**Title: The diversity of tumor-associated cells in glioblastoma and heterogeneous features in recurrent glioblastoma**

# Introduction

## Glioblastoma

Glioblastoma multiforme (GBM) is the most common and malignant tumor presenting in the central nervous system (CNS), and prognosis for recovery remains very poor[1–3]. Despite the development of innovative diagnostics and new therapies, patients diagnosed with GBM have a median survival of only 15 months [4]. Standard GBM treatment includes maximal surgical dissection, followed by radiotherapy and chemotherapy. However, GBM often develops treatment resistance due to tumor heterogeneity[5–7]. Moreover, complete surgical resection is difficult to achieve due to tumor location and its highly invasive nature[8]. Residual tumor cells can lead to malignant progression and recurrence[9].

## Classification of glioblastoma

According to the World Health Organization (WHO) classification in 2016 [10], glioblastoma is divided into two subgroups: primary and secondary GBM. Primary GBM accounts for nearly 90% of total GBM cases and develops rapidly, and is not associated with a mutation in the isocitrate dehydrogenase 1 (IDH 1) gene [11]. The remaining 10% of cases comprise secondary GBM, which progresses from a low-grade diffuse astrocytoma or anaplastic astrocytoma and carries an IDH 1 mutation[12]. Patients afflicted with secondary GBM are usually younger and have a better prognosis[13, 14].

Analysis of the GBM expression profile has been achieved using high-throughput sequencing technologies that have been developed in the last decades [15–17]. Based on genetic differences identified in the Cancer Genome Atlas (TCGA), GBM can be classified into four subgroups: classical, mesenchymal, proneural and neural [18, 19]. Classical glioblastoma is characterized by increased expression of the epidermal growth factor receptor (EGFR) and loss of phosphatase and tensin homolog (PTEN)and cyclin-dependent kinase Inhibitor 2A(CDKN2A)[21], and it is highly responsive to radiotherapy and chemotherapy[20]. The mesenchymal subtype is characterized by mutations in Neurofibromatosis type 1 (NF1), tumor protein 53 (TP53), and PTEN, and is associated with worse outcomes[22, 23]. Signature genetic alterations in proneural glioblastoma tumors include overexpression of platelet-derived growth factor receptor-α (PDGFRA) and mutations in IDH1 [24]. The neural subtype has not been fully characterized, possibly due to contamination with normal cells[25].

## The diverse tumor-parenchymal cells in glioblastoma environment

Increased attention has focused on the interactions between GBM tumor cells and their surrounding cells, including normal brain cells and immigrating cells[26, 27]. GBM recruits several cell types into its tumor environment to promote progression and growth, which may also modify tumor responses to treatment[28–30].

Tumor-associated myeloid cells (TAMs), including microglia and peripheral blood-derived macrophages, accumulate during tumor progression[31, 32]. In a healthy brain, microglia are the primary innate immune cells and regulate brain development and behavioral functions[33]. Bone-marrow-derived macrophages can infiltrate the tumor area due to disruptions in the blood-brain-barrier (BBB). TAMs in glioblastoma are high and can constitute up to 30% of tumor mass[34]. Several factors released by tumor cells, such as colony-stimulating factor 1 (CSF 1) and monocyte chemoattractant protein-1 (MCP-1), attract TAMs, leading to their accumulation, and can convert them into a pro-tumorigenic phenotype[35–37]. Activated TAMs promote metalloprotease (MMP) activity and suppress tissue inhibition of metalloproteinase (TIMP)-2 expression, leading to extracellular matrix degradation and allowing tumor invasion[38]. TAMs can also affect glioma stem cells (GSCs), a small cell population with self-renewal and multi-lineage differentiation properties. Tumor growth factor β (TGF-β), released from TAMs, increases GSCs invasiveness[38].

Glioblastoma is additionally characterized by extensive angiogenesis[39]. A GBM hallmark is a dense network of tortuous and leaky vessels with dilated lumens and abnormally thickened basement membranes[40], including endothelial cells and pericytes. Glioma and immune cells release various factors that promote angiogenesis, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), integrins, and angiopoietins[38, 41–44]. Recent studies show that GSCs present another source of vascular constituents and can differentiate into endothelial cells and pericytes, thus contributing to vessel formation[45–47]. VEGF is highly expressed in gliomas and correlates with tumor malignancy[48, 49]. Endothelial cells express the vascular endothelial growth factor receptor 2 (VEGFR2), which, together with VEGF expression, establish a paracrine signaling loop resulting in endothelial cell proliferation and migration [50]. Ultimately, the increased glioma VEGF signaling pathway results in decreased vessel and vascular integrity due to disruption of the BBB. The BBB is composed of endothelial cells, pericytes, and astrocytes, together forming a neurovascular unit that maintains brain homeostasis by regulating ion and molecular transfer between the brain and blood[51]. The abnormal and disrupted BBB in glioma causes non-uniform permeability and active efflux of various molecules into the tumor tissue, inducing cerebral edema[51]. These changes also attract immune cells, such as macrophages, which promote angiogenesis and inhibit the immune system, increasing blood vessel infiltration into poorly perfused areas. Multiple strategies are currently being developed to improve drug delivery across the BBB to the tumor area [52–54].

Recently, more research has focused on the glioblastoma-neuron interaction[55]. Accumulating studies suggest that glioma arises from either neural precursor cells (NPCs) or oligodendrocyte precursor cells (OPC), whose proliferation is promoted by neural activity [56–59]. Therefore, neurotransmitter release, including brain-derived neurotrophic factor (BDNF), soluble neuroligin-3 (NLGN3), glutamate, and dopamine, can potentially promote glioma proliferation and growth[60]. In turn, gliomas induce increased neuronal activity by promoting synaptogenesis and glutamate release[61, 62].

