

Effect of BMI1 Knockdown on Cell Proliferation, Apoptosis, Invasiveness, and Migration of U251 Glioma Cells

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Abstract : BMI1 belongs to the polycomb-repressive complex 1 (PRC1) family of genes that are conserved chromatin silencers. These are essential for maintaining both the normal and cancerous stem cell state. In this study, we evaluated the effect of siRNA-mediated BMI1 knockdown on tumor cell properties such as invasion, migration, and apoptosis, as well as on cell signaling pathways responsible for tumor progression in the human glioma cell line, U251. Knockdown of BMI1 induced apoptosis by activating cleavage of PARP and caspase-3. It also decreased the expression of anti-apoptotic proteins, survivin, XIAP, Bcl-xL, and Mcl1. Additionally, BMI1 knockdown significantly decreased cell invasion and cell migration ability. BMI1 knockdown also decreased the phosphorylation of Akt/FOXO1/3a signaling proteins. Our results suggest that BMI1 knockdown induces apoptosis and decreases cell invasion and cell migration. Moreover, we believe these phenomena are associated with decreased phosphorylation of Akt signaling proteins, which contributes to cancer progression.

Key words: BMI1, Apoptosis, Invasion, Migration

Introduction

Gliomas are malignant primary brain tumors of glial cells and they represent the most common primary tumor of the central nervous system in humans. Patients with gliomas usually have a poor prognosis, with mean survival of less than 1 year. The current therapeutic procedures used for gliomas include the use of surgery, radiotherapy, and chemotherapy [1,10]. Glioma treatments have many

limitations as glioma tumors can aggressively infiltrate surrounding tissues. The migration, invasion, and proliferation ability of glioma cells primarily determine the process of tumorigenesis and progression [2,3]. Although many studies have focused on developing biomarkers for invasive tumors and effective treatment methods, the molecular mechanisms that regulate tumorigenesis and proliferation of gliomas need to be elucidated.

Tumor progression and metastasis are complicated processes that occur via a coordinated series of cellular and molecular mechanisms. The molecular and biochemical mechanisms that may contribute to phenotypic changes in favor of carcinogenesis include inhibited apoptosis, enhanced tumor cell proliferation, increased invasiveness, and perturbation of cell adhesion [4,5]. Genes related to increased cell motility may provide gene therapy targets

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for suppression of tumor development and metastasis in human cancers.

The B cell-specific Moloney murine leukemia virus insertion site 1 (BMI1) gene is a member of the polycomb-repressive complex 1 (PRC1) family of proteins that are conserved chromatin silencers. BMI1 is involved in cell proliferation, senescence, and tumorigenesis via well-known growth regulatory pathways. Moreover, it plays an important role in self-renewal of hematopoietic stem cells, neural stem cells, and mammary stem cells [7,8]. Recently, overexpression of BMI1 has been implicated in several human malignancies including gastric cancer, colorectal cancer, breast cancer, melanoma, and bladder cancer. Several studies have also shown that dysregulation of BMI1 correlates with advanced tumor invasiveness and poor prognosis in several cancers [2,9]. It has been suggested that BMI1 could be a promising and novel therapeutic target as it is involved in the carcinogenesis of various malignancies including hepatocellular carcinoma, colorectal cancer, nasopharyngeal cancer, and neuroblastoma [10-12]. Therefore, we hypothesized that the silencing of BMI1 expression may be an effective strategy for mitigating tumorigenicity of human glioma cells. In this study, we determined that BMI1 knockdown using siRNA affects tumor cell behavior such as invasion, migration, and apoptosis, and the cell signaling pathways responsible for tumor progression in human glioma cells.

Materials and Methods

1. Cell culture and knockdown of BMI1

The human glioma cell line, U251MG, was obtained from the American Type Culture Collection (Manassa, VA, USA) were from LONZA (Walkersville, MD, USA). Cells were maintained in DMEM (Hyclone, Logna, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA) in a 5% CO₂ humidified incubator. For knockdown of endogenous BMI1 gene, we used small interfering RNAs (siRNA). Scramble siRNA (negative control siRNA) and specific BMI1 siRNA were purchased from Bioneer Inc. (Daejeon, Korea). The siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer's instructions. The knock-

down efficiency of BMI1 was evaluated using RT-PCR and western blotting. For the cell viability assay, transfected cells with siRNA were seeded at density of 5×10^4 cells/mL and then incubated up to 48 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.

2. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). The cDNA was synthesized from total RNA using MMLV reverse transcriptase (Promega, Madison, WI, USA). Genes of interest were amplified with cDNA and specific primers. We used the following primer sequences: BMI1 5'-CGGGGTACCATGCATCGA-3'/5'-GAAGAAGTTGCTGATGACCC-3'; GAPDH 5'-ACCACAGTCCATGCCATCAC-3'/5'-TCCACCACCC TGTGCTGTA-3'. After amplification, PCR products were separated on agarose gels and bands were visualized with ethidium bromide.

3. Western blot analysis

The cells were washed with PBS at the indicated times and lysed in M-PER[®] mammalian protein extraction reagent (Thermo, Rockford, IL, USA). Cytoplasmic and nuclear extracts were prepared using NE-PER nuclear and cytosolic extraction reagents (Thermo). Protein concentrations of cell lysate were quantitated by BCA[™] protein assay (Thermo, Rockford, IL, USA) with BSA as the standard. The proteins were electrophoresed on an SDS-polyacrylamide gel. Blots were transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The specific proteins were blotted with primary antibodies. Antibodies against XIAP, survivin, β -tubulin, and GAPDH were purchased from Santa Cruz Biotechnology (CA, USA). Antibodies against Bcl-XL, Mcl1, Bax, Bak, HtrA2/Omi, Smac/Diablo, Cytochrome C, COXIV, phosphor-Akt, Akt, phosphor-FOXO3a, phosphor-FOXO1/3a, and FOXO1/3a were purchased from Cell Signaling (Danvers, MA, USA). The blots were incubated with secondary antibody-horse-radish peroxidase conjugates (Pierce, Rockland, IL). The detection of specific proteins was carried out using an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL) and a luminescent image analyzer LAS-4000 (Fujifilm, Tokyo, Japan).

4. Cell invasion assay

Cell invasion assay was carried out using Costar 24-well transwell filters with 8- μ M pores (Corning Inc., NY, USA) that were coated with 1% gelatin and dried out at room temperature. BMI1-transfected cells were seeded at a density of 2×10^5 cells in 0.2% BSA medium in the upper chamber. As a chemoattractant, human plasma fibronectin (Calbiochem, La Jolla, CA, USA) was suspended into the lower chamber. After 24 h of incubation, invasive cells that had adhered to the bottom surface of the transwell were fixed with 70% ethanol and stained with Diff Quiksoln (Sysmex, Kobe, Japan). The stained cells were counted from 5 selected fields (each 0.5 mm² in area) under a light microscope. Data are presented as mean \pm SE of the number of cells/field in 3 individual experiments.

5. Cell migration assay

Cell migration was measured using Culture-Inserts (Ibidi, Regensburg, Germany). The siRNA transfected U251 cells were seeded in the Culture-Inserts. To create a cell-free gap, cells were seeded in Culture-Inserts and gently removed with tweezers after 24 h of incubation. The progress of cell migration into the cell-free gap was photographed at 0, 24, and 48 h using an inverted microscope. The migration distance was normalized to 1 cm after capturing three random sites.

6. Flow cytometric analysis

For annexin-V staining, live cells were washed in phosphate-buffered saline (PBS), and incubated with annexin-V APC (R&D Systems, Minneapolis, MN) and 7-AAD. For cell cycle profiles, cells were fixed in 70% ethanol overnight at 4°C, and then were washed in phosphate buffered saline (PBS) with 0.1% BSA. The cells were incubated with RNase A (DNase free) and PI overnight at room temperature in the dark. The proportion of apoptotic cells that were annexin-V positive and at the sub-G1 phase were analyzed using a FACS Calibur flow cytometer (Becton-Dickenson, San Jose, CA) and the Cell Quest software (Becton-Dickinson).

