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Overexpression of eIF4F components in meningiomas and suppression of meningioma cell growth by inhibiting translation initiation

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Abstract

Meningiomas frequently display activation of the PI3K/AKT/mTOR pathway, leading to elevated levels of phospho-4E binding proteins, which enhances protein synthesis; however, it is not known whether inhibition of protein translation is an effective treatment option for meningiomas. We found that human meningiomas expressed high levels of the three components of the eukaryotic initiation factor 4F (eIF4F) translation initiation complex, eIF4A, eIF4E, and eIF4G. The expression of eIF4A and eIF4E was important in sustaining the growth of *NF2*-deficient benign meningioma Ben-Men-1 cells, as shRNA-mediated knockdown of these proteins strongly reduced cell proliferation. Among a series of 23 natural compounds evaluated, silvestrol, which inhibits eIF4A, was identified as being the most growth inhibitory in both primary meningioma and Ben-Men-1 cells. Silvestrol treatment of meningioma cells prominently induced G2/M arrest. Consistently, silvestrol significantly decreased the amounts of cyclins D1, E1, A, and B, PCNA and Aurora A. In addition, total and phosphorylated AKT, ERK and FAK, which have been shown to be important drivers for meningioma cell proliferation, were markedly lower in silvestrol-

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Conflict of interest statement

The authors declare no conflict of interest.

Authorship

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treated Ben-Men-1 cells. Our findings suggest that inhibiting protein translation could be a potential treatment for meningiomas.

Keywords

Protein translation; eIF4F; eIF4A; eIF4E; eIF4G; meningioma; neurofibromatosis type 2 (NF2); merlin; silvestrol

1. Introduction

Meningiomas, accounting for over one third of all primary brain tumors, cause significant morbidity, including cranial nerve palsy, seizures, and brainstem compression, which may lead to paralysis, aspiration pneumonia, and death (Ostrom et al., 2015). Current treatment options for these tumors are limited to surgery and radiation. However, incomplete tumor resection is not uncommon and is one of the main causes of tumor recurrence (Goldbrunner et al., 2016). Even after total gross resection, the 5-year recurrence rate is about 7–23% (Rogers et al., 2015). Meningiomas can occur sporadically or in patients with neurofibromatosis 2 (NF2), which is caused by the inactivation of *NF2*/merlin tumor suppressor function (Rouleau et al., 1993; Trofatter et al., 1993). Importantly, patients with NF2 are at increased risk for multiple meningiomas, which often requires them to undergo several arduous treatment cycles. Development of an effective systemic medical therapy for meningiomas is therefore of urgent clinical need.

In addition to NF2-assocaited meningiomas, about 50% of sporadic tumors also harbor *NF2* mutations. Loss of merlin abnormally activates several mitogenic signals, such as the PI3K/AKT/mTOR pathway (Ammoun et al., 2008; Jacob et al., 2008). Indeed, meningiomas often exhibit elevated levels of phospho-AKT (p-AKT), which can promote protein biosynthesis by stimulating p70 S6-kinase, leading to phosphorylation of the S6 ribosomal protein (Sorrells et al., 1999; De Benedetti and Graff, 2004; Holland et al., 2004; Anjum and Blenis, 2008; Menon and Manning, 2008; Kleiner et al., 2009; Ma and Blenis, 2009; Silvera et al., 2010; Li et al., 2012). Other important signaling proteins downstream of the PI3K/AKT/mTOR pathway include the 4E-binding protein (4E-BP) translational repressors; upon phosphorylation, these proteins are inactivated and protein biosynthesis is facilitated. Consistent with this notion, meningiomas display high p-4E-BP levels (Pachow et al., 2013), suggesting that protein translation initiation is likely enhanced in these tumors.

Initiation of protein translation is strictly-controlled and occurs when the eukaryotic initiation factor 4F (eIF4F) complex is recruited to the 5′ cap structure of mRNA, followed by the unwinding of the secondary structure of its 5′ untranslated region (Jackson et al., 2010; Silvera et al., 2010; Blagden and Willis, 2011). The eIF4F complex is composed of three components: eIF4E binds the 5′ cap; the eIF4A RNA helicase unwinds the secondary structure; and eIF4G is a scaffold for other eIFs and enhances the eIF4A helicase activity. Studies have shown that the 4E-BP proteins repress translation by binding to eIF4G and preventing its association with eIF4E. Phosphorylation of 4E-BPs causes these proteins to dissociate from eIF4G, thus promoting eIF4F assembly. The effective formation of the eIF4F complex may require sufficient levels of each eIF4F component.

As uncontrolled growth of tumor cells often requires a high degree of protein translation, increased expression of the eIF4F components has been reported in several cancer types (Sorrells et al., 1999; De Benedetti and Graff, 2004; Kleiner et al., 2009; Silvera et al., 2010; Li et al., 2012). Overexpression of eIF4E promotes cell transformation (Lazaris-Karatzas et al., 1990) and frequently correlates with high tumor grade and poor patient prognosis (Li et al., 1997; Berkel et al., 2001; Li et al., 2012). While the oncogenicity of eIF4A and eIF4G has not been as well characterized as for eIF4E, elevated eIF4A and eIF4G levels have been reported in hepatocellular and lung carcinomas, respectively (Shuda et al., 2000; Bauer et al., 2001; Comtesse et al., 2007). Also, forced overexpression of eIF4A enhances malignant progression in an acute lymphocytic leukemia mouse model (Wolfe et al., 2014). Furthermore, enhanced protein translation has been described in a spectrum of neurological disorders, including autism spectrum disorders and fragile X syndrome (Silvera et al., 2010; Gkogkas et al., 2013; Gkogkas et al., 2014), suggesting that targeting translation initiation may have therapeutic potential. Recently, we reported elevated expression of all three eIF4F components in *NF2*-deficient vestibular schwannomas, and depletion of these eIF4F components reduced schwannoma cell growth (Oblinger et al., 2016). However, the protein levels of eIF4A, eIF4E, and eIF4G have not been rigorously explored in meningiomas.

In this study, we used meningioma tumors, primary meningioma cell cultures, and the benign *NF2*-deficient meningioma Ben-Men-1 cell line to demonstrate that meningiomas overexpress all three eIF4F components. Overexpression of these components is an important driver of meningioma cell proliferation, as confirmed by shRNA-mediated knockdown and pharmacological inhibition. Our results suggest that inhibition of translation initiation factors should be further evaluated as a potential treatment for these tumors.

