

Valproic acid promotes radiosensitization in meningioma stem-like cells

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ABSTRACT

Although meningioma stem-like cells have been isolated and characterized, their therapeutic targeting remains a challenge. Meningioma sphere cells (MgSCs) with cancer stem cells properties show chemo- and radioresistance in comparison with meningioma adherent cells (MgACs). We tested the effect of valproic acid (VPA), a commonly used anti-epileptic drug, which passes the blood brain barrier, on cultured MgSCs. VPA reduced the viability of MgSCs and MgACs. In MgSCs, treatment with VPA increased radio-sensitivity, expression of p-cdc2, p-H2AX and cleaved caspase-3 and PARP. Anchorage-independent growth (AIG) was reduced by VPA. AIG was further reduced by combined treatment with irradiation. Expression of a stem cell marker, Oct4, was reduced by VPA. Oct4 was further decreased by combined treatment with irradiation. These results suggest that VPA may be a potential treatment for meningioma through targeting meningioma stem-like cells.

INTRODUCTION

The presence of cancer stem cells (CSCs) is well documented in many kinds of human tumors [1]. With the ability of self-renewal [2], CSCs are considered responsible for tumor initiation, maintenance, and metastasis. Several studies have isolated human meningioma stem-like cells and characterized their CSCs properties. Isolated meningioma stem-like cells can form spheres and express the CD133 stem cell marker [3-5]. Moreover, isolated meningioma sphere cells (MgSCs) possess chemo- and radioresistant properties and express multiple drug-resistant genes, compared to meningioma adherent cells (MgACs) [3]. Studies by Kalamarides et al. have also demonstrated that different subtypes of meningioma come from a common prostaglandin D2 synthase-positive progenitor cell [6]. Although the biology of meningioma stem-like cells has been well established [3-6], therapeutic strategies targeting CSCs in meningioma remain unclear.

Meningioma is among the most common intracranial tumors and accounts for 13-26% of all intracranial neoplasms. The recurrence rate is reported

to be 20% for World Health Organization (WHO) grade I, 40% for WHO grade II, and 78% for WHO grade III meningiomas [7]. Aggressive meningiomas are related to the high incidence of recurrence and mortality [8]. Management of recurrent meningioma currently is repeat surgical resection or stereotactic radiosurgery. Patients with aggressive meningiomas are treated with prior surgical resection followed by radiotherapy. However, atypical and anaplastic meningiomas remain challenging tumors to treat. Known risk factors such as a larger tumor size, nuclear atypia, increased mitotic rate, and necrosis are key to recurrence [8]. Since mainstream management of meningioma is surgical resection and stereotactic radiosurgery, anti-cancer drugs are less identified as treatment in management of meningioma. Without doubt, the development of adjuvant therapy is warranted to improve tumor control and minimize recurrence.

Valproic acid (VPA, 2-propylpentanoic acid) has been used extensively as an anti-convulsant for more than 40 years and is a frequent choice for patients with seizures [9]. As a histone deacetylase inhibitor, VPA can induce the differentiation of many kinds of cancer cells *in vitro* and suppress tumor growth and metastasis *in vivo* [10].

It improves the responsiveness of tumors to conventional therapeutic agents and increases the radiosensitivity of esophageal squamous cell carcinoma [11]. VPA has been investigated for its anti-cancer effect in many experimental human cancer models of lung cancer [12], renal cell carcinoma [13], bladder cancer [14], myeloma cell [15], and cervical cancer [16]. Its anti-tumor activity varies depending on the cell type and is conducted through multiple mechanisms, such as cell cycle arrest, apoptosis, angiogenesis, metastasis, differentiation, and senescence [17]. The purpose of this study is to examine the therapeutic potential of VPA through the targeting of MgSCs, and explore the related mechanisms.

RESULTS

Expression of Oct4 in MgSCs versus MgACs

To further characterize the stem-like properties of MgSCs, the expression of stem cell marker was analyzed by immunofluorescence and RT-PCR. Of the induced pluripotent stem (iPS) cell factors, Oct4 was first examined since it could reprogram adult stem cells to iPS

cells as a single factor [18]. Day 2 cultured MgSCs formed spheres (Figure 1A) and were positive for Oct4 (Figure 1B), but daughter MgACs had relatively low expression on Oct4. Oct4 mRNA expressions in MgSCs and MgACs were determined by RT-PCR (Figure 1C).