7. Statistical analysis

All data are derived from at least three independent experiments, and values are presented as mean \pm standard

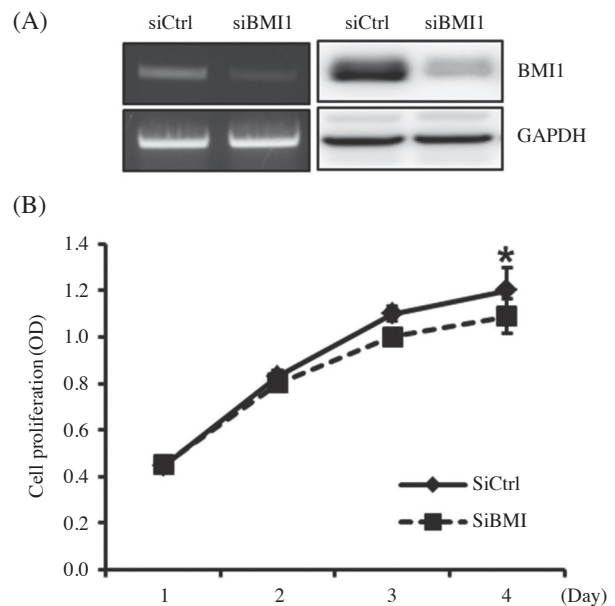


Fig. 1. Effect of BMI1 siRNA on endogenous BMI1 expression and proliferation of U251 cells. (A) mRNA and protein expression of BMI1 decreases after BMI1 knockdown. BMI1 expression was analyzed using RT-PCR and western blot analysis. (B) Knockdown of endogenous BMI1 expression in U251 cells reduced cell proliferation. The optic density (OD) indicating proliferating viable cells, is significantly reduced in BMI1 siRNA-transfected cells (mean \pm SD; $n=3$; * $p < 0.05$, compared to siCtrl). siCtrl: scramble siRNA, siBMI1: BMI1 siRNA.

deviation (SD). Statistical analyses were performed using the Sigma Plot software (Aspire Software International, Ashburn, VA). Significant differences among the groups were determined using the Student's t-test. Values of $p < 0.05$ were considered statistically significant.

Results

1. Knockdown of BMI1 reduces proliferation of U251 glioma cells.

We used BMI1 specific small interfering RNA (siRNA) to knockdown endogenous BMI1 gene expression in U251 cells. RT-PCR and western blot analysis showed that BMI1 siRNA was able to reduce BMI1 mRNA and protein expression in U251 cells (Fig. 1A). To determine the effects of BMI1 knockdown on cell proliferation, MTT proliferation assay was performed after transfection with BMI1 siRNA. Cell proliferation in BMI1 siRNA-transfected U251 cells decreased significantly compared to scramble

