The embryonic stem cell microenvironment inhibits mouse glioma cell proliferation by regulating PI3K/AKT pathway

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Abstract

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Abstract

PURPOSE: To study the effect of the embryonic stem cell (ESc) microenvironment in inhibiting mouse glioma cells and its possible mechanism.

METHODS: Glioblastoma cell line U118 in the brain, was investigated in this study. There were four experimental groups: U118 glioma cells cultured alone as the control group (GB group), U118 cell and ESc co-cultured group(GE group), U118 cell and ESc co-cultured and adding phosphoinositide 3-kinase (PI3K) agonist group (GA group), U118 and temozolomide as the positive control group (GT group); U118 cells were harvested after 72 hours of culture. Cell proliferation, apoptosis, reactive oxygen species (ROS) and vasculogenic mimicry assays, quantitative real-time RT-PCR (qPCR), and western blot (WB) were study, the biological function of U118 glioma cells and the PI3K/AKT signaling pathway were compared the differences between the groups.

RESULTS: Compared with the GB control group, the GE co-culture group and GT chemotherapy group showed reduced cell proliferation, increased apoptosis, increased ROS, decreased or disappearance of vasculogenic mimicry. Expression of cyclin B and D, significantly reduced, while that of BAX, BCL-2, P53, Caspase3, Gsk-3B, P21, and P27, significantly increased. The expression of PI3K, PDK, AKT,
and mTOR, significantly decreased while that of PTEN significantly increased. The expression of positive regulatory factors significantly increased but negative regulatory factors decreased in GA group compared to the GE group.

CONCLUSION: The ESC microenvironment reverse glioma malignancy, partially via inhibition of the PI3K signaling pathway. Our study may have a significant impact and clinical implication on cell therapy in glioma.

Keywords: embryonic stem cells, glioma, PI3K/AKT signaling pathway, microenvironment, Cell therapy
Introduction: Glioblastoma multiforme (GBM) is the primary invasive malignant brain cancer, approximate 16% of all major cancers of Brain and spinal cord.[1] Glioma resulted in poor prognosis due to rapid growth and extensive infiltration into nearby tissue. Current standard therapies include maximum surgical resection, postoperative adjuvant temozolomide (TMZ), and radiation therapy.[2] Because the boundary between GBM and normal brain tissue is unclear, it is not possible to completely remove cancer cells. Even with improvements in surgical techniques, radical resection of the tumor mass is very difficult and incomplete, because infiltrated tumor cells always remain in the surrounding tissue, and the prognosis for GBM patients is poor with a median survival of 15 Month.[3]

The paradigm that mutations cause cancer (the mutation-centric model) has been challenged by recent studies. A representative model “adaptive oncogenesis” was recently introduced by Dr. DeGregori[4]. This model views cancer from an evolutionary biology perspective. It demonstrated that the microenvironment surrounding cancer cells is as significant in causing cancer development as mutations. Healthy microenvironment provides tumor-suppressive signals with tissue homeostasis controlled. However, once tissue homeostasis is uncontrolled, for example by carcinogenic factors such as radiation, smoking, virus infection, and aging, the altered microenvironment can become a potent cancer promoter and favor the growth of cells with cancer-causing mutations [5] The microenvironment can also directly affect the phenotype of malignant cells without altering their genetic makeup. Therefore, in the treatment of cancer, it is not enough to consider only killing the cancer cells while ignoring the microenvironment that causes the cancerous changes. Considering the pivotal role of the microenvironment in cancer initiation, progression, and metastasis, the promising approaches for cancer prevention and treatment from the evolutionary biology view, should through modulating the microenvironment, i.e. shift it from cancer “permissive” to cancer “suppressive” and maintain it.

Previous studies have suggested that the early embryonic microenvironment may reverse the malignant melanoma cells into a non-tumorigenic phenotype [6]. However, it has been demonstrated in chicken embryos [7], mouse blastocysts [8], and
zebrafish embryos [6] that the embryonic microenvironment can reverse tumor cells to be non-tumorigenic. These findings indicate that modulating the microenvironment can reprogram cancer cells without damaging normal cells, thereby avoiding the side effects of normal anti-cancer therapies on normal organisms. However, with the development of the embryo, the ability of the embryo to reverse the transformation of tumor cells into non-tumor phenotypes is reduced and is almost completely lost after birth [8,9]. Attempts to reprogram tumor cells using adult stem cells have proved unsuccessful [10].

