

# Tumor-tumor Cell Fusion Hybrids Obtained by the Modified PHA-DMSO-PEG Method Promote Temozolomide Resistance in Glioma

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## Research

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# Abstract

**Background:** Cell fusion and the subsequent aneuploidy are commonly observed in different kinds of tumor. In glioma, cell fusion and the number of the polyploid giant cells were found to be augmented with the tumor grades (WHO  $\text{I-IV}$ ) and closely related to poor prognosis. Phytohemagglutinin (PHA) holds an ability to induce cell-cell membrane contact and accelerates the cell fusion process mediated by the fusogenic agent polyethylene glycol (PEG). Dimethyl sulfoxide (DMSO) is well known as a cryoprotective agent involving in cell cryopreservation. In this study, we aim to obtain the glioma fusion hybrids by the modified fusion method in vitro, and then investigate the pathological consequences and the related molecular mechanism with the cell hybrids.

**Methods:** Glioma cells were labelled by lentiviruses infection. The PEG fusion efficiency was respectively improved by the addition of PHA and DMSO, and quantified by flow cytometry. Then, fusion hybrids were obtained by puromycin screening and fluorescence-activated cell sorting (FACS). Furthermore, DNA content was analyzed through flow cytometry. Cell proliferation rate and cell viability under temozolomide (TMZ) was detected by CCK-8 assay. Lastly, the related gene expression was measured through qRT-PCR and Western blotting.

**Results:** Glioma cell-cell contact was achieved by adding certain concentration of PHA in vitro. Tumor-tumor cell fusion efficiency was improved by PHA and DMSO. Glioma fusion hybrids were successfully obtained after puromycin screening and FACS. Cell size, DNA content and chromosome numbers of the fusion hybrids were almost twice that of the parental glioma cells. Moreover, glioma fusion hybrids showed an enhanced TMZ resistance potential compared to the parental cells, and also the MGMT expression was up-regulated in the hybrids.

**Conclusions:** We successfully obtained the glioma tumor-tumor cell fusion hybrids through the modified PHA-DMSO-PEG fusion method. Cell fusion may contribute to TMZ resistance in glioma, thus inhibition of cell fusion could be a promising orientation to improve TMZ resistance. Moreover, combining TMZ and MGMT inhibitor could be a beneficial approach in patients with glioma polyploid giant cells.

## Introduction

Glioma cells originate from neuroepithelium and account for almost 40 ~ 50% of primary brain tumors in adults [1]. The World Health Organization classified glioma into four clinical grades (WHO  $\text{I-IV}$ ) according to the histopathology and molecular characteristics. Glioblastoma multiform (GBM), the most progressive type of glioma (WHO  $\text{IV}$ ), is well known for its distal prognosis (median survival time of 14.6 months) despite the advances in comprehensive treatment of surgery followed by chemotherapy and radiotherapy concurrently [2, 3]. This awful outcome may partially attribute to the invasive growth of the glioma cells and the resistance to the chemo- and radio- therapies [4].

Physiological cell fusion rarely occurred in the specialized conditions, including placenta formation, bone homeostasis and especially fertilization, in which the egg and sperm cells fused together to form zygote

for sexual propagation[5], while pathological cell fusion mainly emerge in different kinds of cancer, including prostate cancer, melanoma and glioma [6–9]. Polyploid giant cancer cells and cell aneuploidies associated with cell fusion were commonly observed in tumor tissues and closely related to poor prognosis [8, 10, 11]. In human glioma, the number of the glioma polyploid giant cells (GPGCs) was significantly increased with the grade of glioma (WHO Ⅱ-Ⅳ) [7, 12], indicating cell fusion may participate in glioma evolution and correlate with tumor stage and prognosis. However, the fusion process of glioma tumor-tumor cells and the pathological characteristics of fusion hybrids remain largely elusive.

Cell fusion technology was mostly utilized in the process of antibody or vaccine production [13], in which cell fusion was induced in vitro through chemical fusogenic agent (e.g. polyethylene glycol) or physical electrofusion (e.g. ECM 2001 electronic fusion device) [14, 15]. Phytohemagglutinin (PHA) is able to bind to the specific sugar receptors on the cell surface so as to induce cell-cell membrane contact which is initially necessary for cell fusion [16]. We have previously reported that PHA was able to promote melanoma cell fusion both in the polyethylene glycol (PEG) chemo-fusion and ECM830 electro-fusion method [15–17]. Dimethyl sulfoxide (DMSO) holds many biological effects, and it is well known as a cryoprotective agent involving in cell frozen process [18, 19]. It was reported that DMSO could also enhance the somatic cell member fusion [18]. However, the fusogenic effects of DMSO as well as PHA in glioma cells are not fully elucidated.

Chemotherapy has been utilized in glioma treatment for decades, and currently oral alkylating drug temozolomide (TMZ) is the international standard chemotherapeutic agent in clinic [20]. TMZ could methylate O<sup>6</sup> site of guanine to achieve its cytotoxicity. It could effectively inhibit glioma cell proliferation and induce apoptosis [21, 22]. However, resistance to TMZ and the subsequent tumor recurrence has become a major obstacle for effective treatment of GBM. O<sup>6</sup>-methylguanine DNA methyltransferase (MGMT) could neutralize the cytotoxic effect of TMZ through reversing the alkylation of O<sup>6</sup> site of the guanine [23, 24]. Glioma patients with low MGMT (high MGMT methylation) level tends to have good clinical benefit in TMZ chemotherapy[25]. However, the pathological characteristics of fusion cells in glioma TMZ resistance and the related mechanism remain unclear.

In this study, high-grade U251 and low-grade Hs683 glioma cell lines were utilized in fusion process. Glioma fusion hybrids or polyploid giant cells (GPGCs) were successfully obtained by the modified fusion method in vitro. And the fusion efficiency of glioma tumor-tumor hybrids was highly improved by using PHA and DMSO. Then, cell size, DNA content and chromosome numbers of fusion hybrids were characterized. Furthermore, we explored the pathological characteristics of the fusion hybrids particularly TMZ resistance and the related molecular mechanism, attempting to deeply understand cell fusion in glioma and offer the promising new targets for GBM treatment.

## Materials And Methods

### Cell lines and cell culture

U251 and Hs683 human glioma cells were purchased from the American Type Culture Collection (ATCC, Gaithersburg, MD, USA) and Genechem Co., Ltd (China). U251, U251-F, Hs683 and Hs683-F cells were all cultured with DMEM medium (HyClone, CA, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 100U/mL penicillin and 0.1 mg/mL streptomycin (Gibco, USA) in a humidified 5% CO<sub>2</sub> incubator at 37°C. Cells culture medium was changed every 1-2 days and cells were passaged every 3-4 days.

## Cell labeling

U251 and Hs683 cells were infected with the U6-MSC-Ubiquitin-mCherry-IRES-Puromycin and U6-MSC-Ubiquitin-EGFP lentiviruses (Genechem Co., Ltd China). Cells were cultured to the logarithmic phase and plated in the culture flask. On the following day, cells were infected with lentiviruses when they were in 70% confluence. The fresh medium was replaced 8 h later, and cell fluorescence was observed after 48-72 h.

## Fluorescence analysis and fluorescence activated cell sorting

Cells were collected, washed twice and resuspended in the DMEM medium at a concentration of  $1 \times 10^7$  /mL. Monoclonal positive cells labeled with EGFP or mCherry were collected with a FACS Aria sorter (BECKMAN COULTER MoFlo XDP Beckman, USA) and cultured in a DMEM medium containing 20% FBS and double antibiotics. U251 or Hs683 cells were used as control.

For the fusion efficiency analysis and fusion hybrids selection, double EGFP/mCherry positive cells were analyzed or collected, cells carrying EGFP or mCherry alone was applied as control. Monoclonal and polyclonal cells of the fusion hybrids were all collected.

## Efficacy of PHA to glioma cells

U251 and Hs683 glioma cells were collected, resuspended by DMEM with PHA at different concentration (0, 5, 10, 20, 40, 80 µg/mL) and incubated at 37°C for 20 min. Then, a small number of cells were observed and photographed under the fluorescence microscope, the rest of cells were centrifuged and seeded in the culture dish. The next day, cells were fixed in 4% paraformaldehyde at room temperature for 30min following by stained with 0.1% crystal violet (1mg/mL) for 20 min. Random fields of stained cells were photographed and counted using ImageJ software (NIH, USA).

