treat glioblastoma patients. In order to achieve this goal, Firstly, I investigated on anti-proliferatory effects of PRC2 as a potential therapeutic target against human glioblastoma and found the molecular mechanism of PRC2 during cell cycle regulation in the PRC2-depleted T98G cells. PRC2-depletion increased the stability of CDKN1B protein via down-regulation of MYC in SKP2 pathway resulting in G_1 phase arrest during cell cycle. Secondly, I investigated PRC2 role in the regulation of chemosensitivity in glioblatoma cell line and proved that the PRC2 enhances TMZ-sensitivity via down-regulation of BER

genes such as *PARP1*, *XRCC1*and *LIG3*. Finally, I found that the co-treatment with lobarstin and TMZ enhances TMZ treatment efficiency through down-regulation of MGMT and PARP1 both transcriptionally and translationally.

Taken together, my present data showed that the PRC2 can be considered as a promising target for novel GBM therapeutic strategies and Lobarstin might be used as potential combinatorial drug for GBM therapy.

II. MATERIAL AND METHODS

1. Cell culture

Human glioblastoma T98G cells (generous gift from Dr. S. S. Kang of Gyeongsang National University, Korea) and the human embryonic kidney cell line 293T (generous gift from Dr. H. Y Chung of Hanyang University, Korea) were cultured in DMEM supplemented with 10% fetal bovine serum (J R Scientific, Woodland, CA, USA), 100 units/ml of penicillin and 100 μg/ml of streptomycin sulfate (Welgene, Daegu, Korea).

2. Reagents

TMZ, propidium iodide and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO, USA). RNase A was purchased from Bio Basic (Markham, Ontario, Canada) and Puromycin was purchased from InvivoGen (San Diego, CA, USA). TMZ was dissolved in DMSO. Cyclohexamide and BCNU were dissolved in Ethanol.

3. Plasmid preparation and transfections

3-1. Cloning short hairpin RNA into lenti-viral vector

For gene silencing, shLuc and shSUZ12 targeting CTTCGAAATGTCCGTTCGGTT of firefly luciferase gene and AAGCTGTTACCAAGCTCCGTG of human SUZ12 gene respectively, were cloned into pLB lentiviral vector as described [104]. Packaging vectors were psPAX2 and pMD2.G purchased from Addgene (Cambridge, MA, USA).

3-2. Transfection of lenti-viral vector and transduction of lenti-virus

To generate infectious lenti-viral particles, HEK-293 T cells were cultured in D10 medium (DMEM containing 10% fetal bovine serum, 2 mM L-glutamine (Welgene), 10 mM HEPES (Welgene) and transfected at 70-80% confluence using the calcium phosphate precipitation method and 12 μg lentiviral shRNA construct, 8 μg psPAX2, and 6 μg pMD2.G. Following 12 hrs incubation, the culture medium was replaced with fresh virus production medium (D10 medium containing 1.28% BSA), supernatants containing viral-particles were collected 48 hrs later and centrifuged for 5 min at 3, 000 × g to remove cell debris. T98G cells were infected with lenti-viral supernatant with 8 μg/ml of protamine sulfate (Sigma) for 12 hrs and the medium was replaced with fresh medium. Gene knockdown was examined 48 hrs post-infection.

3-3. Cloning short hairpin RNA into retro-viral vector

For overexpression of MYC, coding sequence cloned into a retroviral expression vector, MFG-IRES-Puromycin (generous gift from Dr. H. Y. Chung of Hanyang University) and named Myc-MFG-IRES Puromycin. pMDG and HIV gag-pol encoding the proteins required for virus packaging were purchased from Addgene (Cambridge, MA, USA)

3-4. Transfection of retro-viral vector and transduction of retro-virus

To generate infectious retro-viral particles, HEK-293 T cells were cultured in D10 medium (DMEM containing 10% fetal bovine serum, 2 mM L-glutamine (Welgene), 10 mM HEPES (Welgene)) and transfected at 70-80% confluence using the calcium phosphate precipitation method and 16 μg retroviral shRNA construct, 6 μg HIV gag-pol and 2 μg pMD.G. Following 12 hrs incubation, the culture medium was replaced with fresh virus production medium (D10 medium containing 1.28% BSA). Virus-containing supernatant was collected 72 hrs later and centrifuged for 5 min at 3, 000 × g to remove cell debris. For infection, the culture medium was replaced by retro-viral supernatant supplemented with 8 μg/ml of polybrene (Sigma), and the cells were incubated at 37°C for 12 hrs. At 48 hrs after infection, virally transduced cells were selected by culture in 1 μg/ml of puromycin (InvivoGen, CA, USA) for 1 week and subjected to further analysis.

4. Cell viability assay

Water-soluble tetrazolium salt (WST) assay was performed with EZ-Cytox (DAEIL LAB SERVICE, DoGen; Seoul, Korea) as instructed by the manufacturer. Primary human fibroblasts and T98G cells were plated into 96 well plates (2×10^3 cells/well), respectively, and incubated overnight at 37°C. The cells were treated with drugs for indicated times, 10 μl of WST solution (EZ-CyTox) was added to the cell, which were further incubated for 1hr. The absorbance at 450 nm was measured with a microplate reader (Bio-rad, Richmond, CA, USA).

5. Cell cycle analysis

The cells were collected by centrifugation at 3, 000 × g for 5 min, fixed in 70% ice-cold ethanol at 4°C for overnight and washed two times with 1 × PBS containing 1% FBS, resuspended in 1 ml of 1 × PBS containing 50 μg/ml of RNase A and 50 μg/ml of propidium iodide (Sigma-Aldrich), incubated in the dark for 10 min at room temperature and then analyzed using FACS canto II and FACSDiva software (BD bioscience, Franklin Lakes, NJ, USA)

6. Reverse transcription-polymerase chain reaction (RT-PCR)

6-1. RNA isolation

Total RNA was isolated according to the method of Chomczynski and Sacchi [105]. Briefly, cells were washed with cold PBS and lysed in solution D (4 M guanidium thiocyanate, 0.1 M ß-mercaptoethanol, 0.5% sodium N-laurylsarcosine, 25 mM sodium citrate, pH 7.0), 0.1 volume of 2 M sodium acetate pH 4.0, 1 volume of water-saturated phenol, and 0.2 volume of chloroform:isoamyl alcohol (25:1) were added. After centrifugation, the aqueous phase was removed and RNA was precipitated with the addition of an equal volume of isopropanol. The RNA pellets were washed twice with 75% ethanol. Following centrifugation, RNA pellets were resuspended in RNase-free dH_2O. RNA quantity and quality were assessed by measuring the optical density at 260 and 280 nm.

6-2. cDNA synthesis and RT-PCR

cDNA was generated with M-MLV Reverse Transcriptase (Elpis Biotech, Daejeon Korea), followed by PCR with HiPi Plus Thermostable DNA Polymerase (Elpis Biotech). Primers used for PCR are as follows.

SKP2-F	CGTGTACAGCACATGGACCT
SKP2-R	GGGCAAATTCAGAGAATCCA
MYC-F	ACC ACC AGC AGC GAC TCT GA
MYC-R	TCC AGC AGA AGG TGA TCC AGA CT
GAPDH-F	CTCAGACACCATGGGGAAGGTGA
GAPDH-R	ATGATCTTGAGGCTGTTGTCATA
MGMT-F	GCAATGAGAGGCAATCCTGT
MGMT-R	GTCGCTCAAACATCCATCCT
PARP1-F	GCT CCT GAA CAA TGC AGA CA
PARP1-R	CAT TGT GTG TGG TTG CAT GA
XRCC1-F	GAG GAT GAG GCC TCT CAC AG
XRCC1-R	TCC TCT GTG TCC CCA GAA TC
LIG3-F	GTG GAT TTG GGC ATG TAT CC
LIG3-R	GCC CAT TCC CCC TAT ACT GT
MPG-F	TGG CAC AGG ATG AAG CTG TA
MPG-R	GTG TCC TGC TCA GCC ACT CT
POLB-F	GAG AAG AAC GTG AGC CAA GC
POLB-R	CGT ATC ATC CTG CCG AAT CT
ACTIN-F	GGC ACC CAG CAC AAT GAA G
ACTIN-R	GCC GAT CCA CAC GGA GTA CT

7. Western blot analysis

Cells were lysed in RIPA buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1% sodium deoxycholate, 0.1% SDS and 1% Triton X-100, pH 7.4) with a protease-inhibitor cocktail (P2850, Sigma-Aldrich). Protein samples were separated by SDS–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences Whatman, Germany). After blocking (5 % non-fat dry milk), the membranes were probed with appropriate primary antibodies and peroxidase-conjugated secondary antibodies and protein bands were detected using a chemiluminescent substrate kit (Elpis Biotech). Immunoblots were visualized with ChemiDoc XRS system (Bio-Rad) and analyzed with ImageJ (NIH, Bethesda, MD, USA).

Western blot analysis was performed as described previously (17). Antibodies against MGMT, CDKN1B, Cyclin E, Cyclin D1, CDK2, CDK4, and SKP2 were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), EZH2, MYC, PARP1, XRCC1 and GAPDH were from Cell Signaling Technology (Danvers, MA, U.S.A.), LIG3, MPG and Polß were from GeneTex (Irvine, CA, U.S.A.), LAMIN B1 was from abcam and HRP-conjugated IgGs were from Jackson ImmunoResearch Laboratories (West Grove, PA, U.S.A.).

8. Immunostaining

Cells were seeded in 6 well plates (SPL Life Science, Pocheon, Korea) a density for 5×10^4 cells per well. The next day, cells were rinsed with ice-cold PBS and fixed with 4% paraformaldehyde and permeabilized with 0.3% triton X-100 and 10% normal goat serum (NGS) in 1× PBS. Following cell fixation, cell were incubated with the appropriate primary antibodies in a solution of 1× PBS with 1% Bovine serum albumin and 0.3% Triton X-100 at 4℃ for overnight. Staining was visualized using anti-rabbit Cy3, anti-mouse Alexa Fluor 594 and the labeled fluorescence was examined by fluorescence microscopy. Nuclei were counterstained using 4, 6-diamidino-d-phenylindole (DAPI; vectorlabs, Burlingame, CA, USA).

9. Alkaline comet assay

Alkaine comet assay was performed with the CometAssay Kit (Trevigen; Gaithersburg, MD, U.S.A.) as instructed by the manufacturer. After trypsinization, cells were washed with ice-cold PBS and suspended in a 0.5% (wt/vol) solution of low temperature-melting agarose in PBS (pH 7.4) at 37°C and immediately layered onto Comet Slides (Trevigen). The agarose was allowed to set for 30 min at 4°C and the slides were incubated in a lysis solution (25 mM NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium lauryl sarcosinate, and 0.01% Triton X-100; provided by Trevigen) at 4°C for 30 min in the dark. For the alkali comet assay, the slides were immersed in alkaline unwinding solution (200 mM NaOH, 1 mM EDTA) and then subjected to electrophoresis in alkali buffer at 1 V/cm at 4°C for 25 minutes. After rinsing twice in distilled water, the slides were dried at 45℃ for 20min and then stained with SYBR Green. Images obtained by fluorescence microscopy (Olympus IX71 from Olympus; Tokyo, Japan) were subjected to analysis with Comet Assay IV v4.3 (Perceptive Instruments; Suffolk, United Kingdom). Statistical analysis of the results was performed as recommended by Bright *et al.*[106]. Briefly, 50 measurements of tail intensity (TI, also known as % tail DNA) obtained per treatment were normalized and expressed as fold-change relative to the vehicle-treated group. Results from three independent experiments were analyzed using a one-way ANOVA, followed by post hoc test using Scheffe (PASW Statistics for

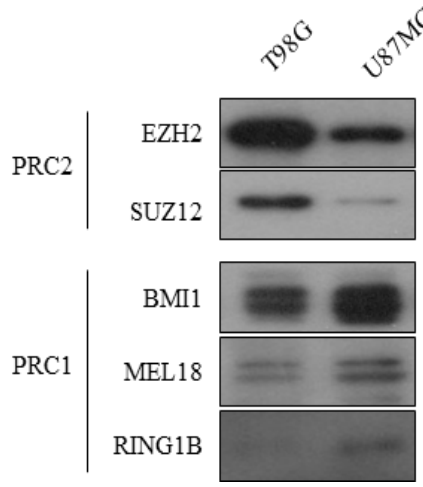
Windows, Version 18.0 from SPSS Inc.; Chicago, IL, USA) to examine for group differences.

III. RESULTS

1. PRC2 regulates cell proliferation positively via modulation of the MYC-SKP2-CDKN1B axis in glioblastoma

1-1. PRC2 expression is up-regulated in T98G glioblastoma cells

PcG proteins such as PRC1 and PRC2 are highly associated with human cancers. The high expression of PRC2 is a hallmark of glioblastoma and implicated in tumorigenic potential and poor prognosis by inhibiting tumor suppressing related genes [64]. However, the role of PRC2 in glioblasma is not clearly understood. In order to investigate the role of PRC2 in glioblastoma progression, I initialized the study by investigating the expression levels of PRC1 and PRC2 proteins in two human GBM cell lines (T98G and U87MG). Previously, it has been shown that U87MG and T98G cells express different expression levels of MGMT protein due to its methylation status and it is directly associated with the degree of sensitivity towards temozolomide [107]. For this purpose, I confirmed the expression level of PRC2 protein was high in MGMT-positive and TMZ-resistant T98G cells when compared to U87MG cell (Figure 1). Due to the facts that the expression level of PRC2 was high in T98G cell lines, I used this cell lines as an experiment models for further studies.