The effect of reprogramming tumor cells using ESC conditioned medium [11] or ESC extracellular matrix [12] to mimic the embryonic microenvironment was much weaker than that observed in early embryos, presumably due to cancer cells and embryo caused by lack of direct interaction between microenvironments. These findings indicate that reprogramming tumor cells requires both the early embryonic microenvironment and cell-cell interactions. Therefore, embryonic stem cells (ESCs) from blastocysts were selected for this study because they can provide a microenvironment similar to early embryos.

Researchers have successfully used the embryonic stem cells to simulate the microenvironment of embryos, confirmed their safety and efficacy in the treatment of leukemic mice,[13] and found that embryonic stem cell microenvironment can reverse the degree of malignancy of uveal melanoma and cutaneous melanoma by downregulating PI3K pathway[14,15]. This study aimed to investigate whether embryonic stem cell microenvironment can reverse the degree of malignancy of human glioma. In addition, the involved mechanism of action was studied to provide a new theoretical basis and treatment directions for the prevention and treatment of glioma.

Materials and Methods:

1. ES culture

E14 mouse embryonic stem cells were generously provided by Professor Peng Xiang of the Stem Cell and Tissue Engineering Research Center. ES cells were inoculated into a 25 cm culture flask at a density of $4 \times 10^4$/cm$^2$, cultured at 37°C, 5% CO$_2$, and passaged at the same density every 2-3 days.

2. U118 cell culture
U118 glioma cells (ATCC® HTB-15TM) were purchased from ATCC. SH-SY5Y cells in good condition were plated in a Petri dish at the ratio of 1:3 (a density of approximately $3 \times 10^4$/cm$^2$), cultured at 37 °C, 5% CO$_2$, and passaged at the same density every 3-5 days.

There were four experimental groups:

GB group (control group): U118 human glioma cells were cultured separately in GBM medium, and the U118 cells of interest were digested directly after 72 hours.

GE group: Embryonic stem cells and U118 cells were mixed at a ratio of 1:3 in GBM medium and seeded in the same flask. After 72 hours after co-culture, the U118 cells of interest were isolated using a flow cytometry sorter.

GA group: Embryonic stem cells and U118 cells were mixed at a ratio of 1:3 in GBM medium and seeded in the same flask. After the cells were adherent for 2 hours, PI3K pathway agonist (PTEN inhibitor) SF1670 (2 uM) was added. After 72 hours after co-culture, the U118 cells of interest were isolated using a flow cytometry sorter.

GT group: After U118 glioma cells became adherent in GBM medium, the chemotherapy agent temozolomide was added (300 µM TMZ was added 2 hours after the cells became adherent), and the U118 cells of interest were digested directly after 72 hours.

3. 3,3'-dioctadecyloxacarbocyanine perchlorate (DIO) cell marker

In a clean bench, use a sterile pipette to remove the cell culture medium from the culture flask as much as possible. The cells were completely covered with the cell stain solution 5ul of DIO reagent (Vybrant® DiD cell-labeling solution, V22887) per 1ml. After incubating for 20 minutes, the stain was discarded and washed three times with fresh U118 medium, the cells were passaged according to conventional U118 cells.

4. Flow cytometry sorting

The cells directly co-cultured in a culture flask, washed twice with PBS and mixed with TrypLE Express for 1 to 2 minutes. centrifuged for 3 minutes at 1500 rpm, discarded the supernatant, and resuspend the cells in the loading buffer. Cell concentration was adjusted to $1 \times 10^7$ cells/mL and filtered through a 40 µm cell sieve. DiO-labeled U118 cells selected by the flow cytometer were collected. The entire
process was conducted away from light as much as possible.

5. Flow cytometric determination of apoptosis

After collecting $1 \times 10^6$ U118 cells from each group, washed cells with PBS twice and centrifuged for 3 minutes at 1000 rpm, and discarded the supernatant. KGI Annexin V-APC/7-aminoactinomycin D (Invitrogen) apoptosis detection kit reagents were added to the sample away from light, and the samples were loaded in the flow cytometer.

6. Cell proliferation experiments

Each group of collected cells were centrifuged, resuspended, counted, and adjusted to a concentration of 5000 cells/mL. 200 µl of cell suspension was added to each well of a 96-well plate, with 5 replicate wells per group. Then placed the plates in an incubator. The culture medium was replaced with fresh cell culture medium containing CCK-8 reagent (Dojindo Molecular Technologies, Japan) (10 µl CCK8 per 100 µl of fresh culture medium). The Optical Density (OD) was measured at 450 nm over seven consecutive days using a multifunction microplate reader. The experiment was repeated in triplicates.

