Glioblastoma is the most common and aggressive primary brain tumor. Despite standard therapy, the average survival time is only 15 months, and the best 5-year survival rate reported is only 9.8%. Although glioblastoma shows characteristics of increased angiogenesis, anti-angiogenic therapy has not consistently resulted in a survival advantage.

Recent studies indicate that glioblastoma stem cells (GSCs) express stem cell markers (cluster of differentiation [CD]133 and sex determining region Y box 2 [Sox2]) and have the capacity to commit to endothelial cells. These glioblastoma-derived endothelial cells (GDEC) are usually abnormal cells, generated through a process termed...
Importance of the study

A new microcirculation pattern, glioma cell-derived vessels, has been recognized by our study. We have identified GDECs in glioblastoma samples. Through 3D culture, we obtained evidence of the differentiation process of GSCs into endothelial cells by live-cell imaging. Our findings therefore suggest that GSCs might serve as a potential target for anti-angiogenic therapy instead of endothelial cells.

“Vasculogenic mimicry” (VM). VM occurs due to the ability of aggressive tumor cells to form vessel-like networks that complement the endothelial cell-dependent vessels. We previously reported the presence of VM in gliomas and demonstrated the correlation between VM channels and the aggressiveness of the tumors. A recent study reported that anti-angiogenic therapy may accelerate VM by increasing the population of breast cancer stem cells. Live-cell imaging allows the study of such complex processes orchestrated by GSCs in great detail, thereby providing an opportunity to fully understand the environmental conditions and molecular makers that promote angiogenesis via VM.

Three-dimensional culture technologies have been used to mimic the in vivo microenvironment in vitro, especially for complex processes such as stem cell differentiation. One method of documenting the stem cell differentiation in a 3D culture environment is the Live-Cell Imaging System (LCIS), which allows real-time analysis of the cellular redistribution of a protein in response to environmental stimulation. We have previously demonstrated that tumor-derived vessels provide a complementary framework to ensure blood supply. In an attempt to understand the role of GSCs in angiogenesis observed in glioblastomas, we characterized the differentiation of GSCs and the tumor cell–derived channel formation using the LCIS and a fluorescent xenograft mouse model.

Methods

Ethics Statement

The study was approved by the research ethics committee of Sun Yat-sen University Cancer Center. All patients provided written informed consent. All animal studies were performed in accordance with institutional ethical guidelines for experimental animal care.

Isolation and Characterization of GSC-1 Cells

Resected glioblastoma tumor tissues were collected from Sun Yat-sen University Cancer Center after receiving informed consent from patients. The tumors were graded pathologically according to the World Health Organization (WHO) criteria. The tumors were processed immediately after surgery as previously described. Briefly, the tumors were cut into small pieces, washed 3 times in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Gibco), digested with trypsin (Gibco), triturated mechanically with surgical scissors, and filtered through a cell strainer (Becton-Dickinson [BD]). The red blood cells were removed with a red cell lysis buffer (Tiangen). The tumor cells were then washed several times with DMEM/F12 and cultured in DMEM with 2% B27 supplement (Gibco), 20 ng/mL epidermal growth factor, and 10 ng/mL basic fibroblast growth factor (In vitro). The cells were dissociated with StemProAccutase (Gibco) and cultured in flasks (BD). These cells were named GSC-1 cells and were used in subsequent experiments.

Generation of RFP–GSC-1 Cells Stably Expressing Red Fluorescence Protein

To establish the red fluorescent protein (RFP)–GSC-1 stable cell line, the GSC-1 cells were transduced with lentivirus (GeneChem) supernatants for 8 to 12 hours in the presence of 5 μg/mL Polybrene. The transduced cells were selected using 5 μg/mL puromycin (Sigma-Aldrich) for 2 days and sorted using a MoFlo cell sorter (Beckman Coulter). The RFP expression in all clones was analyzed by immunostaining and fluorescence microscopy (Olympus). Clones that expressed high levels of RFP were passaged and maintained in growth medium containing 5 μg/mL puromycin. These RFP–GSC-1 cells were used for in vivo and in vitro studies.

Xenografts

Four-week-old female BALB/c nude mice were obtained from the animal experiment center of the Guangdong Academy of Medical Sciences and maintained under specific pathogen-free conditions at 23°–25°C and 40%–50% humidity. The RFP–GSC-1 cells were harvested from cultures, washed with phosphate buffered saline, and resuspended in serum-free DMEM at a density of 1 × 10⁶ cells/μL. Then, 100 μL of the RFP–GSC-1 cell suspension was subcutaneously injected into the right flank of 10 mice. After transplantation, the weight of the animal and the growth of the subcutaneous tumors were assessed daily. Four weeks after the transplantation, the mice were euthanized. Following this, the tumors were resected, formalin fixed, and paraffin embedded for further use.

