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# Mesenchymal stromal cells in malignant glioma

## Functions and therapeutic potential

ANDREAS SVENSSON | FACULTY OF MEDICINE | LUND UNIVERSITY

# Mesenchymal stromal cells in malignant glioma

## Functions and therapeutic potential

Andreas Svensson

2015

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som med vederbörligt tillstånd från Medicinska fakulteten vid Lunds universitet
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Abstract:

The most common malignant brain tumor in adults is a glioma called glioblastoma multiforme (GBM). About 300 persons are diagnosed with GBM every year in Sweden. Unfortunately, it is also the most aggressive brain tumor and as of today, it is not possible to cure it. Despite treating the patients with surgery, radiation and chemotherapy, the median survival is only 15 months. The main problem with GBM is its infiltrative growth. As the tumor cells leave the tumor bulk and migrate into the normal brain parenchyma, it is impossible to reach them with the current standard treatments. Hence, even after treatment, some tumor cells will remain in the brain and eventually give rise to a new tumor.

To be able to reach the migrating cells, new treatment strategies need to be developed. One such strategy is to use stem cells as drug delivery vehicles. It has been shown that mesenchymal stromal cells (MSCs) derived from the bone marrow (BM) have the ability to specifically migrate throughout a glioma. Upon intratumoral transplantation, they spread within the tumor, along its extensions and toward migrating tumor cells that has left the main tumor bulk, making BM-MSCs ideal as transporters of anti-tumoral substances. However, several safety concerns have been raised as MSCs also have shown to mediate tumor growth by acting immunosuppressive and contribute to the tumor stroma and vascularization.

This thesis will discuss 1) the role of endogenous MSCs in malignant glioma and 2) the use of transplanted BM-MSCs as glioma treatment.

We have shown that human malignant gliomas harbor two distinct cell populations that resemble BM-MSCs. We have characterized the cells and conclude that they most likely play an important role in tumor angiogenesis and immunosuppression. Further on, we have seen that MSC-like pericytes within the normal mouse brain are activated by, and migrate into, an orthotopic glioma model. The cells align perivascularly and contribute the majority of all pericytes within the tumor.

To evaluate their tumor-tropism, MSCs were derived from rat bone marrow and transplanted into, and adjacent to, orthotopic rat gliomas. We conclude that even though they show strong tumor-tropic migration capabilities upon intratumoral transplantation they do not migrate when transplanted into the normal brain of tumor bearing animals. We also report that intratumorally transplanted BM-MSCs potentiate the effect of peripheral immunotherapy against malignant gliomas, demonstrating their use in a therapeutic setting.



Key words: glioma, glioblastoma, mesenchymal stromal cell, pericyte, immunotherapy, interferon-γ

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Signature Andreas Svensson Date 2015-08-13

# Mesenchymal stromal cells in malignant glioma

## Functions and therapeutic potential

Andreas Svensson

ACADEMIC DISSERTATION

Lund 2015

Division of Neurosurgery
Department of Clinical Sciences in Lund
Faculty of Medicine, Lund University

Cover: Rat brain tissue section with an N32 glioma in the striatum. The dense tumor is visible to the right, with the ipsilateral ventricle to the left. The blue cell nuclei are visualized by Hoechst staining.

Cover artwork by Andreas Svensson and Fredrik Svensson.

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KLIMATKOMPENSERAT
PAPPER

*Till min familj*

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# Populärvetenskaplig sammanfattning

Den vanligaste formen av elakartad hjärntumör i vuxna kallas glioblastoma multiforme (GBM). Det är tyvärr också den allvarligaste formen av hjärntumör, och trots att man behandlar patienterna med kirurgi, strålning och cellgiftsbehandling är den genomsnittliga överlevnaden bara 15 månader. I dagsläget finns ingen bot att erbjuda de ungefär 300 personer som drabbas varje år i Sverige.

Det främsta problemet med GBM är att den invaderar den normala hjärnvävnaden. Enskilda celler kan lämna tumören och förflytta sig till andra delar av hjärnan, och dessa celler går inte att komma åt med dagens behandlingar. Även om man tar bort tumören, kommer dessa celler till slut att ge upphov till en ny tumör.

För att nå dessa invaderande celler behövs nya behandlingsmetoder. En strategi som undersöks för närvarande är användandet av stamceller. Det har visat sig att stamceller från benmärgen som i normala fall bildar ben, brosk och fettvävnad, så kallade mesenkymala stromaceller (MSCs), sprider sig väldigt effektivt om man transplanterar in dem i en hjärntumör. Den stora fördelen är att de bara sprider sig i tumören, även de invaderande delarna och enskilda tumörceller utanför tumören, utan att gå ut i den normala hjärnvävnaden. Denna tumörspecifika spridning gör att MSCs lämpar sig för att leverera läkemedel till de delar av tumören som dagens behandlingsmetoder inte kommer åt. Man kan till exempel modifiera cellerna genetiskt så att de börjar producera ett tumördödande ämne, eller så kan man fylla cellerna med nanopartiklar som innehåller ett läkemedel.

Ett problem med att transplantera MSCs till hjärntumörer är att det finns en risk för att de förvärrar situationen. Forskning har visat att MSCs kan stimulera tumören så att den växer fortare, till exempel genom att bilda nya blodkärl. De kan också hämma immunförsvaret, så att kroppen får svårare att bekämpa tumören. Dessutom finns det alltid en risk att transplanterade stamceller själva bildar en tumör, då de precis som cancerceller kan dela sig obehindrat.

I den här avhandlingen diskuteras 1) om det finns MSCs naturligt i GBM från människa och 2) om MSCs kan användas för att på ett säkert sätt behandla GBM.

I den första studien samlade vi 14 hjärntumörer från neurokirurgen vid Skånes Universitetssjukhus i Lund. Dessa odlades i cellodlingsflaskor och tumörcellerna undersöktes. Det visade sig att samtliga tumörer innehöll två olika celltyper som liknade MSCs till utseende och beteende. Skillnaden mellan cellerna var att endast

den ena typen hade proteinet CD90 på sin cellyta. När cellerna undersöktes vidare visade det sig att de producerade höga nivåer av två molekyler kallade vaskulär endotelial tillväxtfaktor (VEGF) och prostaglandin $E_2$ ($PGE_2$), där den förstnämnda stimulerar bildandet av nya blodkärl medan den andra hämmar immunförsvaret. Dessa molekyler producerades i högre grad i de celler som saknade CD90 på cellytan. Slutsatsen är att GBM i människor innehåller två celltyper som liknar MSCs och som sannolikt hjälper tumören att växa. Dessa celler skulle kunna utgöra ett mål för framtida läkemedel.

I den andra studien undersökte vi hur MSC-liknande celler i hjärnan, kallade pericyter, reagerade när en hjärntumör växte i hjärnan. Vi använde en genmodifierad mus i vilken hjärnpericyterna uttrycker grönt fluorescerande protein. Det innebär att pericyterna är gröna om man tittar på den i ett mikroskop, vilket gör att man kan särskilja dem från alla andra celler i hjärnan. Vi transplanterade in tumörceller i mushjärnan och undersökte den efter 19 dagar. Då hade det bildats en tumör, och det visade sig att gröna pericyter hade aktiverats i hela hjärnan och börjat vandra in i tumören. Det visade sig dessutom att majoriteten av alla pericyter i tumören var gröna, vilket betyder att de vandrat in från den normala hjärnan och inte bildats av tumörcellerna själva. Pericyterna utgör en del av blodkärlen i tumören, och genom att förhindra att de börjar förflytta sig från den normala hjärnan kanske man kan hämma tumörens förmåga att bilda blodkärl.

I den tredje studien undersökte vi hur MSCs från benmärg i råtta betedde sig om de transplanterades in i, eller utanför, hjärntumörer i råtthjärnan. Det visade sig att de spred sig väldigt väl och specifikt inuti tumören, men att de inte förflyttade sig om de istället placerades utanför tumören i den normala hjärnvävnaden. När MSCs transplanterades till en frisk råtta utan hjärntumör förflyttade de sig inte heller. Vidare undersökte vi om transplanterade MSCs delade sig i tumören, vilket de inte gjorde. Sammataget visar resultaten att MSCs sprider sig väl och tumörspecifikt om de transplanteras in i en hjärntumör. Risken är dessutom låg för att MSCs hamnar på fel ställe i hjärnan, eftersom de inte förflyttar sig genom normal hjärnvävnad. De tycks dessutom inte dela sig, vilket innebär en låg risk för att de själva ska bilda en tumör.

I den fjärde studien undersökte vi om MSCs som transplanteras in i en hjärntumör kan hjälpa kroppens immunförsvar att bekämpa den. Tidigare forskning har visat att man kan vaccinera en råtta med tumörceller som modifierats genetiskt för att producera en interferon-γ (IFNγ), en molekyl som stimulerar immunförsvaret. Det har dessutom visat sig att MSCs som utsätts för IFNγ omvänds från att hämma immunförsvaret till att stimulera det. Detta gjorde att vi transplanterade in MSCs i hjärntumörer i råttor, samtidigt som de vaccinerades med IFNγ-producerande tumörceller. Det visade sig att fler djur överlevde om de behandlades både med MSCs och med vaccination jämfört med om de bara behandlades med en av metoderna. När tumörerna analyserades närmare visade det sig dessutom att det fanns betydligt fler vita blodkroppar i de tumörer som behandlats med både MSCs och med vaccination jämfört med de tumörer som bara behandlades med en av metoderna.

# Original papers

This thesis is based on the following original papers:

Paper I — Identification of two distinct mesenchymal stromal cell populations in human malignant glioma.
**Andreas Svensson**, Sofia Eberstål, Stefan Scheding, Johan Bengzon.
*Manuscript*

Paper II — Endogenous brain pericytes are widely activated and contribute to mouse glioma microvasculature.
**Andreas Svensson**, Ilknur Özen, Guillem Genové, Gesine Paul*, Johan Bengzon*.
*These authors contributed equally to this work.
*PLOS ONE* (2015) 10(4):1-15.

Paper III — Rat multipotent mesenchymal stromal cells lack long-distance tropism to 3 different rat glioma models.
Daniel Bexell, Salina Gunnarsson, **Andreas Svensson**, Ariane Tormin, Catarina Henriques-Oliveira, Peter Siesjö, Gesine Paul, Leif G. Salford, Stefan Scheding, Johan Bengzon.
*Neurosurgery* (2012) 70(3):731-739.

Paper IV — Intratumorally implanted mesenchymal stromal cells potentiate peripheral immunotherapy against malignant rat gliomas.
Salina Ströjby, Sofia Eberstål, **Andreas Svensson**, Sara Fritzell, Daniel Bexell, Peter Siesjö, Anna Darabi, Johan Bengzon.
*Journal of Neuroimmunology* (2014) 274(1-2):240-243.

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# Papers not included in the thesis

Intratumoral IL-7 delivery by mesenchymal stromal cells potentiates IFNgamma-transduced tumor cell immunotherapy of experimental glioma.
Salina Gunnarsson, Daniel Bexell, **Andreas Svensson**, Peter Siesjö, Anna Darabi, Johan Bengzon.
*Journal of Neuroimmunology* (2010) 218(1-2):140-144.

Stem cell-based therapy for malignant glioma.
Daniel Bexell, **Andreas Svensson**, Johan Bengzon.
*Cancer Treatment Reviews* (2013) 39(4):358-365.

Al adjuvants can be tracked in viable cells by lumogallion staining.
Irene Mile, **Andreas Svensson**, Anna Darabi, Matthew Mold, Peter Siesjö, Håkan Eriksson.
*Journal of Immunological Methods* (2015) 422:87-94.

# Abbreviations

| | |
|---|---|
| APC | antigen-presenting cell |
| BBB | blood-brain barrier |
| BM | bone marrow |
| CNS | central nervous system |
| DC | dendritic cell |
| ECM | extracellular matrix |
| eGFP | enhanced green fluorescent protein |
| EGFR | epidermal growth factor receptor |
| GBM | glioblastoma multiforme |
| GCV | ganciclovir |
| GFP | green fluorescent protein |
| GLUT | glucose transporter |
| HIF | hypoxia inducible factor |
| HSV-TK | herpes simplex virus-1 thymidine kinase |
| i.c. | intracerebral |
| i.p. | intraperitoneal |
| i.t. | intratumoral |
| Iba | ionized calcium-binding adapter molecule |
| IDH | isocitrate dehydrogenase |
| IFN | interferon |
| IL | interleukin |
| ISCT | International Society for Cellular Therapy |
| MCP | monocyte chemotactic protein |
| MGMT | $O^6$-methylguanine-DNA methyltransferase |

| | |
|---|---|
| MHC | major histocompatibility complex |
| MMP | matrix-metalloproteinase |
| MSC | mesenchymal stromal cell |
| NG | neuron-glial antigen |
| NK cell | natural killer cell |
| PDGF | platelet-derived growth factor |
| PDGF-R | platelet-derived growth factor receptor |
| $PGE_2$ | prostaglandin $E_2$ |
| PTEN | phosphatase and tensin homolog |
| RGS | regulator of G-protein signaling |
| s.c. | subcutaneous |
| SDF | stromal cell-derived factor |
| SMA | smooth muscle actin |
| SVZ | subventricular zone |
| TCR | T cell receptor |
| TGF | transforming growth factor |
| TMZ | temozolomide |
| TRAIL | tumor necrosis factor-related apoptosis-inducing ligand |
| Treg | T regulatory cell |
| VEGF | vascular endothelial growth factor |
| VEGF-R | vascular endothelial growth factor receptor |
| WHO | World Health Organization |

# Introduction

The most common malignant brain tumor in adults is a glioma called glioblastoma multiforme (GBM). About 300 persons are diagnosed with GBM every year in Sweden. Unfortunately, it is also the most aggressive brain tumor and as of today, it is not possible to cure it. Despite treating the patients with surgery, radiation and chemotherapy, the median survival is only 15 months. The main problem with GBM is its infiltrative growth. As the tumor cells leave the tumor bulk and migrate into the normal brain parenchyma, it is impossible to reach them with the current standard treatments. Hence, even after treatment, some tumor cells will remain in the brain and eventually give rise to a new tumor.

To be able to reach the migrating cells, new treatment strategies need to be developed. One such strategy is to use stem cells as drug delivery vehicles. It has been shown that mesenchymal stromal cells (MSCs) derived from the bone marrow (BM) have the ability to specifically migrate throughout a glioma. Upon intratumoral transplantation, they spread within the tumor, along its extensions and toward migrating tumor cells that has left the main tumor bulk, making BM-MSCs ideal as transporters of anti-tumoral substances. However, several safety concerns have been raised as MSCs also have shown to mediate tumor growth by acting immunosuppressive and contribute to the tumor stroma and vascularization.

This thesis will discuss 1) the role of endogenous MSCs in malignant glioma and 2) the use of transplanted BM-MSCs as glioma treatment.

We have shown that human malignant gliomas harbor two distinct cell populations that resemble BM-MSCs. We have characterized the cells and conclude that they most likely play an important role in tumor angiogenesis and immunosuppression. Further on, we have seen that MSC-like pericytes within the normal mouse brain are activated by, and migrate into, an orthotopic glioma model. The cells align perivascularly and contribute the majority of all pericytes within the tumor.

To evaluate their tumor-tropism, MSCs were derived from rat bone marrow and transplanted into, and adjacent to, orthotopic rat gliomas. We conclude that even though they show strong tumor-tropic migration capabilities upon intratumoral transplantation they do not migrate when transplanted into the normal brain of tumor bearing animals. We also report that intratumorally transplanted BM-MSCs potentiate the effect of peripheral immunotherapy against malignant gliomas, demonstrating their use in a therapeutic setting.

# Glioma

Tumors that originate from glial cells, the supportive tissue of the brain and spinal cord, are called gliomas. After meningiomas, which are benign tumors arising from the meninges of the brain, gliomas are the most common type of tumor arising from the central nervous system (CNS).[1] They constitute about 28% of all benign and malignant primary tumors, i.e. tumors formed *de novo* and not originating from a preceding tumor, and 80% of all primary malignant tumors in the CNS. Gliomas have an annual incidence of approximately 6.3 cases per 100 000 persons, and the vast majority occur in the brain.[2]

Gliomas are categorized based on the type of glial cell they originate from or are histologically similar to; astrocytes, oligodendrocytes and ependymal cells give rise to astrocytomas, oligodendrogliomas and ependymomas, respectively. Gliomas that originate from more than one type of glial cell, for example oligoastrocytomas that contain both oligodendrocyte- and astrocyte-like cells, are called mixed gliomas. Each of these glioma subtypes are then further categorized based on histological properties, such as mitotic activity, neoangiogenesis, necrosis and pleomorphism.[1]

The prognosis for glioma patients depends largely on the type of tumor. Factors such as patient age, tumor location, extent of surgical resection and genetic alterations all affect the estimate of prognosis. To further help predict tumor behavior and patient prognosis, and to facilitate the choice of therapy, the World Health Organization (WHO) has graded all gliomas on a four-tiered scale based on four histological properties: cytological atypia, mitosis, endothelial proliferation and necrosis (Table 1).

**Table 1. WHO classification of gliomas.[1]**

| **Astrocytic tumors** | **WHO grade** | | | | **Oligodendroglial tumors** | **WHO grade** | | | |
|---|---|---|---|---|---|---|---|---|---|
| Pilocytic astrocytoma | I | | | | Oligodendroglioma | | II | | |
| Subependymal giant cell astrocytoma | I | | | | Anaplastic oligodendroglioma | | | III | |
| | | | | | **Oligoastrocytic tumors** | | | | |
| Diffuse astrocytoma | | II | | | Oligoastrocytoma | | II | | |
| Pilomyxoid astrocytoma | | II | | | Anaplastic oligoastrocytoma | | | III | |
| Pleomorphic xanthoastrocytoma | | II | | | **Ependymal tumors** | | | | |
| Anaplastic astrocytoma | | | III | | Subependymoma | I | | | |
| Glioblastoma multiforme | | | | IV | Myxopapillary ependymoma | I | | | |
| Giant cell glioblastoma | | | | IV | Ependymoma | | II | | |
| Gliosarcoma | | | | IV | Anaplastic ependymoma | | | III | |

The WHO grade reflects the tumor malignancy, where grade I are the least aggressive and a grade IV are the most aggressive tumors. Gliomas of WHO grade I and II are collectively called low-grade gliomas and are slowly growing, well-differentiated

tumors with a high proportion of long-term surviving patients. Similarly, gliomas of WHO grade III and IV are called high-grade gliomas, characterized by high proliferation, atypical and poorly differentiated cells and short patient survival. Low-grade gliomas that has been treated often undergo malignant transformation and recur as a secondary, higher-grade glioma with worse prognosis.[1]

## Astrocytoma

The most common glioma subtype, accounting for approximately 75% of all gliomas, is the astrocytoma arising from the star-shaped glial cells called astrocytes.[2]

Among the grade I astrocytomas, the pilocytic astrocytoma is the far most common comprising about 7% of all astrocytomas.[2] It is the most common glioma in children, with the vast majority of all pilocytic astrocytomas occurring in children and young adults with a mean age of 22 years at diagnosis. Pilocytic astrocytomas grow slowly and non-invasively and are generally well-circumscribed and often cystic tumors that can be treated with surgery alone. However, if the tumor location does not allow complete surgical resection, radiation is a common treatment adjuvant. It is extremely rare that pilocytic astrocytomas transform into a higher grade glioma, but rather stabilize and maintain their tumor grade for decades. In rare occasions, the tumor can even spontaneously regress. The prognosis is good, with a 10-year survival of more than 95%. If total surgical resection is accomplished, the patient can be cured.[1,3]

Another astrocytoma designated WHO grade I is the subependymal giant cell astrocytoma. It is a slowly growing, benign tumor associated with tuberous sclerosis complex, a genetic disorder characterized by benign tumors occurring, among others, in the CNS.[1]

Astrocytomas that show nuclear atypia as the only histological grading hallmark are designated grade II astrocytomas, with diffuse astrocytoma being the most common one. It represents approximately 12% of all astrocytomas and most commonly occur in young adults, with a mean age of 34 years at diagnosis.[1,2] Diffuse astrocytomas are further divided into three histological subtypes: fibrillary astrocytoma, gemistocytic astrocytoma and protoplasmic astrocytoma. They are well-differentiated and slowly growing tumors, but generally considered malignant due to their diffuse and infiltrative growth. The normal treatment consists of surgical resection, sometimes with the addition of radiotherapy depending on the tumor location and extent of resection.[1] Nevertheless, the tumor usually recurs 4-5 years after initial treatment, often transformed into a high-grade astrocytoma, resulting in a median survival of 5-8 years for this type of tumor.[1,4,5]

Pilomyxoid astrocytoma is a subtype closely related to pilocytic astrocytoma, but it grows infiltrative and usually recurs after treatment, and thus classifies as a grade II

astrocytoma. It is a rare tumor typically occurring in children, with a mean age of 18 months at diagnosis.[1,6]

A third variant of grade II astrocytoma is pleomorphic xanthoastrocytoma, accounting for less than 1% of all astrocytomas. It usually occurs in children and young adults, but has also been reported in older patients. The prognosis is relatively favorable, with a 10-year survival of 70%.[1]

Grade III astrocytomas are called anaplastic astrocytomas and are characterized by nuclear atypia, high proliferative activity and, as the name suggests, anaplasia. They constitute about 8% of all astrocytomas and usually occur in adults, with a mean age of 46 years at diagnosis.[1,2] Anaplastic astrocytomas often progress from diffuse astrocytomas, but can also form without evidence of a previous, less malignant, tumor. They are highly malignant and infiltrative tumors that are hard to treat. There is no universally accepted standard of care, but surgery followed by radiotherapy is the common treatment. Chemotherapy is sometimes considered, especially for recurrent tumors, but no significant survival benefits have, in contrast to grade IV astrocytomas, been shown when treating primary anaplastic astrocytomas. The tumor usually recurs within 2 years, typically transformed into a grade IV astrocytoma, and the median survival is about 3 years with a 5-year and 10-year survival of approximately 24% and 15%, respectively.[1,7,8]

Grade IV astrocytomas are called glioblastoma multiforme (GBM). It is the most common type of primary brain tumor in adults, constituting about 73% of all astrocytomas, and also the most aggressive with a median survival of only 15 months despite multimodal treatment with surgery, radiation and chemotherapy.[1,2,9] The work in this thesis is mainly concentrated on this glioma subtype, wherefore it is described in more detail on the next page.

