Stem cell based therapy of malignant brain tumors

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Stem cell based therapy of malignant brain tumors

Mesenchymal and neural precursor cells as migratory vehicles in experimental gliomas

av

Daniel Bexell

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Mesenchymal and neural precursor cells as migratory vehicles in experimental gliomas

Abstract Malignant brain tumor is a highly invasive and vascularized cancer. Current forms of therapy are not curative and can, at best, prolong survival for patients with this disease. The mean survival is only around 15 months. The aims of the present thesis have been to develop and explore a stem cell based vector system for delivery of therapeutic transgenes to experimental gliomas.

First, we investigated the frequency and properties of cancer stem cell (CSC)-like cells in the rat glioma models used in the present thesis. Virtually all cells within both glioma models concomitantly express CD133, nestin as well as the neural lineage markers glial fibrillary acidic protein, βIII-tubulin and CNPase *in vitro* and *in vivo*. Unsorted tumor cells displayed very high clonogenic capacity *in vitro* and robust tumorigenicity *in vivo*. Thus, CSC-like cells do not reside within a rare sub-population of cells in these glioma models but constitute most, or all, cells.

We next examined the subventricular zone (SVZ) response to growth of malignant glioma. Tumor growth resulted in decreased numbers of SVZ proliferating cells, increased SVZ neuroblast immunoreactivity, and migration of striatal neuroblasts from the SVZ toward glioma. Although endogenous neuroblast migration toward gliomas was of moderate magnitude, this indicates a region specific reparative mechanism in response to tumor growth. We continued by implanting rat SVZ-derived neural precursor cells (NPCs) and rat multipotent mesenchymal stroma cells (MSCs) into gliomas. Intratumorally implanted NPCs and MSCs migrated specifically within gliomas and largely avoided normal brain tissue. Importantly, implanted NPCs and MSCs did not proliferate within tumors. This indicates a low risk of development of secondary malignancies. A comparative analysis revealed higher survival and better intratumoral migratory capacity of implanted MSCs, compared to NPCs.

Intratumorally implanted MSCs migrated to the majority of the invasive glioma extensions and to a substantial fraction of distant tumor microsatellites. Furthermore, implanted MSCs integrated into tumor blood vessel walls and expressed pericyte markers but not endothelial cell markers. The pericyte marker expression profile and perivascular location of implanted MSCs indicate that these cells act as pericytes within tumors. MSC implantation did not, however, affect tumor microvessel density or the survival of glioma-bearing animals.

In summary, gliomas can attract endogenous striatal neuroblasts from the SVZ. However, intratumoral implantation of NPCs and MSCs yields a much more powerful, and tumor-specific, infiltration within gliomas. In particular, pericyte-like MSCs represent a well suited vector system for the delivery of therapeutic transgenes to vascularized and invasive gliomas.

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by

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Cover: Tissue section of a rat brain demonstrating rat eGFP+ multipotent mesenchymal stroma cells (MSCs, green) implanted into established N29dsRed glioma (red). 10 days after implantation, MSCs migrate along invasive tumor extensions and to distant tumor microsatellites but not to normal brain tissue. Modified version of the original image. Cover artwork by Bengt Mattsson and Daniel Bexell.

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SUMMARY

SUMMARY

Malignant brain tumor is a highly invasive and vascularized cancer. Current forms of therapy are not curative and can, at best, prolong survival for patients with this disease. The mean survival is only around 15 months. The aims of the present thesis have been to develop and explore a stem cell based vector system for delivery of therapeutic transgenes to experimental gliomas.

First, we investigated the frequency and properties of cancer stem cell (CSC)-like cells in the rat glioma models used in the present thesis. Virtually all cells within both glioma models concomitantly express CD133, nestin as well as the neural lineage markers glial fibrillary acidic protein, βIII-tubulin and CNPase *in vitro* and *in vivo*. Unsorted tumor cells displayed very high clonogenic capacity *in vitro* and robust tumorigenicity *in vivo*. Thus, CSC-like cells do not reside within a rare sub-population of cells in these glioma models but constitute most, or all, cells.

We next examined the subventricular zone (SVZ) response to growth of malignant glioma. Tumor growth resulted in decreased numbers of SVZ proliferating cells, increased SVZ neuroblast immunoreactivity, and migration of striatal neuroblasts from the SVZ toward glioma. Although endogenous neuroblast migration toward gliomas was of moderate magnitude, this indicates a region specific reparative mechanism in response to tumor growth. We continued by implanting rat SVZ-derived neural precursor cells (NPCs) and rat bone marrow-derived multipotent mesenchymal stroma cells (MSCs) into gliomas. Intratumorally implanted NPCs and MSCs migrated specifically within gliomas and largely avoided normal brain tissue. Importantly, implanted NPCs and MSCs did not proliferate within tumors. This indicates a low risk of development of secondary malignancies. A comparative analysis revealed higher survival and better intratumoral migratory capacity of implanted MSCs, compared to NPCs.

Intratumorally implanted MSCs migrated to the majority of the invasive glioma extensions and to a substantial fraction of distant tumor microsatellites. Furthermore, implanted MSCs integrated into tumor blood vessel walls and expressed pericyte markers but not endothelial cell markers. The pericyte marker expression profile and perivascular location of implanted MSCs indicate that these cells act as pericytes within tumors. MSC implantation did not, however, affect tumor microvessel density or the survival of glioma-bearing animals.

In summary, gliomas can attract endogenous striatal neuroblasts from the SVZ. However, intratumoral implantation of NPCs and MSCs yields a much more powerful, and tumor-specific, infiltration within gliomas. In particular, pericyte-like MSCs represent a well suited vector system for the delivery of therapeutic transgenes to vascularized and invasive gliomas.

POPULÄRVETENSKAPLIG SAMMANFATTNING

POPULÄRVETENSKAPLIG SAMMANFATTNING

Cancer drabbar ca 50 000 personer varje år i Sverige. En del cancerformer kan man behandla med gott resultat men elakartad hjärntumör är en speciellt farlig och svårbehandlad cancer. Denna sjukdom drabbar ca 400 personer varje år och få patienter överlever mer än 2 år trots befintlig behandling som består av kirurgi, strålning och cellgifter.

Varför kan man inte bota elakartad hjärntumör?
Det finns många orsaker till att befintlig behandling inte kan bota patienter med elakartad hjärntumör. En orsak är tumörens invasiva växtsätt. Detta innebär att så kallade tumörutlöpare, likt bläckfiskarmar, växer från tumören in i den normala hjärnvävnaden och etablerar så kallade tumörmikrosatelliter som är avskilda från huvudmassan av tumören. Förekomsten av tumörutlöpare och tumörmikrosatelliter i kombination med tumörens placering i hjärnan komplicerar behandlingen eftersom man givetvis vill undvika att skada omkringliggande normal hjärnvävnad. Det finns alltså ett stort behov av nya behandlingsformer mot denna sjukdom.

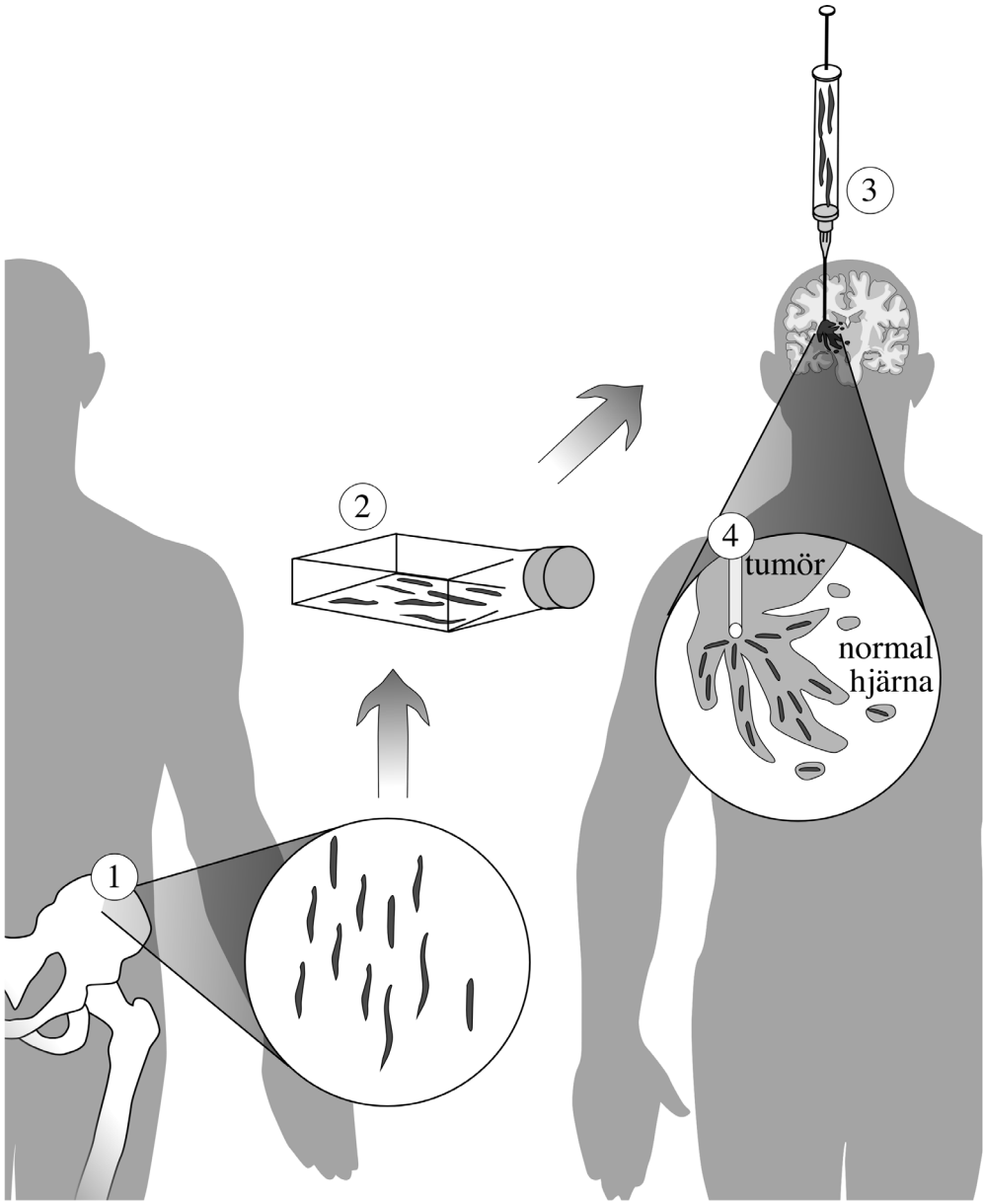
Är stamceller intressanta för behandling av hjärntumör?
Stamceller är celler som har förmåga att dela sig upprepade gånger och därmed förnya sig själva. Dessutom kan en stamcell ge upphov till olika celltyper. Med stamceller försöker man reparera och ersätta skadade celler i kroppen. Vissa typer av stamceller kan också förflytta sig, migrera, specifikt i riktning mot vissa sjukdomsprocesser som till exempel cancer. Därmed skulle stamceller kunna användas som transportmedel för att leverera substanser som kan döda tumörcellerna. Den här avhandlingen undersöker just stamcellers migrerande förmåga i experimentell hjärntumör för att ta reda på om stamceller kan användas i framtida behandling av denna sjukdom.

Hur reagerar hjärnans egna stamceller på förekomst av hjärntumör?
Avhandlingen visar att det sker migration av neuroblaster (celler som härstammar från stamceller och är på väg att utvecklas till nervceller) från ett visst område i hjärnan specifikt mot hjärntumören. Neuroblasterna migrerar normalt sett enbart längs en speciell bana i hjärnan men förekomsten av hjärntumör gjorde att neuroblasterna delvis avvek från banan och sökte sig mot tumören. Vi vet emellertid inte vilken funktion de migrerande neuroblasterna har och om de motverkar eller gynnar tumörväxten. En hypotes är att neuroblasterna skulle kunna ersätta de nervceller som dött pga. tumörens växt. Migration av hjärnans egna neuroblaster mot tumören visade sig dock vara av måttlig mängd. Vi fortsatte därför att undersöka om man istället kan transplantera stamceller in i tumören.

Vad händer om man transplanterar stamceller in i hjärntumören?
Vi undersökte om två olika typer av stamceller, tagna från benmärgens stödjevävnad eller från hjärnan, kan användas som transportmedel av tumöravdödande substanser. Därför isolerade vi och odlade upp stamceller i stora mängder. Stamcellerna transplanterades sedan in i hjärntumörer. Avhandlingen visar att båda typerna av stamceller sprider ut sig snabbt inne i tumören och undviker normal hjärnvävnad. Fynden talar för att stamceller lämpar sig väl som transportmedel av tumöravdödande substanser som kan angripa tumörcellerna specifikt utan att skada normala hjärnceller. Det är också viktigt att veta att de transplanterade stamcellerna i sig inte bildar en ny tumör i hjärnan och förvärrar situationen. Det visade sig att de transplanterade stamcellerna inte fortsätter att dela sig inne i tumören vilket tyder på att det finns en mycket liten risk att stamcellerna ger upphov till en ny tumör. Avhandlingens resultat visar dessutom att benmärgsstamcellerna överlever och migrerar i tumören i större utsträckning än stamcellerna från hjärnan. Därför fortsatte vi att i detalj studera transplantation av benmärgsstamceller till hjärntumör.

Hur kan benmärgsstamceller användas som transportmedel i hjärntumör?
När benmärgsstamceller transplanterades rakt in i hjärntumören migrerade stamcellerna till en stor del av tumörutlöparna och till en mindre del av tumörmikrosatelliterna. Samtidigt undvek stamcellerna nästan helt normal hjärna. Detta visar att stamcellerna har förmågan att söka upp de mikroskopiska delar av tumören dit kirurgisk behandling inte når. Dessutom verkar benmärgsstamcellerna dras specifikt till tumörens blodkärl och i vissa fall integreras de i kärlväggen. Avhandlingen påvisar även likheter mellan benmärgsstamcellerna och en sorts celler i kärlväggen, pericyter, som är involverade i blodförsörjningen till tumören. Blodkärlsnybildningen är nödvändig för tumörens överlevnad och de transplanterade stamcellerna är därmed strategiskt väl placerade för att attackera tumörens kärlförsörjning.

Kommer stamceller att kunna användas i behandling av cancerpatienter?
Den här avhandlingen visar att stamceller som transplanterats in i experimentell hjärntumör sprider ut sig specifikt inne i tumören och förflyttar sig till nybildade tumörblodkärl. Därmed skulle stamcellerna kunna användas som transportmedel av substanser som motverkar tumörens växt. Det återstår att se om transplanterade stamceller migrerar även i mänsklig hjärntumör. Dessutom behövs mer forskning för att ta reda på vilken tumöravdödande substans som stamcellerna ska laddas med och leverera inne i tumören för att på bästa sätt minska tumörväxten. Avhandlingen behandlar specifikt hjärntumör men transplantation av stamceller, som bärare av tumöravdödande substanser, har visat sig framgångsrikt även vid behandling av många andra cancertyper. Därför kan avhandlingens fynd vara av generellt intresse för utvecklingen av nya behandlingsmetoder också mot andra cancerformer. Figur 1 visar ett tänkbart framtida behandlingsscenario för patienter med elakartad hjärntumör.



Figur 1. *Ett tänkbart framtida behandlingsscenario för patienter med elakartad hjärntumör.*
(1) Först tas ett benmärgsprov från en donator. (2) Ur benmärgsprovet isoleras stamceller från benmärgens stödjevävnad fram. Stamcellerna beväpnas med en substans som verkar mot tumörceller. Därefter odlas stamcellerna upp i stora mängder. (3) I samband med operation av hjärntumören sprutas stamcellerna in i kvarvarande tumör. (4) De beväpnade stamcellerna sprider ut sig inne i tumören och även till invasiva tumörceller. Stamcellerna levererar den tumöravdödande substansen och motverkar därmed tumörens växt vilket leder till förlängd överlevnad eller bot av patienten.

LIST OF ORIGINAL ARTICLES

I. CD133+ Tumor Initiating Cells Dominate in N29 and N32 Experimental Gliomas (2008)
Bexell D*, Gunnarsson S*, Siesjö P, Bengzon J‡ and Darabi A‡
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IV. Bone marrow multipotent mesenchymal stroma cells act as pericyte-like migratory vehicles in experimental gliomas (2008)
Bexell D, Gunnarsson S, Tormin A, Darabi A, Gisselsson D, Roybon L, Scheding S and Bengzon J
Molecular Therapy, in press

ABBREVIATIONS

α-sma	α-smooth muscle actin
BBB	blood-brain barrier
BrdU	5-bromodeoxyuridine
CNPase	2′,3′-cyclic nucleotide 3′-phosphodiesterase
CSC	cancer stem cell
DCx	doublecortin
ECM	extracellular matrix
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
EPC	endothelial progenitor cell
ENU	ethyl-nitrosourea
FGF	fibroblast growth factor
FISH	Fluorescence *in situ* hybridization
GBM	glioblastoma multiformae
GEM	genetically engineered model
GFAP	glial fibrillary acidic protein
i.v.	intravenous
MCP-1	monocyte chemoattractant protein-1
MGMT	O^6-methylguanine methyltransferase
MSC	multipotent mesenchymal stroma cell
NG2	neuron glia 2
NeuN	neuronal nuclei
NPC	neural precursor cell
NSC	neural stem cell
OB	olfactory bulb
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor-receptor
PSA-NCAM	polysialylated neural cell adhesion molecule
RECA	rat endothelial cell antigen
RMS	rostral migratory stream
SDF-1α	stromal derived factor-1α
SVZ	subventricular zone
TUNEL	terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labelling
VE-Cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor

BACKGROUND

BACKGROUND

MALIGNANT BRAIN TUMORS

Introduction to malignant brain tumors

A tumor is "an abnormal mass of tissue that results when cells divide more than they should or do not die when they should. Tumors may be benign (not cancerous), or malignant (cancerous)"[1]. Cancer is "a term used for diseases in which abnormal cells divide without control and are able to invade other tissues"[1]. Cancer is characterized by alterations in cell physiology which include self-sufficiency in growth signals (i.e. growth factor production and autocrine stimulation), insensitivity to antigrowth signals, resistance to apoptosis, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis[2]. In Sweden, cancer is the second most common cause of death[3].

The most common types of brain tumors are malignant gliomas, meningiomas, vestibular schwannomas and pituitary adenomas. The malignant gliomas are most common and encompass Glioblastoma multiformae (GBM), anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma and other less common types of malignancies[4]. GBM is the most severe form and accounts for 60-70% of the malignant gliomas[4]. Secondary GBM develops from a previously known less malignant tumor while primary GBM is diagnosed *de novo* without knowledge of a preexisting tumor. It is, however, possible that primary GBM can undergo the same genetic and cellular changes as secondary GBM, but it is not diagnosed until progression to GBM has occurred[5]. The symptoms of GBM vary and include headache, epileptic seizures, focal neurological deficits, confusion, memory loss and personality changes[6].

Histopathology

The World Health Organization classifies, on the basis of histopathological hallmarks, astrocytomas into four prognostic grades where GBM corresponds to grade IV. As the name *glioblastoma multiformae* indicates, this disease exhibits an extremely variable morphology. The histopathological features of GBM are poorly differentiated cells, nuclear atypia, mitotic activity, microvascular proliferation (i.e. endothelial cell proliferation) and necrosis. There are two main types of necrosis: (1) large central necrotic areas which may comprise 80% of the total tumor mass and can be seen as non-enhanced areas by neuroimaging, and (2) multiple small irregular necrotic areas surrounded by densely packed radially oriented tumor cells (pseudopalisades)[7].

Genetics and epigenetics

The malignant transformation of astrocytes/precursor cells into tumor cells is driven by genetic and epigenetic alterations. In the development of high-grade astrocytomas from low-grade tumors there is a sequential acquisition of genetic alterations which means that GBM displays the highest numbers of genetic changes. Common mechanisms in tumor development are over-expression and amplification of proto-oncogenes and inactivation of tumor suppressor genes leading to loss of cell-cycle control. Gliomas are characterized by mutations of tumor suppressor genes PTEN, TP53, $p16^{Ink4a}/p14^{ARF}$, Rb and over-expression and/or amplification of epidermal growth factor-receptor (EGFR) and platelet-derived growth factor-receptor (PDGFR)-α[7]. In addition to genetic mutations, epigenetic changes like DNA methylation and histone deacetylation contribute to silencing of genes involved in DNA repair, tumor suppression and cell-cycle control[8]. Epigenetic silencing by methylation of the DNA repair gene O^6-methylguanine methyltransferase (MGMT), which protects tumor cells from chemotherapy-induced DNA damage, is found in a subset of GBM patients and they show improved response to alkylating agents (e.g. Temozolomide)[9].

Invasive growth

Invasion is a hallmark of malignant brain tumors which makes therapy very challenging. GBM typically grows with infiltrative tumor extensions along white matter fiber tracts and along blood vessels. The tumor extensions can reach far into the normal brain parenchyma. Distant tumor microsatellites, presumably separated from the main tumor, can be found several centimeters away from the main tumor mass. It has been suggested that more than 50% of untreated malignant brain tumors have reached the contralateral hemisphere at the time of diagnosis[10].The process of cancer cell invasion includes cytoskeleton rearrangements, alterations in cell-cell adhesion molecules, changes in integrin expression, production and activation of extracellular matrix (ECM)-degrading proteases and production of specific ECM components which facilitate invasion[2, 11]. Molecules involved in GBM cell invasion include CD44, integrins, cadherins, neural cell adhesion molecules, matrix metalloproteinases 2, 9 and 12, and ECM components tenascin C, vitronectin and laminin[11].

Role of tumor stroma in tumor growth

A solid tumor consists not only of tumor cells but also of tumor stroma. The major stromal components of GBM are reactive astrocytes, immune cells, endothelial cells, pericytes and ECM proteins. The growth and invasion of a malignant tumor is dependent on a close interaction between tumor cells and stromal components. Stromal cells can promote tumor growth and invasion by production of growth factors and ECM-

degrading enzymes but stromal cells might also inhibit tumor growth through other mechanisms[12]. Several studies have shown that therapeutic targeting of tumor stroma can decrease tumor growth. Stromal cells are presumably not genetically unstable (as tumor cells) and these cells might therefore be less prone to develop drug resistance[12]. The GBM vasculature consists of endothelial cells which form the inner lining of vessels and pericytes. Pericytes are perivascular cells which surround endothelial cells and are important for stabilization and functional maturation of newly formed blood vessels for example by production of vascular endothelial growth factor (VEGF)[13].