Furthermore, many other examples of interactions between glioblastoma cells and their surrounding cells, such as T cells and astrocytes, within the tumor microenvironment have been reported[63, 64]. These new insights provide the basis for potential novel therapies for gliomas[65].

## The heterogeneity in glioblastoma

Heterogeneity, which is responsible for tumor progression, treatment resistance, metastasis, and recurrence, is one of the fundamental characteristics in tumors [6, 7]. Genetic alterations, causing tumor transformation, are the primary mechanism leading to inter-tumor heterogeneity[17, 66]. Although glioblastomas are classified into several subtypes according to their genetic alterations, recent studies show spatial variation in GBM transcriptional profiles within the same tumor[5], allowing tumor subdivision into subgroups[67, 68]. Intra-tumor heterogeneity is likely caused by spatial differences in growth factors, oxygen pressure, blood vessel density, and extracellular matrix composition within the tumor microenvironment[69, 70]. Since only a single piece of the tumor is usually used for clinical diagnosis and planning the drug therapy protocol, doctors may receive an incomplete and possibly misleading picture of the tumor resulting from regional heterogeneity[71, 72]. Tumor areas that are not classified according to their gene expression profiles may affect the tumor response to therapy by modifying the microenvironment, and affecting tumor invasiveness, proliferation rate, and angiogenesis[73]. Thus, understanding tumor heterogeneity is essential for developing personalized glioblastoma treatments[74–76].

## Objectives of the study

1. Characterize a new myeloid-like cell population traced in a transgenic mouse model;
2. Assess TAMEP presence and its heterogeneity in human brain tumor tissue;
3. Investigate the heterogeneous features in recurrent glioblastoma.

# Materials

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# Methods

## Cell culture

Murine GBM cell lines GL261 and GL261-HSVTK-GFP were cultured in DMEM containing 10% fetal bovine serum, 1X MEM non-essential amino acids, and 1% penicillin-streptomycin. All cells were maintained at 37℃ in a humidified atmosphere of 95% O2 and 5% CO2.

## Animal experiments

### Animals

All animal experiments were performed in compliance with the German National Guidelines for Animal Protection and conducted with the approval of the local animal care committee of the Government of Oberbayern. Animals were kept in standard cages with access to water and food *ad libitum* in a 12h light/dark cycle at the Walter Brendel Center for Experimental Medicine, LMU Munich. Mice were sacrificed with symptoms or at defined points.

### Tumor inoculation

Mice were anesthetized IP using a weight of 7uL/g of a mixture of 1.02 mL 10% ketamine, 0.36 mL 2% Rompun and 4.86 ml 0.9% NaCl. A middle incision was made on the skin with a scalpel after disinfection with a 10% povidone iodine solution. To prevent the animals’ corneas drying out, their eyes were covered with Bepanthen cream. Mice were immobilized on a stereotactic frame in a flat-skull position. After drilling a hole into the skull with a 23G needle tip (coordinates 1.0 m anterior and 1.5mm right of the bregema), 1μl of cells (1×105 murine GBM cells/μl or 5×104 human GBM cells/μl in a supplement-free medium) was slowly injected within two minutes with a 22G Hamilton syringe at a depth of 3mm (the syringe was vertically inserted 4mm and retracted 1mm). Afterward, the syringe was retracted 1mm/min, and the skin was carefully sutured.

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### Mice tail vein injection

Mice were anesthetized as previously described and placed in the restraining device. The tail was wiped with an alcohol-dampened gauze to disinfect the tail skin and increase vein visibility. The tail was immobilized using the non-dominant hand, and the needle was aligned parallel to the tail with the beveled edge of the needle. The needle was inserted into the tail vein starting from the distal end. When the injection was successful, blood flashed into the syringe and the injected materials flowed smoothly without resistance. If injection was not successful, a new position toward the base of the tail was chosen. The needle was removed after completing the administration, and the injection point was gently pressed with gauze (30–60 seconds) until bleeding stopped.

### Tamoxifen-inducible Cre-LoxP system

The Cre-LoxP system is a widely used technology for tracing cells or gene modifications *in vivo*[77, 78]. The system consists of a single enzyme, Cre recombinase, which recombines a pair of short target sequences called the Lox sequences. The gene Cre can be modified and fused with a mutant estrogen receptor (ERT2), which acts as a tamoxifen specific receptor and does not bind natural estrogens or other physical steroids[79]. In the absence of tamoxifen or hydro-tamoxifen, Cre-ERT2 protein is sequestered to the cytoplasm by heat shock protein 90[80], preventing nuclear recombination events. In transgenic mice that expressed Cre-LoxP in a defined cell population, tamoxifen injection enabled tracing of these cells or their progeny at desired times.

## Single cell preparation

The tumor tissue was microdissected under a Leica M205 FA stereomicroscope. After washing with sterile 1X PBS, the tumor tissue was homogenated on ice using a mortar and pestle. Collagenase A (1mg/ml) and Dnase I (0.1mg/ml) were added and the sample was incubated for 10 minutes at 37℃. Following incubation, the tumor homogenate was centrifuged and the supernatant was discarded. Sediment was resuspended in sterile 1X PBS and rat anti-mouse CD31 microbeads were added. The solution was incubated for 30 minutes at 4℃. The tubes were placed in a magnetic particle separator for two minutes and the supernatant was collected, centrifuged, and suspended in a FACS buffer.

## Histology

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### Mice perfusion and brain tissue preparation

Mice were anesthetized with Nacoren® and intracardially perfused with 10ml 1X PBS, followed by a 15ml 4% paraformaldehyde (PFA) solution. The brain was carefully removed and incubated with 4% PFA at 4℃ for 24 hours and then immersed in 30% sucrose until the brain sank to the bottom of the tube. The brain was then embedded in Cryomatrix and frozen in 2,2,4-Trimethylpentane with liquid nitrogen. Sequential and horizontal 40μm-thick sections were prepared using a sliding microtome. Sections were stored in 24-well plates filled with cryoprotectant (ethylene glycol, glycerol, and 1X PBS with a ratio 1:1；two at pH 7.4) at -20℃ and protected from light.