## PEG cell fusion method

EGFP glioma cells ( $1 \times 10^7$ ) and mCherry glioma cells ( $1 \times 10^7$ ) were mixed together and washed twice by DMEM the supernatant was removed completely and cells were vibrated in order to be loose, then 1mL

PEG (Roche, Germany) were joined in slowly within 60 s at 37°C water bath, stayed steadily for 90 s. After that, 10 mL DMEM (preheated in 37°C) was added gently within 3 min to terminate the reaction and incubated at 37°C for 5 min. Finally, cells were centrifuged, resuspended by culture medium and plated in petri dishes. The next day, Random fields of stained cells were photographed and analyzed using ImageJ software (NIH, USA).

## PHA-PEG cell fusion method

Cells were collected, washed twice and suspended in PHA (Sigma Aldrich, Germany) at certain concentrations (10µg/mL for U251 cells and 20 µg/mL for Hs683 cells) in DMEM, and incubated at 37°C for 20 min, then centrifuged to remove the supernatant completely, also cells were vibrated in order to be loose. After that, 1mL PEG were joined in slowly within 60 s at 37°C water bath, stayed steadily for 90 s. The next steps were the same to the PEG fusion method.

## PHA-DMSO-PEG cell fusion method:

Cells were collected, washed twice and suspended in PHA to incubate for 20 min, then centrifuged to completely remove the supernatant. After that, cells were vibrated in order to be loose following slowly joined in 1 mL DMSO-PEG (1% DMSO) within 60 s at 37°C water bath and stayed steadily for 90 s. The next steps were the same to the PEG fusion method.

## Puromycin drug screening

Puromycin (Gibco, USA) was added to the cells 24 h after cell fusion at certain concentration 2 µg/mL in U251 and 1.5 µg/mL in Hs683 cells, respectively and sustained for certain days (7 days in U251 and 10 days in Hs683 cells, respectively). Cell culture medium was changed every 3 days. Hybrids cell clones were observed and photographed under the fluorescence microscope, and total clone (over 20 cells) number along the axis of the petri dish (Fig6. B, line 1, 2 and 3) was counted to represent the clone numbers per dish.

## Cell volume measurement

Glioma cells at logarithmic phase were collected and washed twice by PBS, re-suspended in DMEM at a concentration of  $1 \times 10^5$ /mL and cell volume was analyzed by the Beckman Counter Multisizer 3.

## DNA content assay

Glioma cells were collected, washed twice by PBS and fixed by 75% ethanol overnight. The next day, cells washed twice by PBS to discard the ethanol completely and treated with RNAase (Sigma-Aldrich, USA) for 30 min at 37°C. After that, they were stained by propidium iodide (0.5 mg/mL, Sigma-Aldrich, USA) for 30 min in dark condition, and DNA content was detected through flow cytometry.

## Karyotype analysis

Glioma cells and glioma hybrids were cultured to the logarithmic phase. Then, colchicine (Solarbio, China, 0.2 µg/mL) was added to the culture medium and sustained in 37°C incubators for 4 h. After that, cells were collected, washed by PBS and suspended by 5 mL KCl (Solarbio, China, 0.075 mol/L) hypotonic solution (Solarbio, China, 37°C preheated), incubated at 37°C for 20 min. Then, they were centrifuged and resuspended by 5 mL Carnoy fixative solution (methyl alcohol: glacial acetic acid = 3:1, fresh) gently, fixed for 10 min and centrifuged to discard the Carnoy fixative solution, this process was repeated twice until 0.4 mL Carnoy fixative solution was added to suspended cells. After that, 1-2 drops of the cell liquid was dropped to the glass slide, fixed by flame quickly and dried in the 80°C oven for 1h. Finally, cells were processed by DAPI (Solarbio, China) according to the manufactures instruction.

## Cell proliferation and viability assay

Cell proliferation rate was detected by the Cell Counting Kit-8 (CCK-8 kit, Dojindo Laboratories, Japan). U251, U251-F, Hs683, Hs683-F cells were seeded in the 96 well plates ( $n = 5$ ) and cultured for 1, 2, 3, 4 and 5 days. At various time points, CCK8 (10 µL/100µL medium ) was added to each well and continually cultured for 2 h, the spectrometric absorbance of each well was measured by a microplate reader (PerkinElmer, USA) at 450 nm. For the cell viability assay, cells were seeded in the 96 well plates ( $n = 5$ ) and treated with TMZ (Sigma-Aldrich, USA) at variants concentrations for 72 h. After that, CCK8 was added to each well and the absorbance was determined.

## Colony formation assay

Glioma cells were collected and seeded into the 100 mm culture dish at a density of 1000 cells per dish. After that, cells were cultured in vitro for several days (7 days for U251 group and 12 days for Hs683 group, respectively), fixed with methanol and stained with 0.1% crystal violet (1mg/mL). The number of the colony was detected and analyzed by ImageJ software (NIH, USA).

## Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from U251, U251-F, Hs683 and Hs683-F cells using TRIzol (Invitrogen, USA) according to the manufacturer's instruction. Then, RNA was reverse-transcribed to cDNA using the Reverse Transcription System Kit (A3500, Promega, USA) according the protocol provided by the

manufacture. After that, qPCR was performed using the SYBR-Green PCR Master Mix (Applied Biosystems, USA). Oligonucleotide primers were synthesized to detect MGMT with  $\beta$ -actin as an internal control. The primer sequences were as follows: human MGMT forward (CACCGTTTGC GACTTGGTACTT) and reverse (AGACCCTGCTCACAACCAGACA),  $\beta$ -actin forward (GCCATGCCAATCTCATCTT), and reverse (ACCTGTACGCCAACACAGTG).

## Cell fractionation and Western blotting

Cells were harvested by certain volume of lysis buffer (2% SDS, 0.1 mol/L DTT, 10% glycerol and 60 mmol/L Tris, PH 6.8) according to the cell numbers, and protein lysates were boiled at 98°C for 10min. Then, proteins were separated by the 10% SDS-PAGE and transferred to the PVDF members (Millipore, Billerica, MA, USA). The members were then blocked by 5% non-fat milk in TBST buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% Tween-20, pH7.5) at room temperature for 1h, followed by incubated with the appropriate primary antibodies overnight at 4°C. After that, the members were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody and detected with an ELC detection system. The first antibody used was as follows: MGMT (1:500, Bioworld technology, China),  $\beta$ -actin (1:2000, Cell Signaling Technology, USA).

## Statistical analysis

All data were in a normal distribution, and variance was similar among groups. Data are presented as mean  $\pm$  SD. The difference between two groups was evaluated using a two-tailed Student's *t* test for single comparisons or one-way ANOVA test for multiple comparisons. All statistical analyses were performed by the GraphPad Prism software. A *p* value < 0.05 was considered to be statistically significant.

## Results

### Glioma cell labeling

In order to obtain the glioma tumor-tumor fusion hybrids, high grade and low grade human glioma cells (U251 and Hs683) were initially labeled with EGFP or mCherry tag by lentiviruses U6-Ubiquitin-EGFP or U6-Ubiquitin-mCherry-IRES-Puromycin with MOI=1 for U251 and MOI=2 for Hs683 cells, respectively. Positive monoclonal cells were selected by the fluorescence active cell sorting (FACS) and termed U251-EGFP (Hs683-EGFP) or U251-mCherry (Hs683-mCherry) cells (Fig.1).

### Glioma cell-cell contact by PHA

PHA was reported to promote cell-cell membrane contact which was firstly achieved in cell fusion process. Thus, we applied PHA to agglutinate glioma cells together to improve the fusion efficiency. Different concentrations of PHA were added to U251 or Hs683 glioma cells respectively. Then, glioma cells were fixed and dyed to analyze the effect and cytotoxicity of PHA. The results showed that glioma cells could agglutinate together and retain active at certain concentration of PHA (PHA  $\leq$  10 $\mu$ g/mL in U251 and PHA  $\leq$  20 $\mu$ g/mL in Hs683 cells) (Fig. 2a and Fig. 3a). The agglutination effect was enhanced with the increase of PHA concentration, gradually cells began to form cell clusters and cell hypertrophy (PHA > 10 $\mu$ g/mL in U251 and PHA > 20 $\mu$ g/mL in Hs683 cells, Fig. 2a and Fig. 3a). Cell survival rate also began to sharply decrease with the increase of PHA concentration (PHA > 10 $\mu$ g/mL in U251 and PHA > 20 $\mu$ g/mL in Hs683 cells, Fig. 2b, c and Fig. 3b, c). So the proper PHA concentration for U251 or Hs683 cells was respectively determined to be 10 $\mu$ g/mL or 20 $\mu$ g/mL in cell fusion process.

