Malignant gliomas can be converted to non-proliferating glial cells by treatment with a combination of small molecules

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Abstract. Gliomas, the most highly malignant central nervous system tumors, are associated with an extremely poor patient survival rate. Given that gliomas are derived from mutations in glial precursor cells, a considerable number of them strongly react with glial precursor cell-specific markers. Thus, we investigated whether malignant gliomas can be converted to glial cells through the regulation of endogenous gene expression implicated in glial precursor cells. In the present study, we used three small-molecule compounds, [cyclic adenosine monophosphate (cAMP) enhancer, a mammalian target of rapamycin (mTOR) inhibitor, and a bromodomain and extra-terminal motif (BET) inhibitor] for glial reprogramming. Small-molecule-induced gliomas (SMiGs) were not only transformed into exhibiting a glial-specific morphology, but also showed positive reactions with glial-specific markers such as glial fibrillary acidic protein (GFAP), 2',3'-cyclic nucleotide 3'-phosphohydrolase (CNP) and anti-oligodendrocyte (RIP). A microarray analysis indicated that SMiGs exhibited a marked increase in specific gene levels, whereas that of a malignant cancer-specific gene was greatly decreased. Moreover, proliferation of the cells was markedly suppressed after the conversion of malignant glioma cells into glial cells. Our findings confirmed that malignant gliomas can be reprogrammed to non-proliferating glial cells, using a combination of small molecules, and their proliferation can be regulated by their differentiation. We suggest that our small-molecule combination (with forskolin, rapamycin and

I-BET151) may be the next generation of anticancer agents that act by reprogramming malignant gliomas to differentiate into glial cells.

Introduction

Several studies have reported that mouse or human fibroblasts can be directly reprogrammed into neurons, neural stem cells, or glial cells by the introduction of cell-specific transcription factors (1-9). Malignant gliomas can also be converted into functional neurons with the aid of neural-cell-specific transcription factors (10,11). These studies suggest the possibility that direct reprogramming technology can change the fate of a cell, irrespective of the cell type.

Direct reprogramming studies have mostly used the lentivirus system to introduce cell-specific transcription factors into donor cells (8); this may, however, lead to the insertion of the host chromosome. However, in a previous study, a direct reprogramming technology was developed based on small-molecule compounds to overcome the critical problem of the virus platform (12,13). This new technology enabled the conversion of mouse and human somatic cells into neurons, neural stem cells, or glial cells, without inserting the host chromosome. Therefore, these small-molecule compounds can replace the transcription factors in direct reprogramming.

Previous studies have reported that gliomas are derived from glial precursor cells (14), and a significant portion of gliomas strongly react with glial precursor cell-specific markers (15). However, whether or not gliomas can be transformed into glial cells has not been explicitly investigated. Therefore, we hypothesized that regulating the glial-specific endogenous gene expression of gliomas by a combination of small-molecule compounds could affect glial reprogramming.

Materials and methods

Cell culture. In the present study, rat C6 and human U87MG glioma cells (of unknown origin) (HTB-14™; The American Type Culture Collection, Manassas, VA, USA) were used. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, A, USA) containing 10% fetal bovine serum (FBS; HyClone Laboratories; GE Healthcare, Chicago,

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IL, USA), 0.1% β-mercaptoethanol (Life Technologies; Thermo Fisher Scientific, Inc.), 1% penicillin/streptomycin (P/S) (Life Technologies; Thermo Fisher Scientific, Inc.), 1% non-essential amino acids (NEAA; Life Technologies; Thermo Fisher Scientific, Inc.) and 1% sodium pyruvate (Life Technologies; Thermo Fisher Scientific, Inc.), and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Differentiation and maturation of glial cells. Glioma cells were seeded on 1% basement membrane matrix-coated plates (BD Biosciences, San Jose, CA, USA) at a density of $1x10^3$ cells/cm^2. After incubating the cells at 37°C for 24 h, we replaced the medium with glial differentiation medium, which consisted of neurobasal medium:advanced DMEM/F12 (1X) (1:1), 1% N2 supplement (100X), 2% B27 supplement (50X), 0.05% bovine serum albumin (BSA), 1% P/S, 1% glutamax-I (100X), 0.1% β-mercaptoethanol (1000X) (all of which were from Life Technologies; Thermo Fisher Scientific, Inc.), BDNF (10 ng/ml), GDNF (10 ng/ml) and NT-3 (10 ng/ml) (all from PeproTech, Inc., Rocky Hill, NJ, USA). We then added a combination of small molecules, composed of either forskolin (Tocris Bioscience, Bristol, UK) and rapamycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) or forskolin, rapamycin and T3 (Sigma-Aldrich; Merck KGaA). After 7 days, we replaced the medium with only I-BET151 (Tocris Bioscience) for maturation of the differentiated cells. The cells were then incubated at 37°C for 7 additional days. We replaced the media every 2-3 days. In some experiments, we used temozolomide (TMZ; 50 μM) to compare the growth inhibition effect of the small-molecule combination.