Cell viability of MgSCs and MgACs was reduced by VPA

VPA could induce apoptosis in tumors but not in non-malignant cells [19, 20]. To investigate cell toxicity of VPA on MgSCs and MgACs, cells were treated with VPA for 72 h and the cell viability was determined by MTS assay. At 1 mM ~16 mM, VPA reduced cell viability on both MgSCs and MgACs in a dose-response manner ($p < 0.001$) (Figure 2A), but not on human adipocyte-derived stem cells (hASCs), which served as a non-malignant control, compared to untreated cells. MgSCs were more susceptible to VPA than MgACs at 2 and 4 mM. There were no differences in cell viability between MgSCs and MgACs at 16 mM VPA (Figure 2A). Representative microphotographs are shown in Figure 2B. These results showed that both MgSCs and MgACs were susceptible to VPA treatment, especially MgSCs.

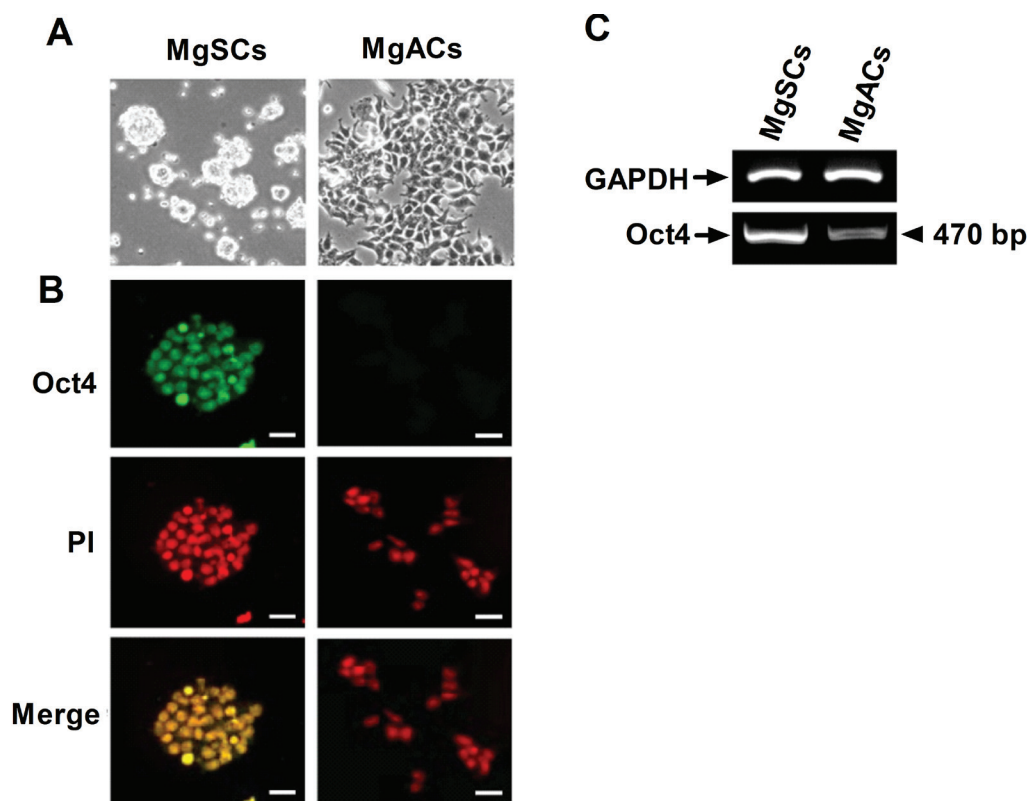


Figure 1: Expression of Oct4 in meningeoma sphere cells (MgSCs) and meningeoma adherent cells (MgACs). (A) Representative bright field micrographs showed growth of cultured MgSCs and MgACs in stem cell culture medium or DMEM with 10% FBS, respectively. (B) Oct4 expressions in MgSCs and MgACs were examined by immuno-fluorescence staining with anti-Oct4 antibody. Magnification, 100x. PI staining indicated the nucleus (red). Merged PI and Oct4 are shown in yellow. Bars: 100 μ m. (C) Oct4 mRNA expression was examined by RT-PCR, with GAPDH as an internal control. Data are representative of 3 independent experiments.