Gliosarcoma is a variant of GBM displaying both glial and mesenchymal differentiation. It constitutes about 2% of all GBMs and the tumors are overall similar in patient outcome.[1]

A second subtype of GBM is called giant cell glioblastoma, characterized by multinucleated giant cells. It accounts for approximately 5% of all GBMs and has been reported to have a better clinical outcome than ordinary GBM due to less infiltrative behavior.[1]

## Experimental glioma models

To be able to study glioma in experimental settings *in vitro* and *in vivo*, it is vital to have glioma models that mimic the properties of human gliomas as close as possible. Several cells lines have been established over the last decades, both of human (such as the U87)[10], rat (such as C6, 9L RG2, N32, N29 and CNS-1)[11,12] and mouse (such as GL261)[13-15] origin. Beside cell lines, primary glioma cells can be studied, either

*in vitro* or transplanted into immunodeficient mice (xenograft models), and genetically engineered mouse models can be used where the animal develop spontaneous tumors.[16] In the studies of this thesis, the GL261 mouse model and N29, N32 and RG2 rat models are used.

The GL261 mouse glioma cell line is the most commonly used model in mice. It was induced already in 1939 by implantation of methylcholanthrene into the brains of mice and has been widely studies since. This model is shares several histopathological properties and molecular alterations with human GBM. GL261 tumors are invasive and display poorly differentiated, pleomorphic cells with atypical nuclei and mitotic activity. They show necrotic areas, with pseudopalisading cells, and marked vascularization. Further on, they carry point mutations in the H-ras, K-ras and N-ras oncogenes as well as the p53 gene. It is a moderately immunogenic model.[13-15]

The N29 and N32 rat glioma cell lines were established in the 1990s by transplacental injection of N-ethyl-N-nitrosourea to pregnant rats, giving rise to tumors in the offspring. The N29 model resembles human GBM, with invasive growth, tumor extensions and invading micro-satellites, whereas the N32 model is more circumscribed and rarely spread into the normal brain parenchyma. They are weakly immunogenic and dominated by cells expressing the stem/progenitor markers CD133 and nestin and the neural markers glial fibrillary acidic protein, βIII-tubulin and CNPase.[12,17,18]

# Glioblastoma multiforme

Glioblastoma multiforme (GBM) is the most malignant brain tumor in adults, corresponding to a grade IV astrocytoma in the WHO grading system. It is also the most common primary malignant brain tumor, accounting for about 73% of all astrocytomas, 55% of all gliomas and 15% of all primary CNS tumors. The annual incidence is about 3.2 cases per 100 000 persons, meaning that about 300 persons are diagnosed with GBM every year in Sweden.[1,2]

GBM can occur at any age but is more common in adults, with a mean age of 62 years at diagnosis and a male:female ratio of 1.34.[1] The only established risk factor is exposure to ionizing radiation, whereas evidence of other causes, such as smoking, exposure to electromagnetic fields and the use of cell phones, is inconclusive.[19-21] However, 5% of all patients diagnosed with a malignant glioma have a family history of gliomas.[19]

In case of a primary GBM, the clinical history is usually less than 3 months. Common symptoms are headache, nausea and vomiting as a result of the increased intracranial pressure. Moreover, some patients experience epileptic seizures, focal

neurologic deficits, confusion, memory loss and personality changes, depending on the location of the tumor.[1,19,22]

More than 90% of all diagnosed GBMs are primary tumors, developing rapidly without clinical or histological evidence of a preceding, less malignant tumor. The rest, less than 10%, are secondary GBMs developing from grade II or III astrocytomas. Secondary GBM is more common in younger patients, with a mean age of 45 years at diagnosis.[1]

## Treatment

Due to rapid and infiltrative growth, GBMs are often large at the time of diagnosis. The standard treatment of GBM consists of surgery, radiotherapy and chemotherapy. Due to the infiltrative nature of the tumor it cannot be completely resected by surgery, but maximal surgical resection is desirable to reduce symptoms caused by the increased intracranial pressure and to provide tissue for histologic diagnosis. However, depending on location of the tumor, surgical resection may not always be possible. Advances in brain tumor surgery, such as intraoperative magnetic resonance imaging, brain mapping and fluorescence-guided surgery, have improved the extent of resection, but it is not clear whether this affects the patient survival.[1,19,23,24]

The addition of radiotherapy, generally 60 Gy of irradiation delivered in fractions of about 2 Gy over six weeks, further increases the survival of the patients from about 3 months to a range of 7 to 12 months. Attempts to increase the radiation dose, for example with stereotactic radiosurgery, have not improved patient outcome.[19,25]

Temozolomide (TMZ) is an alkylating prodrug with the ability to cross the blood-brain barrier. In 2005, a large clinical study concluded that six weeks of radiotherapy with concomitant TMZ treatment (75 mg/m$^2$ per day) followed by adjuvant administration of TMZ (150 to 200 mg/m$^2$ per day for 5 days every 28 days for 6 cycles) increased the survival of GBM patients with about 2.5 months, hence becoming part of the standard treatment for GBM.[9,19,26]

Several investigational molecular therapies have emerged as the understanding of the GBM biology has increased. A lot of focus has been given inhibitors targeting receptor tyrosine kinases, such as epidermal growth factor receptor (EGFR)[27], platelet-derived growth factor receptor (PDGF-R)[28] and vascular endothelial growth factor receptor (VEGF-R)[29], and signal-transduction inhibitors targeting the mammalian target for rapamycin[30] and farnesyltransferase[31]. However, such therapies have been of limited success, possibly due to the redundant signaling pathways and multiple tyrosine kinases exhibited by GBM.[19] Further on, due to the vivid neovascularization within GBMs, angiogenesis inhibitors targeting VEGF and VEGF-R are being explored.[32] Other investigational therapies include novel chemotherapeutic drugs, gene therapy, immunotherapy and treatment with antibodies.[19]

Treated GBM eventually recurs, usually within 2 cm of the original location,[33] with a median time to progression of 6.9 months after treatment with radiation and temozolomide.[9] Resistance to conventional therapy is mainly due to 1) poor drug delivery due to the blood-brain barrier and high intratumoral pressure, 2) genome instability leading to clonal populations of cells resistant to single therapies, 3) invasive tumor cells, 4) stem-like cells with resistance mechanisms different from the rest of the tumor cells and 5) DNA-repair properties. Infiltrating tumor cells often form micro-satellites localized at a distance from the contrast-enhancing tumor bulk, and consequently evade treatment with surgical resection and radiation.[1] Further on, it has been shown that migration and cell proliferation are mutually exclusive, suggesting that migrating tumor cells do not proliferate. As GBM therapy primarily target dividing cells, this indicates a further protection of infiltrating cells.[34,35] Other mechanisms for escaping treatment include activation of DNA-damage-response pathways,[36] overexpression of $O^6$-methylguanine-DNA methyltransferase (MGMT),[37] upregulation of drug resistance genes and inhibition of apoptosis.[38-40]

## Prognosis

Despite treatment with surgery, radiation and chemotherapy, the median survival of GBM is only 14.6 months, with a 2-year survival of 27.2% and a 5-year survival of 9.8%.[9,41] Prognostic factors include age, where patients younger than 50 years at diagnosis have a better prognosis, extent of necrosis, where less necrosis correlates with longer survival, and presence of MGMT methylation.[1,37] Epigenetic silencing of the DNA-repair gene MGMT by promoter methylation has shown to decrease the DNA repair activity, thus increasing the susceptibility of the tumor cells to TMZ. About 45% of all patients display a methylated MGMT promoter, resulting in a median survival of almost 22 months, compared to patients without MGMT promoter methylation with a median survival of only 12.7 months.[37] Further on, a good initial Karnofsky performance score correlates with a better prognosis.[42] Several genetic alterations, such as p53 mutation, EGFR amplification and mutation in the phosphatase and tensin homolog (PTEN), have been investigated but not correlated to patient outcome. However, loss of heterozygosity on chromosome 10q has been associated with reduced survival, whereas mutation of isocitrate dehydrogenase 1 (IDH1) correlates to longer survival.[1,43] Further on, it was recently shown that GBMs can be divided into a proneural, neural, classical and mesenchymal subclass based on gene expression, where the classical subtype is associated with better survival and the proneural and mesenchymal subclass is associated with worse survival.[44,45]

## Location and infiltration

The most frequent location of GBM is the subcortical white matter of the cerebral hemispheres, whereas the basal ganglia, thalamus, brain stem, cerebellum and spine are rare sites. The tumor often infiltrates the surrounding cortex and commonly extends to the contralateral hemisphere through corpus callosum, forming a bilateral butterfly glioma.[1]

This extensive infiltration is a typical characteristic for malignant glioma (Figure 1), and GBM is a particularly invasive tumor spreading along the perivascular space and myelinated structures of the white matter. Despite this, GBM seldom metastasize outside the brain. Invasion of the subarachnoid space is unusual, and hence tumor cells rarely spread via the cerebrospinal fluid. Further on, hematogenous spread to extraneural tissue and penetration of the dura or bone is very uncommon.[1,33]

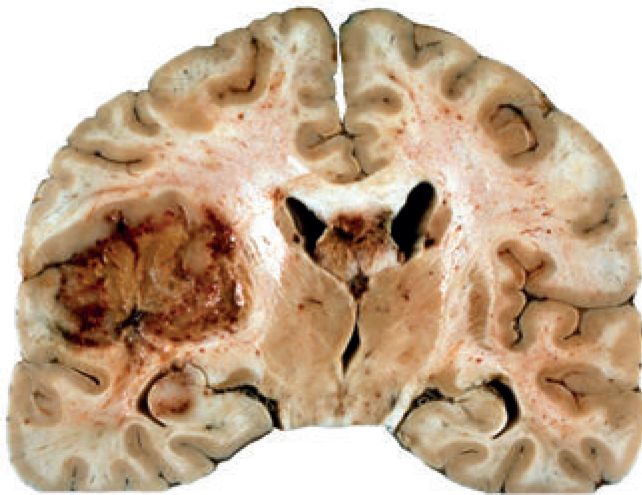

**Figure 1. Macroscopic image of glioblastoma multiforme.**
Coronal section of a human brain specimen with an invasive, partly necrotic GBM growing in the left hemisphere.
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To obtain invasive properties, the tumor cells undergo several biological processes to alter their shape and gain the ability to interact with, and degrade, the extracellular matrix (ECM). The ECM offers anchoring ligands, giving the tumor cells handles to facilitate their motility, but to pass the physical barriers they also need to degrade the ECM proteins. In this process, matrix-metalloproteinases (MMPs) have been reported to play an important role, and the upregulation of several MMPs have been shown to correlate with GBM invasiveness. Other factors and signaling pathways associated with GBM invasiveness are transforming growth factor-β (TGF-β), hypoxia inducible factor-1α (HIF-1α), PI3K/Akt, Wnt and sonic hedgehog-GLI1.[1,33]

## Histopathology

As the term "multiforme" indicates, GBMs are heterogeneous tumors with extremely variable histopathology. They are anaplastic, cellular tumors displaying pleomorphic cells with marked nuclear atypia and mitotic activity, prominent microvascular proliferation and necrosis, all occurring at different degrees in different tumors resulting in a remarkable regional heterogeneity.[1]

GBM is one of the most vascularized tumors in humans. Several mechanisms facilitate the vascularization, such as by sprouting of pre-existing vessels by endothelial cell proliferation and adoption of pre-existing vessels by migrating tumor cells. A major driving force in GBM angiogenesis is hypoxia, resulting from the rapid tumor growth and dysfunctional vasculature. HIF-1$\alpha$ has been shown to activate several genes that control angiogenesis and cellular metabolism, apoptosis and migration. One of the most important HIF-1$\alpha$ induced factors is vascular endothelial growth factor (VEGF), known to promote angiogenesis, increase the vascular permeability and recruit bone marrow-derived cells that might participate in vessel formation into the perivascular space.[1,46-48]

Necrosis is one of the main characteristics of GBM, and high degree of necrosis correlates to worse patient survival. It can be presented as large areas of non-viable tumor tissue, where the central necrosis may occupy up to 80% of the total tumor mass, or as small irregularly-shaped foci surrounded by radially oriented and densely packed pseudopalisading tumor cells (Figure 2). The mechanisms behind necrosis formation have not been clarified, but involvement of tumor necrosis factor has been proposed.[1,49]

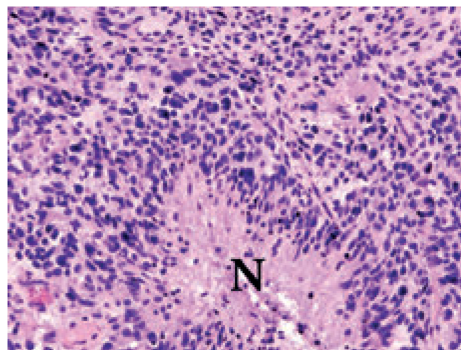


**Figure 2. Histopathological image of glioblastoma multiforme.**
Hematoxylin and eosin staining of cerebral GBM showing characteristic nuclear atypia and high cell density. A necrotic focus (N) is surrounded by pleomorphic, pseudopalisading tumor cells.
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### Genetics

The malignant transformation of normal glial cells into tumor cells is driven by the sequential acquisition of several genetic alterations, where GBM is the astrocytic tumor with the highest number of genetic changes. Primary GBM commonly features EGFR amplification and mutation, loss of heterozygosity of chromosome 10q, deletion of PTEN on chromosome 10 and p16 deletion. However, secondary GBMs are characterized by mutations in the p53 suppressor gene, overexpression of PDGF-R, abnormalities in the p16 pathway, and loss of heterozygosity of chromosome 10q. Despite these differences, primary and secondary GBM respond similarly to conventional therapy.[1,19] However, it has been shown that epigenetic silencing of the MGMT gene promoter methylation decreases the DNA repair activity and correlates with better survival.[1,37]

## Multipotent mesenchymal stromal cells

Multipotent mesenchymal stromal cells (MSCs), sometimes referred to as mesenchymal stem cells, are a heterogeneous population of non-hematopoietic progenitor cells traditionally found in the bone marrow.[50-52] MSCs were first described more than four decades ago,[53] and were long thought upon as stem cells. However, more recent research has revealed that the previously entitled mesenchymal stem cells in fact are a homogenous population in which not all cells conform to the strict stem cell definition, *i.e.* long-term self-renewal and ability to differentiate into more mature cells *in vivo*. Hence, the term multipotent mesenchymal stromal cell has been proposed for multipotent bone marrow-cells isolated by plastic adherence, whereas the term mesenchymal stem cell should be reserved for the uniform subset of these cells that are actual stem cells.[50,54]

In 2005, due to an increasing inconsistency within the research field in how to define, isolate and characterize MSCs, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed a standard set of criteria to define human MSCs *in vitro*[55]:

- MSCs should adhere to plastic when maintained under standard culture conditions.
- MSCs should express the surface markers CD73, CD90 and CD105 but lack expression of the hematopoietic surface markers CD11b or CD14, CD19 or CD79α, CD34, CD45 and HLA-DR.
- MSCs should be able to differentiate into adipocytes, osteoblasts and chondrocytes.

In the search for a less complex definition of the MSC phenotype, or ideally a single surface marker specifically expressed by MSCs, several other markers and marker combinations have been suggested for isolation of MSC. Commonly used markers are STRO-1[56,57], CD271[58], CD146[59], stage-specific embryonic antigen-4[60], GD2[61], CD 56[62], CD140b, and CD200.[63,64]

## MSC source and functions

The original source of MSCs was the bone marrow, where they constitute 0.01-0.001% of all cells.[65] Today however, MSCs have been shown to exist in a variety of tissues, such as adipose tissue[66], lung[67], skin[68], placenta[69] and umbilical cord blood[70].

The functions of MSCs are several, being precursors for adipocytes, osteoblasts and chondrocytes, contributing to homeostasis of the hematopoietic compartment and provide modulatory signals to hematopoietic progenitors. Further on, MSCs have a remarkable immunosuppressive activity. By secreting mediators such as interleukin-10 (IL-10), prostaglandin $E_2$ ($PGE_2$), nitric oxide and TGF-β, MSCs have shown to inhibit T cell activation, B cell proliferation and dendritic cell (DC) differentiation as well as impair the cytolytic potential of natural killer cells.[71] Due to the hematopoietic regulation and immunosuppressive properties, MSCs have been used to facilitate engraftment of transplanted hematopoietic stem cells and treat graft-versus-host disease.[72] However, it was recently reported that MSCs can be polarized into a pro-inflammatory type as well,[73,74] and they have been shown to acquire an immunostimulatory phenotype and antigen-presenting properties upon exposure to interferon-γ (IFNγ).[153-156] This MSC polarization could be possibly advantageous when using MSCs in the treatment of gliomas.

## MSC tumor tropism

In 2000, Aboody et al. demonstrated that neural stem cells show tumor-tropic properties when transplanted into malignant gliomas.[75] It was later shown that MSCs possess the same capacity and display a superior tumor-specific tropism upon intratumoral transplantation, migrating extensively throughout the tumor, along its extensions and to distant tumor microsatellites, while avoiding the normal brain parenchyma.[76-78] They are easy to obtain through bone marrow puncture and to expand *in vitro*, making them promising candidates for cell-based gene therapy where they can act as specific delivery vehicles of tumoricidal substances.[79,80] However, several safety concerns have been raised regarding transplantation of MSCs into malignant gliomas. It has been reported that MSCs can promote tumor growth as they might act immunosuppressive[81,82], contribute to the tumor stroma[83,84] and vascularization[85,86] and undergo malignant transformation[87,88].

The mechanisms behind MSC migration are not completely elucidated, but several tumor components have been reported as important regulators. Inflammatory factors, such as IL-8[89], monocyte chemotactic protein-1 (MCP-1)[90], stromal cell-derived factor-1α (SDF-1α)[91] and hepatocyte-growth factor[92] have all been reported to attract MSCs.[93] Further on, the tumor angiogenesis plays a major part in MSC migration, and tumor angiogenesis-associated factors such as PDGF-BB, PDGF-D, VEGF-A, TGF-β1 and neutrophin-3 have been reported to mediate MSC recruitment.[48,77,94] The intratumoral MSC migration occurs preferentially along tumor vessels, suggesting angiogenic signaling might be involved in intratumoral migration as well.[78] It has also been shown that MSCs remodel the ECM during migration, and upregulation of MMP-1 has been shown to increase MSC migration towards gliomas.[95,96]

## MSCs in glioma therapy

Several studies have utilized MSCs as tumor-specific cellular vehicles to deliver anti-tumoral substances to gliomas, with encouraging results in experimental models.

Delivery of pro-inflammatory cytokines is an approach intended to enhance the immunological response to the tumor, and MSCs transduced to produce IFN-β[77], IL-7[97] or IL-23[98] have shown potential to prolong survival in glioma-bearing animals. Further on, it has been reported that the therapeutic effect can be potentiated when combined with systemic immunotherapy.[97]

Another investigated factor is tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). It can selectively target tumor cells and induce apoptosis through activation of the pro-apoptotic death receptors 4 and 5.[99,100] TRAIL-producing MSCs have shown therapeutic effect against glioma in several studies.[91,101,102]

Suicide gene therapy with enzymes converting inactive pro-drugs into toxic substances has been studied in glioma, but with little success due to the use of viral vectors with limited intratumoral distribution.[103] However, MSCs transduced to express a pro-drug converting enzyme and migrating throughout a glioma can convert a systemically administered pro-drug and deliver the active product to large parts of the tumor.[104,105] The pro-drug is activated within the MSC and then transferred to neighboring cells through gap junctions, a process termed the bystander effect.[106-109] A widely studied pro-drug system is the herpes simplex virus-1 thymidine kinase (HSV-TK) combined with the guanosine analogue ganciclovir (GCV). HSV-TK activates GCV by phosphorylation, and it is then transferred to adjacent cells and incorporated in their DNA, disrupting the DNA synthesis and leading to cell death. Other studied pro-drug systems that work in a similar manner are cytosine deaminase combined with the pyrimidine analogue 5-fluorocytosine and *Drosophila melanogaster* deoxyribonucleoside kinase combined with the cytidine analogue gemcitabine.[110-113]

Another currently investigated field is bionanotechnology. Nanoparticles give the potential to use a single vehicle for both diagnosis and customized therapeutic drug delivery, and MSCs are currently explored as containers to efficiently deliver and spread drug-loaded nanoparticles throughout the tumor.[114-116] Such treatment is dependent on that the nanoparticles can be incorporated in the MSCs and that they do not change the migratory capability of the cells.[117,118]

Therapy with oncolytic viruses has shown promising results, but limitations in vector distribution within the tumor poses a problem. Further on, the immune system often neutralizes the viral particles within the tumor.[119] However, several studies have suggested that MSC-mediated oncolytic virus delivery can improve the survival of glioma-bearing animals.[120-122]

MSC-mediated delivery of anti-tumor antibodies might reduce side-effects caused by systemic antibody delivery and potentiate the therapy efficacy.[123] It has been shown that MSCs expressing a cell surface-bound single-chain antibody against EGFR variant III (EGFRvIII) reduces the tumor vascularization and increases survival of glioma-bearing mice.[124]

## Pericytes

Pericytes are a heterogeneous population of perivascular cells that line the microvasculature throughout the body, forming the basement membrane of the microvessels together with the endothelial cells. Pericytes are contractile cells that play an important role in stabilizing the blood vessels and regulating the blood flow, but they are also involved in vessel formation, communicating with the endothelial cells through gap junctions and paracrine signaling pathways. It has been shown that pericytes and MSCs are biologically related, as MSCs can be found in the perivascular compartment and share several characteristics with pericytes.[125-127]

The phenotype of pericytes depends on the tissue in which they are located, but commonly used markers are PDGFR-β, α-smooth muscle actin (α-SMA), neuron-glial antigen 2 (NG2), nestin and regulator of G-protein signaling 5 (RGS5).[125,128]

The brain is one of the most pericyte-dense organs of the body, as pericytes constitute an important part of the blood-brain barrier (BBB). The BBB is a selectively permeable space between the blood vessels and the cells of the brain, protecting the brain cells from potentially toxic blood-derived factors. In turn, pericytes protect the BBB from disruption and have been reported to have macrophage-like properties within the brain.[129]

# Tumor immunology

The immune system plays a critical role in recognizing and eliminating early tumors, a process termed immunosurveillance. However, it has also been shown to promote tumor growth factors by producing pro-angiogenic factors, cytokines and growth factors, thus playing a dual role in tumor development. Hence, immunosurveillance has been suggested to be part of a broader process called immunoediting, describing how the immune system on one hand eliminates tumors, but on the other hand promotes the development of less immunogenic tumors.[130,131]

## Immunoediting

The dynamic process of immunoediting is defined as three phases: elimination, equilibrium and escape.