2. Materials and Methods

2.1. Tissue acquisition and cell cultures

The Ohio State University (OSU) Institutional Review Board (IRB) approved the human subjects protocols for the acquisition of meningioma specimens, the diagnosis of which was confirmed by a pathologist. Fresh meningioma tissues were finely minced and digested with collagenase/dispase at 37°C overnight as previously described (Chang and Welling, 2009). Primary meningioma cells, normal human meningeal cells (ScienCell), and *NF2*-deficient benign meningioma Ben-Men-1 cells were grown in Dulbecco's modified Eagle medium (DMEM) and 10% fetal bovine serum (FBS) (Thermo Fisher) as described previously (Burns et al., 2013). All primary cell cultures were used at early passages (less than five passages).

2.2. Natural compound treatment, cell proliferation assays, and flow cytometry

A series of 23 natural compounds, including silvestrol (Figure 3A), was isolated and their structures and absolute configurations were previously reported (Kinghorn et al., 2011; Oblinger et al., 2016). Purified compounds were dissolved in dimethyl sulfoxide (DMSO) to 1~10 mM and then diluted for cell proliferation assays. Briefly, Ben-Men-1 and primary meningioma cells were seeded in 96-well plates at 4,000 cells/well and treated the next day with various concentrations of each natural compound or DMSO as a control. After three

days, cell proliferation was assessed using resazurin, and the IC_{50} values were determined (Burns et al., 2013).

Cell cycle analysis of drug-treated Ben-Men-1 cells was performed as previously described (Oblinger et al., 2016). A FACSCalibur flow cytometer (Becton Dickinson) was used to analyze samples after gating around the diploid population (FL2-A/FL2-W). Histograms and cell cycle distribution were determined using ModFit LT software (Verity Software House).

2.3. Western blots

Subconfluent Ben-Men-1 cells were treated with the indicated concentrations of silvestrol or DMSO vehicle for 24 hours, followed by cell lysis and Western blotting according to Oblinger *et al.* (Oblinger et al., 2016). The antibodies used included anti-eIF4A1 (2490), AKT (9272), phospho-AKT [p-AKT(Ser^{473})] (4060), extracellular signal-regulated kinases 1 and 2 (ERK1/2; 4695), p-ERK (4370), proline-rich Akt substrate of 40 kDa (PRAS40; 2691), p-PRAS40(Thr^{246}) (2997), focal adhesion kinase (FAK; 3285), cyclin B1 (4138), cyclin D1 (2978), cyclin E1 (4129), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 5174), merlin (12888) (all from Cell Signaling Technology), proliferating cell nuclear antigen (PCNA; sc-56), cyclin A (sc-751), eIF4A2 (sc-137148), and eIF4G (sc-133155) (all from Santa Cruz Biotechnology), p-FAK(Tyr^{397}) (700255 from Thermo Fisher), and eIF4E (ab33768 from Abcam). Protein bands were detected using an HRP-conjugated secondary antibody and the ECL Plus Western Blotting Substrate (Thermo Fisher) or a fluorescently-labeled secondary antibody and the Odyssey CLx imaging system (LI-COR Biosciences).

2.4. Lentiviral-mediated short-hairpin RNA (shRNA) transduction

Ben-Men-1 cells were seeded in 6-well plates at 9,000 cells/well. The next day, cells were fed with fresh DMEM/10% FBS containing 8 μg/mL polybrene and then incubated overnight with MISSION shRNA lentiviruses targeting *EIF4A1* (TRCN0000288729; target sequence 5′-GCCGTAAAGGTGTGGCTATTA-3′), *EIF4A2* (TRCN0000051869; target sequence 5′-CGGGAGAGTGTTTGATATGTT-3′), *EIF4E* (TRCN0000062573; target sequence 5′-CCACTCTGTAATAGTTCAGTA-3′) or shNon-targeting lentivirus (#SHC002V, insert sequence 5′-CAACAAGATGAAGAGCACCAA-3′; all from Sigma) at a multiplicity of infection (MOI) of 10. Two days following transduction, puromycin (Corning) was added to the culture medium to a final concentration of 2.5 μg/mL. Cells were grown for another 8 days before counting on a hemocytometer. Subsequently, the cells were centrifuged, and the resulting pellets were lysed in 2% SDS with protease and phosphatase inhibitors (Sigma). Equal amounts of protein were analyzed by Western blot for eIF4A1, eIF4A2, eIF4E, and GAPDH (Oblinger et al., 2016). Fluorescently-labeled protein bands were captured on an Odyssey CLx Imager (LI-COR BioSciences), quantitated using Image Studio, and calculated as percent of the nontargeting control after normalization to GAPDH.

2.5. Immunohistochemistry (IHC)

Archived formalin-fixed, paraffin-embedded sections of human meningioma tissues were acquired, deparaffinized, and immunostained for eIF4AI-II (sc-50354) and eIF4G (sc-133155) (both from Santa Cruz) and eIF4E (ab33768 from Abcam) as previously described (Burns et al., 2013). Quantification of immunostaining signal was performed by a

pathologist and an investigator using visual analysis based on the intensity and percentage of immunopositive cells.

3. Results

3.1. Meningiomas expressed elevated levels of eIF4A, eIF4E, and eIF4G compared to normal meningeal cells

To determine the expression levels of the eIF4F components, we performed immunohistochemical staining for eIF4A, eIF4E and eIF4G on 16 meningiomas, including six grade I sporadic tumors, four grade I NF2-associated tumors, five grade II sporadic tumors, and one grade III sporadic tumor. All of these meningiomas showed more intense labeling of eIF4F components compared to adjacent normal meningeal tissues (Figure 1 and Table 1). Fifty percent of the meningioma specimens (8/16) were scored as having 1+ or greater labeling for eIF4A, 94% of the tumors (15/16) were scored as having 1+ or greater labeling for eIF4E, and 75% percent of the tumors (12/16) were scored as having 1+ or greater labeling for eIF4G. Notably, all four NF2-associated tumors had intense staining for eIF4A and eIF4E (Table 1).

The RNA helicase eIF4A had prominent perinuclear staining, consistent with its role in translation initiation and localization to the rough endoplasmic reticulum. Staining for eIF4E occurred more diffusely throughout the cytoplasm, with faint nuclear labeling (Rosenwald et al., 1995; Culjkovic et al., 2008). The labeling pattern for eIF4G was diffusely cytoplasmic, with some conspicuous rimming around the nucleus that resembled the perinuclear concentrations observed with eIF4A. While the sample size was limited, the six high grade meningiomas (five grade II and one grade III) tended to show higher labeling in eIF4G, when compared to grade I tumors (Table 1).