The small molecules were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich; Merck KGaA) and diluted in glial differentiation medium to the following final concentrations: forskolin, 100 μM; rapamycin, 100 nM; T3, 100 nM; I-BET151, 1 μM; and TMZ, 50 μM. We tested the glial cell differentiation into glioma in three independent experiments.

MTT assay. For measuring cell proliferation, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich; Merck KGaA). Cells from each group, containing the same volume of DMSO as that in the differentiation media, were differentiated for 7 or 14 days in 24-well plates. Then, we added 5 mg/ml MTT solution to the media and incubated them at 37°C for 3 h. The media were removed, and formazan crystals were dissolved using DMSO. The samples were then incubated at 37°C for 10 min and transferred to 96-well plates, and the absorbance was measured at 490 nm using VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). Results were acquired from three independent experiments.

Immunofluorescence and cell quantification. Media were removed and the cells were washed with phosphate-buffered saline (PBS; HyClone Laboratories; GE Healthcare). The cells were then fixed with 4% paraformaldehyde (Millipore, Temecula, CA, USA), pH 7.2, for 10 min at room temperature. Subsequently, they were washed thrice with 0.3% Tween-20 (Life Technologies; Thermo Fisher Scientific, Inc.) in PBS for 3 min. The blocking procedure was performed in PBS with 10% normal donkey serum and 0.3% Triton-X 100 for 30 min at room temperature. Primary antibodies, diluted in the blocking buffer, namely, mouse monoclonal anti-Nestin (dilution 1:1,000; cat. no. ab6142; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-NG2 (dilution 1:250; cat. no. ab5320; Millipore), rabbit polyclonal anti-Olig2 (dilution 1:100; cat. no. ab42453; Abcam), rabbit polyclonal anti-PDGFRa (dilution 1:500; cat. no. sc-338; Santa Cruz Biotechnology, Santa Cruz, CA, USA;) mouse monoclonal anti-MBP (dilution 1:1,000; cat. no. ab2404; Abcam), mouse monoclonal anti-CNP (dilution 1:1,000; cat. no. NE1020; Millipore), mouse monoclonal anti-oligodendrocyte (RIP; dilution 1:50,000; cat. no. MAB1580; Millipore), rabbit polyclonal anti-GFAP (dilution 1:1,000; cat. no. ab7260; Abcam), mouse monoclonal anti-GFAP (dilution 1:1,000; cat. no. G3893; Sigma-Aldrich; Merck KGaA), mouse monoclonal anti-O4 (dilution 1:100; cat. no. MAB345; Millipore) and rabbit polyclonal anti-Ki-67 (dilution 1:250; cat. no. ab15580; Abcam), were allowed to react with the samples for 60 min at room temperature. After washing with PBS with 0.3% Tween-20, secondary antibodies diluted in the blocking buffer were added and allowed to react for 30 min at room temperature: FITC donkey anti-rabbit (cat. no. 711-095-152) or anti-mouse (cat. no. 715-095-151) and Cy3 donkey anti-rabbit (cat. no. 711-165-152) or anti-mouse (cat. no. 715-165-151) (dilution 1:500; all of them are from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) were used as secondary antibodies. The samples were then washed thrice and stained using 4',6'-diamino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). The samples were then covered with glass coverslips and examined using a confocal laser scanning microscope (LSM 700; Carl Zeiss, Oberkochen, Germany).

Flow cytometry. To determine the number of positive cells stained by each antibody (GFAP, CNP, RIP and Ki-67), we performed flow cytometric analyses. Briefly, the cells were harvested using 0.25% trypsin/EDTA (Life Technologies; Thermo Fisher Scientific, Inc.). For fixation, 4% paraformaldehyde was added to the cell pellet and incubated for 10 min. After centrifugation at 400 x g for 5 min, the supernatant was discarded and the cell pellet was washed twice with 0.3% Tween-20 in PBS for 3 min. Next, the primary antibodies (GFAP, 1:1,000; CNP, 1:1,000; RIP, 1:50,000; and Ki-67, 1:250) were added to the pellet and allowed to react for 60 min at room temperature. Following a second wash using the same procedure, secondary antibodies (FITC donkey anti-rabbit or anti-mouse and Cy3 donkey anti-rabbit or anti-mouse; dilution 1:500) were added to the cell pellet and allowed to react for 30 min at room temperature. Finally, the pellet was washed and re-suspended in PBS. Samples were analyzed using a FACSCalibur (Becton-Dickinson; BD Biosciences, San Jose, CA, USA) and CellQuest software (Becton-Dickinson; BD Biosciences). Results were acquired from three independent experiments.