Angiogenesis

Solid tumors are dependent of neovascularization to grow beyond a certain size[14]. Initially, GBM cells grow by *co-option* of pre-existing blood vessels. As the tumor grows, blood vessels become compressed and destabilized leading to reduced perfusion. Subsequently, hypoxia and cell death occurs which induce *angiogenesis*, i.e. new blood vessel formation from pre-existing endothelial cells. Up-regulation of hypoxia inducible factor 1-α and VEGF are key regulators in this process. In addition, circulating bone marrow-derived cells are believed to form new tumor vessels, a process called *vasculogenesis*[13]. Impaired recruitment of bone marrow-derived endothelial cells leads to inhibition of tumor angiogenesis and decreases tumor growth[15]. Depletion of tumor pericytes causes dysfunctional tumor vessels and increased apoptosis of tumor endothelial cells[16]. However, the contribution of bone marrow endothelial cells to tumor endothelium is controversial with reports ranging between 0 – 90%[17-20]. Rajantie et al. showed that the main contribution of bone marrow to tumor vessels is to provide periendothelial vascular mural cells and not endothelial cells[21]. This was further supported by Bababeygy et al. showing that hematopoietic stem cell-derived pericytic cells become integrated in glioma vessels[22].

GBM blood vessels are tortuous, disorganized and highly permeable/leaky which results in an abnormal and non-uniform blood flow. The abnormality of GBM vessels has been ascribed to transcellular and intercellular openings of endothelial cells[23] and to deficiencies in pericyte coverage[13, 24]. Vessel leakiness causes increased interstitial fluid pressure and heterogenous tumor blood flow. This makes efficient delivery of systemically administered drugs to tumors complicated[13]. Infiltrating tumor cells often make use of the existing brain vasculature (i.e. co-option) which has an intact blood-brain barrier[25].

EXPERIMENTAL ANIMAL MODELS OF BRAIN TUMORS

The clinical significance of the results from experimental animal studies depends on the models used to mimic the disease. Several experimental tumor models do not necessarily correlate with clinical features and may therefore yield false-positive results[26]. In fact, most experimental glioma therapies, which have shown very potent results in animal models, have shown no or minimal effects in clinical GBM trials[27, 28]. Accordingly, it is important to use tumor models that are relevant for the scientific question in focus.

The major strategies used to model malignant brain tumors *in vivo* are 1) chemical mutagen-induced models, 2) xenograft transplantation models, and 3) genetically engineered models (GEMs). Each model has its advantages and limitations and the choice of model should depend on the nature of the study[29].

In most chemical mutagen-induced models, gliomas are induced either by direct or transplacental exposure of a nitrosourea-derivate. Nitrosoureas are DNA alkylating agents which cause point mutations resulting in glioma formation. Rat glioma cell lines developed by nitrosourea injections include 9L, C6, F98, RG2, N29, and N32[30, 31]. The genetic mutation that caused the tumors as well as the cell-of-origin are unknown although recent data suggest that cells from the subventricular zone (SVZ) are involved in tumor formation following nitrosourea injection[32].

In the present thesis, the N29 and N32 glioma models are used. The models were originally developed by transplacental injection of ethyl-nitrosourea (ENU) to pregnant Fischer344 rats[33]. The N29 tumor arose in the right hemisphere of a female offspring and the N32 tumor arose in the right hemisphere of a male offspring[33]. Both N29 and N32 tumor cells proliferate rapidly *in vitro* and *in vivo*. N29 tumors exhibit GBM-like features including nuclear atypia, mitotic figures, neovascularization, necrosis and display a highly invasive growth pattern with infiltrative tumor extensions and distant tumor microsatellites[31]. N32 tumor cells give rise to a less invasive, although highly vascularized, tumor which resembles some of the features of human anaplastic astrocytoma[31]. Like human malignant brain tumors, N29 and N32 tumor cells produce the immunosuppressive factors nitric oxide and transforming growth factor-β[31].

A major limitation using rat glioma cell lines to model GBM is that tumor formation does not resemble the process of human GBM initiation and subsequent development. Human GBM acquires sequential genetic changes during months or years and exhibits a broad genetic heterogeneity. Rat glioma cell lines are thought to lack extensive genetic heterogeneity[34, 35]. The advantages of using these models include high penetrance, high reproducibility and rapid tumor development[29]. N29 and N32 have, in contrast

to many other experimental glioma models like the rat C6 glioma, a syngeneic host (i.e. the Fischer344 rat). This implies that N29 and N32 gliomas are non or only very weakly immunogenic *in vivo*. While findings from the use of the C6 glioma might be associated with immunogenicity of implanted tumor cells, N29 and N32 gliomas are much less associated with this risk.

In xenograft transplantation models, primary tumor cells or cell lines are implanted subcutaneously or orthotopically (i.e. into native tumor site). Human tumor cells are often implanted into immunosuppressed or immunodeficient mice. Xenograft models are reproducible and can form tumors rapidly. Human cells used in xenograft studies have often been passaged for years and these cells do, due to selection pressure, therefore not represent the genetics and histology of the original tumor[36]. However, human xenografts taken directly from patient explants can potentially represent the genetic hetereogeneity of human malignant brain tumors[37]. A limitation in using xenograft models is that immunological interactions between tumor and host are lacking[29].

GEMs can form spontaneous tumors due to GBM-associated gene mutations. Using GEMs, the initiating genetic lesion is known, the mice are immunocompetent and GEMs can recapitulate some of the complex biological processes involved in GBM formation and growth. However, many GEMs display low reproducibility, low tumor penetrance and latency of tumor formation[29].

STEM CELLS

Introduction to adult stem cells

Adult stem cells exhibit two fundamental criteria; *self-renewal* (the ability to divide and generate an identical daughter cell), and *multipotency* (multipotent differentiation capacity)[38, 39]. Stem cells have an unlimited/very high capacity for self-renewal and the ability to give rise to multiple mature cell types. A stem cell can generate a *progenitor*, which is a cell with limited self-renewal capacity and a more restricted differentiation potential. Progenitor cells are usually highly proliferative and can therefore expand the pool of newly formed cells. The progenitors in turn generate differentiated cells. Sometimes the term *precursor* is used to more vaguely describe a cell with self-renewal and multipotency capacity that includes both stem cells and progenitors.

Multipotent mesenchymal stroma cells

Multipotent mesenchymal stroma cells (MSCs), or mesenchymal stem cells, are thought to be the precursors of the bone marrow stroma. Cultured MSCs possess the capacity to differentiate into osteoblasts, adipocytes and chondrocytes following exposure to spe-

cific differentiation supplements. MSCs can be isolated by their adherent growth in culture and by expression of certain surface markers. There is, at present, no single marker that specifically identifies MSCs. A combination of markers including CD73, CD90, CD105, CD146, CD271, STRO-1, SH2 and SH3 are often used to identify MSCs[40]. In addition, MSCs lack expression of the hematopoietic antigens CD34 and CD45[40].

It has been suggested that human MSCs constitute approximately 0,001 – 0,01% of the cells of the bone marrow[41]. The lack of specific MSC markers and the low frequency of MSCs *in vivo* make studies of MSCs complicated and most data is based on *in vitro* experiments of expanded cells, which are presumed to act as MSCs *in vivo*. In addition to bone marrow, MSCs have also been isolated from most other organs including adipose tissue, the synovial membrane, the dental pulp, umbilical cord blood and fetal liver and lung[40].

There are multiple functions of MSCs; bone marrow MSCs form the hematopoietic microenvironment which supports the long-term maintenance and differentiation of hematopoietic stem cells[42]. MSCs also possess immunosuppressive properties by modulation of cytotoxic T-cells, antigen-presenting cells, natural killer cells and B-cells[43]. Because of the immunosuppressive properties, MSCs have been successfully used for treatment of severe graft-versus-host disease in cancer patients following hematopoietic stem cell transplantation[44]. Furthermore, MSCs can promote angiogenesis and contribute to wound repair by production of pro-angiogenic factors like VEGF[45, 46].

Adult neural stem cells and neurogenesis

Adult neural stem cells (NSCs) are undifferentiated cells which divide and can generate neurons, astrocytes and oligodendrocytes[47]. The formation of new neurons from neural stem cells is called *neurogenesis*, a process that consists in division of a precursor cell, migration, differentiation, survival and functional maturation. NSCs reside in two main neurogenic regions of the adult brain; the SVZ and the subgranular zone in the dentate gyrus of the hippocampus[48]. This thesis focuses on the use of endogenous and exogenous SVZ-derived NSCs and neural precursor cells (NPCs).

The SVZ, or the subependymal layer, lines the lateral wall of the lateral ventricles. The wall of the lateral ventricles consists of a layer of ciliated ependymal cells (E-cells). These cells can express CD133 and are mainly quiescent but can be activated and possibly act as NSCs upon brain injury[49]. The B-cell is slowly-dividing, expresses the intermediate filament protein glial fibrillary acidic protein (GFAP) and is by many regarded as the adult NSC[50]. The B-cell generates the C-cell which is the rapidly dividing transit-amplifying progenitor cell and thus expands the pool of newly formed cells. The

C-cell gives rise to the A-cell, the migrating neuroblast (neuronal progenitor), which expresses the neuronal lineage markers Doublecortin (DCx) and Polysialylated neural cell adhesion molecule (PSA-NCAM). The A-cells migrate from the SVZ, along the rostral migratory stream (RMS), towards the olfactory bulb (OB), where these cells differentiate into granular or periglomerular interneurons[48, 51]. Many of the newly formed cells die within the SVZ, RMS or in the OB, but a fraction of these cells survive and display electrophysiological characteristics of neuronal maturation in the OB[52].

The E-B-C-A system has been described in the context of the adult mouse brain. In the adult rat brain, the B-cell is thought to directly generate the A-cell[53]. The adult human SVZ consists of a ribbon of GFAP+ cells that proliferate *in vivo* and display multipotency capacity (i.e. the formation of neurons, astrocytes and oligodendrocytes) *in vitro*[54]. In the adult human brain, proliferating PSA-NCAM+ neuroblasts migrate from the SVZ along the RMS and towards the OB[55].

In the intact rodent brain, migration of SVZ neuroblasts (A-cells) is thus restricted to the RMS / OB. Following a lesion in the rodent brain (e.g. ischemic stroke in the striatum), there is an increase in SVZ cell proliferation and the newly formed neuroblasts leave the SVZ / RMS and migrate towards the lesion in the striatum[56, 57]. Here, the newly formed cells differentiate into mature neurons and display the same phenotype as the neurons that died due to the insult, suggesting that endogenous SVZ precursors contribute to neuronal cell replacement[56].

Less is known about the SVZ response to growth of malignant brain tumors. Glass et al. found that nestin+ cells surround and infiltrate experimental gliomas. A fraction of these cells were reported to co-express the neuronal markers DCx and PSA-NCAM[58]. Findings from *in vitro* experiments suggest that NPCs could induce apoptosis of glioma cells[58]. In contrast, Staflin et al. found no evidence of endogenous NPC migration toward experimental gliomas[59].

CANCER STEM CELLS

Cancer stem cells in human brain tumors

The previously held assumption on the growth of tumors was that most, if not all, tumor cells can maintain tumor growth. The cancer stem cell (CSC) hypothesis implies that only a distinct subset of tumor cells are capable of extensive self-renewal and tumor maintainance and that most cells within the tumor do not have this ability[60, 61]. A CSC can be defined as "a cell within a tumor that possess the capacity to self-renew and to cause the heterogenous lineages of cancer cells that comprise the tumor"[62]. The term tumor-initiating cell refers to the cell from which the cancer arose. This cell might be

distinct from the CSC. That CSCs exist in solid tumors is, however, a hypothesis and remains to be proven[61, 63]. If the hypothesis turns out to be correct, it would have very important implications for the development of novel cancer therapy.

Putative CSCs, have been isolated from human malignant brain tumors including GBM, medulloblastoma and ependymoma[64-66]. Singh et al. have shown that 100 CD133+ human GBM cells were sufficient to initiate tumor growth following injection into immunodeficient mice, whereas injection of 10^5 CD133-negative cells was not[64]. The established tumor recapitulated the histology of the original tumor[64]. This indicates that there is a sub-population of GBM cells (i.e. CD133+ cells) that are responsible for the maintenance of GBM growth. However, other groups have reported the existence of CD133-negative tumor-initiating GBM cells[67, 68]. The value of CD133 as a specific CSC marker of GBM is therefore questionable.

The CSC hypothesis is thus based on experimental findings where a subset of human tumor cells have been isolated and transplanted to immunodeficient rodents where the cells give rise to tumors that recapitulate the features of the original tumor. One inherent limitation of these studies is that tumor-initiating capacity *in vivo* has been assessed by xeno-implantation of human cancer cells into immunodeficient rodents. Tumor growth is dependent on the tumor microenvironment[12] and putative CSCs interact with their microenvironment within the tumor[69]. There is, however, a lack of appropriate interaction between putative human CSCs and tumor microenvironment in glioma-bearing immunocomprised mice[29]. The findings of putative CSCs therefore rather demonstrate the capacity of a population of human (CD133 positive or negative) cancer cells that possess the capacity to engraft and establish tumors in a foreign environment (i.e. immunodeficient mice or rats)[63]. These cells are not necessarily the same cells that propagate tumor growth in patients. Furthermore, a concern has been raised that preparation of cell suspensions with enzyme treatment could affect the marker expression of genetically unstable tumor cells[63]. In addition, the putative CSC marker CD133 is expressed in up to 70% of human GBM cells[70]. This is inconsistent with the view that CSCs are a rare subset of cells within the tumor. In contrast to the CSC hypothesis, tumor growth might be sustained by one dominant clone or multiple clones of tumor cells[63, 71].

If the CSC hypothesis is correct, it would have very important implications for the development of novel glioma therapy. Conventional treatment targeting dividing cells in general can decrease tumor growth by killing the majority of cancer cells within the tumor. However, putative CSCs might be more resistant to conventional treatment and these cells can therefore survive treatment and contribute to tumor re-growth despite eradication of the main tumor mass. For instance, putative CSCs are predicted to be slowly-dividing and to express high levels of drug export proteins making them re-

sistant to conventional treatment[61]. Novel therapy should thus be specifically directed against putative CSCs[60]. Complete eradication of human tumors based on targeting of specific markers, signaling pathways or genes in a defined subset of tumor cells, would provide final proof for the CSC hypothesis.

For long, the glioma cell-of-origin was thought to be a dedifferentiated astrocyte. Similarities between neural stem/progenitor cells and glioma cells have led to the hypothesis that the glioma cell-of-origin is a transformed SVZ precursor cell. However, it is presently unclear which cell or cells initiate GBM growth in humans[61].

Cancer stem cells in experimental glioma models

Support for the notion that experimental glioma models contain a rare subset of CSCs was presented in the rat 9L gliosarcoma model and in the mouse GL261 glioma model[72, 73]. In both studies, sphere formation was used to enrich for CSC-like cells[72, 73]. It was claimed that implantation of sphere-forming cells, as compared to adherently growing cells, forms larger tumors *in vivo* and results in shorter survival of animals. Furthermore, sphere-generated 9L cells were reported to exhibit increased expression of anti-apoptosis and drug resistance related genes[72]. Mouse GL261 glioma cells grown as spheres display increased expression of nestin and of radial glia related genes as compared to adherently growing cells. In addition, cells within the GL261 line were reported to down-regulate nestin expression and up-regulate expression of neural lineage markers upon growth in differentiation conditions *in vitro*. Rat 9L cells were found to aberrantly co-express the stem/progenitor marker Nestin with GFAP and the neuronal lineage marker MAP2[72].

Both studies indirectly suggested the existence of a sub-population of tumor cells within the cell line that exhibit certain characteristics of CSC-like cells[72, 73]. However, in contrast to studies of putative CSCs in human brain tumors, the authors were not able to identify and isolate a subset of tumor cells that are the only cells that can propagate/initiate tumor growth.

Contradictory results were described in the C6 rat glioma model where the vast majority of cells were found to possess CSC-like features. Single C6 glioma cells consistently generated clones *in vitro* and the clone-forming cells were capable of tumor formation upon xenografting into immunodeficient mice. CSC features were found in both CD133+ and CD133- cells[74].

TREATMENT OF MALIGNANT BRAIN TUMORS

Challenges in the treatment of malignant brain tumors

There have been few significant advances in the treatment of GBM for the last decades[75]. The shortcomings of GBM treatment have been ascribed to 1) the localization of the tumor in the brain; 2) the invasive growth pattern; 3) increased interstitial fluid pressure within tumor leading to low concentrations of systemically delivered drugs; 4) intrinsic drug resistance of tumor cells; 5) acquired drug resistance; and 6) neurotoxicity of treatment[75].

The blood-brain barrier (BBB) constitutes a formidable barrier for systemic drug delivery to the brain. Breakdown and dysfunction of the BBB is a well-known feature of GBM but invasive tumor cells are often protected by an intact BBB[25]. Although several attempts have been made to overcome the difficulties of BBB penetration, the barrier still presents a challenge for systemic administration of drugs directed against GBM[76].

Another challenge in the development of successful GBM therapy is that several experimental glioma models constitute suboptimal models for human GBM; *in vitro* models of glioma (i.e. glioma cell lines) do not exhibit the complex genetic heterogeneity found in human GBM[34]. In addition, xenograft models do not accurately resemble the growth, invasion, gene expression profiling and stromal microenvironment interactions of human GBM. In animal studies, there is also often a short interval (often only a few days) between tumor implantation and treatment. This time-course is completely different from the clinical situation where tumors have been present for months or years and thus exhibit broad heterogeneity. Many preclinical glioma models are therefore non-predictive for the evaluation of certain clinical treatment modalities[27, 28].

Successful GBM therapy should 1) target the invasive tumor cells; 2) target tumor cells with different genetic aberrations; and 3) selectively target tumor cells relative to normal neural cells[75, 76].

Conventional treatment

Standard treatment of GBM patients is surgical resection, radiotherapy and chemotherapy[4]. Surgery of GBM implies resection of the main tumor mass (i.e. debulking). Complete surgical resection is almost impossible to achieve due to microscopic infiltrative tumor extensions and tumor microsatellites located far away from the main tumor mass. Recurrence of the tumor in the contralateral hemisphere has been observed even following a procedure as extensive as hemispherectomy[77]. Surgery is, however, necessary to manage the acute symptoms of intracerebral tumor growth and to obtain biopsies for diagnosis. The long-term benefit of extensive tumor resection alone is dis-

puted[78], but evidence suggests a beneficial effect on survival[79]. In addition, debulking of the main tumor mass will likely be important to facilitate other treatment modalities which can target the remaining tumor cells.

Focal radiotherapy is administered to a volume defined by magnetic resonance imaging and to an additional rim of 2 cm outside the tumor mass in order to encompass most of the infiltrating tumor cells. Radiotherapy can prolong survival for GBM patients undergoing surgical resection but tumor growth recurs in almost every patient. Several attempts have been made to sensitize tumor cells to radiotherapy but none of these attempts have been clinically successful[25].

The present choice of chemotherapy is Temozolomide, an alkylating agent that crosses the blood-brain barrier. Temozolomide in combination with radiotherapy prolong median survival of GBM patients to 14,6 months compared to 12,1 months for radiotherapy alone[80]. The 2-year survival rate has improved from 10,4% for radiotherapy alone to 26,5% with temozolomide plus radiotherapy[80]. A subset of GBM patients whose tumors contain epigenetic silencing of the DNA repair gene MGMT benefit more from Temozolomide and their median survival is 21,7 months[9].

Accordingly, conventional treatment can prolong survival for GBM patients by several months. However, treatment outcome is still unsatisfactory. Current treatment is also non-specific, which means that it targets both malignant and non-malignant cells. Therefore, there is clearly a great need to develop novel specific and effective therapies for malignant brain tumors.

Immunotherapy

Human gliomas evade and suppress the patient's antitumor immune response through several mechanisms, e.g. through production of immunosuppressive factors[6]. In glioma immunotherapy, attempts are made to enhance the endogenous immune response against the tumor. One example of glioma immunotherapy is peripheral immunization with irradiated autologous tumor cells transduced to overexpress the pro-inflammatory cytokine interferon-γ. Immunization leads to increased infiltration of cytotoxic T-cells and NK-cells into tumors and concomitant decreased tumor growth[81, 82]. An ongoing clinical GBM trial using immunization with interferon-γ producing autologous tumor cells has demonstrated increased immune cell infiltration into tumors. The results point to a prolonged survival of patients[83].

Molecular targeting

Novel insights into the molecular biology of GBM have provided targeted therapy against critical signaling pathways that regulate tumor cell proliferation, invasion, apoptosis, tumor angiogenesis and sensitivity to chemo- and radiotherapy[76]. The EGFR is overexpressed in human GBM and there is a constitutively active variant of the EGFR (EGFR vIII). Several EGFR-tyrosine kinase inhibitors have therefore been developed but they show limited effect on GBM. Other targeted therapies include monoclonal antibodies against VEGF, VEGF-receptor-tyrosine kinase inhibitors, inhibition of the PDGFR and of various intracellular signaling pathways. In light of the genetic heterogeneity of GBM, successful targeted therapy should most likely include a combination of molecular targets[76]. In addition, proteins involved in downstream signaling are often mutated in GBM. These proteins may drive GBM signaling pathways despite inhibition of their upstream receptors[84].

Gene therapy

Gene therapy has been extensively explored as a treatment for experimental glioma and human GBM. Viral vectors have been administered either systemically or directly into the tumor following surgical resection. Transgenes that inhibit tumor angiogenesis, stimulate an anti-tumor immune response, correct genetic defects (e.g. introduction of the tumor suppressor TP53 gene), and encode prodrug-activating enzymes have been used in glioma gene therapy[85, 86].

Gene therapy utilizing viral vectors as delivery vehicles have been successful in many experimental glioma studies, but clinical studies have had very limited success[28]. Inefficient spread of vectors to the invasive tumor cells and low transduction efficiency of tumor cells are two reasons for this failure[28]. However, gene therapy clinical trials have often been initiated at a very late stage of tumor development when patients already are "beyond cure"[28]. Earlier onset of treatment could potentially improve the outcome of gene therapy in clinical GBM trials.

Stem cell tropism to gliomas

A novel approach to deliver cytotoxic substances to gliomas through intracranial implantation of genetically modified NSCs was presented by Aboody et al. and Benedetti et al.[87, 88]. Implanted NSCs were shown to migrate throughout normal brain tissue toward experimental gliomas where NSCs delivered a cytotoxic substance, which could decrease tumor growth[87, 88]. Aboody et al. made use of a suicide gene system; immortalized murine C17.2 NSCs were engineered to deliver the pro-drug converting enzyme cytosine deaminase which converts the systemically administered non-toxic substance

5-Fluorocytosine to the cytotoxic drug 5-Fluorouracil within tumors[87]. Benedetti et al. showed that implantation of interleukin-4 producing NSCs prolong survival of glioma-bearing animals[88].

The above-mentioned initial studies were followed by several studies using either neural, bone marrow-derived mesenchymal, endothelial, hematopoietic, umbilical-cord blood derived and skin-derived precursor cells as therapeutic delivery vehicles in glioma therapy[89-100].