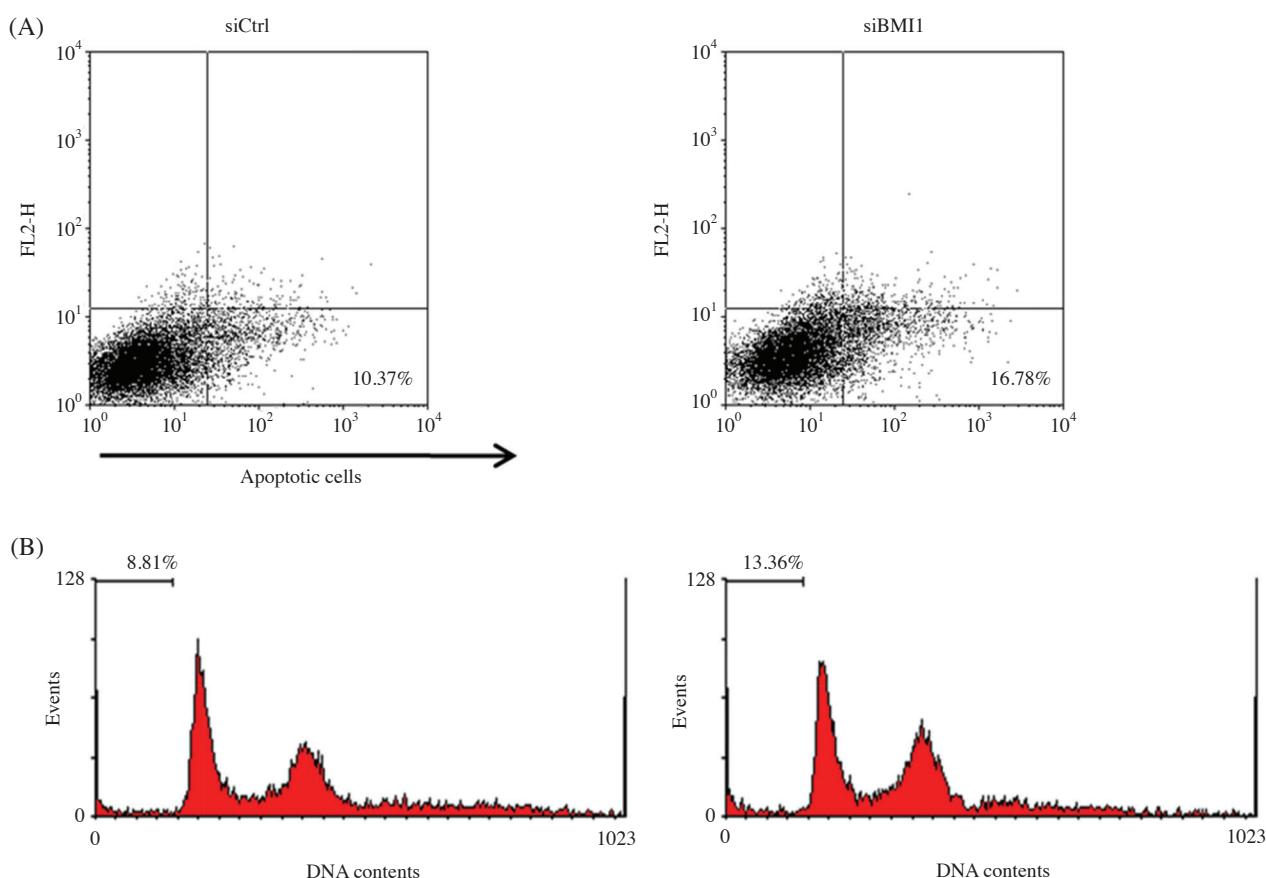


Fig. 2. Effect of BMI1 knockdown on apoptosis and cell cycle arrest in U251 cells. (A) The proportion of apoptotic cells induced by transfection of BMI1 siRNA was greater than that induced by transfection of scramble siRNA in U251 cells. (B) Cell cycle arrest in the subG1 phase increased in BMI1 siRNA-transfected cells compared to scramble siRNA-transfected cells. siCtrl: scramble siRNA, siBMI1: BMI1 siRNA.

siRNA-transfected U251 cells at day 4 ($p < 0.05$) (Fig. 1B). These results indicate that treatment with BMI1 specific siRNA efficiently decreases cell proliferation.

2. Knockdown of BMI1 increases apoptosis in U251 glioma cells

To evaluate the role of BMI1 in apoptosis, we performed FACS analyses with Annexin-V and PI stains. The apoptotic effects of BMI knockdown in U251 cells were determined by estimating the proportion of Annexin-V positive cells and sub-G1 DNA content. The proportion of Annexin-V positive cells after transfection of BMI1 siRNA was greater than after transfection with scramble siRNA (16.78 vs. 10.37%) in U251 cells (Fig. 2A). The sub-G1 DNA content increased in BMI1 siRNA-transfected cells compared to scramble siRNA-transfected cells (8.81 vs. 16.78%; Fig. 2B).