Combined treatment with VPA and irradiation induced cell cycle arrest, apoptosis, and DNA damage in MgSCs

Despite the chemoresistance of MgSCs to vincristine [3], VPA induced more severe cell death in MgSCs than in MgACs (Figure 2). The MgSCs were treated with an IC_{50} dose of VPA (2 mM) and irradiation (5Gy) alone or in combination, and were subjected to MTS assay. The results revealed that MgSCs pre-treated with VPA had reduced cell viability with the use of irradiation compared to the untreated control ($p < 0.01$) (Figure 3A), indicating that VPA increased the radio-sensitivity of MgSCs. Moreover, p-cdc2 (Tyr15), which was elevated in the G2/M phase of the cell cycle [21], was significantly induced by the combined treatment, compared to the untreated control ($p < 0.01$) (Figure 3B). The cleavage of apoptotic proteins, caspase-3 and PARP was also induced by the combined treatment (Figure 3C). DNA damage-inducing p-H2AX [22] was also significantly induced by the combined treatment, compared to the untreated control ($p < 0.001$) (Figure 3D). These findings indicate that VPA enhanced the radiosensitivity of MgSCs and that combined treatment decreased MgSCs survival through mechanisms

like cell cycle arrest, apoptosis and DNA fragmentation.

Combined VPA and irradiation decreased the colony formation of MgSCs

Since VPA could induce cell differentiation and anchorage-independent growth (AIG) was a key criterion for tumor metastasis [17], the effects of VPA and irradiation on MgSCs AIG were determined by soft-agar assay. The number of colony formations in MgSCs treated with radiation or VPA alone was significantly reduced ($*p < 0.05$ and $^{\#}p < 0.001$, respectively) (Figure 4). Moreover, combined treatment significantly reduced colony formation compared to both the untreated control ($^{\#}p < 0.001$) and irradiation alone ($**p < 0.01$).

Combined treatment with VPA and irradiation down-regulated Oct4 expression

To investigate the stem-like properties of MgSCs after treatment with VPA or irradiation alone or in combination, Oct4 expression was examined by immunofluorescence and immunoblotting. The expression of Oct4 in MgSCs was significantly reduced by VPA, but not by

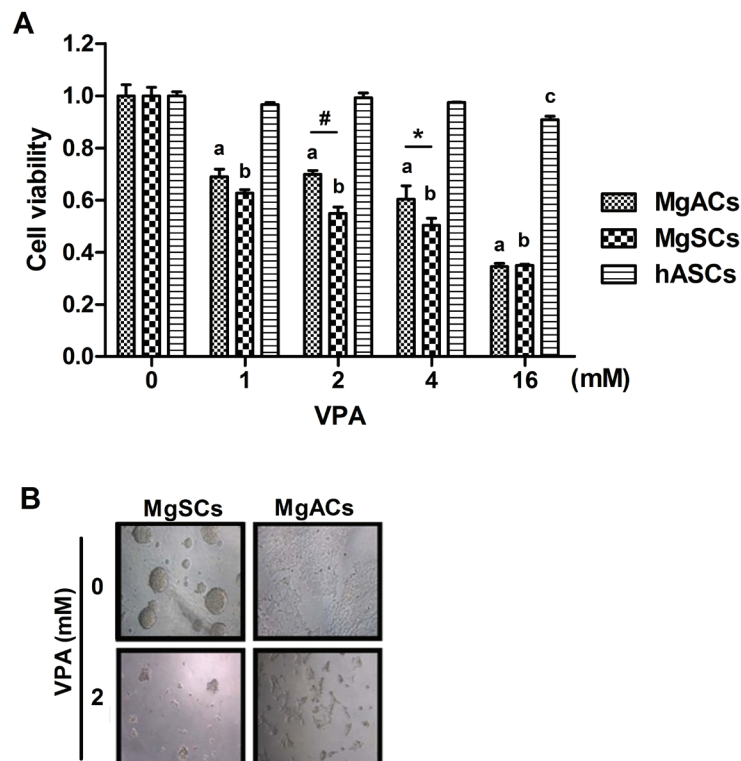


Figure 2: VPA decreased the cell viability of MgSCs and MgACs. (A) MgSCs, MgACs, and hASCs were treated with 1, 2, 4, and 16 mM of VPA for 72 h, and cell viabilities were determined by MTS assay. (B) Bright field micrographs showed the cytotoxicity of VPA on MgSCs and MgACs. Bars, mean \pm SEM; a, $p < 0.001$ compared to MgACs without VPA; b, $p < 0.001$ compared to MgSCs without VPA; c, $p < 0.05$ compared to hASCs without VPA; $*p < 0.05$, $^{\#}p < 0.001$ showed significant differences. Data are representative of 3 independent experiments.