## **PEG fusion efficiency was significantly improved by PHA and DMSO in glioma tumor-tumor cell fusion**

To increase the fusion efficiency in vitro, PHA was first utilized to promote cell-cell membrane contact. Moreover, DMSO was added into PEG to further improve fusion efficiency in vitro. EGFP/mCherry double positive cells (U251-F or Hs683-F cells) were detected under the microscope and analyzed 24 h after cell fusion. The results showed that the proportion of U251 (Hs683) fusion hybrids in the PHA-PEG group was significantly increased compared to the PEG group (Fig. 4a-c), while the proportion of U251 (Hs683) hybrids in the PHA-DMSO-PEG (termed PD-PEG) group was more significantly improved compared to the PHA-PEG group (Fig. 4a-c). Moreover, we quantified the fusion efficiency by flow cytometry analysis. The results showed that the fusion efficiency was also significantly increased in the PHA-PEG group compared to the PEG group, meanwhile the fusion efficiency in the PD-PEG group was significantly improved compared to the PHA-PEG group (Fig. 5a-c), similar to the results under the microscope. Furthermore, puromycin was added in the culture medium to screen the hybrids. There were only double positive fusion hybrids and mCherry positive cells left in the petri dish after certain days of puromycin screening (6 days for U251 and 10 days for Hs683 cells, respectively). Afterwards, fusion hybrid clones carrying EGFP marker in the dish were counted and analyzed (Fig. 6a, b). The results showed that the clone numbers of U251 (Hs683) hybrids in the PHA-PEG group were significantly increased compared to the PEG group (Fig. 6c, d), while the clone numbers of U251 (Hs683) hybrids in the PD-PEG group were significantly increased compared to the PHA-PEG group (Fig. 6c, d), which was consistent with the previous data. All these results demonstrated that PEG fusion efficacy could be improved by PHA and DMSO in vitro.

## **Obtainment of the monoclonal and polyclonal glioma tumor-tumor cell fusion hybrids**

In order to obtain the stable glioma fusion hybrid, U251 and Hs683 monoclonal and polyclonal fusion cells were obtained by FACS and cultured in vitro for almost a month to construct the stable fusion glioma cell lines (Fig. 7a, b).

## **Glioma fusion hybrids converted from hyperdiploidy to hypertetraploidy**

Cell size, DNA content and karyotyping analysis were evaluated to characterize the obtained polyclonal glioma fusion hybrids. The results showed that the cell size of the U251 (Hs683) glioma fusion hybrids (termed U251-F or Hs683-F) were significantly enlarged compared to the U251 (Hs683) cells (Fig. 8a, b). The DNA content of the U251 (Hs683) glioma fusion hybrids was almost twice than that of the parental U251 (Hs683) cells (Fig. 8c). Karyotyping analysis showed that the chromosome numbers of the U251 (Hs683) glioma fusion hybrids were almost doubled compared to the parental U251 (Hs683) cells (Fig. 8d), and converted from hyperdiploidy to hypertetraploidy (Fig. 8e, f).

## **Cell fusion contributes to temozolomide resistance in glioma cells**

We then evaluated and analyze the pathological characteristics of the polyclonal glioma fusion hybrids. The results showed that the proliferation rate of the U251 and Hs683 fusion hybrids were both significantly decreased compared to U251 and Hs683 parental cells (Fig. 9a, b). Cell cloning formation experiment also showed that the clone numbers of the U251 (Hs683) hybrids were also significantly decreased compared to parental U251 (Hs683) cells (Fig. 9c-e). On the other hand, the cell viability under TMZ was significantly increased in U251 (Hs683) fusion hybrids compared to the parental U251 (Hs683) cells (Fig. 9f, g), indicating glioma fusion hybrids obtained enhanced temozolomide resistance potential compared to the parental cells.

## **The up-regulated MGMT expression in glioma fusion hybrids**

MGMT, a DNA repair enzyme, is a main culprit contributing to TMZ resistance. So, we naturally detected the MGMT expression in polyclonal glioma fusion hybrids. Interestingly, qRT-PCR (Fig 10a, b) and Western blotting (Fig 10c) results all showed that MGMT was significantly up-regulated in U251 (Hs683) fusion hybrids compared to the parental U251 (Hs683) cells, indicating MGMT may contribute to TMZ resistance in glioma fusion hybrids.

## **Discussion**

Cell fusion and associated polyploid giant cancer cells with augmented cell volume, multinuclear and polymorpho-nuclear are commonly observed in the glioma tissue especially in high grade glioma (GBM) [7, 11, 26, 27]. However, glioma cell fusion hybrids and its specific pathological characteristics remain unclear due to the lack of fusion cell model in vitro and in vivo. In this study, we successfully obtained the glioma homogenous fusion hybrids by the modified PHA-DMSO-PEG fusion method. Then, we investigated its pathological characteristics and the corresponded molecular mechanisms.

PEG fusion method, characterized as the limited the fusion efficiency, was the traditional fusion method commonly used in antibody production [28]. In our previous research, PHA was utilized to improve cell fusion efficacy through promoting cell-cell membrane contact and agglutination [17]. In this study, PHA was also certified to greatly increase the PEG cell fusion efficiency in glioma cells. Moreover, the cryogen DMSO was also proved to further improve the glioma cell fusion efficiency. Lipidic DMSO may play a role in promoting member-member fusion. In the BTX ECM2001 electronic fusion approach, cells are arranged in a bead chain and contact together under an alternating current wave (AC), and then cell members fuse through a direct current (DC) [15]. PHA and DMSO, analogous to the AC and DC in electronic fusion method, were utilized in our study so as to improve the cell fusion efficiency. Therefore, we demonstrate a convenient and effective PHA-DMSO-PEG fusion method that could be applied in many fields such as antibody production and cancer research. We detected the survived fusion hybrids 24 hours after cell fusion. Many dead fusion cells can be observed in the supernatant medium at this time point. This phenomenon indicated that many fusion hybrids couldn't survived in the following cell division process due to the chromosomal and genomic instability, so the survival rate of hybrids is relative low in this cell fusion process [10].

Previous studies revealed that the number of polyploid giant cancer cells was associated with tumor grade and progression in human glioma [7]. Moreover, macrophage-glioblastoma cell fusion hybrids entail glioblastoma invasion [29]. In this study, we focused on the homogenous tumor-tumor cell fusion in glioma and investigated the pathological characteristics in the polyclonal fusion hybrids. In vivo, two cells could also be in close contact to accomplish cell fusion [30]. During macrophage cell fusion, a fusion receptor and its ligand CD47 was involved[31]. The fusion tendency of cancer cells may be due to their high expression of the fusion-associated member receptors and ligands [32-34]. Identification of more member receptors and ligands involved in glioma cell fusion may pave the way for a new therapeutic strategy for GBM treatment.

Cell proliferation and TMZ resistance are two most important criteria to evaluate the pathological behavior of glioma [35]. In this study, glioma fusion hybrids showed a low proliferation rate but considerably enhanced TMZ resistance potential. The decreased proliferation rate was similar to the previous reports in the fusion between B16 and B16 cancer cells as well as between cancer cells and normal cells [36], and this phenotype maybe explained by the enhancement of the tumor suppressor genes (e.g. *p53* and *PTEN*) in the fusion hybrids [17, 37]. On the other hand, we found that tumor-tumor cell fusion hybrids acquired enhanced temozolomide resistance potential in glioma. Moreover, MGMT was up-regulated in the fusion hybrids. MGMT, a DNA repair enzyme and a main culprit contributing to

TMZ resistance, could remove the methyl group in the O<sup>6</sup>-methylguanine to invalid TMZ-induced DNA damage and cytotoxic lesion [38, 39]. So TMZ resistance induced by cell fusion could be ascribed to MGMT dependent approach. Furthermore, the methylation status of the cysteine-phosphate-guanine (CpG) island in the MGMT promoter site was directly correlated with the MGMT expression level [38]. So the therapeutic approaches for glioma patients with pathological GPGCs may premeditate to combine TMZ with the MGMT inhibitors (e.g. O<sup>6</sup>-BG, SP600125 and AS602801) for more clinical benefits [40]. Other underlying mechanisms of TMZ resistance in the glioma fusion hybrids should be further investigated to identify more targets for glioma treatment.

As it was well known, cell fusion and polyploid giant cells contribute to tumor heterogeneity [41-43]. Prior study revealed that different clones of the fused rat intestinal epithelial cells (ICE-6) exhibited different phenotype and genotype during serial passage of the hybrids [10]. We obtained dozens of monoclonal glioma fusion hybrids in this study. Further phenotype and genotype investigation should be carried out with these generated hybrids to better understand cell fusion and tumor heterogeneity [44, 45]. Both the monoclonal and polyclonal glioma fusion hybrids obtained could prove to be useful in more pathological characteristics studies, including glioma invasion and glioma stem cell traits, etc.