3. Apoptosis induced by BMI1 knockdown is associated with modulation of apoptotic regulatory proteins in U251 glioma cells

During cellular apoptosis following BMI1 knockdown, the expression of various apoptosis-associated proteins in U251 cells were analyzed by western blotting. BMI1 knockdown significantly increased the activation of caspases 3 and PARP, which are key mediators of apoptosis (Fig. 3A). As shown in Fig. 3B, levels of anti-apoptotic proteins, survivin, XIAP, Bcl-xL, and Mcl1 decreased, whereas the pro-apoptotic protein Bax increased in response to BMI1 knockdown. Apoptotic stimuli induces the release of mitochondrial proteins such as Smac/DIABLO, Omi/HtrA2, and cytochrome c from the mitochondria to the cytosol. To detect the release of mitochondrial proteins after BMI1 knockdown, a western blot was performed for proteins in the cytosolic and mitochondrial fractions. The release of

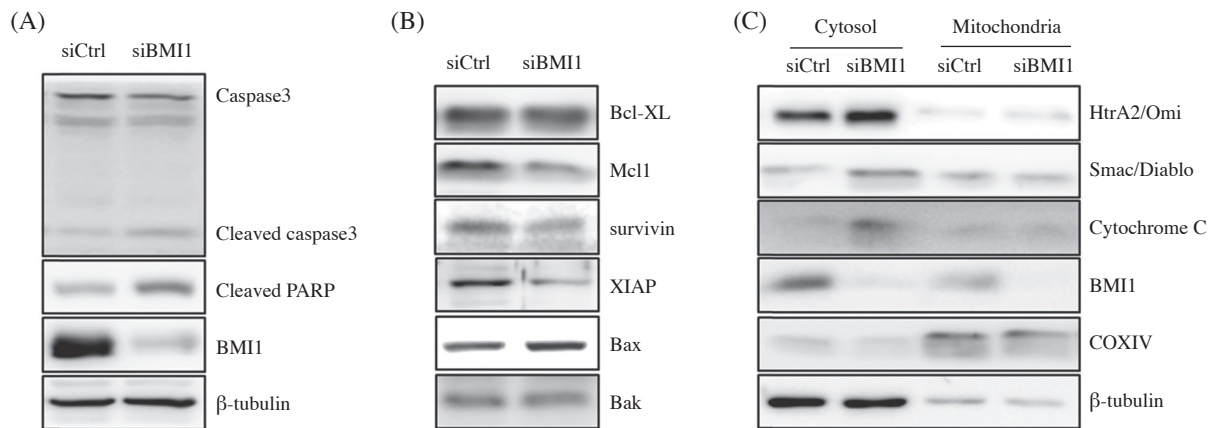


Fig. 3. BMI1 knockdown alters apoptotic regulatory proteins in U251 cells. (A) BMI1 knockdown led to increased apoptotic protein activity, caspase 3, and PARP. (B) BMI1 knockdown in U251 cells regulated the expression of apoptosis related proteins. Anti-apoptotic proteins such as survivin, XIAP, BCL-XL, and Mcl1 decreased, whereas pro-apoptotic protein Bax increased following BMI1 knockdown. (C) Apoptotic mitochondrial release proteins in the cytosol of BMI1 siRNA-transfected cells increased. siCtrl: scramble siRNA, siBMI1: BMI1 siRNA.

Smac/DIABLO, Omi/HtrA2, and cytochrome c into the cytosol was induced by BMI1 knockdown (Fig. 3C).

4. Knockdown of BMI1 inhibits invasion and migration of U251 glioma cells

To investigate the effects of BMI1 knockdown on the biological properties of U251 cells, we evaluated their invasion and migration ability after BMI1 knockdown. The number of invasive cells derived from BMI1 siRNA-transfected cells were 90.0 ± 2.3 , whereas 143.5 ± 2.9 cells were observed for scrambled siRNA-transfected cells. The difference between the two groups was significant (Fig. 4A). Migration distance of scrambled siRNA-transfected cells was 27% greater than that of BMI1 siRNA-transfected cells at 24 and 48 h (Fig. 4B). These results showed that BMI1 knockdown downregulated cellular motility during migration and invasion.

5. BMI1 knockdown decreases phosphorylation of Akt/FOXO1/3a signaling proteins in U251 glioma cells

To explore the underlying mechanisms that regulate the effects of BMI1 knockdown in U251 cells, we examined Akt/FOXOs signaling essential for tumor development and carcinogenesis. The phosphorylation levels of Akt, FOXO1, and FOXO3a decreased after BMI1 knockdown in U251 cells (Fig. 5). Akt inhibition through BMI1 knock-

down inhibits transcriptional functions of FOXOs and affects motility and apoptosis of tumor cells.