T98G : PRC2 > PRC1
U87MG : PRC2 < PRC1

		T98G	U87MG
PRC2	EZH2	++	+
	SUZ12	+	-
PRC1	BMI1	+	++
	MEL18	+	+
	RING1B	+	+

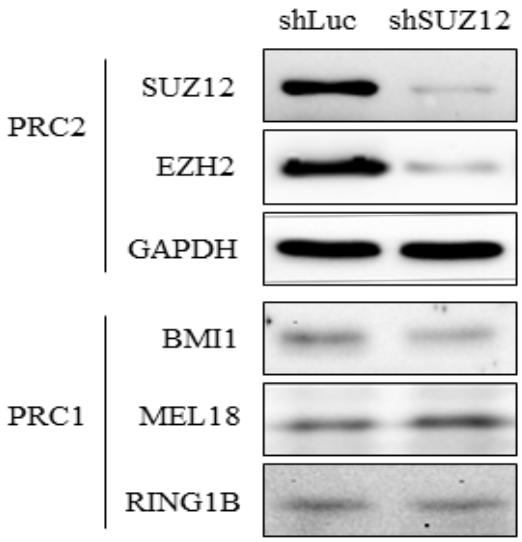
Figure 1. Expression of polycomb group genes in human Glioblastoma cell lines.

The expression levels of the PRC1 (BMI1, MEL18, RING1B), and PRC2 (EZH2, SUZ12) proteins in MGMT-positive T98G and MGMT-negative U87MG cells were analyzed by immunoblotting. The table in right panel represents the quantification of protein expression detected through immunoblotting. Results are representative of three independent experiments.

1-2. PRC2-depletion inhibits cell proliferation of GBM cells.

Previously, it was reported that the SUZ12-knockdown strongly impaired the EZH2 activity as well as its expression level [52, 67, 68]. Based on this report, I established shSUZ12-mediated PRC2 silencing system to investigate the role of PRC2 in glioblastoma progression. The result showed that the shSUZ12 effectively down-regulated the expression of SUZ12 as well as EZH2 in T98G cells (Figure 2A). I then investigated the effect of PRC2-depletion on the T98G cell proliferation, the depleted expression of PRC2 in T98G cells showed significant inhibition in glioblastoma cell proliferation (Figure 2B).

A


shLuc
shSUZ12
PRC2
SUZ12
EZH2
GAPDH
PRC1
BMI1
MEL18
RING1B

B

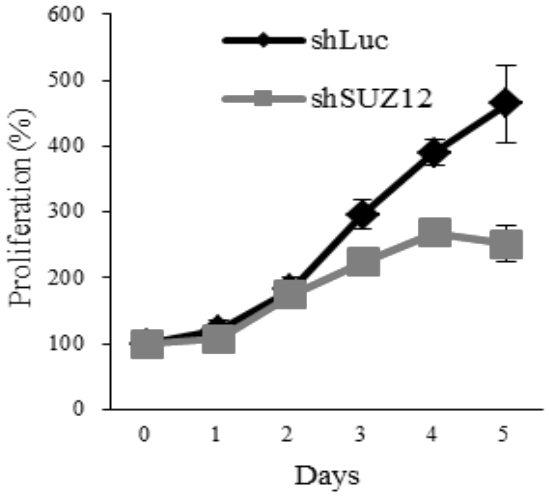

600
500
400
300
200
100
0
Proliferation (%)
shLuc
shSUZ12
0
1
2
3
4
5
Days

Figure 2. PRC2-depletion inhibits the proliferation of T98G glioblastoma cells.

T98G cells were infected with shLuc (left lanes) or shSUZ12 virus (right lanes) for 48 hrs. Cells were harvested and subjected to immunoblotting to confirm the PRC2 depletion. (A) Expressions of PRC1 and PRC2 proteins were assessed with respective antibodies (individual proteins are indicated). GAPDH was used as an internal control. (B) T98G cells infected with shLuc (blackline) or shSUZ12 virus (gray line) for 48 hrs and seeded at 2,000 cells per well in 96-well flat-bottomed plates containing 0.1 ml medium was incubated up to 5 days. Cell proliferation was assessed every day up to 5 days by water-soluble tetrazolium salt (WST) assay. Data shown are representative of at least three independent experiments. Each bar represents the mean ±S.D. from five separate experiments.

1-3. PRC2-depletion attenuates cell cycle progression at G_1 phase

To investigate the molecular mechanism of PRC2-mediated inhibition of cell proliferation, the cell-cycle analysis was performed using flow cytometry. The results showed that PRC2-depletion led to the cell accumulation in the G_1 phase. Eighty-four percent of PRC2-depleted T98G cells were at G_1 phase, as compared with 70% in control T98G cells (Figure 3A). These data indicated that the PRC2-depletion inhibits the T98G cell proliferation by inducing G_1 arrest. Next, the immunoblotting was performed to elucidate the protein expression levels of cell cycle regulators which are responsible for the G_1 arrest. As shown in Figure 3B, up regulation of Cyclin D1 and CDKN1B proteins were detected in the PRC2-depleted T98G cells. Taken together, these results support the notion that PRC2-depletion is sufficient to inhibit the proliferation of T98G glioblastoma cells.

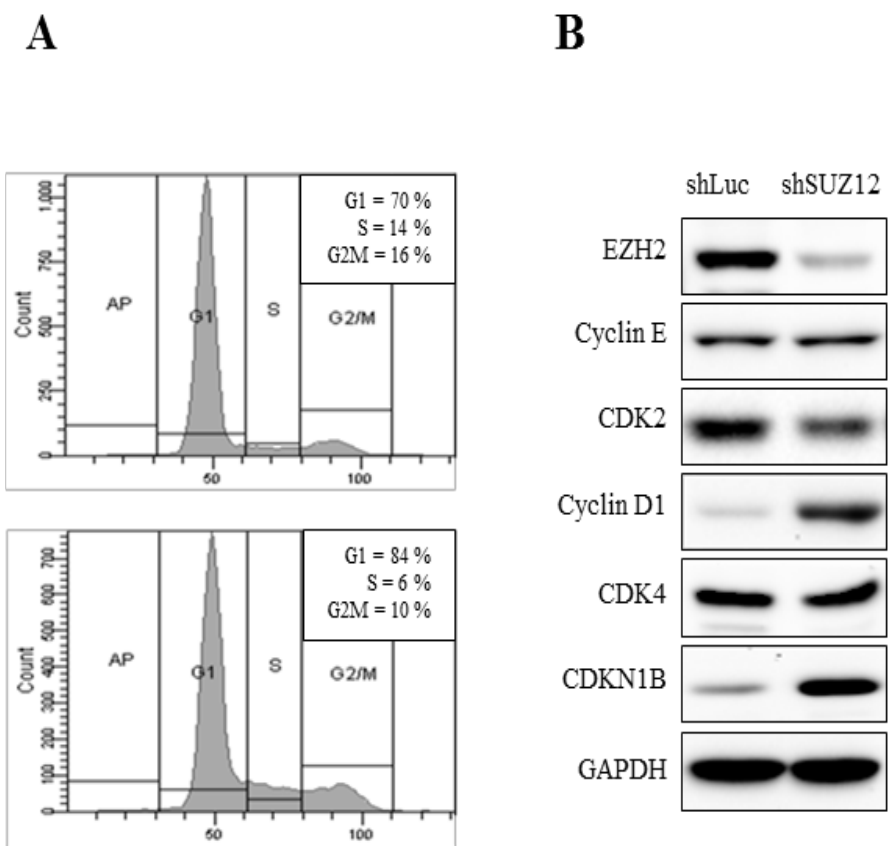


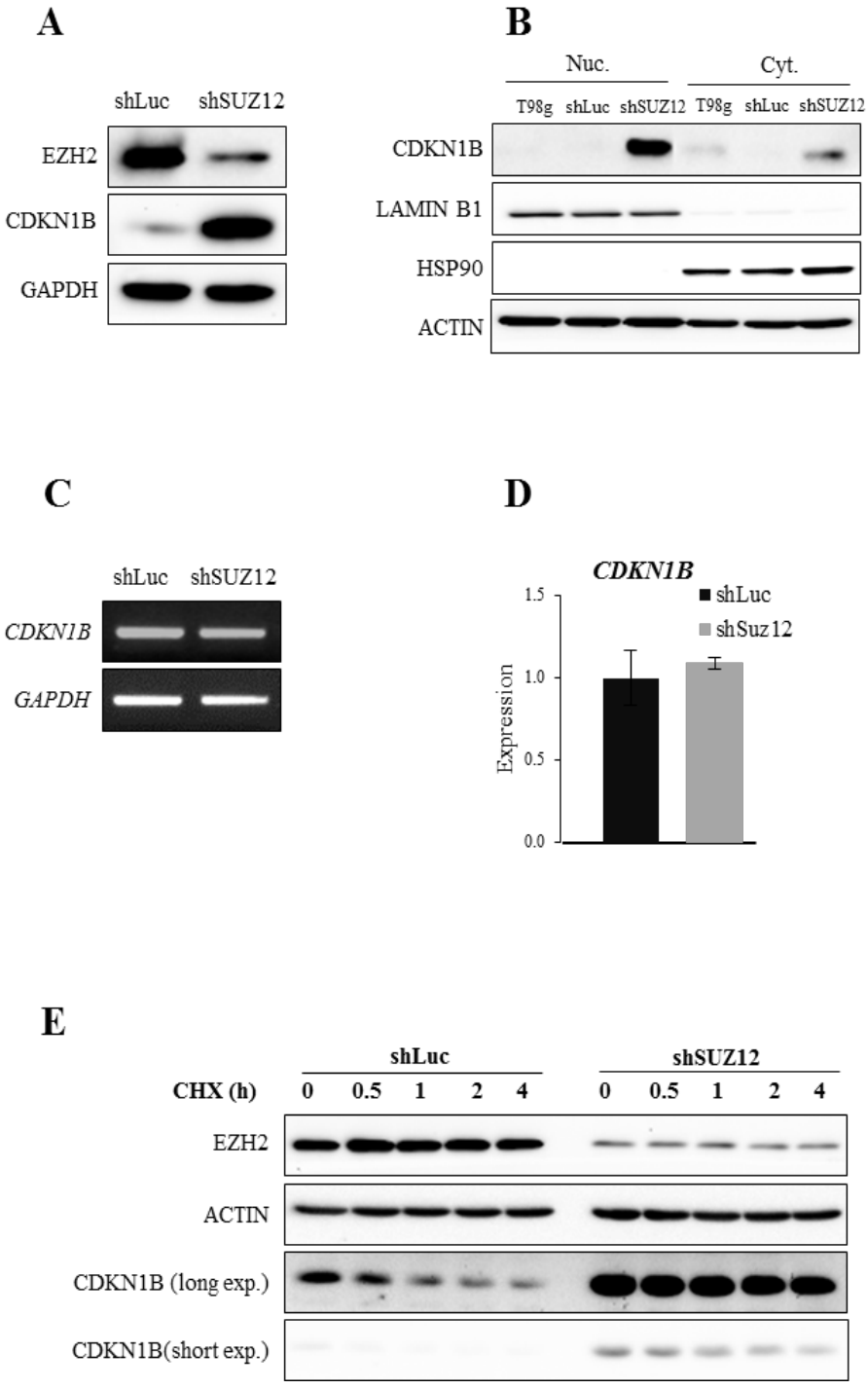
Figure 3. PRC2-depletion attenuates cell cycle progression at G_1 phase of glioblastoma cells