## Conclusions

In this study, we demonstrated that PEG fusion efficiency could be improved PHA and DMSO in glioma cells. We successfully obtained glioma tumor-tumor fusion hybrids that can be applied in many fields, particularly tumor heterogeneity and pathological characteristics research. The hybrids of fusion between glioma cells acquired an increased TMZ resistance potential, and the MGMT was up regulated in the fusion hybrids. We provided a novel insight in cell fusion and glioma progression, which suggested that blockage of cell fusion could be a promising orientation to improve TMZ resistance. Moreover, combining TMZ and MGMT inhibitor could be a beneficial approach in patients with glioma polyploid giant cells.

## Abbreviations

GBM: Glioblastoma; PHA: PEG: phytohemagglutinin; polyethylene glycol; DMSO: Dimethyl sulfoxide; FACS: fluorescence-activated cell sorting; TMZ: temozolomide; WHO: world health organization; GPGCs: glioma polyploid giant cells; MGMT: methylguanine DNA methyltransferase; ATCC: American Type Culture Collection; CCK-8: Cell Counting Kit-8.

## Declarations

## Acknowledgements

Not applicable

## Authors' contributions

RFM performed experimental design, conception, data acquisition, data analysis and draft. JYJ, MXL, MMZ participated in the experiments and reversion of the manuscript. JWZ participated in the statistical analysis. FSL contributed to research funding and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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## Availability of Data and materials

The datasets used and analyzed in this study are available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

Not applicable

## Consent for publication

All authors agreed to publication

## Competing interests

The authors declare that they have no conflicts of interest.

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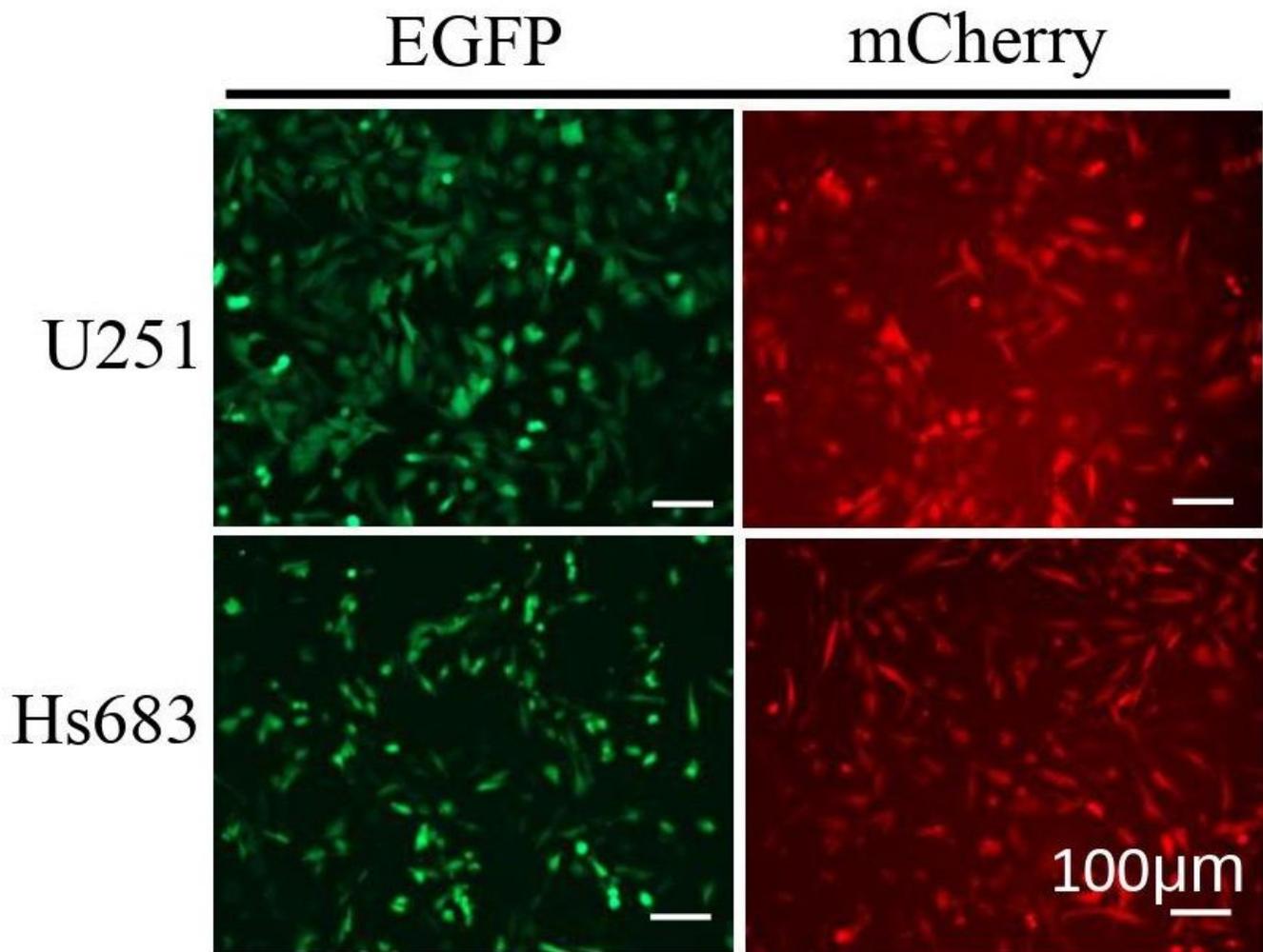
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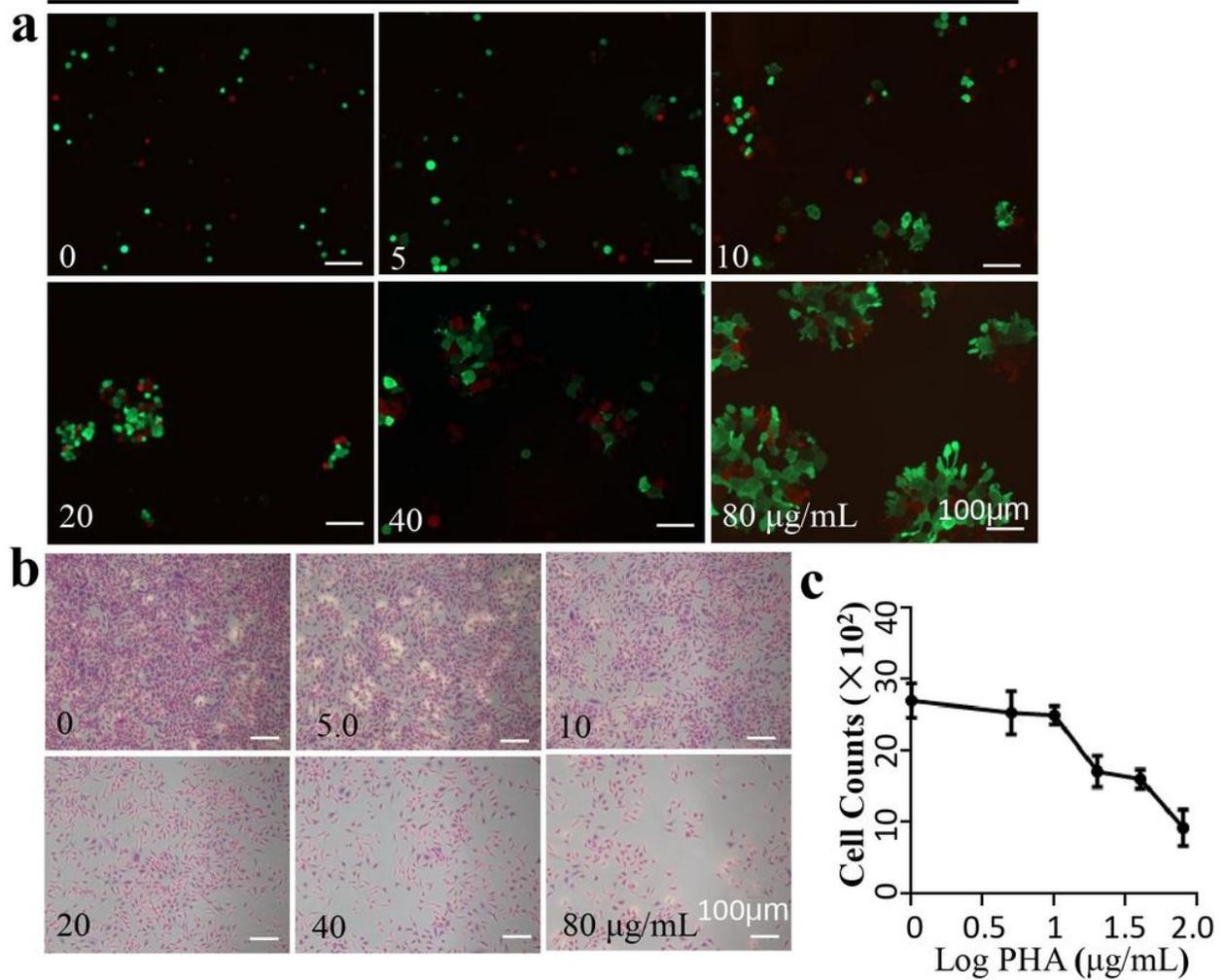
## Figures



**Figure 1**

U251 and Hs683 glioma cells were labeled with EGFP or mCherry makers. U251 and Hs683 glioma were infected by U6-Ubiquitin-EGFP or U6-Ubiquitin-Cherry-IRES-Puromycin lentivirus. Positive EGFP or mCherry monoclonal cells were selected by the FACS and cultured in vitro ( $\times 100$ ).