Nakamura et al. were the first to demonstrate a therapeutic effect of genetically modified bone marrow-derived MSCs[97]. Implanted rat MSCs, engineered to produce enhanced green fluorescent protein (eGFP), were reported to prolong survival of glioma-bearing animals. The therapeutic effect was further enhanced by implantation of interleukin-2 producing MSCs which can support an immune response within tumors. This study demonstrated proof-of-principle that rat MSCs can be used as delivery vehicles to gliomas[97]. The implantation of MSCs was, however, performed either by co-injection with tumor cells or three days after glioma cell implantation. This makes it difficult to assess the true potential of MSCs in glioma therapy.

The use of MSCs in glioma treatment was further strengthened by a study of Nakamizo et al. demonstrating glioma tropic migratory capacity of human MSCs. Human MSCs distributed themselves specifically within gliomas of immunocompromised mice following injections into the ipsilateral or the contralateral carotid artery. In contrast, intra-arterial injections of fibroblasts resulted in death of the animals. Intra-arterially injected mouse MSCs were also attracted to gliomas suggesting that the findings were not due to species-specific interactions between human glioma cells and human MSCs in mice, but rather to inherent properties of MSCs. Furthermore, a beneficial therapeutic effect by interferon-β producing MSCs was observed upon intratumoral and intra-arterial injections into glioma-bearing mice[96].

Factors involved in stem cell tropism to gliomas

Most studies investigating the mechanisms of stem cell migration toward gliomas have utilized an *in vitro* transwell assay to determine which factors can attract stem cells. The transwell assay is an artifical and simplified system which does not resemble the complex microenvironment of the tumor and its surroundings. Despite this, some clues as to which factors that govern stem cell migration to and within tumors have emerged. Glioma-produced factors that attract NSCs *in vitro* are VEGF[101], scatter factor/hepatocyte growth factor, fibroblast growth factor (FGF)-2, transforming growth factor-α[102] and stromal derived factor-1α (SDF-1α)[103]. Tumor-produced ECM proteins

(e.g. laminin and tenascin-C) have been suggested to facilitate NSC tropism towards tumor[104]. Growth factors that mediate tropism of human bone-marrow derived MSCs towards glioma are platelet-derived growth factor (PDGF), epidermal growth factor (EGF), SDF1α[96], VEGF-A[105], interleukin-8, transforming growth factor-β1 and neurotrophin-3[106]. In addition, soluble chemoattractants produced by inflammatory cells might direct implanted stem cells towards the tumor. Activated microglial cells within gliomas[107] have the capacity to attract NPCs[108]. Monocyte chemoattractant protein-1 (MCP-1), produced by activated microglia, plays an important role in directing neural progenitors to sites of neuroinflammation[109].

Novel insights into the molecular factors and pathways modulating the tumor tropism of stem cells have contributed to the development of genetically modified stem cells that display increased migratory capacity towards malignant brain tumors. Bone marrow-derived MSCs transfected with the EGFR exhibit enhanced *in vitro* and *in vivo* migration towards glioma as compared to non-transfected MSCs[110]. Over-expression of the chemokine receptor CXCR3 in HiB5 cells (a neural progenitor cell line derived from the embryonic hippocampus) results in enhanced glioma-specific migratory capacity of these cells as compared to non-CXCR3 over-expressing cells[111]. These abovementioned studies demonstrate an approach to enhance the migratory capacity of implanted stem cells in glioma therapy.

Requirements of stem cells in glioma therapy

The concept of stem cell therapy of gliomas can be broken down into three critical requirements. Stem cells should be able to 1) migrate extensively within tumors and specifically to invasive glioma cells, 2) deliver a tumoricidal substance which can decrease tumor growth, and 3) not cause any substantial harm to the patient. There has been an intense focus on finding novel tumoricidal substances that stem cells can deliver, and on elucidating factors that mediate stem cell migration to tumors. However, in light of the shortcomings of multiple gene therapy glioma trials, the concept of stem cell therapy of gliomas raises and falls with the migratory capacity of stem cells within tumors. While initial studies[87, 97] reported that stem cells potentially could track invasive glioma cells, the extent of this crucial property has not been investigated. Furthermore, it is unknown which cell-type would act as the most efficient vector, or which mode of administration would provide the most extensive infiltration of tumors.

AIMS

AIMS

The general aims of the present thesis have been to develop and explore a cellular vector system for the treatment of experimental malignant glioma.

The specific aims were:

1. to examine the clonogenic capacity, *in vivo* tumorigenicity, phenotype and differentiation capacity of N29 and N32 glioma cells in order to estimate the frequency of CSC–like cells in the glioma models used in papers II-IV (paper I);

2. to explore the subventricular zone neurogenic response to growth of experimental malignant glioma with emphasis on endogenous NPC migration toward glioma (paper II);

3. to examine and compare the magnitude and specificity of migration of NPCs and MSCs upon implantation into or at a distant site to experimental malignant gliomas (paper III);

4. to determine the proliferation rate of intratumorally implanted NPCs and MSCs (paper III);

5. to investigate the capacity of intratumorally implanted MSCs to migrate specifically to invasive glioma cells (paper IV);

6. to explore the phenotype of MSCs *in vitro* and upon implantation into gliomas (paper IV); and

7. to determine the association between intratumorally implanted MSCs and glioma vasculature, the effects of implanted MSCs on tumor microvessel density, and the effect of implanted MSCs on survival of glioma-bearing animals (paper IV).

RESULTS AND COMMENTS

RESULTS AND COMMENTS

Here follows a summary of the results of the thesis. Please refer to the original articles for further details.

1. Cancer stem cell-like cells in N29 and N32 rat glioma models (paper I)
The N29 and N32 rat glioma models have previously been explored with respect to their histopathological features and immunosuppressive properties[31]. Here, we assessed the frequency of CSC-like cells of the N29 and N32 glioma models.

N29 and N32 glioma cells both formed free-floating tumorspheres upon growth in stem cell medium (including EGF and FGF-2) and following extensive confluent growth in ordinary cell culture medium. Tumorspheres were mechanically dissociated into single cells, which could form new spheres. Spheres were passaged multiple times and the new spheres retained their protein expression profile and *in vitro* growth pattern.

We next analyzed expression of certain stemness- and lineage related proteins. Surprisingly, both N29 and N32 glioma cells expressed the putative CSC marker CD133, neural stem and progenitor marker nestin concomitantly with the neural lineage markers DCx, βIII-tubulin (neuronal markers), GFAP (astrocyte and NSC marker) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase, oligodendrocyte marker). Immunocytochemistry and flow cytometric analysis revealed that the expression profile was homogenously found in the vast majority, if not all, of the tumor cells. There was no difference in the expression profile between adherently growing tumor cells and tumorspheres.

In order to elucidate potential differentiation capacity of N29 and N32 glioma cells, tumorspheres or adherently growing cells were exposed to differentiation-promoting conditions (i.e. removal of growth factors and addition of serum). Neither cell type changed phenotype or morphology when subjected to differentiation conditions *in vitro*. To determine differentiation capacity *in vivo*, tumor cells were implanted into syngeneic Fischer344 rats. The established tumors largely retained their phenotype *in vivo*.

To functionally assess N29 and N32 glioma cells with respect to CSC-like properties, we investigated *in vitro* clone-forming capacity and *in vivo* tumorigenicity upon orthotopic implantation. In order to investigate clone-forming capacity, adherently growing N29 and N32 cells were diluted to achieve the concentrations of 0.5, 1 and 5 cells per

well. The percentage of N29 colony formation was 33% for the concentration of 0.5 cells, 55% for the concentration of 1 cell and 94% for 5 cells per well. N32 colony formation was 30% for the concentration of 0.5 cells per well, 54% for 1 cell and 99% for 5 cells per well. *In vivo* tumorigenicity was examined by stereotactic intracerebral inoculation of N29 and N32 cells. Intracerebral tumors were established in 100%, 78% and 75 % of the animals upon implantation of 1000, 100 and 10 N29 cells, respectively, and in 100%, 25% and 25% of the animals receiving 1000, 100 and 10 N32 cells. Accordingly, as few as 10 adherently grown unsorted N29 and N32 cells, respectively, were sufficient to establish intracerebral tumors. This demonstrates powerful tumorigenic abilities of N29 and N32 glioma cells.

The results indicate that the N29 and N32 glioma models consist of homogenous populations of tumor cells. Importantly, both tumor cell types are highly clonogenic *in vitro* and tumorigenic *in vivo*.

2. Characterization of the subventricular zone neurogenic response to malignant brain tumor (paper II)

We investigated the endogenous response of the SVZ to growth of N32 malignant brain tumor in the striatum. For this purpose, N32 tumor cells were transduced to express the dsRed protein which allows for visualization *in vivo*. N32dsRed tumors were established in the striatum of adult Fischer344 rats. The SVZ neurogenic response was investigated on days 9, 16, 23 and 30 following tumor cell implantation.

We found high numbers of nestin+ cells that surrounded the periphery of tumors and scattered nestin+ cells infiltrated into tumors. Animals receiving cell medium inoculations also displayed nestin immunoreactivity along the needle track. The numbers of nestin+ cells within the SVZ did not differ between animals with tumors and animals receiving medium inoculations. In addition, there was no increase in migratory nestin+ cells in the area between the SVZ and the peritumoral rim of nestin+ cells. Nestin+ cells that surrounded and infiltrated tumors exhibited a stellate morphology and were co-labelled with the astrocytic marker GFAP, suggesting that they were reactive astrocytes[112].

We next assessed changes in SVZ cell proliferation and cell death in response to glioma growth. The numbers of cells expressing the cell-cycle marker Ki-67 within the SVZ were counted. In addition, 5-Bromodeoxyuridine (BrdU) was given once a day for seven days in order to estimate the numbers of SVZ proliferating cells. Both Ki-67 and BrdU labeling demonstrated that striatal brain tumor growth caused a decrease in the numbers of proliferating cells in the SVZ. The effect was noticeable first on day

30 when tumors occupied most of the striatum. Hence, the decrease in SVZ cell proliferation seems to depend on tumor size or on proximity of tumor to the SVZ. Tumor growth caused, however, no apparent effect on SVZ apoptotic cell death, as detected by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labelling (TUNEL) immunoreactivity.

Despite the decrease in SVZ cell proliferation, we found a concomitant increase of DCx and PSA-NCAM expression in the SVZ. These findings represent increased areas of neuroblast immunoreactivity and not necessarily increased numbers of neuroblasts within the SVZ. The actual numbers of neuroblasts are difficult to assess due to lack of specific nuclear markers. However, there was no apparent difference in the numbers of Hoechst-stained nuclei (representing all cells) in the SVZ between glioma-bearing animals and animals receiving cell medium inoculation.

In line with the increased neuroblast immumoreactivity, we found DCX+ and PSA-NCAM+ cells in the area between the SVZ and the tumor. DCX+ and PSA-NCAM+ cells displayed neuroblast morphology (i.e. bipolar cells with elongated processes). Migratory neuroblasts were mainly located in the striatum but also along the corpus callosum and on the lateral side of the tumor in the striatum. Infiltration of neuroblasts into tumors was rare. Most DCx+ cells outside the SVZ expressed Pbx, a transcription factor for immature striatal neurons. Because of the higher density of neuroblasts found in proximity to the SVZ, it is likely that the neuroblasts originated from the SVZ. Furthermore, previous tracing experiments have shown that neuroblasts attracted to ischemic stroke are derived from the SVZ[113]. Taken together, striatal tumors are capable of attracting region-specific neuroblasts, most likely originating from the SVZ.

The region-specific phenotype of the neuroblasts suggests that they are on their way to differentiate into mature striatal neurons. We found, however, no signs of neuroblast differentiation into mature neurons. In this experiment, BrdU was administered twice daily for 14 days following tumor cell implantation and animals were sacrificed after an additional 14 days. We did not observe any BrdU+ cells that co-localized with NeuN (a marker for mature neurons) in the striatum, pointing against substantial neuronal maturation. However, because of limitations in the experimental set-up, we cannot exclude the possibility that tumor-attracted SVZ-derived neuroblasts can undergo neuronal maturation.

Figure 2 shows the endogenous cellular response to the growth of malignant brain tumor.

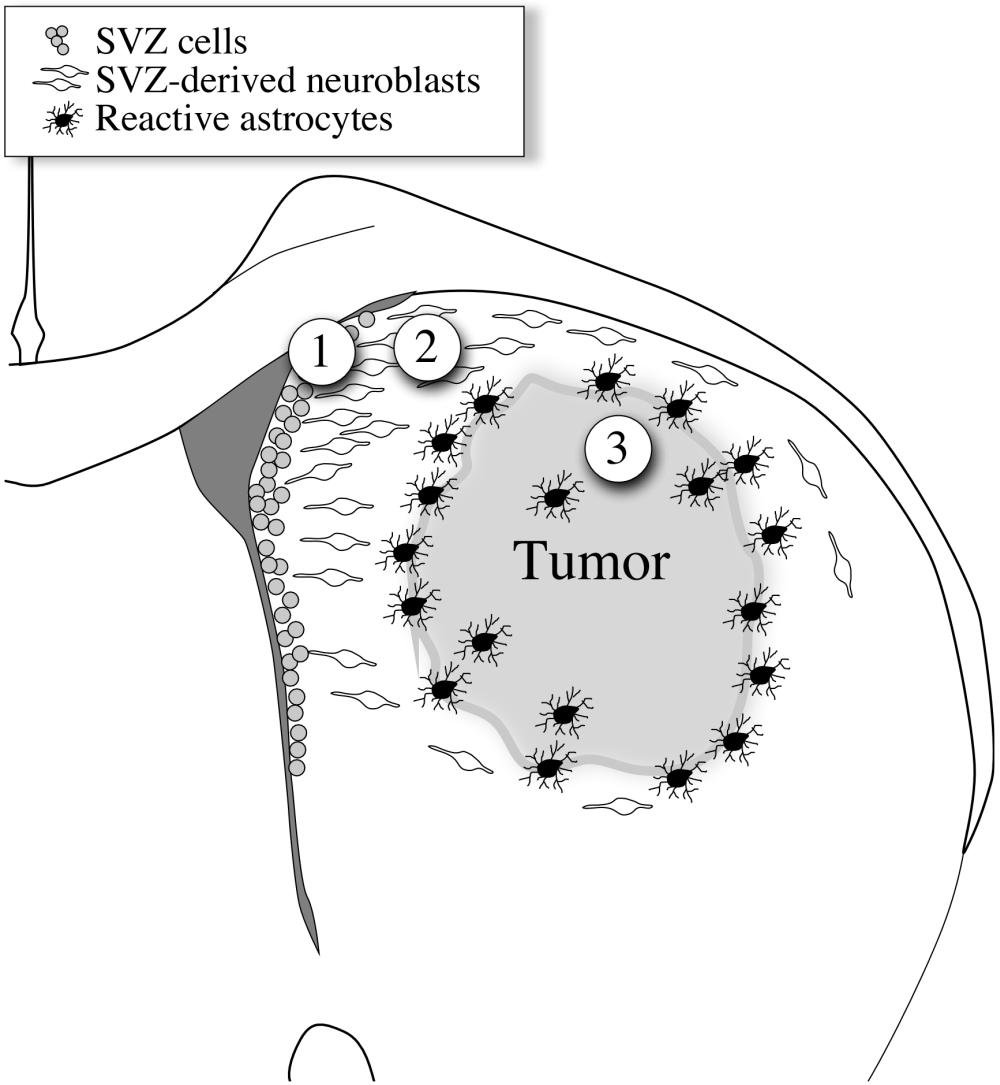

Figure 2. *Multifaceted endogenous cellular response to the growth of malignant brain tumor.*
The figure shows a coronal section of a rat cerebral hemisphere with malignant brain tumor growth in the striatum. (1) Tumor growth results in decreased numbers of SVZ proliferating cells. (2) DCx+ neuroblasts migrate from the SVZ towards the tumor area but neuroblasts do not infiltrate into tumors. (3) Nestin+/GFAP+ reactive astrocytes accumulate around and within gliomas. The endogenous cellular response to gliomas also includes microglia activation, infiltration of blood-borne immune cells, and infiltration of bone marrow-derived perivascular cells into tumors.

3. Comparative analysis of rat bone marrow- and subventricular zone- derived precursor cells as cellular vectors in glioma gene therapy (paper III)

Although both MSCs and NPCs have been used for delivery of therapeutic transgenes to gliomas, the efficiency of these two vector systems has not been compared. In order to choose the most efficient vector system, we therefore analyzed the migratory patterns and survival of intratumorally implanted bone marrow-derived MSCs and SVZ-derived NPCs. In addition, we determined the proliferation rate of intratumorally implanted MSCs and NPCs.

Isolation and characterization of adult rat MSCs is described in paper IV. Adult rat SVZ-derived NPCs were grown as neurospheres and expressed stem/progenitor marker nestin *in vitro*. Importantly, both MSCs/NPCs and tumor cells were syngeneic to the host. For visualization *in vivo*, MSCs and NPCs were transduced to express enhanced green fluorescent protein (eGFP) using a retroviral vector. More than 90% of the transduced MSCs and NPCs expressed eGFP *in vitro*.

eGFP+ MSCs or eGFP+ NPCs were implanted on day 14 into established N32 gliomas. One day after implantation, MSCs/NPCs were found mainly within the graft at the inoculation site. On day four, MSCs and NPCs were seen within the graft and at the tumor periphery. An additional four days later, MSCs and NPCs had localized both in the tumor periphery and in the tumor core. Importantly, migration of intratumorally implanted cells into normal brain tissue was extremely rare. Thus, intratumorally implanted MSCs and NPCs displayed extensive migratory capacities specifically within gliomas.

Semiquantitative analysis showed that the numbers of eGFP+ MSCs within tumors were much higher compared to the numbers eGFP+ NPCs. For further comparison, we quantified the amount of eGFP within tumors. This showed that the amount of eGFP within tumors receiving MSCs was approximately 11-fold higher compared to tumors receiving NPCs. The analysis performed gives an estimation of the relative cell numbers within tumors and cannot be used for detection of minor differences in cell numbers. Nevertheless, the magnitude of the difference in GFP amounts clearly suggests that implanted eGFP+ MSCs persist in much higher numbers within tumors as compared to eGFP+ NPCs.

In contrast to the widespread migration of intratumorally implanted cells, we found virtually no migration of eGFP+ MSCs or eGFP+ NPCs toward tumors upon implantation at a distant site in normal brain tissue. Instead, implanted eGFP+ cells localized at the implantation site. In addition to the analysis of eGFP+ cells, we analyzed the expression of certain endogenous markers that are expressed by the implanted cells (neuron

glia 2, NG2, and α-smooth muscle actin, α-sma, for MSCs, and nestin and DCx for NPCs). There was no evidence of NG2 or α-sma expressing cells with spindle-shaped morphology migrating from MSC grafts toward tumors. Neither did we detect any nestin or DCx+ cells with migratory morphology between NPC grafts and tumors. Taken together, implanted MSCs and NPCs do not seem to migrate throughout normal brain tissue toward gliomas.

In order to evaluate tumorigenicity of implanted precursor cells, we assessed their proliferation rate within tumors. For this purpose, confocal microscopy was used to determine co-expression of the cell-cycle marker Ki-67 and eGFP. 100 eGFP+ MSCs and NPCs, respectively, were analyzed. We did not find a single eGFP+ cell that expressed Ki-67. Accordingly, more than 99%, if not all, of the intratumorally implanted MSCs and NPCs are non-cycling.

4. Bone marrow multipotent mesenchymal stroma cells act as pericyte-like migratory vehicles in experimental gliomas (paper IV)

In light of the findings of paper III, we continued by investigating the use of bone marrow-derived MSCs as cellular vectors in the invasive N29 glioma model. In addition, we studied the association of implanted MSCs with tumor blood vessels.

MSCs were derived from the bone marrow of adult Fischer344 rats and isolated based on their adherent growth *in vitro*. MSCs displayed spindle-shaped morphology and possessed the capacity to differentiate into adipocytes and osteoblasts *in vitro*. Flow cytometric analysis revealed that MSCs were positive for CD73 and CD90 and negative for the hematopoietic marker CD45.

In order to assess the capacity of MSCs to migrate to tumor extensions and to distantly located tumor micro satellites, we implanted eGFP+ MSCs into established N29dsRed tumors. MSCs were implanted intratumorally 10 days after tumor cell implantation. MSC migratory capacity was evaluated 5, 10 and 15 days later. Serial section analysis revealed that a single MSC injection results in MSC migration to approximately 70% of the tumor extensions and to approximately 30% of the distant tumor micro satellites. Importantly, implanted MSCs were largely retained within tumor tissue and only single eGFP+ MSCs were found within normal brain tissue. MSC tumor specific capacity was further validated by Fluorescence *in situ* hybridization (FISH) where male-derived MSCs (carrying the Y chromosome) were implanted into female hosts carrying N29 tumors (established in a female animal). Expression of eGFP was unfortunately lost due to heavy enzymatic pretreatment of the tissue sections. We were therefore unable to co-localize eGFP with signals from the Y chromosomes (i.e. MSCs). However, we

could, based on signals from the Y chromosomes, differentiate MSCs from N29 tumor cells and host cells. Findings from FISH analysis support the finding that intratumorally implanted MSCs are restricted within tumors.
Co-labeling with GFP and rat endothelial cell antigen (RECA) revealed that high numbers of implanted eGFP+ MSCs closely localized to glioma vasculature. To investigate whether MSCs contribute to tumor endothelium, we used confocal microscopic analysis to determine co-expression of eGFP with RECA and vascular endothelial cadherin (VE-Cadherin). We found no co-expression of eGFP with RECA or VE-Cadherin *in vitro* or *in vivo*. Thus, implanted MSCs migrate along, and possibly integrate into, tumor vessels but do not directly contribute to tumor endothelium.

The phenotype of MSCs was further investigated. We found that more than 99% of the MSCs expressed pericyte markers α-sma, NG2 and PDGFR-β *in vitro*. The expression pattern was largely retained upon intratumoral implantation. The pericyte marker expression profile and perivascular location of implanted MSCs indicate that these cells might act as pericytes within tumors. Because of the functional effects of pericytes on tumor vessels[24], we studied the effect of MSC implantation on the tumor vasculature. We found, however, no effect of MSCs on tumor microvessel density. Neither did intratumoral MSC implantation apparently affect survival of glioma-bearing animals.

To shed further light on MSCs association with tumor vessels, we implanted MSCs into gliomas and treated the animals p.o. with Sunitinib, which is an anti-angiogenic drug. Specifically, Sunitinib inhibits multiple tyrosine kinase receptors including PDGFR-α and -β, VEGF-receptor-1 and-2, stem cell factor receptor, KIT, and Fms-like tyrosine kinase-3 receptor[114]. Treatment with Sunitinib caused a decrease in the number of tumor microvessels as well as in the volume of tumors. Concomitantly, the number of MSCs migrating from the graft at the site of implantation to tumor periphery decreased. In addition, implanted MSCs migrated from the graft to the tumor core in vehicle-treated animals while virtually no MSCs were found within the tumor core in Sunitinib-treated animals. Because of the broad inhibitory effect of Sunitinib we cannot exclude that the observed effect was, in part, due to decreased survival of implanted MSCs following Sunitinib treatment. Nevertheless, MSCs persisted in high numbers at the implantation site and their migratory pattern was radically changed by Sunitinib. This indicates that angiogenic signaling factors and, possibly, tumor vascularization *per se* are important mediators of MSC migration within tumors.