### H&E staining

H&E staining combined two histological stains (hematoxylin and eosin). Nuclei was stained blue by hematoxylin, and the cell cytoplasm was stained pink by eosin

providing a clear view of the tissue structures. Staining was performed as follows: sections were: mounted on glass slides and air-dried for 15 minutes; dehydrated in 100% ethanol for 30 seconds; stained in a hematoxylin solution for two minutes; rinsed in running water for five minutes; stained in 0.5% eosin for one minute and rinsed briefly in distilled water; dehydrated using a graded series of ethanol (70%, 96%, 100%) for one minute each time; cleared twice with xylene; and covered with an Entellan® mounting medium.

### Tumor volume quantification

Tumor volume was quantified according to the Cavalieri principle. Every 12th section was inspected under a microscope, and the tumor region was measured using the Axiovision Rel. 4.9 software. Stereotactical coordinates of mice brain sections were used to calculate the tumor Z-axis. Volume was calculated by multiplying the Z-axis with the average tumor area.

### Tumor invasiveness quantification

GBM cell invasion scores were calculated as previously described (PMID: **32545380**). Every eighth tumor section was assigned an invasive score from 0 to 3 according to the following parameters: a score of 0 means no histological cell invasion from the tumor mass; a score of 1 represents a more extensive, connected group of invading GBM cells; a score of 2 describes smaller scattered groups of invading GBM cells; and a score of 3 indicates single, scattered, highly invasive GBM cells.

## Immunofluorescence staining and quantification

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### Immunofluorescence staining of mouse brain sections

Floating sections were washed three times for five minutes in PBT (0.1% Tween-20 in 1X PBS) and then incubated in a blocking buffer (5% normal donkey serum and 0.3% Triton X-100 in 1X PBS) for one hour at room temperature. Samples were incubated with primary antibodies (Table 2.5) overnight at 4℃, washed with PBT three times for five minutes, and incubated with secondary antibodies (Table 2.6) for two hours at room temperature. All antibodies were diluted in a blocking buffer. Nuclei were stained with DAPI (1:10,000) for two minutes after washing three times in PBT. Finally, sections were mounted in a fluorescent mounting medium after washing.

### Immunofluorescence staining for paraffin-embedded sections

Tissue sections were deparaffinized in ROTI ® Histol for 20 minutes at room temperature. Slides were taken out and fixed in 70% acetone for 10 minutes at -20°C. After washing with PBT for five minutes three times, antigen retrieval was performed by immersing the sections in a citrate buffer and microwaving for 20 minutes. After slides cooled down, they were washed with PBT three times for five minutes, followed by blocking for 30 minutes (5% donkey serum and 0.3% Triton-X in 1× PBS). Sections were then incubated with goat anti-Sox2 (1:200) and rabbit anti-PU.1 (1:100) antibodies overnight at 4℃. The next day, sections were washed three times in PBT for five minutes and incubated with the secondary antibodies donkey anti-rabbit AF594 (1:200) and donkey anti-goat AF488 (1:200) for two hours at room temperature. All antibodies were diluted in a blocking buffer. Nuclei were stained with DAPI (1:10,000) for two minutes after washing three times in PBT. Finally, sections were mounted in Fluorescent Mounting Medium after washing.

### Quantification of total vessel length

Mouse sections stained with CD31 were photographed to quantify vessel length and density within the tumor area. For each mouse, three or four sections with good quality containing a tumor were prepared, and nine 40X magnification images per section were taken using a TCS SP8 microscope. Vessel length density was analyzed using AngioTool 0.6 software.

## Statistical analysis

All statistical analyses in this thesis were performed using GraphPad Prism 7 software. An unpaired Student’s t-test was used when two independent groups were compared. The Log-rank (Mantel-Cox) test was used to determine statistical significance in survival experiments. The criteria for statistically significant differences was *p* < 0.05. P-values as shown in figures are: \*, *p*＜0.05; \*\*, *p*＜0.01; \*\*\*, *p*＜0.001; \*\*\*\*, *p*＜0.0001; and NS, not significant.

# Results

## Tracing a new myeloid-like cell population in glioblastoma

### Using a nestin-RFP mouse model to trace two RFP+ cell types in glioblastoma

The mouse model Nestin::CreER2:R26-RFP (abbreviated as Nes-RFP mice, Fig 4.1.1-A) allows tracing of nestin-expressing cells (nestin+) and their progeny in a glioblastoma microenvironment. Traced RFP+ cells were classified into two subgroups according to their position relative to tumor vessels (Fig 4.1.1-B). The first RFP+ subgroup (Fig 4.1.1-B, arrow), defined as vascular RFP+ cells, were close to the vessels and wrapped around the endothelial cells (Fig 4.1.1-B, CD31+ cells). Immunofluorescence staining for platelet-derived growth factor receptor B (PDGFRβ) identified vascular RFP+ cells as pericytes. The second RFP+ cell subgroup (Fig 4.1.1-B, arrowhead), defined as avascular RFP+ cells, did not show close contact with vessels and did not express PDGFRβ.

**Figure 4.1.1 Traced vascular and avascular RFP+ cells in a Nes-RFP GBM mouse model. (A)** Nes-RFP mice were inoculated with the murine GBM cell line GL261 at day 0. TAM was injected intraperitoneally at a dose of 75mg/kg at days 1, 2, and 3. Mice brains were harvested seven days post-operatively (DPO). **(B)** Immunofluorescence staining for PDGFβ (pericyte marker) and CD31 (endothelial cell marker) in 7DPO GBM tissue. Vascular RFP+ cells were located close to the vessel and PDGFRβ positive (arrow). Avascular RFP+ cells were PDGFRβ negative. The scale bar is 20 µm.