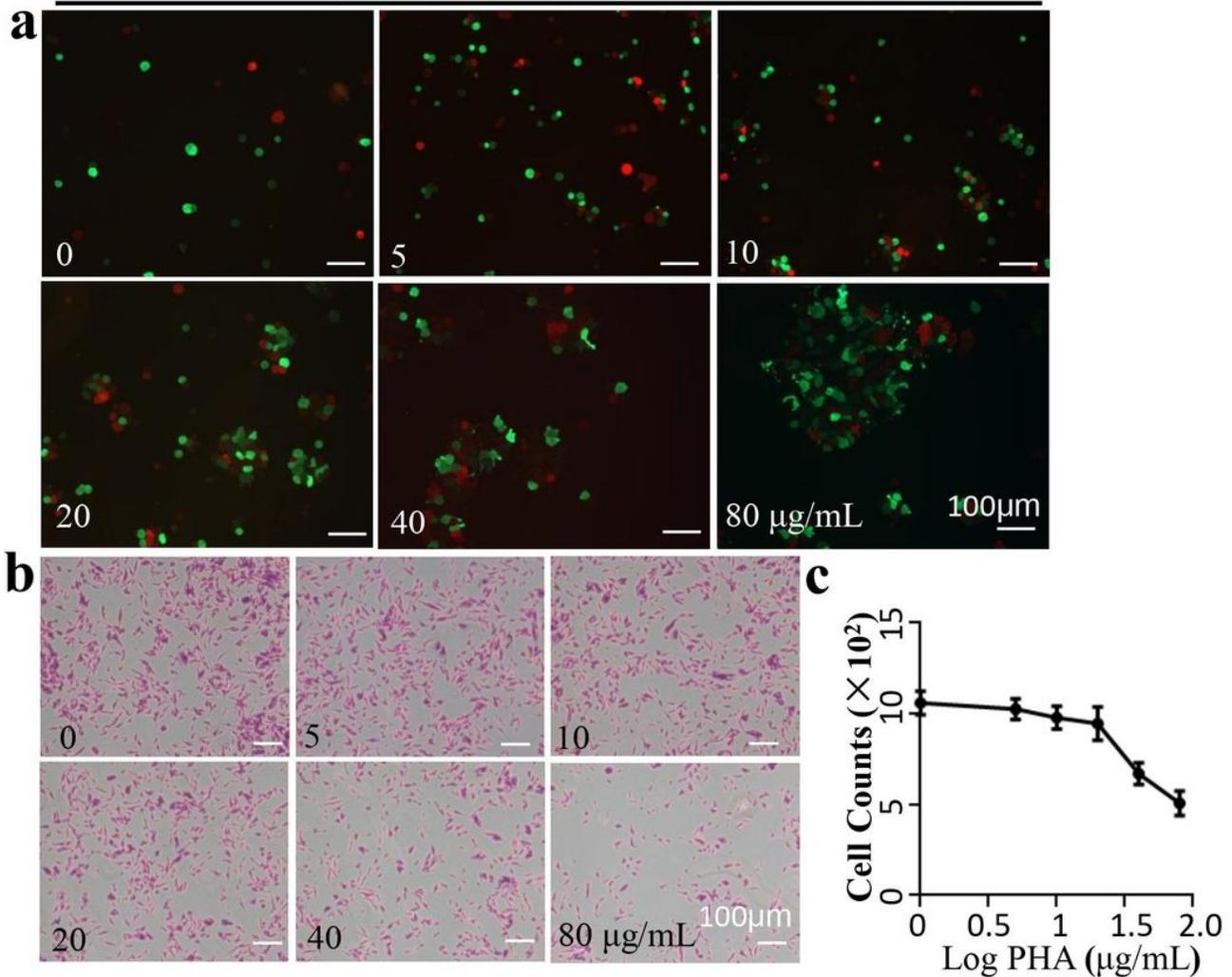
### Analysis of PHA efficacy in U251 cells



**Figure 2**

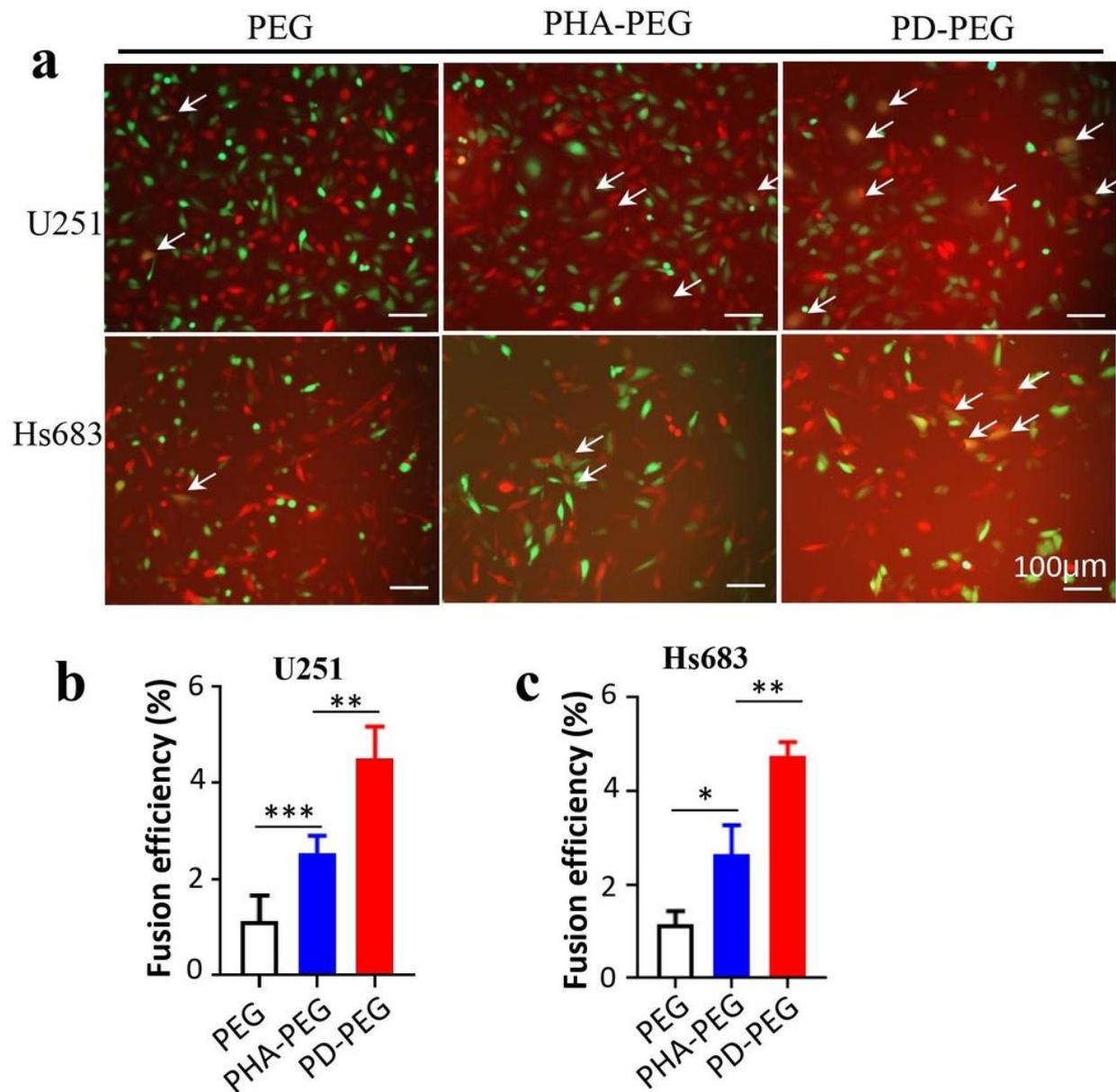
The efficacy of PHA in U251 glioma cells. a U251 glioma cells could agglutinate together at certain concentration of PHA ( $\leq 10\mu\text{g/mL}$ ) and cells began to form cell clusters and cell hypertrophy (PHA  $> 10\mu\text{g/mL}$ ) ( $\times 100$ ). b Glioma cells were dyed by crystal violet 24 h after applying PHA ( $\times 100$ ). c Cell number was also sharply decreased when the PHA concentration was bigger than  $10\mu\text{g/mL}$ . Data is representative of three independent experiments.