7. Reactive oxygen species (ROS) measurement

2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) was diluted to a final concentration of 10 µmol / L in serum-free medium. Collected cells at a concentration of $1 \times 10^6$ cells / mL and resuspended in pre-diluted DCFH-DA. Incubated in a 37°C incubator for 20 minutes, washed cells three times with serum-free cell culture medium to completely remove DCFH-DA that did not enter the cells. Each group of GBM cells was examined by flow cytometry within 30 minutes.

8. Angiogenesis mimetic experiment

After pre-cooling the 96-well plate, pipette, and tip at -20°C, melt on Matrigel ice, place 96-well plate on ice, add 30 ul Matrigel to each well. After 10 minutes in the refrigerator, place it in the incubator for 30 minutes. Collected U118 cells, adjust the cell density to $8 \times 10^5$ cells/ml, and take 200 ul of the cell suspension into the spare wells coated with Matrigel, each group of 3 complexes. After 12 hours, observed the formation of angiogenic mimicry with a light microscope, and photographed and recorded the number of tubular structures in the same size of 5 medium-magnification
fields. This experiment was repeated 3 times.

9. RNA extraction and semi-quantitative RT-PCR analysis

Extracted the total RNA of the cells using Trizol reagent, and used the housekeeping gene ACTIN as an internal reference. First, by using a cDNA synthesis kit in a 20ul reaction system, synthesized single-stranded cDNA from 1 mg of total RNA. The mRNA expression of $\Delta$ Np63, ABCG2, integrin $\beta$ 1, CK3 and other genes were detected by semi-quantitative PCR. Primers were designed using Primer 5 software. See Table 1 for details.

10. Western blot

After treatment of each group of cells for 3 days, the collected cells were tested for expression of PI3K, AKT, mTOR, Bcl, Bax, etc., and were operated 33 according to the previously described method. The antibodies used were as follows: anti-PTEN (Ab32199, Abcam), anti-P53 (2542 CST), anti-PI3K (ab186612, Abcam), anti-PDK2 (NBP1-878787307, Novusbio), anti-AKT1/2/3 (Ab179463, Abcam), anti-mTOR (2983 CST), anti-Cyclin B1 (ab181593, Abcam), anti-Bcl (Ab32124, Abcam), anti-Cyclin D1 (ab134175, Abcam), anti-Bax (ab77566, Abcam), Anti-P21 (Ab109520, Abcam), Anti-Caspase3 (ab32351, Abcam), Anti-GSK-3$\beta$ (Ab75814, Abcam), anti-Beclin1 (Ab207612, Abcam), Anti-P27 (Ab32034, Abcam).

Statistical analysis

All data in this study were analyzed using SPSS 11.0 software. All data were expressed as mean $\pm$ standard deviation. Group data were statistically analyzed using the ANOVA method, and $p < 0.05$ was considered statistically significant (* $p < 0.001$).
Results:

ES microenvironment inhibits the growth of U118 cells

As observed using an inverted microscope, embryonic stem cells cultured alone grew as round or elliptical colonies with smooth and clear margins. The cells in the clonal colonies were small with large nuclei and little cytoplasm, and with a tight arrangement and unclear boundaries between cells. U118 cells cultured alone had an irregular long fusiform shape and were translucent with clear cell boundaries. According to Figure 1A, in the GE co-culture group, embryonic stem cell clonal colonies were irregular in shape with rough boundaries, which were faintly visible. U118 cells in TMZ group were in fair condition, and there were significantly more dead cells than in the control group. Similarly, U118 cells co-cultured with ES had poor translucency, and the number of dead cells significantly increased.

To investigate whether the role of ES microenvironment on U118 cells was down-regulated by PI3K pathway, we examined PI3K pathway-related genes and found that there was a decrease in the expression of PI3K, AKT, and mTOR in U118 cells while the expression of PI3K inhibitor PTEN increased after co-culture with ESC according to Figure 1B and 1C. When PI3K pathway agonist (PTEN inhibitor) SF1670 was added to GE co-culture group, the expression of cell proliferation-promoting proteins PI3K, AKT, and mTOR increased.

ES microenvironment inhibits the proliferative ability of U118 cells

According to Figure 2A, the results of CCK8 proliferation assays showed the proliferation ability and cell growth curve of U118 cells in each group. In the incubation period, when growth was slow, the growth of the four groups of U118 cells was no significant difference. The growth curve of U118 cells was significantly smaller in the GE co-culture group than in the control group, but there was no significant difference between the GT chemotherapy-positive control group and the co-culture group.