Elimination corresponds to immunosurveillance, where cells surrounding an early tumor become affected by its expansion and start to release factors that attract the innate immune system. Natural killer (NK) cells, DCs and macrophages start to eliminate the tumor cells, releasing tumor antigens that attract the professional antigen-presenting cells (APCs) of the adaptive immune system. They in turn activates T cells by displaying the phagocytized antigens in the draining lymph nodes, where after the T cells home to the tumor and eliminate the remaining tumor cells.[130]

Equilibrium is the phase where the lymphocytes no longer are able to eradicate the tumor cells, but rather contain them. During this phase, the remaining tumor cells are able to mutate and give rise to new cells with decreased immunogenicity.[130]

Escape is the last phase, where the tumor cells have acquired resistance to detection and attacks from the immune cells, allowing them to expand and give rise to a tumor.[130]

## Immunosuppression

To avoid recognition and elimination by the immune system, tumor cells have several ways to suppress it. They can down-regulate their expression of major histocompatibility complex (MHC) molecules to avoid displaying tumor antigens.[130] They can also secrete immunosuppressive factors such as TGF-β[132], IL-10[133] and $PGE_2$[134]. Further on, it has been shown that the tumors can secrete factors to attract regulatory T cells (Tregs)[135], myeloid derived suppressor cells[136] and tumor-associated macrophages[137], all exerting an immunosuppressive effect.

## Immunotherapy against malignant glioma

Immunotherapy against malignant gliomas aims at stimulating the immune system to destroy the tumor cells and counteract their immunosuppressive mechanisms.[138]

Passive immunotherapy is based on a concept where immune cells are isolated from the patient, activated, expanded and sometimes genetically modified *ex vivo*, and then injected back into the patient.[139] It can also involve administration of antibodies targeting a specific glioma antigen, such as EGFRvIII.[140] A drawback of passive immunotherapy is that it does not provide a long-term antitumor response.[139]

Active immunotherapy on the other hand is based on activation of the endogenous immune system, resulting in long-term antitumor effects. This helps eliminating the tumor, but also decreases the risk of tumor recurrence. It is mediated through administration of T cells, DCs or macrophages activated *ex vivo*, or by vaccine therapy with tumor antigens.[139] As GBMs are very heterogeneous tumors and no common antigen shared between all tumor cells has been discovered, it has been suggested that whole tumor cells, or tumor cell lysate, should be used as vaccine.[141,142] The effect of the immunization can be further enhanced by using an adjuvant, a molecule, such as aluminum-based salts, that activates APCs and NK cells.[143,144] APCs phagocytize the injected antigens and present them to naïve T cells, thereby activating them, in lymph nodes or the spleen. The T cells are transported to the tumor via the circulation and enter through the vessel wall, where they subsequently stimulate other immune cells to react to the tumor.

Several immunostimulatory factors have been reported to potentiate the effect of immunotherapy, such as IFNγ[18], IL-2[145], IL-7[97], IL-12[146] and IL-23[98]. IFNγ is a cytokine secreted by T cells, NK cells and DCs, inducing upregulation of the MHC class I and II molecules as well as other immunostimulatory factors. Almost all cell types have receptors for IFNγ.[147,148] IL-2 is a cytokine affecting several immune cells, being reported to induce T cell proliferation as well as reverse T cell anergy.[149] IL-7 is a cytokine important for T cell development, proliferation and survival.[150,151] While it stimulates expansion of $CD4^+$ and $CD8^+$, it has the beneficial effect of decreasing the percentage of Tregs.[152] IL-12 is a cytokine produced by phagocytic and antigen-presenting cells, activating NK cells and inducing IFNγ production.[153] IL-23 is a cytokine sharing a subunit with IL-12. It is produced by activated DCs and has, unlike IL-12, been reported to induce proliferation of memory T cells.[154]

# Aims of the thesis

The general aims of this thesis were to investigate multipotent mesenchymal stromal cells in the context of malignant glioma; their presence and functions within the tumors and their potential as cellular vectors in experimental malignant glioma therapy.

More specifically, the aims were:

- To study the presence of endogenous MSCs, as defined by the ISCT, within human malignant gliomas (paper I).
- To investigate the production of VEGF and $PGE_2$, critical factors for tumor angiogenesis and immunosuppression, by endogenous MSCs within human malignant gliomas (paper I).
- To examine *in vivo* recruitment of endogenous, MSC-like pericytes in the mouse brain to an experimental, orthotopic mouse glioma (paper II).
- To investigate the migratory properties of BM-MSCs in a syngeneic rat model after transplantation into, or at a distance to, an experimental, orthotopic glioma or into a healthy brain (paper III).
- To examine the proliferation capacity of BM-MSCs transplanted into an experimental malignant rat glioma (paper III).
- To investigate if intratumoral BM-MSC transplantation can increase animal survival and intratumoral T cell infiltration when combined with peripheral immunotherapy with IFNγ producing tumor cells against an experimental malignant rat glioma (paper IV).

# Results and discussion

## Paper II: Endogenous brain pericytes are widely activated and contribute to mouse glioma microvasculature.

A major reason for the poor GBM prognosis is the rapid and infiltrative growth of the tumor, largely facilitated by extensive neovascularization.[1] Pericytes constitute an important cellular component of this GBM vasculature, where they mediate immunosuppression and promote endothelial cell survival.[82,167,168] The source of pericytes in GBM is not known, but both generation from tumor stem cells[169] and recruitment from the bone marrow[170] have been reported. In this study, we investigated the contribution of normal brain pericytes to GBM vasculature. We used a knock-out/knock-in C57BL/6 mouse line expressing GFP under the pericyte-specific RGS5 promoter[171,172] and the syngeneic orthotopic mouse glioma model GL261. It is a widely used glioma model as it is well characterized and closely mimics the invasiveness and angiogenesis of human GBM.[13-15]

Tumors were established by stereotactic injection of 5000 GL261 cells into the caudate nucleus of the mice, and after 19 days the animals were cardially perfused with 4% paraformaldehyde. The pericytes were visualized with GFP-DAB in a light microscope, or, when investigating co-localization with other markers, immunofluorescent GFP staining in a confocal microscope.

Under pathological conditions pericytes can become activated and get a more prominent cell body (Figure 4A-B),[128] and our results show that, in response to the orthotopic GL261 glioma, the number of activated $GFP^+$ pericytes within the cerebral cortex was significantly increased compared to non-tumor-bearing mice (mean: 127 ± 4.97 and 48.5 ± 2.28, respectively; $p<0.001$) (Figure 4C). The increase was observed both in the ipsilateral and the contralateral hemisphere, indicating widespread pericyte activation. Activated pericytes were also found in the ipsilateral subventricular zone (SVZ), an active and proliferative area of the brain known to be reactive and produce neuroblasts in response to glioma.[173,174]

The pericytes were not only present in the cerebral cortex but also found within the tumor, exhibiting a different morphology compared to the pericytes in the normal brain parenchyma (Figure 4D). While the pericytes in the cortex were elongated and small, though with a prominent cell body, the intratumoral pericytes showed either a flattened cell body with elongated processes or a prominent cell body with retracted finger-like projections (Figure 4E-F).

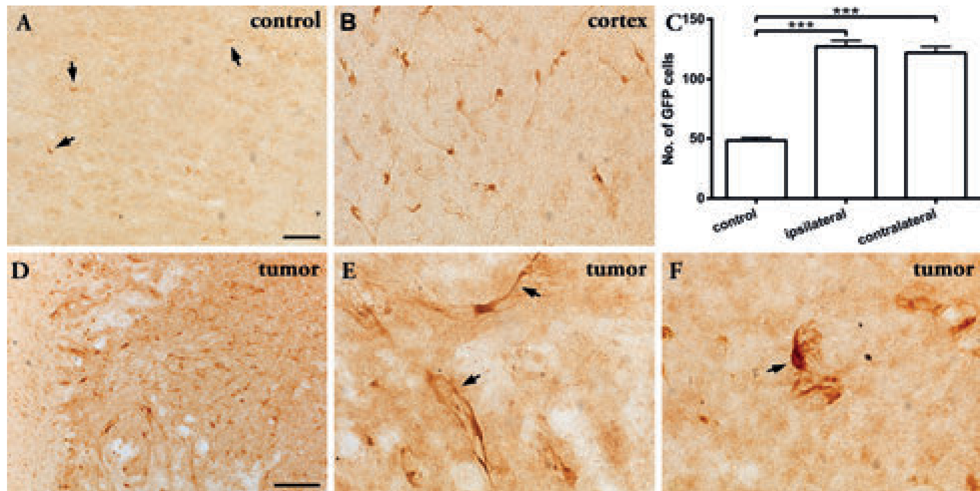


**Figure 4. Brain pericytes are activated in response to intracerebral glioma.**
GFP-DAB staining of brain pericytes. (A) In the normal brain, quiesent pericytes show a flat mophology with a small cell body (arrows). (B) However, in response to a GL261 glioma, the pericytes in the cerebral cortex become activated and get a more prominent cell body. (C) The number of GFP+ pericytes was significatnly increased in response to the tumor, both in the ipsilateral and contralateral hemisphere, compared to normal mouse brain (n = 3, mean ± SEM, *** = p<0.001, ANOVA). (D) The pericytes infiltrated the GL261 tumor and displayed a different morphology compared to pericytes in the cortex, with either (E) a flattened cell body with elongated processes (arrows) or (F) a prominent cell body with tuft-like projections (arrow). Scale bar in A, B, E and F is 20 µm and scale bar in D is 100 µm.

Laminins are important for glioma cell invasion and growth,[175] and immunofluorescent staining showed that the GL261 gliomas expressed laminin at high levels. Migrating GFP+ pericytes were located in close proximity to these laminin+ cells, adjacent to but also at laminin+ tumor microsatellites distant from the main tumor bulk (Figure 5), but no overlap between laminin and GFP was seen.

Several molecular regulators for pericyte activation and function have been defined,[129,176] but the mechanism behind this widespread pericyte activation in response to an intracerebral glioma is unknown. Possible activation mechanisms include parenchymal diffusion of tumor produced factors, systemic exosomes derived from the tumor or hypoxia and edema resulting from elevated intracranial pressure.[177-179] Our results suggest that the GL261-induced activation, and possibly recruitment,

of normal brain pericytes might depend on the interaction with the laminin-rich vascular basement membrane of the tumor.

However, as pericytes were also activated at a significant distance from the tumor, we investigated the involvement of hypoxia. It is a well-known characteristic of malignant gliomas,[1] and HIF-1α has been shown to be involved in the attraction of pericyte progenitors to GBM in mice.[170] Immunofluorescent staining for glucose transporter 1 (GLUT1), associated with tumor hypoxia due to the increased need for glucose,[180] revealed that $GFP^+$ pericytes were primarily localized at hypoxic, but also at normoxic, regions of the tumor. The $GLUT1^+$ areas were mainly localized in the tumor periphery, where $GFP^+$ pericytes seemed to form a stream of migrating cells from the surrounding normal brain tissue. This further highlights hypoxia as an important element for pericyte recruitment. No $GLUT1^+$ cells were found within or near the SVZ.

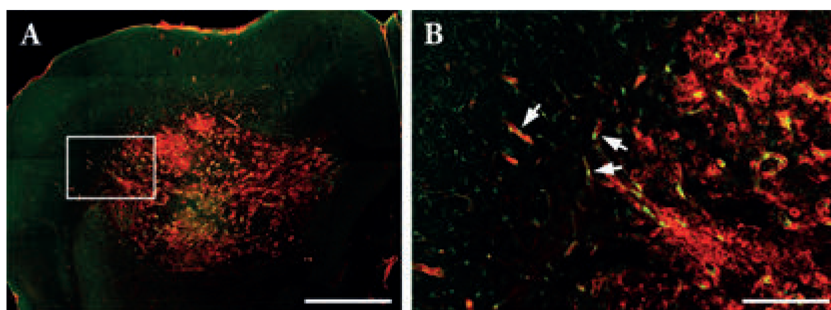


**Figure 5. Laminin-expressing GL261 tumor with recruited pericytes.**
(A) Overview image of GL261 tumor expressing high levels of laminin (red). (B) Higher magnification of the area marked in A, showing $GFP^+$ pericytes (green) associated with laminin-expressing tumor satellites (arrows). Scale bar in A is 1000 μm and scale bar in B is 200 μm.

Tumor vessels were visualized with immunofluorescent staining for CD31 and VEGF-R,[47,181] where the latter is an important factor for angiogenesis, and possibly invasiveness, of gliomas.[182] We found that approximately three quarters of all VEGF-$R^+$ tumor vessels were covered by $GFP^+$ pericytes recruited from the host. The pericytes aligned close to the VEGF-$R^+$ cells but did not express VEGF-R themselves.

PDGFR-β is expressed on the vast majority of all pericytes and has been reported as an important factor for pericyte recruitment to tumor vessels.[129,183-185] We found that all intratumoral $GFP^+$ pericytes also expressed PDGFR-β, but more interestingly, constituted 57 ± 6.6% of all PDGFR-$\beta^+$ cells within the tumor. This indicates that the normal brain vasculature contributes the majority of the pericytes in GL261 gliomas, which contradicts another recent study concluding that the majority of the intratumoral pericytes is derived from glioma stem cells.[169] Of course, our findings do not rule out the possibility that a proportion of the pericytes are derived from the tumor itself, and the diverse results between the studies might be explained by

differences in plasticity and differentiation potential between different tumor models. In fact, the GL261 mouse glioma model is a heterogeneous cell line containing subpopulations of cells with retained differentiation capacity that might give rise to the $GFP^-PDGFR\text{-}\beta^+$ pericytes we observe within the tumors.[15,186,187] However, as we use non-labeled tumor cells, no conclusions can be drawn regarding the source of these cells. Further experiments are needed to clarify their origin, and whether the pericyte recruitment we observe here is present in other glioma models and, importantly, in human malignant gliomas as well.

Beside PDGFR-β, expressed on all $GFP^+$ pericytes, the presence of several other markers associated with pericytes were investigated by immunofluorescent staining. NG2 is a marker associated with activated pericytes and angiogenesis.[188] It was expressed on 55 ± 12% of the intratumoral $GFP^+$ pericytes, indicating that the tumor also harbors non-activated pericytes. Further on, CD13, a marker for pericytes and mesenchymal stromal cells,[126,185,189] was expressed on 26 ± 15% of all $GFP^+$ pericytes within the tumor. However, CD13 was more commonly expressed on $GFP^+$ cells outside the tumor, suggesting a phenotypic shift as the pericytes enter the tumor. The majority of the intratumoral $GFP^+$ pericytes, 86 ± 7.7%, weakly expressed the pericyte marker α-SMA.[129,185] All markers were expressed on both types of morphologically different pericytes, but CD13 seemed to be primarily expressed on flat, elongated cells. The variation in marker expression on the intratumoral pericytes reflects that they are a heterogeneous cell population, known to alter their phenotype depending on the surrounding tissue and under pathological conditions.[125] For example, NG2 has been shown to be upregulated in response to angiogenesis,[188] and α-SMA becomes upregulated in the CNS in response to a tumor.[190] Hence, as it is likely that the intratumoral pericytes are recruited to the tumor at different time points, it is also likely that they do not show the same marker expression.

A subset of the $GFP^+$ pericytes within the tumor, 16 ± 1.7%, expressed the proliferation marker Ki67, suggesting that pericyte proliferation also contributes to the process of glioma vascularization. However, the extent of this contribution is unknown as Ki67 only labels actively proliferating cells at the exact time of tissue perfusion.[191] Hence, our results might be an underestimation of the contribution of proliferating pericytes, as opposed to recruitment of already existing pericytes, to the tumor vasculature.

It was recently shown that $RGS5^+$ brain pericytes become activated microglia after recruitment to ischemic stroke.[192] To investigate whether pericytes recruited into GL261 gliomas adopt a different phenotype, they were immunofluorescently stained for S100 calcium binding protein B, expressed by mature astrocytes surrounding blood vessels,[193] and ionized calcium-binding adapter molecule 1 (Iba1), expressed by microglia.[194] Both markers were widely expressed within the tumors, but not co-expressed by the intratumoral $GFP^+$ pericytes. However, $Iba1^+$ microglia cells were found in very close proximity to the pericytes, suggesting possible juxtracrine-like communication.

# Paper III: Rat multipotent mesenchymal stromal cells lack long-distance tropism to 3 different rat glioma models.

MSCs have an inherent tropism for gliomas. Their ability to specifically migrate throughout the tumors and track single tumor cells makes them promising candidates for cell-based glioma gene therapy where they can act as delivery vehicles for tumoricidal substances.[76-78] Studies have shown that MSCs can migrate throughout normal brain parenchyma if implanted at a distance from the tumor,[76,77] but their migratory capacity has not been investigated in detail using adult MSCs syngeneic to both the glioma and the host tissue. In this study, we investigated MSC migration and proliferation after transplantation into, or outside, syngeneic orthotopic rat gliomas. Further on, the migratory capabilities of MSCs transplanted into partially resected tumors was elucidated. We used adult rat BM-MSCs and the three orthotopic rat glioma models N29, N32 and RG2, all syngeneic to the Fischer 344 rat.[12,17,195,196]

Tumors were established by stereotactic injection of 1000 or 3000 tumor cells into the right striatum or right frontal corpus callosum of the rats and the animals were cardially perfused with 4% paraformaldehyde before the brains were analyzed. Previously characterized[78] rat BM-MSCs were transduced to express enhanced green fluorescent protein (eGFP)[197] and visualized by immunofluorescent GFP staining and epifluorescence or confocal microscopy. In the present study, immunofluorescent staining also revealed that the MSC-GFPs express the mesenchymal markers CD73 and CD90 and desmin.[198,199]

To investigate MSC migration within malignant rat gliomas, 250 000 MSC-GFPs were transplanted into orthotopic N32 gliomas on day 14 after tumor inoculation. One day after the transplantation, the majority of the grafted MSCs were found at the inoculation site and a few cells were localized in the tumor periphery. At day 4 after MSC transplantation, a large proportion of the MSCs had entered the tumor periphery and single cells were located in the tumor center, whereas the majority of the cells were spread throughout the whole tumor at day 8 after transplantation (Figure 6). At all time points, only a few MSCs were located in the normal brain parenchyma.

To better mimic the clinical scenario, we investigated MSC migration in N32 gliomas after partial surgical resection. Tumor cells were inoculated into the striatum, and fourteen days later the established tumor was partially resected. At the time of surgery, 250 000 MSC-GFPs were transplanted into the remaining tumor mass. Seven days later, the MSCs had migrated extensively throughout the remaining tumor tissue, whereas only a few cells were found in the adjacent normal brain tissue.

Next, we analyzed whether MSC-GFPs grafted into the normal brain parenchyma would migrate toward an N29, N32 or RG2 tumor. Seven days after tumor inoculation into the striatum, 250 000 MSC-GFPs were transplanted 4 mm caudally and 2 mm lateral to the tumor inoculation coordinates. Further on, tumor cells were inoculated into corpus callosum, a known migration route,[1,76] and seven days later 250 000 MSC-GFPs were transplanted to the corresponding coordinates on the contralateral side. On day 14 after MSC transplantation, no MSCs showed any signs of specific migration through the striatum or along corpus callosum toward any of the three tumor models. Instead, the vast majority of the transplanted MSCs were still located at the inoculation site, whereas a few cells had spread to its immediate vicinity.

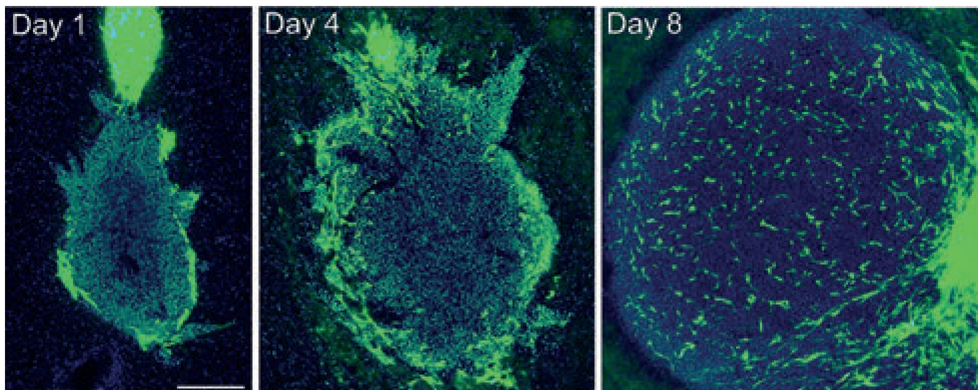


**Figure 6. Migration of intratumorally transplanted BM-MSCs.**
Migration pattern of MSC-GFPs (green) transplanted into a syngeneic, orthotopic N32 rat glioma (blue). One day after MSC transplantation, the cells were mainly localized within the graft, and to some extent in the tumor periphery. Four days after transplantation, the MSCs had started to migrate into the tumor, and after eight days they were found throughout the whole tumor. Scale bar is 200 μm.

The best route of administration in MSC-based glioma therapy remains to be determined. Several studies have suggested intraarterial[77,200] or intravenous[201] cell injections, but we have not seen any evidence of intratumoral MSC-GFPs after intravenous MSC injection in our syngeneic model.[78] Further on, systemic administration of MSCs is associated with severe side effects, such as pulmonary embolism.[202,203] Instead, the present study shows that intratumoral MSC injection might be the preferred route of administration as it results in extensive tumor-specific migration even when parts of the tumor have been surgically resected. In contrast to previous studies,[76,77] we found no evidence of long-distance MSC migration throughout normal brain tissue.

The different results obtained in these studies might be explained by the use of different types of models.[76,77] Interactions between cells of different species might influence migration, and different glioma models might produce different levels of

migration-mediating factors within and outside the tumor.[48,89,94,95,204,205] Further on, different subpopulations of BM-MSCs might have different tumor-tropic migratory properties.[206] The interpreting of the results is also dependent on the cell labeling technique. Whereas labeling-dyes can be unspecifically transferred between adjacent cells with retained fluorescence,[207] genetic labeling, such as eGFP transduction, should be less likely to unspecifically spread to the surrounding tissue. We have previously shown that the expression pattern of eGFP *in vivo* correlates with fluorescence in situ hybridization analysis of the Y chromosome in male MSC-GFPs transplanted into female hosts.[78]

To study long-term survival and migration of MSCs in the healthy brain, MSC-GFPs were transplanted into the striatum or corpus callosum of non-tumor-bearing animals. After 118 days, low numbers of MSCs were found at the site of transplantation, but at no other location of the brain. Additionally, the presence of MSC-GFPs was evaluated within the liver, spleen and cervical lymph nodes, but no $eGFP^+$ cells were found in these organs.