### Avascular RFP+ cells have a myeloid-like expression profile

In order to uncover the avascular RFP+ cell identity, tumors were dissected under a microscope at 7 and 21 days post-operatively (DPO, Fig 4.1.2-A). After tumor dissociation, avascular RFP+ cells were purified (Fig 4.1.2-B) and analyzed by single-cell RNA sequencing (scRNA-seq). Due to their tight association with tumor vessels, vascular RFP+ cells were removed during the isolation procedure (Fig 4.1.2-B)[81]. The isolated avascular RFP+ cells were compared with the expression profiles of over 3,000 neural and non-neural mouse cells [82] within the t-distributed stochastic neighbor embedding (t-SNE) plot, which demonstrated that they were a homogeneous cell population (Fig 4.1.2-C) and that their expression profile was different from other known mouse brain cell populations. A random forest algorithm indicated that traced avascular RFP+ cells at 7 and 21DPO had a high statistical similarity to microglia (Fig 4.1.2-D).

**Figure 4.1.2 Purified avascular Nes-RFP+ cells from orthotopic GBM show a myeloid-like expression profile. (A)** Experimental setup: 7DPO and 21DPO tumor tissues from Nes-RFP transgenic mice were microdissected and dissociated. **(B)** Scheme depicting avascular RFP+ cell purification. Rat anti-mouse CD31 microbeads were added to the tumor homogenate. Vascular RFP+ cells that are tightly conjugated to endothelial cells were removed using the magnetic instrument. The remaining avascular RFP+ cells were purified by fluorescence-activated cell sorting (FACS). **(C)** A t-SNE plot showed purified avascular RFP+ cells (red) with a scRNAseq analysis showing a distinct and homogenous cell population. **(D)** A random forest algorithm indicated a similar expression profile of 7DPO and 21DPO avascular RFP+ cells with microglia. The scRNAseq analysis was performed by Philipp Janssen, Wolfgang Enard, and Ines Hellmann.

### Characterization of traced avascular RFP+ cells

Tumor tissue of the 14DPO type was dissected under a microscope and dissociated (Fig 4.1.3-A). Vascular RFP+ cells, which are tightly conjugated with vessels, were excluded, as previously described. Flow cytometry of traced avascular RFP+ cells indicated that they express the myeloid cell marker-CD11b (Fig 4.1.3-B). The Spi1 gene encodes the transcription factor PU.1 and is required for both early differentiation and the functioning of mature myeloid cells and some lymphocytes[83]. We crossed the Nes-RFP mice and Spi1-GFP mice to identify further avascular RFP+ cells with a myeloid appearance (Fig 4.1.3-C). However, Iba1, a canonical marker of tumor- associated myeloid cells, was not detected in traced avascular RFP+ cells (Fig 4.1.3-D). SOX2 is a crucial stem cell transcription factor, and its levels correlate with patient survival[84]. Immunofluorescence showed that while traced avascular RFP+ cells could express SOX2, vascular RFP+ cells do not (Fig 4.1.4-E). Taken together, these results suggest that the traced avascular RFP+ cells constitute a newly identified cell population in the glioblastoma environment, with a myeloid-like expression profile (denominated: tumor-associated cells with a myeloid-like expression profile [TAMEP]).

**Figure 4.1.3 Characterization of traced avascular RFP+ cells. (A)** Experimental setup: 14DPO Nes-RFP tumor tissue was microdissected and dissociated. **(B)** FACS analysis of the myeloid cell marker CD11b in avascular RFP+ cells from 14DPO tumor tissue (representative data of nine independent FACS experiments). **(C)** PU.1 transcription factor expression in avascular RFP+ cells taken from a Nes-RFP,Spi1-GFP glioma model (arrowhead points to a single cell is shown in orthogonal view). **(D)** Immunofluorescence staining shows that Iba1 is not expressed in avascular RFP+ cells purified from an Nes-RFP tumor section. **(E)** SOX2 is expressed in avascular RFP+ cells (arrowhead, orthogonal view) but not in vascular RFP+ cells (the vessel is indicated by the dashed line). The scale bar is 20 µm.

### TAMEP are not derived from microglia, macrophages, endothelial cells, or pericyte

The Cx3cr1::creER2, R26-RFP transgenic mouse model is a useful model for tracing tumor-associated myeloid cells (TAMs) [85]. Microglia can be specifically traced using tamoxifen pulse-chase protocols (Fig 4.1.4-A, abbreviated as microglia-RFP) due to the high self-renewal rates of peripheral macrophages[86]. Immunofluorescence staining indicated that glioblastoma microglia do not express SOX2, suggesting that tumor- associated cells with a myeloid-like expression profile (TAMEP) do not originate from CNS-resident microglia (Fig 4.1.4-B). We injected TAMs at different time points to trace all TAMs (Fig 4.1.4-C). SOX2 was also not expressed in the traced TAMs (Fig 4.1.4-D), indicating that TAMEP is also not derived from peripheral macrophages. Glioblastoma endothelial cells, marked in aVE-cadherin::creER2, R26-RFP mouse model [87], also did not express SOX2 (Fig 4.1.4-F), excluding them as a source for TAMEP.

The transgenic mouse model PDGFRβ::creER2, R26-RFP (abbreviated as PDGFRβ-RFP) is widely used to trace pericytes. Traced pericytes did not express SOX2 (Fig 4.1.4-H) or the myeloid cell markers PU.1 (Fig 4.1.4-I) and CD11b (Fig 4.1.4-J). These immunofluorescence results substantiated TAMEP was also not derived from pericytes.