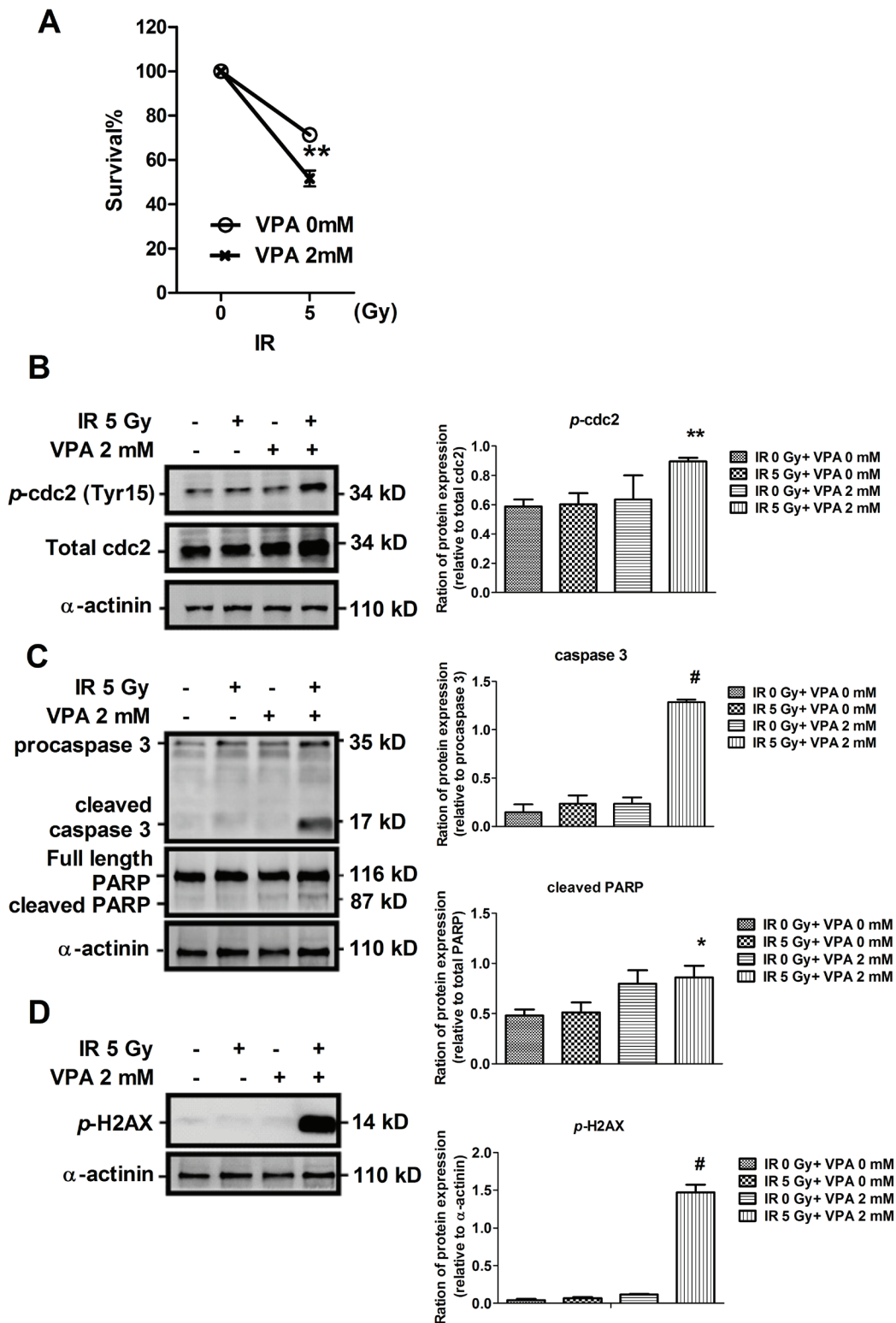


Figure 3: Effects of VPA and irradiation on cell viability and on cell cycle, apoptotic, and DNA damaging protein expressions in MgSCs. MgSCs were treated with or without 2 mM VPA for 24 h followed by irradiation. (A) 24 h after irradiation, the cell viabilities were determined by MTS assay. Immuno-blot showed the (B) protein expressions of *p*-cdc2, (C) cleavage of caspase-3 and PARP, and (D) expression of *p*-H2AX of MgSCs treated with VPA and irradiation. α -actinin, loading control. The quantification results are shown in the right panel. Bars, mean \pm SEM; * p < 0.05, ** p < 0.01, and # p < 0.001 showed significant differences. Data are representative of 3 independent experiments.

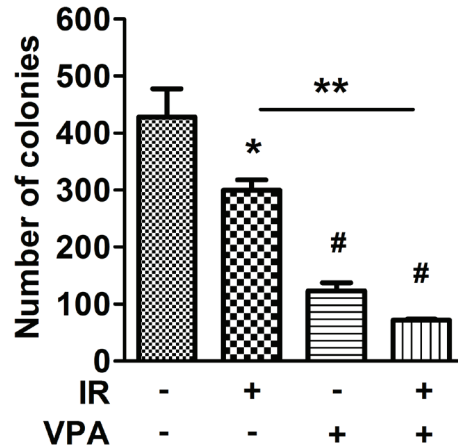