Discussion

Gliomas are the most common and malignant central nervous system tumors in humans. Studies focused on the molecular mechanism of migration and apoptosis in glioma cells can potentially reveal targets for glioma treatment. For this study, we used the U251 glioma cell line, which is derived from highly aggressive tumors of the nervous system and is routinely used for studying the biology of gliomas [13,14]. BMI1 is essential for maintaining both the normal and cancerous stem cell state. It is also involved in multiple biological processes such as motility, apoptosis, proliferation, and invasion of cancer cells [15,16]. Recent studies have investigated BMI1 overexpression in many malignant tumors such as colorectal cancer, prostate cancer, non-small cell lung cancer, and hepatocellular carcinoma (HCC) [17,18]. BMI1 overexpression can enhance invasion of prostate cancer, promote cancer progression, and is associated with poor prognosis [9]. Silencing of BMI1 can change the phenotype of malignant tumors, inhibit cancer cell proliferation, and increase sensitivity of cancer cells to chemotherapy [19]. Several studies have determined a relationship between BMI1 and development of gliomas. Liang J et al. reported

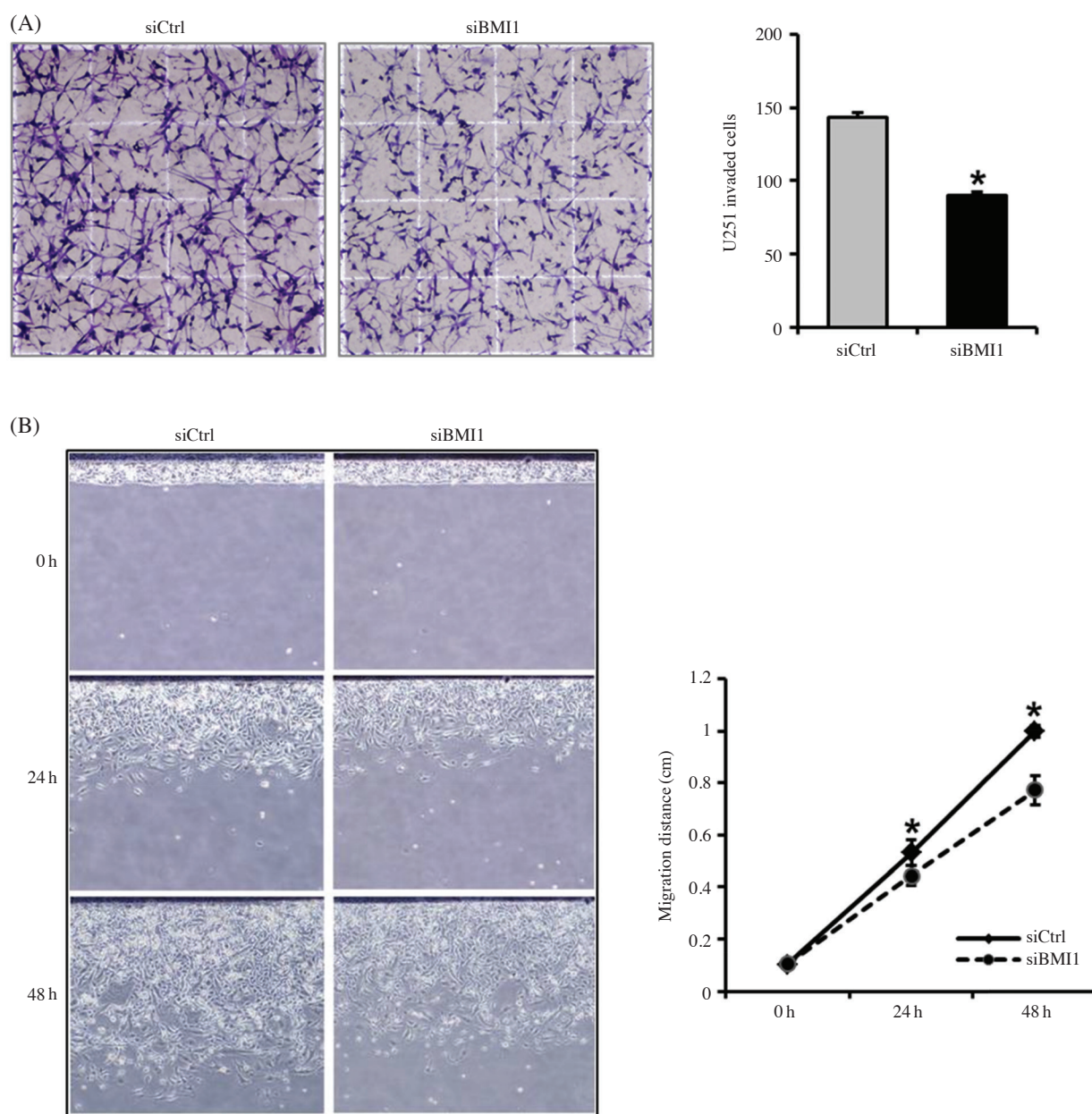