We found that SOX2 plays an important role during TAMEP differentiation and maturation. A conditionalSox2 knockout in traced avascular RFP+ cells caused a decrease in TAMEP amount, thereby reducing GBM expansion (Roland K et al., unpublished data). We also investigated the effect of conditional Sox2 knockouts in microglia (Cx3cr1::creER2, R26-RFP, Sox2fox/flox), endothelial cells (VE-cadherin::creER2, R26-RFP, Sox2fox/flox), and pericytes (PDGFRβ::creER2, R26-RFP, Sox2fox/flox) on GBM expansion. Conditional Sox2 knockouts in microglia, endothelial cells, and pericytes did not reduce tumor size relative to the control group (*Sox2*WT/WT) (Fig 4.1.4-K).

Together, we used a series of transgenic mouse models to show that TAMEP is not derived from TAMs, endothelial cells, or pericytes.

**Figure 4.1.4 TAMEP is not derived from microglia, macrophages, endothelial cells, or pericytes. (A–B)** A pulse-chase experiment inCx3cr1::creER2, R26-RFP model was performed to trace microglia. TAM was given for three consecutive days. Tumor cells were inoculated four weeks later. SOX2 expression was not detected in traced microglia (an arrow indicates a single traced cell), tumor-associated myeloid cells (microglia and macrophage) (**C–D),** or endothelial cells **(E–F)**.

**Figure 4.1.4** **(G–J)** Traced pericytes are SOX2 negative and do not express the myeloid cell markers PU.1 and CD11b. **(K)** Quantification of tumor size in the control group (Sox2WT/WT) and conditional Sox2 knockouts in microglia (Microglia-RFP, Sox2flox/flox), endothelial cells (VE-cadherin-RFP,Sox2flox/flox), and pericytes (PDGFRβ::creER2, R26-RFP, Sox2fox/flox). Statistical significance was determined using a Student’s t-test (NS representing no significant difference). Values are reported as the mean ± SEM. Each dot represents one mouse. The scale bar is 20 µm.

## 4.2 TAMEP are detected in other GBM mouse models and human brain tumor tissue

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### Using the co-expression of SOX2 and PU.1 to identify TAMEP

We used the Nes-RFP transgenic mouse model with an orthotopic implantation tumor to observe the new TAMEP cell population. Previous results showed TAMEP expresses both SOX2 and myeloid cell markers, including PU.1 (Fig 4.2.1-A). We used the TAMEP-specific co-expression of SOX2 and PU.1 to extend our study to other models and human brain tumors. Since both SOX2 and PU.1 are nuclear, immunohistochemistry can easily be used for cell identification. Crossing Sox2::IRES-creER2 and R26-RFP mice (Sox2-RFP)[88] withCx3cr1-GFP mice[89] resulted in traced cells (SOX2 positive cells and progeny) having a myeloid appearance in glioblastoma (Fig 4.2.1-B). This result suggests that TAMEP is also present in other glioblastoma transgenic mouse models. Additionally, PU.1 was also expressed in the Sox2-traced cells (Fig 4.2.1-C), confirming that SOX2 and PU.1 co-expression identify TAMEP. Based on these results, we suggest that combinatorial immunofluorescence detection of SOX2 and PU.1 is useful for identifying TAMEP in other GBM mouse models and, possibly, also in human tissue, since human co-expression of SOX2 and PU.1 has been reported only in some forms of leukemia[90].

**Figure 4.2.1 SOX2 and PU.1 were co-expressed in TAMEP. (A)** SOX2 and GFP co-expression in traced avascular RFP+ cells in a Nes-RFP,GFP glioma model. **(B)** Sox2:: IRES-creER2, R26-RFP, Cx3cr1-GFP glioma model corroborated that GFP was expressed in traced cells. **(C)** Immunofluorescence staining of PU.1 in traced cells in a Sox2::IRES-creER2, R26-RFP glioma model. The scale bar is 20 µm.

###  TAMEP in genetically engineered GBM mouse models

We next turned to a novel, genetically engineered GBM mouse model for TAMEP identification. In this mouse model, subventricular zone (SVZ) stem cells of young cdkn2a-/- mice were transduced with the proto-oncogene PDGFB and transformed into GBM (Fig 4.2.2-A). Sox2 immunofluorescence staining was strong in the tumor area (Fig 4.2.2-B, dashed line) but not in the tumor-free, uninduced contralateral side (Fig 4.2.2-C). An abundance of TAMEP (Fig 4.2.2-D, arrowheads) was also detected in the regional tumor area.

**Figure 4.2.2 Tracing TAMEP in a genetically engineered GBM mouse model. (A)** A retroviral vector containing the PDGFB gene, pseudotyped with a VSV-G envelope, was injected into the SVZ of young (postnatal day 30 [P30]) Cdkn2a-/- mice. PDGFB expression was under glial fibrillary acidic protein (GFAP) promoter control. The SVZ transduced stem cells transform into GBM cells due to tumor suppressor gene Cdkn2a knockout and PDGFB upregulation. **(B–C)** Immunostaining for SOX2 and PU.1 in the tumor and contralateral sides. The GBM area (dashed line), lateral ventricle (dotted line), and choroid plexus (CP) are indicated. **(D)** Many TAMEP (arrowheads) were observed in the GBM area, magnified in orthogonal view (arrows).The scale bars represent 400µm (B–C), and 20 µm (D).