To confirm these findings, we compared eIF4A, eIF4E and eIF4G protein expression in four primary grade I meningioma cultures prepared from two sporadic (samples #1 and 2 in Figure 2A) and two NF2-associated tumors (samples #3 and 4) and the telomerase-immortalized Ben-Men-1 benign meningioma line with those in primary meningeal cells. All four primary cultures of meningioma cells and Ben-Men-1 cells uniformly exhibited higher levels of all three eIF4F components relative to normal meningeal cells (Figure 2A). As previously reported (Burns et al., 2013), Ben-Men-1 cells did not express merlin protein. In addition, merlin was not detected in three of the four primary meningioma cultures and was greatly reduced in the fourth meningioma culture. Collectively, these results indicate increased expression of eIF4A, eIF4E and eIF4G in meningiomas.

3.2. Inhibition of eIF4A and eIF4E impaired meningioma cell proliferation

To verify the importance of eIF4F components in meningioma cell growth, we used shRNA-containing lentiviruses to silence eIF4A and eIF4E expression in Ben-Men-1 cells. Consistent with the knockdown efficiencies (Figure 2B), depletion of eIF4A2 or eIF4E dramatically reduced cell numbers by about 87% and 80%, respectively, relative to control cells that were transduced with a nontargeting shRNA construct (Figure 2C). Silencing eIF4A1 gave rise to partial growth suppression, which was commensurate with a moderate

knockdown efficiency (Figures 2B and 2C). These results indicate that silencing of either eIF4A1 or eIF4A2 impairs meningioma cell proliferation. We also noted that knockdown of any individual eIF resulted in modest decreases in the other eIF components. In these conditions, the levels of eIF4E seemed to be particularly sensitive, decreasing by 53% and 36% after depletion of eIF4A1 or eIF4A2, respectively.

As an FDA-approved medical therapy is currently not available for the treatment of meningiomas, identification of new therapeutics for patients with meningiomas is of great clinical interest. The growth inhibition observed from the eIF4A and eIF4E RNA interference experiments (Figures 2B and 2C) suggested that meningioma cells may be susceptible to pharmacological inhibition of these eIF4F components. Interestingly, we found that among a series of botanical compounds evaluated, the eIF4A inhibitors silvestrol and episilvestrol, an isomer of silvestrol, were the most potent in inhibiting proliferation of Ben-Men-1 cells and primary meningioma cells based on the IC_{50} values and maximal killing effects (Figure 3A). Silvestrol consistently inhibited meningioma cell proliferation at very low IC_{50} values (~10 nM in Ben-Men-1 and ~25 nM in primary meningioma cultures) (Figures 3A and 3B). These results highlight the importance of eIF4F components in the growth of meningioma cells.

3.3. Silvestrol treatment induced G_2/M arrest and reduced the levels of several cell-cycle and mitogenic proteins

Consistent with previous reports (Mi et al., 2006; Oblinger et al., 2016), silvestrol induced cell cycle arrest at the G_2/M phase. Ben-Men-1 cells treated with the IC_{50} dose of silvestrol for three days had an over two-fold increase in the proportion of cells in G_2/M (untreated, 5.68% versus silvestrol-treated, 12.86%; Figure 4). This effect became even more pronounced at twice the IC_{50} dose, in which over half the cell population was in the G_2/M fraction. To further examine the effects of silvestrol on the cell cycle, we profiled the expression of various cell cycle proteins. Ben-Men-1 cells treated with silvestrol exhibited sharp reductions in all examined cyclins (D1, E1, A, and B1) as well as the mitotic Aurora A kinase (Figure 5). PCNA, which acts during S phase to facilitate DNA synthesis, was also markedly reduced. This is consistent with the finding that silvestrol treatment of Ben-Men-1 cells reduced the S phase fraction (Figure 4).

NF2-deficient tumors often exhibit increased phosphorylation of the AKT, ERK 1/2, and FAK kinases (Poulikakos et al., 2006; Jacob et al., 2008; Hilton et al., 2009; Endo et al., 2013; Ammoun et al., 2014), and activation of these kinases can lead to enhanced protein biosynthesis (Silvera et al., 2010). We found that silvestrol treatment decreased both the total and phosphorylated AKT as well as its downstream substrate PRAS40 in Ben-Men-1 cells (Figure 5). Likewise, both the total and phosphorylated ERK1/2 and FAK were suppressed in silvestrol-treated meningioma cells. Taken together, these results suggest that silvestrol exerts its antiproliferative action by simultaneously reducing multiple pro-growth signaling molecules.

4. Discussion

Despite being the most frequent brain tumors, the biology of meningiomas is not well understood and an FDA-approved medical therapy is currently not available for these tumors. While alterations in the *NF2* gene frequently occur, recent molecular genetic studies have identified mutations in *AKT1*, *SMO*, *KLF4*, *TRAF7*, *POLR2A* in non-NF2 meningiomas (Brastianos et al., 2013; Clark et al., 2013; Clark et al., 2016). In addition, gene expression analyses have identified AKT as one of the key drivers for meningioma growth (Wang et al., 2012; Hilton et al., 2016). Activation of AKT promotes protein translation initiation, which is dependent upon expression and assembly of several eIF complexes (Jackson et al., 2010; Silvera et al., 2010). Intriguingly, high levels of eIF4E correlate with meningioma grade (Tejada et al., 2009). Meningiomas lacking *NF2* frequently have more abundant eIF3C than *NF2*-expressing tumors (Scoles et al., 2006). We now report that all three components of the eIF4F complex are overexpressed in meningiomas. While the number of meningiomas that we analyzed is small, the NF2-associated grade I meningiomas that we analyzed exhibited high levels of these proteins, particularly eIF4A and eIF4E, when compared to sporadic grade I tumors (Table 1 and Figure 2A). Similarly, we recently reported that *NF2*-associated vestibular schwannomas also appeared to have more eIF4F components (Oblinger et al., 2016). In addition, we observed that the six high grade (II and III) tumors expressed higher amounts of eIF4E and eIF4G, compared to sporadic grade I tumors (Table 1). In line with this tumor study, our primary cultures of meningioma cells also showed elevated levels of eIF4F components compared with normal meningeal cells. It would be interesting to extend our study to a larger cohort of tumors to see if these findings can be confirmed and if they correlate with any clinical outcome parameters.