Microarray. Microarray analysis was performed according to the manufacturer's instructions. After total RNA isolation, cDNA was synthesized using the GeneChip WT (Whole Transcript) Amplification kit (Thermo Fisher Scientific, Inc.). Labeled DNA target was hybridized to the Affymetrix

GeneChip Array (Affymetrix; Thermo Fisher Scientific, Inc.). Hybridized arrays were washed and stained on a GeneChip Fluidics Station 450 and scanned on a GCS3000 Scanner (Thermo Fisher Scientific, Inc.). Analysis was performed using Affymetrix® GeneChip Command Console® Software (AGCC; Affymetrix; Thermo Fisher Scientific, Inc.). Microarray data were deposited in a public database (https://www.ncbi.nlm.nih.gov/geo/), GSE101337 (For undifferentiated C6 and SMiG), and 101338 (For single clone no. 4 and single clone no. 8).

Cell counting. To determine the proliferation rate of untreated glioma cells, i.e., those without the small molecules, we seeded them at a density of $1x10^3$ cells/cm^2 on a 24-well plate. When the cells reached confluence, they were harvested using 0.25% trypsin/EDTA. After being centrifuged at 268 x g for 3 min and re-suspended in 1 ml culture medium, the cells were diluted to half concentration in trypan blue (Life Technologies; Thermo Fisher Scientific, Inc.) and counted using the manual cell counting method. All the cells were then seeded on 12-well plates (Corning Inc., Corning, NY, USA). The cells were counted when they reached confluence, and were re-plated on wider plates, using the same method. Results were acquired from three independent experiments.

Single colony selection. We performed a serial dilution of glioma cells for isolation of a single colony. Briefly, we added 100 µl culture media into all the wells of a 96-well plate. Approximately 100 cells were mixed with 100 µl culture medium and added into the first well of a 96-well plate. Then, 100 µl was taken from the first well, and then serially diluted to the next well, and so on. After confirming the single cell isolation, cells were incubated until a colony was formed. After 14 days, each colony was treated with trypsin for 5 min, and maintained in the culture media.

We tested the glial differentiation efficiency with a few isolated glioma. We used the clone no. 4 and 8 glioma for the present study.

Statistical analysis. A two-tailed Student's t-test was performed to assess differences between two groups. Two-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to assess differences among three groups or more. Data are presented as mean ± standard error of the mean (SEM) and P-values <0.05 were considered to indicate a statistically significant result.

Results

Direct conversion of malignant glioma cells into non-proliferating glial cells by treatment with a combination of small molecules. A schematic diagram was provided to facilitate understanding of the present study (Fig. 1A). First, we confirmed that undifferentiated glioma cells were positive for specific markers of glial progenitor cells, namely, Nestin, NG2, Olig2 and PDGFRa (Fig. 1B). However, the glioma cells were negative for other markers of glial cells, namely, GFAP, CNP, RIP and O4 (Fig. 1B).

Therefore, we aimed to ascertain whether T3, which is typically used to differentiate glial progenitor cells into oligodendrocytes, could convert gliomas into glial cells (i.e., oligodendrocytes). However, the T3-treated glioma cells were not transformed into a glial-specific morphology (Fig. 1C). Next, we added T3 and forskolin to the glioma cells, but could not confirm glial-specific morphology until 7 days of incubation (Fig. 1C). Nevertheless, we persisted in our efforts to convert C6 glioma cells into glial cells by adding rapamycin to T3 and forskolin, and incubating the cells for 7 days. With this, we were finally able to observe glial-specific morphology (Fig. 1C), as well as glial-specific markers such as GFAP, CNP, and RIP (Fig. 1D).

After several experiments, we confirmed that this glial-specific morphology could be maintained using medium containing rapamycin, T3 and I-BET151 (Fig. 1E). To further confirm whether the converted cells were fully differentiated into glial cells, they were cultured in the absence of small molecules for 3 days. Consequently, we observed that the glial-specific morphology was retained; the GFAP-, CNP- and RIP-positive cells did not react with Ki-67 (Fig. 1F).

Next, we investigated the growth inhibitory effect of the small molecules. The undifferentiated glioma cells grew rapidly, despite a very low cell density (Fig. 1G). In contrast, treatment with a combination of forskolin, T3 and rapamycin conspicuously decreased the cell proliferation (Fig. 1H). Further experimentation confirmed that I-BET151 played a major role in the growth inhibition after glial induction (Fig. 1I).

Glial differentiation of malignant glioma cells through optimal treatment with small molecules. To determine the major compound among forskolin, T3, rapamycin and I-BET151, responsible for glial induction, we performed further experiments. We confirmed that glioma cells were converted into GFAP-, CNP-, RIP- and O4-positive cells with glial-specific morphology, even in the absence of T3 (Fig. 2A and B). These converted cells did not react with Ki-67 (marker for dividing cells) (Fig. 2B). Thus, T3 did not affect the glial conversion of glioma cells.

We further confirmed that glial-specific morphology was maintained in the presence of I-BET151 after induction of glial differentiation using forskolin and rapamycin, for 7 days (Fig. 2C). In contrast, it was difficult to maintain glial-specific morphology in the absence of I-BET151 (Fig. 2D). As a quantitative result, the number of CNP-, RIP- and GFAP-positive cells clearly increased in the SMiGs converted using forskolin, rapamycin and I-BET151 (Fig. 2E). We also observed that glial-specific morphology was retained even when small molecules were withdrawn for 3 days (Fig. 2F).