### Human GBM tissue express heterogenous TAMEP

We also identified TAMEP in human primary and recurrent GBM tissue (Table 4.2). TAMEP (defined as SOX2 and PU.1 double-positive cells) was detected (Fig 4.2.3 A–E) in various primary GBM tissues; however, the amount of TAMEP varied. While some primary GBM tissues expressed a low amount of TAMEP per area (Fig 4.3.3 A–C), others exhibited high TAMEP levels (Fig 4.2.3 D–E). Even within the same GBM biopsy, the number of TAMEP was different in discrete areas (Fig 4.2.3 E–F). In the recurrent GBM tissue, TAMEP distribution was similar to that of primary GBM tissue, while the amount also varied. Recurrent GBM tissues exhibited anywhere between no TAMEP, low levels, or high levels (Fig. 4.2.3 G–J),

**Figure 4.2.3 Detection of TAMEP in human GBM tissue. (A**–**D)** TAMEP Sox2 and PU.1 double-labeled cells in primary GBM tissue. **(E)** Sox2 and PU.1 positively-labeled cells and **(F)** Sox2 and PU.1 negatively-labeled cells in the same primary GBM tissue. **(G–H)** Sox2 and PU.1 positively-labeled cells in recurrent GBM tissue. **(I–J)** Sox2 and PU.1 positively-labeled (I) and negatively-labeled (J) cells in the same recurrent GBM tissue. The scale bar is 20 µm.

### TAMEP can be detected in other brain tumor types

Since TAMEP is detected in human GBM tissue, we sought to determine whether TAMEP exists in other human brain tumor tissue (Table 4.2). We stained a human brain tumor tissue array (human brain tumor tissue from different patients in one slide) for SOX2 and PU.1. SOX2/PU.1 double-positive cells were detected in low-grade glioma (Fig 4.2.4-A), medulloblastoma (Fig 4.2.4-B), and in metastatic brain tumors (Fig 4.2.4-C). We also observed areas with abundant TAMEP in metastatic brain tumors (Fig 4.2.-C).

**Figure 4.2.4 Detection of TAMEP in a human brain tissue array.** Sox2 and PU.1 double-labeled cells in **(A)** Grade II glioma, **(B)** medulloblastoma and, **(C)** brain metastatic tumor. The scale bar is 20 µm.

Table 4.2 Human brain biopsies

## 4.3 Establishing a novel recurrent GBM mouse model

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### Ganciclovir can induce GL261-HSVTK-GFP cell death

Ganciclovir (GCV) application can induce cell death in a GL261 cell line transfected with the herpes simplex virus thymidine kinase (HSVTK) by inhibiting DNA synthesis (Fig 4.3.1-A)[91]. We used this HSVTK/GCV system to establish a new recurrent GBM mouse model. Experiments were performed according to the experimental setup in Figure 4.3.1 A. Ganciclovir was injected intraperitoneally in orthotopic GBM induced by the GL261-HSVTK-GFP cell line. GCV significantly reduced tumor mass with residual tumor cells continuing to grow, leading to GBM recurrence.

To test the HSVTK/GCV system’s efficiency, we first conducted an in vitro experiment. The transgenic glioma cells were cultured in 24-well plates and treated with GCV. GCV-treated cells died rapidly, while control cells proliferated (Fig 4.3.1-B).

**Figure 4.3.1 Schematic diagram of the recurrent GBM model and in vitro test. (A)** HSVTK expression causes cells to phosphorylate GCV, which interferes with DNA replication and induces apoptosis. GCV was injected intraperitoneally during tumor growth, which largely eliminated the tumor, but the few residual cells continued to grow and eventually caused tumor recurrence **(B)** Representative microscopy images for the in vitro HSVTK/GCV experiment.The **s**cale bar is 200 µm.

### Ganciclovir strongly reduces tumor size and prolongs survival *in vivo*

We next investigated the effect of the HSVTK/GCV system in vivo according to the setup presented in Fig 4.3.2-A. In the experiment group, GCV (50mg/kg) was injected intraperitoneally from day 14 to day 17, and mice were sacrificed at 21DPO. Tumor size was significantly decreased at 21DPO (Fig 4.3.2-B), compared to the control group. Next, we analyzed mouse survival after GCV injection (experimental setup in Fig 4.3.2-C). Mice were sacrificed when symptoms manifested, and the survival day was recorded. The control group’s median survival was 25 days, while mice treated with GCV survived longer, with a median survival of 45.5 days (*p*=0.0015 compared to the control, Fig 4.3.2-D).

Figure 4.3.2 **HSVTK/GCV system in vivo. (A)** A schematic diagram depicting the GCV application *in vivo*. GCV (50mg/kg) was injected at days 14–17 and mice were sacrificed 21DPO. **(B)** Quantification of tumor volume and representative microscopy images of the control and GCV-treated groups, showing a significant difference between the two groups. **(C**–**D)** Schematic design of GCV application in the survival experiment. GCV (50mg/kg) was injected at days 14–17 and mice were sacrificed once they became symptomatic. The median survival time was 25 days in the control group (n=11)and 45.5 days in the GCV-treated group (n=7, *p*=0.0015).Statistical significances were calculated using a Student’s t-test (B) or a Log-rank (Mantel-Cox) test (D), \*\*\* *p* < 0.005. Values (B) are reported as the mean ± SEM. Each dot represents one mouse. The scale bar is 1mm.

### Tumor recurrence after GCV application

In order to observe how GCV treatment affects GBM growth after treatment, tumor size was quantified at different time points following GCV injections (21 DPO, 28 DPO, 35 DPO, mice symptomatic) according to the experimental setup in Fig 4.3.3-A. GCV decreased tumor volume up to 28DPO (Fig 4.3.3-B), after which the tumor began to regrow. The GCV injection lasted four days in total and the effect of GCV-induced tumor cell death was sustained for nearly two weeks. This experiment mimicked the clinical manifestation of tumor recurrence, in which tumor mass is decreased immediately after treatment but then recovers, leading to tumor recurrence.

**Figure 4.3.3** **Tumor volume initially decreases after GCV application but later increases. (A)** Schematic diagram of the experimental setup in which tumor volume was measured at different time points. **(B)** Quantification of the tumor. Each dot represents the average value at different time points.