We injected MSCs intravenously (i.v.) via the tail vein to glioma-bearing animals on day 14 after tumor cell implantation. We found no evidence of eGFP+ MSCs within the tumors 2 or 7 days later. Thus, i.v. administration seems inefficient as a delivery route of MSCs to gliomas.

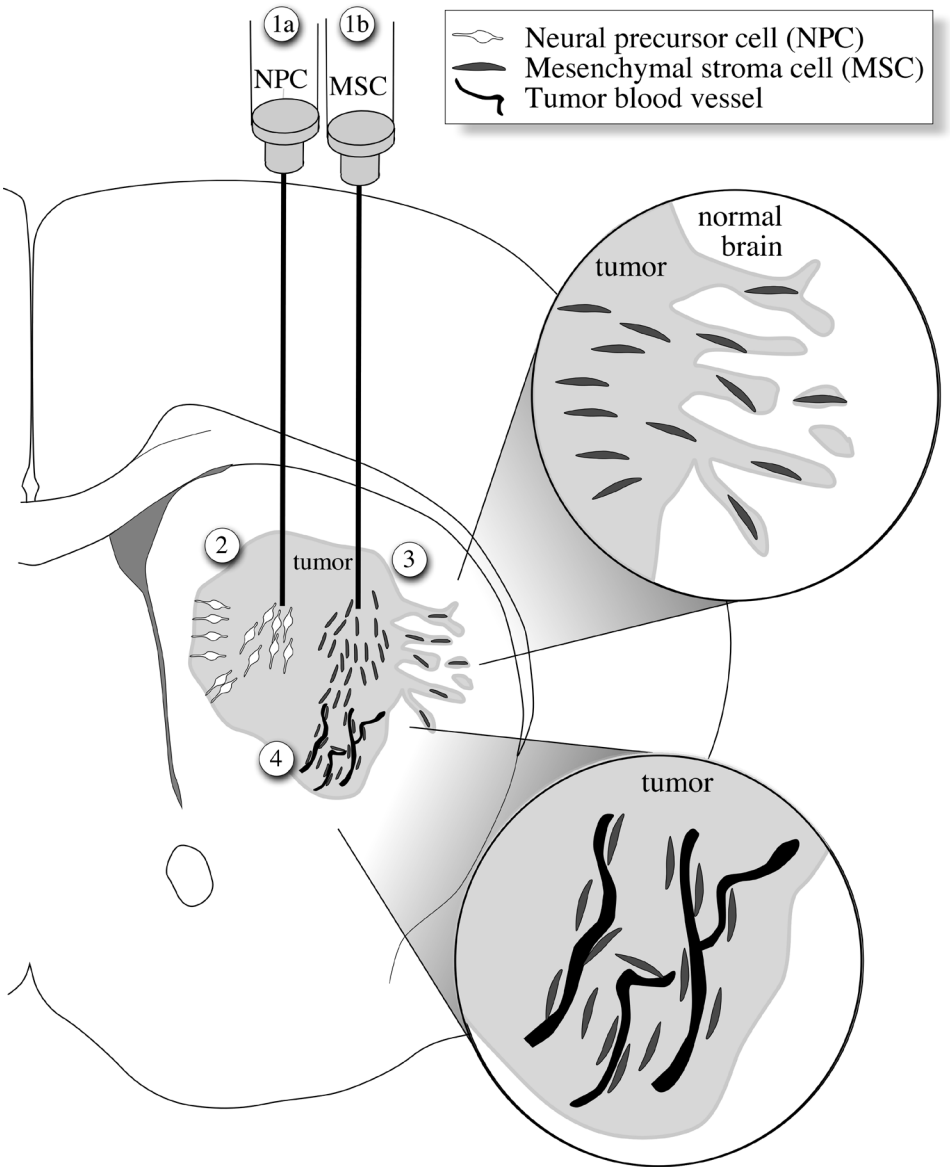

Figure 3. *Towards a stem cell based therapy of malignant brain tumors.*
Either NPCs (1a) or MSCs (1b), are implanted directly into experimental malignant glioma. (2) Implanted NPCs and MSCs migrate within the tumor but not to normal brain tissue. MSCs persist in higher numbers than NPCs. (3) MSCs migrate to invasive tumor extensions and to distant tumor microsatellites. (4) Implanted MSCs migrate along, and possibly integrate into, tumor blood vessels. MSCs could be used as migratory vehicles for delivery of therapeutic transgenes to invasive and vascularized tumors.

Figure 3 summarizes the findings of papers III and IV.

DISCUSSION AND FUTURE PERSPECTIVES

DISCUSSION AND FUTURE PERSPECTIVES

Methodology: Can the results be trusted?

The tracking of implanted cells *in vivo* is associated with certain methodological issues. In the work process of the present thesis, MSCs and NPCs were transduced to express eGFP using a retroviral vector which infects dividing cells. However, transgene silencing upon intracranial implantation of *in vitro* transduced precursor cells is common[115, 116]. Hence, there is a potential risk to underestimate the number of implanted MSCs and NPCs within and outside tumors.

We used two methods to examine whether potential eGFP-transgene silencing *in vivo* contributed to erroneous interpretations. First, FISH was performed to label male-derived MSCs implanted into female hosts carrying female-derived tumors. Male-derived MSCs were discerned from female-derived tumor cells and host cells based on signals from the Y chromosome. Results from the FISH analysis supported the finding that intratumorally implanted MSCs are restricted within tumors. Second, we analyzed *in vivo* expression of endogenous markers (α-sma and NG2 for MSCs[117] and nestin and DCx for NPCs) that are expressed by the implanted cells. Although the expression of the endogenous markers can be down-regulated in vivo, this assay provides an independent method which supports the findings of the eGFP-expression pattern. Nevertheless, it is important to remember that the implanted cells that are analyzed in many experiments in papers III and IV are the cells that express eGFP. MSCs and NPCs that potentially have down-regulated eGFP are not included in the analysis.

Notably, findings from our studies using N29 and N32 glioma cells transduced to produce the DsRed protein[117, 118], which allows visualization *in vivo*, were validated by similar findings obtained by the use of N29wt and N32wt tumor cells (own unpublished results). Accordingly, production of the DsRed protein by N29 and N32 glioma cells did not significantly influence the results presented in papers II and IV.

Can cancer stem cells be assessed in experimental glioma cell lines?

The analysis of putative CSC-like cells in N29 and N32 glioma models was associated with a number of conceptual issues. First, it has been suggested that a sub-population of CSC-like cells reside in the experimental glioma cell lines rat 9L and mouse GL261[72, 73]. However, with respect to murine experimental glioma cell lines, there is yet no evidence for the existence of a rare subset of tumor cells that are the only cells that propagate tumor growth *in vivo*. In contrast, based on clonogenic and tumorigenic properties, Zheng et al. argued that the rat C6 line was composed of a dominant clone

of CSC-like cells[74]. The latter findings are in line with our results from the N29 and N32 glioma models. The five above-mentioned glioma cell lines have been cultured in serum-containing medium for decades. Lee et al. has shown that serum-culturing of Glioblastoma cells favors the development of a clone of tumor cells and not a heterogenous population[119]. This could explain our findings of a homogenous population of tumor cells in the N29 and N32 glioma models. However, it also raises questions on how a fraction of CSC-like cells can persist in the 9L and GL261 glioma models despite years of culturing in serum-containing medium.

Second, putative CSCs in human tumors exhibit aberrant differentiation properties. It has been suggested that rat 9L cells and mouse GL261 cells also can be induced to exhibit a neuronal and glia-like phenotype upon differentiation conditions in vitro. In contrast, our findings show that the N29 and N32 cell lines do not change their phenotype upon growth in differentiation conditions in vitro or upon in vivo implantation. In line with our findings, it has been shown that serum-culturing of Glioblastoma cells causes an aberrant phenotype and irreversible loss of differentiation capacity[119]. It would be interesting to understand how serum-cultured 9L and GL261 glioma cells can maintain their capacity for differentiation after years in culture. In addition, the fact that all, or almost all, N29 and N32 cells already express common differentiation markers makes it problematic to assess differentiation in our model system.

Third, we found that most, if not all, N29 and N32 cells express both nestin and CD133 as well as DCx, BIII-tubulin, GFAP and CNPase[120]. The presence of undifferentiated markers and lineage markers in the same cells might look confusing in the eyes of a neural stem cell biologist. However, the expression of DCx in human brain tumors is associated with invasiveness[121]. DCx expression may thus indicate invasive properties rather than a neuronal phenotype of glioma cells. Similarly, nestin expression is found in many tumors and correlates with high-grade malignancy and invasiveness[122, 123]. βIII-tubulin is commonly used as a neuronal specific marker but βIII-tubulin is also expressed in many types of human non-neural cancers including breast cancer, lung cancer and ovarian cancer[124-126]. Thus, protein expression in cancer cells does not necessarily represent the same phenotype as in normal neural cells. Accordingly, conclusions based on the expression of certain “stemness” or “lineage” associated proteins in glioma cell lines and in human cancers should be interpreted with caution.

Fourth, it has been suggested that CSC-like cells in the 9L and GL261 models accumulate in free-floating spheres as compared to monolayers. As discussed by Singec et al., nearly all dividing cells can form spheres when grown in serum-free media. This implies that neurospheres consist not only of stem cells but also of proliferative progenitors[127]. At best, the studies of 9L and GL261 cell lines demonstrate that the cell lines

consist of a heterogenous set of tumor cells which respond and behave differently with regard to sphere growth as compared to adherent growth[72, 73]. We found that adherently growing cells are highly clonogenic, and that as few as 10 unsorted adherently growing cells can initiate tumors upon orthotopic implantation. This indicates that sphere formation is not crucial for enrichment of CSC-like cells. However, direct comparisons between sphere-forming cells and monolayer cells, with respect to clonogenic capacity and *in vivo* tumorigenicity, were not performed in our study.

Fifth, putative CSCs in human solid tumor are supposed to possess self-renewal capacity. N29 and N32 glioma cells have been cultured for decades, still being highly proliferative *in vitro* as well as *in vivo*. Hence, N29 and N32 glioma cells do certainly possess extensive, and possibly unlimited, proliferative capacities. Moreover, glioma cells could be passaged multiple times and still retain their phenotype and *in vitro* growth pattern. One could possibly infer that the N29 and N32 models possess self-renewal capacity with respect to *in vitro* clonogenicity. On the other hand, one could also argue that assessment of self-renewal becomes relevant first in the context of a heterogenous population of cells (such as a GBM cell suspension). Further experiments to assess self-renewal include serial single-cell transplantation of tumor cells between animals.

Irrespectively of whether N29 and N32 glioma cells form free-floating spheres, possess true self-renewal capacity or express certain markers, the most important functions of the models are that they display extensive proliferative capacity *in vitro* and very robust tumorigenicity *in vivo*.

Are N29 and N32 rat gliomas relevant models of human brain tumors?

A comparison between human GBM and N29 and N32 experimental gliomas is presented in table 1. In both N29 and N32 cell lines, all cells, or a dominant clone of cells, are capable of tumor formation upon orthotopic implantation[120]. This is in contrast to the CSC hypothesis where only a subset of cells can sustain tumor growth. Alternatively, it has been suggested that the growth of human tumors is also sustained by a dominant clone or by all cells[128]. Irrespectively of whether the CSC hypothesis is correct or not, the models are highly tumorigenic *in vivo* and curative treatment directed against N29 and N32 gliomas probably needs to eradicate virtually all tumor cells.

The significance of the results obtained by *in vivo* implantation of glioma cells is associated with one arbitrary factor, which is the time-interval between tumor cell implantation and initiation of treatment. In our studies, precursor cells were implanted or injected usually between 10 and 14 days following tumor cell implantation[117, 129]. At these time-points, N29 tumors display characteristic GBM growth (i.e. invasive

Table 1. *The table shows a comparison between human GBM and N29 and N32 experimental gliomas.*

	GBM	N29	N32
lethal to host	yes	yes	yes
infiltrative growth	yes	yes	no
highly proliferative	yes	yes	yes
neovascularization	yes	yes	yes
central necrosis	yes	yes	yes
tumor stroma	yes	yes	yes
cellular heterogeneity	yes	no	no
genetic heterogeneity	yes	no	no
immunosuppression	yes	yes	yes
refractory to radio- and chemotherapy	yes	likely (similar ENU-induced models are)	likely (similar ENU-induced models are)

growth, neovascularization and often central necrosis). Our findings that approximately 70% of the tumor extensions and 30% of the distant tumor microsatellites were infiltrated by implanted MSCs must be interpreted in light of the time-interval between tumor cell implantation and MSC implantation. MSC implantation three days after tumor cell implantation would most likely yield higher numbers of MSCs migrating to invasive tumor cells. Conversely, extending the time-interval would likely result in lower numbers of tumor extensions infiltrated by MSCs. In most studies using experimental glioma cell lines, there is often a very short interval (between one to five days) between tumor cell implantation and onset of treatment. At these time-points, the tumor is often poorly developed and does not display the characteristic features of GBM. The outcome of these studies is most likely very different from when treatment is initiated around day 10 or later when the tumor has established its typical growth. Thus, the clinical relevance of the findings obtained when using experimental glioma models increases with increasing the time-interval between tumor cell implantation and onset of treatment.

Arguably, the genetic heterogeneity and genetic instability of human GBM lead to development of treatment-associated resistance through selection pressure of specific clones. The lack of heterogeneity found in experimental glioma cell lines complicates the prediction of the efficiency of certain treatment modalities, e.g. molecular targeting or gene therapy of specific GBM-associated genetic changes. Experimental glioma cell lines may instead be more useful for studies on general GBM features like neovascularization and immunosuppression, and for the development of anti-angiogenic therapy and immunotherapy.

To summarize, the clinical relevance of the use of N29 and N32 glioma models depends on the scientific question and the experimental set-up.

What is the magnitude of endogenous neuroblast migration toward gliomas?
Glass et al. reported abundant numbers of endogenous SVZ-derived neuroblasts that completely surround and infiltrate G261 mouse malignant brain tumors[58]. We found moderate numbers of endogenous neuroblasts in the area between the SVZ and N32 tumors. Virtually no neuroblasts were infiltrating into tumors. In both studies, high numbers of nestin+ cells were found to surround tumors. Nestin is an intermediate filament protein expressed in a variety of cells, including NSCs, endothelial cells and reactive astrocytes[112, 130]. Glass et al. suggested that the nestin+ cells that surround tumors are neuroblasts originating from the SVZ, based on uptake of BrdU and on DiI tracing experiments[58]. However, BrdU+/nestin+ cells might as well represent proliferating nestin+ reactive astrocytes residing in the parenchyma. In addition, no migratory DiI-labelled cells (presumed to originate from the SVZ) were found in the area between the SVZ and tumor[58]. Furthermore, Glass et al. estimated that 10% of the nestin+ cells co-expressed DCx and 30% of nestin+ cells co-expressed PSA-NCAM[58]. These results are surprising in light of the finding that nestin is largely down-regulated in migrating neuroblasts[131] and that DCx expression almost completely overlaps with the expression of PSA-NCAM[132]. In contrast, we counted the actual numbers of nestin+ cells in the area between the SVZ and tumors at four time-points. We found no increase of nestin+ cells in the area between the SVZ and the tumor. This implies that the absolute majority of nestin+ cells surrounding malignant brain tumors originate from the brain parenchyma rather than from the SVZ. Furthermore, based on their star-shaped morphology and concomitant expression of GFAP, we considered the nestin+ cells to represent reactive astrocytes. Our finding of moderate numbers of DCx+ neuroblasts in the area between the SVZ and tumors points to a more limited SVZ response to tumors.

Another study by Staflin et al. found no evidence of DCx+ neuroblasts migrating from the SVZ toward N29 brain tumor[59]. Our findings when using N32 tumors indicate that the SVZ neurogenic response to tumors is dependent on tumor size and on the proximity of the tumor to the SVZ. It would be interesting to investigate the SVZ response to N29 tumor growth at later time-points than was done by Staflin et al.

The different results from the above-mentioned studies are probably due to differences in methodology and experimental set-up. Alternatively, endogenous neuroblast migration toward tumors may be dependent on the tumor type.

What are the possible functions of endogenous neuroblast migration toward gliomas?

NPCs anti-glioma effect, as described by Glass et al.[58], gained attention because of the potential therapeutic implication. In this study, the authors argued that the increased tumor volume, found in old animals as compared to young animals, was caused by a concomitant decrease in nestin+ cells (putative NPCs) around tumors[58]. The increased tumor growth found in aged animals can, however, be associated with many factors including a declining anti-tumor immune response. In addition, our findings indicate that the vast majority of the tumor-associated nestin+ cells are not NPCs but reactive astrocytes[118]. Additional experiments conducted by Glass et al. demonstrate anti-glioma properties of NPCs under certain experimental conditions, but they do not say anything about the effect of endogenous NPCs on tumor growth *in vivo*. In contrast, it has been hypothesized that SVZ-derived NPCs can contribute to glioma growth, directly or indirectly by production of growth factors[133]. As pointed out in this article, this is a theoretical proposal not supported by experimental evidence[133].

Our findings demonstrating that the majority of the migrating neuroblasts express Pbx, a region-specific marker of immature neurons, could point to an attempt by the SVZ to regenerate neurons that died as a result of tumor growth[118]. Migratory tumor-associated neuroblasts could potentially contribute to neuronal replacement, as has been shown for SVZ-derived neuroblasts following ischemic stroke[56]. Obviously, neuronal replacement is not the primary interest for the GBM patient of today. However, given the development of successful glioma therapy, neuronal replacement by endogenous NPCs could provide a role in the prevention or treatment of tumor-associated neurological complications.

In summary, limited numbers of studies of glioma-attracted endogenous neuroblasts exist and notions of their possible effect*s in vivo* are speculative. Still, if migratory endogenous neuroblasts exhibit a direct anti-tumor effect, as suggested by Glass et al., one would like to modify the endogenous SVZ response in order to increase the numbers of neuroblasts with anti-tumor effects. This would need to be very carefully designed in order to avoid stimulation of glioma cell growth or migration, and to avoid negative effects on the SVZ. For instance, intracerebroventricular infusions of EGF lead to formation of hyperplasias in the SVZ[134]. Identification of signalling factors that specificially stimulate NPC migration and not glioma cell migration would facilitate such functional studies.

Which type of cell is most efficient as a migratory delivery vehicle in gliomas?
Current knowledge in the field has mainly emerged from the use of immortalized NSC lines, such as the murine NSC line C17.2 or the human fetal NSC line HB1.F3[135]. In our and other studies, primary isolated precursor cells, derived from the adult SVZ or the adult bone marrow, were used[97, 117, 129]. The present thesis suggests that adult intratumorally implanted MSCs are more efficient as migratory vehicles than adult NPCs. This may be due both to superior survival and migration of implanted MSCs.

Immortalized NSCs can migrate throughout the normal brain parenchyma toward a distant tumor[87, 99]. Our results show that adult primary NPCs and MSCs, syngeneic to the host and tumor, need to be implanted into or in close proximity to tumors to achieve widespread migration within tumors. We found, however, that NPCs, when implanted into the corpus callosum, can migrate along the corpus callosum toward a distant tumor (own unpublished observations). NPC migration along the corpus callosum was also found when NPCs were implanted intracranially in the absence of tumor, implying that their migratory capacity throughout normal brain tissue was not specific to tumors. It is not clear from the initial study by Aboody et al. if migration of immortalized NSCs along the corpus callosum is specific for tumors or if it also occurs in the absence of tumor growth. Still, the capacity of immortalized NSCs to migrate throughout normal brain tissue toward tumors makes them potentially more efficient delivery vehicles compared to adult MSCs and NPCs. However, implantation of immortalized NSCs into the brain can evoke an immune response. This implies that the immune system might neutralize implanted cells and negatively effect their survival and migration to tumor microsatellites. HLA-matched allogeneic stem/precursor cells, as used in our studies, are immunologically compatible and this may favor their survival and migration within tumors. Future studies need to compare the efficiency in survival and intratumoral migration between adult MSCs and immortalized NSCs.

In addition, alternative types of precursor cells, not investigated in the present thesis, might serve as potent vehicles in gliomas. Human skin-derived stem cells can migrate to human gliomas in immunodeficient mice, reduce tumor angiogenesis and decrease glioma growth. Interestingly, a fraction of the implanted skin-derived stem cells adopt a pericytic phenotype[98]. I.v. injected endothelial progenitor cells (EPCs) can integrate into tumors but intracranial implantation of EPCs does not result in migration toward gliomas[98].

Why do stem cells migrate toward and within tumors?
Our results demonstrate that implanted MSCs migrate along tumor blood vessels *in vivo*. Furthermore, inhibition of signaling factors involved in tumor vessel formation is associated with decreased numbers of implanted MSCs within tumors[117]. Previous

findings using *in vitro* assays have shown that angiogenic cytokines like VEGF-A, interleukin-8, transforming growth factor-β1 and neurotrophin-3 are involved in the recruitment of MSCs to gliomas[105, 106]. The pericyte-like phenotype of MSCs indicates that endothelial interactions and specifically PDGF-β, sphingosine-1-phosphate and angiopoietin-1 mediate MSC recruitment to tumors since these factors contribute to recruit pericytes to tumor vessels[24, 136]. Recent findings have demonstrated strong *in vitro* and *in situ* links between human MSCs and human pericytes[137]. This study shows that MSCs are members of the pericyte family, although all pericytes are not MSCs. Thus, MSCs reside within a perivascular niche in the bone marrow and localize to the perivascular niche upon implantation to gliomas. It is conceivable that similar factors attract MSCs to their bone marrow perivascular niche and to glioma vessels. MSCs also home to wounds where they contribute to wound healing[46, 138]. Tumors, which can be considered as "wounds that never heal"[139], provide a site with neovascularization, high numbers of inflammatory cells and cytokines that can attract MSCs. Hence, it is possible that the extensive intratumoral migration capacity of implanted MSCs represents a stereotypic response to injury-induced inflammation.

Why do NPCs migrate within tumors? The literature suggests that VEGF, hepatocyte growth factor, SDF-1α and MCP-1 are important factors[102, 109]. We found that implanted NPCs are able to localize closely to tumor vessels, but not to the same extent as MSCs do (own unpublished observations). Interestingly, it has been suggested that tumor-produced ECM proteins (such as laminin and tenascin-C) facilitate NSC migration to tumors[104]. This could explain why implanted stem and precursor cells possess the capacity to migrate to invasive tumor cells and avoid normal brain tissue.

How should stem cells be administered?