The mechanism behind the tumor-tropic MSC migration is not clear, but several tumor-related factors have shown to attract stem and progenitor cells to gliomas.[93,208] In this study we show that MSCs need contact with the tumor to migrate, suggesting that the tumor microenvironment, with active neoangiogenesis and inflammation, is required for migration of transplanted MSCs.

Tumor formation from grafted stem cells is a major concern in stem cell-based therapy,[209] and we evaluated the proliferation rates of intratumorally transplanted MSC-GFPs. The cells were grafted into established orthotopic N32 gliomas and analyzed for expression of the proliferation marker Ki67 at day 8 and 16 after MSC-GFP transplantation.[191] In each tumor, 100 MSC-GFPs were randomly chosen throughout the tumor and at the graft site, but none of them expressed Ki67. This suggests that the absolute majority of the intratumorally grafted MSCs do not proliferate 8 and 16 days after transplantation, indicating a low risk for secondary malignancies.

## Paper IV: Intratumorally implanted mesenchymal stromal cells potentiate peripheral immunotherapy against malignant rat gliomas.

Peripheral immunotherapy using IFNγ-secreting tumor cells has shown great potential in the treatment of experimental gliomas.[210-212] We hypothesized that intratumorally (i.t.) transplanted MSCs, generally considered immunosuppressive but shown to acquire an immunostimulatory phenotype upon IFNγ exposure,[73,74,213-216]

could potentiate such glioma immunotherapy. The tumor-tropic properties of MSCs give them the possibility to reach and exert their immunostimulatory effect throughout the whole tumor and at migrating tumor microsatellites.[76-78] We used the N32 rat glioma model[12,17] and eGFP-transduced rat BM-MSCs[197], both syngeneic to the Fischer 344 rat.

To investigate whether our MSC-GFPs could adopt an immunostimulatory phenotype *in vitro*, 100 000 cells were cultured with recombinant IFNγ (rIFNγ, 0-10 000 U/ml). After 24 hours, the levels of the immunosuppressive factors $PGE_2$[161,163,164] and IL-10[133,217] in the supernatants were analyzed with ELISA, and the expression of MHC class I and II on the cells was analyzed by flow cytometry. It has been previously reported that MSCs upregulate MHC expression and get antigen-presenting capabilities in response to IFNγ.[213-215] Low levels of both $PGE_2$ (mean: 81 pg/ml) and IL-10 (mean: 2 pg/ml) were naturally produced by the MSC-GFPs, but neither of them was significantly downregulated upon rIFNγ treatment. This treatment, however, resulted in a significant upregulation of MHC class I and II by the MSC-GFPs.

To assess whether the MSC-GFPs could potentiate the effect of peripheral immunotherapy with IFNγ-producing tumor cells, 3000 N32 cells were inoculated into the right striatum of Fischer 344 rats. On day 1, 14, and 28 after tumor inoculation, 3 000 000 irradiated (80 Gy) IFNγ–producing N32 (N32-IFNγ)[210] cells were injected subcutaneously (s.c.) on the right thigh, and on day 7 and 17, 250 000 MSC-GFPs were transplanted into the established N32 tumors (Figure 8A). The treatment with intratumoral MSCs significantly increased the animal survival (54% survivors) compared to treatment with immunotherapy (21% survivors; $p<0.01$) or MSC-GFPs (0% survivors; $p<0.001$) alone (Figure 7).

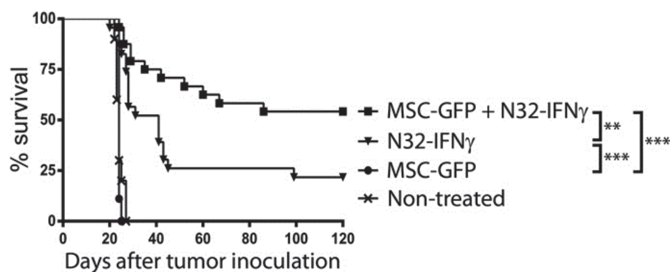


**Figure 7. Kaplan-Meier survival graph.**
Treatment with intratumoral MSCs and peripheral immunotherapy increased animal survival compared to treatment with MSC transplantation or immunotherapy alone. Groups include 9-24 animals and were compared using log-rank test.

Last, we investigated the amounts of infiltrating T cells, known to be associated with GBM patient survival and a major source of IFNγ.[147,218,219] It has previously been shown that immunotherapy alone increases the plasma IFNγ levels as well as systemic

and intratumoral levels of T cells,[211,212,220-223] and MSCs have previously been shown to induce $CD8^+$ T cells when treated with IFNγ.[214] Tumors were inoculated by stereotactic injection of 3000 N32 cells into the right striatum of Fischer 344. On day 4 and 14 after tumor inoculation, 3 000 000 irradiated N32-IFNγ cells were injected intraperitoneally (i.p.), and on day 7 and 11, 250 000 MSC-GFPs were transplanted into the established N32 tumors (Figure 8B). At day 25 after tumor inoculation, the brains were snap frozen, immunohistochemically stained for T cell receptor αβ (TCRαβ) and CD8α, expressed on T cells,[224] and analyzed on a light microscope. The results showed that the amount of intratumoral $TCR\alpha\beta^+$ cells was significantly increased in animals receiving the combined therapy (mean: 9.0%) compared to animals treated with immunotherapy (2.1%; $p<0.01$) or MSC-GFPs (0.5%; $p<0.001$) alone. Similar results were obtained for $CD8\alpha^+$ cells, where the combination therapy caused the highest infiltration (mean: 8.0%) compared to treatment with immunotherapy (3.5%; $p<0.05$) or MSC-GFPs (1.6%; $p<0.001$) alone.

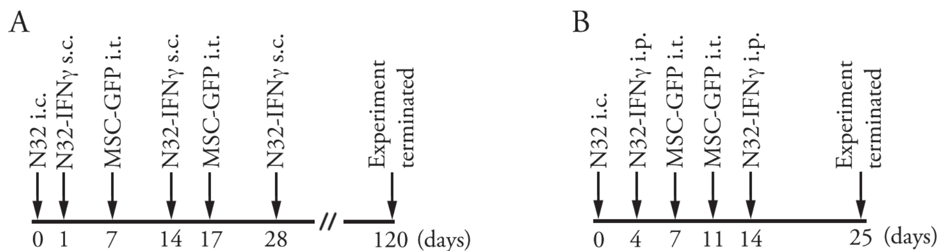


**Figure 8. Experimental setup of *in vivo* experiments.**
(A) Experimental setup of survival study. (B) Experimental setup of T cell infiltration study.

The present study suggests that intratumorally transplanted BM-MSCs can enhance the pro-inflammatory tumor-microenvironment generated by peripheral immunotherapy. The mechanism of this enhancing effect is not completely clear, but the MSCs might act as antigen presenting cells or induce $CD8^+$ T cells.[213-215] Moreover, we have previously shown that intratumorally transplanted MSCs act as pericytes,[78] cells reported to normalize tumor vessels and increase the amount of infiltrating T cells in response to immunotherapy.[225] However, the MSCs have no effect when they are intratumorally transplanted without preceding immunotherapy, possibly because those cells are grafted into a non-primed, immunosuppressive environment. In this scenario, they might even exert an immunosuppressive function within the tumor.[164,226] Another possible explanation for the enhancing effect of immunotherapy is that the immunogenic GFP protein expressed by the transplanted MSCs affects the immune system.[227-229] However, as the animals treated with MSC-GFP transplantation alone did not show an increased survival, GFP immunogenicity is most likely of limited effect.[230]

# Conclusions

The main conclusions of this thesis are:

- Human malignant gliomas harbor two distinct MSC-like cell populations differing in their expression of the CD90 surface marker (paper I).
- The $CD90^-$ population produces higher amounts of VEGF and $PGE_2$ compared to its $CD90^+$ counterpart, suggesting a more active involvement in tumor vascularization and immunosuppression (paper I).
- Brain pericytes become activated in widespread areas of the brain in response to orthotopic GL261 mouse gliomas (paper II).
- The activated pericytes infiltrate the glioma extensively, integrate with the glioma vasculature, and constitute the majority of all pericytes in the GL261 glioma model (paper II).
- Rat BM-MSCs efficiently and specifically spread throughout experimental rat gliomas upon intratumoral implantation. However, they do not migrate to the liver, spleen and cervical lymph nodes and do not proliferate, indicating a low risk of developing secondary malignancies (paper III).
- Rat BM-MSCs do not migrate through the striatum or across corpus callosum when transplanted outside an intracerebral N29, N32 or RG2 rat glioma, suggesting intratumoral transplantation as the best route of administration for MSC-based glioma therapy (paper III).
- Intratumoral implantation of BM-MSCs potentiates immunotherapy with IFNγ-producing tumor cells, leading to increased intratumoral T cell infiltration and survival of glioma-bearing rats (paper IV).

# Future perspectives

GBM is one of the most challenging diseases in oncology, and even after surgery, radiation and chemotherapy the prognosis for the patients remains poor. Despite large research efforts over the last decades, treatment outcome has only slightly improved and the last significant advancement, the introduction of chemotherapy with TMZ, was introduced more than ten years ago.[9] New treatment strategies need to be explored, and cell-based therapy with MSCs is an approach that shows promising progress. The tumor-tropic properties and migratory capabilities of MSCs make them excellent carriers of antitumoral substances specifically targeting the tumor cells that conventional treatment cannot reach.[76-78]

To develop a treatment based on intratumorally transplanted MSCs, all related safety concerns have to be thoroughly investigated. MSCs have been reported to facilitate tumor growth by promoting neoangiogenesis and mediate immuno-suppression,[81,82,85,86] and it has been shown that human malignant gliomas with a mesenchymal gene expression profile correlate with short patient survival.[44,45] In the first study of this thesis, we show that human malignant gliomas harbor MSC-like cells that express high levels of $PGE_2$ and VEGF. This indicates that malignant gliomas contain MSCs that might facilitate tumor growth, but their effect on the tumor cells remains to be determined. *In vitro* co-culture with glioma-derived MSC-like cells and a human glioma cell line, such as the widely used U87,[10] could determine if the MSCs affect tumor cell growth. Similarly, *in vitro* bioassays can be used to further assess their immunosuppressive function.[231] Further on, transplantation of glioma-derived MSC-like cells into human gliomas established in immunodeficient mice could be used to investigate their influence on tumor growth *in vivo*. Depending on future findings, the MSC-like subpopulations in human malignant gliomas might emerge as a novel therapy target to disrupt the tumor vascularization and reduce its immunosuppression.

Another important question is the origin of the glioma-derived MSC-like cells. They might originate from the tumor cells, being derived from tumor stem cells or transformed through epithelial-mesenchymal transition, a process where tumor-transformed epithelial cells convert their phenotype into mesenchymal-like cells.[88,169,232-234] Moreover, they might be recruited from normal host tissue such as the surrounding brain or the bone marrow.[83,94] Genetic analysis of the isolated cells could reveal tumor-specific genetic alterations, suggesting a tumor origin, or a normal gene profile, indicating recruitment of healthy cells.

In the second study of this thesis, we investigate the recruitment scenario further in an animal model where the MSC-like pericytes of the brain were genetically labelled with GFP.[172] In response to an intracerebral GL261 glioma, these pericytes were activated in the whole brain, recruited into the tumor and integrated with the tumor vasculature. As pericytes derived from the normal brain constituted a major part of the total pericyte coverage within the tumor vasculature, this recruitment is believed to play an important role in tumor development. The next step is to determine the mechanisms behind the activation and recruitment and to clarify how they affect the tumor and its microenvironment. If the recruitment can be blocked, tumor development without involvement of normal brain pericytes can be investigated. PDGFR-β might be a good target for pharmacological blockage, as it was expressed on all brain-derived pericytes in our experiments and PDGF-β is an important factor for initiation of pericyte proliferation and migration.[235,236] Such drug-mediated blockage might be a favorable way to treat malignant gliomas and target them already at the developmental stage. Beneficial effects have been reported from anti-angiogenic therapy when directing it towards toward both endothelial cells and pericytes.[167]

Several safety issues have to be thoroughly investigated before MSCs can be used as glioma therapy. Their effect on tumor growth needs to be clarified, as MSCs have been reported to exert both suppressive and stimulatory effects on gliomas.[76,81,82,237] The risks of secondary malignancies, often associated with stem cell transplants,[209] and the effect of the MSCs after completed treatment need to be further investigated. In the third study of this thesis, we showed that rat BM-MSCs transplanted into an experimental, orthotopic rat glioma did not proliferate within the tumor and did not migrate to the liver, spleen and cervical lymph nodes. Further on, they did not migrate when transplanted into the brains of non-tumor-bearing animals. We also showed that BM-MSCs lack long-distance tropism to three different rat glioma models syngeneic to both the MSCs and the host, contradicting other studies reporting that transplanted MSCs migrate toward gliomas through normal brain tissue.[76,77] Our results suggests intratumoral transplantation as the preferred route of MSC administration. Other studies have suggested intraarterial or intravenous administration,[77,200,201] which on one hand would be a less invasive procedure, but on the other hand mean less efficient and less specific MSC distribution. In our previous experiments, no MSC infiltration was detected in the tumor after intravenous administration.[78] Hence, MSC-based glioma therapy needs further investigation in other models, not the least in a human setting, to elucidate the safety of intratumoral MSC administration in terms of MSC proliferation and migration through normal tissue. For example, the mechanism behind the tumor tropism of MSCs is not completely clear, and animal models come with limitations as they are considerably more homogenous than human malignant gliomas.[1,238] However, angiogenesis[48,77,94], hypoxia[170] and inflammation[89,239] are all properties that are associated with MSC migration and, importantly, properties present in both animal models and human

tumors.[1,15] Nevertheless, to study human MSCs in human glioma tissue, organotypic tumor slice cultures might be used to model intratumoral MSC transplantation.[240,241]

MSCs have been reported to adopt an immunostimulatory phenotype with antigen-presenting properties if they are exposed to IFNγ,[73,74,213-216] and in the fourth study of this thesis we conclude that intratumorally transplanted MSCs actually potentiate peripheral immunotherapy with IFNγ-producing tumor cells. Hence, if the MSCs do promote tumor growth, the effect can be overridden by their immunostimulatory properties in this therapeutic setting. In fact, MSCs grafted into orthotopic rat gliomas did not alter animal survival if immunization was omitted, suggesting that, at least in this rat glioma model, BM-MSCs *per se* do not affect the tumor growth. However, the mechanism behind their enhancing effect is not completely clear. In this context, it is of importance to elucidate the MSCs antigen-presenting capabilities as they might play a major role in their enhancing effect. It is also important to clarify the biology behind the phenotypic switch and investigate if proinflammatory and non-tumor-promoting MSCs can be obtained from humans.[74] This might be done either by isolation of a subpopulation of cells from the bone marrow exerting the desired properties, or by polarizing the cells by exposure to cytokines, such as IFNγ, or possibly low-dose radiation.[242]

The treatment of malignant gliomas needs to be updated with novel therapies, and MSC-based gene therapy is a promising approach. Several safety concerns need to be addressed, but encouraging results in animal studies point toward MSCs as a tumor-specific tool that can target the infiltrative parts of the tumor that cannot be reached with standard treatment. Regarding the safety of such treatment, human malignant gliomas contain endogenous MSCs that probably constitute a pro-tumor component facilitating glioma growth. Nevertheless, we see no evidence that intratumorally transplanted rat BM-MSCs favor tumor growth in any of our rat glioma models, suggesting that at least a subpopulation within bone marrow-derived cells are appropriate candidates for MSC-based therapy. Furthermore, MSCs can adopt a tumor-suppressive phenotype, further enhancing the possibilities to develop a safe treatment with MSCs. Future studies will have to address the compatibility of these MSCs with the standard treatments. It has been shown that intratumoral transplantation of TRAIL-secreting MSCs in combination with TMZ results in a more efficient treatment compared to each therapy alone.[102] Further on, we show that the migratory potential of the MSCs is retained even when they are transplanted into a partially resected tumor. The next question is how to use the MSCs in a clinical setting, and several other approaches have been suggested.[79,80] The vascular preference of the MSCs makes anti-angiogenic therapy a feasible choice.[201] Furthermore, immunostimulatory cytokines,[77,97,98] apoptosis-inducing factors,[91,102,243] oncolytic viruses,[120,121,244] anti-tumoral antibodies,[124] drug-carrying nanoparticles[116,245] and suicide gene therapy[104,105] are all therapeutic strategies that might benefit from the tumor-specific delivery capabilities of MSCs.

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# Paper II

PLOS | ONE

RESEARCH ARTICLE

# Endogenous Brain Pericytes Are Widely Activated and Contribute to Mouse Glioma Microvasculature

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## Abstract

Glioblastoma multiforme (GBM) is the most common brain tumor in adults. It presents an extremely challenging clinical problem, and treatment very frequently fails due to the infiltrative growth, facilitated by extensive angiogenesis and neovascularization. Pericytes constitute an important part of the GBM microvasculature. The contribution of endogenous brain pericytes to the tumor vasculature in GBM is, however, unclear. In this study, we determine the site of activation and the extent of contribution of endogenous brain pericytes to the GBM vasculature. GL261 mouse glioma was orthotopically implanted in mice expressing green fluorescent protein (GFP) under the pericyte marker regulator of G protein signaling 5 (RGS5). Host pericytes were not only activated within the glioma, but also in cortical areas overlying the tumor, the ipsilateral subventricular zone and within the hemisphere contralateral to the tumor. The host-derived activated pericytes that infiltrated the glioma were mainly localized to the tumor vessel wall. Infiltrating GFP positive pericytes co-expressed the pericyte markers platelet-derived growth factor receptor-β (PDGFR-β) and neuron-glial antigen 2. Interestingly, more than half of all PDGFR-β positive pericytes within the tumor were contributed by the host brain. We did not find any evidence that RGS5 positive pericytes adopt another phenotype within glioma in this paradigm. We conclude that endogenous pericytes become activated in widespread areas of the brain in response to an orthotopic mouse glioma. Host pericytes are recruited into the tumor and constitute a major part of the tumor pericyte population.

## Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain tumor in adults, with a median survival of only 14.6 months even when all available treatment is given

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[1]. One major reason for this poor survival is the rapid and infiltrative growth pattern of the tumor, facilitated by extensive angiogenesis and neovascularization [2]. An important cellular component of the GBM vasculature is the pericytes. Tumor pericytes mediate immunosuppression [3] and promote endothelial cell survival [4,5], thus facilitating tumor growth. Pericytes aligning glioma vessels are often abnormal and scarcer compared to pericytes on normal vessels [6,7], resulting in a dysfunctional vasculature and blood-brain barrier.

The source of pericytes in GBM remains controversial. A proportion of pericytes in GBMs are generated from tumor stem cells residing within the GBM itself [8] or recruited from the bone marrow [9]. However, whether brain-derived pericytes contribute to the tumor vasculature is not known.

Here we investigate the contribution of normal brain pericytes to GBM vasculature using an orthotopic mouse glioma model. We have recently shown [10] that pericytes in the human brain resemble perivascular multipotent mesenchymal stromal cells that share characteristics of both pericytes and mesenchymal stromal cells [11]. Recent observations in several tissues indicate that pericytes are versatile and have the ability to respond to environmental stimuli such as stroke [12]. Furthermore, mesenchymal stromal cells, similar to pericytes, have a strong tumor tropism and migratory capabilities, and integrate with the tumor vessels as pericyte-like cells upon intratumoral implantation [13].

In the present study we used mice where green fluorescent protein (GFP) is expressed under the pericyte-specific promoter regulator of G protein signaling 5 (RGS5) [14,15] and thus labels host-derived pericytes. We implanted GL261 mouse glioma cells [16,17] into these mice and show that endogenous pericytes are activated in widespread areas of the brain, recruited into established intracerebral GL261 gliomas and integrate with the tumor vessels. Quantification revealed that more than half of all platelet-derived growth factor receptor-β (PDGFR-β) positive pericytes within the glioma are host brain-derived.

## Materials and Methods

### Ethics Statement

All animal procedures were approved by the Committee of Animal Ethics in Lund-Malmö, Sweden (permit number: M259-12).

### Cell Line and Culture

The GL261 mouse glioma cell line [16,17], syngenic to the C57BL/6 mouse strain, was a kind gift from Dr. Géza Sáfrány, Hungary. The cells were cultured in R10 medium (RPMI 1640 medium supplemented with 300 μg/ml L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 50 μg/ml gentamicin (Life Technologies, Carlsbad, CA, USA) and 10% fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany)) at 37°C in a humidified atmosphere containing 5% $CO_2$. For *in vivo* inoculation, cells were resuspended in R0 medium (R10 medium without gentamicin and FBS).

### GL261 Tumor Cell Inoculation *in vivo*

We used the reporter rgs5$^{GFP/+}$ mice, a knock-out/knock-in C57BL/6 mouse line expressing GFP under the pericyte-specific RGS5 promoter [15]. Heterozygote rgs5$^{GFP/+}$ females between 7–17 weeks of age, or 14 weeks old wild-type C57BL/6 females, were anaesthetized with isoflurane (Forene, Abbott Scandinavia AB, Solna, Sweden) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). They received local anaesthetic by subcutaneous injection of 0.025 ml Marcain with adrenaline (2.5 mg/ml bupivacaine, 5 μg/ml epinephrine, AstraZeneca

AB, Södertälje, Sweden) on the skull. A hole was drilled and 5000 GL261 tumor cells in 5 μl R0 medium were injected at 1 μl/min into the caudate nucleus using a 10 μl Hamilton syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) at the following coordinates: 1.5 mm lateral and 1.0 mm anterior of bregma, 2.75 mm ventral of the skull bone. After injection, the needle was left in the brain for 5 minutes before it was slowly retracted. The hole in the skull was sealed with bone wax. At day 19 after tumor inoculation, animals were cardially perfused with 0.9% NaCl solution (Merck KGaA, Darmstadt, Germany) followed by 4% paraformaldehyde (PFA, Electron Microscopy Sciences, Hatfield, PA, USA). Brains were removed and postfixed in 4% PFA at 4°C overnight before being transferred to 30% sucrose solution (Merck KGaA). The brains were sectioned in 40 μm thick coronal sections with a Leica SM200 R sliding microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany) and stored at -20°C in anti-freeze solution (30% etylen glycol and 30% glycerol (both from VWR International, Radnor, PA, USA) in 0.012 M $NaH_2PO_4 \cdot H_2O$ and 0.031 M $Na_2HPO_4 \cdot 2H_2O$ (both from Sigma-Aldrich, Stockholm, Sweden)) for subsequent histological staining.