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## Characterization of recurrent glioblastoma

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### Recurrent GBM are more invasive

In the GCV-treated mice, GBM recurred at both the original tumor site (Fig 4.4.1 A and C, arrowhead) and in other locations (Fig 4.4.1 A and C, arrow). This distant recurrence indicates that the GCV-treated GBM cells became more invasive.

We assessed the extent of the GCV-treated GBM invasion by scoring the degree of invasiveness on a scale of 0–3 (0 represents no histological invasion, 1 shows the appearance of a larger and connected group of tumor cells, 2 describes smaller scatted groups of invading tumor cells, and 3 indicates single scattered highly invasive tumor cells). In the primary GBM mouse model, 7DPO, 14DPO, and 21DPO tumors (mice usually get symptomatic around 21DPO) represent early, middle, and late tumor growth periods, respectively. The adjusted time points in the recurrent GBM group were determined according to tumor size and were set at 28DPO, 35DPO, and at symptom appearance. In the early stage tumor period, untreated 7DPO and GCV 28 DPO tumor volume were similar. However, the invasive score was significantly higher in GCV treated mice (1.77 VS 0.17, Fig 4.4.1 C). The invasive score of recurrent GBM was also significantly higher than that of primary GBM in both the middle (1.47 VS 0.17) and late stages (1.30 VS 0.16, Fig 4.4.1 E), although the recurrent tumor size was smaller than the primary GBM.

**Figure 4.4.1** Increased invasiveness in **GCV-treated tumor cells. (A–D)** Hematoxylin and Eosin staining (H&E staining) show local (arrowheads) and distal tumor recurrence (arrows). Distant recurrence **(B and D)** was confirmed by GFP immunofluorescence. The scale bars are 1mm in A and C, 100 µm in B and D.**Figure 4.4.1** **(E–G)** Tumor size and invasive score were quantified in the control and GCV-treated groups at different tumor stages. At all stages, the invasive score in recurrent GBM was higher than in primary GBM. Representative images of HE staining showed round and smooth tumor borders in primary GBM (dashed line) and irregular infiltrative edges (dotted line) in recurrent GBM. Statistical significance was calculated according to a Student’s t-test, \* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.0005, \*\*\*\* *p* < 0.0001. Each dot represents one mouse. Values are reported as the mean ± SEM. The scale bars are 1 mm in (A), (C), (E–G), 100 µm in (B) and (D).

### Recurrent GBM is less angiogenic

Angiogenesis levels in primary and recurrent GBM were evaluated by immunostaining for CD31 (Fig 4.4.2 A–C). Total vessel length was measured in treated and untreated groups at different time points (as selected according to tumor growth, see the previous section). In the tumor’s early, middle and late stages, the total vessel length in recurrent GBM was significantly decreased compared to the corresponding stage in primary GBM (Fig 4.4.2 A–C), suggesting that recurrent GBM tumors are less angiogenic.

**Figure 4.4.2 Recurrent GBM show decreased vascularization. (A**–**C)** Tumor size and total vessel length per 40X vision were quantified in the control and GCV-treated group at different tumor stages. The total vessel length was lower in recurrent GBM than in primary GBM at all tumor stages. Representative images of immunostaining for CD31 show fewer vessels in recurrent GBM. Statistical significance was calculated according to a Student’s t-test, \* *p* < 0.05, \*\* *p* < 0.01, \*\*\* *p* < 0.0005, \*\*\*\* *p* < 0.0001. Each dot represents one mouse. Values are reported as the mean ± SEM. The scale bars are 1 mm in (A), (C), (E–G), 100 µm in (B) and (D).

# Discussion

We investigated a novel cell population in the glioblastoma environment by using a lineage-tracing transgenic Nes-RFP mouse model. Single cell gene expression analysis of this population indicates high similarity with microglia. The transcription factor SOX2, known to be expressed in neural stem/progenitor cells and GSC[92, 93], is also expressed in the traced cell population. Several studies have found that SOX2 was expressed in neural stem/progenitor cells and GSC[92, 93]. In glioblastoma, SOX2 is highly expressed in GSC with levels correlating to tumor aggressiveness[94, 95]. We identified this cell population as avascular RFP+ cells (under Nestin promoter control and characterized with SOX2-expression), in which both of them were recognized as immature cells, and which then differentiated into cells with a myeloid-like expression profile. Immunostaining results show that this new cell population does not express all TAM markers. The canonical myeloid cell marker Iba1 was not detected in traced avascular RFP+ cells, distinguishing them from microglia. Although some studies have identified Iba1-negative microglia in brain diseases [96, 97], the results from our study that traced microglia in glioblastoma did not express SOX2, and indicated that conditional SOX2-loss in microglia did not affect tumor expansion, suggest that the traced avascular RFP+ cells are not originated from microglia.

In the healthy brain, microglia are the primary innate immune cells and form the blood brain barrier that prevents the entry of bone-marrow-derived macrophages into the brain parenchyma[98]. However, peripheral macrophages can infiltrate into glioblastoma due to disruptions in the blood brain barrier. Tumor-associated myeloid cells include resident microglia and peripheral macrophages, which have mostly overlapping marker profiles in the glioblastoma microenvironment[29, 35]. Tumor-associated myeloid cells (assessed using the myeloid cell tracing model, Cx3cr1::creER2, R26-RFP) do not express Sox2, indicating that the traced avascular RFP+ cells in the Nes-RFP model are not peripheral macrophages. Previous work has suggested that Nestin is expressed in proliferative endothelial cells[99]. However, since we have determined that endothelial cells do not express SOX2, they are unlikely to serve as a source for the traced avascular RFP+ cells in the Nes-RFP model.