### Analysis of PHA efficacy in Hs683 cells



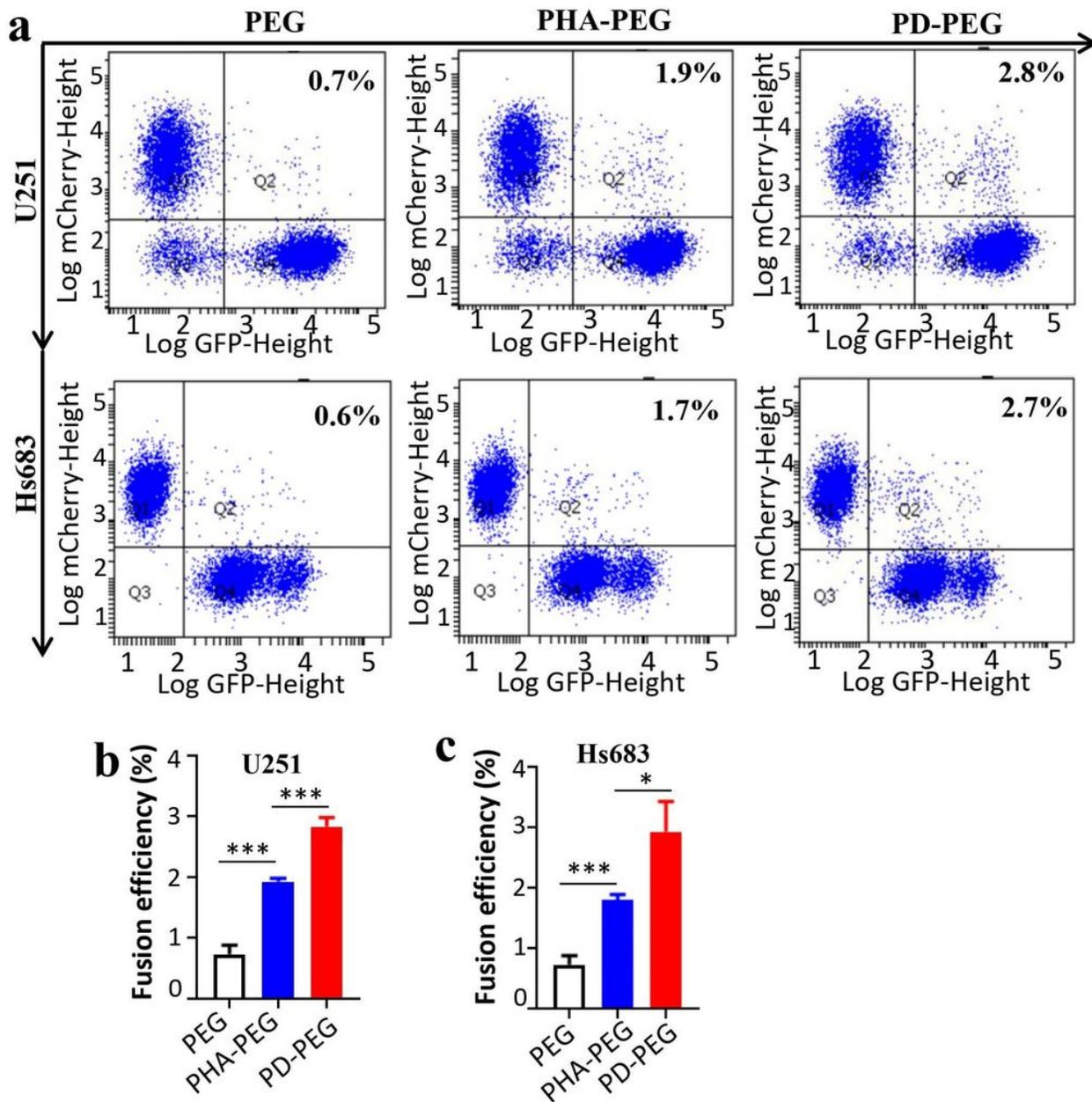
**Figure 3**

The efficacy of PHA in Hs683 glioma cells. a Hs683 glioma cells could agglutinated together at certain concentration of PHA ( $\leq 20\mu\text{g/mL}$ ) and form cell clusters and cell hypertrophy (PHA  $> 20\mu\text{g/mL}$ ) ( $\times 100$ ). b Glioma cells were dyed by crystal violet 24 h after applying PHA ( $\times 100$ ). c Cell number was also sharply decreased when the PHA concentration was bigger than  $20\mu\text{g/mL}$ . Data is representative of three independent experiments.



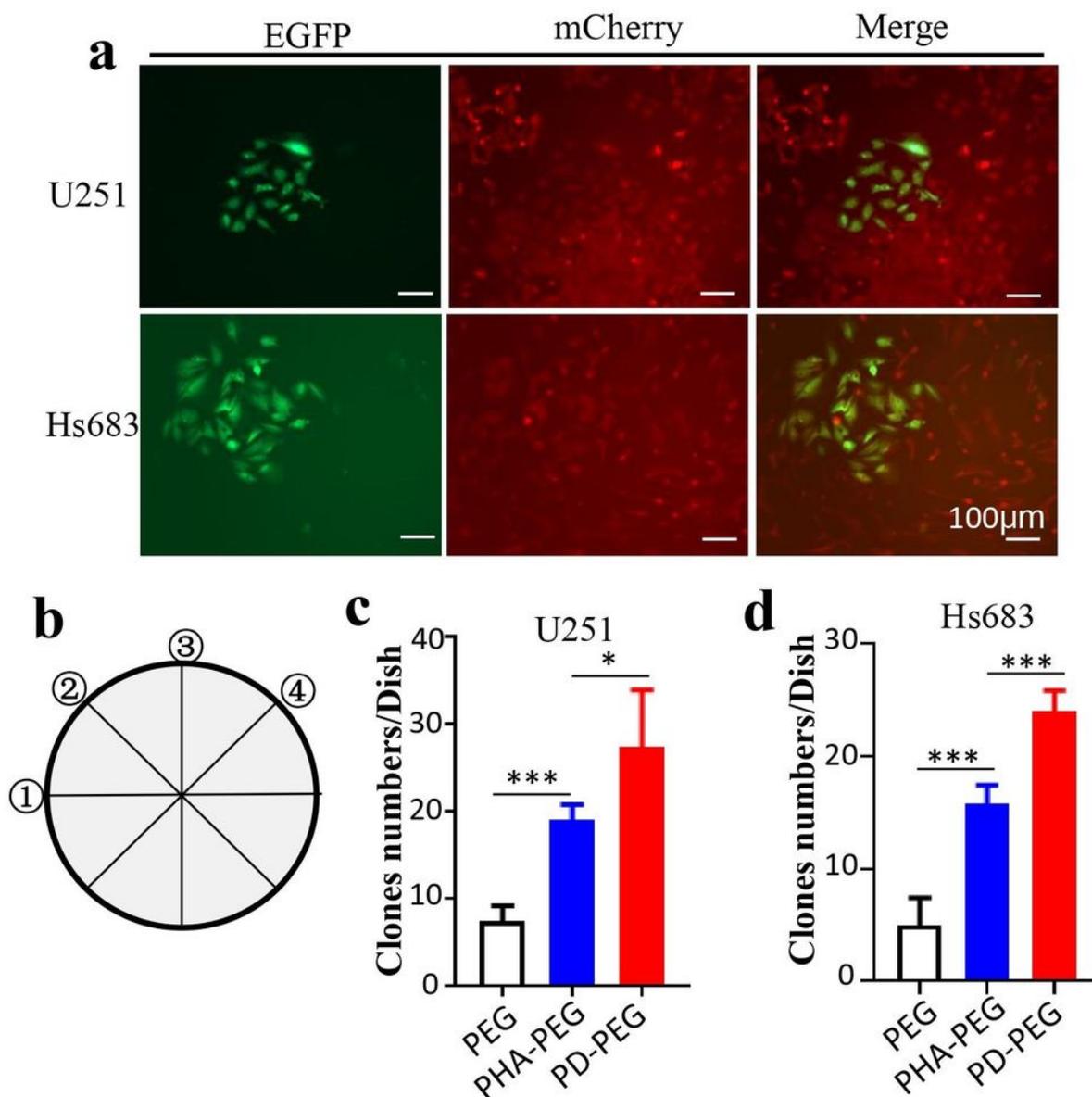
**Figure 4**

The fusion efficiency detected by the microscope. a EGFP/mcherry double positive hybrids were detected by the fluorescence microscope ( $\times 100$ ). b Fusion efficiency of U251 cells was analyzed by ImageJ software ( $n = 5$ ). c Fusion efficiency of Hs683 cells was analyzed by ImageJ software ( $n = 5$ ). Data is representative of three independent experiments,  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$