Fig. 4. Effect of BMI1 knockdown on cell invasion and migration in U251 cells. (A) Invasive cells in the cell invasion assay were stained and counted. The comparison between two groups of cells has been represented as a graph. BMI1 siRNA-transfected cells resulted in significantly lower number of invasive cells compared with those from scramble siRNA-transfected cells (mean \pm SE, $n = 6$; $*p < 0.05$, compared to siCtrl). (B) A migration assay using siRNA-transfected cells was performed and graphs represent migration distances of cells from the two groups. Cell migration was significantly affected in BMI1 siRNA-transfected cells (mean \pm SD, $n = 3$; $*p < 0.05$) compared with siCtrl-transfected cells. siCtrl; scramble siRNA, siBMI1; BMI1 siRNA.

that motility of glioma cells was affected by BMI1 [20]. BMI1 has also been associated with the development of radioresistance in glioma cells through suppression of senescence [21] and Jiang L linked BMI-1 to glioma angiogenesis [22]. In this study, we demonstrated that silencing

BMI expression using BMI1 siRNA could attenuate proliferation, migration, and invasion of glioma cells. These results suggest that BMI1 expression is associated with the promotion of tumor cell proliferation, invasion, and migration, which are processes crucial for tumor metastasis.

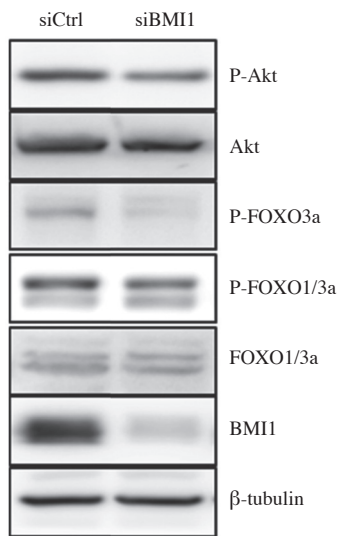


Fig. 5. Effect of BMI1 knockdown on intracellular signaling in U251 cells. BMI1 knockdown leads to downregulation of p-PEN, p-Akt, p-Foxo1/3a, and p-Foxo3a. siCtrl: scramble siRNA, siBMI1: BMI1 siRNA.