## Immunofluorescence

Free-floating sections were washed three times in phosphate-buffered saline (PBS, Life Technologies) and Fc receptors were blocked with Innovex Fc Receptor Blocker (Innovex Biosciences Inc., Richmond, CA, USA) in accordance to the manufacturer's instructions. Sections were blocked with 5% normal goat serum (NGS, Jackson ImmunoResearch Europe Ltd., Suffolk, United Kingdom) and 0.5% Triton X-100 (Sigma-Aldrich) in PBS (PBTX) and then incubated with chicken anti-GFP antibody (1 μg/ml, Abcam, Cambridge, United Kingdom) in 0.5% PBTX supplemented with 3% NGS at room temperature overnight. The next day, the sections were washed three times in PBS and subsequently incubated with biotinylated goat anti-chicken antibody (6 μg/ml, Vector Laboratories Ltd., Peterborough, United Kingdom) in 0.5% PBTX supplemented with 3% NGS at room temperature for 2 hours. The sections where then washed three times in PBS and incubated with Alexa Fluor 594-conjugated streptavidin (7.2 μg/ml, Jackson ImmunoResearch Europe Ltd.) in 0.5% PBTX supplemented with 3% NGS at 4°C for 2 hours.

All sections were washed three times with PBS. Sections stained for CD13 and CD31 were blocked with 5% normal donkey serum (NDS, Jackson ImmunoResearch Europe Ltd.) in 0.5% PBTX. Sections stained for PDGFR-β and glucose transporter 1 (GLUT1) were incubated in 10 mM citrate buffer (10 mM trisodium citrate dehydrate supplemented with 0.05% Tween 20 (both from Sigma-Aldrich), pH 6.0) at 80°C for 30 minutes for antigen retrieval and then washed three times in PBS. Sections were incubated with either rabbit anti-laminin antibody (1.2 μg/ml, Abcam), rabbit anti-Ki67 antibody (2.5 μg/ml, Abcam), rabbit anti-PDGFR-β antibody (diluted 1:200, Cell Signaling Technology Europe, B.V., Leiden, The Netherlands), rabbit anti-GLUT1 antibody (5 μg/ml, Abcam), rabbit anti-vascular endothelial growth factor receptor 2 (VEGF-R) antibody (diluted 1:200, Cell Signaling Technology Europe), rabbit anti-α-smooth muscle actin (α-SMA) antibody (2 μg/ml, Abcam), rat anti-CD13 antibody (2 μg/ml, AbD Serotec, Kidlington, United Kingdom), rabbit anti-ionized calcium binding adapter molecule 1 (Iba1) antibody (0.25 μg/ml, Wako Chemicals GmbH, Neuss, Germany), rabbit anti-S100 calcium binding protein B (S100B) antibody (diluted 1:500, Abcam), rabbit anti-neuron-glial antigen 2 (NG2) antibody (2 μg/ml, Merck Millipore, Billerica, MA, USA) or rat anti-CD31 antibody (0.04 μg/ml, BD Biosciences, Heidelberg, Germany) in 0.5% PBTX with either 3% NDS or 3% NGS overnight. The anti-laminin, anti-GLUT1, anti-CD13 and anti-CD31 antibodies were incubated at 4°C and the rest at room temperature. After incubation, the sections were washed three times in PBS and then incubated with secondary Alexa Fluor 647 goat anti-rabbit, Alexa Fluor 647 donkey anti-rat or DyLight 649 donkey anti-mouse antibody (3 μg/ml,

all from Jackson ImmunoResearch Europe Ltd.) in 0.5% PBTX at room temperature for 2 hours. After incubation, the sections were washed three times in PBS and then stained with Hoechst 33342 (8.1 μM, Life Technologies) for 10 minutes. The sections were washed three more times and then mounted on SuperFrost Plus glasses (Thermo Fisher Scientific Inc., Waltham, MA, USA) with DABCO (Sigma-Aldrich) and coverslipped.

Brains from five tumor-bearing mice were used for the GLUT1 analysis and brains from three tumor-bearing mice were used for the analysis of the other markers.

## DAB Staining

Free-floating sections were washed three times in PBS and then quenched in PBS supplemented with 3% $H_2O_2$ (Merck KGaA) and 10% methanol (J.T.Baker, Avantor Performance Materials B.V., Deventer, The Netherlands) for 15 minutes. Sections were blocked with 5% NGS and 1% Tween 20 in PBS and then incubated with chicken anti-GFP antibody (2 μg/ml) in PBS supplemented with 3% NGS at room temperature overnight. The next day, the sections were washed three times in PBS and subsequently incubated with biotinylated goat anti-chicken antibody (6 μg/ml) in PBS supplemented with 3% NGS at room temperature for 2 hours. The sections where then washed three times in PBS and the biotinylated antibody was visualized with the VECTASTAIN Elite ABC Kit and the DAB Peroxidase Substrate Kit (both from Vector Laboratories Ltd.) in accordance to the manufacturer's instructions. The sections were washed three more times and then mounted on gelatin-coated glasses (Thermo Fisher Scientific Inc.), left to dry overnight and coverslipped using DPX mounting medium (Sigma-Aldrich).

Brains from three tumor-bearing mice were used for the DAB analysis and tumor-free brains from three mice were used as control.

## Confocal Microscopy

The immunofluorescent tissue sections were analyzed on a Zeiss LSM 780 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Hoechst 33342 was excited with the 405 nm laser and light was collected between 410–476 nm. Alexa Fluor 594 was excited with the 561 nm laser and light was collected between 588–642 nm. Alexa Fluor 647 and DyLight 649 were excited with the 633 nm laser and light was collected between 654–755 nm. To determine the amount of autofluorescence from the 561 nm laser, the sections were also excited with the 488 nm laser and light was collected between 491–571 nm or 589–643 nm. No specific staining was visualized with these settings, but the autofluorescent parts could be defined and subtracted from the real Alexa Fluor 594 staining.

## Digital Image Processing

The immunofluorescence images of Alexa Fluor 594 were digitally enhanced by removing autofluorescent elements and noise in Adobe Photoshop CS5.1 (Adobe Systems Inc., San Jose, CA, USA). Autofluorescence was removed using two separate single channel images. The first image was excited with the 561 nm laser and contained true antibody-mediated staining as well as autofluorescence. The second image was captured with the 488 nm laser, which only slightly excited Alexa Fluor 594, and hence only contained the non-specific autofluorescence. The images were overlayed in Adobe Photoshop and the exposures were adjusted to match. Each pixel value of the 561 nm image was then compared to the corresponding pixel value of the 488 nm image, and the difference was calculated. A new single channel image was created, where each pixel value was the difference between the corresponding pixel values of the original images. This was done using the *Difference* blend mode. The result was an image where elements present in both

original images, i.e. autofluorescence, were removed while elements only present in one of the images, i.e. antibody-mediated staining, were preserved. Finally, the *Dust & Scratches* filter was applied to reduce image noise.

### Stereology

Imaging and quantification of the DAB stained sections was conducted with an Olympus BX53 system microscope (Olympus, Shinjuku, Tokyo, Japan). Each brain was divided into two regions of interest (ROIs); one outside the tumor and one in the corresponding location in the contralateral hemisphere. Three images at 20x magnification, resulting in a total area of 0.277 $mm^2$, were taken from each ROI and the GFP positive cells were manually counted in each image.

To quantify the number of cells positive for GFP, PDGFR-β, NG2, CD13, α-SMA and Ki67, confocal z-stacks from three different tumors were taken at 20x magnification. Each z-stack consisted of 10 sequential, 1.14 to 1.77 μm thick, optical sections showing the expression of GFP and one additional marker. The area counted in each tumor was 0.181 $mm^2$. To count the number of cells expressing each marker, all optical sections were analyzed in Adobe Photoshop CS5.1 one by one. The cells, visualized by fluorescent staining, were manually counted. To avoid counting the same cell several times, each cell was marked in a transparent layer that was moved between all the optical sections within the z-stack. At last, the number of cells expressing GFP alone, one of the other markers alone, or co-expressing GFP with one of the other markers was determined for each z-stack.

### Statistics

The cell counting analysis was performed using ANOVA, where $p<0.05$ was considered statistically significant.

## Results

### Pericytes are Activated in Widespread Areas of the Brain in Response to Local Glioma Growth

Under normal conditions, GFP positive pericytes show a flat morphology with a small cell body indicating a quiescent state (Fig 1A and 1B). However, under pathological conditions they can become activated and show a more prominent cell body [18]. In brains containing glioma, the number of activated GFP positive pericytes within the cerebral cortex, adjacent to and overlying the tumor, was significantly increased compared to the corresponding region of rgs5$^{GFP/+}$ mice not harboring tumor (mean: 127 ± 4.97 and 48.5 ± 2.28, respectively; $p<0.001$) (Fig 1C–1E). The number of activated GFP positive pericytes was also significantly increased within the contralateral hemisphere of tumor-bearing rgs5$^{GFP/+}$ mice, indicating a widespread activation of perivascular cells (Fig 1C, 1F). Similar to cerebral cortex, activated GFP positive pericytes were consistently found in the rostral subventricular zone (SVZ) of the lateral ventricle ipsilateral to the glioma as compared to the SVZ contralateral to the tumor (Fig 1G–1I). The morphology of the GFP positive pericytes in the host cortex was consistent with activated pericytes (Fig 1E and 1F), while the GFP positive pericytes within the tumor were found to have different morphological profiles (Fig 1J–1L). They had either a flattened cell body with elongated processes (Fig 1K) or a prominent cell body with retracted finger-like projections (Fig 1L). Both cell types were found throughout the whole tumor with no area-specific distribution pattern. However, the flattened morphology was mainly localized close to the tumor

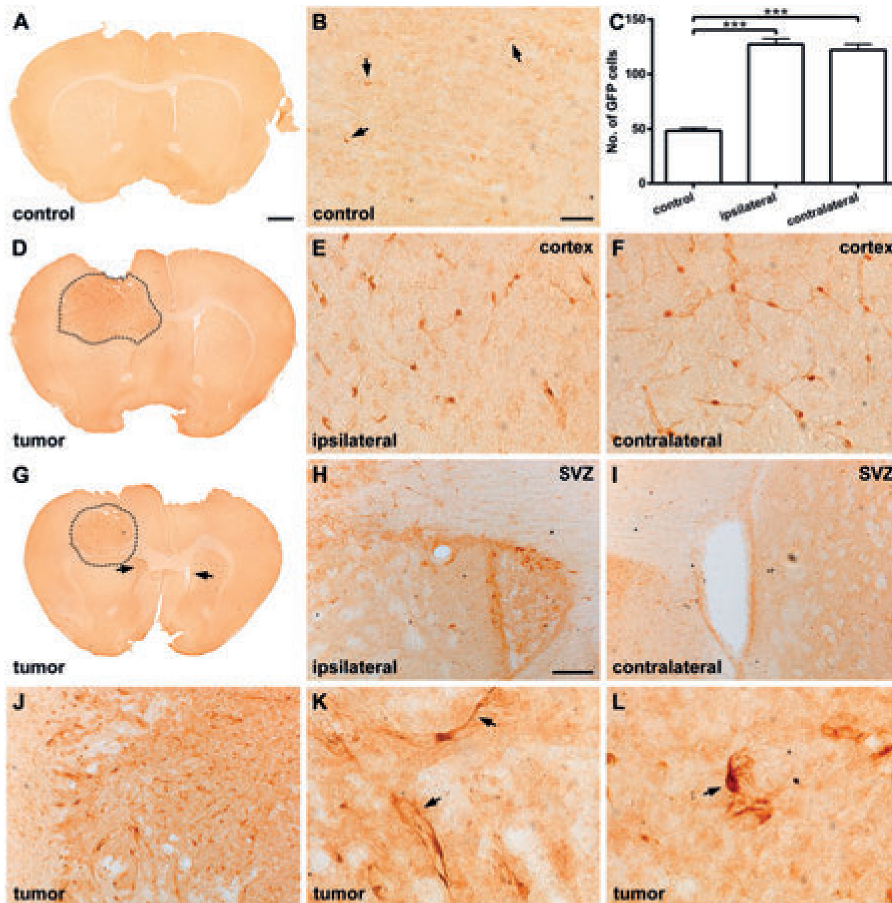


**Fig 1. Pericytes are activated around glioma.** (A) Low magnification photomicrograph of a normal rgs5$^{GFP/+}$ mouse brain, scale bar is 500 μm. (B) Under normal conditions, quiescent GFP positive pericytes showed a flat morphology with a small cell body (arrows), scale bar is 20 μm. (C) However, in response to a GL261 glioma, the number of GFP positive pericytes within the cerebral cortex was significantly increased, both in the ipsilateral and contralateral hemisphere, compared to a normal mouse brain without tumor (n = 3, mean ± SEM, ***, p<0.001, ANOVA). (D) Low magnification photomicrograph of a representative GL261 tumor (dashed) in the rgs5$^{GFP/+}$ mouse brain, scale bar as in A. Pericytes showing a morphology consistent with activated pericytes were found in the cerebral cortex both in the (E) ipsilateral and (F) contralateral hemisphere, scale bars as in B. (G) Low magnification photomicrograph of a representative GL261 tumor (dashed) in the rgs5$^{GFP/+}$ mouse brain showing the SVZ (arrows), scale bar as in A. Activated pericytes are present (H) in the SVZ ipsilateral to the tumor but not (I) in the SVZ contralateral to the tumor, scale bar is 50 μm. (J) The morphology of the GFP positive pericytes inside the tumor was different compared to the pericytes in the cortex, with either (K) a flattened cell body with elongated processes (arrows) or (L) a prominent cell body with tuft-like processes (arrow). Scale bar in J as in H and scale bars in K-L as in B.

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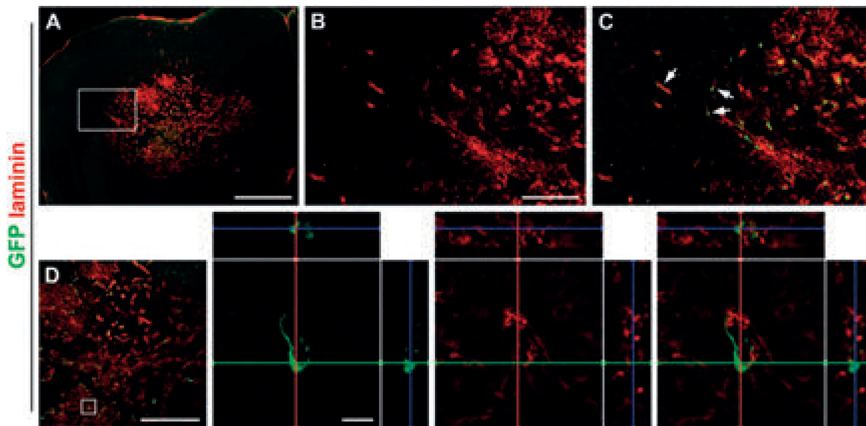


**Fig 2. Pericytes are associated with laminin-expressing tumor satellites.** (A) Overview image of GL261 tumor expressing high levels of laminin, scale bar is 1000 μm. (B) Higher magnification of laminin expression around the tumor border, scale bar is 200 μm. (C) GFP positive pericytes localize close to laminin-expressing tumor microsatellites outside the tumor (arrows), scale bar as in B. (D) However, they do not express laminin themselves. Scale bar is 500 μm in the low magnification image and 20 μm in the high magnification images.

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border whereas cells with a large cell body and prominent projections were evenly distributed within the tumor.

## Activated Pericytes are Associated with Laminin Positive Tumor Microsatellites

Recent studies of glioma have shown that laminins are important for glioma cell invasion and growth [19]. Given these findings, we next examined the expression of laminin in GL261 tumors in rgs5$^{GFP/+}$ mice 19 days after tumor inoculation and found laminin to be highly expressed by these tumors (Fig 2A and 2B). Laminin positive glioma microsatellites were found at the glioma/brain interface at some distance from the main tumor bulk. Migrating GFP positive pericytes were located close to these laminin positive satellites, not only adjacent to the tumor but also at a distance to its margin (Fig 2C). The GFP positive pericytes did not co-express laminin (Fig 2D).

## Activated Pericytes are Attracted to Hypoxic Tumor Regions

Hypoxic regions are a well-known characteristic of malignant gliomas. Next, we investigated whether the recruitment of activated pericytes was related to hypoxia by staining for GLUT1, a transport protein upregulated at hypoxic conditions due to the increased need for glucose [20]. Although GFP positive pericytes were found at both normoxic and hypoxic regions of the tumor, they were clearly more numerous around areas of GLUT1 immunoreactivity. The GLUT1 positive tumor areas were mainly localized in the periphery of the tumor. Close to these hypoxic areas at the interface between brain and tumor, GFP positive pericytes appeared to form a stream of migrating cells from the brain into the penumbra

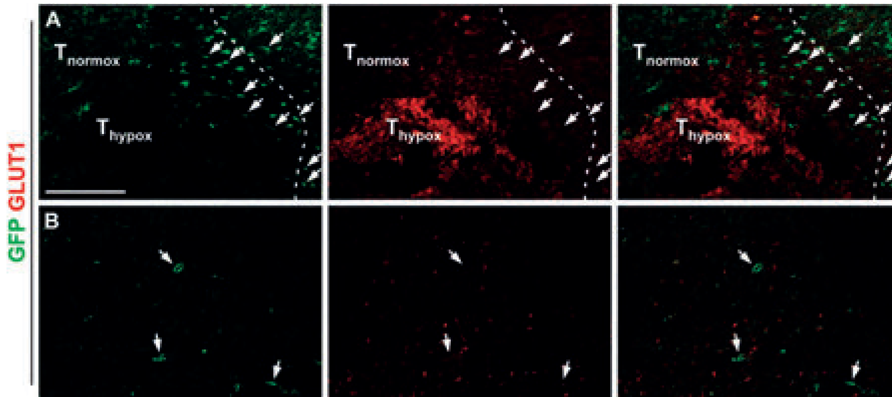


**Fig 3. GFP positive pericytes are found preferably within hypoxic regions of glioma.** (A) GFP positive cells (arrows) are attracted more numerous within the penumbra zone around GLUT1 positive hypoxic regions close to the GL261 tumor border (dashed), scale bar is 200 μm. Normoxic and hypoxic parts of the tumor are marked with $T_{normox}$ and $T_{hypox}$, respectively. (B) Few GFP positive pericytes (arrows) are found at normoxic regions within the tumor, scale bar as in A.

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zone around the GLUT1 positive areas (Fig 3A and 3B). No GLUT1 positivity was observed within or near the SVZ.

## The Majority of the PDGFR-β Positive Pericytes within the Tumor are Host-Derived

Glioma vasculature consists of dilated and tortuous vessels expressing markers such as CD31 (Fig 4A) and VEGF-R (Fig 4B) [21,22]. Approximately three quarters of all VEGF-R expressing tumor vessels in the GL261 glioma in the present study were covered by GFP positive pericytes.

Interestingly, out of all PDGFR-β positive cells associated with microvessel walls within the GL261 glioma, 57 ± 6.6% co-labeled for GFP (Fig 5A and 5B). A subpopulation of the GFP positive cells inside the tumor expressed NG2 (55 ± 12%) and CD13 (26 ± 15%), markers associated with activated pericytes and mesenchymal stromal cells (Fig 5C and 5D) [23]. Further on, the majority of the intratumoral GFP positive pericytes weakly expressed the pericyte marker α-SMA (86 ± 7.7%; Fig 5E). In these cells, α-SMA immunoreactivity was visualized as patches within the cytoplasm in close contact with the plasma membrane. All pericyte markers were represented among both morphologically different pericyte types, although CD13 seemed to be expressed predominantly on flat, elongated cells. A subset of the GFP positive pericytes labeled for Ki67 (16 ± 1.7%), indicating active proliferation (Fig 6A and 6B).

## Pericytes Do Not Label with Stromal Tumor Cell Markers or Inflammatory Cell Markers

Finally, we examined whether pericytes adopt a different phenotype within the tumor. GFP positive pericytes did not express the astrocyte marker S100B (Fig 7A) and all GFP positive pericytes within the tumors were negative for the microglia marker Iba1 (Fig 7B). However, Iba1 positive

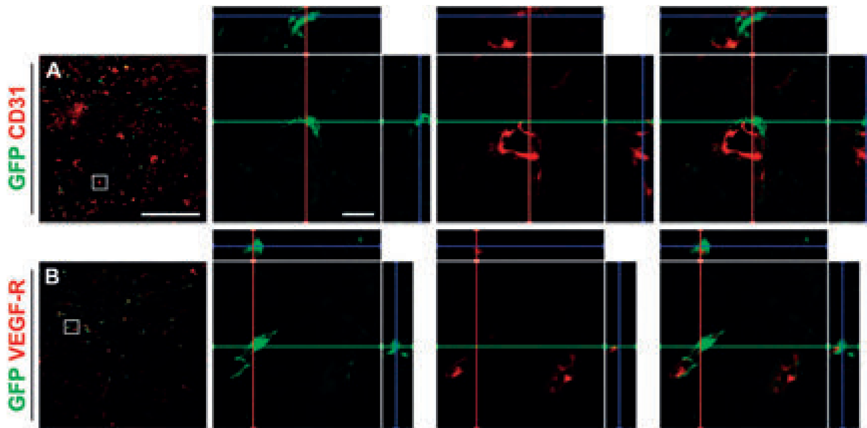


**Fig 4. Pericytes within the tumor localize adjacent to vessels.** The GFP positive cells within GL261 tumors are localized near cells expressing (A) CD31 and (B) VEGF-R, but do not express the markers themselves. Scale bar is 500 μm in the low magnification images and 20 μm in the high magnification images.

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microglia cells were found in very close proximity to pericytes indicating a possible juxtracrine-like communication.

## Discussion

Here we provide evidence that the normal brain vasculature contributes the majority of pericytes to GL261 mouse glioma vasculature. Using a pericyte reporter mouse where activated pericytes express GFP [15], we show that the tumor vasculature of grafted glioma contains a high proportion of host-derived GFP positive cells. Furthermore, in response to unilateral growth of an intracranial tumor, a significant increase of activated pericytes was observed within the cortex of both the ipsilateral and contralateral hemisphere as well as in the ipsilateral SVZ, indicating a significant influence of local glioma growth on widespread areas of the mouse brain.