Gene expression profile in undifferentiated glioma cells and SMiGs. We performed microarray analysis to compare the gene expression pattern between glioma cells and SMiGs. In a microarray analysis, gene expression profile related to oligodendrocyte differentiation and myelination was significantly induced in the SMiGs. In contrast, gene expression profiles relating to cell division and mitosis, or extracellular matrix (ECM) and vessel development were markedly upregulated in glioma cells (Fig. 2G). Gene Ontology related to glial differentiation, including oligodendrocyte differentiation and myelination, was significantly different between undifferentiated glioma cells and SMiGs (Fig. 2H). These results revealed that specific characteristics of malignant gliomas
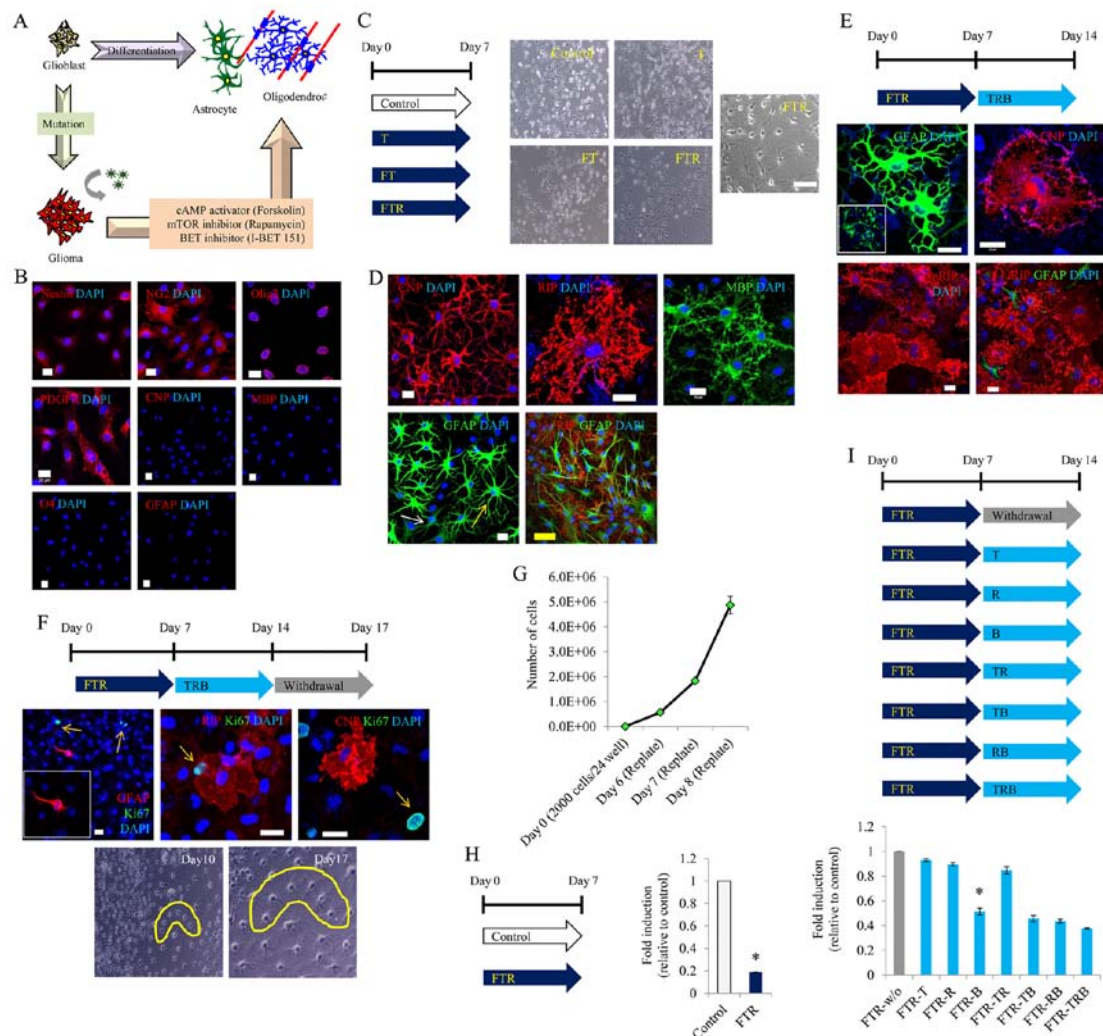
Figure 1. Glial differentiation of malignant glioma cells by treatment with small molecules. (A) Schematic diagram. (B) Representative image of undifferentiated glioma cells stained with Nestin, NG2, PDGFR, Olig2, GFAP, CNP, RIP and O4. Scale bar, 20 μm. (C) Representative image 7 days after induction of glial differentiation. T (T3), FT (forskolin/T3), FTR (forskolin/T3/rapamycin). Original magnification, x100; Scale bar, 100 μm. (D) Representative image of GFAP, CNP and RIP staining 7 days after induction of glial differentiation. Scale bars, 20 μm (white) and 50 μm (yellow). (E) Representative image of GFAP, CNP and RIP staining 14 days after induction of glial differentiation. Scale bars, 20 μm. TRB, T3/rapamycin/I-BET151. (F) Representative image of Ki-67, GFAP, CNP and RIP staining after 3 days of small molecule withdrawal following 14 days of glial differentiation. Scale bar, 20 μm. (G) Quantitative result of cell proliferation in basal media. (H) Quantitative result of cell density 7 days after induction of glial differentiation. (I) Quantitative result of cell density 14 days after induction of glial differentiation. *P<0.05. Data are presented as the mean ± SEM.