Clone formation assays showed that the clone formation rate of the GE group (10 ± 0.26%) was lower than that of the control group (29 ± 0.2%); the difference was statistically significant ($p < 0.001$). This indicates that ES microenvironment can inhibit the clonogenicity of U118 cells according to Figure 2B.
To observe the role of ES microenvironment on the cell cycle of U118 cells, we used flow cytometer to measure the cell cycle distribution of each group of U118 cells. The results showed a significant reduction in the proportion of U118 cells in GE co-culture group that exited the G1 phase and entered the replication-proliferation phase (S+G2 phase). In particular, the number of cells entering the S phase (14.91 ± 10.84%) was significantly lower than that in the control group (25.50 ± 19.92%, p < 0.05) according to Figure 2C. To understand the effectors that influence cell cycle, we examined a number of factors are related to the cell cycle. RT-PCR results showed the expression of cell cycle proteins cyclin B and cyclin D decrease in U118 cells co-cultured with ES, and a significant increase in the expression of P27, P21 and GSK-3β negative cell cycle regulators. The changes in these genes were consistent with cell cycle measurement results. Western blotting analysis confirmed that a decrease expression of cyclin D and cyclin B cell cycle proteins in U118 cells co-cultured with ES, and the negative cell cycle regulators P27, P21, and GSK-3β significantly increased according to Figure 2D,2E. There was no change cell cycle-related protein expression of U118 cells in the GT chemotherapy group.

**ES microenvironment promotes apoptosis of U118 cells**

ROS promote apoptosis through the P53/Bax pathway, ultimately leading to cell death. Flow cytometer was used to detect the expression level of ROS in each group according to Figure 3A. It could be seen that the level of ROS was significantly higher in GE co-culture group than that in the control group (p < 0.01). The status of apoptosis and necrosis of U118 cells in each group was determined. The apoptosis rates of U118 cells of the GB control group, GE co-culture group, and GT chemotherapy group were 12.64 ± 0.285%, 39.6 ± 5.015%, and 48.1 ± 4.9529%, respectively. The apoptosis rate of U118 co-cultured with ES significantly increased, whereas the apoptosis rate of U118 cells was the highest in GT chemotherapy group, indicating that ES microenvironment can promote the apoptosis of U118 cells according to Figure 3B. ESC microenvironment blocked U118 cell vasculogenic mimicry formation according to Figure 3C.

We measured the changes in genes associated with apoptosis. In GB group the pro-
apoptosis genes Bcl-2, p53, Bax and Caspase3 was significantly higher than that in the control group, but there was no significant difference compared to the chemotherapy group. This indicated that GE co-culture group promote tumor cell apoptosis consistent with the effects of chemotherapy agents. Western blotting confirmed that the expression of pro-apoptosis genes Bcl-2, p53, Bax and Caspase3 in U118 cells of GE co-culture group was significantly higher than the control group according to Figure 3D,3E.

Discussion:

Gliomas are tumors derived from neuroepithelial cells and account for 40-50% of all intracranial tumors. It has a high incidence and recurrence rate, low cure rate and high mortality [16]. TMZ is currently used in the clinical treatment of glioma [17]. Studies found that TMZ can inhibit the proliferation of glioma cells, effectively promoting apoptosis and blocking the cell cycle. This study found that the CCK8 proliferation test of U118 cell clones in the TMZ chemotherapy group showed a significant decrease in cell proliferation, and the proportion of apoptosis confirmed by ROS and apoptosis tests increased. These recurrent GBM tumors are usually resistant to TMZ. Although patients are relatively well tolerated to TMZ, there are still dose-dependent adverse reactions such as severe bone marrow suppression, which limits the TMZ treatment dose. [18]. The embryonic microenvironment can effectively regulate the biological behavior of tumor cells and completely reverse tumor cells into benign cells as well. The ability of the embryonic microenvironment to reverse tumor cells gradually diminishes as the embryo develops and differentiates. Gerschenson et al [19] injected melanoma cells into E10 mouse embryos, E14 mouse embryos, and the dorsal skin of newborn mice. The tumor formation rates were 0 (0/5), 73% (32/44), and 80% (16/20), respectively. Cucina et al. [20] found that extract from late zebrafish blastocysts could inhibit growth and proliferation and promote the apoptosis of human rectal cancer cells, while extract from embryos at the gastrula stage had no such effect. Postovit et al. [21] found that treatment with adult bone marrow stem cell, amniotic fluid stem cell, and trophoblast cell microenvironments did not have tumor-reversing effects. This indicates that only the embryonic microenvironment has tumor-reversing
effects. Previous studies have found that ES cells can recapitulate the microenvironment of embryos [13-15]. We attempted to use ES cells on a mouse leukemia model and we found that ES cells could significantly improve myelography of mice and prolong their survival. In addition, we found that ES could reverse the degree of malignancy of uveal melanoma and cutaneous melanoma in vitro. Therefore, to simulate this early embryonic microenvironment more efficiently, ES cells were selected in our study instead of other types stem cells, for example the bone marrow mesenchymal stem cells, as the effector cells to simulate the embryonic microenvironment. The study showed that tumor growth significantly reduced, apoptotic cells increased, and clonogenicity decreased after co-culture with ES.