The present study uses the GL261 mouse glioma model. It carries both p53 and K-ras mutations, as does many of its human counterpart, GBM [17]. The model was chosen for the present study because it represents one of the very few mouse brain tumor models syngenic to the C57BL/6 mouse strain and it is widely used because of its well characterized similarities to GBM. In particular, the invasive and angiogenic properties of GL261 closely mimic that of human GBM [24].

The vast majority of pericytes in normal as well as pathological tissues, such as tumors, express PDGFR-β [25]. Interestingly, a majority of the PDGFR-β positive pericytes within the tumor were co-labeled for GFP, indicating that they are recruited from the host. This contrasts the recent work of Cheng et al. stating that a majority of the pericytes within GBMs are derived from the tumor itself [8]. In that study, *in vivo* cell lineage tracing demonstrated that glioma stem cells generate the majority of vascular pericytes in mouse and human GBM. The present study does not rule out the possibility that a proportion of pericytes within the tumor are derived

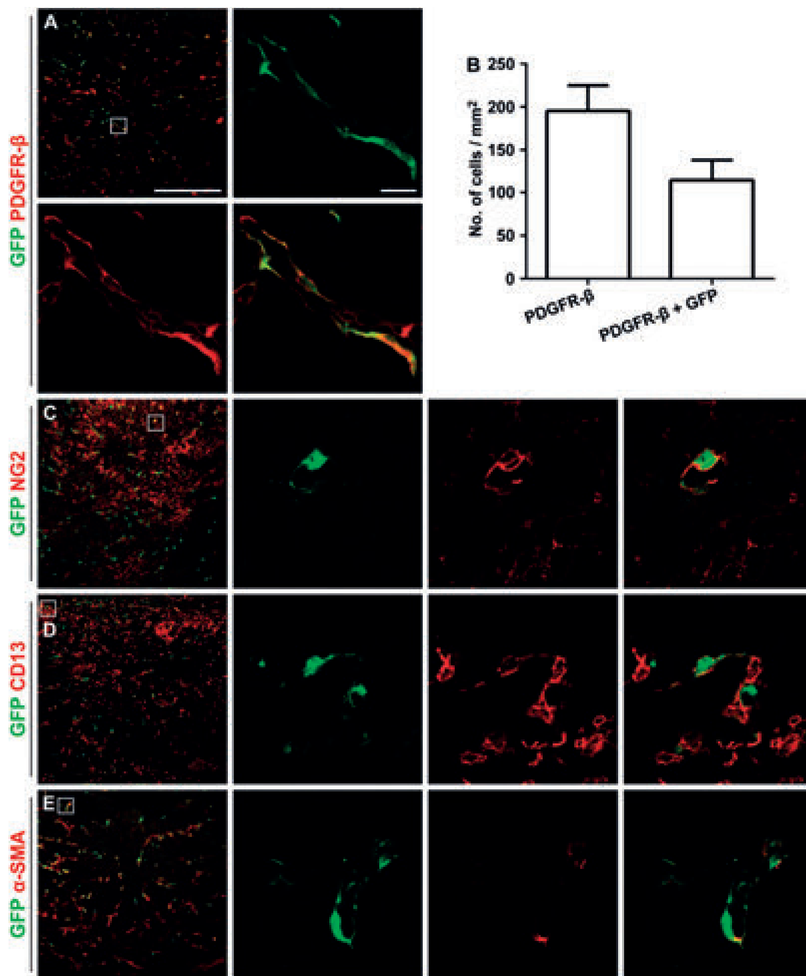


**Fig 5. GFP positive cells express pericyte markers.** (A) All GFP positive cells within the GL261 tumor are clearly positive for PDGFR-β. (B) Out of all PDGFR-β positive cells within the tumor, 57 ± 6.6% are host-derived GFP positive cells (n = 3, mean ± SEM). (C) A proportion of the cells are positive for the activation marker NG2. The majority of the GFP positive cells lack expression of the mesenchymal stromal cell marker CD13. (D) GFP positive cell at the tumor border expressing CD13. (E) The majority of the cells weakly express the pericyte marker α-SMA. Scale bar is 500 μm in the low magnification images and 20 μm in the high magnification images.

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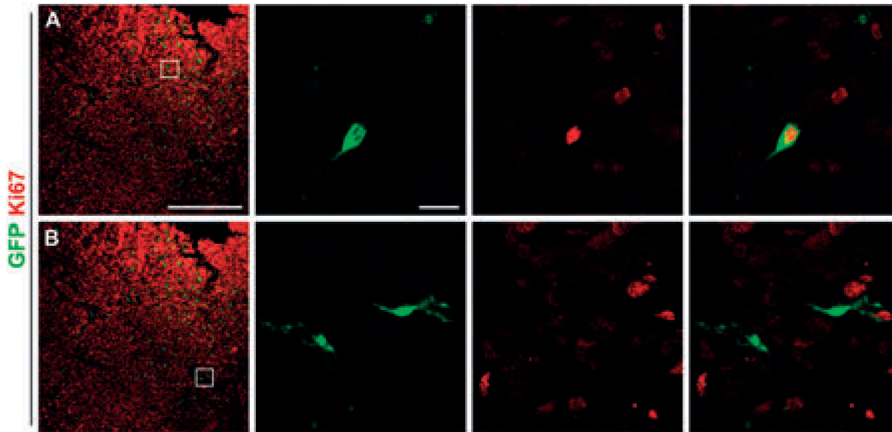


**Fig 6. A proportion of the pericytes proliferate intratumorally.** (A) A subset of the GFP positive cells within GL261 tumors express the proliferation marker Ki67, (B) whereas a majority of the cells do not. Scale bar is 500 μm in the low magnification images and 20 μm in the high magnification images.

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from the glioma cells. Even though the GL261 mouse glioma model mainly consists of differentiated cells, it is a heterogeneous cell line containing subpopulations of cells with retained differentiation capacity [24,26,27]. In fact, the occurrence of GFP negative PDGFR-β positive pericytes, although at a considerably lower frequency than in [8], within mouse glioma would lend some

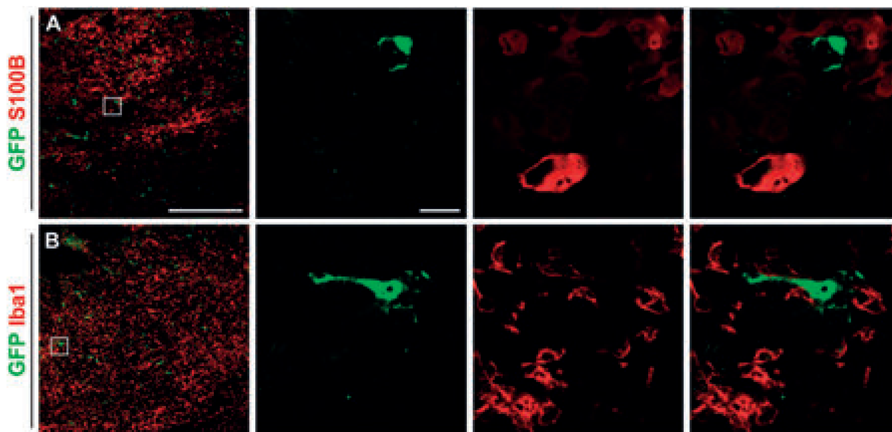


**Fig 7. GFP positive cells become neither astrocytes nor microglia.** None of the GFP positive cells express (A) S100B or (B) Iba1, ruling out the possibility that they become astrocytes or microglia. Scale bar is 500 μm in the low magnification images and 20 μm in the high magnification images.

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support to such a clonal origin. Resolution of the discrepancy between the findings of the present study and that of Cheng et al. cannot be obtained at this stage, however, a differential capacity of tumor cell plasticity and differentiation potential between different models of glioma might be a contributing factor. Since we have used non-labeled tumor cells that cannot be traced, we are not able to draw any further conclusions about the origin of the GFP negative PDGFR-β positive pericytes. Furthermore, and importantly, whether brain pericytes are activated and recruited into other animal models of glioma and into highly malignant glioma in humans remains to be clarified.

Although many key molecular regulators of pericyte function and activation have been previously defined [28], the mechanism of widespread pericyte activation in response to local tumor growth at a considerable distance remains unknown. Principally, pericyte activation could result from the widespread parenchymal diffusion of factors produced locally by the tumor, from glioma-derived factors such as exosomes delivered by the systemic circulation [29] or the cerebrospinal fluid or, alternatively, from the action of elevated intracranial pressure and resulting hypoxia [30] or brain edema [31]. Interestingly, GFP positive pericytes were found in close vicinity of laminin positive tumor vessel microsatellites. This may indicate that pericyte activation and recruitment into glioma requires specific interaction with the laminin-rich vascular basement membrane of the GL261 tumor. However, in the present study, the activation of pericytes included structures also located in the contralateral hemisphere, at a considerable distance from the main tumor. The molecular basis for glioma-induced recruitment of pericytes from another distant site, the bone marrow, has been elucidated in some detail. Hypoxia-inducible factor-1α (HIF-1α), a direct mediator of tumor hypoxia, has been shown to mobilize and lead to tumor incorporation of bone marrow-derived vascular modulatory cells, including a small portion of pericyte progenitor cells [9]. This effect is mediated through the HIF-1 target stromal-derived factor-1α and recruitment is dependent on the presence of matrix metalloproteinase-9 and its ability to mobilize sequestered VEGF within the tumor. The importance of tumor hypoxia as a critical trigger of pericyte recruitment into glioma is further substantiated by the findings of large numbers of pericytes specifically in the penumbra around areas of hypoxia in the present paper.

Interestingly, also pericytes in the rostral SVZ were activated by tumor growth in the ipsilateral striatum. The SVZ is an active proliferative zone within the brain and this region has previously been shown to be reactive and produce nestin and doublecortin positive neuroblasts in response to glioma [32,33]. Furthermore, in response to local cerebral ischemia, precursors of pericytes within the SVZ proliferate and migrate to the infarcted area where they are incorporated into new vessels of the peri-infarct regions [34]. Whether pericyte activation in the SVZ facilitates this process remains to be established.

A portion of the GFP positive cells infiltrating the tumor were co-labeled for the proliferation marker Ki67, indicating that proliferation of endogenous brain pericyte precursor cells is actively involved in the process of glioma vascularization. Although proliferation is part of this process, the present study was not designed to clarify to what extent proliferation of perivascular progenitors contributes. Only a subset of the GFP positive cells within the tumor was co-labeled for Ki67. However, only actively proliferating cells at the exact time of tissue perfusion are labeled by this marker. Thus, these results might be an underestimation of the contribution of pericyte stem- or precursor cell proliferation as opposed to pericyte recruitment by the mechanism of tumor-tropic migration of existing, post-mitotic pericytes.

A subset of cells did not express NG2 showing that non-activated pericytes also reside within the tumor [23]. Furthermore, a majority of the GFP positive cells inside the tumor were negative for the pericyte marker CD13, also expressed by mesenchymal stromal cells. In contrast, GFP positive pericytes outside the tumor expressed CD13, thereby indicating a phenotypic shift as

the cells enters the tumor. Interestingly, even though intratumoral RGS5 positive pericytes aligned close to cells expressing the pro-angiogenic factor VEGF-R, known to play a major role in glioma angiogenesis and possibly invasiveness [35], pericytes activated in response to glioma growth did not express VEGF-R themselves.

The intratumoral GFP positive pericytes were all positive for the pericyte marker PDGFR-β, but only a proportion of the cells expressed the pericyte markers NG2, CD13 and α-SMA. It is known that pericytes constitute a heterogeneous cell population with a marker expression that varies depending on the surrounding tissue [36]. Furthermore, the marker expression can be altered under pathological conditions. For example, α-SMA is upregulated on pericytes in the central nervous system in response to a tumor [37], and NG2 becomes upregulated in response to angiogenesis [23]. Thus, it is likely that intratumoral pericytes, possibly recruited at different time points to the tumor, do not share the same expression marker profile.

To exclude the possibility that the GFP positive cells within the tumor become astrocytes, they were stained for S100B that is expressed by mature astrocytes surrounding blood vessels [38]. A considerable amount of S100B positive cells were seen within the tumor, however none of the cells co-localized with GFP. We also investigated whether the recruited pericytes could become activated microglia, as we have recently shown in ischemic stroke [12]. However, although a large number of Iba1 positive microglia was present in the GL261 tumors, none of the cells co-expressed GFP indicating that the recruited pericytes do not become microglia within the mouse glioma tumor or peritumoral microenvironment.

Taken together, our findings show that pericytes become activated in widespread areas of the brain in response to GL261 mouse gliomas. Non-tumor-derived pericytes infiltrate the glioma extensively and integrate with the vasculature. The findings thus strongly support that this glioma model constitutes a mosaic of host-derived and tumor-derived cells rather than being predominantly of a single cell clonal origin. If these results are confirmed in human glioma, the findings may provide a rational basis for targeting pericyte activation in glioma therapy.

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## Author Contributions

Conceived and designed the experiments: AS IÖ GP JB. Performed the experiments: AS IÖ. Analyzed the data: AS IÖ GP JB. Contributed reagents/materials/analysis tools: GG GP JB. Wrote the paper: AS IÖ GG GP JB.

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# Paper III

# Rat Multipotent Mesenchymal Stromal Cells Lack Long-Distance Tropism to 3 Different Rat Glioma Models

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**BACKGROUND:** Viral gene therapy of malignant brain tumors has been restricted by the limited vector distribution within the tumors. Multipotent mesenchymal stromal cells (MSCs) and other precursor cells have shown tropism for gliomas, and these cells are currently being explored as potential vehicles for gene delivery in glioma gene therapy.

**OBJECTIVE:** To investigate MSC migration in detail after intratumoral and extratumoral implantation through syngeneic and orthotopic glioma models.

**METHODS:** Adult rat bone marrow–derived MSCs were transduced to express enhanced green fluorescent protein and implanted either directly into or at a distance from rat gliomas.

**RESULTS:** We found no evidence of long-distance MSC migration through the intact striatum toward syngeneic D74(RG2), N32, and N29 gliomas in the ipsilateral hemisphere or across the corpus callosum to gliomas located in the contralateral hemisphere. After intratumoral injection, MSCs migrated extensively, specifically within N32 gliomas. The MSCs did not proliferate within tumors, suggesting a low risk of malignant transformation of *in vivo* grafted cell vectors. Using a model for surgical glioma resection, we found that intratumorally grafted MSCs migrate efficiently within glioma remnants after partial surgical resection.

**CONCLUSION:** The findings point to limitations for the use of MSCs as vectors in glioma gene therapy, although intratumoral MSC implantation provides a dense and tumor-specific vector distribution.

**KEY WORDS:** Gene delivery, Glioma, Mesenchymal stem cell, Multipotent mesenchymal stromal cell, Tumor

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Glioblastoma multiforme is an aggressive, invasive neoplasm in the brain. This tumor remains one of the most lethal forms of human cancer. Fewer than 3% of the 77 000 patients diagnosed each year with glioblastoma multiforme in the United States and Europe will survive > 5 years. Glioblastoma multiforme infiltrates crucial structures in the brain, preventing curative surgical resection. Radiation and chemotherapy offer only modest benefits and remain essentially palliative.[1] Gene therapy using viral vectors to target malignant gliomas is a potentially promising approach to improve glioblastoma multiforme treatment. However, clinical trials have had only very limited success so far. The main reason is the inefficient spread of viral vectors *in vivo*, and effective and sustained gene delivery into brain tumors still presents a major obstacle.[2]

**ABBREVIATIONS: FBS,** fetal bovine serum; **MSC,** multipotent mesenchymal stromal cell; **PBS,** phosphate-buffered saline

Implanted stem and precursor cells have emerged as an alternative vector system for gene delivery to gliomas and other tumors. Malignant gliomas have been reported to attract implanted and injected multipotent mesenchymal stromal cells (MSCs), neural precursor cells, endothelial cells, and hematopoietic stem and progenitor cells.[3-7] The previously reported capacity of these cells to track and home to malignant brain tumors would make these cells potentially more efficient than viral vectors for local delivery of therapeutic tumoricidal substances.[8]

It has been reported that MSCs migrate throughout normal brain parenchyma towards brain tumors after implantation at a distance from tumors in the ipsilateral or contralateral hemisphere.[5,6] However, a detailed analysis of the migratory capacity of adult MSCs, syngeneic to both glioma and host tissue, has not yet been reported but is certainly critical because the concept of using cells as gene vectors to tumors depends on their tumor-tropic migratory capacity.

Therefore, we implanted adult rat bone marrow–derived MSCs either directly into or at a distance from orthotopic and syngeneic rat gliomas and investigated MSC intratumoral infiltration and migration toward 3 different glioma models. We provide striking alternative findings compared with previous reports using MSCs as therapeutic delivery vehicles.

Our objectives were (1) to describe MSC migration in detail after intratumoral and extratumoral implantation to experimental syngeneic and orthotopic gliomas, (2) to investigate proliferation rates of implanted MSCs within gliomas, and (3) to elucidate MSC migratory pattern within glioma tissue after partial surgical tumor resection.

## METHODS

### Rat Glioma Cell Lines

The rat glioma cell lines D74(RG2), N32, and N29, syngeneic with the Fischer 344 rat, were originally induced by transplacental injection of ethyl-*N*-nitrosourea to a pregnant rat, the offspring of which developed malignant brain tumors.[9,10] N29 and N32 tumor cells were maintained in R10 medium consisting of RPMI 1640 medium (1 ×) with L-glutamine supplemented with 10% fetal bovine serum (FBS; VWR, West Grove, Pennsylvania), 10 mmol/L HEPES buffer solution, 1 mmol/L sodium pyruvate, and 50 mg/mL gentamicin. D74 cells were maintained in Dulbecco's Modified Eagle Medium with 10% FBS and 50 mg/mL gentamicin (all chemicals except FBS from GIBCO, Invitrogen, Carlsbad, California). Cells were detached with trypsin-EDTA (0.25% trypsin with EDTA 4Na) 1 × (Invitrogen). Cells were incubated at 37°C in a humidified atmosphere containing 6.0% $CO_2$. Before inoculation *in vivo*, cells were washed and resuspended in medium without FBS and gentamicin (referred to as R0 medium).

### Establishment and Culture of Bone Marrow–Derived Rat MSCs

The MSC cultures were derived from bone marrow of a Fischer 344 male rat (8 weeks old) as previously described.[11] Briefly, MSCs were generated by adherent culture of Ficoll-isolated nucleated bone marrow cells in NH expansion medium (Miltenyi Biotech, Bergisch Gladbach, Germany) or minimum essential medium-α supplemented with 10% FBS and 1% antibiotic antimycotic solution (Sigma-Aldrich, St. Louis, Missouri). Nonadherent cells were removed after 3 days, and culture medium was changed weekly thereafter.

### Enhanced Green Fluorescent Protein Retroviral Production and Transduction of MSCs

To visualize the MSCs, cells were genetically modified to express enhanced green fluorescent protein (eGFP). The MSCs were transduced with a Moloney leukemia–based retroviral vector, which has the characteristic of infecting dividing cells. The Moloney leukemia retroviral vector pCMMP-IRES2eEGFP-WPRE used in this study has been described elsewhere.[12] The viral particles were produced from the producer cell line 293VSVG.[13] Concentrated particles were resuspended in 0.5 mL of Dulbecco modified Eagle medium (Sigma-Aldrich). The titer was measured by FACSCalibur analysis, based on eGFP reporter gene expression, 3 days after infection of the HT1080 cells and varied from $0.7 \times 10^9$ to $1.2 \times 10^9$ TU/mL, depending on the batches. When at 60% to 70% confluence, MSCs were transduced at a multiplicity of infection of 5. To increase transduction efficiency, protamine sulfate was added to the medium at a final concentration of 1 mg/mL (Sigma). More than 90% of the cells expressed eGFP as assessed in an inverted microscope 4 days after transduction.

### Animal Procedure and Experimental Design

Adult male Fischer 344 rats (8-9 weeks old; from Scanbur, Stockholm, Sweden) were used. Animal procedures were approved by the Ethical Committee for Use of Laboratory Animals at Lund University, Lund, Sweden. Rats were anaesthetized with isoflurane (2.5% in $O_2$, Forene) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, California). The following coordinates were used for tumor cell inoculation into the right striatum (relative to bregma): anterior-posterior, +1.7; medial-lateral, −2.5; and dorsoventral to dura, −5.0. The tooth bar was set at −3.3 mm. Coordinates for tumor cell inoculation into the corpus callosum were as follows: anterior-posterior, +0.7; medial-lateral, −2.0; and dorsoventral to dura, −3.0. The tooth bar was set at −3.3 mm. Tumor cells were inoculated at 1 μL/min with a 10-μL Hamilton syringe. The MSCs were grafted at 0.5 μL/min with a 10-μL Hamilton syringe with a glass micropipette attached to the needle tip. After cell inoculation, the micropipette was kept in place for 5 minutes before being retracted slowly.

For studying MSC intratumoral infiltration, 3000 N32wt tumor cells were inoculated into the right striatum of male rats (n = 14) on day 1. The $eGFP^+$ MSCs ($2.5 \times 10^5$ cells) suspended as single cells in 5 μL cell medium were grafted on day 14 using the same coordinates as for tumor cell inoculation. Animals were killed on days 15, 18, and 22 after tumor cell inoculation, which correlate to days 1, 4, and 8 after MSC grafting (n = 4-5 at each time point).

For examination of proliferation rates of grafted MSCs, $eGFP^+$ cells were grafted intratumorally into established N32 intracerebral tumors (n = 3). Animals were killed on day 8 or on day 16 after MSC grafting.

In the experiment studying MSC migration throughout the normal brain parenchyma toward a distant malignant brain tumor, we established D74 (RG2; 3000 cells), N32 (1000 cells), and N29 (3000 cells) tumors in the right striatum. Seven days later, $eGFP^+$ MSCs ($2.5 \times 10^6$ cells, n = 5 in each group) were grafted 4.0 mm caudally and 2.0 mm lateral to the inoculation coordinates of the tumor. Animals were killed on day 22 after tumor cell inoculation, corresponding to day 14 after MSC grafting. In a parallel experiment, D74 (RG2; 3000 cells), N32 (1000 cells), and N29 (3000 cells) tumors were injected into the right mediofrontal corpus callosum. Seven days later, $eGFP^+$ MSCs ($2.5 \times 10^6$ cells, n = 5 in each group) were grafted into the corpus callosum at the corresponding coordinates on the contralateral (left) side. Animals were killed on day 21 after tumor cell inoculation.

To study long-time survival and migration in the adult normal brain, $eGFP^+$ MSCs were grafted into either the striatum (n = 4) or the corpus callosum (n = 4) of non–tumor-bearing animals. Animals were killed on

day 118 after MSC grafting (Figure 1B). The brain, liver, spleen, and cervical lymph nodes were analyzed for presence of $eGFP^+$ MSCs.