The work in the present thesis focuses primarily on administration of precursor cells through intratumoral delivery. This mode of delivery seems to be an efficient and tumor specific way for delivery of precursor cells to and within gliomas[117, 129]. Nakamizo et al. reported incorporation of human MSCs into human gliomas in immunodeficient mice upon intra-arterial injections via the ipsilateral or contralateral carotid artery[96]. No systematic study has been performed to compare the efficiency of MSC distribution upon intratumoral *vs*. intra-arterial delivery. Neither is it known whether intra-arterial injections of MSCs result in infiltration to distantly located tumor microsatellites with an intact blood-brain barrier.

Work with the NSC line C17.2 suggest that these cells possess the capacity to infiltrate gliomas following i.v. delivery, although at a low frequency[87]. We performed MSC i.v. injections into glioma-bearing animals and we found no evidence of MSC incorpora-

tion in vascularized gliomas[117]. In contrast, Studeny et al. reported that i.v. administered MSCs home to subcutaneously growing melanomas. Infiltration into melanomas was inefficient since five MSC injections during a 20-day period infiltrated only a fraction of the established tumors[140]. Intratumoral delivery seems more promising compared to i.v. delivery in light of the low efficiency and the potential side-effects of i.v. injected MSCs. Intra-arterial administration, although also possessing potential side-effects, remains a possible alternative to intratumoral delivery.

How does implantation of multipotent mesenchymal stroma cells affect tumor growth?

Implantation of MSCs, not modified to produce a therapeutic transgene but often modified to allow for visualization e.g. by eGFP transduction, has been reported to either decrease tumor growth, favor tumor growth or to give no effect. We found no apparent effect on tumor growth following intratumoral implantation of eGFP+ MSCs[117]. Nakamura et al. reported that implanted rat GFP+ MSCs prolong survival of glioma-bearing animals, but no mechanistic effect was presented[97]. In another study, conditioned media from human MSCs was found to inhibit human cancer cell proliferation *in vitro* by down-regulation of the transcription factor NF-κβ[141].

In contrast, MSCs can favor tumor growth through production of immunosuppressive factors and inihibition of lymphocyte proliferation[142]. Additionally, one could imagine that implantation of pericyte-like MSCs would favor tumor angiogenesis and thereby facilitate tumor growth, but our results indicate that MSC implantation has no apparent effect on tumor microvessel density within gliomas[117]. It is, however, possible that implanted MSCs can influence tumor blood flow without affecting the number of tumor vessels.

The different effects of implantations/injections of MSCs on tumor growth are striking. It is possible that heterogeneity of cultured MSCs (i.e. multiple MSC sub-populations with different properties)[143], differences in tumor cells and models, species-specific interactions between tumor cells, MSCs and hosts account for some of the different effects on tumor growth. The findings illustrate the complexity of the role of MSCs in cancer. Our findings indicate that MSCs need to be genetically modified to express a therapeutic transgene in order to decrease tumor growth[117], but the opposing results from other groups justify further studies.

Can implanted stem cells form secondary malignancies?

Transplantation of stem cells into the pathological brain is associated with potential risks of tumor formation[144]. MSCs are candidate cells for the origin of sarcomas, and SVZ-derived NPCs might be associated with glioma development[145, 146]. In addition, a concern when using retroviral vectors is the risk of insertional mutagenesis and tumorigenic development of the transduced cells[147].

Importantly, we found no signs of development of secondary malignancies of retrovirally transduced adult eGFP+ MSCs/NPCs after implantation into established malignant brain tumors or into normal brain tissue. Using confocal microscopy to detect co-labelling of GFP and the cell-cycle marker Ki67, we found no evidence of proliferation of implanted MSCs or NPCs within highly proliferative tumors up to 30 days after MSC/NPC implantation[129]. This findings speaks against tumor formation of MSCs and NPCs although one cannot exclude transformation of these cells *in vivo* at later time-points. In contrast, Shah et al. reported that approximately 2% of implanted immortalized NSCs continue to proliferate following implantation into malignant brain tumors[148]. There is an obvious risk that expression of the gene (e.g. the proto-oncogene v-*myc*) used to immortalize the cell line can contribute to malignant transformation of stem cells.

In addition, long-term culture of stem cells can be associated with malignant transformation. It has been shown that human MSCs can be cultured safely for standard expansion periods (6-12 weeks)[149, 150]. However, long-term (i.e. five months) *in vitro* culturing can transform MSCs into a malignant phenotype[150]. Conflicting data exist regarding transformation of expanded NPCs. Foroni et al. demonstrated that mouse adult SVZ-derived NSCs can be propagated for more than a year and still maintain a stable phenotype[151]. In contrast, another group showed that rat SVZ-derived NPCs can develop into tumorigenic cells briefly upon *in vitro* expansion[152]. The different results may be explained by heterogeneity of the isolated cells, species differences, and by differences in culturing methods.

What should stem cells deliver to gliomas?

MSCs association and integration into tumor vessels offer several opportunities for therapeutic targeting of gliomas. The tumor neovasculature is critical for tumor growth[14] and implanted MSCs may act as vehicles for delivery of anti-angiogenic substances to vascularized tumors. Furthermore, combinatorial targeting of both tumor endothelium and tumor pericytes can diminish tumor vascularization in a synergistic way and concomitantly decrease tumor growth[153]. The vessel association of MSC implies that an approach to locally target both tumor endothelial cells and tumor pericytes is possible.

Implanted MSCs are also strategically located to deliver substances that can attack putative CSCs, which may reside within a perivascular niche[69].

The close association of implanted MSCs with tumor vessels may also explain their robust capacity to attract blood-borne immune cells to tumors. MSCs, genetically modified to produce pro-inflammatory substances like interleukin-2, interferon-β and interleukin-23 can decrease tumor growth and prolong survival of tumor-bearing animals[96, 97, 154]. The therapeutic effect correlates with increased infiltration of blood-borne immune cells, e.g. cytotoxic T-cells and NK-cells, into tumors[96, 97, 154]. It would be interesting to see if systemic immunotherapy in combination with intratumoral implantation of immunomodulatory MSCs could act synergistically in glioma treatment.

The capacity of MSCs to target infiltrative tumor cells suggests that implanted or injected MSCs can be used to deliver anti-invasive substances to prevent further disseminating growth. Identification of substances that specifically can target migration of glioma cells without affecting intratumoral MSC migration would be important for this purpose. Alternatively, an inducible drug delivery system, like a pro-drug converting enzyme, would allow MSCs to migrate to invasive tumor extensions and then, upon induction, deliver anti-invasive substances.

Can MSCs achieve therapeutic benefit without delivery of a tumoricidal substance? The pericytic phenotype and perivascular location of MSCs indicate that implanted MSCs could possibly function as pericytes within tumors. Tumor pericytes contribute to vascular normalization which in turn enhances the influx of immune effector cells, which can lead to decreased tumor growth[155]. Accordingly, it would be interesting to see if implanted MSCs can contribute to normalization of tumor vasculature and facilitate immunotherapy of tumors. In addition, Xian et al. demonstrated that tumor pericytes can limit tumor cell metastasis[156]. In contrast, another group showed that, when mixed with breast cancer cells, MSCs can increase the metastatic potential of the cancer[157]. Because of the infiltrative growth of human GBM, it will be important to investigate the role of implanted MSCs in glioma cell invasion.

Stem cell based therapy of malignant brain tumors – will it work in patients?

The literature provides abundant reports of novel successful experimental glioma therapies but the clinical outcome remains unsatisfactory. As discussed above, it is clear that most experimental glioma models do not exhibit the same genetic heterogeneity as human GBM. Experimental scientists often treat a one to five days old brain tumor while clinicians indeed face a more challenging task. So, why would implantation of genetically modified stem/precursor cells prevail against brain tumors? The present

thesis shows that MSC intratumoral migration is associated with a general GBM feature, i.e. tumor neovascularization, and not with specific genetic pathways. In addition, efficient and tumor specific migratory capacity of MSCs and NPCs was demonstrated after implantation into gliomas that previously were allowed to grow for a certain time-period and establish their characteristic growth pattern. It is therefore conceivable that implanted human precursors will migrate within vascularized tumors of patients too. Importantly, implanted MSCs can, in contrast to most other experimental glioma therapies, specifically target the invasive tumor cells. The success of stem cell implantation in glioma therapy will, however, depend not only on their migratory capacity but also on availability, safety issues and the choice of therapeutic transgenes.

A prospect for stem cell based therapy of glioma patients includes implantation of genetically modified stem cells into the remaining tumor at the time of surgical resection. This would probably involve injections at multiple co-ordinates within the tumor. Subsequent intratumoral implantations of stem cells could be undertaken at later time-points using image-guided stereotaxic techniques. Future studies need to determine the migratory and proliferative capacity of implanted human MSCs or NPCs within gliomas of patients. In addition, concomitant radio- and chemotherapy will most likely influence the survival and migration of implanted precursor cells. Interestingly, a recent study shows that tumor irradiation enhances the migration of implanted MSCs toward tumors[158], suggesting a combined, and possibly synergistic, approach to the treatment of gliomas.

Finally, implantation and injection of genetically modified stem and precursor cells have shown promise in different types of experimental cancer models, including malignant melanoma, ovarian cancer, Kaposi´s sarcoma, melanoma brain metastases and lung metastases[135, 159, 160]. The findings of the present thesis may therefore contribute to the development of therapy not only against primary brain tumors but also against other types of cancers.

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CD133+ TUMOR INITIATING CELLS DOMINATE IN N29 AND N32 EXPERIMENTAL GLIOMAS

I

CD133+ Tumor Initiating Cells Dominate in N29 and N32 Experimental Gliomas

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The present study was designed to critically evaluate the notion that cancer stem cell (CSC)-like cells constitute a sub-population of cells within experimental gliomas. Virtually all cells within the N29 and N32 rat glioma models homogenously expressed the putative CSC marker CD133, the stem/progenitor marker nestin as well as the neural lineage markers glial fibrillary acidic protein, βIII-tubulin, and CNPase *in vitro*. The phenotype was largely retained upon exposure to conditions promoting differentiation *in vitro* and after intracranial implantation of tumor cells into syngeneic hosts. Unsorted adherently grown cells displayed very high clonogenicity *in vitro* and robust tumorigenicity *in vivo*. Single N29 and N32 tumor cells invariably formed clones *in vitro*, and intracerebral inoculation of as few as 10 adherently growing N29 and N32 tumor cells, respectively, gave rise to a tumor. These results provide an alternative view on CSC-like cells in glioma models: sphere-formation is not a prerequisite for accumulation of tumorigenic cells, and CSCs do not reside within a rare sub-population of cells in these glioma models. N29 and N32 gliomas may be used for the development of treatment strategies directed specifically against a practically pure population of brain tumor-initiating CSC-like cells.

Keywords: cancer stem cell, glioma, rat, tumor
Abbreviations: AP, anterior-posterior; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; CC, corpus callosum, CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; CSC, cancer stem cell; DV, dorso-ventral; ECL, ependymal cell layer; EGF, epidermal growth factor; ENU, ethylnitrosourea; FBS, fetal bovine serum; GBM, glioblastoma multiformae; GFAP, glial fibrillary acidic protein; LV, lateral ventricle; ML, medial-lateral; NSC, neural stem cell; RECA, rat endothelial cell antigen, SCM, stem cell medium; SVZ, subventricular zone

It has been proposed that glioblastoma multiforme (GBM) in humans contain a subset of cells, so called cancer stem cells (CSCs), which are responsible for maintenance of the entire tumor[1, 2]. Although experimental findings suggest the existence of CSCs within human GBM, this hypothesis is not proven[3-5]. Alternatively, all or the majority of cancer cells might maintain brain tumor growth[6]. If the CSC hypothesis is correct, it implies that successful tumor treatment can only be achieved if this cell population is eliminated[5]. This is a therapeutic challenge since putative CSCs seem to be more resistant to conventional treatment strategies such as radiotherapy and chemotherapy, compared to the majority of the GBM cells[7-10].

CSCs within experimental glioma models have recently been investigated. Support for the idea that

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CSCs reside within a small fraction of cells were found in the rat 9L gliosarcoma and the mouse GL261 glioma models. Formation of free-floating tumor cell spheres was used as a selection tool to enrich cells with CSC properties in experimental gliomas[11, 12]. In the 9L cell line, sphere-forming cells expressed stem cell markers nestin and Sox2 and displayed increased tumor growth *in vivo*, but slower growth *in vitro*, as compared to 9L cells grown as monolayers. Sphere-generated 9L cells also seemed to be more chemoresistant and exhibited increased expression of anti-apoptosis and drug resistance related genes[11]. Mouse GL261 glioma cells grown as spheres displayed increased protein expression of nestin and expression of radial glia related genes as compared to adherently growing GL261 cells. Sphere-forming cells were capable of clone formation and generated larger tumors *in vivo*[12]. However, contradictory results were found in the C6 rat glioma model where most cells were found to possess CSC-like features[13].

We chose to address the issue of CSC-like cells in experimental glioma models using two rat brain tumor models, N29 and N32, previously induced by *in utero* transplacental ethyl-nitrosourea (ENU) administration[14]. The N29 glioma model mimics the histological appearance of human GBM and exhibits an invasive growth pattern, large areas of necrosis and neovascularization. The N32 tumor is highly proliferative and displays a more circumscribed growth pattern. Both N29 and N32 glioma cells are syngeneic to the host when implanted into Fischer344 rats. The tumor cells are therefore non or only very weakly immunogenic following implantation *in vivo* and the models can be used for the development of e.g. glioma immunotherapy[15, 16]. Here, we characterized the expression profile of CD133, nestin and a selection of neural lineage markers *in vitro* and *in vivo*, clonogenic capacity *in vitro* and *in vivo* tumorigenicity of these glioma models. With respect to these parameters, our study provides striking alternative findings compared to other experimental glioma models.

MATERIALS AND METHODS

Tumor cell lines and culture

The rat glioma cell lines N29 and N32, syngeneic with the Fischer344 rat were originally induced by transplacental injection of ENU to pregnant rats whose offsprings developed malignant brain tumors[14]. The cells were maintained in R10 medium, consisting of: RPMI 1640 medium (1X) with L-glutamine supplemented with 10% fetal bovine serum (FBS) (VWR, West Chester, PA, USA), 10 mM HEPES buffer solution, 1 mM sodium pyruvate and 50 μg/ml gentamicin (all chemicals except FBS from GIBCO-Invitrogen, Carlsbad, CA, USA).

Cells were thawed and transferred to cold R10 medium, spun down and re-suspended in 5 ml R10 medium and transferred to a T25 flask. Cultured cells were washed with phosphate buffered saline (PBS) and detached using trypsin-EDTA (0.25% trypsin with EDTA) 1X (GIBCO-Invitrogen), re-suspended in R10 medium and transferred to a new flask at every other passage. R10 medium was changed every 2-3 days and passaged about twice a week when kept in culture. Cells were incubated at 37°C in a humidified atmosphere containing 6.0% CO_2. Before inoculation *in vivo* the cells were washed and re-suspended in medium without FBS and gentamicin (referred to as R0 medium).

Tumor sphere culture

The N29 and N32 cells were detached from monolayer cultures in R10, washed two times in R0 and re-suspended in stem cell medium (SCM) for sphere culture. The SCM consists of DMEM, F-12, B27, 10 mM HEPES, Penicillin-Streptomycin (GIBCO-Invitrogen), NaHCO3 7.5%, (Sigma-Aldrich, Saint Louis, Missouri, USA), basic fibroblast growth factor (bFGF) 10 ng/ml, and epidermal growth factor (EGF) 20 ng/ml (R&D Systems, Minneapolis, M.N, USA). Fresh medium was added to the cultures every second day, and spheres were mechanically passaged every fifth day.

Subventricular zone neurosphere culture

Brains of male Fischer 344 rats (8 weeks old) were dissected, placed into ice-cold Pipes-buffer and kept on ice. The subventricular zone (SVZ) was dissected under sterile conditions and pieces collected in ice-cold Pipes-buffer. Tissue pieces were enzymatically digested in papain solution for 30 minutes at 37°C. The supernatant was carefully removed and the tissue resuspended in 1 ml control media containing DMEM/F12 (GIBCO-Invitrogen), 30% glucose, 1 M HEPES, N2 and penicillin/

streptomycin and 1 mg/ml DNAse, mechanically triturated and spun for 10 minutes at 1000 RPM and then resuspended in 1 ml proliferation media. Proliferation medium contains control medium, bovine serum albumin, heparin solution and 10 ng/ml bFGF and 20 ng/ml EGF.

Cells were counted using a Bürker chamber and plated at 100 000/ml on uncoated T25 flasks in proliferation medium and incubated at 37°C and 5.0% CO_2. Cells were grown as neurospheres and new growth factors were added every other day. Spheres were collected with media, allowed to settle, the supernatant was aspirated and 1 ml fresh medium was added. Cells were mechanically triturated, counted and plated at desired density in T25 flasks for further studies.

Differentiation assay of spheres

Spheres obtained from culturing in SCM were seeded into multi-chamber culturing slides (BD Pharmingen, Franklin Lakes, N.J, USA) in either SCM devoid of mitogens with the addition of 1.5% FBS or R10. After 4-5 days the cells were stained using immunocytochemistry.

Immunocytochemistry

Tumor cells and SVZ derived cells were grown for 4-5 days in multi-chamber culture slides (BD Pharmingen) before staining. Cells were fixed in 4% paraformaldehyde for 30 minutes, and permeabilized using 0.3% Triton X-100 for 5 min. The cells were blocked with 5% goat serum (Jackson ImmunoResearch Laboratories Inc., West Grove, P.A, USA) for 20 minutes and incubated with the primary antibodies; rabbit polyclonal anti-CD133, 10μg/ml, (Abcam, Cambridge, U.K), rabbit polyclonal anti-nestin, 5μg/ml, (Abcam), mouse monoclonal anti-CNPase, 10μg/ml, (Chemicon, Temecula, C.A. USA), mouse monoclonal anti-glial fibrillary acidic protein (GFAP), 5μg/ml, (BD Pharmingen), mouse monoclonal anti-ß III-tubulin, 5μg/ml, (Sigma-Aldrich, Saint-Louis, Missouri, USA), mouse anti-rat TCR$\alpha\beta$, 5μg/ml (BD Pharmingen), and mouse anti-rat endothelial cell antigen (RECA), MCA970R, 5 μg/ml, (Serotec, Oxford, UK) for 2.5 hours at 37°C. Cells were incubated for 30 minutes at 37°C with the secondary antibodies; goat anti-mouse or goat-anti rabbit conjugated to Alexa 488 or Alexa 594, 5μg/ml, (Molecular Probes, Eugene, Oregon, USA). The chamber-slides were mounted wet using Pro-Long Gold anti-fading reagent, (Molecular Probes), with nuclear staining (DAPI). PBS was used in all washing steps as well as a diluent for reagents. As negative control, the primary antibody was omitted.

Flow cytometry

Adherent N29 and N32 cells were detached and at least 400.000 cells were used for flow cytometric analysis. Cells were fixed and permeabilized using a BD Cytifix/Cytoperm™-kit (BD Pharmingen) according to manufacturers instruction before being stained with the same primary primary antibodies as stated above. Cells were washed twice in BD Perm/Wash™ Buffer before the secondary antibodies (goat-anti rabbit-FITC, Jackson ImmunoResearch Laboratories Inc., USA) was added for 30 minutes at 4°C. Goat serum (5%) (Jackson ImmunoResearch Laboratories Inc.) was included in all steps to avoid unspecific binding of the secondary antibody. As negative control, the primary antibody was omitted. Fluorescence was measured on a FACSCalibur flow cytometer (BD, Heidelberg, Germany) and analysis of the data was performed using CellQuest software (BD).

Single-cell cloning

The N29 and N32 cells were grown in R10 until semi confluent. Cells were then detached, counted and diluted to achieve the concentrations of 0.5 cell, 1 cell and 5 cells respectively in 200 μl R10 per well in 96-well plates. Cell suspensions were counted using a Bürker chamber at high magnification before seeding into 96-well plates and no cell aggregates were visible, only cells. Each well in the 96-well plate dish was examined microscopically to validate the presence of single cells. The cells were incubated for 22 days in 37°C in a humidified atmosphere containing 6.0% CO_2 in order for colonies to form. Single-cell cloning was done twice.

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, Sweden. Animals were anaesthetized with Isoflurane (2.5% in O_2, Forene, Abbott Scan-

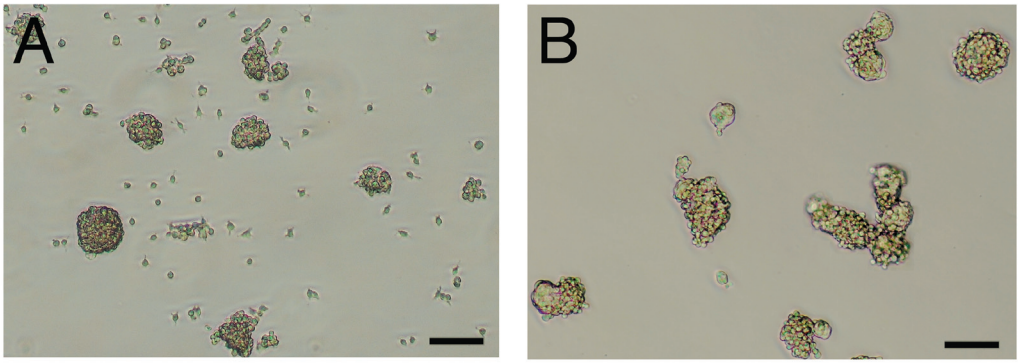


Figure 1. *N29 and N32 tumor cells exhibit sphere formation.*
Tumor spheres were obtained by culturing the N29 (A) and the N32 (B) rat glioma cells in SCM. Spheres were visible after 4-5 days in culture and could be mechanically dissociated into single cells and passaged for at least five times. Scale bar is 100μm.

dinavia AB, Solna, Sweden) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, USA). The following coordinates were used for tumor cell inoculation, (relative to the bregma): anterior-posterior (AP): +1.7, medial-lateral (ML) -2.5 and dorso-ventral (DV) -5.0. The tooth bar was set at -3.3 mm. Either 10, 100, or 1000 N29 tumor cells or 10, 100 or 1000 N32 tumor cells in 3 μl medium were inoculated at 0.5 μl/min, using a 10 μl Hamilton syringe. After tumor cell inoculation, the needle was kept in place for 5 minutes before being slowly retracted. Animals were observed for a minimum of 28 days and then sacrificed.

Immunohistochemistry

Brains were removed and fixed for two minutes in Isopentane at a temperature of -50˚C. Brains were then kept in -80˚C until coronal sectioning on a freezing cryotome was performed. Six μm thick sections were cut and mounted directly onto glass slides. To investigate presence of tumor, every third section was stained with Mayer's Hematoxylin (DakoCytomation, Glostrup, Denmark) and examined using light microscopy. For immuno-histochemical staining of tumors, sections were fixed in room-tempered acetone for 5 minutes and rinsed in PBS. To permeabilize the tissue, PBS containing 0.1% saponin (Riedel-de Haen, Seelze, Germany) was used as washing buffer and diluent in all steps. The cells were blocked with 5% goat serum (Jackson ImmunoResearch Laboratories Inc.) for 20 minutes and incubated with the same primary and secondary antibodies as stated above for 60 minutes in room temperature (RT). Sections were then washed and incubated for 30 minutes in RT with the secondary antibodies. The slides were mounted wet using ProLong Gold anti-fading reagent, (Molecular Probes), with nuclear staining (DAPI). As negative control, the primary antibody was omitted.