Primary GBM has three core signaling pathways: receptor tyrosine kinase (RTK) / phosphoinositide 3-kinase (PI3K) / mitogen activated protein kinase (MAPK), retinoblastoma and tumor protein p53. The RTK/PI3K/MAPK pathway contains mutations in 90% of cases of GBM [22]. MAPK and PI3K promote the growth of many cancers, including migration, proliferation and survival [23,24]. The tumor suppressor PTEN affects cell functions such as cell growth, proliferation, survival, and migration, and is a core negative regulator of the PI3K / Akt signaling pathway. Loss of PTEN also fails to block RTK inhibitors to turn off PI3K signaling, but may reduce the likelihood that a single drug will cause cancer to respond to these treatments. [25,26]. The present study revealed that in ES and U118 GE co-cultured group of glioblastoma cells, PTEN expression significantly increased and PI3K and AKT expression significantly reduced. Embryonic stem cells can promote PTEN expression and inhibit PI3K signaling pathway activation, thereby reversing the degree of malignancy of U118 glioma cells. At the same time, PTEN expression decreased in the GA group with the addition of PI3K agonist, but it increased in the control group. The expression of PI3K, AKT, and mTOR was higher than that in the GE co-culture group, indicating that the embryonic stem cells inhibited PI3K/AKT signaling pathway to reduce the degree of malignancy of U118 cells.

ROS promotes apoptosis through the P53 / Bax pathway [27] and leads to cell death. p53 protein inhibits cell cycle progression and induces apoptosis, and Akt activation
inhibits p53-mediated apoptosis [28]. When Akt is inactivated, cell mitochondrial membrane potential is lost, activating caspase-9 and caspase-3 leading to apoptosis [29]. TMZ can inhibit the normal function of the mitochondrial respiratory chain by interfering with oxidative phosphorylation, leading to ATP deficiency, ROS formation, and oxidative stress. In the present study, both ES co-culture group and TMZ induction group were found to promote the apoptosis of U118 cells. ES microenvironment could promote the increase in ROS in U118 glioma cells, leading to oxidative damage of the cells, and increase in pro-apoptosis proteins P53, Bax and Caspase3, leading to increased apoptosis of U118 cells. In addition, when PI3K agonist was added to the co-culture system, the expression of ROS in tumor cells significantly reduced, indicating that ES microenvironment reduced oxidative damage in U118 cells by downregulating the PI3K pathway.

GSK-3β can inhibit tumor transformation and development, which is considered to be a "tumor suppressor" [30]. Hepatocellular carcinoma cells are resistant to etoposide and camptothecin after treatment with the GSK-3β inhibitor lithium. However, the expression of GSK-3β activated by LY294002 and exogenous Ser9 GSK-3β can increase these drugs to induce hepatocellular carcinoma cells apoptosis [31]. Other reports suggest that GSK-3β activation can promote sensitivity of human breast cancer cells to paclitaxel, 5-fluorouracil, cisplatin, and prodigiosin. [32]. In addition, studies show that inhibition of GSK-3β phosphorylation can significantly reduce tumor aggressiveness [33]. The cell cycle proteins cyclin D1 and cyclin B1 play important roles in cell cycle tumorigenesis and regulation. Cyclin B1 is a G2 phase cyclin, which increases the acceleration of G2/M phase transition and causes abnormal cell proliferation. Cyclin D1 is the most important regulatory factor at the G1/S checkpoint, and acts in G1 phase to promote cell entry into the S phase in conjunction with many associated proteins as well as promotes cell division and proliferation. The present study found that the ES microenvironment could promote the expression of cell cycle-associated proteins that inhibit proliferation by significantly increasing the expression of GSK-3β, P21, and P27, while reducing the expression of cell cycle proteins associated with tumor proliferation such as cyclin D and cyclin B. After the addition of PI3K
agonist, the expression of cell cycle-associated proteins that inhibit the proliferation of GSK-3β, P21 and P27 in tumor cells significantly decreased, while the expression of cell cycle proteins associated with tumor proliferation such as cyclin D and cyclin B significantly increased. This demonstrates that the ES microenvironment can inhibit the cell cycle of U118 cells by downregulating PI3K.