Figure 4: Effects of VPA and irradiation on colony formation of MgSCs. MgSCs were treated with or without 2 mM VPA for 24 h, followed by irradiation 5 Gy. 24 h after irradiation, MgSCs were transferred and cultured in soft agar for 16 days. The colony formations were determined by soft agar assay and the quantitative results are shown. Bars, mean±SEM; * $p < 0.05$, ** $p < 0.01$, and # $p < 0.001$ showed significant differences. Data are representative of 3 independent experiments.

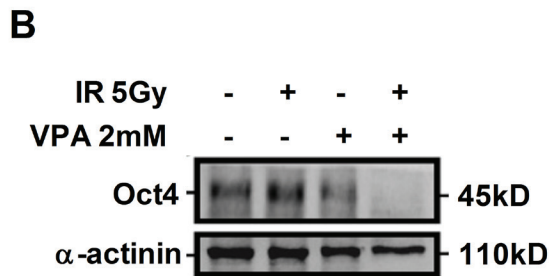
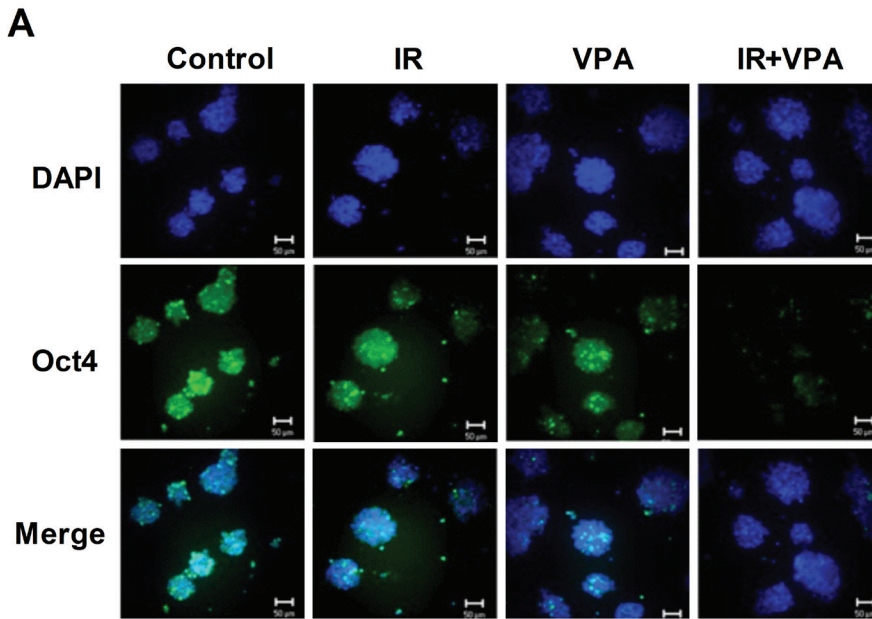


Figure 5: Effects of VPA and irradiation on Oct4 expression. MgSCs were treated with or without 2 mM VPA for 24 h, followed by irradiation 5 Gy. 24 h after irradiation, the Oct4 expression was determined by (A) immuno-fluorescence and (B) immunoblotting. DAPI, nucleus; α -actinin, loading control. Scale bar: 50 μ m.