Both RNA silencing and pharmacological inhibition verified that these eIF4F components are critical for meningioma cell growth. Using shRNA-mediated knockdown in Ben-Men-1 cells, we found that silencing eIF4A1, eIF4A2, or eIF4E was sufficient to inhibit cell growth. Curiously, eIF4A2 appeared to be as important for proliferation as eIF4A1 since eIF4A2 knockdown profoundly impaired proliferation of meningioma cells (Figure 2C). Previously, we observed similar results in malignant peripheral nerve sheath tumor (MPNST) cells (Oblinger et al., 2016). However, the role of eIF4A2 on cell proliferation may depend upon the cell type (Galicia-Vázquez et al., 2012). In a similar context, it would be also interesting to investigate the role of eIF4G in meningioma cell growth. As isoforms exist in eIF4G (Silvera et al., 2010), the contribution of each isoform should be evaluated as we did with eIF4A1 and eIF4A2. Unexpectedly, we did not observe that depletion of eIF4A1, eIF4A2, or eIF4E resulted in a compensatory increase of the other two eIF4F components, but instead, we saw modest decreases in the levels of these components (Figure 2B). Our previous study in MPNST cells also showed reduced eIF4E levels following knockdown of eIF4A1 or eIF4A2 protein (Oblinger et al., 2016), suggesting coordinate regulation of these eIF4F components.

The importance of eIF4A activity for meningioma cell proliferation was also validated by the identification of the eIF4A inhibitor silvestrol as being profoundly growth inhibitory in both primary meningioma and Ben-Men-1 cells. Consistent with previous reports (Mi et al.,

2006; Oblinger et al., 2016), silvestrol treatment resulted in a prominent G_2/M arrest in meningioma cells (Figure 4). Cyclin levels are induced during specific phases of the cell cycle and are well-known for having a fast protein turnover rate (Pines, 1996). While tumor cells frequently exhibit abnormal G_1 checkpoint, sharp declines in multiple cyclins following silvestrol treatment render them liable to G_2/M arrest. Additionally, the pro-growth signaling molecules AKT, ERK1/2 and FAK are frequently phosphorylated and activated in NF2-related tumor cells (Poulikakos et al., 2006; Jacob et al., 2008). Reducing the levels of these mitogenic signals would be expected to reinforce silvestrol's growth inhibitory properties.

With its high potency in several tumor types, silvestrol has been rigorously investigated as a potential cancer therapeutic (Pan et al., 2014). While it is well tolerated in mice, studies are ongoing to determine dosing schedules and toxicity in large animals. We have demonstrated that silvestrol exhibits consistent strong suppression of the growth of meningioma cells in culture (Figure 3). Previous pharmacokinetic analysis showed that silvestrol requires intraperitoneal or intravenous delivery for maximal bioavailability (Saradhi et al., 2011). However, even with these delivery methods, the distribution to the brain is relatively low, suggesting that silvestrol may not readily cross the blood-brain barrier. Silvestrol has a bulky sugar-like dioxanyl ring, which has been shown to confer susceptibility to the multi-drug resistance protein 1 (MDR1) transporter (Gupta et al., 2011). This characteristic may limit the distribution of silvestrol to the brain. Intriguingly, rocaglaol, a silvestrol-related compound lacking this ring, possesses antitumor activity and a synthetic analog of rocaglaol displays activity in drug-resistant breast carcinoma cells that overexpress MDR1 (Mi et al., 2006; Pan et al., 2013). The finding that the growth-suppressive effects of rocaglaol are also mediated by inhibiting protein translation indicates that the dioxanyl ring of silvestrol is dispensable for eIF4A inhibition (Ohse et al., 1996). Moreover, rocaglaol analogs may exhibit cardioprotective and/or neuroprotective effects (Bernard et al., 2011; Thuaud et al., 2011). A halogenated rocaglaol derivative has been shown to be capable of crossing the blood-brain barrier (Fahrig et al., 2005; Thuaud et al., 2011). In addition, our preliminary study has identified several silvestrol-related rocaglates that lack the dioxanyl ring but exhibit potent growth-inhibitory activity in meningioma cells (data not shown). Nevertheless, it should be noted that meningiomas may disrupt the blood-brain barrier. Experiments are in progress to evaluate the anti-tumor effects of silvestrol and a series of silvestrol-related rocaglates lacking this dioxanyl ring in an orthotopic mouse model for meningioma (Burns et al., 2013). Identification of an effective treatment for meningioma would significantly advance our efforts to improve clinical care and long-term treatment outcomes for these patients.

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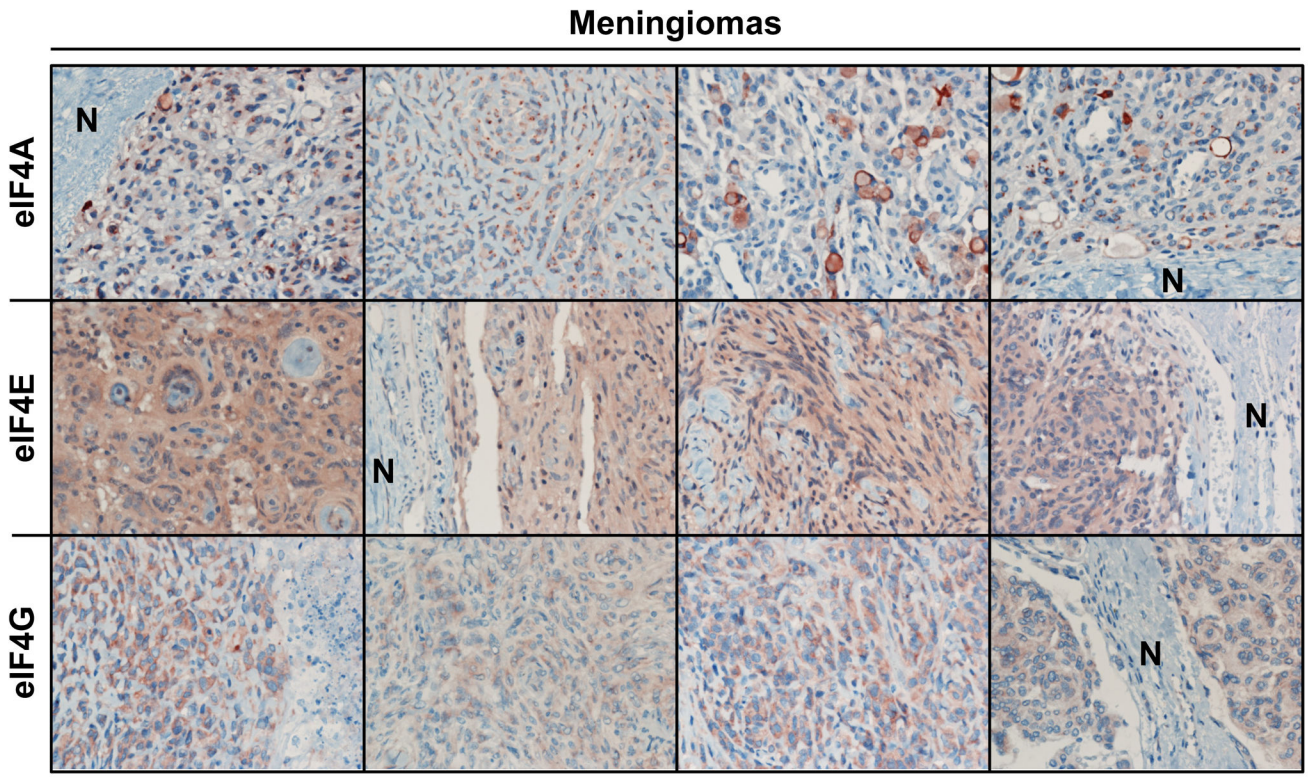

Figure 1.