Stem cells have the ability to homing to intracranial lesions, making them more suitable for residual and primary GBM [34]. Using tumor-specific therapeutic proteins and advanced imaging agents has proven that both human and mouse stem cells can home to GBM and play a therapeutic role [35,36]. In a recent study, the use of fluorescent and bioluminescent protein markers could observe the growth and resection of malignant GBM cells in vivo in real time, thus mimicking the clinical status of GBM resection. The main barriers that prevent many drugs from reaching brain tumor cells are the blood-brain barrier (BBB) [37] and tumor vascular dysfunction [38]. One solution to intracranial tumor drug administration is to provide new tumor-specific drugs in situ. This study shows that the embryonic stem cell microenvironment can significantly promote tumor cell apoptosis and inhibit tumor cell proliferation. The ES co-culture group and TMZ chemotherapy group promotes ROS expression, and PI3K/AKT/mTOR promotes decreased expression of tumor proliferation proteins and inhibits the increased expression of tumor proliferating proteins P21/P27/GSK.

Conclusion: The present study found that the ES microenvironment could inhibit the PI3K/AKT pathway to promote apoptosis and inhibit U118 tumor cell proliferation. These findings provide a new approach to the development of ES transplantation in cancer treatment by reversing the microenvironment of cancer survival rather than directly killing tumor cells.

Abbreviations:
ESc: embryonic stem cells; PI3K: phosphoinoside-3-kinase; GB group: U118 glioma cells cultured alone as the control group; GE group: U118 cell and ESc co-cultured; GA group: U118 cell and ESc co-cultured and adding PI3K agonist; GT group: U118 and temozolomide as the positive control group; ROS: Reactive oxygen species; RT-qPCR: reverse transcription polymerase chain reaction; WB: western blot;
GBM: Glioblastoma multiforme; TMZ: temozolomide; OD: optical density; RTK: receptor tyrosine kinase; MAPK: mitogen activated protein kinase; BBB: Blood-brain barrier; PDK2: pyruvate dehydro- genase kinase isoform 2; mTOR: mechanistic target of rapamycin; AKT: protein kinase B; DiO: 3,3'-dioctadecyloxacarbocyanine perchlorate; DCFH-DA: 2,7-Dichlorodihydrofluorescein diacetate; Bcl : B-cell lymphoma; Caspase3, GSK-3β; PTEN: phosphate and tension homology.

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The manuscript does not report on or involve the use of any animal or human data or tissue, and not contain data from any individual person.

Competing Interests
The authors have declared that no competing interest exists.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends:

**Figure 1.** ES environment inhibits the proliferative ability of U118 cells by downregulating PI3K pathway; Figure 1A, Condition of each group of cells observed by inverted microscope, ES: Embryonic stem cells; GB: U118 cells; GE: U118 Co-culture; ES: U118 Co-culture ES plus PTEN inhibitor; GT: U118 plus temozolomide Figure 1B and 1C. The expression of cell proliferation-promoting proteins PI3K, AKT, and mTOR in different group.

**Figure 2.** ES microenvironment inhibits the proliferative ability of U118 cells

ES: Embryonic stem cells; GB: U118 cells; GE: U118 Co-culture; ES: U118 Co-culture ES plus PTEN inhibitor; GT: U118 plus temozolomide; Figure 2A, ES microenvironment can inhibit the clonogenicity of U118 cells according to Figure 2B.
the proliferation ability and cell growth curve of U118 cells in each group according to Figure 2C. these cycle-related protein expression of U118 cells in the different group Figure 2D, 2E;

**Figure 3:** ES microenvironment promotes apoptosis of U118 cells

Flow cytometer was used to detect the expression level of ROS in each group according to Figure 3A. the apoptosis rate of U118 cells in each group according to Figure 3B. ESC microenvironment blocked U118 cell vasculogenic mimicry formation according to Figure 3C. the pro-apoptosis genes Bcl-2, p53, Bax and Caspase3 in each group according to Figure 3D, 3E.