Table 1: Summary of ongoing, or completed clinical trials using valproic acid as a single agent or in combination with IR, or other agents.

Cancer types	Phase of Trials	Serum level or dosage of VPA	Combined therapies	Outcome/ Status	Reference/ ClinicalTrials.gov Identifier
High Grade Gliomas and Brain Tumors	II	25 mg/kg/day, BID	TMZ + IR	Ongoing	NCT00302159 [§]
Recurrent High-Grade Glioma	II	NA	Tosylate, Sildenafil Citrate	Recruiting	NCT01817751 [§]
High Grade Gliomas	II	15 mg/kg/day	Bevacizumab	Recruiting	NCT00879437 [§]
Brain Metastasis	I	20~50 mg/kg/day	TMZ + IR	NA	NCT00437957 [§]
Refractory Solid or CNS Tumors	I	TID, trough level of 75 to 100 µg/mL	VPA as a single agent	Well tolerated	Su JM et al.[49]
Neuronal Tumors and Brain Metastases	I	10 mg/kg/day	Etoposide	NA	NCT00513162 [§]

BID: Twice a day; NA: Not available; TID: three times daily; TMZ: Temozolomide; IR: irradiation

[§]ClinicalTrials.gov Identifier number from website of <https://clinicaltrials.gov/>

irradiation, compared to the untreated control (Figure 5). Combined treatment with VPA and irradiation further reduced the expression of Oct4 in MgSCs, compared to VPA alone.

DISCUSSION

Using isolated meningioma stem-like cells, this study investigated the therapeutic potential of VPA and irradiation on drug-resistant meningioma. MgSCs treated with VPA prior to irradiation show reduced radio-resistance and anchorage-independent growth. Activation of signaling pathways, including apoptosis, cell cycle arrest, and DNA damage, were observed with combined treatment with VPA and irradiation, along with reduced Oct4 expression. These results suggest that combined treatment with VPA and irradiation might be a novel therapeutic strategy in the treatment of radioresistant meningiomas.

As CSCs are considered the major cause of chemo-resistance, radioresistance, and early recurrence of tumor, the induction of differentiation by VPA may hold promise

in cancer therapy. Supporting this hypothesis, this study found that VPA reduces Oct4 expression in MgSCs (Figure 5). Moreover, AIG is greatly impaired by VPA treatment (Figure 4), suggesting the loss of stemness of MgSCs. The finding of the differentiation-promoting activity of VPA on MgSCs is consistent with previous studies on various cancer cells, including neuroblastoma [23], glioblastoma [24], head and neck cancer [25], thyroid cancer [26], and uveal melanoma [27]. Furthermore, recent studies using isolated glioblastoma-derived stem cells treated with VPA showed a reduced proliferation rate and expression of stem cell markers, including Oct4, indicating cell differentiation [28].

VPA also promotes cell self-renewal and reprogramming in a cancer type-dependent manner. For example, inclusion of VPA in the reprogramming procedure can significantly increase the efficiency of iPS cells induction in cells over-expressing Oct4, Sox2, and Klf-4 [29]. Moreover, VPA increases the proliferation and self-renewal of normal hematopoietic stem cells (HSCs) to expand the HSC pool [30]. Mechanistic investigation by Teng et al. revealed that VPA activates the hormone

response element on the Oct4 promoter through the PI3K/AKT/mTOR pathway and exhibits a pluripotency-promoting effect in myogenic cells [31, 32]. Studies on isolated breast cancer stem-like cells showed that VPA promotes cell de-differentiation via WNT/ β -catenin signaling [33], suggesting the effect of VPA on CSCs was in a cancer type-dependent manner.

The divergent effect of VPA on cell differentiation has also been reported in previous studies, suggesting an association with cell differentiation level and underlying genetic alternation [34]. Since the functional proteomic expression varies with different cell types, the identification of specific molecular targets activated by VPA for promoting MgSCs differentiation may shed light on the application of VPA for cancer therapy.