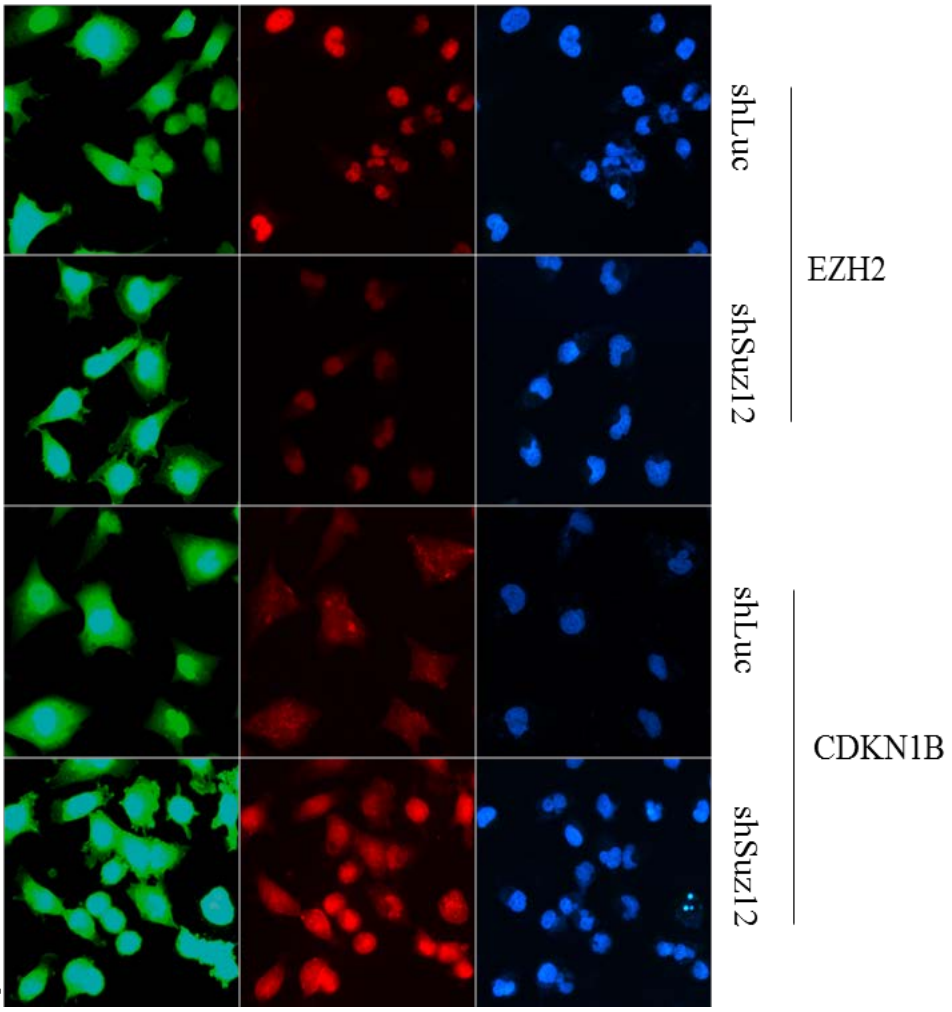
(A) Analysis of T98G cell cycle distribution by flow cytometry after shLuc and shSUZ12 infection. Cells were infected with shLuc or shSUZ12 virus for 48 hrs and stained with propidium iodide (PI) and the effect on cell cycle progression was monitored by FACS analysis. (B) Expression of the cell cycle regulators at G_1 phase was analyzed by immunoblotting. Results were obtained from the identical samples of (A). GAPDH was used as an internal control.

1-4. PRC2-depletion stabilizes and induces the nuclear accumulation of CDKN1B

I examined the protein levels of CDKN1B in cytoplasm and nucleus of PRC2-knockdown cells, because it is known that the CDKN1B acts as a CDK2 inhibitor, regulating cell cycle progression through the G_1 arrest. As shown in Figure 4A and 4B, PRC2-depletion caused a remarkable accumulation of CDKN1B in nucleus suggesting PRC2 depletion-mediated G_1 arrest was mainly associated with increased CDKN1B expression. To cross confirm this data, I performed immuofluorescence staining to estimate the expression level and distribution of CDKN1B in the PRC2-depleted T98G cells. The faint staining of CDKN1B observed in the control T98G cell; whereas a strong nuclear staining of CDKN1B observed in the PRC2-depleted T98G cells (Figure 4F). The CDKN1B expression patterns were inversely proportional to EZH2 expression. EZH2 is an epigenetic regulator and directly involved in transcriptional repression of several genes [108, 109]. In order to confirm whether CDKN1B is a direct target of EZH2 in the PRC2-depleted T98G cells, I measured the protein level of CDKN1B by Immunoblotting (Figure 4B) and the level of CDKN1B mRNA by RT-PCR (Figure 4C), and RT-q PCR (Figure 4D)**.** The result showed that the levels of CDKN1B protein dramatically increased in the depleted expression of PRC2, though the CDKN1B mRNA expression level remained unchanged. To understand how CDKN1B was regulated post-transcriptionally in the PRC2-depleted T98G cell, I treated cells with protein

synthesis inhibitor cycloheximide **(**10 μg/mL; CHX) at different time intervals (0, 0.5, 1, 2, 4 hrs) and analyzed the CDKN1B expression level by Immunoblotting. As shown in Figure 4E, in the PRC2-depleted T98G cells, the degradation level of CDKN1B did not reduced by the cyclohexamide treatment when compared to the control cell. These results show that the depletion of PRC2 leads to the stabilization of CDKN1B protein through enhancing its half-life.


A
shLuc
shSUZ12
EZH2
CDKN1B
GAPDH
B
Nuc.
Cyt.
T98g
shLuc
shSUZ12
T98g
shLuc
shSUZ12
CDKN1B
LAMIN B1
HSP90
ACTIN
C
shLuc
shSUZ12
CDKN1B
GAPDH
D
CDKN1B
shLuc
shSuz12
Expression
1.5
1.0
0.5
0.0
E
shLuc
shSUZ12
CHX (h)
0
0.5
1
2
4
0
0.5
1
2
4
EZH2
ACTIN
CDKN1B (long exp.)
CDKN1B(short exp.)



F

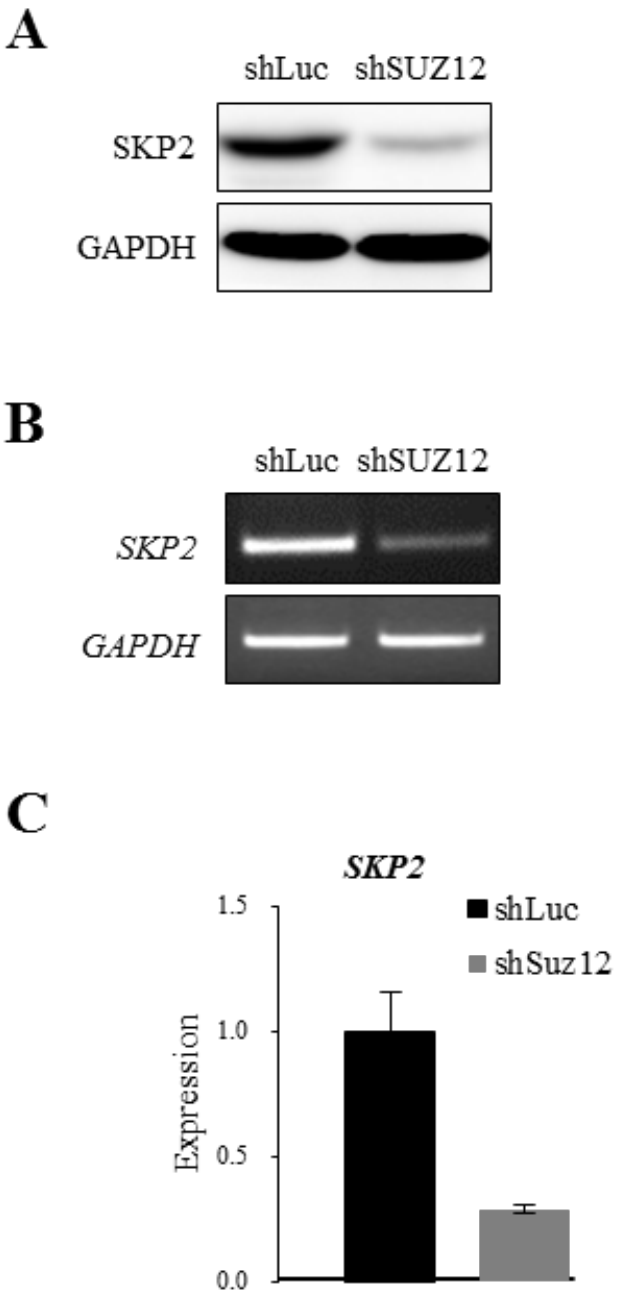
Figure 4. PRC2-depletion stabilizes and enhances nuclear accumulation of CDKN1B protein

(A) T98G cells were infected with shLuc or shSUZ12 virus for 48 hrs and expression of the EZH2 and CDKN1B were analyzed by immunoblotting, (C) RT-PCR (D) and RT-qPCR. (B) T98G cells were infected with shLuc or shSUZ12 virus for 48 hrs and then cells were harvested for isolation of cytosolic and nuclear proteins. Expression level of CDKN1B protein was analysed by immunoblotting. LAMIN B1 (nuclear protein) and HSP90 (cytosolic protein) expression were analyzed for successful fractionation. (E) Cycloheximide treated in a time dependent manner to analysis CDKN1B stability. Cells were harvested at 0, 0.5, 1, 2, 4 hrs after cycloheximide (10 μg/mL) addition and were analysed for CDKN1B protein levels by immunoblotting. ACTIN was used as an internal control. (F) T98G cells were infected with shLuc or shSUZ12 virus for 48 hrs and cells were fixed and immunostained with antibodies against EZH2, CDKN1B. Nuclei was stained with DAPI.

1-5. PRC2-depletion represses MYC-dependent SKP2 expression.

SKP2, the component of the SCF^{SKP2} E3 ubiquitin ligase, is required for the ubiquitination and degradation of phosphorylated CDKN1B protein which is expressed during the G_1 phase of the cell cycle [78, 90]**.** To determine whether PRC2-depletion has an effect on SKP2 expression level, I examined the expression of SKP2 protein and mRNA in the PRC2-depleted T98G cell lines. In reduced expression of PRC2, the SKP2 expressions were decreased both at the mRNA and protein levels (Figure 5). To identify the candidate genes for the SKP2 regulation, *MYC* gene was tested due to its direct association with SKP2 and also known to involve in MYC-dependent regulation of the SKP2 pathway which leads to CDKN1B degradation in leukemia cells [110]. To determine the effect of PRC2 in the regulation of MYC expression, I examined both at protein and mRNA levels of MYC in the PRC2-depleted T98G cells. This result showed that the MYC expressions were down-regulated both transcriptional and translational in the PRC2-depleted T98G cells (Figure. 6). Due to the fact that MYC is the upstream gene in the MYC-SKP2-CDKN1B axis [90] and depletion of PRC2 leads to MYC suppression, I hypothesized that the MYC could directly regulate SKP2 expression in T98G cells. To this end, I ectopically overexpressed MYC in T98G cell and investigated whether it alters any PRC2 depletion effect on SKP2 expression. Interestingly, in the MYC-overexpressed PRC2-depleted T98G cells, SKP2 inhibition completely rescued, in contrast, the levels of CDKN1B were markedly

inhibited (Figure 7). Taken together, these results suggest that PRC2 is involved in the regulation of MYC-SKP2-CDKN1B axis in glioblastoma cells.


A
shLuc
shSUZ12
SKP2
GAPDH
B
shLuc
shSUZ12
SKP2
GAPDH
C
SKP2
1.5
1.0
0.5
0.0
Expression
shLuc
shSuz12

D

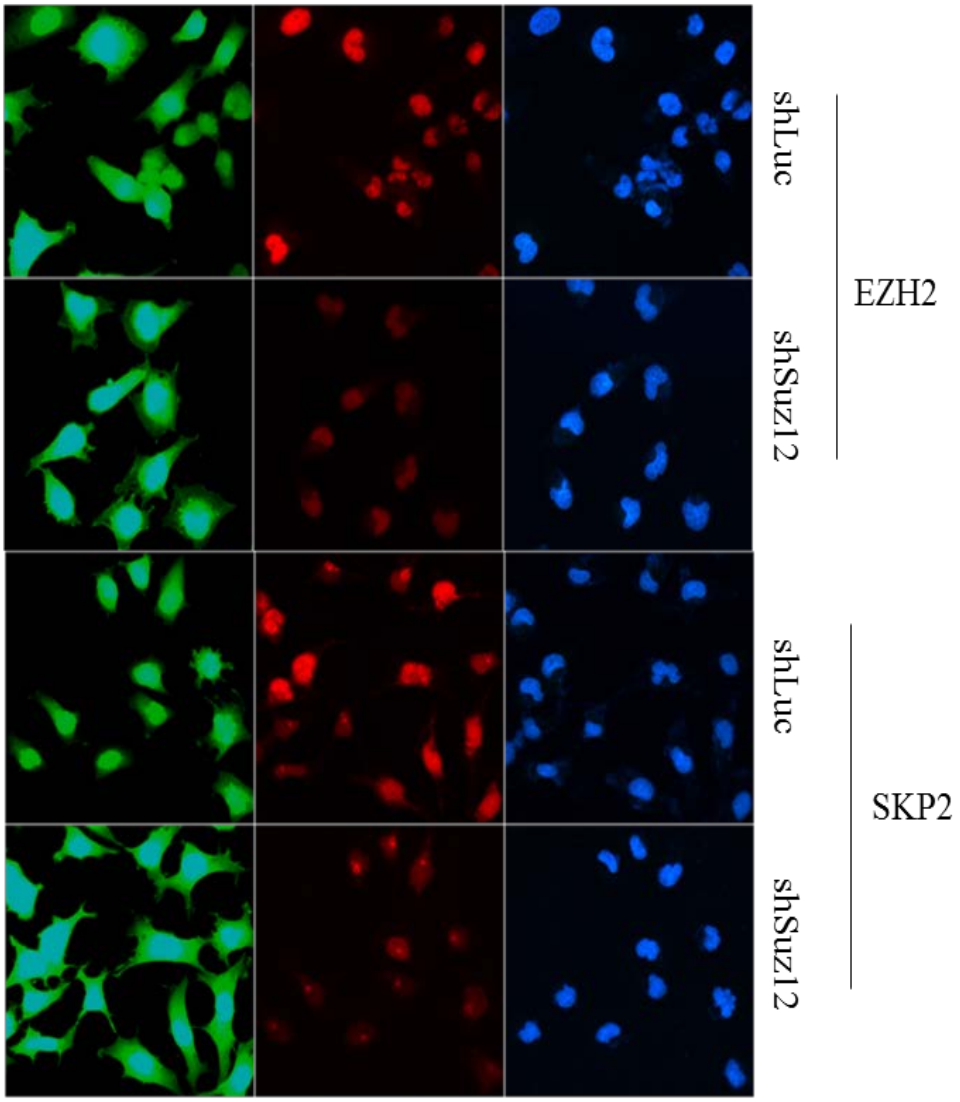


Figure 5. PRC2-depletion represses SKP2 expression

(A) T98G cells were infected with shLuc or shSUZ12 virus for 48 hrs and expressions of the SKP2 were analyzed by immunoblot, (B) RT-PCR (C) and RT-qPCR. GAPDH was used as an internal control. (D) T98G cells were infected with shLuc or shSUZ12 virus for 48 hrs and cells were fixed and immunostained with antibody against EZH2, SKP2. Nuclei were stained with DAPI.