Meningioma tissues overexpressed all three components of the eIF4F complex. Shown are representative images of meningioma sections immunostained for eIF4A, eIF4E and eIF4G. "N" indicates adjacent normal meninges. The staining scores for all tumors are reported in Table 1.

Figure 2A

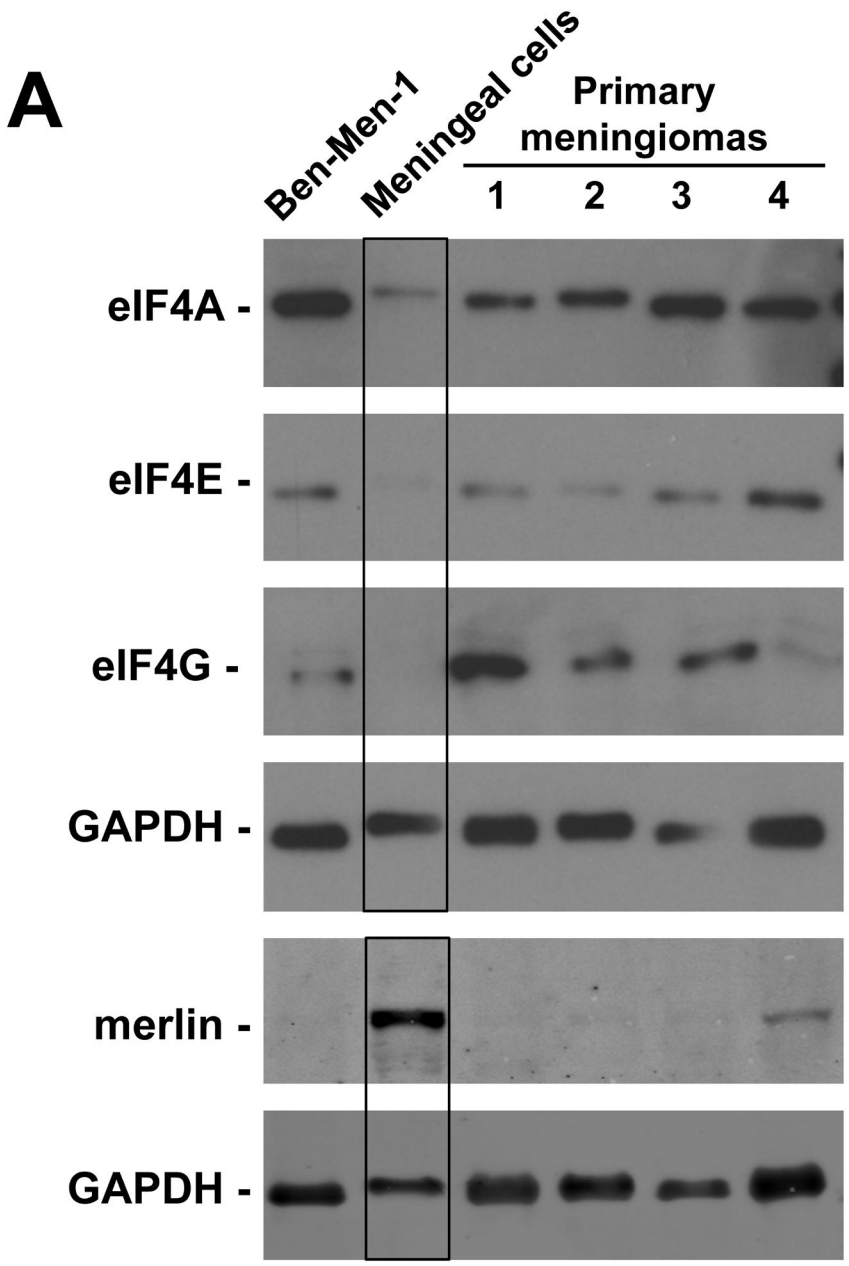

Figure 2B

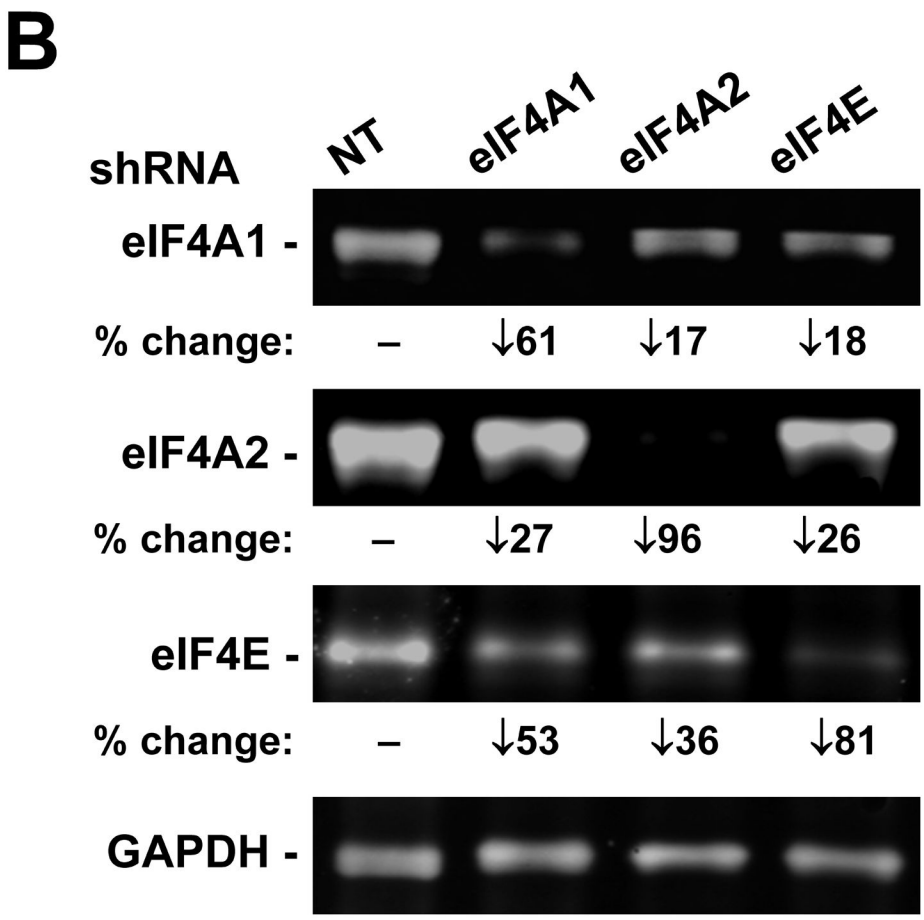

Figure 2C

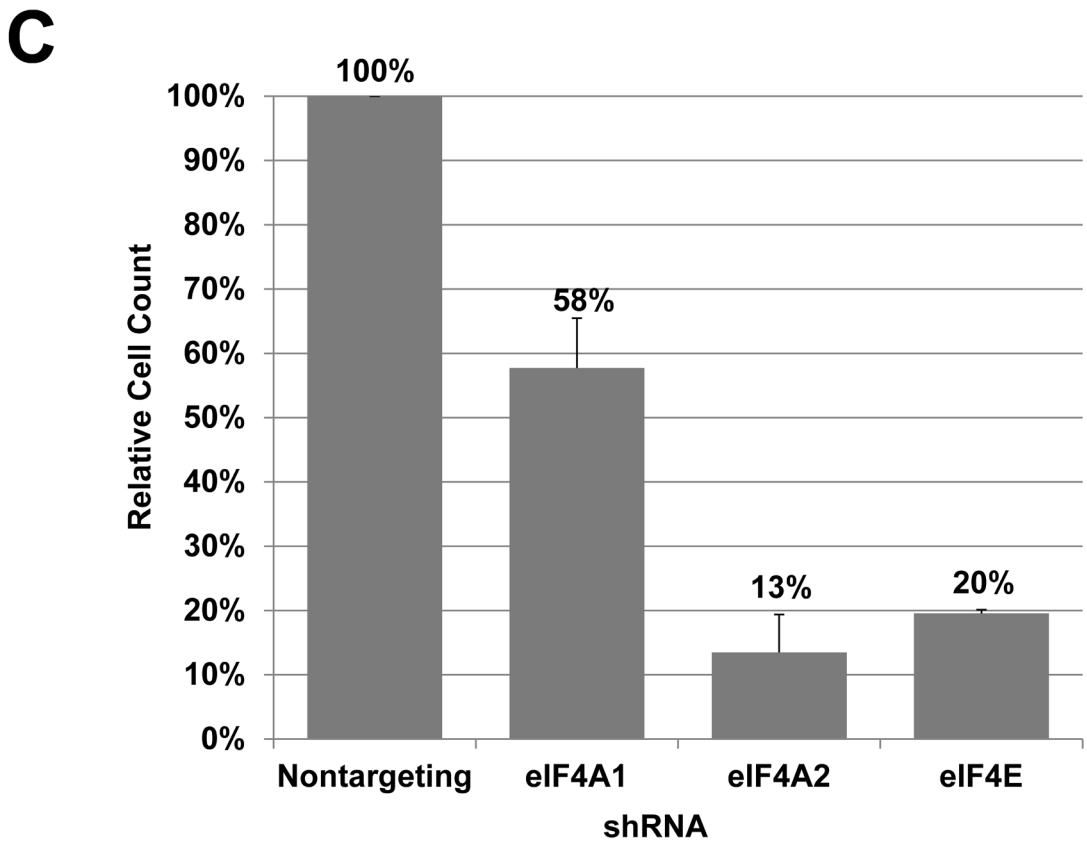

Figure 2.

Meningioma cells expressed higher levels of eIF4F components. (A) Overexpression of eIF4F components in primary meningioma cells and the Ben-Men-1 benign meningioma cell line. Total cell lysates from four primary cultures of meningioma cells, normal human meningeal cells, and Ben-Men-1 cells were resolved by SDS-PAGE and probed for eIF4E, eIF4A, eIF4G, merlin, and GAPDH (loading control). The four meningiomas used to prepare primary cell cultures included two sporadic (#1 and #2) and two NF2-associated (#3 and #4) grade I tumors. (B) Silencing each indicated eIF4F component by shRNA. Ben-Men-1 cells were transduced with 10 MOI of lentiviruses expressing the indicated shRNAs (Materials and Methods). After 8 days, cells were lysed and analyzed by Western blotting for eIF4A1, eIF4A2, eIF4E, and GAPDH. Fluorescently-labeled protein bands were