The anti-cancer effects of VPA include cell cycle arrest, apoptosis, and DNA damage, and vary according to cell types [17]. In MgSCs, 2 mM VPA cannot significantly alter the activation of the apoptotic protein caspase-3 and PARP, cell cycle arrest protein *p*-cdc2, and DNA-damage inducing *p*-H2AX. However, the possibility that 2 mM VPA can induce cell death in MgSCs through cell apoptosis, cell cycle arrest, and DNA damage cannot be completely ruled out, since limited protein targets have been examined. In addition, previous studies have demonstrated that VPA also induces cell death through a caspase-independent pathway [35] and by autophagy [36]. Whether VPA induced MgSCs cell death through these pathways needs further investigation.

Combined treatment significantly induces *p*-cdc2, cleavage of caspase-3, cleavage of PARP, and *p*-H2AX expression with reduced cell viability, indicating a synergistic effect on signaling activation to promote cell death. VPA-induced radiosensitization has also been reported in various cancers, including colon cancer [37], esophageal squamous carcinoma [11], prostate cancer [38], and glioma [39]. With regard to combination therapies for CSCs, Jokinen et al. proposed drug ablation of the ALK oncogene using the ALK tyrosine kinase inhibitor, TAE684, in combination with PI3K inhibitor, or salinomycin. The features of CSCs were inverted with reduction of colony formation, indicating combination drug therapies can suppress CSCs features in acquired and adaptive resistance [40].

In vivo phase III clinical trials have been conducted or are ongoing using VPA alone or in combination with irradiation or other chemotherapeutic drugs for the treatment of cancers such as advanced cervical cancer (Table 1) [41]. Since VPA can pass the blood brain barrier, the above strong *in vivo* evidence from human clinical trials further supports the rationale for the potential application of VPA in the treatment of brain meningiomas. Our study clarifies the mechanism of action of VPA in meningioma stem-like cells, and further supports the evidence from the above *in vivo* clinical trials.

Novel strategies based on identifying new

mechanisms of old drugs may open new windows for developing chemotherapeutic agents targeting CSCs. Despite inducing CSCs differentiation, a novel concept targeting the conserved mitochondrial biogenesis pathway among CSCs to reduce its clonal expansion was also reported for FDA-approved antibiotics [42].

In summary, the present study suggests VPA is a potentially effective drug in the treatment of high recurrence meningioma. MgSCs and MgACs are both sensitive to VPA, which significantly reduces the radioresistance and anchorage-independent growth of MgSCs. VPA increases the susceptibility of MgSCs to irradiation. Oct4 expression in MgSCs was dramatically reduced by combined treatment with VPA and irradiation. These results also provide a novel insight into the development of an effective therapeutic strategy using a lower drug dosage and irradiation for the treatment of meningioma.

MATERIALS AND METHODS

Cell culture

The protocol for the maintenance of meningioma stem-like cells was as described previously [3]. In brief, meningioma stem-like cells were cultured in medium to obtain MgSCs from primary meningioma cells, while the control MgACs were maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (Harlan–Serlab, Belton, UK) on coating dishes.

Stem cell culture medium contained serum-free stem cell culture neurobasal DMEM/F12 medium (Gibco, CA, USA), B27 supplement, fresh aliquots of growth factors, 10 ng/ml recombinant human epidermal growth factor (EGF; Peprotech Rocky Hill, NJ, USA), and 10 ng/ml recombinant human basic fibroblast growth factor (bFGF; Peprotech, Rocky Hill, NJ, USA). The human adipose-derived stem cells (hASCs) were provided by S.M. Huang [43] and maintained in DMEM-low glucose (DMEM-LG; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin-streptomycin (Invitrogen) to form sphere cells as non-tumor control cells. The cells were maintained in culture dishes at 37°C in 5% carbon dioxide.

The medium was changed every 3 days. When the cells grew to 70-90% confluence, they were trypsinized (0.25% trypsin; Sigma), and then neutralized by culture medium. The cells were passed at a ratio of 1:3. The characterization of human ASCs was as described previously [44].