can be converted into those of glial cells by treatment with a combination of forskolin, rapamycin and I-BET151.

Proliferation of malignant glioma cells is abrogated by a combination of small molecules. We investigated whether the strong proliferation potency of malignant gliomas can be inhibited by glial conversion. On day 7 after treatment with a combination of forskolin and rapamycin, we confirmed that the proliferation of glioma cells was significantly reduced compared to that of the single-molecule treatment group, and the rate of Ki-67-positive cells was significantly decreased compared to that in the DMSO group (Fig. 3A). We also confirmed that the proliferation of U87MG glioma cells was significantly reduced compared to that of the single-molecule treatment group (Fig. 3B). Further treatment with I-BET151 was more effective in inhibiting cell proliferation (Fig. 3C and D). This pattern was similar even in the absence of the small molecules (Fig. 3E). These results

indicated that the strong proliferation capacity of malignant glioma cell can be controlled by glial conversion.

Specific cell type responds to the small-molecule combination for glial conversion. Some cells were not positive for GFAP, CNP, or RIP after glial induction by small molecules (Fig. 4A). We, therefore, selected each single colony considering the glioma's heterogeneity (Fig. 4B). Of the many colonies we isolated, colony no. 4 did not react with GFAP, CNP or RIP after glial induction with forskolin, rapamycin and I-BET151; it was only positive for Ki-67 (Fig. 4C). However, most of colony no. 8 was positive for GFAP, CNP