To study migration of grafted MSCs in gliomas after surgical resection, 3000 N32 glioma cells were inoculated into the striatum on day 1 (n = 5). Fourteen days later, established gliomas were partially resected. Subsequently, $2.5 \times 10^5$ $GFP^+$ MSCs were inoculated directly into the remaining tumor mass. Animals were killed 7 days later, and the brains were analyzed for the presence of $eGFP^+$ MSCs.

## Immunohistochemistry

The rats were deeply anaesthetized and perfused through the ascending aorta with phosphate-buffered saline (PBS), pH 7.4, followed by cold 4% paraformaldehyde in PBS. The brains were removed, postfixed in cold 4% paraformaldehyde overnight, and then transferred to 20% sucrose solution in PBS. Transversal sectioning of the brains was performed on a freezing microtome (40 μm), and the sections were put in antifreeze solution. Free-floating sections were rinsed 3 times in potassium PBS. Sections were blocked with 5% normal goat serum and 5% normal donkey serum in 0.25% Triton X-100 solution and then incubated with the primary antibodies chicken anti-GFP (1:1500; Chemicon, Temecula, California), mouse anti-Ki67 (1:50; Novocastra, Newcastle Upon Tyne, United Kingdom), mouse anti-NG2 (1:500; Chemicon), and rabbit anti-α-smooth muscle actin (1:400; Abcam) at 4°C overnight. The next day, the sections were rinsed 3 times in appropriate sera in potassium PBS and incubated for 2 hours with 1 or 2 of the following secondary antibodies: Alexa488 goat anti-chicken (1:400; Molecular Probes), Alexa594 goat anti-rabbit (1:400, Probes), or Cy3 donkey

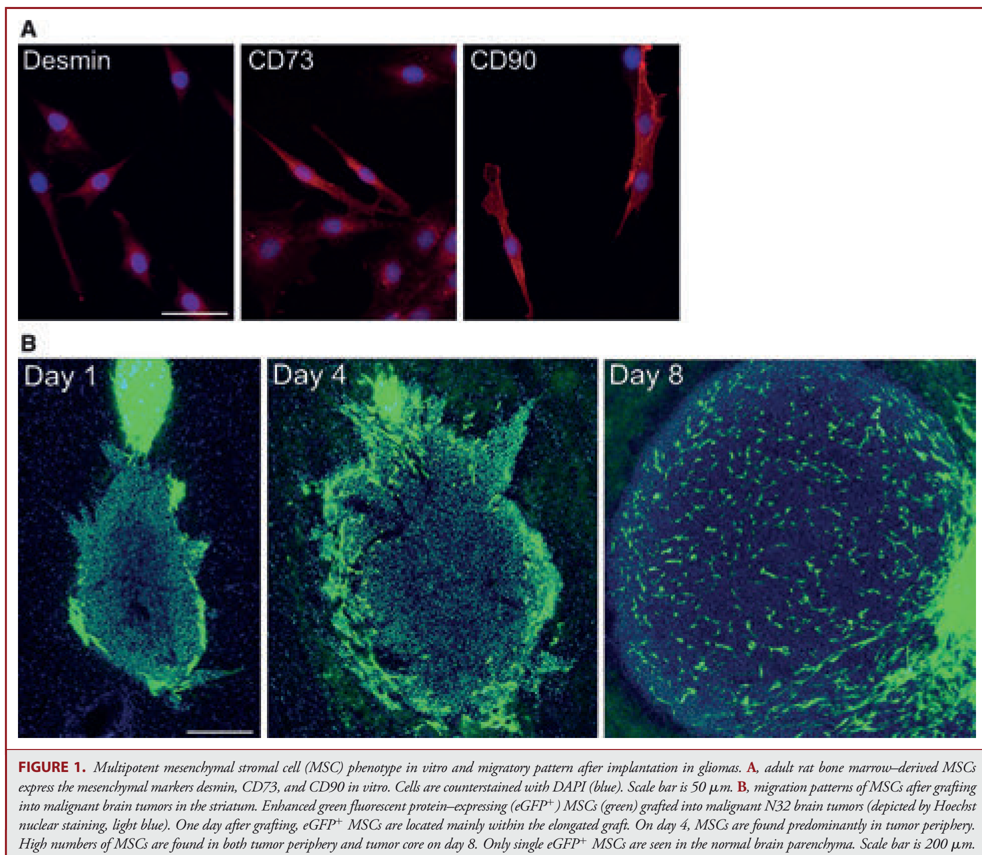


**FIGURE 1.** *Multipotent mesenchymal stromal cell (MSC) phenotype in vitro and migratory pattern after implantation in gliomas.* **A**, *adult rat bone marrow–derived MSCs express the mesenchymal markers desmin, CD73, and CD90 in vitro. Cells are counterstained with DAPI (blue). Scale bar is 50 μm.* **B**, *migration patterns of MSCs after grafting into malignant brain tumors in the striatum. Enhanced green fluorescent protein–expressing ($eGFP^+$) MSCs (green) grafted into malignant N32 brain tumors (depicted by Hoechst nuclear staining, light blue). One day after grafting, $eGFP^+$ MSCs are located mainly within the elongated graft. On day 4, MSCs are found predominantly in tumor periphery. High numbers of MSCs are found in both tumor periphery and tumor core on day 8. Only single $eGFP^+$ MSCs are seen in the normal brain parenchyma. Scale bar is 200 μm.*

anti-mouse (1:400, Jackson). The sections were counterstained with Hoechst nuclear staining to visualize tumors, mounted onto glass slides, and covered with a coverslip with DABCO mounting medium.

### Image Analysis

Sections were analyzed with an Olympus TX60 light microscope (Olympus, Tokyo, Japan), an Olympus BX60 epifluorescence microscope, or a confocal laser scanning microscopy (Leica Microsystems, Mannheim, Germany).

### Statistical Analysis

A Student unpaired *t* test was used for comparison between groups. Data are presented as mean ± SEM, and data are considered significant at $P < .05$.

## RESULTS

### Extensive and Tumor-Specific Intratumoral MSC Migration

We have previously characterized the MSCs used in the present study by their adherent growth, surface marker profile, and differentiation capacity. Using FACS, we have shown that the MSCs display the mesenchymal markers CD73, CD90, and CD105 but not the hematopoietic markers CD34 and CD45. Thus, the MSCs express MSC-associated markers and no hematopoietic stem cell–associated markers. Furthermore, the MSCs possess the capacity to differentiate into osteoblasts and adipocytes on exposure to conditions promoting differentiation.[11] In the present study, adult rat bone marrow–derived MSCs were found to be positive for the mesenchymal markers desmin, CD73, and CD90 by immunocytochemistry (Figure 1A).

To determine the capacity of MSCs to migrate within gliomas after intratumoral implantation, we established N32 gliomas in the striatum (n = 14). The MSCs, transduced to express eGFP, were grafted into tumors, and the migration pattern was assessed at 3 time points (n = 4-5 for each time point) after grafting. On day 1 after grafting, eGFP$^+$ MSCs were found within a well-defined elongated cluster at the inoculation site adjacent to or surrounding the tumor. Single MSCs were located in the outer periphery of the tumor or in the normal brain parenchyma, but the absolute majority of the grafted cells were seen at the inoculation site (Figure 1B). Already on day 4 after grafting, numerous MSCs were found within the peripheral zone of the tumor (Figure 1B). Single MSCs were also found in the core of the tumor. On day 8 after grafting, abundant MSCs were located in both the tumor periphery and core (Figure 1B). The vast majority of MSCs were located within the graft or within the tumor, and only a few MSCs were found in the normal brain parenchyma at any time point.

### No Evidence of Ongoing Proliferation of MSCs *In Vivo*

Confocal microscopy analysis of eGFP and Ki67 expression was used to investigate the cell-cycle state of MSCs grafted into N32 malignant brain tumors. Ki67 is a marker of cells in the G1, S, M, and G2 cell-cycle phases, ie, dividing cells. The MSCs were grafted into established N32 tumors and analyzed on days 8 and 16 after grafting. One hundred eGFP$^+$ cells in each tumor model were randomly chosen and analyzed by confocal microscopy for expression of Ki67. Grafted eGFP$^+$ cells located within the core of the graft and migratory eGFP$^+$ cells located intratumorally but far from the graft core were analyzed. Proliferating tumor cells expressing Ki67 were used as positive controls. We did not find a single eGFP$^+$ MSC that expressed Ki67. Representative examples of non–Ki67-expressing eGFP$^+$ MSCs and MSCs within highly proliferative tumors are shown in Figure 2A and 2B. We conclude that the absolute majority of intratumorally grafted MSCs are in a noncycling state 8 and 16 days after MSC grafting.

### No Long-Distance MSC Migration After Grafting Into the Normal Brain Parenchyma

We investigated whether MSCs, grafted at an ipsilateral but distant site to established N32, N29, and D74 tumors, would migrate through the normal brain parenchyma toward tumors. The eGFP$^+$ MSCs were grafted 4 mm behind and 2 mm lateral to striatal tumors established 7 days earlier. On day 14 after MSC grafting, eGFP$^+$ cell distribution was assessed. In contrast to the MSC distribution pattern after intratumoral grafting, grafting into the normal brain parenchyma did not result in directed MSC migration toward any of the tumor types (Figure 3); MSCs were seen in a coherent cluster of cells at the inoculation site. Scattered cells were also observed in the immediate vicinity outside the cluster (Figure 3). In addition, MSC grafting was performed into the frontal corpus callosum contralateral to N32, N29, and D74 tumors. No tumor-tropic MSC migration along the corpus callosum was observed (Figure 4). In addition to the analysis of eGFP$^+$ cells, we analyzed the expression of endogenous markers expressed by grafted MSCs (NG2 and α-smooth muscle actin). We found no evidence of spindle-shaped cells expressing NG2 or α-smooth muscle actin that migrated from the MSC graft toward tumors (data not shown). These results confirm the absence of MSC migration throughout normal brain tissue toward tumors.

### Long-term MSC Migration in the Intact Brain

To study long-time survival and migration in the adult normal brain, eGFP$^+$ MSCs were grafted into either the striatum or the corpus callosum of non–tumor-bearing animals. Animals were killed 118 days after intracranial MSC grafting. Low numbers of MSCs were seen preferentially at the site of the inoculation coordinate. We did not find any MSCs at any other location of the analyzed sections. To elucidate tropism to other organs, the liver, spleen, and cervical lymph nodes were analyzed for the presence of eGFP$^+$ MSCs. Using immunofluorescence microscopic analysis, we did not find any eGFP$^+$ MSCs in sections from the liver, spleen, or cervical lymph nodes.

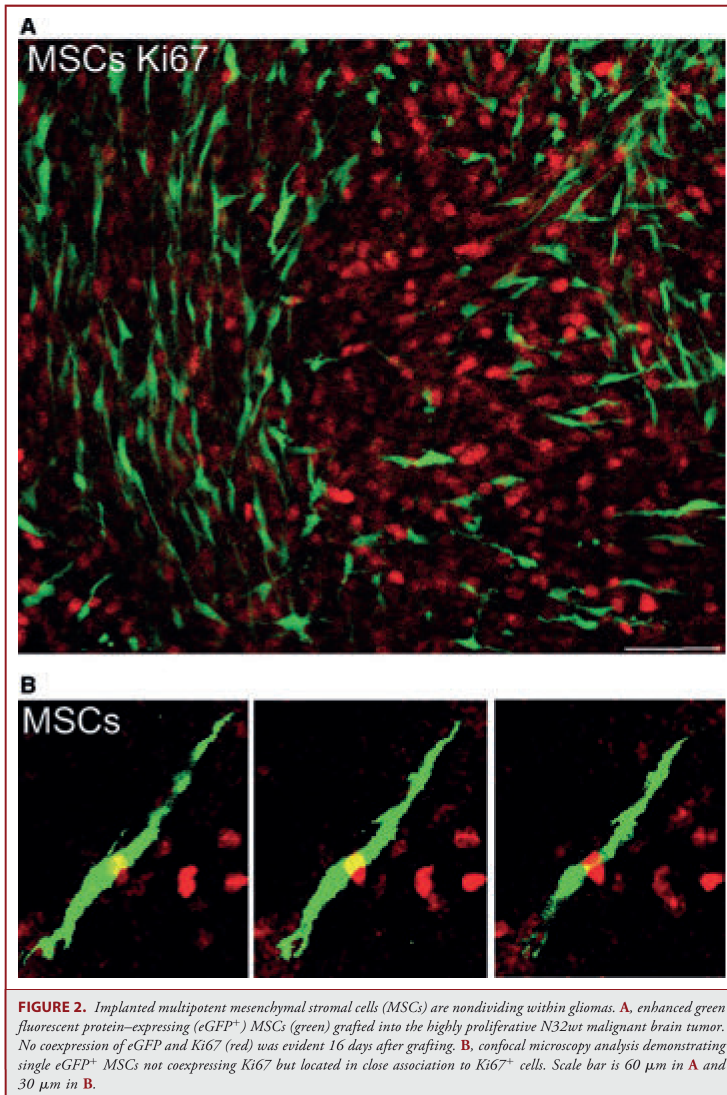


**FIGURE 2.** *Implanted multipotent mesenchymal stromal cells (MSCs) are nondividing within gliomas.* **A**, *enhanced green fluorescent protein–expressing (eGFP$^{+}$) MSCs (green) grafted into the highly proliferative N32wt malignant brain tumor. No coexpression of eGFP and Ki67 (red) was evident 16 days after grafting.* **B**, *confocal microscopy analysis demonstrating single eGFP$^{+}$ MSCs not coexpressing Ki67 but located in close association to Ki67$^{+}$ cells. Scale bar is 60 μm in* **A** *and 30 μm in* **B**.

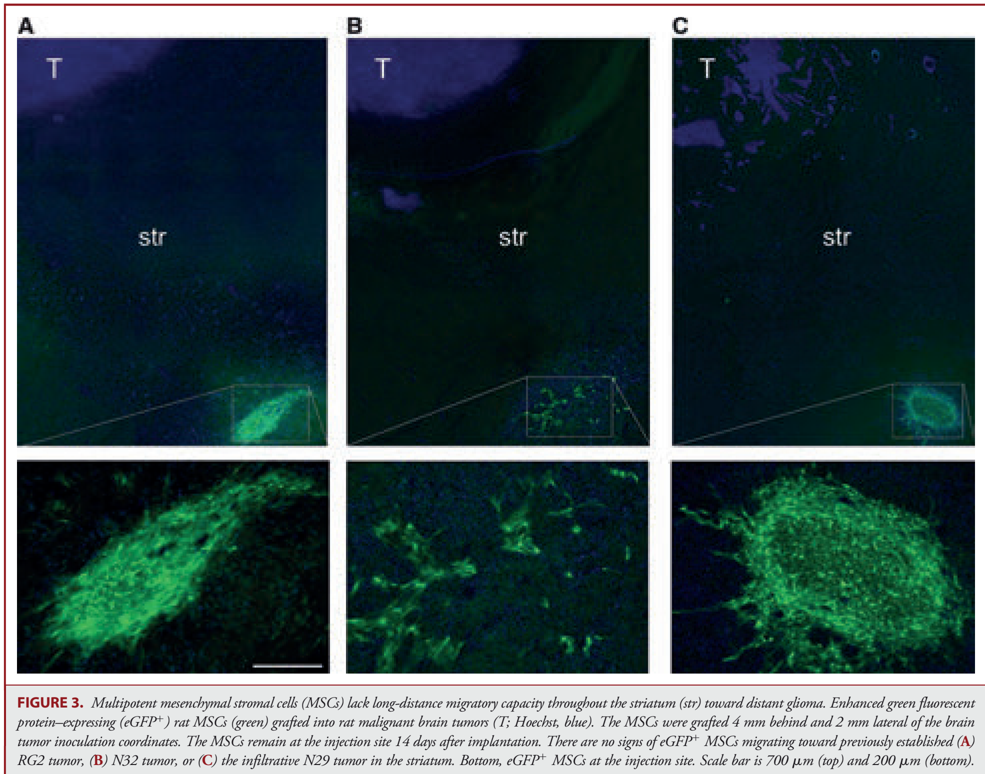


**FIGURE 3.** *Multipotent mesenchymal stromal cells (MSCs) lack long-distance migratory capacity throughout the striatum (str) toward distant glioma. Enhanced green fluorescent protein–expressing (eGFP$^+$) rat MSCs (green) grafted into rat malignant brain tumors (T; Hoechst, blue). The MSCs were grafted 4 mm behind and 2 mm lateral of the brain tumor inoculation coordinates. The MSCs remain at the injection site 14 days after implantation. There are no signs of eGFP$^+$ MSCs migrating toward previously established (**A**) RG2 tumor, (**B**) N32 tumor, or (**C**) the infiltrative N29 tumor in the striatum. Bottom, eGFP$^+$ MSCs at the injection site. Scale bar is 700 μm (top) and 200 μm (bottom).*

### Grafted MSCs Migrate After Surgical Resection

To examine grafted MSC migration in a glioma surgical treatment model, we first established a glioma model that included partial surgical resection of established N32 gliomas (Figure 5A). We then used this model to investigate whether grafted MSCs migrate within tumor remnants after partial surgical resection. Brains were analyzed for the presence of eGFP$^+$ MSCs 7 days after tumor resection and MSC implantation. We found extensive MSC survival and migration within the remaining glioma tissue and only minimal MSCs in the surrounding normal brain tissue (Figure 5B).

## DISCUSSION

Here, we describe in detail the migratory patterns of grafted rat MSCs using 3 different rat orthotopic glioma models syngeneic to the Fischer 344 rat. Grafting was performed either into or at a distance from gliomas. In contrast to previous data,[5,6] we found no evidence of long-distance MSC migration throughout the corpus callosum toward gliomas located in the contralateral hemisphere. In addition, there were no signs of long-distance MSC migration through normal brain tissue toward distant gliomas located in the ipsilateral hemisphere. In contrast, MSCs migrate efficiently and specifically within gliomas after intratumoral grafting. Our results imply that MSC migration toward tumors in normal brain tissue is severely restricted and that MSCs should be implanted by intratumoral injection for efficient distribution within tumors. Furthermore, using a glioma surgical treatment model, we found that intratumorally grafted MSCs migrate efficiently within glioma remnants after partial surgical resection.

The discrepancy between our results and previous studies[5,6] might be due to species-specific interactions (ie, between human MSCs and human glioma xenografts in mice) and/or

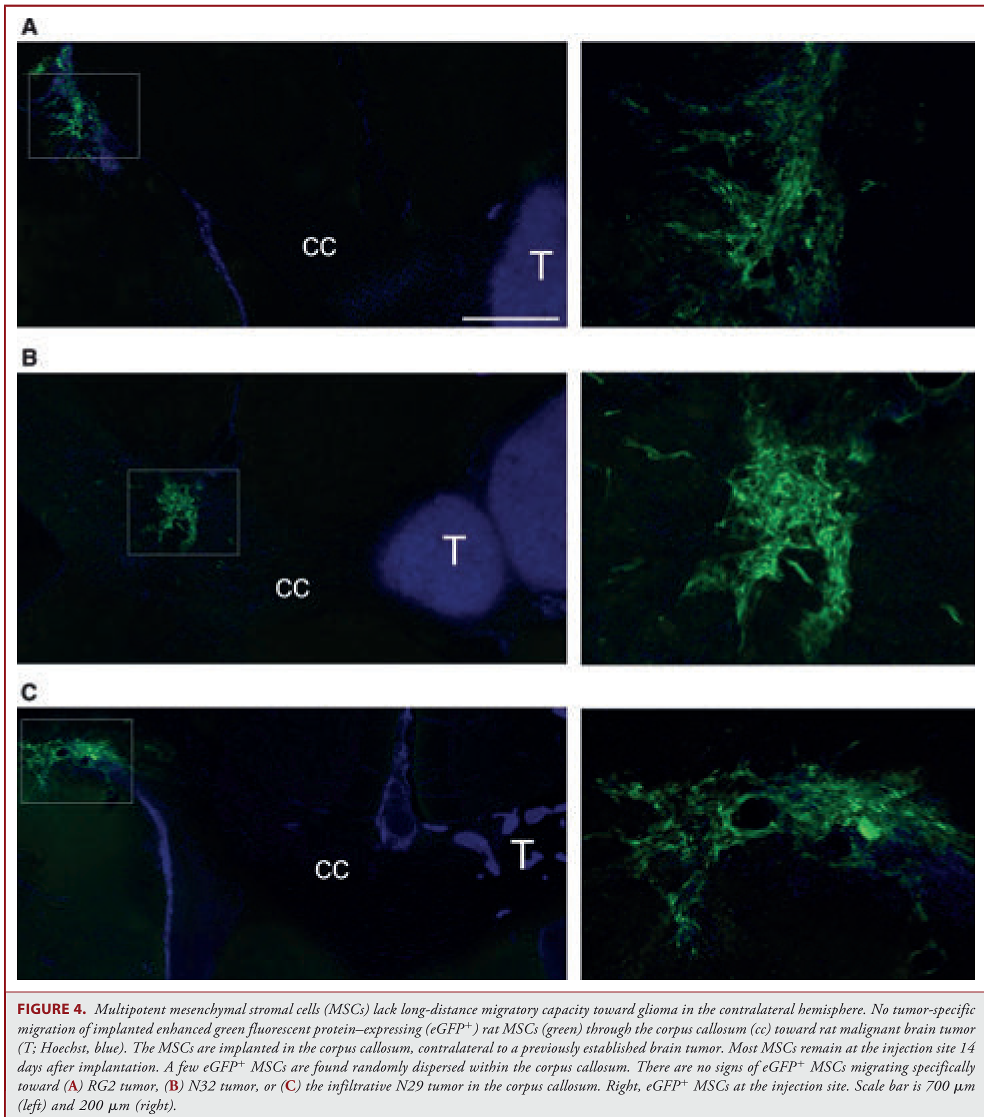


**FIGURE 4.** *Multipotent mesenchymal stromal cells (MSCs) lack long-distance migratory capacity toward glioma in the contralateral hemisphere. No tumor-specific migration of implanted enhanced green fluorescent protein–expressing (eGFP$^+$) rat MSCs (green) through the corpus callosum (cc) toward rat malignant brain tumor (T; Hoechst, blue). The MSCs are implanted in the corpus callosum, contralateral to a previously established brain tumor. Most MSCs remain at the injection site 14 days after implantation. A few eGFP$^+$ MSCs are found randomly dispersed within the corpus callosum. There are no signs of eGFP$^+$ MSCs migrating specifically toward (***A***) RG2 tumor, (***B***) N32 tumor, or (***C***) the infiltrative N29 tumor in the corpus callosum. Right, eGFP$^+$ MSCs at the injection site. Scale bar is 700 μm (left) and 200 μm (right).*

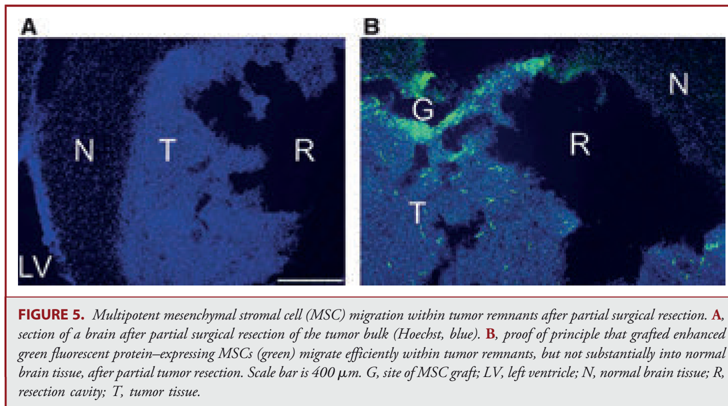


**FIGURE 5.** *Multipotent mesenchymal stromal cell (MSC) migration within tumor remnants after partial surgical resection.* **A**, *section of a brain after partial surgical resection of the tumor bulk (Hoechst, blue).* **B**, *proof of principle that grafted enhanced green fluorescent protein–expressing MSCs (green) migrate efficiently within tumor remnants, but not substantially into normal brain tissue, after partial tumor resection. Scale bar is 400 μm. G, site of MSC graft; LV, left ventricle; N, normal brain tissue; R, resection cavity; T, tumor tissue.*

to different tumor-tropic migratory properties between different subpopulations of MSCs. Tumor model–specific factors such as the production of growth factors and molecules involved in angiogenesis[5,14-17] and the levels of chemokines and cytokines[15,18-21] in the vicinity of the tumor could also play a decisive role in the attraction of grafted MSCs. The discrepant results might also be due to differences in graft labeling techniques. Interpretation of survival and migratory behavior of grafted cells is critically dependent on the sensitivity and specificity of graft labeling. Nongenetic labeling techniques such as fluorescent dye or iron labeling may result in unspecific labeling after the death of grafted cells and the uptake of dye in resident host microglia, macrophages, and rapidly dividing tumor cells. Importantly, we have previously shown that the MSC eGFP expression pattern *in vivo* correlates with findings from fluorescence in situ hybridization analysis in which Y chromosomes carrying male MSCs were implanted into female hosts.[11] Thus, our results are derived from 2 independent analyses of implanted MSCs.