Digital capturing of stained cells

Stained cells and tissues were analyzed by the use of a light microscope (BX-60, Olympus America Inc., Melville NY, USA), equipped with a mercury lamp and filters for fluorescence (U-MWG, U-MWB, U-MWU, Olympus). Images were taken in 10× or 40× magnification for tissue sections and 20× magnification for stained cells using an Olympus Color View digital camera and captured using the analySIS® Image analysis software (Olympus).

RESULTS

The N29 and N32 rat glioma cells form free-floating tumor spheres

Cancer stem cell-like cells possess the capacity to form free-floating tumor cell spheres when the cells are grown in specialized serum free medium with the addition of the growth factors bFGF and EGF. To determine if N29 and N32 glioma cells formed spheres, cells were cultured in stem cell medium (SCM, see Materials and Methods). After 4 days, both cell types had formed free-floating

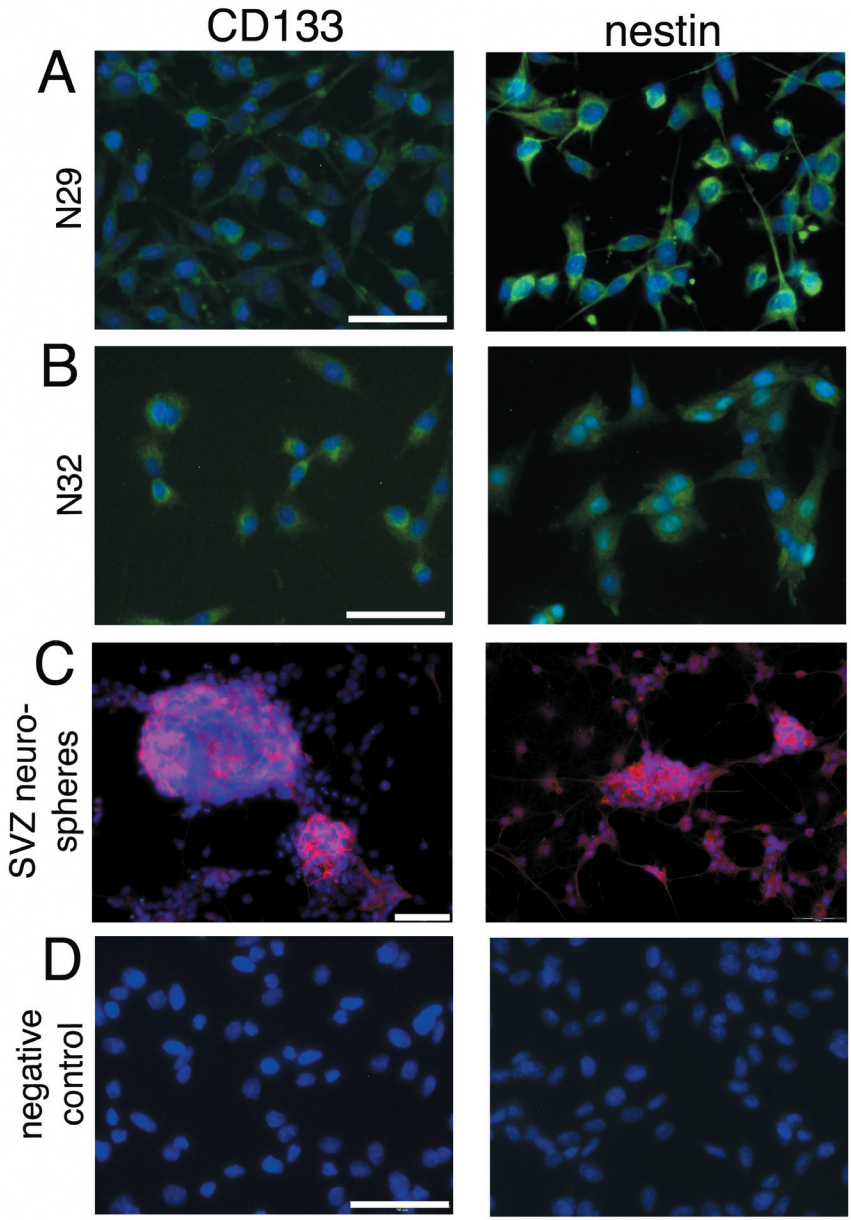


Figure 2. *N29 and N32 tumor cells express stem/progenitor cell markers.*
Expression of the putative cancer stem cell marker CD133 (A, B, green, C, red) and the neural stem/progenitor marker nestin (A, B, green and C, red) was determined using immunocytochemistry. SVZ-derived neurospheres, obtained from normal rat brain, were used for comparison (C, red). As negative control, the primary antibody was omitted (D). Cells were counterstained with DAPI (A-D, blue). All images were taken in 20× magnification. Scale bar is 50μm.

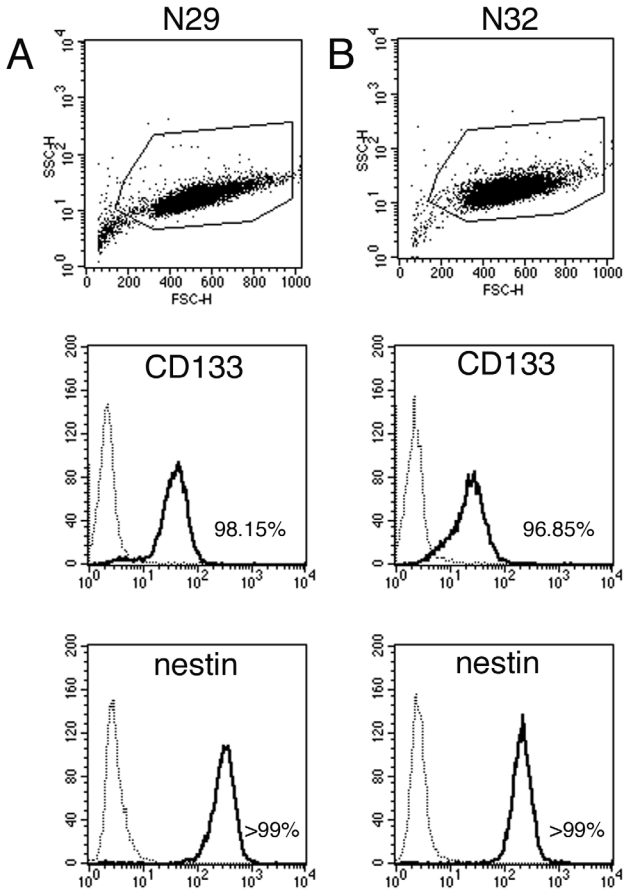


Figure 3. *The majority of N29 and N32 tumor cells express CD133 and nestin.*
Flow cytometric analysis was used to quantitatively assess CD133 and nestin staining of adherent N29 and N32 cells. More than 96% of the N29 and N32 cells stained positive for CD133 (Fig. 3A, B, peak to the right). More than 99% of N29 as well as of N32 cells stained positive for nestin (Fig. 3A, B, peak to the right).

tumor spheres (Fig. 1). Due to a slower proliferation rate of N29 compared to N32, a few more days were required for all N29 cells to form spheres. The spheres could be dissociated mechanically and passaged for at least five times. The N29 and N32 cells grow as an adherent layer when kept in cell culturing medium containing 10% FBS (R10, see Materials and Methods). Interestingly, both N29 and N32 glioma cells consistently form free-floating spheres following extensive confluent growth in R10 medium. Thus, both N29 and N32 rat glioma cells exhibit sphere-formation capacity and this was independent of the growth conditions tested.

N29 and N32 tumor cells co-express stem/progenitor cell markers

The expression profile of the putative cancer stem cell marker CD133 and neural stem/progenitor marker nestin *in vitro* was analyzed. Both tumor cell lines exhibited a homogenous staining of CD133 and nestin (Fig. 2A, B). N29 cells clearly displayed a stronger immunopositivity of nestin compared to N32 cells, while the intensity of CD133 was similar between the two tumor types. There was no difference in the staining intensity or in the number of stained cells between tumor spheres and the adherently growing cells, therefore only the adherent cells are shown in figure 2. Normal SVZ-derived NSCs grown as neurospheres were used as positive control for CD133 and nestin staining. Normal SVZ-derived neurospheres contained a heterogenous population of cells that were either clearly labeled by CD133 and nestin or devoid of labeling.

Flow cytometric analysis was performed to verify and quantitatively assess CD133 and nestin expression of N29 and N32 cells from adherent cultures. The absolute majority (>96%) of the N29 and the N32 cells were positive for CD133 and nestin with a higher mean fluorescence intensity of nestin as compared to CD133 (Fig. 3 A, B).

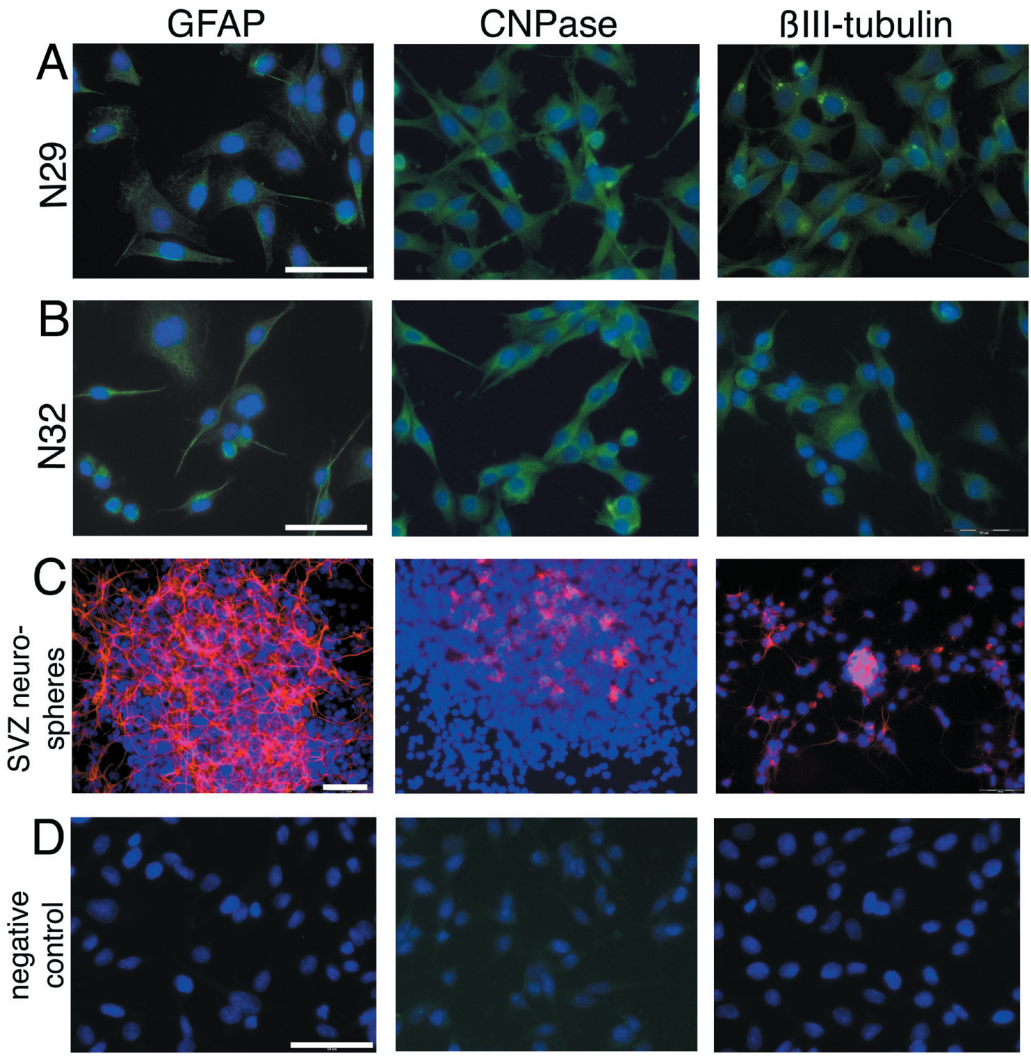


Figure 4. *N29 and N32 tumor cells express neural lineage markers*.
Immunocytochemistry was used to determine the expression of the astrocyte marker glial fibrillary acidic protein (GFAP) (A, B, green and C, red), the oligodendrocyte marker CNPase (A, B, green and C, red) and the neuronal marker ßIII-tubulin (A, B, green and C, red). For comparison, SVZ-derived neurospheres obtained from normal rat brain were used (C, red). As negative control the primary antibody was omitted (D). Cells were counterstained with DAPI (A-D, blue). All images were taken in 20× magnification. Scale bar is 50μm.

N29 and N32 tumor cells express neural lineage markers

Next, we assessed the immunolabeling profile of different neural lineage markers *in vitro*; the astrocytic marker GFAP, the oligodendrocyte marker CNPase and the neuronal marker ßIII-tubulin. Both tumor types were immunopositive for CNPase and ßIII-tubulin (Fig. 4A, B). Both N29 and N32 cells also displayed weak labeling of GFAP (Fig. 4A, B) as compared to SVZ cells (Fig. 4C). The staining intensity was generally distributed homogenously

on all tumor cells. Within SVZ spheres, cells with different levels of staining of the markers were seen. SVZ-derived cells that displayed a strong immunopositivity within the sphere had a characteristic morphology, for example cells positive for CNPase had a typical oligodendrocytic appearance and the morphology of ßIII-tubulin positive cells resembled neuronal cells with long processes (Fig. 4C). There was no difference in the staining intensity or in the number of stained cells between tumor spheres and the adherently growing cells, therefore only the adherent cells are shown in figure 4. In contrast to the broad expression pattern of neural stem- and lineage markers, N29 and N32 glioma cells did not express the endothelial cell marker RECA or the T lymphocyte marker TCRαβ (data not shown).

In conclusion, N29 and N32 glioma cells co-express both stem/progenitor and neural lineage markers and the expression is homogenously distributed on all cells. There was no difference in the expression pattern between tumor spheres or adherently growing tumor cells.

N29 and N32 glioma cells retain their phenotype upon exposure to conditions promoting differentiation in vitro

Multipotentiality of neural stem cells (NSCs) implies that the cells have the capacity to differentiate into astrocytes, neurons and oligodendrocytes. Differentiation capacity can be confirmed by exposing stem cells to growth conditions deprived of the growth factors bFGF and EGF and with addition of FBS. It has been suggested that CSC-like cells also have the capacity to differentiate into cells of multiple lineages when subjected to differentiation conditions[11]. We subjected N29 and N32 tumor spheres to various differentiation conditions and compared the staining profile of the putative cancer stem cell marker CD133, as well as the stem/progenitor cell marker nestin with the adherently growing tumor cells as well as to differentiated neurospheres from SVZ.

Neither N29 nor N32 glioma cells, grown adherently or as tumor spheres, changed their phenotype or morphology when subjected to differentiation conditions. In contrast, SVZ-derived neurospheres contained cells with different levels of immunolabeling, when subjected to differentiation conditions. Cells that migrated from the SVZ neurospheres, and hence had started to differentiate, displayed a weaker staining (Fig. 2C). Thus, both N29 and N32 tumor cells retained their phenotype when subjected to various conditions promoting differentiation *in vitro*.

N29 and N32 glioma cells express stem- and lineage markers in vivo

The marker expression profile of N29 and N32 tumors *in vivo* was investigated. For this purpose, 1000 tumor cells of either cell type were inoculated intracranially and animals were sacrificed on day 28 following tumor cell inoculation. At the time of sacrifice, tumors had grown to a considerable size, occupying a large part of the striatum. Immunohistochemistry of tissue sections demonstrated clear CD133 expression within N29 and N32 tumors (Fig. 5A, B). CD133-positive ependymal cells along the lateral ventricles were used as positive control[17] (Fig. 5C). In contrast, normal neural tissue was negative for CD133. Nestin immunolabeling was more intense in N29 compared to N32 tumors (Fig. 5A, B). Endogenous nestin positive cells were also present in the SVZ of the normal brain (Fig. 5C). Weak expression of GFAP, CNPase and ßIII-tubulin was found within both N29 and N32 tumors (Fig.6A, B). Endogenous expression of GFAP, CNPase and ßIII-tubulin was found within the SVZ, corpus callosum (CC) and striatum respectively (Fig. 6C).

The N29 and N32 rat glioma cells are clonogenic in vitro

To investigate the glioma cells capacity of clonogenicity *in vitro*, a single-cell cloning assay was performed. Unsorted N29 and N32 cells were counted and diluted to achieve concentrations of 0.5, 1 as well as 5 cells in 200 μl per well in 96-well plates. Colony formation was calculated. After 22 days, the percentage of N29 colony formation was 33% for the concentration of 0.5 cells, 55% for the concentration of 1 cell and for 5 cells 94%, and N32 colony formation was 30% for 0.5 cells, 54% for the concentration of 1 cell and for 5 cells 99% (Table 1).

The N29 and N32 rat glioma cells display tumorigenic properties in vivo

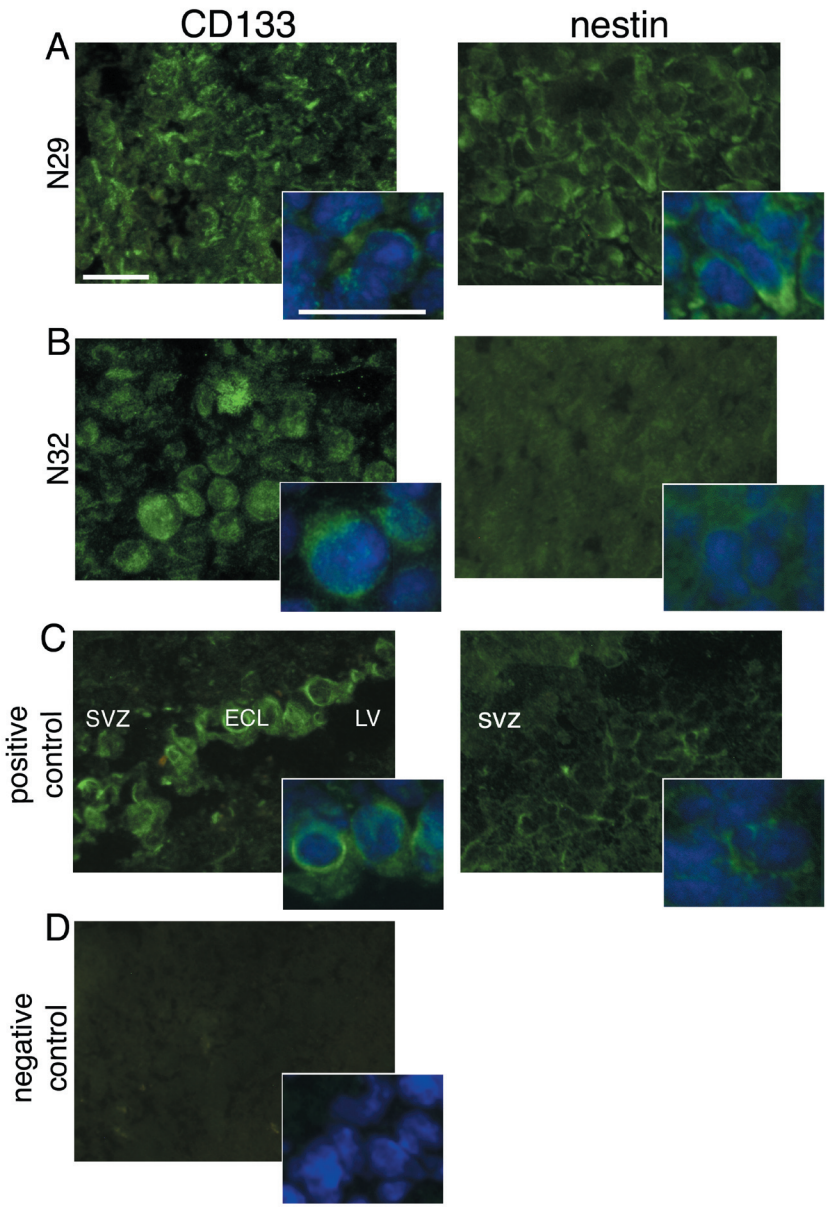


Figure 5. *Expression of CD133 and nestin is retained in intra-cerebral N29 and N32 tumors.*
Immunohistochemistry was used to determine the expression of CD133 and nestin in intra-cerebral N29 (A) and N32 (B) tumors. CD133-expressing cells in the ependymal cell layer (ECL) and nestin-expressing cells in the subventricular zone (SVZ) served as positive controls (LV, lateral ventricle) (C). As negative control the primary antibody was omitted (D). Cells were counterstained with DAPI (inserted figures A-D). All images were taken in 40× magnification. Scale bar is 20μm.

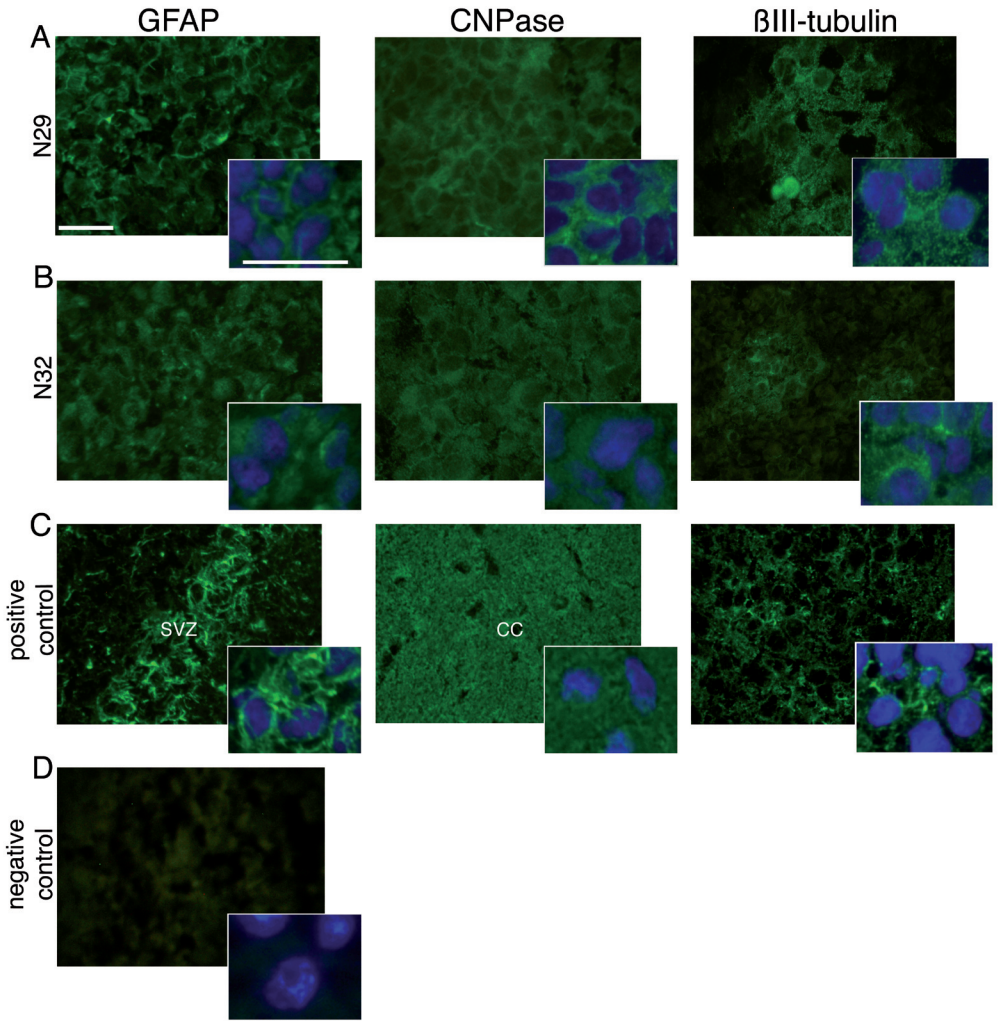


Figure 6. *Expression of neural lineage markers is retained in N29 and N32 intra-cerebral tumors.*
Immunohistochemistry was used to determine the expression of GFAP, CNPase and βIII-tubulin in intra-cerebral N29 (A) and N32 (B) tumors. Cells within the SVZ, the corpus callosum (CC) and normal brain tissue were used as positive controls (C). As negative control the primary antibody was omitted (D). Cells were counterstained with DAPI (inserted figures A-D). All images were taken in 40× magnification. Scale bar is 20μm.