Immunofluorescence Staining

To analyze the endothelial differentiation in clinical samples, glioblastoma tissues were collected from 64 patients who underwent surgery at Sun Yat-sen University Cancer Center between 2000 and 2012. The tumors were graded and histological assessment was made by 2 neuropathologists.
who verified the diagnosis according to the WHO 2007 classification standards for CNS tumors. The formalin-fixed, paraffin-embedded sections of samples from mice and glioblastoma patients were deparaffinized and rehydrated and then incubated in 3% hydrogen peroxide for 20 minutes to quench the endogenous peroxidase activity. The sections were then blocked with 5% bovine serum albumin (diluted with phosphate buffered saline) for 1 hour. The sample sections from animals were incubated with anti-CD34 antibody or anti-CD31 antibody (rabbit immunoglobulin G [IgG], 1:1000; Abcam). The patient sample sections were incubated with anti-CD34 (rabbit IgG, 1:1000; Abcam) and anti-GFP (mouse IgG, 1:1000; Abcam) antibodies, or anti-CD31 antibody (rabbit IgG, 1:1000; Abcam) and anti-GFP (mouse IgG, 1:1000; Abcam) antibodies. The sections were first incubated with the primary antibodies overnight at 4°C and then with the secondary antibodies, Alexa 488–conjugated goat anti-rabbit IgG and Alexa 594–conjugated goat anti-mouse IgG (1:1000; Abcam), at room temperature for 1 hour. All images were acquired using an Olympus confocal laser scanning microscope.

We have compared the utility of CD31 and CD34 in determining endothelial cells in glioblastoma samples and found that the results came out similar. Our previous study also found that CD34 results in a more distinct labeling and easier determination of endothelium compared with other markers. Thus we choose CD34 as the endothelium marker in this study.

The RFP–GSC-1 cells were placed on adhesion microscope slides (Citoglas), fixed with 4% paraformaldehyde, and incubated overnight at 4°C with primary antibodies against CD133 (rabbit IgG, 1:1000; Abcam) and Sox2 (rabbit IgG, 1:1000; Abcam). Following this, the samples were incubated with Alexa Fluor 488–conjugated secondary antibodies (1:1000; Abcam) for 1 hour at room temperature, followed by incubating with 4′,6-diamidino-2-phenylindole (1:1000; Abcam) for nuclear staining.

The immunofluorescence staining of RFP–GSC-1 cells was performed before and after the 3D culture for 6 hours. For this purpose, the RFP–GSC-1 cells at 0 and 6 hours of 3D culture were fixed with 4% paraformaldehyde, incubated with a primary antibody against CD34 (rabbit IgG, 1:1000; Abcam), and then incubated with Alexa Fluor 488–conjugated secondary antibody (1:1000; Abcam).

Live-Cell Imaging

The preparation of cells and imaging have been described elsewhere. Briefly, the GSC-1 cells were plated onto a glass-bottom dish (Nest) after incubating the basement membrane (Trevigen) in the dish for 30 minutes at 37°C for solidification. The DMEM/F12 in the dish was replaced with DMEM supplemented with 10% fetal bovine serum (Gibco). The cells were labeled with fluorescein isothiocyanate mouse anti-human CD34 (1:1000; BD) and were stimulated with 2 ng/mL vascular endothelial growth factor (VEGF; Invitrogen). The dish was then placed on a confocal scanner box (CV1000; Yokogawa) featuring a culture system maintained at 37°C and 5% CO2. Wide-field fluorescence images were acquired using a 10× objective lens. Five different focal planes were imaged at 2-µm intervals. Time-lapsed image sequences were acquired for up to 6 hours with 2- to 3-minute time intervals. The GSC-1 cells were visualized by white light and RFP. The CD34 was labeled using fluorescein isothiocyanate mouse anti-human CD34 antibody.

The Proportion of GDEC Vessel Assessment

The tubelike structures (considered as blood vessels) containing cells doubly positive for CD34-GFAP or CD31-GFAP were defined as GDEC vessels. First we examined GDEC-positive samples in all 64 glioma samples. Then the blood vessels (CD34+ or CD31+ vessels, and CD34-GFAP or CD31-GFAP double-positive GDEC vessels) were counted in one observation area of each GDEC+ sample. The ratio of GDEC vessels (average number) to blood vessels (average number) was calculated.

Results

Evaluation of CD34-GFAP or CD31-GFAP Protein Coexpression in Glioblastoma Cells

To examine whether CD34-GFAP or CD31-GFAP were coexpressed in glioblastoma, 64 human glioblastoma samples were analyzed using dual immunofluorescence staining. These glioblastoma cells were scanned at low magnification and confirmed at high magnification. The results revealed that CD34-GFAP or CD31-GFAP were coexpressed in 30 (out of 64) glioblastoma samples (46.9%). Notably, only GDEC expressed CD34-GFAP or CD31-GFAP (Fig. 1). The GDEC were 5.03 ± 10.6 per high-power field in CD34-GFAP coexpression samples and 5.09 ± 2.16 per high-power field in CD31-GFAP coexpression samples.