and RIP after glial induction with forskolin, rapamycin and I-BET151 (Fig. 4C). GFAP-, CNP- and RIP-positive cells did not react with Ki-67 (Fig. 4C). We, then, analyzed the gene expression patterns between colony no. 4 and 8, using a microarray analysis (Fig. 4D). The top 10 most-expressed genes in colony no. 8 were *prkcb, postn, mpz, pros1, cd55, ct55, plxdc2, antxr1, fmr1nb* and
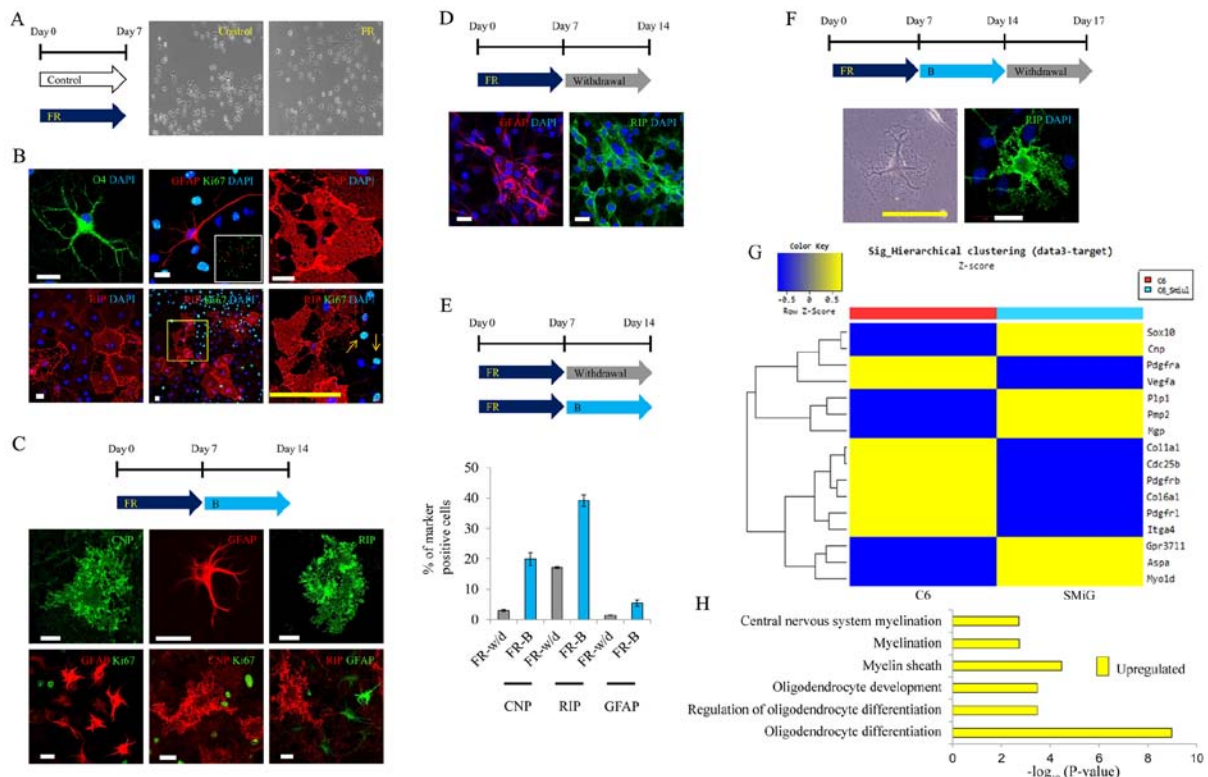
Figure 2. Glial differentiation of malignant glioma cells through optimal treatment with small molecules. (A) Representative image 7 days after induction of glial differentiation. (original magnification, x100). FR, forskolin/rapamycin. (B) Representative image of GFAP, CNP, RIP, O4 and Ki-67 staining 7 days after induction of glial differentiation. Scale bars, 20 µm (white) and 100 µm (yellow). B, I-BET151. (C) Representative image of GFAP, CNP, RIP and Ki-67 staining 14 days after induction of glial differentiation. Scale bars, 20 µm. (D) Representative image of GFAP and RIP staining after 7 days of small molecule withdrawal following 7 days of glial differentiation. Scale bars, 50 µm. (E) Quantitative result of the glial-specific marker-positive cells after glial differentiation for 14 days. (F) Representative image of RIP staining after three days of small molecule withdrawal following 14 days of glial differentiation. Scale bars, 20 µm (white) and 200 µm (yellow). (G) Lists of upregulated or downregulated genes in small-molecule-induced gliomas (SMiGs). (H) Upregulated Gene Ontology terms related to glial differentiation in SMiGs.
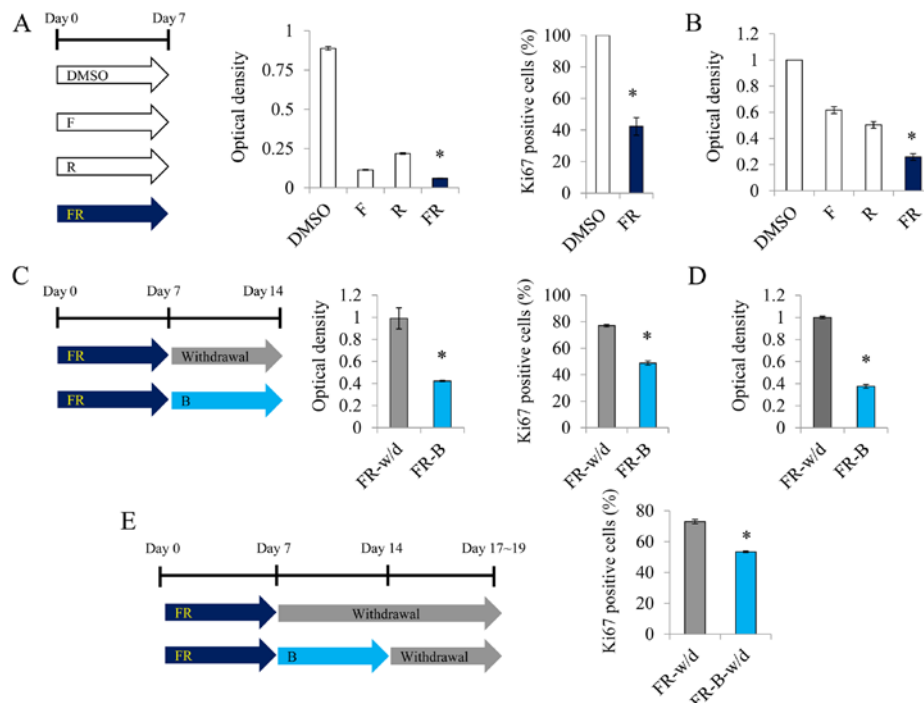