detected on the Odyssey CLx and quantitated in Image Studio. Shown below the blots is the % decrease in the eIF4A1, eIF4A2, or eIF4E protein level in shRNA-transduced cells relative to the nontargeting (NT) control. (C) Suppression of meningioma cell growth following depletion of eIF4A or eIF4E. Ben-Men-1 cells transduced as described in (B) were counted. Shown are the means and standard deviations (SDs) from two independent experiments run in technical duplicates.

Figure 3A

A

Compound \ IC_{50}	Ben-Men-1 cells	Primary meningioma cells
Silvestrol	10 nM	25 nM
Episilvestrol	32 nM	25 nM
Bruceantin	200 nM	256 nM
Bruceine A	960 nM	1190 nM
13-Acetoxyrolandrolide	800 nM	1 μM
2α,13-diacetoxy-4α-hydroxy-8α-methacryloyloxybourbonen-12,6α-olide	> 10 μM	> 10 μM
Cucurbitacin D	200 nM	200 nM
Dichamanetin	27 μM	27 μM
Antidesmone	4 μM	36 μM
(-)-Isogaudichaudiic acid	7 μM	15 μM
Artonin O	13 μM	18 μM
Artobiloxanthone	30 μM	32 μM
Artorigidin A	7 μM	12 μM
α-Mangostin	> 18 μM	> 18 μM
1,3,7-trihydroxy-2,4-diisoprenylxanthone	30 μM	30 μM
Cochinchinone A	24 μM	22 μM
3,4′-dimethoxy-5,7,3′-trihydroxyflavone	> 4 μM	> 4 μM
Goyazensolide	1 μM	> 4 μM
[3-(3,5-dimethoxy-4-hydroxyphenyl)]propanol-trans-coumarate	> 45 μM	45 μM
CAPE	> 20 μM	ND
Curcumin	7 μM	ND
Resveratrol	> 30 μM	ND
Sulforaphane	8 μM	ND