It has been reported that human MSCs can be found in human glioma xenografts in immunocompromised mice after intracarotid injections.[5] In contrast, we previously found no evidence of intravenously injected MSCs within intracranial gliomas after a single injection,[11] although we used a rat syngeneic transplantation model. However, as shown in the present study, intratumoral injections of MSCs result in substantial tumor-specific migration throughout the entire tumor. Furthermore, keeping in mind that systemic vascular administration of stem and progenitor cells carries a risk for a high frequency of serious systemic side effects such as pulmonary embolism,[22,23] our data suggest that the best administration route for MSCs in glioma therapy may be by intratumoral implantation rather than by systemic injections.

A large number of soluble and membrane-bound factors produced by tumor cells, tumor vasculature, and inflammatory cells can attract stem and progenitor cells to gliomas (see elsewhere[24] for review). The substantial intratumoral MSC migration and virtual absence of MSC migration in normal brain tissue suggest that the tumor microenvironment, eg, tumor vasculature and inflammatory cells, is permissive for migration of grafted MSCs. Active neoangiogenesis and/or inflammation are presumably required for MSC attraction to gliomas.

A major safety issue in the development of stem cell therapies for neurological disorders is the risk of tumor formation of grafted stem cells.[25] Bone marrow–derived mouse MSCs have been implicated in the development of Ewing sarcoma.[26] In our experiments, we found no indication of MSC proliferation *in vivo* 8 and 16 days after grafting into the highly proliferative N32 tumor. These results point to a low risk for the development of secondary malignancies from grafted MSCs. Noteworthy in terms of safety, no infiltration of cervical lymph nodes, liver, or spleen was seen in the present study after grafting of MSCs intratumorally.

## CONCLUSION

Rat MSCs effectively spread out in experimental rat glioma tumor tissues after intratumoral implantation. In contrast to previous reports, we found no evidence of long-distance MSC migration across the corpus callosum or through the striatum toward malignant gliomas. Our results indicate that intratumoral implantation may be the method of choice for MSC-based treatment approaches of malignant brain tumors.

### Disclosure

This work was supported by the Swedish Cancer Society, the Swedish Childhood Cancer Foundation, Hans and Märit Rausing Charitable Trust, Segerfalk Foundation, Crafoord Foundation, Elsa Schmitz Foundation, Magnus Bergvall Foundation, Lund University Hospital Foundation, Gunnar Nilsson Cancer Foundation, and the Royal Physiographic Society in Lund. The authors

have no personal financial or institutional interest in any of the drugs, materials, or devices described in this article.

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## COMMENT

This study shows that the long-distance migration of mesenchymal stem cells (MSCs) to tumors may not be a universal phenomenon. Because MSCs are being developed by many groups as vectors for therapy, this is an important point, and the biological basis for the difference needs to be better understood. In the present study, a syngeneic rat model is used, which offers some advantages over human xenografts, most notably an intact host immune system. The sound evidence shown in this article, which supports the conclusion that the autologous MSCs in this model do not migrate to a tumor over long distances, means that we need to know more about the signals emanating from these rat glioma cells and human cells used in other studies in which such migration was demonstrated. At the same time, the authors show us very encouraging data of MSCs that are delivered close to the tumor mass and broadly infiltrate it. Even more relevant to the clinical scenario is their finding that MSCs migrate into a partially resected tumor. Perhaps the biggest challenge in controlling glioblastomas is targeting the dispersed, treatment-resistant cells beyond the magnetic resonance imaging signal, and it is likely that a broad distribution of MSCs will be needed to do so effectively. In this scenario, the long-distance tracking by MSCs may not be as important as their ability to follow tumor cells at short range. Defining exactly what that range is will be critical to making such therapies effective.

**Oliver Bogler**
*Houston, Texas*

# Paper IV

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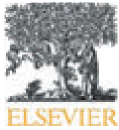

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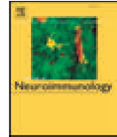

Short communication

# Intratumorally implanted mesenchymal stromal cells potentiate peripheral immunotherapy against malignant rat gliomas

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ABSTRACT

Bone marrow-derived mesenchymal stromal cells (MSCs) target glioma extensions and micro-satellites efficiently when implanted intratumorally. Here, we report that intratumoral implantation of MSCs and peripheral immunotherapy with interferon-gamma (IFNγ) producing tumor cells improve the survival of glioma-bearing rats (54% cure rate) compared to MSC alone (0% cure rate) or immunotherapy alone (21% cure rate) by enforcing an intratumoral $CD8^+$ T cell response. Further analysis revealed that the MSCs up-regulate MHC classes I and II in response to IFNγ treatment in vitro and secrete low amounts of immunosuppressive molecules prostaglandin $E_2$ and interleukin-10.

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## 1. Introduction

The prognosis for patients with high-grade gliomas remains very poor despite extensive surgical resection and adjuvant chemo- and radiotherapy (Lamborn et al., 2008; Grossman et al., 2010). Treatment failure is mainly ascribed to the infiltrative capacity of the tumor cells, which form microsatellites deep within the normal brain.

Bone marrow-derived multipotent mesenchymal stromal cells (MSCs) display inherent tumor-tropic properties and constitute a novel treatment approach with the potential to target tumor microsatellites. Following intratumoral implantation, the MSCs migrate extensively throughout experimental brain tumors, whereas no infiltration of the normal brain has been detected (Bexell et al., 2009). This glioma-specific tropism has been exploited to deliver anti-neoplastic agents such as pro-drug converting enzymes, oncolytic viruses and pro-inflammatory cytokines (Yong et al., 2009; Gunnarsson et al., 2010; Matuskova et al., 2010).

Several reports show that MSCs can be polarized into an immunostimulatory/anti-tumoral phenotype when exposed to e.g. interferon-gamma (IFNγ) (Le Blanc et al., 2003; Stagg et al., 2006; Romieu-Mourez et al., 2007, 2009; Waterman et al., 2012). In this context, peripheral immunotherapy using cytokine-secreting tumor cells can eradicate experimental gliomas by inducing a pro-inflammatory tumor-microenvironment (Visse et al., 1999, 2000; Fritzell et al., 2013b) and in the present study we investigated the combinatorial effect of intratumoral MSCs and immunotherapy against rat gliomas.

## 2. Material and methods

### 2.1. Cells

The N32 rat glioma cell line is syngeneic with Fischer 344 rats and resembles anaplastic astrocytoma (Janelidze et al., 2009). The cells have been transduced to express IFNγ (N32-IFNγ) (Visse et al., 1999) and both cell lines were cultured as described elsewhere (Eberstal et al., 2012).

MSCs were isolated from the bone marrow of male Fischer 344 rats. Previously, the cells have been characterized and transduced to express enhanced green fluorescent protein (GFP; MSC-GFP) and were cultured as earlier described (Bexell et al., 2009).

All cells were maintained at 37 °C in the presence of 6.0% $CO_2$.

### 2.2. Flow cytometry and ELISA

100,000 MSC-GFP cells were cultured with recombinant IFNγ (rIFNγ; 0–10,000 U/ml, Miltenyi Biotec Norden AB, Lund, Sweden) for 24 h. Afterwards, cells were pre-incubated with anti-CD16/CD32 and stained using PE-RT1A (OX-18; MHC I), PE-RT1B (OX-6; MHC II) or

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PE-$IgG_1$ κ isotype control (BD Biosciences, Stockholm, Sweden). Fluorescence was analyzed using a C6 Flow Cytometer (Accuri Cytometers, Inc., Ann Arbor, USA).

Supernatants were assessed for the production of interleukin (IL)-10 (BD Biosciences) and prostaglandin $E_2$ ($PGE_2$) (Cayman Chemicals, Larodan Fine Chemicals AB, Malmö, Sweden) using ELISA.

### 2.3. Survival study

All animal procedures were performed according to the practices of the Swedish board of Animal Research and were approved by the Committee of Animal Ethics in Lund-Malmö, Sweden.

3000 N32 tumor cells were inoculated intracerebrally (i.c.) into the right striatum of male Fischer 344 rats (8–9 weeks old; NOVA-SCB AB, Sollentuna, Sweden), as previously described (Bexell et al., 2009). On days 1, 14 and 28, animals were immunized subcutaneously (s.c.) with 3,000,000 irradiated (80 Gy) N32-IFNγ cells into the right thigh. On days 7 and 17, rats received 250,000 MSC-GFP cells i.c.

Animals were euthanized when neurological symptoms appeared and post-mortem examinations confirmed i.c. tumors.

### 2.4. Tumor-infiltration study

3000 N32 tumor cells were inoculated i.c. into male Fischer 344 rats. On days 4 and 14, 3,000,000 irradiated N32-IFNγ tumor cells were injected intraperitoneally (i.p.) and on days 7 and 11, animals received 250,000 MSC-GFP cells i.c. Animals were euthanized on day 25 and brains were snap frozen and cut into 6 μm sections.

Sections were fixed in acetone (10 min) and endogenous peroxidase was blocked using peroxidase blocking solution (Dako, Glostrup, Denmark). 5% donkey serum was added for 20 min (Jackson ImmunoResearch Laboratories, West Grove, USA) and afterwards, sections were incubated 60 min with anti-rat TCRαβ (R73) or anti-rat CD8α (OX-8, 5 μg/ml; BD Biosciences). Later, sections were incubated for 30 min with donkey anti-mouse-biotin or donkey anti-rabbit-biotin (5 μg/ml), followed by streptavidin–peroxidase for 30 min (5 μg/ml; Jackson ImmunoResearch). Finally, sections were stained with AEC (Dako) for 5 min before Mayer's Hematoxylin (30 s). As negative controls, the primary antibodies were omitted.

Tumor sections were analyzed using a light microscope (BX-60, Olympus America Inc., Melville, USA) and images were taken at 10× magnification. T cell infiltration was calculated as percent stained cells/tumor area using analySIS® software (Olympus).

### 2.5. Statistics

In vitro statistical analyses were performed using paired samples *t*-test. Log-rank test was used for calculating differences between groups in the survival curve and the Mann–Whitney *U*-test was used for comparison between two unpaired groups in vivo. Statistical analyses were performed using GraphPad Prism Software (GraphPad Software, San Diego, USA), where $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. The MSC-GFP cells acquire an immunostimulatory phenotype in response to IFNγ

First, we assessed whether treatment with IFNγ could induce MSC-GFP cells with an immunostimulatory phenotype and addition of rIFNγ (0–10,000 U/ml) significantly up-regulated major histocompatibility complex (MHC) classes I and II ($p < 0.01$; Fig. 1A–B). Moreover, the MSC-GFP cells secreted low levels of the immunosuppressive factors $PGE_2$ (mean: 81 pg/ml) and IL-10 (mean: 2 pg/ml), however neither factor was significantly reduced upon treatment with rIFNγ (Fig. 1C–D).

### 3.2. Improved survival by intratumoral MSCs and peripheral IFNγ-immunotherapy

Next, we investigated the impact of intratumorally grafted MSC-GFP cells against rat gliomas either as monotherapy or in combination with peripheral IFNγ-immunotherapy. As shown in Fig. 2, MSC-GFP and immunotherapy significantly increased the cure rate of glioma-bearing rats (54%) compared with MSC-GFP alone (0%; $p < 0.001$) or immunotherapy only (21%; $p < 0.01$).

### 3.3. The combination therapy increases tumor-infiltrating $TCR^+$ and $CD8^+$ cells

The degree of tumor-infiltrating T cells in glioblastoma patients correlates positively with survival (Lohr et al., 2011; Kmiecik et al., 2013) and here we found that the amounts of tumor-infiltrating $TCR\alpha\beta^+$ cells were significantly elevated in animals receiving the combination therapy (mean: 9.0%) compared with MSC-GFP alone (mean: 0.5%; $p < 0.001$) or immunotherapy alone (mean: 2.1%; $p < 0.01$) (Fig. 3B–C). The levels of $CD8\alpha^+$ cells were also elevated in animals receiving the combination therapy (mean: 8.0%) compared with MSC-GFP alone (mean: 1.6%; $p < 0.001$) or immunotherapy alone (mean: 3.5%; $p < 0.05$) (Fig. 3B, D).

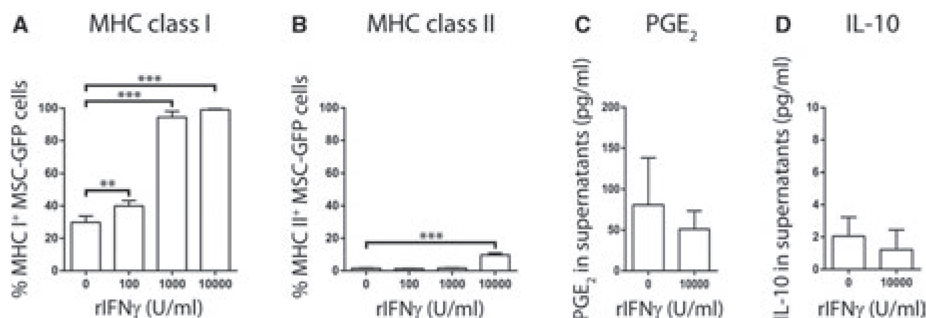


**Fig. 1.** MSC-GFP cells up-regulate MHC I and II upon IFNγ treatment and secrete low levels of $PGE_2$ and IL-10. 100,000 MSC-GFP cells were cultured for 24 h in the presence of rIFNγ (0–10,000 U/ml). The expression of (A) MHC class I and (B) MHC class II were determined in duplicates in three separate experiments using flow cytometry. The levels of (C) $PGE_2$ and (D) IL-10 were determined in supernatants in duplicates in three separate experiments using ELISA. Differences between groups were determined using paired samples *t*-test.

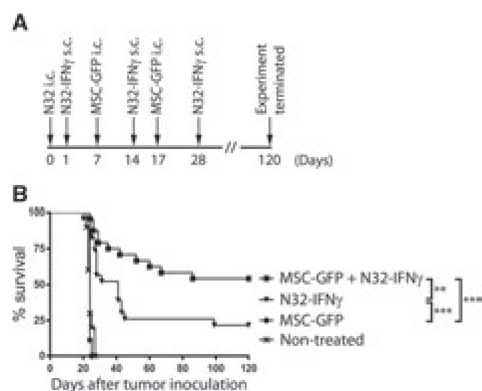


**Fig. 2.** Increased survival in glioma-bearing rats treated with MSC-GFP i.c. and peripheral IFNγ-immunotherapy. N32 tumor cells were inoculated i.c. into rats. On days 1, 14 and 28, animals were immunized s.c. with irradiated N32-IFNγ cells. On days 7 and 17, rats were injected i.c. with MSC-GFP cells. (A) Experimental setup. (B) Kaplan–Meier survival graph. Groups include 9–24 animals and were compared using Log-rank test.

## 4. Discussion

In the current study, we demonstrate that intratumorally grafted MSC-GFP cells enhance an IFNγ-based immunotherapy and induce cure in glioma-bearing rats, whereas immunotherapy only was less effective and MSC-GFP alone had no effect. In this context, we previously reported that IL-7-producing MSCs (MSC-IL-7) and immunotherapy could eradicate rat gliomas (Gunnarsson et al., 2010). In that study however, MSC-IL-7 alone was sufficient to attenuate tumor growth, indicating that immunostimulatory MSCs are required when used as monotherapy, as reported by Waterman et al. (2012).

Immunotherapy per se induces an immune response associated with elevated plasma IFNγ levels and $CD4^+$ and $CD8^+$ T cells systemically and intratumorally (Visse et al., 2000; Eberstal et al., 2012; Fritzell et al., 2013a, b; Eberstal et al., 2014). IFNγ is predominately produced by activated T cells and thus, intratumorally implanted MSCs are most likely exposed to significant levels of IFNγ. MSCs are reported to induce MHC expression and antigen presentation capabilities in response to IFNγ (Le Blanc et al., 2003; Romieu-Mourez et al., 2007) and we demonstrate that the MSC-GFP cells up-regulate MHC classes I and II upon IFNγ treatment in vitro. Moreover, the cells secrete low levels of the immunosuppressive molecules $PGE_2$ and IL-10.

MSCs are reported to induce cytotoxic $CD8^+$ T cells in response to IFNγ (Stagg et al., 2006) and presence of intratumoral T cells has been associated with prolonged survival in high-grade glioma patients (Lohr et al., 2011; Kmiecik et al., 2013). In this context, we here show that the combination therapy resulted in elevated amounts of tumor-infiltrating $TCR^+$ and $CD8^+$ cells compared to both monotherapies.

Our results suggest that immunotherapy generates a pro-inflammatory tumor-microenvironment where transplanted MSC-GFP cells can become immunostimulatory in vivo, thereby contributing to tumor clearance by e.g. acting as antigen presenting cells or by inducing $CD8^+$ T cells. Furthermore, intratumorally grafted MSCs display a pericyte-like phenotype (Bexell et al., 2009) and pericytes are reported to normalize the tumor vasculature and increase the tumor-infiltrating T cells following immunotherapy (Hamzah et al., 2008). When MSC-GFP is applied as monotherapy however, the cells are transplanted into a non-primed immunosuppressive environment where the MSCs rather contribute to tumor growth by e.g. suppressing T cell proliferation (Kraman et al., 2010; Najar et al., 2010). The observed effect might also be a consequence of the GFP expression itself due to the protein's immunogenicity (Rosenzweig et al., 2001; Re et al., 2004). However, no animal receiving MSC-GFP alone survived and the tumors contained very low amounts of tumor-infiltrating T cells. Hence, we conclude that the therapeutic effect is not solely due to immune reactivity against GFP.

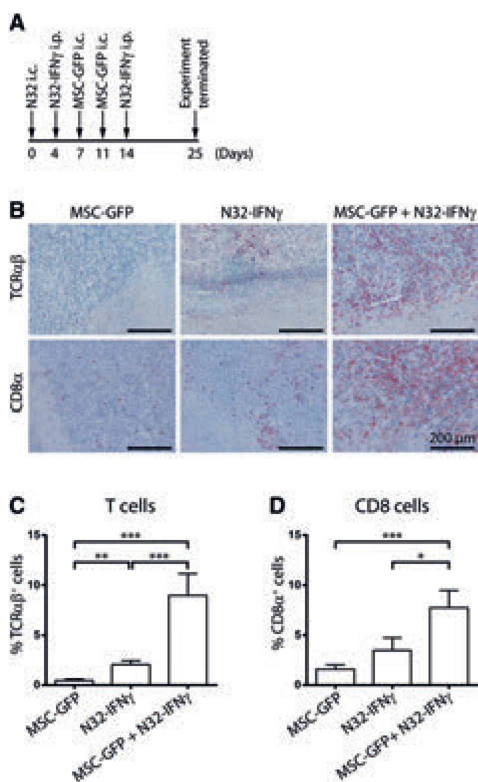


**Fig. 3.** Increased intratumoral infiltration of $CD8^+$ and $TCR^+$ cells in rats receiving the combination therapy. Tumor-bearing rats were immunized i.p. with irradiated N32-IFNγ cells on days 4 and 14. On days 7 and 11, rats were injected i.c. with MSC-GFP cells. Animals were euthanized on day 25 and the brains were sectioned and analyzed for the presence of $TCR\alpha\beta^+$ or $CD8\alpha^+$ cells and hematoxylin (nuclear staining). (A) Experimental setup. (B) Images were taken at 10× magnification at the tumor border. (C–D) Quantitative analysis of intratumoral (C) $TCR\alpha\beta^+$ and (D) $CD8\beta^+$ cells by calculating percent stained cells/tumor area. Groups include 5–11 animals and differences between groups were determined using the Mann–Whitney *U*-test.

In conclusion, the present study shows that intratumoral implantation of MSCs and peripheral IFNγ-immunotherapy can induce cure in glioma-bearing rats. These results underscore the central role of targeting the glioma-microenvironment when turning an indolent immune response into an effective anti-tumor response.

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