Traced vascular RFP+ cells in the Nes-RFP model were identified as mature pericytes in glioblastoma. Pericytes play a vital role in glioblastoma growth and invasion by regulating the blood-brain barrier, promoting angiogenesis, and weakening the extracellular matrix[100, 101]. Some studies have shown that pericytes can differentiate into neural and myeloid lineages[102–104]. Using a pericyte lineage tracing model, we showed that glioblastoma pericytes are negative for SOX2 and CD11b and are, therefore, not the source for the traced avascular RFP+ cells. We further found that the number of traced vascular RFP+ cells can constitute up to nearly 30% of all glioblastoma pericytes (Roland K et al., unpublished data), suggesting that local progenitor cells can produce a large number of pericytes to support tumor growth. Although glioblastoma stem cells have been reported to differentiate into the majority of pericytes in the tumor tissue to assist tumor growth and GSC self-renewal[47, 105], these studies used nude mice, which have an incomplete immune system, and thus may account for the differences in our study. Taking all these factors together, we report a novel cell population of traced avascular RFP+ cells in a Nes-RFP model termed TAMEP.

TAMEP expression in transgenic mouse models varies according to tumor-parenchymal cell diversity and tumor environment complexity. The glioblastoma environment consists of tumor cells, TAMs, and other immune cells, vessels, extracellular matrix, neurons, and astrocytes[106]. We also detected TAMEP in human glioblastoma tissue with levels differing not only between patients but also between tumor locations within the same patient. These results show both inter- and intra-tumor heterogeneity. TAMEP could represent a potent regulator of tumor angiogenesis and glioblastoma expansion (Roland K et al., unpublished data), making it a promising target for glioblastoma treatment, especially in recurrent GBM. Furthermore, detection of TAMEP in other brain tumors (medulloblastoma and metastatic brain tumors) extend the significance of our study to other brain tumors, suggesting a similar mechanism in different brain tumor types that can be targeted in a common treatment. Indeed, TAMEP is not detected in normal brain tissues, cerebrum, and tumor-adjacent normal brain tissues, indicating that TAMEP might be specifically associated with the disease and, therefore, only exist in the tumor environment.

Glioblastoma recurrence after treatment is widespread [107]. Therefore, establishing a recurrent GBM model for investigating the mechanisms underlying recurrence is of the utmost importance. While Mitomu et al. reported an orthotopic recurrent GBM mouse model[108], the mice used in this study were nude mice with an inhibited immune system that cannot wholly duplicate the tumor microenvironment. Furthermore, the radiotherapy inducing tumor regression presented in this model is significantly different from the standard clinical treatment [108]. Shinichi et al. also described patient-derived recurrent glioblastoma models[109] in which the cells injected into the mice were derived from a relapsed glioblastoma after clinical treatment. In this model, recurrent GSCs were inoculated in intact and untreated mice brain, which differed from the human treated brain. Since our study uses immunocompetent mice with a complete immune system, and tumor cells were killed by the HSVTK system, which mimics clinical tumor debulking, our model has clear advantages over the previously reported models.

In our recurrent GBM models, both local and distant recurrences were observed, much like the clinical recurrence pattern[110]. Furthermore, recurrent GBM is less angiogenic compared with primary GBM. Kim et al. showed that while local recurrent tumors share the majority of gene mutations detected in the original tumor, distant recurrent tumors are genetically distinct from the original tumor [111]. These results suggest that distant recurrence arises from a divergent evolution, in which the new tumor cells experience high clonal selection pressure during treatment. Combined with our findings, these results showed inter- and intra-tumor heterogeneity. Our recurrent GBM mouse model could advance the understanding of recurrent glioblastoma heterogeneity and provide novel directions for personalized and effective therapies, especially in distant recurrent glioblastoma.

# Summary

Glioblastoma progression and recurrence are supported by tumor-parenchymal cells in the tumor environment. The diversity of tumor-associated cells and tumor heterogeneity in glioblastoma affect tumor therapies, prognosis, and recurrence rates. This study investigated a novel cell population, termed TAMEP, and established a new recurrent glioblastoma model, which imitates clinical recurrence. We used this model to investigate recurrent glioblastoma heterogeneity.

First, we traced two types of cells in glioblastoma using a transgenic lineage-tracing mouse model. Immunostaining for PDGFRβ (pericyte marker) and their position in relation to vessels identified as traced vascular RFP+ cells as mature pericytes. Single-cell RNA sequencing analysis of purified traced avascular RFP+ cells from 7DPO and 21DPO tumors showed a homogenous cell population. Random forest algorithm indicated that the expression profile of traced avascular RFP+ cells is highly similar to microglia. Immunostaining results confirmed that traced avascular RFP+ cells express myeloid cell markers, such as PU.1 and CD11b, but not the canonical tumor-associated myeloid cell marker, Iba1. Furthermore, analysis of SOX2 expression characterized traced avascular RFP+ cells as a novel cell population. Using a series of cell lineage tracing models, we found that this new cell population does not originate from microglia, endothelial cells, or pericytes. We termed this new cell population of tumor-associated cells with myeloid-like expression profile as TAMEP.

TAMEP was detected in both human primary and recurrent glioblastoma, demonstrating the diversity of glioblastoma-associated cells. The finding that the number of TAMEP varies not only between different human GBM tissues but also between areas in the same GBM tissue indicates glioblastoma heterogeneity. TAMEP is also detected in human medulloblastoma and metastatic brain tumors, extending the significance of our study to other brain tumor types.

Recurrent glioblastomas often harbor different genetic mutations from the initial tumor. In order to investigate the heterogeneous features in recurrent glioblastoma, in the second part of our study, a new recurrent glioblastoma mouse model was established. Distant and local recurrence identified in this model showed characteristics similar to clinical observations. It was also found that recurrent GBM is more invasive but less angiogenic than the initial tumor. These findings, aided with the development of the new mouse model, has allowed us to further explore the heterogeneity of recurrent glioblastoma and may help guide personalized treatment in the future.