Figure 3. Growth inhibition effect of the glial differentiation-based drug. (A) Quantitative results of cell density of C6 glioma at day 7 after glial differentiation. Percentage of Ki-67-positive cells 7 days after glial differentiation. (B) Quantitative results of cell density of U87MG glioma at day 7 after glial differentiation. (C) Quantitative results of cell density of C6 glioma 14 days after glial differentiation. Percentage of Ki-67-positive cells 14 days after glial differentiation. (D) Quantitative results of cell density of U87MG glioma 14 days after glial differentiation. (E) Percentages of Ki-67-positive cells three days after withdrawal of the small-molecule compounds following 14 days of glial differentiation. *P<0.05. Data are presented as the mean ± SEM. F, forskolin; R, rapamycin; B, I-BET151.
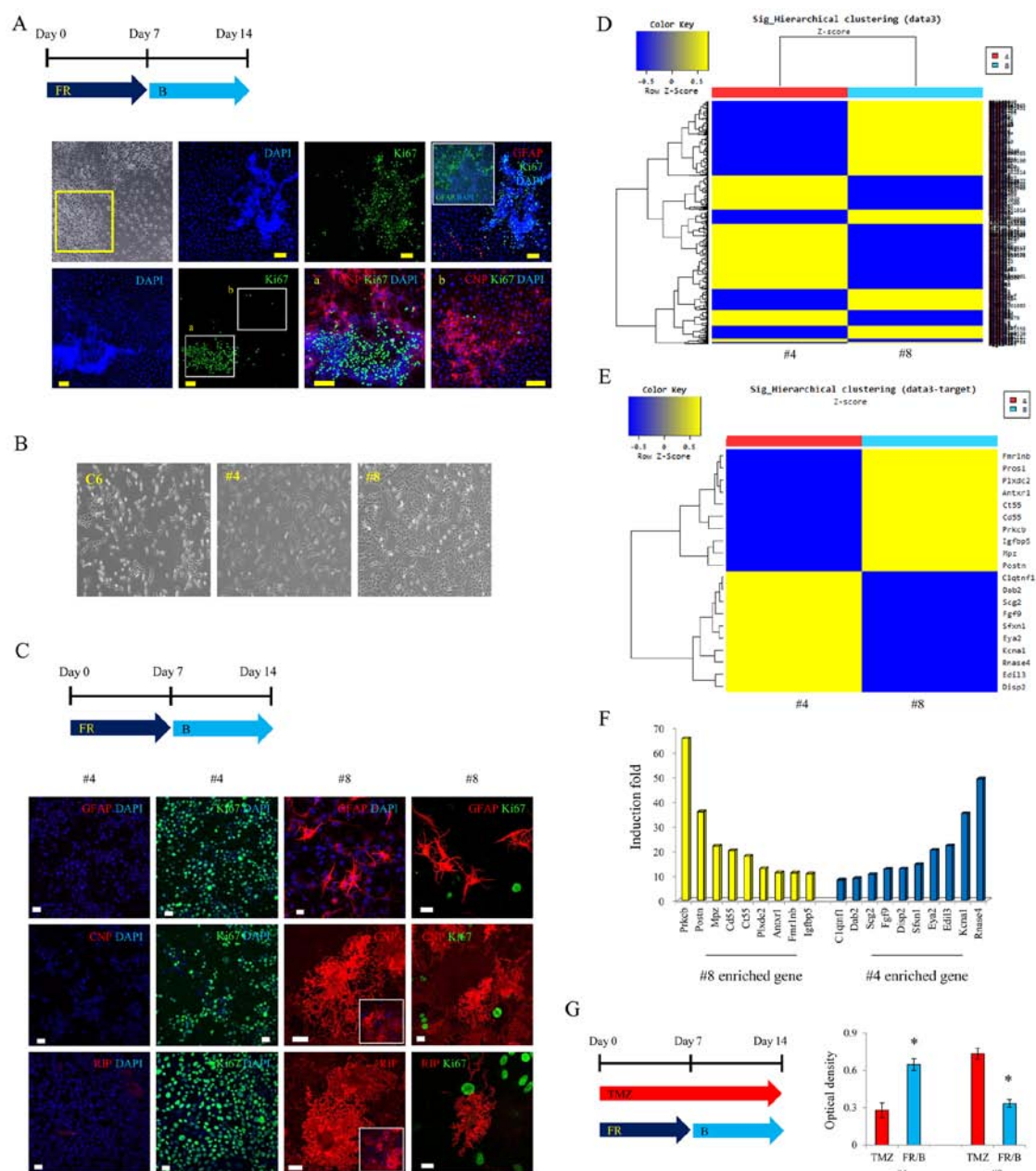
Figure 4. Effect of the glial differentiation-based drug in each clone derived from the glioma. (A) Representative image of Ki-67/GFAP or Ki-67/CNP staining in glioma cells. Scale bars, 200 μm. (B) Representative image of single clones derived from glioma cells. (C) Representative image of Ki-67/GFAP, Ki-67/CNP and Ki-67/RIP staining 14 days after glial differentiation using forskolin (F), rapamycin (R) and I-BET151 (B). Scale bars, 20 μm. (D) Gene expression profiles of two different single clones derived from glioma cells. Heat map of the significant gene in single clones derived from glioma cells. (E) Expression of the top 10 genes in the single clones derived from glioma cells. (F) Fold induction of the expression of the top 10 genes in the single clones derived from glioma cells. (G) Quantitative results of cell density 14 days after treatment with both temozolomide (TMZ) and the glial differentiation-based drug in single clones derived from glioma cells. *P<0.05. Data are presented as the mean ± SEM.

igfbp5 (Fig. 4E and F). We then compared the proliferation-inhibition effect of the small-molecule combination with that of a standard medication such as temozolomide (TMZ). For colony no. 4, cell proliferation was reduced by TMZ, but not by the small-molecule combination (Fig. 4G), although, our small molecules inhibited proliferation more effectively than TMZ in colony no. 8 (Fig. 4G).