Morphological Characteristics and Quantification of GDEC Vessels

Histologically, GDEC appears within the GFAP+ networks and can be recognized by the expression of an endothelial cell marker. To verify GDEC vessel structures in clinical samples, the tubelike structures (considered as blood vessels) containing cells doubly positive for CD34-GFAP or CD31-GFAP were defined as GDEC vessels (Fig. 2B). We examined 64 clinical samples and found glioblastoma cells expressing CD34-GFAP or CD31-GFAP in 30 samples (46.88%). The CD34-GFAP coexpressing cell number was 5.03 ± 10.6 per high-power field in those 30 samples with CD34-GFAP coexpressing cells. The CD31-GFAP coexpressing cell number was 5.09 ± 2.16 per high-power field in the 30 samples. Among 30 GDEC+ samples (with CD34-GFAP or CD31-GFAP coexpressing cells), GDEC vessel structures were detected in 21 (70%). In those samples (with GDEC vessels), the CD34+ GDEC vessels were counted at ~14.16% of total vessels and CD31+ GDEC vessels were counted at ~18.08% of total vessels (Fig. 2).

Characterization of RFP–GSC-1 Cells

The GSC-1 cells isolated from human glioblastoma tissues were round in shape, with round and elliptical
nuclei. After transducing with lentivirus, the cells were sorted by fluorescence-activated cell sorting (FACS). The result of FACS analysis revealed that 98.6% of the transfected cells were RFP+ (Fig. 3A). The results of immunofluorescence staining showed that a subpopulation of cells expressed both Sox2 and CD133 (Fig. 3B and D). These cells displayed differentiation potential characteristics of stem cells. Six hours after stimulation with VEGF, the cells spread well. Most of these cells had a spindle-shaped or polygonal morphology and grew into a confluent monolayer. The cells hardly expressed Sox2 and CD133 (Fig. 3C and E).

**RFP–GSC-1 Cells Have the Capacity to Form New Blood Vessels**

To evaluate the angiogenic potential of the RFP–GSC-1 cells, we established a xenograft model. For this purpose, 10 mice were transplanted with RFP–GSC-1 cells. Endothelial cells without RFP expression could be observed (Fig. 4A). Immunofluorescence analysis with endothelial cell–specific antibodies revealed that the transplanted cells expressing RFP were immunoreactive for CD34/CD31 (Fig. 4B), confirming the presence of human cells expressing the endothelial cell marker in many areas. The vessels derived from
RFP–GSC-1 were characterized by the tubular networks of cells of endothelial phenotype (Fig. 4B). Of the 10 mice xenografts, 7 contained GDEC, and 4 out of 10 mice showed vessels formed from RFP–GSC-1 cells.

**Live-Cell Imaging of Vascular Structure Formation in 3D Gel Cultures of RFP–GSC-1 Cells**

The RFP–GSC-1 cells were able to organize into capillary-like tubes in 3D collagen gel cultures. The tubes formed after VEGF stimulation as a network. The capillary-like structure was composed of 2 or 3 cells, with adjacent cells overlapping extensively. The lumen of the tube was irregular. Upon treatment with VEGF, the RFP–GSC-1 cells formed capillary-like structures inside the basement membrane (Fig. 5).

The effect of VEGF on the angiogenic potential of RFP–GSC-1 cells was also analyzed by monitoring the expression of the endothelial cell marker CD34 (Fig. 5A), a single-pass transmembrane sialomucin protein family member that is...
Fig. 3  Confirmation of successful RFP transduction by FACS. The transduced cells were collected, and the purity of RFP–GSC-1 cells was 98.6% (A). Sox2 and CD133 protein expression in RFP–GSC-1 before (B, D) and after (C, E) treatment with VEGF for 6 hours. Compared with their expression at 0 hour, the CD133 and Sox2 expression was undetectable at 6 hours. Scale bar, 50 μm.
expressed in early hematopoietic and vascular-associated tissues. Cells incubated for 6 hours in 3D gels with VEGF expressed CD34 (Fig. 5A and B). The cell number was counted in 5 random areas of high-power fields of each sample, which was shown as mean value. The cells were CD34-negative at 0 hour and expressed CD34 (9.46%) after 6 hours of differentiation (Supplementary videos S1 and S2). This was consistent with the results of immunofluorescence staining performed to analyze CD34 expression in these cells (Fig. 5B). VEGF significantly enhanced the angiogenic potential of RFP–GSC-1 cells (Fig. 5C) and promoted their differentiation into endothelial cells.