Figure 3B

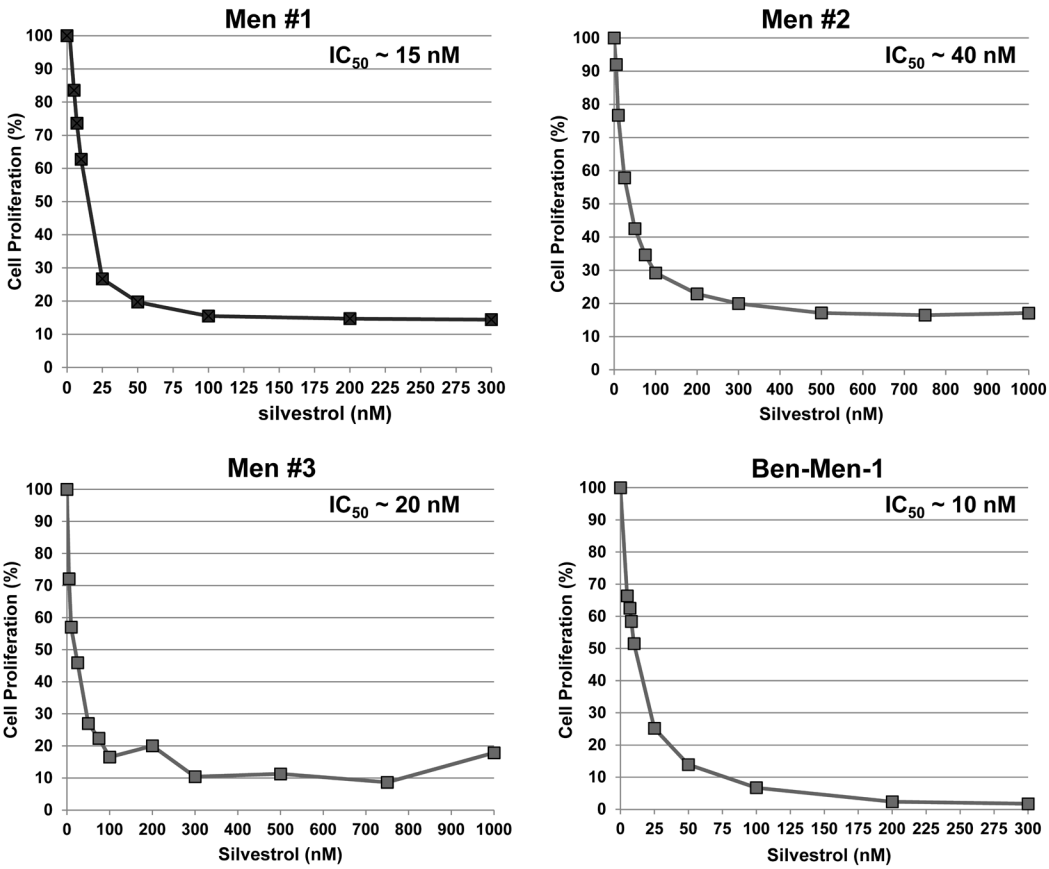

Figure 3.

Silvestrol potently inhibited proliferation of meningioma cells. (A) The natural compounds used in this study and their IC_{50} values in Ben-Men-1 and primary meningioma cells are summarized. Data shown are the mean IC_{50} values from three independent experiments. (B) Resazurin assays for cell proliferation were performed on primary meningioma cells and Ben-Men-1 cells treated with various concentrations of silvestrol for three days. Primary meningioma cells were derived from three separate tumors (Men #1~3) and assays were performed in six replicate wells; the IC_{50} values for each experiment are shown in the graph insets. The mean IC_{50} value for primary meningioma cells across the three experiments was ~25 nM. Experiments on Ben-Men-1 cells were performed in six replicates, and the experiments were independently repeated three times. Data shown are the mean of the six replicate wells from one representative experiment. The mean IC_{50} value for Ben-Men-1 cells across all three experiments was ~10 nM.

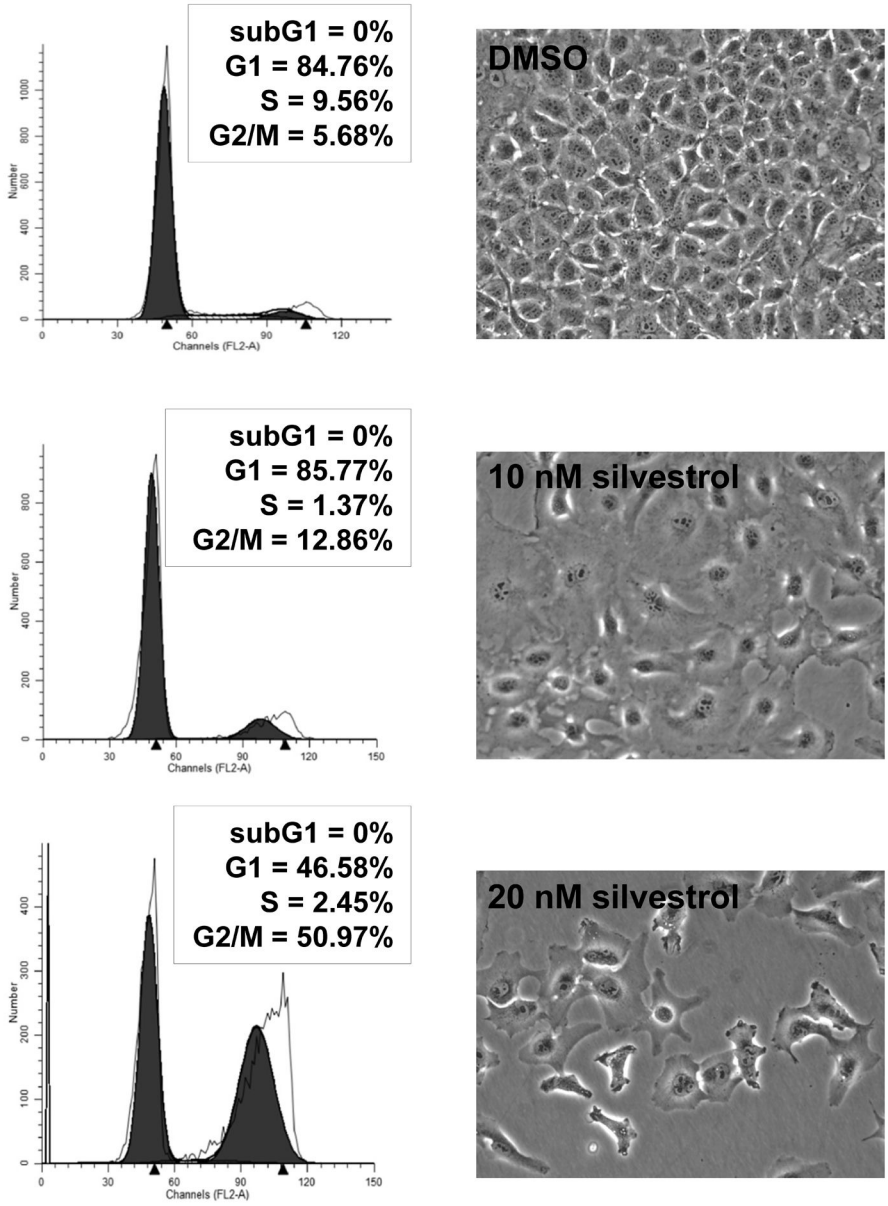

Figure 4.