Discussion

In our previous study, we demonstrated that malignant gliomas can be reprogrammed into functional neurons, using a combination treatment of forskolin and CHIR99021 (GSK3 inhibitor). It is very exciting to report that the fate of gliomas can be changed diversely, into non-proliferating neurons or glial cells, depending on the small molecule combinations chosen.

In this preliminary study, we demonstrated the conversion of malignant glioma cells to glial cells, using small-molecule compounds that have been widely used for glial differentiation. Unexpectedly, the malignant glioma cells did not acquire the glial-specific morphology in the presence of T3 and forskolin, which are known to induce glial differentiation (16). After various experiments, we determined that the glioma cells can be transformed to glial-specific morphology using only forskolin

and rapamycin, without T3. We assume that the characteristics and differentiation mechanism of malignant gliomas are not the same as those of glial precursor cells; however, gliomas are derived from glial precursor cells, and a significant portion of gliomas still strongly react with glial precursor cell-specific markers (14,15). A previous study reported that mTOR inhibition prevents oligodendrocyte differentiation (17). mTOR inhibition was shown to prevent the conversion of glial precursor cells into gliomas (14). However, the role of mTOR inhibition in glioma reprogramming to glial cells remains unknown. This also indicates that the differentiation mechanism of malignant gliomas may not be the same as those of glial precursor cells, and the synergistic effect of cAMP activation and mTOR inhibition may play a major role in the conversion of glioma into glial cells.

BET inhibitor was classified as an anticancer agent after clinical trials in the United States and Europe (18,19). In this study, a BET (bromodomain and extra-terminal motif) inhibitor, I-BET151, was added to the media 7 days after the induction of glial differentiation, using a combination of forskolin and rapamycin. Treatment with I-BET151 not only helped to maintain the glial-specific morphology, but also strongly inhibited cell proliferation (Fig. 2C-F). According to a previous study, the BET inhibitor accelerates the differentiation of mouse primary glial progenitors into oligodendrocytes (20). To the best of our knowledge, this is the first study that reports the effect of I-BET151 on the differentiation of a malignant glioma into glial cells.

The heterogeneity of glioblastomas has been previously reported to affect their drug response, with each single clone responding differently (21). In this study, we observed that some cell populations did not convert to a glial-like morphology even after treatment with forskolin, rapamycin and I-BET151 while most of the population remained positive to Ki-67. Thus, we compared the gene expression profiles in single clones such as no. 4 or 8. In the microarray analysis, the malignancy- or metastasis-related genes were found to be highly expressed in no. 8 clone (Fig. 4E and F) (22-29).

In addition, expression of *pdgfrb* and *sox2* was higher in no. 8 than in no. 4 (data not shown). The expression and amplification of gene in glioma have been reported (30). In brain tumors, *sox2* expression was found to be positively correlated with the grade of malignancy (31,32). *pdgfrb* expression has been reported to be correlated with the metastatic behavior in cancer (33); *pdgfrb* is preferentially expressed in glioma stem cells and its activation promotes glioma stem cell self-renewal (34).

The genes *mpz* and *plp1* were found to be highly overexpressed in no. 8. These genes are reported to be closely related to myelin and oligodendroglioma (35,36). Given that no. 8 clone showed a good response to our drug, this indicates that the above genes may be specific markers for our small-molecule combination. Future studies may need to investigate whether the expression levels of the above genes are implicated in glial induction through a combination treatment of our small molecules.

Moreover, *pdgfra, pdgfrb, pdgfrl, met, vegfa* and *colla1* have been implicated in cancer invasion and proliferation (37,38). Our results showed that the expression of these genes was downregulated by treatment with the specific small molecules (Fig. 2G), thereby indicating that the glial differentiation may influence cancer growth.

The existence of differentiation-resistant cells suggests that this combination of small molecules is neither sufficient nor robust to convert all the glioma cells. However, the standard drug for glioblastoma, temozolomide (TMZ), is also not effective for all heterogeneous glioblastoma; in analogy, our combination of small molecules was found to be quite effective on some types of glioma. It is noteworthy that our small-molecule combination inhibited the proliferation of glioma cells more efficiently than standard anticancer drugs such as TMZ. We demonstrated that the combination treatment inhibited the proliferation of glioma no. 8 more efficiently than TMZ, thereby suggesting that this combination may be effective on TMZ-resistant cells. Thus, a combined therapy, using both TMZ and our molecules, can be applied to TMZ-resistant cells for effective treatment. Thus, extensive research using patient-derived gliomas would be required to confirm this hypothesis.

Our findings suggest that malignant gliomas, derived from mutations in glial precursor cells, can be converted to non-proliferating glial cells with glial-specific characteristics. Moreover, the proliferation of malignant cells can be highly suppressed by the combination treatment of a cAMP activator, mTOR inhibitor and BET inhibitor. In future, we will continue to investigate the genes that are specifically involved in glial differentiation of glioma.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JO and YK designed the study, performed the experiments and wrote the paper. YH was involved in the conception of the study, wrote, reviewed and edited the manuscript. DB performed the data analysis, reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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