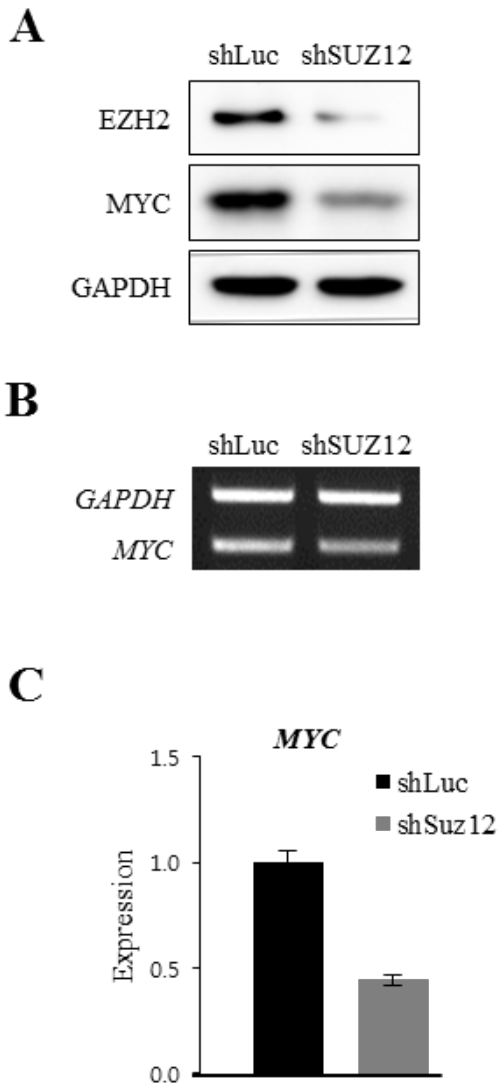
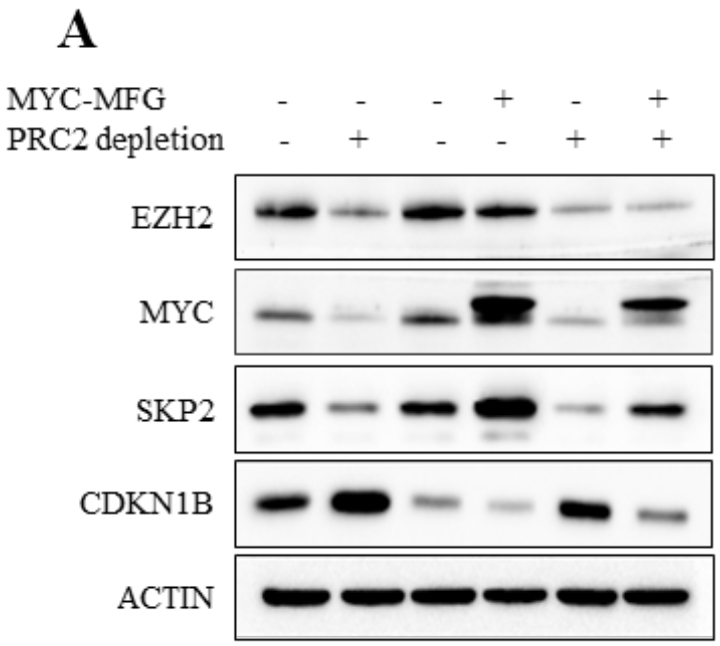


Figure 6. PRC2-depletion down-regulates MYC-dependent SKP2 expression.

(A) T98G cells were infected with shLuc or shSUZ12virus for 48 hrs and expression of the MYC were analyzed by immunoblotting, (B) RT-PCR (C) and RT-qPCR. GAPDH was used as an internal control.


A
MYC-MFG - - - + - +
PRC2 depletion - + - - + +
EZH2
MYC
SKP2
CDKN1B
ACTIN

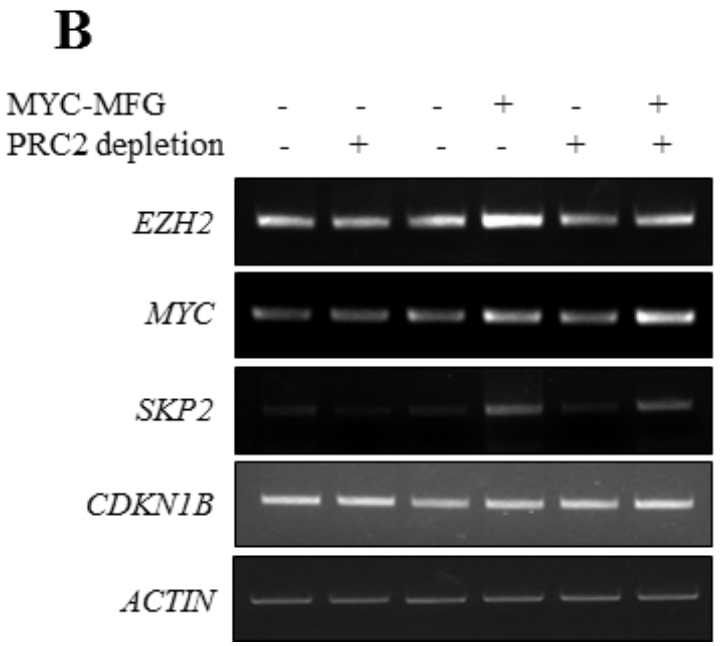

B
MYC-MFG - - - + - +
PRC2 depletion - + - - + +
EZH2
MYC
SKP2
CDKN1B
ACTIN

Figure 7. PRC2-depletion alters the expression of MYC-SKP2-CDKN1B axis in T98G cells.

First round of infection to T98G cells were carried out with MFG-IRES-puromycin (*Puro*) or MFG-*Myc*-IRES-puromycin (*Myc*) retroviruses. After 72 hrs of infection, selection was performed by puromycin (1 μg/ml) for 1 week, and then sencond round of infection was performed using shLuc or shSUZ12 virus for 48 hrs. Next, the cells were harvested to analyze (A) protein levels by immunoblotting. (B) The identical samples of (A) were analyzed by RT-PCR for indicated genes.

1-6. Comparison of the expression levels of EZH2, MYC, CDKN1B in human glioblastoma with normal specimens.

It is know that the high expressions of EZH2 [64, 111] and MYC [112] are related to disease progression in patients with glioblastoma [64, 111]. On the other hand, strongly reduced expression of CDKN1B correlates with progression of glioblastoma [113]. Therefore, the expression levels of EZH2, MYC and CDKN1B were estimated by immunoblotting from human the glioblastoma patient's brain samples and normal brain samples. As previously reported, glioblastoma patients with up-regulated EZH2 expression showed enhanced MYC and reduced CDKN1B (Figure 8). Taken together, these results suggested that PRC2-depletion is responsible for the repression of glioblastoma cell proliferation via down-regulation of MYC-SKP2-CDKN1B axis (Figure 9).

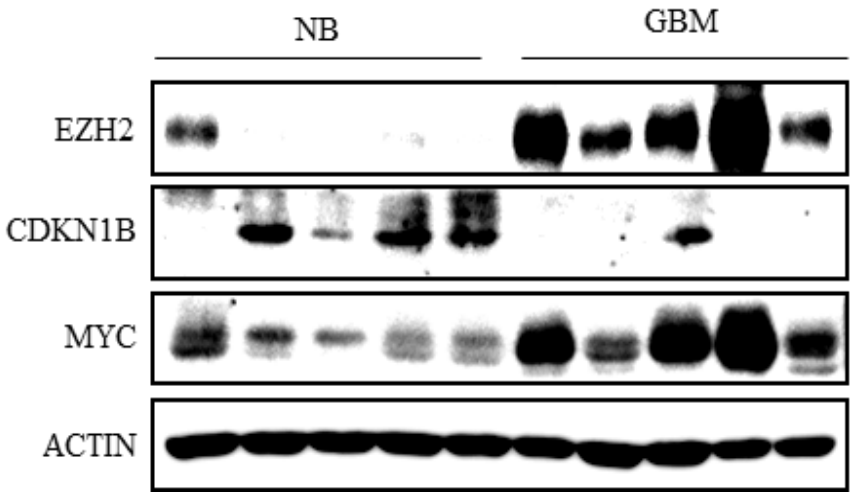


Figure 8. Comparison of expression levels of selected proteins between human glioblastoma and normal brain samples.

Western blot analysis to determine the expression of EZH2, CDKN1B MYC from the glioblastoma patient's brain samples and normal brain samples. Actin was used as an internal control.

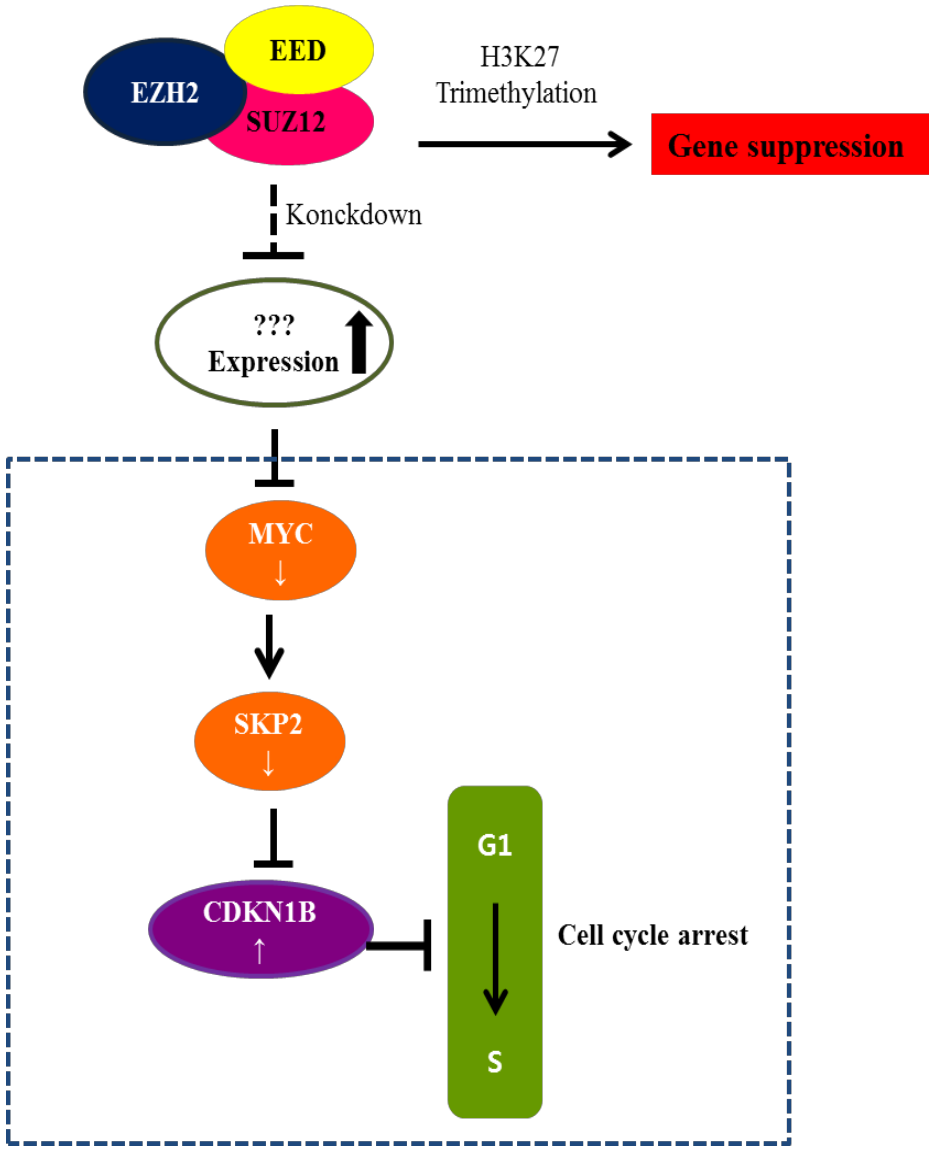


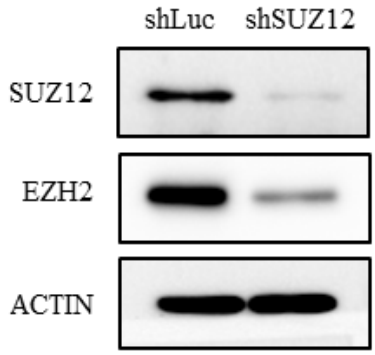
Figure 9. Hypothetical figure representation of PRC2 regulating MYC-SKP2-CDKN1B axis in human glioblastoma cells

2. PRC2-depletion increases chemosensitivity of drug-resistant human glioblastoma cells by repressing DNA repair system

2-1. PRC2-depletion enhances sensitivity for chemotherapeutic agents

Based on my previous observation that the knock down of PRC2 inhibits cell proliferation in glioblastoma, I hypothesized that the reduced PRC2 expression level might enhance chemosensitivity in glioblastoma when it is co-treated with chemotherapeutic agents. For this purpose, I established the PRC2-depleted cell lines using shSUZ12 to investigate the function of PRC2 in chemoresistance of glioblastoma. The generated PRC2-depleted cell lines were first validated for EZH2 and SUZ12 expression and found their expression levels were greatly reduced (Figure 10A). Then, I checked the effect of alkylating agent in both control cells and PRC2-depleted T98G cells. Results showed that the shLuc-infected T98G cells were relatively resistant to the alkylating agent, whereas PRC2-depleted T98G cells exhibit increased sensitivity for TMZ and BCNU treatment ($P<0.05$; Figure 10B) suggesting PRC2 as a potential chemotherapeutic target for glioblastoma.