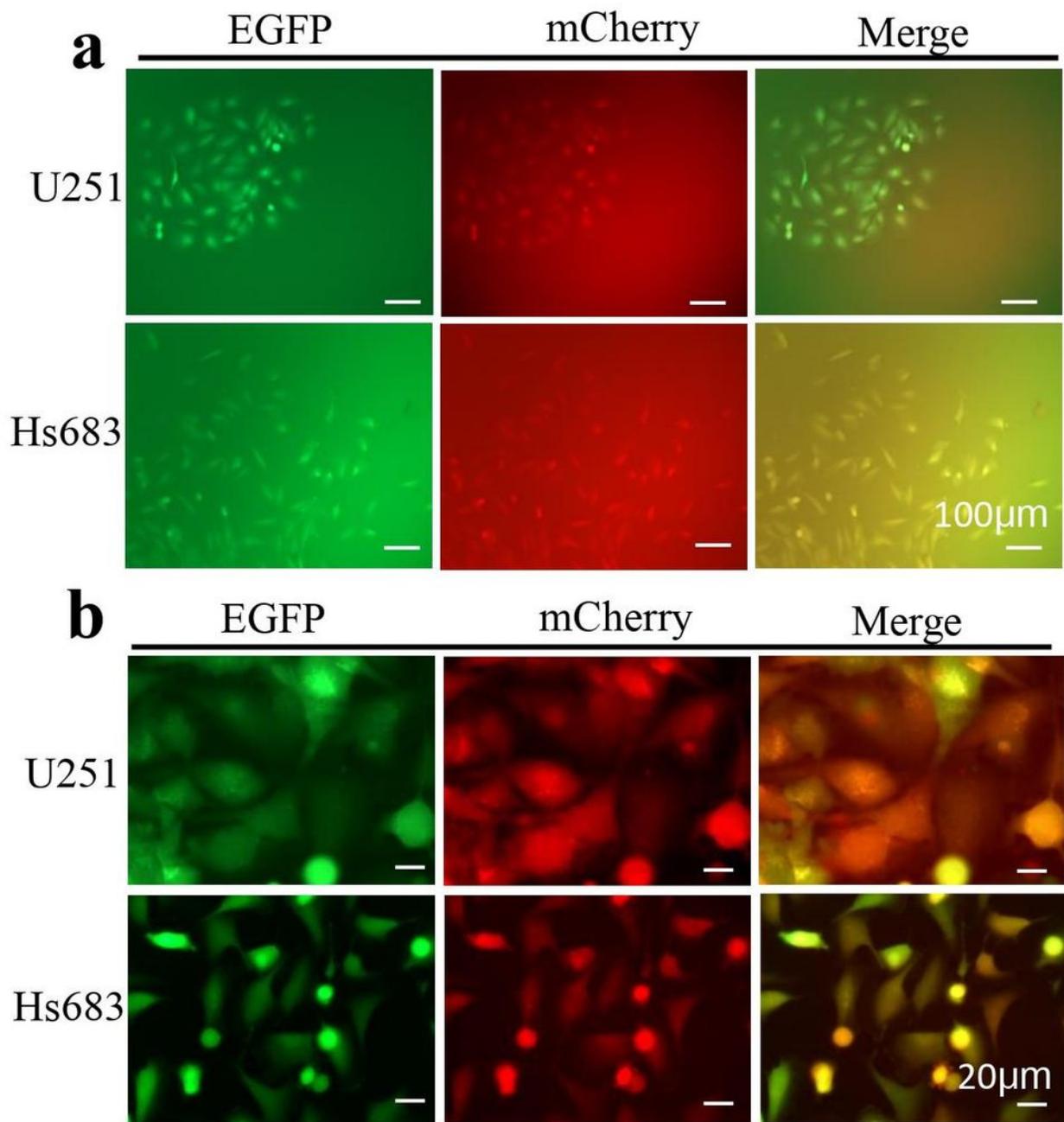
**Figure 5**

The fusion efficiency analyzed by flow cytometry. a Fusion efficiencies of PEG, PHA-PEG and PD-PEG method in U251 or Hs683 cells were detected by flow cytometry, respectively. b,c Fusion efficiency of PEG, PHA-PEG and PD-PEG method in U251 or Hs683 cells was quantified and analyzed (n = 3). Data is representative of three independent experiments. \*P < 0.05, \*\*\*P < 0.001



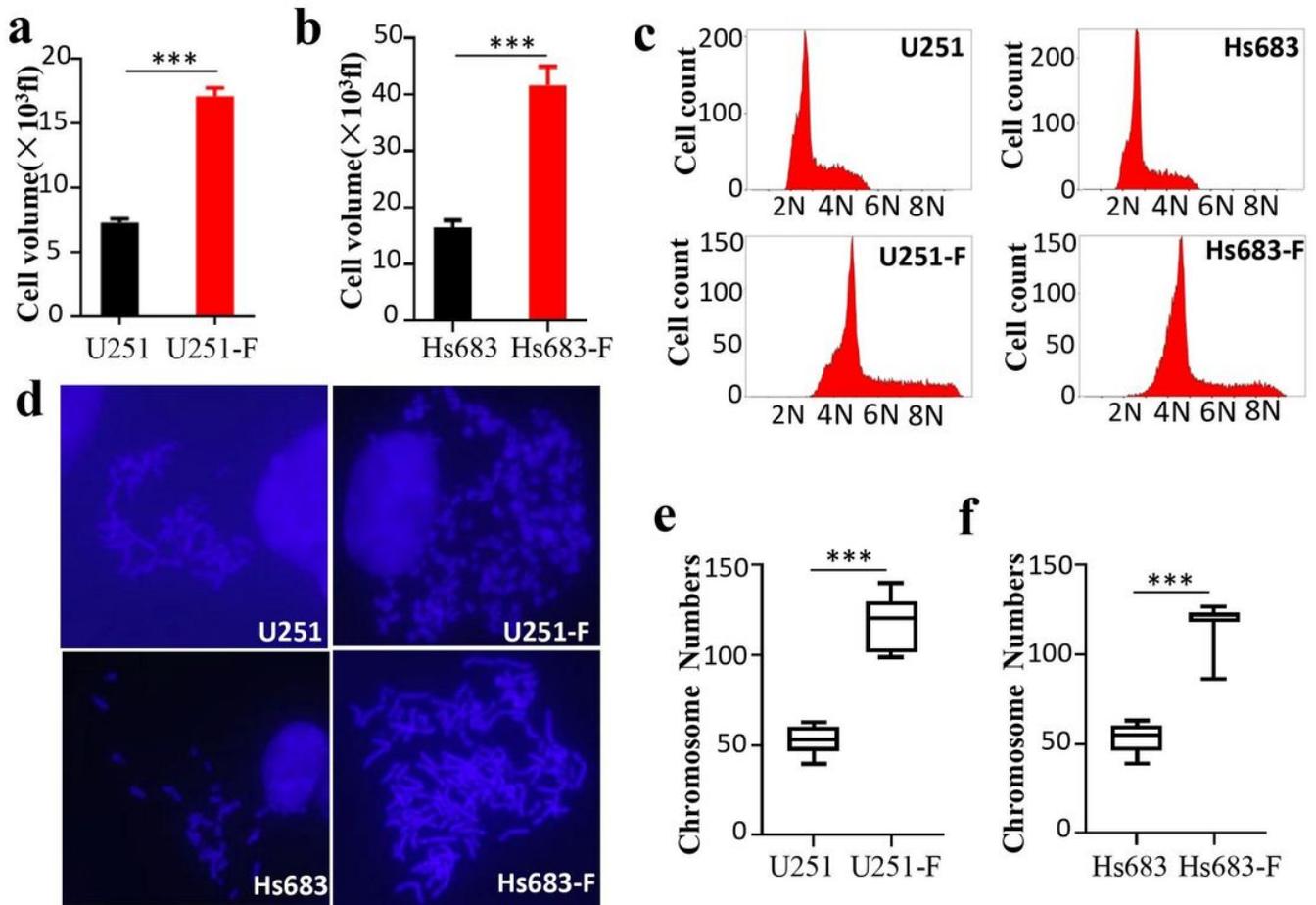
**Figure 6**

The clone number of fusion hybrids screened by puromycin was increased by PHA and DMSO. **a** The fusion hybrid clones carrying the EGFP marker was detected by fluorescence microscope ( $\times 100$ ). **b** The total clone number along the diameters (①, ②, ③, and ④) of the petri dish was quantified. **c,d** The screened U251 and Hs683 clone numbers in the PEG, PHA-PEG, and PD-PEG groups ( $n = 4$ ). Data is representative of three independent experiments. \* $P < 0.05$ , \*\*\* $P < 0.001$