Apoptosis is tightly regulated by a complex process that controls expression and degradation of key molecules including B cell leukemia/lymphoma (Bcl)-2 family proteins, inhibitor of apoptosis protein (IAPs) family, mitochondria released protein, and caspase [23-26]. The Bcl-2 family proteins act as primary regulators of the apoptotic process and are divided into anti-apoptotic and pro-apoptotic members. The anti-apoptotic Bcl-2 proteins include Bcl-2, Bcl-xL, Bcl-w, and myeloid cell leukemia1 (Mcl1), whereas pro-apoptotic Bcl-2 proteins include Bax, Bak, Bad, Bid, Bim, and PUMA. The balance between the levels of anti-apoptotic and pro-apoptotic proteins determines fate of cell survival [24,27]. Our study showed that BMI1 knockdown induced cleavage of apoptotic factors caspase-3 and PARP. We also observed that protein levels of anti-apoptotic factors Bcl-xL and Mcl1 reduced, and protein levels of pro-apoptotic factor Bax increased following knockdown of BMI1 in glioma cells. In particular, Bax overexpression was associated with apoptotic cell death, as reported previously [28]. Furthermore, we determined that BMI1 knockdown induced the release of cytochrome C, Smac/DIABLO, and Omi/HtrA2 from the mitochondrial membrane to the cytosol in glioma cells. Upon induction of the death signal, cytochrome C, Smac/DIABLO, and Omi/HtrA2 were released into the cytosol and facilitated apoptosis by binding and neutralizing IAP

proteins. These proteins are known to negatively regulate apoptosis by inhibiting caspase activity directly [29,30]. Small molecule inhibitors of IAPs that block caspase binding effectively induce apoptosis in a variety of cancer cell lines [31]. We also found that BMI1 knockdown induced the production of IAP proteins such as XIAP and survivin.

AKT signaling pathways play a pivotal role in tumorigenesis, enhanced cell proliferation and migration, and inhibition of apoptosis in various human cancers. The phosphorylation of Akt is associated with cancer progression in humans [32,33]. Phosphorylated Akt promotes cell survival, proliferation, and possibly other malignant properties such as motility and invasiveness by phosphorylating downstream targets such as anti-apoptotic protein Bax and forkhead box protein (FOXO) transcriptional factors [34,35]. The FOXO factors function as transcriptional activators and their activation regulates apoptotic responses, cell cycle arrest, and detoxification of reactive oxygen species as well as repair of damaged DNA [36-38]. Here, we did not study the role of BMI1 in the regulation of the Akt pathway. However, previous studies have suggested that BMI1 overexpression contributes to tumor invasion and metastasis by increasing the expression of MMP-2, MMP-9, and VEGF via the Akt pathway [39]. The expression of MMP-2, MMP-9, and VEGF were related with growth, invasion, and metastasis of cancerous cells [40]. In further studies, we will attempt to explore the relationship between BMI1 and the Akt pathway. Additionally, we have not discussed the off-target effects of BMI1 siRNA since previous studies have suggested that off-target effects do not obstruct RNAi experiments [41].

In conclusion, BMI1 knockdown induces apoptosis and decreases cell invasion and cell migration. These phenomena are associated with a decrease in phosphorylation of components of the Akt signaling pathway, which ultimately contributes to cancer progression in humans.

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U251 신경교종세포의 증식, 세포사멸, 침투 및 이동에 BMI1 발현억제가 미치는 영향

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간추림 : BMI1 유전자는 크로마틴 사일런서를 보존시켜 주며 정상 및 암 줄기세포의 단계를 유지하는 데 필수적인 polycomb-repressive complex 1 (PRC1) 단백질의 한 종류이다. 이번 실험에서, siRNA를 이용한 BMI1 유전자 발현 억제가 사람신경교종세포주인 U251에서 종양세포의 침투, 이동 및 세포사멸과 같은 종양작용과 종양진행과 관련된 세포 신호에 어떠한 영향을 미치는지에 대해 알아보았다. 그 결과, BMI1 유전자 발현 억제는 cleaved PARP와 caspase-3의 활성화와 항세포사멸 단백질, survivin, XIAP, Bcl-xL, Mcl1 단백질들의 발현감소를 통해 세포사멸을 증가시켰다. 또한 BMI1 발현억제는 세포 침투 및 이동 능력을 현저히 감소시켰으며 Akt/FOXO1/3a 신호단백질들의 인산화작용의 감소 역시 관찰되었다. 이상의 연구 결과는 BMI1 유전자의 발현억제가 U251 신경교종세포의 세포사멸을 증가시키고, 침투 및 이동을 감소시켰으며 이러한 현상들은 사람 신경교종세포의 진행에 영향을 미치는 Akt신호체계와 연관이 있음을 시사한다.

찾아보기 낱말 : BMI1, 세포사멸, 침투, 이동