A. Western



B. Short term Cytotoxicity (MTS.)

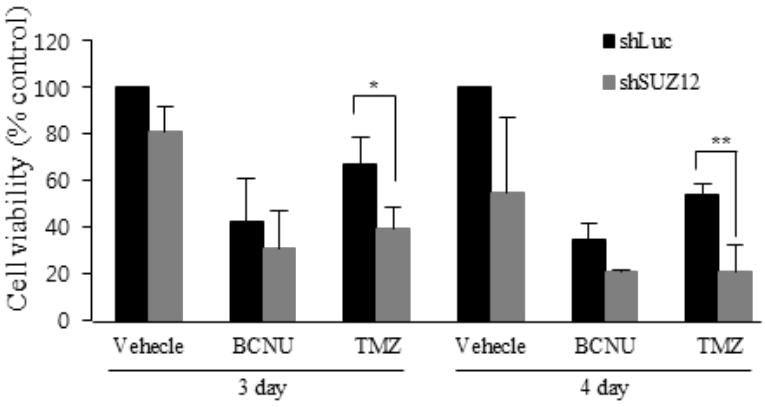


Figure 10. PRC2 depletion in T98G cells dramatically increases the sensitivity for chemotherapeutic agents.

(A) T98G cells were infected with shLuc (left lanes) or shSUZ12 virus (right lanes) for 48 hrs. Cells were harvested and subjected to immunoblotting for confirm the PRC2 depletion. ACTIN was used as an internal control. (B) A cell viability assay was conducted to determine the effect of the PRC2-depletion and examine whether there was a synergetic effect with BCNU or TMZ in T98G cells.

2-2. The potentiation of TMZ by PRC2 depletion is associated with reduced DNA damage recovery

TMZ is known to induce the DNA damage through methylation of O^6, N^7-position of guanine, and the N^3-adenine residues [12, 114]. I concerned with determining the influence of PRC2 during TMZ-induced DNA damage and repair. For this purpose, I studied the extent of DNA damage and repair by alkaline comet assay in shLuc and shSUZ12 infected T98G cells (Figure 11). The principle underlying the comet assay is that undamaged DNA remains tightly coiled and migrates slower under an electrophoretic field and resides in nucleoid, whereas damaged DNA (denatured and cleaved DNA fragments) migrate out of the nucleoid producing the comet tail. The tail intensity allows for assessment of the amount of DNA damage [106]. PRC2-depletion alone had minimal effect on DNA damage, as the tail intensity was similar to that of control T98G cells (Figure 11, Vehicle). I next examined the effect of PRC2 depletion on DNA damage and repair by treating T98G cells with 500 μM TMZ for 2 hrs (TD). After 2 hrs of TMZ treatment cells were washed and incubated with fresh culture medium for 8 h T (R_8h), 24 h T (R_8h), to measure recovery from DNA damage. Results showed that the DNA repair rate was significantly decreased in the PRC2-depleted cells treated with TMZ compared to the controls (Figure 11). Taken together, these results suggest that the potentiation of TMZ efficiency was achieved by the knockdown of PRC2 leading to reduction in DNA repair activity in the PRC2-depleted T98G cells.

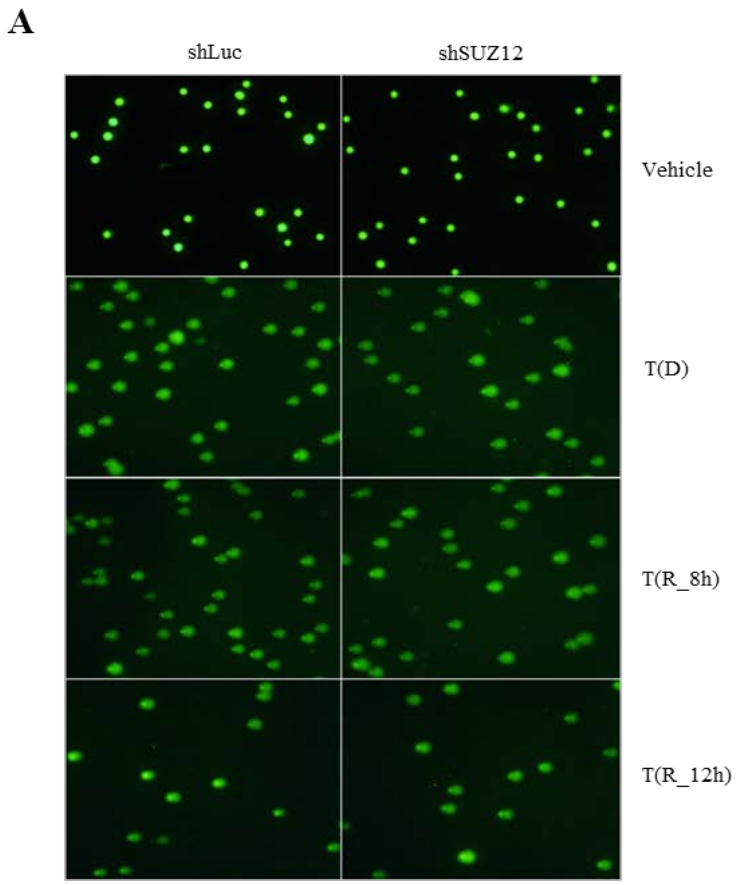

A
shLuc
shSUZ12
Vehicle
T(D)
T(R_8h)
T(R_12h)
B
shLuc
shSUZ12
250
200
150
100
50
0
Fold-change
V
T (D)
T (R 8h)
T (R 8h)

Figure 11. Potentiation of TMZ by PRC2-depletion is associated with reduced DNA damage recovery.

shLuc and shSUZ12 virus infected T98G cells were treated with 500 μM TMZ. After 2 hrs, media were replaced with fresh media for indicated time points and followed by alkaline comet assay. (A) Fluorescent image of cells subjected to alkaline comet assay. [V, vehicle; T (D), TMZ (damage); T (R), TMZ (recovery)] (B) Graphical representation of alkaline comet assay results. Results are shown as average of fold-change relative to control T98G from 50 measurements obtained per treatment and three independent experiments (total of 150 measurements) and standard deviation as error bars.

2-3. PRC2-depletion down-regulates the expression level of DNA repair related genes

In my previous results, PRC2-depletion enhanced the cytotoxicity of TMZ and decreased the repair activity in glioblastoma. It is well-known that the role of MGMT and BER in repairing the TMZ-induced DNA lesions [11, 115]. In order to investigate the effects of PRC2-depletion on DNA damage and repair, MGMT expression level was validated in the PRC2-depleted T98G cells along with control cell. Interestingly, there was no significant difference in MGMT protein level in both PRC2-depleted T98G cells and control cells (Figure 12A).

Next, I estimated the expression levels of several BER genes known to be involved in DNA repair and caused by TMZ-induced DNA damages (N3, N7-methyl adduct). Among these genes, the expressions of PARP1, XRCC1, and Lig3 were significantly down-regulated in the PRC2-depleted T98G cells both protein (Figure 12B) and mRNA level (Figure 12C), however other BER protein levels remain unaltered. These data indicated PRC2-depletion regulated the base excision repair during TMZ-induced DNA damage. In addition, it is well known that the PARP1 is a component of the base excision repair and plays an important role in the repair of TMZ-induced DNA damage in nucleus. I also showed that the nuclear PARP1 expression was significantly reduced in the PRC2-depleted cells during TMZ-induced DNA damage (Figure 13).

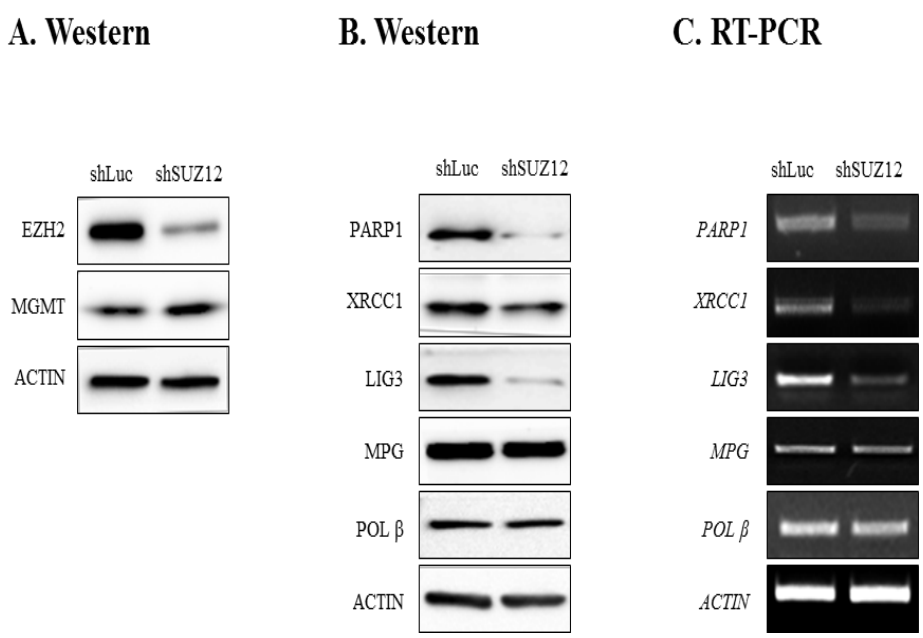


Figure 12. PRC2 depletion in T98G cells inhibits the expression of DNA repair associated genes.

(A) T98G cells were infected with shLuc (left lanes) or shSUZ12 virus (right lanes) for 48 hrs. The expressions of MGMT and EZH2 were confirmed by immunoblot.ting (B) Expressions of the base excision repair genes were analyzed by immunoblotting and (C) by RT-PCR. ACTIN was used as an internal control.

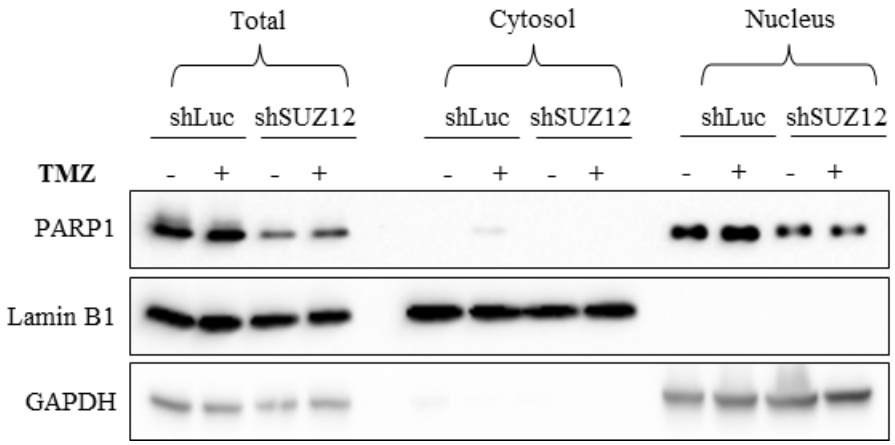
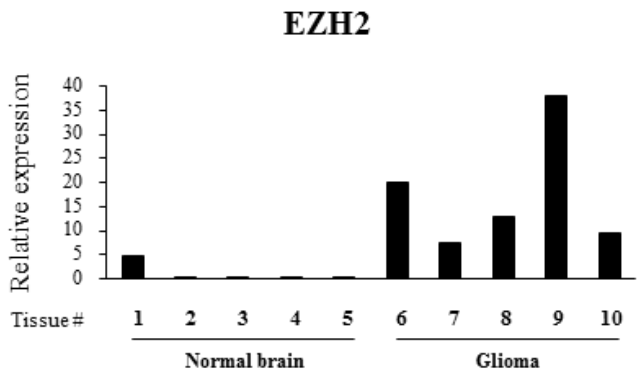


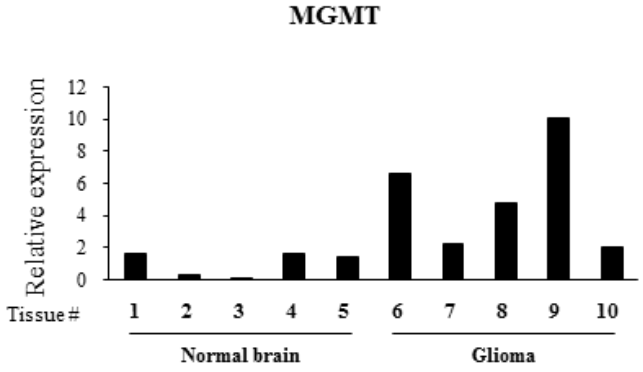
Figure 13. PRC2-depletion inhibits PARP1 expression in nucleus.