MTS assay

The CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) was used for MTS assay. MgSCs, MgACs, and hASCs (2×10^4) were grown in 96-well plates with fresh culture medium. Following treatment with control solvent or VPA, 20 μ l MTS solution was added to each well and the plate was allowed to incubate for 4 h at 37°C. The same dilution of MTS solution in DMEM/F12 medium alone was used as the background.

After incubation, absorbance was recorded at 490 nm. For data analysis, background values were subtracted from all sample values. The calculated absorbance was directly proportional to the number of living cells in the culture.

Reagents and irradiation treatment

VPA was obtained from Sanofi-Aventis (Paris, France). Antibody against Oct4, *p-cdc2*, total *cdc2*, and PARP were purchased from Cell Signaling (Danvers, MA). Antibody against caspase-3 and *p-H2AX* were purchased from Abcam, Inc. (Cambridge, MA). Antibody against α -actinin was purchased from Santa Cruz Biotech, Inc. (Santa Cruz, CA). For the radioresistance assay, the cells were irradiated using a CyberKnife radio-surgery system (Accuray, USA) to deliver different doses.

Immunofluorescence staining

The immunofluorescence staining followed the previous method [3, 21, 45, 46]. The MgACs and MgSCs were fixed using 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 min, blocked with bovine serum albumin for 1 h, washed with PBS containing 0.1% Tween 20, and stained with Oct4 antibody, nuclear dye propidium iodide (PI) (Sigma, St Louis, MO, USA), or nuclear dye 4',6-diamidino-2-phenylindole (DAPI) for 1 h at 4°C. Immunofluorescence was detected after incubation with the appropriate secondary antibodies conjugated with FITC (eBioscience, San Diego, CA, USA) at room temperature for 45 min. After mounting cover-slips with SlowFade Light anti-fade reagent (Molecular Probes, Eugene, OR, USA), immunofluorescent pictures were acquired using a CCD camera (Zeiss). The scale bar was labeled using the SPOT RT3 software (Diagnostic Instruments, Sterling Heights, MI, USA).

Cell lysate preparation and Western blots

Cells were harvested by centrifugation at 1000g for 10 min and lysed by RIPA buffer (100mM Tris-HCl, 150mM NaCl, 0.1% SDS, and 1% Triton-X-100) at 4°C for 10 min. The cell lysates were harvested by centrifugation at 15000 rpm for 10 min to obtain the supernatants for Western blotting. In brief, aliquots of 20 μ g proteins from each group were applied to 10% sodium dodecyl sulfate polyacrylamide gels and electrophoresed for 3 h at 80 V. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore) and blocked with 5% bovine serum albumin in PBS for 2 h at room temperature. Band detection was conducted by enhanced chemiluminescence (Millipore) and an LAS-3000 imaging system (Fujifilm, Tokyo, Japan). Band densities were measured with the gel analysis system (BioSpectrumAC Imaging System Vision Work LS software; UVP, Upland, CA, USA) [47].

RNA isolation and RT-PCR

Total RNA was extracted using EasyPure Total RNA reagent (Bioman, Taiwan, ROC) according to the manufacturer's instructions. Total RNA (1.0 μ g) was reverse transcribed (RT) with MMLV Reverse Transcriptase (Epicentre Biotechnologies, USA) according to the manufacturer's instructions. The primer pairs used were: Oct4 forward, 5'-GAGAATTTGTTCTCCTGCAGTGC -3' and reverse, 5'-GTTCCCAATTCCTTCCTTAGTG -3' [48] and GAPDH forward, 5'-CTTCATTGACCTCAACTAC-3' and reverse, 5'-GCCATCCACAGTCTTCTG-3'. The PCR products were subjected to 1.5% agarose gel and visualized with UV light after ethidium bromide staining.

Anchorage-independent growth in soft agar assay

Cell suspensions (1×10^4) were incubated in an upper layer of 0.3% agar (Difco, Detroit, MI, USA) in DMEM with 2% FBS. This was overlaid on 0.5% basal agar with 2% FBS. Cultures were maintained for 16 days, replenishing the upper medium layer twice a week, and then staining with methylene blue diluted in ethanol. Colonies were counted by microscopy (CK2; Olympus, Tokyo, Japan).

Statistical analysis

All data were calculated using IBM SPSS statistics 20. Data were expressed as mean \pm standard error of the mean (SEM) and differences between counts were determined using One way Anova. Statistical significance was set at $p < 0.05$.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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