**Discussion**

Angiogenesis, formation of vessels from the preexisting ones, has been considered the main mechanism of glioblastoma vascularization. However, our previous study revealed that a new mechanism of vascularization—vasculogenic mimicry—was also found in glioblastomas. Our finding is consistent with studies indicating that GSCs are able to form the VM vessels. Furthermore, VEGF enhanced the GDEC phenotype in GSCs and promoted the tubelike structure formation in 3D gel cultures. Our results reveal that RFP–GSC-1 cells were able to differentiate into VM-related GDEC and form GDEC vessels and that the key trigger in the context of our experiments was the real-time observation of the LCIS and fluorescent xenograft model. These results suggested that VM is potentiated by the microenvironment as observed in the tube formation assay. This finding is consistent with the report of cancer stem cells promoting VM in triple-negative breast cancer. Extending these findings, we identified that transdifferentiation of RFP–GSC-1 cells into endothelial cells is an alternative angiogenic mechanism responsible for tumor cell–derived VM in glioblastomas.

We observed CD34+ glioblastoma cells and GDEC vessels in RFP–GSC-1 cell xenografts in vivo and CD34-GFAP co-localization in glioblastoma tissue. This evidence contradicts the traditional concept that the formation of GDEC and VM are simply accidental events that occur during aggressive tumor growth and supports the opinion that these are specific biological processes that may contribute to neovascularization within glioblastoma tissue. First, RFP–GSC-1 cells transplanted into mice gave rise to a cell population with an endothelial phenotype that was rich in GDEC, which was a component of vessels. Second, immunofluorescence analysis of the glioblastoma samples revealed that a small cross section of the microvasculature that expressed elevated levels of CD34 also expressed GFAP. Third, transdifferentiation of a small fraction of the
RFP–GSC-1 cells was identified using the LCIS, which dynamically tracked cell surface marker expression in each cell. Thus, our results indicate that RFP–GSC-1 cells may differentiate into GDEC to promote VM in glioblastomas. This transdifferentiation indicates the plasticity of RFP–GSC-1 cells which shows characteristics of both tumor cells and endothelial cells. Furthermore, this plasticity of the RFP–GSC-1 cells is illustrated by the multipotency of GSCs, which allows them to differentiate into endothelial cells, pericytes, and astrocytes. A model illustrating the differentiation of RFP–GSC-1 cells is shown in Fig. 6.

A recent study demonstrated that cancer stem cells limit the effectiveness of anti-angiogenic therapy. Bevacizumab prolongs progression-free survival but not overall survival in newly diagnosed glioblastoma cases. Because of their ability to survive under anti-angiogenic treatment pressure and to self-renew once activated from dormant glioblastoma tissue, GSCs may contribute to alternative neovascularization, forming tumor cell–associated networks and supporting the tumor blood supply. Indirect evidence supports this hypothesis. Next, we examined the ability of RFP–GSC-1 cells, the majority of them expressing stem cell markers (CD133 and Sox2), to form independent vasculature. Our results confirmed that these cells directly contributed to VM by differentiating into GDEC.

Our previous study showed that VM existed in glioma and according to Maniotis et al, VM consisted of non–endothelial cell–lined microvascular channels. There are two types of VM vessels: one is a periodic acid–Schiff (PAS)–positive CD34-negative vessel and the other is a tumor cell lining vessel. In this investigation, we focused on the tumor cell lining type and attempted to reveal its development. The conventional concept was that there were no endothelial cells lining VM channels. However, the observation of GDEC vessels revealed a novel type of VM channel lined by endothelial cells which actually were developed from glioblastoma cells. Glioblastoma cells formed tumor cell lining vessels and differentiated into endothelial cells which formed “endothelial cell” vessels. Our data indicated that the GDEC vessel exhibited a new tumor angiogenesis role except host sprouting angiogenesis. The tumor cell lining vessels could alternate into GDEC vessels during tumor cell endothelial differentiation. We have not explored the PAS-positive vessel in the current study, although it is also defined as a VM vessel. The present results demonstrated that VM occurs through the transdifferentiation of GSCs into endothelial cells, while the clinical evidence of endothelial phenotype in glioblastoma cells is hardly identified. We examined 64 clinical glioblastoma samples and found that the differentiated glioblastoma cells are important for VM
and tumor development. This is consistent with the results obtained from the VEGF-stimulated in vitro 3D gel cultures of RFP–GSC-1 cells. The current study refers to the controversy that VEGF appears to be crucial for VM while also having the potential risk to enhance the development of cancer stem cells. However, a clinical trial reported that bevacizumab showed uncertain benefits when used for treating glioblastoma patients. Treatments targeting the GDEC may improve the efficiency of anti-angiogenic therapy and increase the survival rate among patients.

Supplementary Material
Supplementary data are available at Neuro-Oncology online.

References