In vivo tumorigenicity of unsorted N29 and N32 tumor cells were determined by stereotactic intra-cranial inoculation of 10, 100 or 1000 N29 and N32 cells, respectively. The percentage of established N29 tumors following inoculation of 1000 cells was 100% (n=2), 100 cells: 78% (n=9) and 10 cells: 75% (n=4). For N32 tumors, the corresponding percentage of established tumors was for 1000 cells: 100% (n=2), 100 cells: 25% (n=8) and for 10 cells: 25% (n=4), (Fig. 7A-C).

DISCUSSION

The present study was designed in order to challenge the current iteration of the CSC hypothesis that purports the existence of a low frequency of CSC-like cells in experimental glioma models. Both N29 and N32 tumor models gave rise to free-floating tumor spheres when cultured in the presence of bFGF and EGF in serum-free medium. However, sphere-formation was not necessary to enrich for tumorigenic cells. Both tumor types showed a homogenous and uniform immunostaining for the putative CSC marker CD133, as well as the stem/progenitor cell marker nestin *in vitro*. Immunopositivity for the neural lineage markers GFAP (astrocyte and putative NSC marker), ßIII-tubulin (neuronal marker) and CNPase (oligodendrocyte marker) was also found in both tumor types concomitantly with the expression of CD133 and nestin. Interestingly, neither N29 nor N32 tumor cells changed their phenotype upon exposure to differentiation conditions in culture. The tumors largely retained the expression of CD133, nestin and neural lineage markers following intracranial implantation of tumor cells into syngeneic hosts. Single unsorted N29 and N32 tumor cells formed clones *in vitro*, and intracerebral inoculation of as few as 10 N29 and N32 tumor cells, respectively, resulted in tumor growth. Thus, in contrast to most previous findings in other experimental glioma models, N29 and N32 tumors are composed of a homogenous population of tumorigenic CSC-like cells.

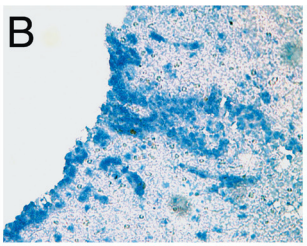
Table 1. *Colony formation of N29 and N32 tumor cells following single-cell cloning.*
N29 and N32 tumor cells were diluted to achieve concentrations of 0.5, 1 and 5 cells per well and distributed in 96-well plates. Colony formation was calculated following 22 days of incubation.

No. of cells	5	1	0.5
N29	94%	55%	33%
N32	99%	54%	30%

The existence of cells with CSC properties in experimental gliomas has recently been addressed. Tumor-initiating cells were claimed to be enriched using sphere formation as a selection tool in the rat 9L model[11] and the mouse GL261 model[12]. We show that when subjected to conditions proposed to promote enrichment of CSC-like cells, both N29 and N32 tumor cultures readily form free-floating spheres. However, when adherently growing N29 and N32 cells loose adherence due to overgrowth, spheres are also formed in ordinary cell culture media containing 10% FBS. As discussed by Singec et al.[18], nearly all dividing cells form spheres when grown in serum-free media. Furthermore, the commonly used neurosphere assay, which has been used as a tool to isolate and expand NSCs and CSCs, has clear limitations. For example, it has been shown that the majority of cells within a neurosphere

A

Number of tumor cells inoculated	N29 tumor growth	N32 tumor growth
10	75%	25%
100	78%	25%
1000	100%	100%



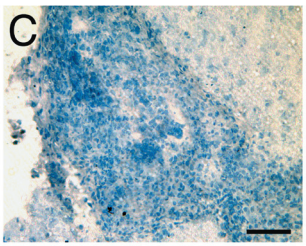


Figure 7. *In vivo tumorigenicity of low numbers of N29 and N32 glioma cells.*
Table showing the percentage of established intracerebral tumors following inoculation of 10, 100 or 1000 cells of N29 and N32, respectively (A). N29 tumor growth in the cortex, 42 days following inoculation of 10 N29 tumor cells. N29 tumors display a disseminated growth pattern with invasive tumor extensions and distant tumor microsatellites separated from the main tumor mass (B). N32 tumor growth in the cortex, 35 days following inoculation of 10 N32 tumor cells (C). N32 tumor displays a more circumscribed growth pattern compared to the N29 tumor, although the growth pace of N32 is higher. Scale bar is 100 μm.

are non-stem cells and that neurospheres readily fuse, hence making it difficult to assay the sphere-forming capacity of individual cells unless single cells are plated in separate wells[18, 19]. Considering the abovementioned limitations of *in vitro* sphere formation in assessing CSC properties of tumor cells, we pursued to study tumor cell clonogenicity *in vitro* and *in vivo* tumorigenicity. Single-cell cloning of adherently growing unsorted tumor cells resulted in colony formation from the vast majority of single cells. The clonogenic capacity at single-cell level is in line with findings from Zheng et. al., demonstrating high clonogenicity of rat C6 glioma cells *in vitro*[13]. Furthermore, intracerebral inoculation of as few as 10 N29 and N32 tumor cells grown adherently, respectively, gave rise to intracerebral tumors. In light of the clonogenic capacity of single cells and the robust tumorigenic ability of very low numbers of adherently growing N29 and N32 tumor cells, we suggest that sphere-forming capacity is not necessary to determine presence and clonogenic potential of CSC-like cells in experimental gliomas. We propose that *in vivo* tumorigenicity constitutes a relevant assay to identify CSC-like cells in experimental glioma models.

The N29 and the N32 tumor cells were found to express the putative CSC marker CD133 as well as the stem/progenitor cell marker nestin. CD133 has been suggested to specifically label human CSCs, however CD133-negative cells with CSC characteristics have also been derived from human GBM[1, 2, 20, 21]. The importance of CD133 expression in the initiation and maintenance of brain tumors is thus not clear. Nestin is expressed by NSCs[22] and is also found in human malignant brain tumors, predominantly in high-grade gliomas (including GBM)[23-25], and a correlation between nestin expression within tumor and patient survival has been shown[25].

In the serum-cultured 9L and GL261 glioma cell lines, sphere-derived cells were reported to differentiate upon growth in differentiation conditions in vitro[11, 12]. In contrast, Lee et al. has demonstrated that serum-culturing of glioblastoma cell lines causes irreversible loss of differentiation capacity[26]. In line with this, we found no noticeable change in tumor cell phenotype of serum-cultured N29 and N32 glioma cells after growth in differentiation conditions *in vitro* or following intracranial implantation of tumor cells. The neural lineage markers GFAP, ßIII-tubulin and CNPase, which also are expressed in human glial tumors and human glioma cell lines[27, 28], were found to co-exist with the stem/progenitor cell markers within the same tumor cells. The immunolabeling profile of N29 and N32 glioma cells showed marked difference as compared to the labeling pattern of non-malignant neural cells. For instance, CD133 and ßIII -tubulin are not co-expressed in the normal brain. However, ßIII-tubulin is expressd in many types of human non-neural cancers including breast cancer, lung cancer and ovarian cancer[29-31]. The expression of ßIII-tubulin in N29 and N32 glioma cells does therefore not necessarily imply a neuronal phenotype of these cells. We therefore avoid far-drawn conclusions concerning the nature of the N29 and N32 glioma cells (such as naming these cells "cancer stem cells") purely based on marker protein expression profile. However, proteins expressed in GBM and associated with high-grade malignancy and tumor cell migration[23-25] are also expressed by the N29 and N32 rat glioma cells.

In brief, N29 and N32 rat tumor cells grow as tumor spheres, express GBM associated stem/progenitor cell markers, are clonogenic *in vitro* and highly tumorigenic *in vivo*. Our results indicate that neither sphere growth nor expression profile of stem/progenitor cell markers per se define CSC-like cells in experimental gliomas. CSC-characteristics in experimental gliomas should rather be based on functional properties such as clonogenic capacity *in vitro* and *in vivo* tumorigenicity. While it is not clear whether CSCs exist in human brain tumors[3, 6], the N29 and N32 glioma models consist of homogenous populations of clonogenic and tumorigenic cells. Experimental therapy using these glioma models is thus directed against a practically pure population of tumorigenic glioma cells. It has been shown that serum-culturing of glioblastoma cells favors the development of a clone of tumor cells and not a heterogenous population[26]. This could explain our findings of a homogenous population of tumor cells in the N29 and N32 glioma models. However, it also raises questions on how a fraction of CSC-like cells can persist in the 9L and GL261 glioma models despite years of culturing in serum-containing medium. The lack of heterogeneity within these glioma models needs to be recognized and may complicate for prediction of therapeutic

efficacy of certain experimental treatment modalities like gene therapy of specific GBM-associated gene defects. The models may be more useful for studies on general GBM features such as neovascularization and immunosuppression, and for the development of anti-angiogenic therapy and immunotherapy.

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CHARACTERIZATION OF THE SUBVENTRICULAR ZONE NEUROGENIC RESPONSE TO RAT MALIGNANT BRAIN TUMORS

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CHARACTERIZATION OF THE SUBVENTRICULAR ZONE NEUROGENIC RESPONSE TO RAT MALIGNANT BRAIN TUMORS

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Abstract—The subventricular zone (SVZ) is one of the neurogenic regions of the adult brain. We characterized the neurogenic response of the SVZ to the growth of brain tumors in the rat striatum. Abundant nestin positive cells, most likely representing reactive astrocytes, were found surrounding the tumor. However, we observed no substantial migration of nestin positive cells from the SVZ toward the tumor. Tumor growth resulted in *decreased* numbers of bromodeoxyuridine positive and Ki-67 positive proliferating cells and a concomitant *increase* in doublecortin and polysialylated neural cell adhesion molecule immunoreactivity within the SVZ. Neuroblasts were observed in high numbers in the area between the SVZ and the tumor, most likely pointing to the SVZ as the principal source of these cells. Neuroblasts located between the SVZ and the tumor expressed the transcription factor Pbx, a marker for immature striatal neurons. However, no evidence of neuroblast differentiation into fully mature neurons was found. This study thus demonstrates increased neuroblast immunoreactivity within the SVZ ipsilateral to a brain tumor in the striatum. SVZ-derived neuroblasts attracted by the tumor adopt an immature striatal phenotype indicating a region specific reparative mechanism in response to a malignant tumor.

Key words: brain tumor, neuroblast, doublecortin, glioma, rat, neurogenesis.

Glial tumors within the CNS present challenging clinical and scientific problems. Glioblastoma (WHO grade IV) is the most common and aggressive form of primary brain tumor. Due to the diffuse and invasive growth pattern of this tumor, complete surgical resection is, with few exceptions, impossible. Glioblastoma is also highly resistant to radiotherapy and chemotherapy and the mean survival time for patients with this disease is therefore only about 12 months with the currently offered therapy (Salcman, 1996; Surawicz et al., 1998). In the past few years, a number of studies have focused on transplantation of neural (and mesenchymal) stem cells carrying various therapeutic transgenes as experimental therapy for this disease, see e.g. (Aboody et al., 2000; Benedetti et al., 2000; Nakamizo et al., 2005), however, the endogenous neurogenic response of the brain to malignant tumor growth has only recently started to gain attention.

Newly formed neurons, generated in the subventricular zone (SVZ), have a potent migratory capability and potential to replace damaged neurons following lesions of the adult rodent brain (Alvarez-Buylla et al., 2000; Arvidsson et al., 2002). Research in the past years has largely been directed toward clarifying how new neurons from the SVZ are generated in response to brain lesions such as cerebral ischemia, epileptic seizures and mechanical trauma, see e.g. (Hallbergson et al., 2003) for review. Much less is known about the SVZ response to the growth of a malignant brain tumor. Duntsch et al. (2005) found an upregulation of glial progenitors, and Glass et al. (2005) reported an increase of nestin+ cells, of which a fraction co-expressed neuronal markers, in response to experimental brain tumors.

The present paper focuses on the neurogenic response of the SVZ to malignant tumor growth in the striatum of the adult rat and the main objectives of the study were: 1) to correlate proliferation in the SVZ with tumor growth; 2) to analyze SVZ neuroblast marker expression relative to tumor growth; 3) to analyze neuroblast migration toward the tumor at different time points and, finally, 4) to clarify the degree of differentiation of neuroblasts generated in the presence of a tumor.

EXPERIMENTAL PROCEDURES

Cell lines and culture

The rat glioma cell line N32, syngeneic with the Fischer 344 rat and originally induced by ethyl-*N*-nitrosourea *in vivo* as previously described (Siesjo et al., 1993), were kindly provided by Anna Darabi (Glioma Immunotherapy Group, BMC, Lund University, Lund, Sweden). The cells were maintained in R10 medium, consisting of: RPMI 1640 medium (1×) with L-glutamine supplemented with 10% fetal calf serum (FCS) (VWR, Chester, PA, USA), 10 mM Hepes buffer solution, 1 mM sodium pyruvate and 50 μg/ml gentamicin (all chemicals except FCS from GIBCO, Invitrogen, Carlsbad, CA, USA).

Cells were thawed and transferred to 2 ml cold R10 medium, after 5 min another 2 ml R10 medium was added, and finally 5 ml R10 medium was added before the cells were spun down and

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Abbreviations: BrdU, 5-bromodeoxyuridine; DCx, doublecortin; DsRed2, *Discosoma* red fluorescent protein; FCS, fetal calf serum; GFAP, glial fibrillary acidic protein; MOI, multiplicity of infection; NeuN, neuronal nuclei; PBS, phosphate-buffered saline; Pbx, transcription factor; PFA, paraformaldehyde; PSA-NCAM, polysialylated neural cell adhesion molecule; RMS, rostral migratory stream; S.E.M., standard error of the mean; SVZ, subventricular zone; TdT, terminal deoxynucleotidyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labeling; VEGF, vascular endothelial growth factor.

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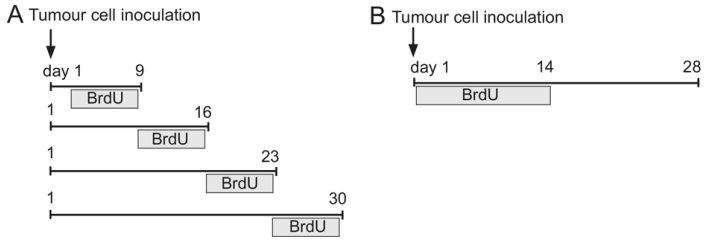

Fig. 1. Experimental design of the time-course study (A) and the study of neuronal differentiation (B). (A) For the time-course study, the animals (n=40) were inoculated with either 500 N32DsRed2 tumor cells (n=4–6 per group) or cell medium (n=4 per group) into the striatum of the right hemisphere on day 1. Animals were perfused at four different time-points (on days 9, 16, 23 and 30) following tumor cell inoculation. Daily i.p. injections of BrdU were administered to all of the animals for 7 days prior to killing. (B) In the experiment for neuronal differentiation, 50 N32DsRed2 tumor cells were inoculated into the striatum on day 1 (n=6). BrdU was given twice daily during days 1–14. Animals were perfused on day 28.

resuspended in 5 ml R10 medium and transferred to a T25 flask. Cultured cells were washed with phosphate-buffered saline (PBS) and detached using trypsin–EDTA (0.25% trypsin with EDTA 4Na) 1× (Invitrogen), resuspended in R10 medium and transferred to a new flask at the second passage. R10 medium was changed every 2–3 days and passaged about twice a week when kept in culture. Cells were never kept in culture for more than 6 weeks. Before inoculation *in vivo* the cells were washed and resuspended in medium without FCS and gentamicin (referred to as R0 medium). Cells were counted and resuspended in a volume corresponding to the target concentration. Cells were incubated at 37 °C in a humidified atmosphere containing 6.0% CO_2.

Discosoma red fluorescent protein (DsRed2)-labeling of N32 tumor cells

At 80% confluency, tumor cells were transduced in R10 medium at different multiplicity of infection (MOI), from MOI=1–10, with a Moloney leukemia-derived virus. Cells were transduced with viruses encoding the DsRed2 under control of the CMV promoter (pCMMP-IRES2Dsred2-WPRE) (kindly provided and performed by Laurent Roybon, courtesy of Professor Patrik Brundin, Neuronal Survival, Wallenberg Neuroscience Center, Lund University, Lund, Sweden). Protamine sulfate was added to the medium to increase the transduction efficiency (4 μg/ml). Cells were incubated at 37 °C in a humidified atmosphere containing 6.0% CO_2. The day after addition of viral vector, tumor cells started to express the fluorescent protein DsRed2. Transduced cells were then purified through single cell cloning. Cells were counted and diluted to obtain concentrations of 5 and 0.5 cells/200 μl R10 medium per well in 96-well plates. An incubation period for 10 days followed in order for colonies to form. DsRed2 positive colonies formed from single cells were washed with PBS, trypsinated, resuspended in R10 medium and collected in a six-well plate. After a few days, when the cells had reached a higher confluence, cells were transferred to a T25 flask in 5 ml R10 medium. The single cell cloning resulted in a DsRed2 expression *in vitro* of 90% as analyzed by FACS.

Surgical procedure and experimental design

Fischer 344 male rats (8–9 weeks) from Scanbur, Stockholm, Sweden were used. Animal procedures were approved by the Ethical Committee for Use of Laboratory Animals at Lund University, Sweden. All experiments conformed to the Helsinki declaration on the ethical use of animals, and every effort was made to minimize the number of animals used and their suffering. Animals were anesthetized with isoflurane (2.5% in O_2, Forene, Abbott Scandinavia AB, Solna, Sweden) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). The following coordinates were used for tumor cell inoculation and R0 cell medium inoculation (control group) into the right striatum, (relative to bregma): anterior–posterior: +2.2, medial–lateral: −2.5 and dorsoventral: −5.0. Tumor cells were inoculated at 1 μl/min, using a 10 μl Hamilton syringe (Hamilton, Reno, NV, USA). After tumor cell inoculation, the needle was kept in place for 5 min before being slowly retracted.

For the time-course study (Fig. 1A), either 500 N32DsRed2 tumor cells in 1 μl R0 medium (tumor group) or 1 μl R0 medium (control group) were inoculated in the right striatum. Rats were killed at different time-points (days 9, 16, 23 and 30) following tumor inoculation. At each time-point, tumor-bearing rats (n=4–6) as well as rats receiving control lesion (n=4) were killed. For the study of neuronal differentiation (Fig. 1B), 50 N32DsRed2 tumor cells in 1 μl R0 medium (only 1 μl R0 medium in control group) were inoculated. The animals were killed on day 28 following tumor inoculation.

5-Bromodeoxyuridine (BrdU) administration

The animals were given BrdU, (i.p. injections, 50 mg/kg, 20 mg/ml, Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS, pH 7.4. In the time-course study, BrdU was injected once a day during the last 7 days before killing. In the experiment for neuronal differentiation, BrdU was given twice daily on days 1–14.

Immunohistochemistry

The rats were deeply anesthetized and perfused through the ascending aorta with PBS, pH 7.4, followed by cold 4% paraformaldehyde (PFA) in PBS. The brains were removed and postfixed in cold 4% PFA overnight and then transferred to 20% sucrose solution in PBS. Coronal sectioning of the brains was performed on a freezing microtome (40 μm) and the sections were transferred to antifreeze solution. Free-floating sections were rinsed three times in PBS. For BrdU immunohistochemistry, sections were denaturated by incubation in 1 M HCl at 65 °C during 30 min. The sections were blocked with either 5% normal donkey serum or 5% normal goat serum in 0.25% Triton X-100 solution. Primary antibodies were mouse anti-rat nestin (1:1000, Chemicon, Temecula, CA, USA), rabbit anti-GFAP (1:1000, DAKO, Glostrup, Denmark), goat anti-doublecortin (DCx) (1:150, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-transcription factor Pbx 1/2/3 (1:300, C-20, Santa Cruz), mouse anti-Ki-67 (1:100, Novocastra, Newcastle upon Tyne, UK), rat anti-BrdU (1:100, Oxford Biotechnology, UK), mouse anti-neuronal nuclei (NeuN) (1:100, Chemicon) and mouse anti–polysialylated neural cell adhesion molecule (PSA-NCAM) (1:500, Chemicon). Sections were incubated with primary antibodies at 4 °C overnight. The next day, sections were rinsed three times in appropriate sera in PBS and

incubated during 1 h with a 1:400 solution of one or two of the following secondary antibodies: Alexa488 donkey anti-goat (Molecular Probes, Eugene, OR, USA), Alexa488 goat anti-mouse (Probes), Alexa594 goat anti-rabbit (Molecular Probes), Cy3 donkey anti-mouse (Jackson Immunoresearch, West Grove, PA, USA), Cy3 donkey anti-rat (Jackson) or Cy5 goat anti-rabbit (Jackson). Sections were counterstained with Hoechst 33342, 10 μg/ml for 5 min (Molecular Probes), mounted onto glass slides and coverslipped with DABCO mounting medium.

In situ detection of fragmented DNA was performed by terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labeling (TUNEL). Free-floating sections were mounted on glass slides and dried. Sections were pretreated with 4% PFA for 20 min, 95% methanol for 30 min, proteinase K (10 μg/ml PBS) for 10 min, 4% PFA for 5 min and 0.1% Triton X-100 in 0.1% sodium citrate for 2 min. Between each step, sections were rinsed in PBS. Sections were incubated at 37 °C for 60 min in terminal deoxynucleotidyl transferase (TdT) buffer, containing 20 μl TdT solution and 150 μl label solution with fluorescein-conjugated dUTP (Roche Diagnostics, Mannheim, Germany). Sections were counterstained with Hoechst 33342, 10 μg/ml for 10 min (Molecular Probes) and coverslipped with DABCO mounting medium.