Silvestrol induced G2/M arrest in meningioma cells. Ben-Men-1 cells were treated with the indicated concentrations of silvestrol for three days, and phase contrast images of treated cells were taken, followed by cell harvesting for flow cytometry analysis as described in the Materials and Methods. Cell cycle histograms of propidium iodide-labeled cells revealed a prominent increase in the G_2/M peak after silvestrol treatment.

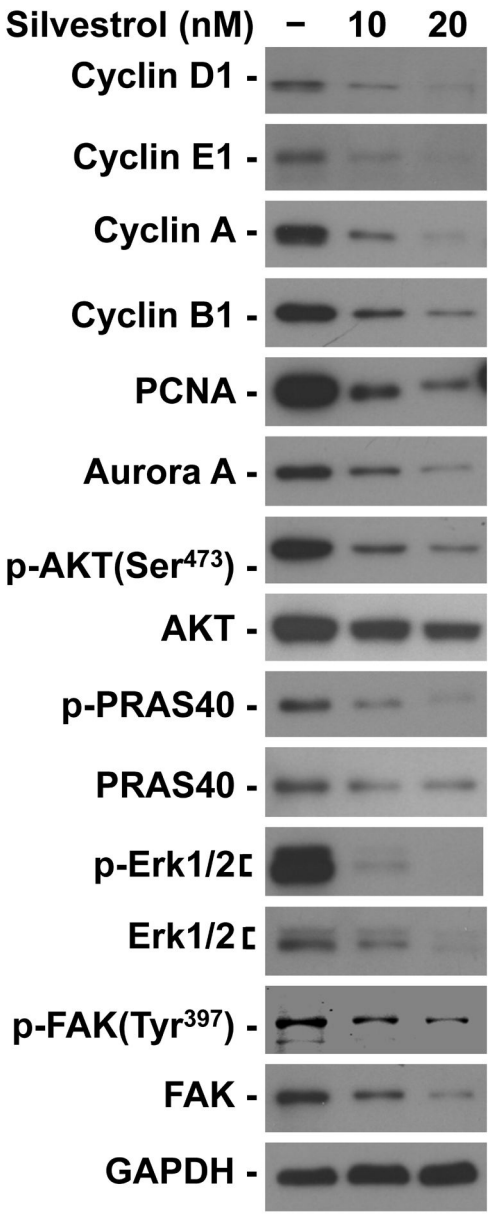

Figure 5.

Silvestrol suppressed the expression of multiple cell cycle proteins and mitogenic kinases. Ben-Men-1 cells were treated with 1x and 2x the IC_{50} dose of silvestrol for 24 hours followed by cell lysis and Western blotting for the indicated proteins. GAPDH served as a loading control.

Table 1

Clinicopathological information of meningiomas and their relative IHC staining signals for eIF4A, eIF4E, and eIF4G

Meningioma tumor sections were processed for immunohistochemistry analysis as described in the Materials and Methods. Immunostaining signals were quantified using visual analysis based on the intensity and percentage of immunopositive cells (0 as negative, 0.5 = weak positive, 1 = moderate positive, 2 = strong positive, and 3 = very strong positive). Adjacent normal meningeal cells showed little to no staining and were scored as 0 (Figure 1).

Tumor ID.	Gender	Tumor grade	Histological type	Clinical diagnosis	Tumor size (cm)	IHC staining signals		
						eIF4A	eIF4E	eIF4G
Men01	F	WHO grade I	Meningothelial	Sporadic	3.3 × 2.7 × 1.6	0.5+	1+	0 ~ 0.5+
Men02	F	WHO grade I	Meningothelial	Sporadic	2.0 × 1.0 × 1.0	1 ~ 2+	0.5+	1 ~ 2+
Men03	F	WHO grade I	Fibrous	Sporadic	3.2 × 2.6 x1.3	0.5+	2+	0.5 ~ 1+
Men04	F	WHO grade I	Meningothelial	Sporadic	2.5 × 2.0 × 1.8	0.5+	2+	1 ~ 1.5+
Men05	N/A*	WHO grade I	Meningothelial	Sporadic	2.5 × 1.5 × 1.0	1 ~ 2+	1 ~ 2+	1 ~ 2+
Men06	F	WHO grade I	Meningothelial	Sporadic	2.5 × 1.8 × 0.9	0.5+	2+	0.5 ~ 1+
Men07	M	WHO grade I	Meningothelial	NF2	2.0 × 1.2 × 0.8	1 ~ 3+	2 ~ 3+	2 ~3+
Men08	F	WHO grade I	Meningothelial	NF2	3.4 × 2.8 × 1.7	0.5 ~ 1+	1 ~ 2+	0.5+
Men09	M	WHO grade I	Meningothelial	NF2	3.2 × 2.9 × 3.4	1 ~ 3+	1 ~ 2+	0.5+
Men10	M	WHO grade I	Meningothelial	NF2	2.0 × 2.2 × 2.5	2 ~ 3+	1 ~ 2+	0.5+
Men11	F	WHO grade II	Atypical	Sporadic	3.2 × 1.4 × 2.1	0.5 ~ 1+	1 ~ 3+	1 ~ 2+
Men12	F	WHO grade II	Atypical	Sporadic	9.0 × 4.0 × 3.0	0.5+	1 ~ 1.5+	1 ~ 2+
Men13	F	WHO grade II	Atypical	Sporadic	3.0 × 2.5 × 1.1	0.5+	2+	1+
Men14	M	WHO grade II	Atypical	Sporadic	1.5 × 1.0 × 0.8	0.5+	0.5 ~ 1+	0.5 ~ 1+
Men15	F	WHO grade II	Chordoid	Sporadic	6.5 × 6.0 × 3.0	0.5 ~ 1+	1+	1+
Men16	M	WHO grade III	Anaplastic	Sporadic	5.8 × 4.0 × 3.1	0.5+	1+	1 ~ 1.5+

*N/A, not available.