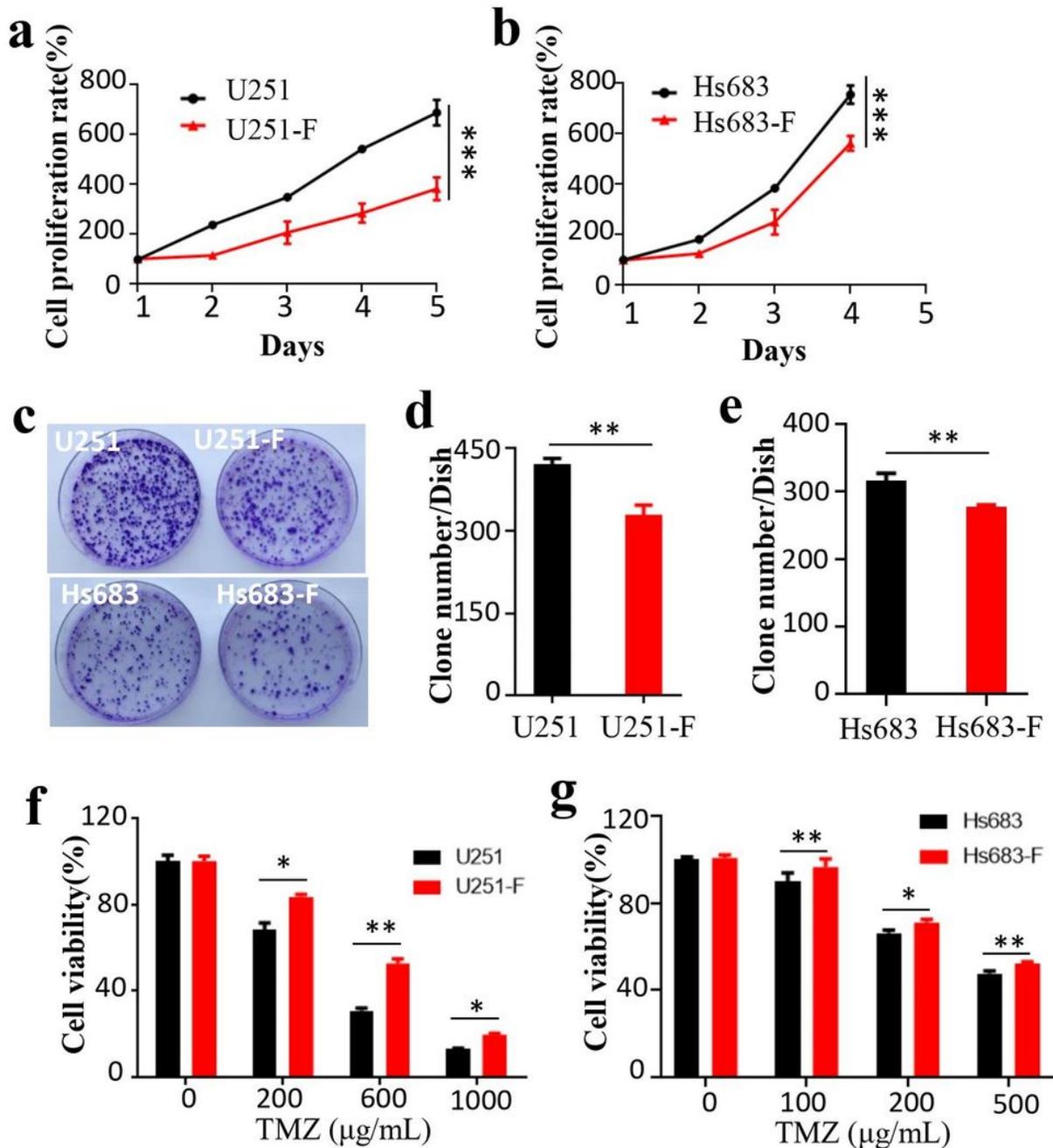
**Figure 7**

Monoclonal and polyclonal fusion hybrids was obtained by FACS. a Monoclonal fusion hybrids of glioma U251 and Hs683 cells ( $\times 100$ ). b Polyclonal fusion hybrids of glioma U251 and Hs683 cells ( $\times 400$ ).



**Figure 8**

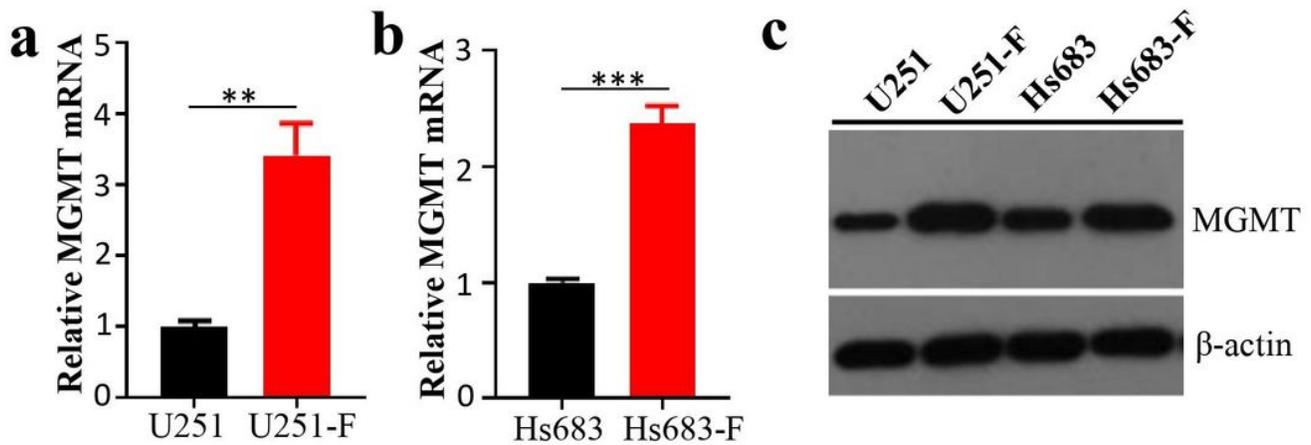
Glioma fusion hybrids converted from hyperdiploidy to hypertetraploidy. a,b Cell volume of the U251 or Hs683 fusion hybrid was enlarged compared to the parental cells ( $n = 3$ ). c DNA content of the U251 or Hs683 fusion hybrid was twice that of the parental cells ( $n = 3$ ). d Karyotyping analysis of the glioma fusion hybrids and the parental cells ( $\times 630$ ). e,f Karyotyping analysis showed that the chromosome number of the U251 or Hs683 fusion hybrid was almost twice that of the parental cells ( $n = 5$ ). Data is representative of three independent experiments. \*\*\* $P < 0.001$



**Figure 9**

The pathological characteristics of the glioma fusion hybrids. a,b The proliferation rate of U251 (Hs683) fusion hybrids was significantly decreased compared with parental U251 (Hs683) cells (n = 5). c Colonies formed by the U251 (Hs683) glioma or glioma hybrid cells were stained. d,e Colony numbers of U251 (Hs683) fusion hybrids was significantly decreased compared with parental U251 (Hs683) cells (n = 3). f,g The cell viability under TMZ was significantly increased in U251 (Hs683) fusion hybrids compared to

parental U251 (Hs683) cells (n = 3). Data is representative of three independent experiments. \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001



**Figure 10**

The up-regulated MGMT in glioma fusion hybrids. a,b Both qRT-PCR and Western blotting results showed that MGMT was significantly up-regulated in U251 (Hs683) glioma fusion hybrids compared to the parental U251 (Hs683) glioma cells. Data is representative of three experiments. \*\*P < 0.01, \*\*\*P < 0.001