T98G cells were infected with shLuc or shSUZ12 virus for 48 hrs and treated with 500 μM TMZ for 24 hrs. Followed by cytosol/nuclear extraction, the expression level of PARP1 was analysed by immunoblotting. Lamin B1 (nuclear protein) and GAPDH (cytosolic protein) expression confirmed successful fractionation.

2-4. Comparison of expression levels of EZH2, MGMT and PARP1 in human glioblastoma samples with normal brain samples.

The expression of EZH2 is highly related to disease progression in patients with glioblastoma [64, 111] and PARP1 protein is detectable in GBM cells, but is undetectable in normal brain tissue [116]. MGMT expression level showed association with TMZ resistance in the GBM cell line. Thus, I measured EZH2, MGMT and PARP1 expression levels in human glioblastoma and normal brain tissue by immunoblotting. Results showed that the EZH2 expression level was up-regulated in glioblastoma tissue, and also enhanced PARP1 and MGMT expression (Figure 14). Taken together, these result suggested the notion that PRC2-depletion enhances TMZ efficiency via down-regulation of BER genes such as PARP1, XRCC1, LIG3 (Figure 15).


EZH2
Relative expression
40
35
30
25
20
15
10
5
0
Tissue #
1 2 3 4 5 6 7 8 9 10
Normal brain
Glioma


MGMT
Relative expression
12
10
8
6
4
2
0
Tissue #
1 2 3 4 5 6 7 8 9 10
Normal brain
Glioma

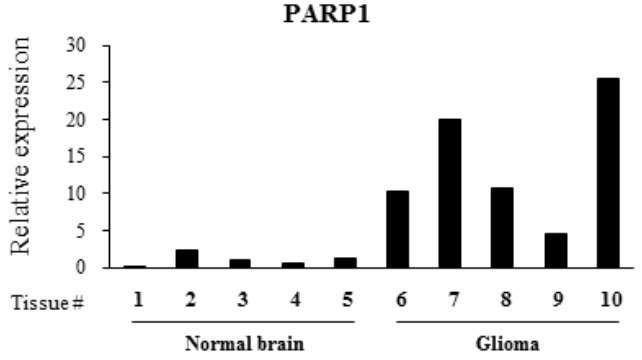

PARP1
Relative expression
30
25
20
15
10
5
0
Tissue #
1 2 3 4 5 6 7 8 9 10
Normal brain
Glioma

Figure 14. Comparison of expression levels of EZH2, MGMT and PARP1 between human glioblastoma samples and normal brain samples.

I estimated the expressions levels of EZH2, MGMT and PARP1 from glioblastoma multiforme patients samples and compared with that of normal brain tissues samples by immunoblotting. Analysis of protein expression was normalized within each sample to the expression of ACTIN as controls.

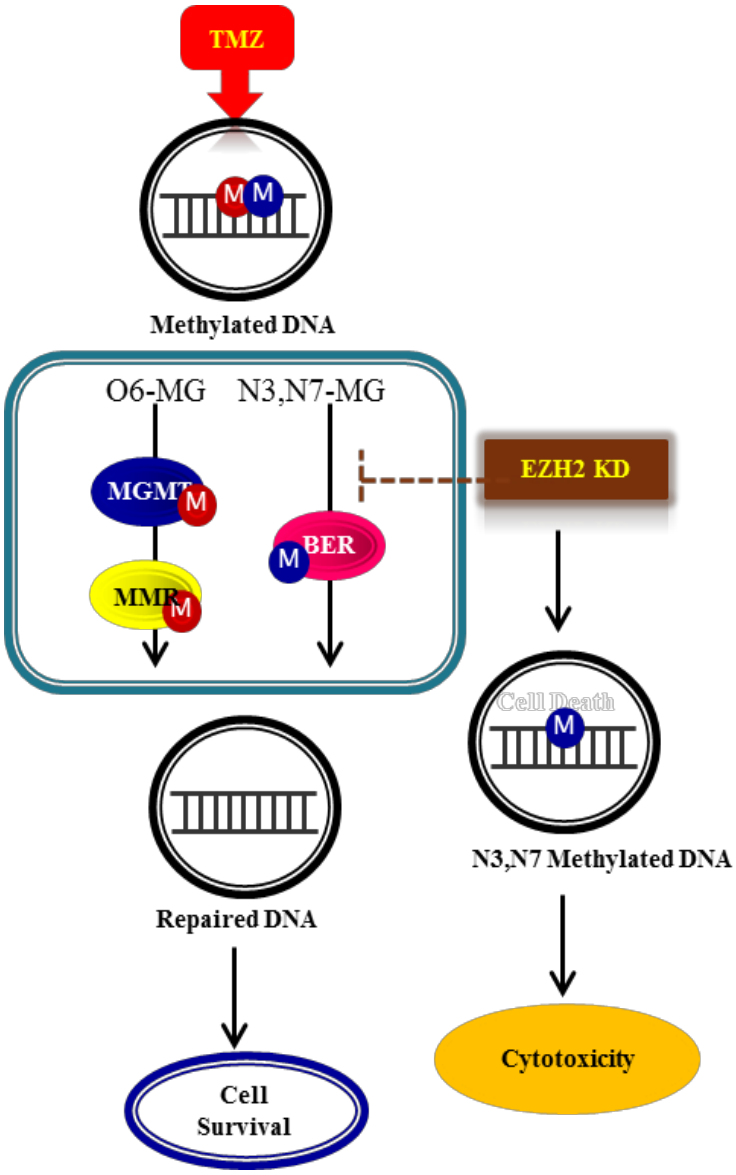


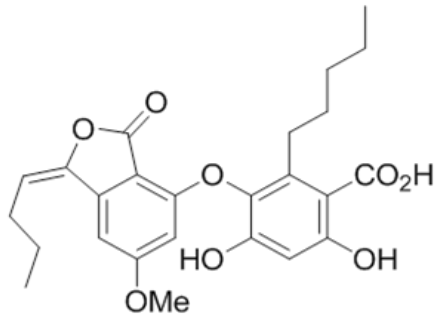
Figure 15. Hypothetical figure representation of PRC2 regulating base excision repair in human glioblastoma cells

3. Lobarstin enhances chemosensitivity in human glioblastoma T98G cells

3-1. Lobarstin inhibits the cell viability of glioblastoma cell

Treatment of lobarstin (Figure 16A) was performed at increasing doses for three days on T98G glioblastoma cells. As seen in Figure 16B, lobarstin showed cytotoxic effect on T98G at a concentration as low as 10 μM (n=5, p=0.002, Student's t-test). Whereas the cytotoxic effect of lobarstin was not observed on human normal fibroblast even at 40 μM (Figure 16B; n=3, p=0.108, Student's t-test). Since, the dose at 40 μM was relatively toxic to T98G cells (n=5, p=5.16E-05, Student's t-test) while it was a tolerated dose for normal fibroblasts, I used 40 μM as the treatment dose concentration for my further experiments.

A



B

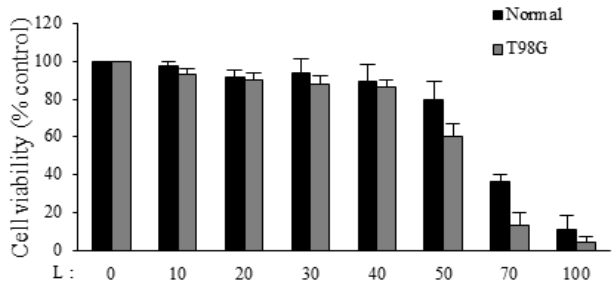
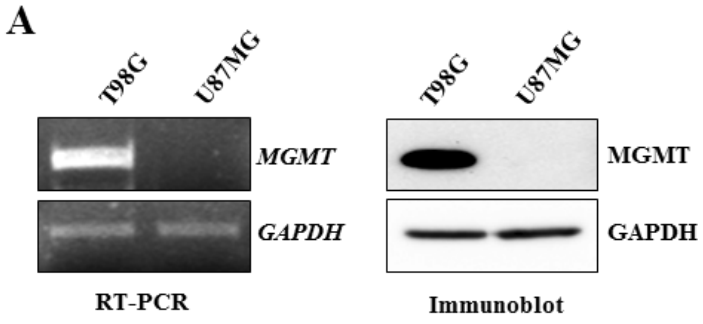


Figure 16. Chemical structure and cytotoxicity of lobarstin. (A) Chemical structure of lobarstin. (B) Primary human fibroblasts (Normal) and T98G (Glioblastoma) cells were tested for cell viability with lobarstin as described in Materials and methods. Cells were treated with indicated doses of lobarstin for 72 hrs. Results were shown as average of three (Normal) and five (T98G) independent experiments with standard deviation as error bars.

3-2. Co-treatment of lobarstin with TMZ enhances cytotoxicity

In general, TMZ is used as a standard chemotherapeutic agent for glioblastoma, but it is less effective to the patients expressing MGMT, a gene responsible for repairing alkylation induced by TMZ at the O^6-position of guanine, than those who do not [117]. Initially, I checked the expression level of MGMT in T98G and U87MG cell lines and found that the expression level was significantly high in T98G cell lines (Figure 17A). Therefore, I wished to pursue my research on T98G cells expressing high MGMT, rendering more resistant to TMZ to investigate therapeutic effect of lobarstin [118]. TMZ treatment on T98G cells at increasing concentration showed statistically significant toxicity in all the conditions tested (Student's t-test, not shown), particularly the toxicity was more prominent at high doses of 500 and 750 μM (Figure 17B). Co-treatment of lobarstin with high doses of TMZ on T98G cells resulted in enhanced toxicity when compared with only TMZ treatment (Figure 17C). These results suggest that lobarstin might provide additional toxicity effect along with TMZ or enhances toxicity effect of TMZ on cells.


A
T98G
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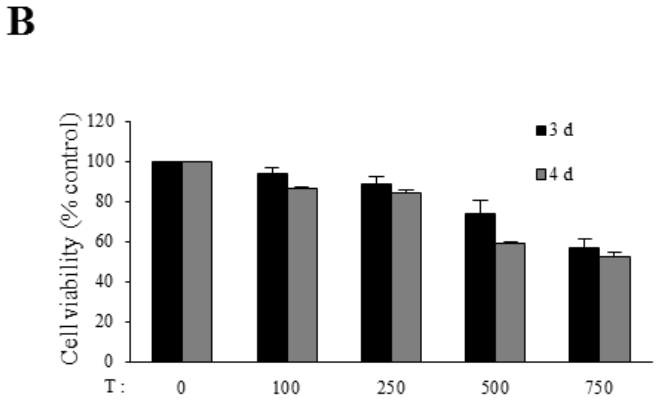
MGMT
GAPDH
RT-PCR
T98G
U87MG
MGMT
GAPDH
Immunoblot


B
Cell viability (% control)
120
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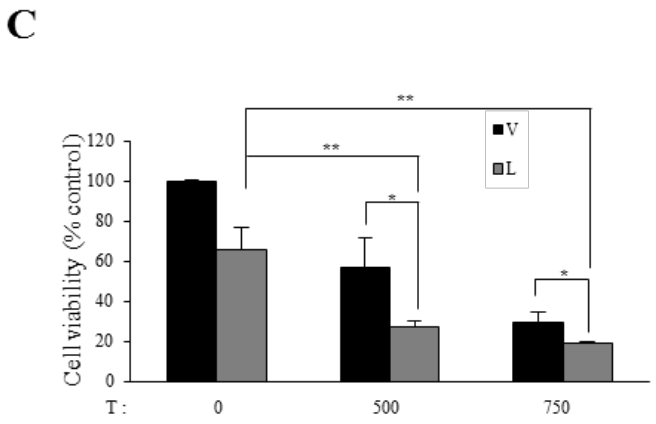
C
Cell viability (% control)
120
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**
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Figure 17. Lobarstin potentiates the sensitivity of TMZ in MGMT-positive T98G cells.

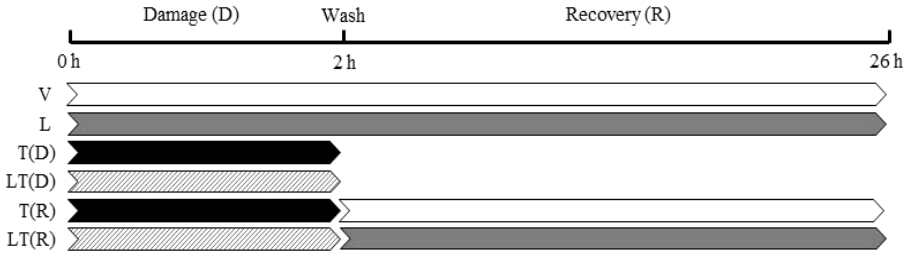
(A) Expression of MGMT in T98G cells. Expression at mRNA (left panels, RT-PCR) and protein (right panels, Immunoblot) levels were shown. MGMT-negative U87MG cells were used as negative control for MGMT expression. GAPDH was used as an internal control. (B) Cytotoxicity of TMZ on T98G cells. T98G cells were treated with indicated doses of TMZ for 72 hrs (3 d) or 96 hrs (4 d). (C) The effect of concomitant treatment of lobarstin and TMZ on T98G cell viability. Cells were treated with indicated combination of drugs for 72 hrs. T, 500 μM TMZ; V, vehicle; L, 40 μM lobarstin. Student's *t*-test, **P*<0.05, 9dla***P*<0.01. Results were shown as representative (A) or average of three independent experiments with standard deviation as error bars (B and C).

3-3. Reduced recovery from TMZ-induced DNA damage by lobarstin.

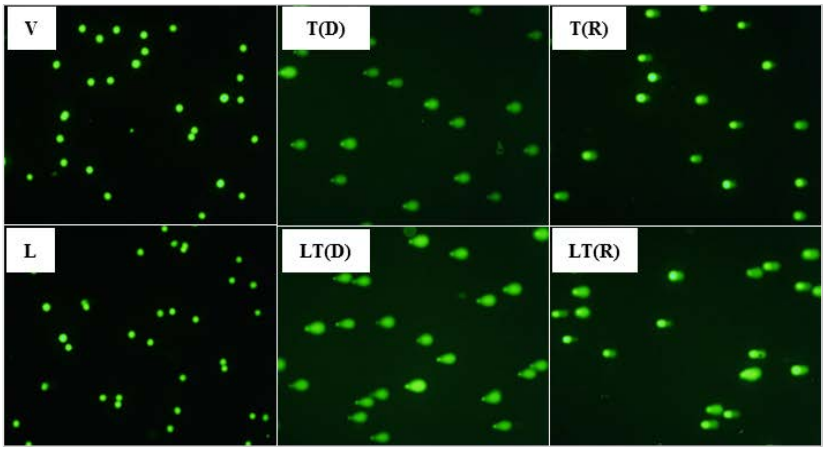
TMZ is known to damage DNA by methylating guanine (at O^6 and N^7 positions) and/or adenine (at N^3 position) residues [114], Firstly, I wished to check the effect of lobarstin on DNA damage. Therefore, I quantified DNA damage exhibited by the treatment of only TMZ or along with lobarstin by the alkaline comet assay (Figure 18). My result showed that the lobarstin-alone at 40 μM for 26 hrs had minimal effect on DNA damage, as the tail intensity (TI) was similar to that of vehicle-treated cells for 26 hrs (Figure 18, L vs. V). Then, I examined the effect of co-treatment of lobarstin with TMZ on DNA damage, by treating T98G cells with 500 μM TMZ-alone or with lobarstin for 2 hrs. Treatment duration was fixed to 2 hrs because in this 2hrs treatment, both TMZ and TMZ/lobarstin treated conditions showed similar-extent of DNA lesion [Figure 18, T(D) vs. LT(D)] and the time was long enough to induce DNA damage but short enough not to overlap with the DNA repair system induced upon DNA damage [Figure 18A;Damage(D)]. Therefore, cells were washed after 2 hrs of drug treatment and incubated with fresh culture medium to measure recovery from DNA damage [Figure 18A; Recovery(R)]. When damaged cells were challenged with fresh medium for 24 hrs, the cells incubated with lobarstin-containing medium showed higher TI than those with vehicle treated cells [Figure 18, T(R) vs. LT(R)]. Using one-way ANOVA to examine the group differences, statistical significance was seen between the groups [$F(1,5) = 4058.828$, $p<0.001$]. Results obtained by utilizing the post-hoc test using Scheffe were as follows: (1) Significant difference observed between V and T(D), and between T(D) and T(R),

$p<0.001$; (2) significant difference between L and LT(D), and between LT(D) and LT(R), $p<0.001$; (3) no significant difference between V and L, $p=1.000$; (4) no significant difference between T(D) and LT(D), $p=0.998$; and (5) significant difference was observed between T(R) and LT(R), $p<0.001$. Taken together, these results suggest that lobarstin-alone may not induce DNA damage, but the DNA damage induced by TMZ may be sustained in the presence of lobarstin.

A


Damage (D)
Wash
Recovery (R)
0 h
2 h
26 h
V
L
T(D)
LT(D)
T(R)
LT(R)

B


V
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LT(D)
LT(R)

C

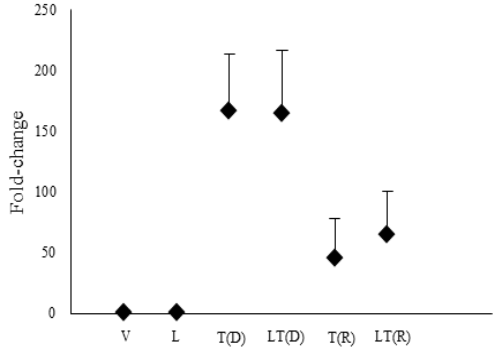
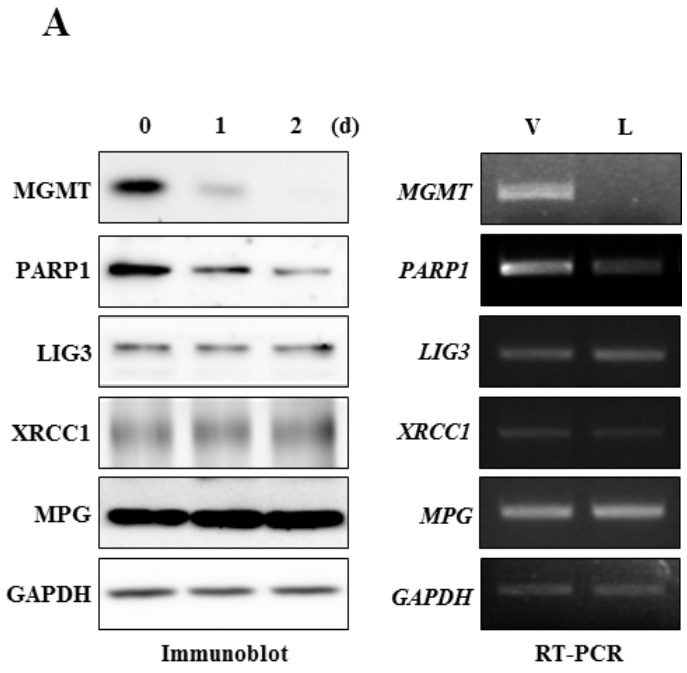

250
200
150
100
50
0
Fold-change
V
L
T(D)
LT(D)
T(R)
LT(R)

Figure 18. Effect of lobarstin on DNA damage and recovery. (A) Experimental paradigm of lobarstin and/or TMZ treatment for alkaline comet assay. Damage (D) and recovery (R) were defined in the solid line shown on the top (not drawn to scale). Six different experimental conditions were shown underneath the solid line as arrow [V, vehicle; L, lobarstin; T (D), TMZ (damage); LT (D), lobarstin and TMZ (damage); T (R), TMZ (recovery); and LT (R), lobarstin and TMZ (recovery)]. Alkaline comet assay was performed at the end of each arrow. (B) Fluorescent image of cells subjected to alkaline comet assay. (C) Summary of alkaline comet assay. Results are shown as average of fold-change relative to the vehicle-treated group from 50 measurements obtained per treatment and three independent experiments (total of 150 measurements) and standard deviation as error bars.

3-4. Down-regulation of DNA repair related genes by lobarstin.

As I observed that the lobarstin-treated cells showing reduced recovery from DNA damage in the alkaline comet assays, I hypothesized that the effect of lobarstin might affect the expression level of DNA repair related genes. To this end, I checked whether the treatment of T98G cells with lobarstin-alone for up to 48 hrs and found that the expression of MGMT and PARP1 which are implicated in DNA repair was dramatically decreased, in a time-dependent manner (Figure 19A, left panels). In addition, reduced mRNA expression was also observed for MGMT and PARP1 indicating negative regulation of lobarstin on these genes at its transcription level (Figure 19A, right panels). Moreover, co-treatment of lobarstin with TMZ resulted in significant reduction in MGMT, PARP1, LIG3 and XRCC1 protein levels (Figure 19B). Taken together, these results show that the increased chemotherapeutic sensitivity of glioblastoma by lobarstin might be due to the down regulation of DNA repair related genes (Figure 20).


A
0
1
2
(d)
MGMT
PARP1
LIG3
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MPG
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Immunoblot
V
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LIG3
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RT-PCR

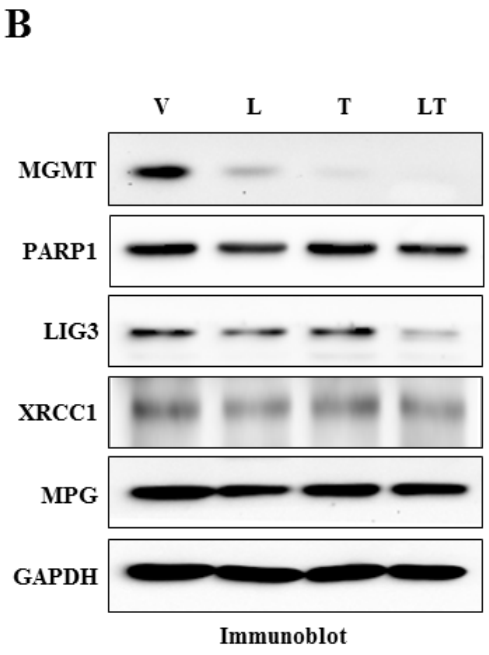
B
V
L
T
LT
MGMT
PARP1
LIG3
XRCC1
MPG
GAPDH
Immunoblot

Figure 19. Effect of lobarstin on the expression of DNA repair genes.

(A) The effect of lobarstin on DNA repair genes. Cells were treated with 40 μM lobarstin for indicated times (left panels) or for 24 hrs (right panels) and subjected to immunoblot and RT-PCR, respectively. (B) The effect of 40 μM lobarstin and/or 500 μM TMZ on DNA repair genes. Cells were treated with indicated drugs for 24 hrs and subjected to immunoblot. Shown are representative results of three independent experiments. V, vehicle; L, lobarstin only; T, TMZ only; LT, lobarstin and TMZ.

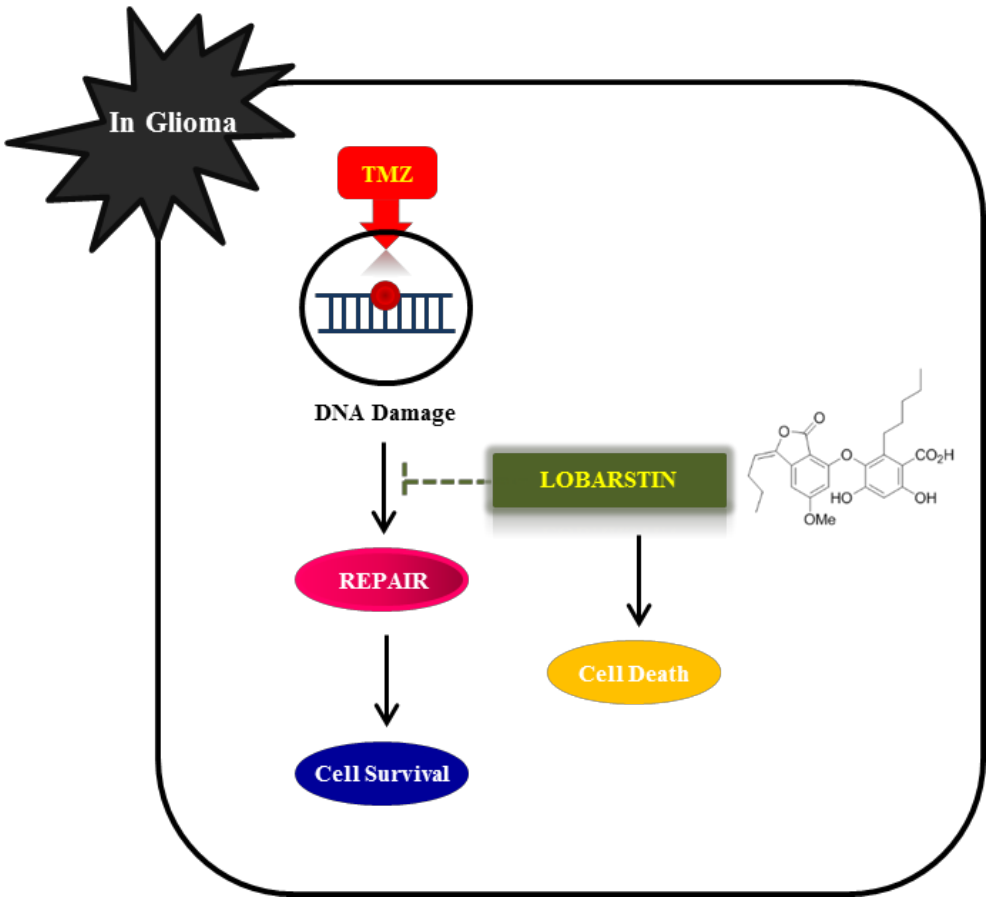

Figure 20. Hypothetical figure representation of lobarstin regulating repair system in human glioblastoma cells

IV. DISSCUSSION

Epigenetic gene regulation by polycombgroup proteins is essential for embryonic development and its maintenance [31, 32]. PcG proteins were found to be overexpressed in various cancers through down-regulation of tumor suppressor genes involved in the carcinogenesis and poor prognosis. PcG is reported to be involved in self-renewal of cancer stem cells as well as normal cell [119-125], its mechanism involve in the negative regulation of locus CDKN2A/CDKN2B (INK4A/ARF) [73-77, 126]. Recently, several reports have been suggested that the microRNA (miRNA) regulates PcG roles such as cancer cell proliferation, migration, maintenance of stemness, angiogenesis, etc [127-131]. In addition, PcG also shows a characteristic of anti-cancer drug resistance in several cancer cells [47-49]. Based on the previous reports, there could be high possibility of controlling cancer cell growth by regulating the PcG expression levels. Thus, further research on alteration of PcG expressions and its effect on cancer cells are much needed.

EZH2, SUZ12 and EED are the components of PRC2, is one of the two classes of PcG proteins [32] which are mainly involved in transcriptional repression [32, 44]. Among these proteins, EZH2 acts mainly as a gene silencer which is involved in chromatin condensation by transferring a methyl group to H3K27 [47-49, 132]**.** SUZ12 is mainly required to stabilize EZH2 protein. It has been reported that the knock-down of SUZ12 by small interfering RNA led to down-regulation of EZH2

expression, and subsequently leading to the inhibition of EZH2-mediated histone methyltransferase activity [48, 68, 133]. In order to investigate whether PRC2 could serve as a therapeutic target against GBM, I generated PRC2-depletion system using shSUZ12 lentivirus because shSUZ12 was more effective than the shEZH2 on PRC2-mediated gene regulation in my experiments.

CDKN1B has a critical role in G_0 and early G_1 phase of the cell cycle inhibiting the activity of Cyclin/Cdk complexes [80]. In glioblastoma, reduced expression of CDKN1B is associated with poor prognosis and increased cell proliferation [113, 134]. *SKP2* has oncogenic properties and plays a critical role in cell cycle progression through degradation of cell cycle regulator such as CDKN1B, Cyclin D1 [135]. Firstly, I investigated the anti-proliferation activity of PRC2 on T98G glioblastoma cells by depleting the PRC2 expression level using shSUZ12 lentivirus and observed that the cell growth was dramatically decreased in T98G cells. It is well known that PRC2-depletion down-regulates *MYC* expression and further *SKP2* is a direct target to *MYC* [90]. In line with previous reports, my results showed that the PRC2-depletion induces G_1 phase cell arrest via regulation of *MYC-SKP2-CDKN1B* axis resulting in the stabilization and enhancement of CDKN1B protein. Recently, it has been reported that the up-regulation of EZH2 expression was regulated by *MYC* [136], however, there was no evidence whether the EZH2 leads to the down-regulation of MYC expression. In my result, PRC2 act as a negative regulator or the gene regulating MYC expression levels even though PRC2 is not a direct target to *MYC*. Therefore, identification of the negative regulator that

regulates *MYC* under PRC2 depletion would be necessary.

Recent studies have shown that the malignant transformation is correlated with the overexpression of *MYC, SKP2* and down-regulation of CDKN1B. In this study, EZH2 depletion leads to high CDKN1B protein level via repression of *MYC*-dependent *SKP2* pathway. My study also proves the anti-proliferation activity of PRC2 as a potential therapeutic target against human glioblastomas. However, further research has to be carried out to investigate the candidates involved in regulating *MYC* in the PRC2-depleted cells.

Treatment of GBM often fails because these tumors are highly resistant to the current anticancer therapy such as chemotherapy and radiation therapy [137, 138]. Currently, TMZ is most widely used as a standard chemotherapeutics for glioblastoma [8]. However, TMZ-induced DNA damage resistant was also reported in a number of cases due to active DNA repair systems [139]. Therefore, various approaches are in progress in order to modulate or control proteins expression levels that are related to DNA repair system to overcome the TMZ resistance, among them are MGMT inhibitor [140], poly(ADP-ribose) polymerase 1 (PARP1) inhibitor [116, 141], N-methylpurine DNA glycosylase (MPG), DNA polymerase β (Polβ) [10, 142] and AP endonuclease (APE-1) inhibitors [143]. The DNA repair mechanisms in TMZ-induced DNA damage mainly includes BER, MGMT and MMR and also it is evident that T98G cells show strong expression of these repair pathways. Among these repair systems, I focused mainly on BER because of the high MGMT expression levels in the PRC2-depleted T98G cells. A key component of base excise

repair is *PARP1* which is activated in response to DNA damage and binds to the single-stranded DNA breaks and function as a DNA damage sensor [11, 144, 145]. PARP1 catalyze the poly (ADP-ribosylation) from NAD+ to itself and to nuclear acceptor proteins, generating long chains of poly ADP-ribosylated polymers [146] and subsequently recruits the DNA repair proteins such as scaffolding protein XRCC1, Polβ, LIG3 to the damaged sites and then accomplish the repair mechanism [142, 147-149]. In an attempt to develop effective anticancer drugs for glioblastoma therapy, the role of PRC2 and the effects of lobarstin was examined in T98G glioblastoma cells. In this study, I reported that PRC2-depleted T98G cells were more sensitive to TMZ than in T98G control cells. Additionally, I observed the down-regulation of several BER proteins such as *PARP1*, *XRCC1*, and *LIG3* in the PRC2-depleted T98G cells suggesting the suppression of base excision repair mechanism.

It has been reported that the increase in chemosensitivity of cancer cells by alkylating agents was through regulation of the BER pathway suggesting the possibility of modulating BER pathway as a potential target for chemotherapeutics. In line with the previous studies, my study suggested that the PRC2-depleted T98G cells showed increased chemosensitivity to TMZ via down-regulation of BER despite exhibiting the high MGMT expression levels. In conclusion, I suggest that the PRC2 is a target protein responsible for enhancing the TMZ cytotoxicity of GBM cells. However, further studies are essential to determine the direct targets for PRC2 which are responsible for regulating the inhibition of *PARP1, XRCC1*, *Lig3.*

Thus, there could be a possibility of developing PRC2 as a potential target for overcoming the problem of drug resistance in GBM patient.

Finally, I reported molecular functions of lobarstin in enhancing the TMZ-sensitivity in chemoresistant glioblastoma T98G cells. I observed reduced recovery of DNA damage in cells co-treated with lobarstin and TMZ, indicating that the lobarstin is mainly involved in the down-regulation of DNA repair related gene expressions. Suppression of MGMT expression followed by TMZ treatment has been reported previously [150]. In contrast, induction of MGMT expression by TMZ treatment has been reported as well [151]. Such discrepancies may be attributed due to differences in the TMZ treatment conditions, as prolonged exposure to low-dosage of TMZ is predicted to acquire chemoresistance [152]. Although I used a high dose of TMZ at 500 μM in my present study, long-term effect of lobarstin treatment on the MGMT expression levels and its regulation on DNA repair related genes are yet to be investigated. Furthermore, it would be intriguing to investigate the effect of lobarstin with other clinically-utilized therapies, such as radiotherapy [153], carmustine [154] and bevacizumab [155]. Despite standard therapy, GBM is known to present an extremely high incidence of recurrence (~90%) [156]. It has been suggested that a sub-population of therapy-resistant cells with stem cell-like characteristics are responsible for tumor re-growth [157]. Although these cells may be highly heterogeneous, thus extremely difficult to characterize, eradication of these tumor-initiating cells would be pivotal in the complete cure of this fatal disease [157, 158]. It would be very interesting to examine the effect of lobarstin in a

population of cells called side-population, which behaves like stem cells and is resistant to chemotherapeutic treatments [159]. Because multiple gene expression was up or down regulated by lobarstin, I can predict that lobarstin may modulate the expression and/or activity of critical transcription factor(s). GATA4 may be an interesting candidate to examine its correlation with lobarstin for future research, because the expression of the BER enzyme alkylpurine DNA N-glycosylase was reduced by GATA4 [160]. Although, lobarstin reported as a metabolite isolated from *Stereocaulon alpinum*, which act as a potent inhibitor of protein tyrosine phosphatase N1 (PTPN1) [97], the exact biological activity of lobarstin remains to be elucidated. Further, testing for the possibility of lobarstin as a phosphatase inhibitor would be intriguing, as PTPN1 is actively pursued as a drug target for various diseases, including cancer [161, 162]. In conclusion, I showed the functions of lobarstin in enhancing the sensitivity of GBM cells to TMZ. I also suggested a possible molecular mechanism of lobartin in down regulating DNA repair related gene expressions. These results open the possibility of lobarstin as a potential factor for GBM combination therapy.

As compared with normal brain samples, EZH2 expressed predominantly high in glioblastoma patient samples suggesting the epigenetic PRC2 activity may participate in the progression of tumorigenesis and cell growth rate of GBM. My study results showed that shSUZ12-mediated PRC2-depletion leads to glioblastoma growth inhibition and increased TMZ potentiation. However, how PRC2 regulate the *MYC* and BER proteins remains to be examined because the PRC2 is a

methyltransferase and play a critical role as transcriptional repressor. Therefore, further research including identification of target genes that are positively regulated by PRC2 complex would be essential**.** Taken together, my research results provide an informative theoretical foundation to develop PRC2 depletion as a potential strategy for GBM therapy and lobarstin as a combination drug for GBM treatment.

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국문 요약

다형성아교모세포종은 중추신경계의 아교세포와 그 전구세포에서 기원하는 악성 원발성 뇌종양이다. PcG 단백질 중 BMI1, SUZ12, EZH2 등은 다양한 암종에서 과발현되어 암 억제 유전자의 발현을 억제시켜 암을 유발하는 것으로 알려져 있다. 본 논문에서는 발암 기전과 항암제 활성을 증진시킬 수 있는 두 가지 다른 접근을 통해 다형성아교모세포종의 치료를 위한 연구를 진행하였다. 첫째로 다형성아교모세포종에서 Polycomb Repressive complex 2 (PRC2) 의 역할을 규명하여 뇌세포의 마스터 조절장치 (master regulator)로서 가능성을 연구하였다. 둘째로 남극 지의류인 *Stereocaulon alpinum* 로부터 분리한 lobarstin이 기존의 항암제와 병행 투여 시 뇌암 치료의 효과를 상승 시킬 수 있는지 그 가능성을 연구하였다. 실제 다형성아교모세포종의 세포주인 T98G 에서 PRC2 유전자를 억제 하였을 때 G_1기에 성장이 멈춰진 세포가 증가하고 세포의 증식이 억제되는 것을 확인 할 수 있었다. 기전을 살펴보게 되면 PCR2 억제는 MYC-SKP2 경로를 억제하여 SKP2에 의해 분해되는 CDKN1B 단백의 안전성을 증가시켰다. 또한 동일한 세포주에 MYC 유전자를 과발현 시켰을 때 같은 기전이 조절되는 것을 확인할 수 있었다. 이러한 사실은 인체 뇌종양에서도 PRC2와 MYC의 과발현 CDKN1B의 발현 감소로 같은 양상이 나타남을 확인할

수 있었다. 한편으로는 PRC2가 항암제의 효과를 증진 시킬 수 있는지 T98G 세포를 이용한 *in vitro* 연구와 인체시료에서의 검정을 통해 고찰하고자 하였다. 실제 PRC2가 억제 된 T98G 세포주에서 항암제 민감성이 증가 하였다. 이는 PRC2 제어에 의해 Base Excision Repair (BER) 관련 유전자인 PARP1, XRCC1, Ligase3의 감소에 따른 따른 것이다. 따라서 Temozolomide (TMZ: 표준치료제) 처리로 손상된 DNA가 회복이 억제 되어 세포 독성이 증가하는 것으로 판단된다. 마지막으로, 남극 지의류인 *Stereocaulon alpinum*로부터 분리된 lobarstin이 정상세포주보다 T98G 뇌암 세포주에서 높은 독성을 보임을 확인하였다. 또한 TMZ와 병행 처리시 세포 독성이 증가되는 것을 확인할 수 있었다. 이에 따른 설명으로 TMZ에 의해 손상된 DNA 병변을 회복하는 기전에 작용하는 것으로 확인하였고, 특히나 GBM에서 항암제 저항성에서 가장 이슈가 되고 있는 MGMT 유전자의 감소뿐 아니라 BER 관련 유전자들의 발현이 감소됨을 확인하였다. 이러한 결과들을 종합해보면 PRC2 억제는 뇌암 세포의 증식 억제와 표준항암제의 효과를 증진 시킬 수 있음을 알 수 있다. 또한 lobarstin은 TMZ 에 의해 손상된 DNA를 회복하는 두 가지 기전을 동시에 억제하여 병행치료제로서 효용 가능성을 보여 주고 있다.