Microscopic analysis

All sections were examined with an Olympus BX60 epifluorescence microscope using a ×40 objective (Olympus, Tokyo, Japan). Quantification of cell numbers and SVZ and tumor area was performed on three coronal sections per animal and expressed as mean value per section. The first section for quantification was chosen at around +2.2 mm anterior from bregma (at the level of tumor inoculation) and from this section, the eighth rostral and the eighth caudal section were included.

SVZ area immunoreactive for DCx and PSA-NCAM and tumor area were manually delineated and quantified using the image analysis program analySIS (Soft Imaging System). The numbers of Ki-67+ and BrdU+ nuclei within the SVZ and the numbers of DCx+ cells in the striatum and in corpus callosum were manually counted using a ×40 objective. For quantification of nestin+ cells in close proximity to tumor, a 0.2×0.2 mm large grid was placed adjacent to the medial border of the tumor, facing the SVZ. For quantification of nestin+ cells in the area between the SVZ and tumor, the same grid was placed in the center between the SVZ and the medial border of the tumor. The numbers of nestin+ cell bodies within the grid were manually counted in three coronal sections per animal as described above.

Co-localization of DCx and Pbx, and nestin and GFAP, was assessed using confocal laser scanning microscopy (Leica Microsystems, Mannheim, Germany).

Statistical analysis

Student's unpaired *t*-test was used for comparison between groups. Data are presented as means±standard error of the mean (S.E.M.) and data are considered significant at $P<0.05$. Correlation between tumor area and SVZ cell proliferation was assessed using Spearman rank correlation test.

RESULTS

Nestin expression in response to malignant brain tumor growth

Although not invasively growing into the SVZ or the rostral migratory stream (RMS), the N32 malignant tumor occupied the major part of the striatum on day 30 and probably caused mechanical compression of the SVZ at this late stage. We observed nestin immunoreactivity in SVZ cells and in cells surrounding the tumor. Blood vessels in the normal brain and newly formed blood vessels within the tumor were also weakly immunoreactive for nestin and these cells were, based on morphology and microscopical appearance, excluded from our analysis. Abundant nestin+ cells were spread out homogenously around the periphery of the tumor and scattered nestin+ cells were seen infiltrating deep into the tumor at all time points (Fig. 2A and D). The vast majority of the nestin+ cells surrounding the tumor were of stellate morphology, indicating an astrocytic phenotype. Indeed, double-staining for nestin and the astrocyte marker glial fibrillary acidic protein (GFAP) revealed that the absolute majority of the nestin+ cells in proximity to tumor co-labeled with GFAP, demonstrating that these cells were reactive astrocytes (Fig. 2B). Fig. 2C shows the typical stellate morphology of a nestin+ cell located in close proximity to tumor and Fig. 2D demonstrates co-expression of nestin and GFAP by the same cell.

The numbers of nestin expressing cells in close proximity to the medial side of the tumor were highest on day 9 following tumor inoculation. At later time-points, significantly lower numbers of nestin+ cells were observed and these numbers did not differ from the numbers of nestin+ cells found in animals receiving control lesion (Fig. 2E). Thus, increasing tumor growth did not increase nestin expression in proximity to the tumor.

In order to investigate tumor tropism of nestin+ cells from the SVZ, we counted the number of nestin+ cells in the area between the SVZ and the tumor. In contrast to the high numbers of nestin+ cells surrounding the tumor, only single nestin+ cells were found between the SVZ and the tumor at any given time-point. On day 9, when nestin expression peaked in the peritumoral rim, not even a single nestin+ cell was observed between the tumor and the SVZ.

Thus, at any given time-point, there were high numbers of nestin expressing cells in proximity to the tumor, but not in the area between the SVZ and the peritumoral rim of nestin+ cells (Fig. 2E). The animals in the control group also displayed nestin immunoreactive astrocyte-like cells along the needle track. Based on these findings, the vast majority of the nestin+ cells surrounding the tumor were most likely reactive astrocytes, originating from the brain parenchyma rather than from the SVZ.

Cell proliferation and cell death in the SVZ

For quantification of SVZ cell proliferation at the time of perfusion, cells expressing the cell-cycle marker Ki-67 within the SVZ were counted. To estimate cell proliferation during the week prior to killing, daily BrdU injections were given to the animals during 7 days prior to killing and the numbers of BrdU+ cells in the SVZ were counted. Malignant brain tumor growth significantly decreased the number of proliferating cells in the ipsilateral SVZ (Fig. 3), as analyzed both with BrdU (Fig. 3A, B, E, G) and with Ki-67 (Fig. 3C, D, F, H)). The number of BrdU+ cells per section in the SVZ was, on day 30, 64±48 on the ipsilateral side in tumor group as compared with 221±20 in the control

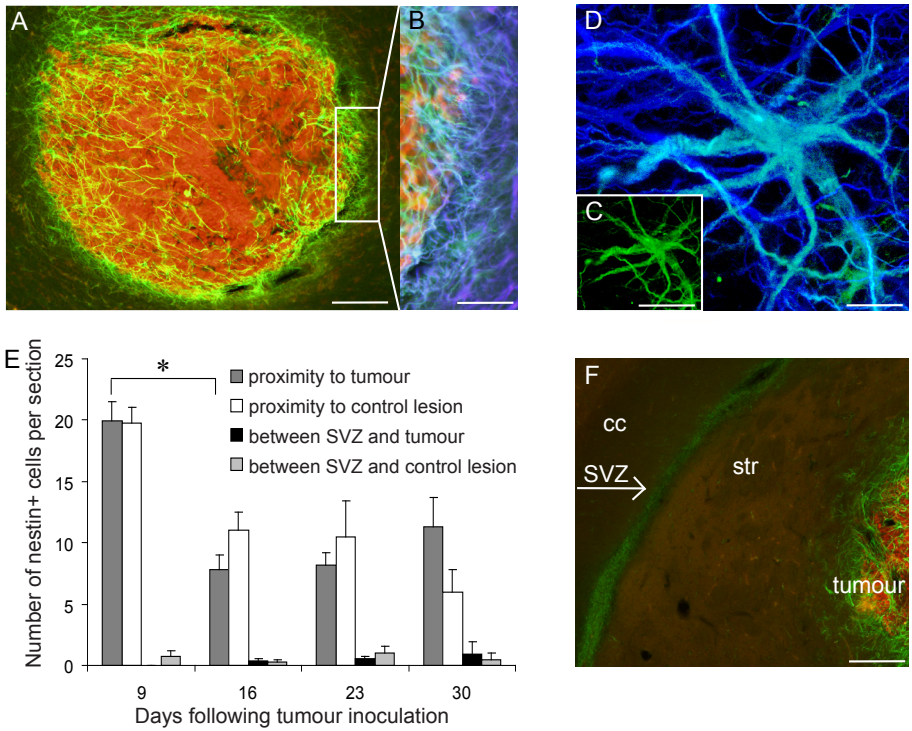

Fig. 2. Nestin expression in response to N32DsRed2 malignant brain tumor growth in the striatum. (A) Nestin+ cells (green) surround and infiltrate N32DsRed2 malignant brain tumor (red) in the striatum on day 23 following tumor inoculation. (B) Magnification of the marked area in A. Double-immunostaining for nestin (green) and GFAP (blue) demonstrates that the vast majority of the nestin+ cells in the peritumoral rim express GFAP. Weak nestin immunoreactivity is also found in tumor blood vessels. (C) High power magnification of a nestin+ cell located in proximity to tumor. (D) Nestin (green) and GFAP (blue) co-labeling of the same cell as shown in C. The stellate morphology and the expression of GFAP strongly speak for an astrocytic phenotype of this cell. The absolute majority of the nestin+ cells in tumor proximity exhibited a similar astrocyte-like morphology. (E) Numbers of nestin+ cells per section in close proximity to tumor, in close proximity to control lesion, in the area between the SVZ and tumor and in the area between the SVZ and control lesion. Note that high numbers of nestin+ cells are present in proximity to tumor and that only single nestin+ cells are found in the area between the SVZ and tumor. (F) Strong nestin immunoreactivity (green) is seen around and within the tumor (red). Weaker nestin immunoreactivity is seen in the SVZ. However, no nestin+ cells with migratory morphology can be found in the area between the SVZ and the peritumoral rim of nestin+ cells on day 23 following tumor inoculation. str, striatum; cc, corpus callosum. Scale bar=200 μm (A, F), 100 μm (B), 40 μm (C) and 15 μm (D).

group, (Fig. 3E; $P<0,05$, $n=4$ in both groups, un-paired *t*-test). The number of Ki-67+ cells in the SVZ was, on the ipsilateral side in the tumor group, 58±42 compared with 239±27 in the control group (Fig. 3F; $P<0,05$, un-paired *t*-test). The Spearman rank correlation test revealed a strong negative correlation between tumor area and the number of proliferating cells in the SVZ (correlation coefficient $r_s=-0.96$ for BrdU and $r_s=-0.95$ for Ki-67, $P<0,001$, Fig. 3G and H). Animals killed on days 23 and 30 were included in the correlation test.

We investigated whether the decrease in SVZ cell proliferation on day 30 was coupled to a change in SVZ cell death. TUNEL detects fragmented DNA in apoptotic cells and was used as a marker for cell death. Massive TUNEL immunoreactivity in the tumor core served as positive control. There was, however, no difference in the numbers of TUNEL+ cells in the SVZ between the tumor and the control group on day 30. The number of TUNEL+ cells (per section) in the SVZ was in the tumor group 2.8±1.2 and in the control group 4.6±1.6.

Increase of DCx and PSA-NCAM immunoreactivity in the SVZ

In the present study, we used DCx and PSA-NCAM immunostaining to detect neuroblasts. DCx, a phosphoprotein associated with microtubuli (Gleeson et al., 1999), has been validated as a specific immunohistochemical marker of newly generated neuroblasts within the CNS (Rao and Shetty, 2004; Couillard-Despres et al., 2005). PSA-NCAM is the polysialylated form of the neural cell adhesion molecule and is expressed by migrating neuronal precursors

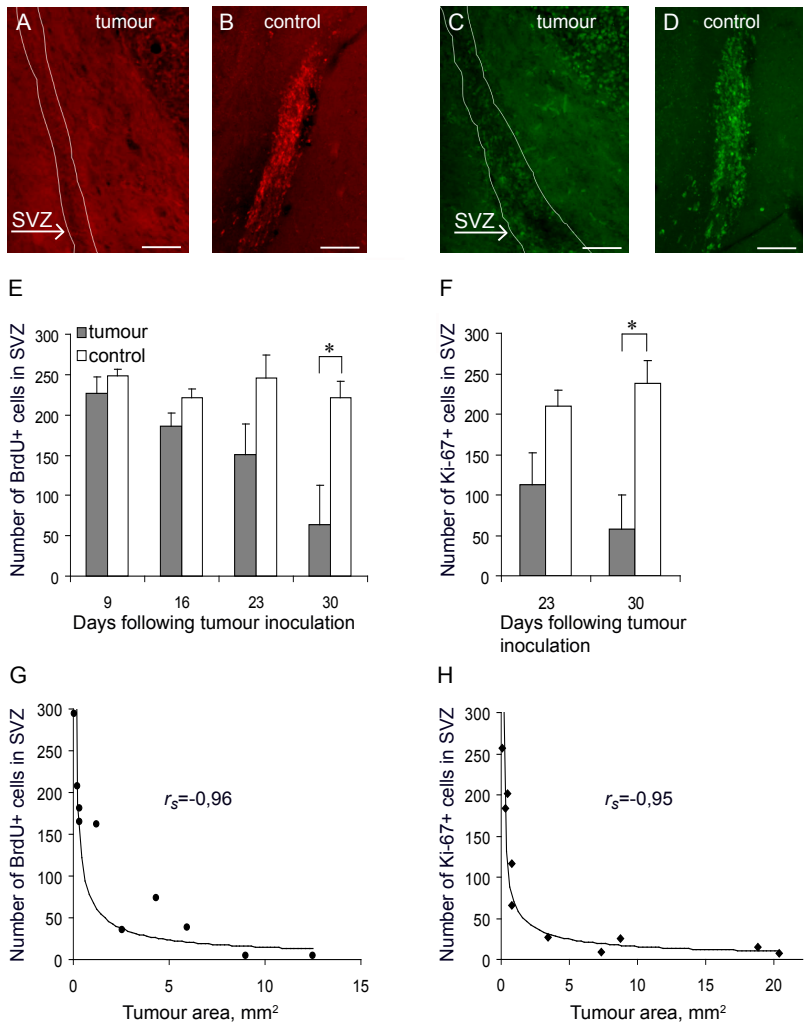

Fig. 3. Decreased cell proliferation in the SVZ in response to N32DsRed2 malignant brain tumor growth in the striatum. SVZ cells immunoreactive for BrdU (A, B) and for Ki-67 (C, D) in the SVZ on day 30. (A) Low numbers of BrdU+ cells (red) in the ipsilateral SVZ in response to tumor growth. (B) High numbers of BrdU+ cells are seen in the SVZ on day 30 following control lesion. (C, D) The numbers of Ki-67+ cells (green) in the SVZ also decreased significantly in tumor-bearing animals compared with controls on day 30. (E, F) Number (means±S.E.M.) of BrdU+ cells and Ki-67+ cells per section in the SVZ on the ipsilateral side in the tumor group compared with the ipsilateral side in animals with control lesion. * $P<0.05$, un-paired *t*-test. (G, H) Spearman rank correlation test was used to assess the correlation between tumor area and the numbers of proliferating cells in the SVZ. Animals killed on days 23 and 30 were included in the correlation test. Spearman rank correlation coefficient revealed a strong negative correlation; for BrdU, $r_s=-0.96$ (G) and for Ki-67, $r_s=-0.95$ (H), $P<0.001$. Scale bar=200 μm.

within the SVZ and the RMS (Doetsch and Alvarez-Buylla, 1996). We quantified the area of the SVZ immunoreactive for these markers. At early time-points, there was no difference between tumor and control groups. However, on day 30, there was a significant increase in the area of SVZ containing DCx+ cells and PSA-NCAM+ cells ipsilateral to the tumor compared with the ipsilateral SVZ in the control group. On day 30, the area of DCx+ cells in the

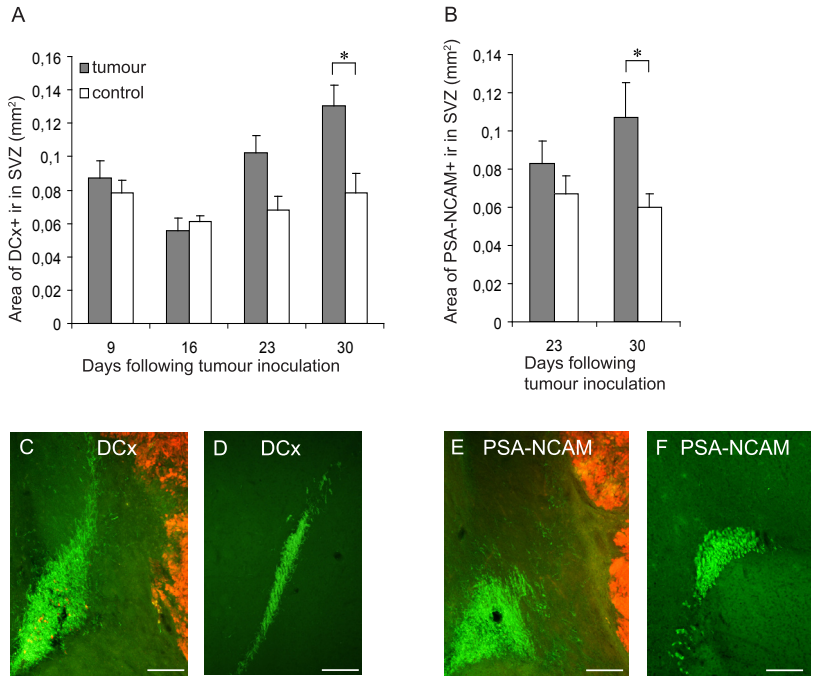

Fig. 4. Increased DCx and PSA-NCAM immunoreactivity (ir) in the SVZ in response to N32DsRed2 malignant brain tumor growth in the striatum. (A, B) The area of DCx ir and PSA-NCAM ir in the SVZ following tumor growth or control lesion. Data are shown as means±S.E.M. * $P<0.05$. (C, D) Increased DCx immunoreactivity (green) in the SVZ in response to tumor growth (red) compared with the SVZ on the ipsilateral side following control lesion. (E, F) Increased PSA-NCAM expression (green) in the rostral SVZ following tumor growth compared with the rostral SVZ in the control group. Scale bar=200 μm. Tumor group, $n=4–6$. Control group, $n=4$.

SVZ was 0.13±0.01 mm^2 for the tumor group and 0.08±0.01 mm^2 for the control group (Fig. 4A, C, D; $P<0,05$, un-paired *t*-test). The area of PSA-NCAM+ cells within the SVZ was 0.11±0.02 mm^2 for the tumor group and 0.06±0.01 mm^2 for the control group (Fig. 4B, E, F; $P<0,05$, un-paired *t*-test. Quantification of Hoechst-stained cell nuclei revealed that the total number of cell nuclei within the ipsilateral SVZ did not differ significantly at any time-point between tumor and control group.

Neuroblast migration toward tumor in the striatum

There was a significant increase of DCx+ cells in the striatum and the corpus callosum following striatal brain tumor growth (Fig. 5). On day 30, the number of DCx+ cells in the ipsilateral striatum and corpus callosum per section was 26±3 in tumor group, compared with 11±2 in the control group (Fig. 5A; $P<0,05$, un-paired *t*-test). The DCx+ cells were mainly located in the striatum between the SVZ and the tumor (Fig. 5B). In this area, we also found a similar increase in the numbers of PSA-NCAM+ cells (Fig. 5C). The DCx+ and PSA-NCAM+ cells were found at highest density in the proximity to the SVZ and in the area between the SVZ and the tumor, but there were also lower numbers of DCx+ and PSA-NCAM+ cells with migratory morphology in the corpus callosum and even on the lateral side of the tumor (Fig. 5D for DCx). Infiltration of neuroblasts into the tumor was sparsely noticed. DCx+ and PSA-NCAM+ cells located between the SVZ and the tumor displayed neuroblast morphology i.e. bipolar cells with elongated processes (Fig. 5E, F). Only a minority of the DCx+ cells outside the SVZ was co-labeled by BrdU. To further characterize the identity of the DCx+ cells, we performed double-staining with DCx and the transcription factor Pbx (a marker for immature striatal neurons). We found that the majority (81%) of the DCx+ cells co-localized with Pbx (Fig. 5G).

No evidence of neuroblast differentiation into mature neurons

In another experiment, we investigated whether DCx+ neuroblasts differentiated into mature neurons. BrdU was given twice daily during days 1–14 following tumor inoculation and the animals were killed on day 28. In the striatum, where the DCx+ cells were located, we found no cells

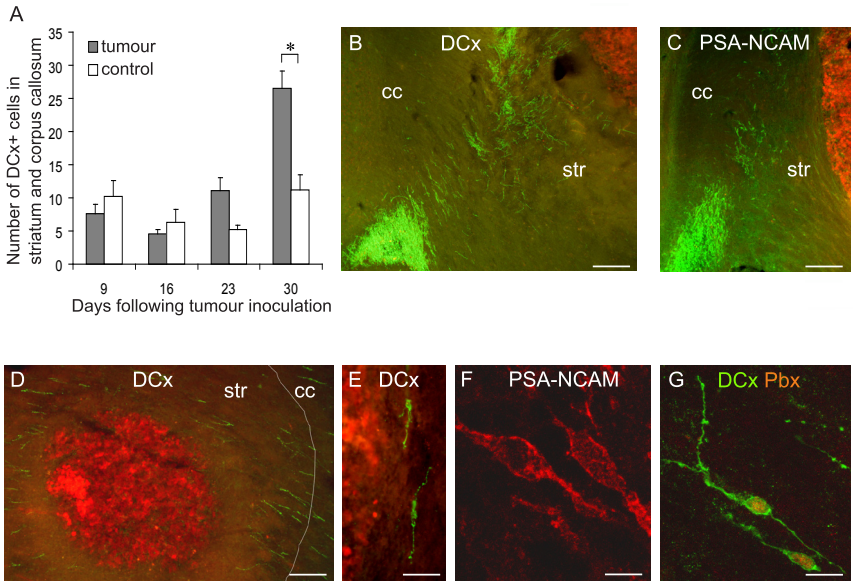

Fig. 5. Neuroblast migration from the SVZ toward malignant brain tumor growth in the striatum. (A) Number of DCx+ cells per section in the str and cc in brain tumor–bearing rats compared with the ipsilateral side of animals with control lesion. Means±S.E.M., * $P<0.05$. (B, C) A large number of (B) DCx+ cells (green) and (C) PSA-NCAM+ cells (green) are found in the area between the rostral SVZ and the N32DsRed2 malignant brain tumor (red) in the str. (D) DCx+ cells were also seen to surround the tumor (located in the striatum). (E) DCx+ cells with typical neuroblast morphology (bipolar cells with elongated processes) were found in close proximity to the tumor. However, infiltration of DCx+ cells into the tumor was minimal. (F) Migratory morphology of PSA-NCAM+ cells (red) located in the proximity to tumor. (G) The majority of the DCx+ cells (green) also expressed Pbx (red), a marker for immature striatal neurons. Scale bar=200 μm for B, C and D, for E 40 μm, for F 10 μm and for G 20 μm. Tumor group, $n=4–6$. Control group $n=4$. str, striatum; cc, corpus callosum.

expressing the mature neuronal marker, NeuN, that were co-labeled with BrdU. A few NeuN+ cells, located in the cerebral cortex, displayed weak BrdU immunoreactivity. However, the weak BrdU immunoreactivity and the location of these cells in the cortex (where no DCx+ cells were found), suggest that these cells were undergoing DNA repair or aberrant DNA synthesis rather than cell division. Thus, at the time-point investigated, there was no evidence of differentiation of neuroblasts into mature neurons.

DISCUSSION

This study demonstrates a marked neurogenic response of the SVZ to a malignant brain tumor. Proliferation within the SVZ, as measured by BrdU incorporation and Ki-67 immunostaining, decreased with tumor growth. Thus, there was a negative correlation between the tumor size and the number of Ki-67- and BrdU-labeled cells in the SVZ. However, the size of the SVZ, as assessed by DCx and PSA-NCAM immunostaining, increased markedly with growth of the tumor. Neuroblasts were observed in high numbers in the area between the SVZ and the tumor, and in the immediate vicinity to the tumor, most likely pointing to a directed migration of neuroblasts from the SVZ toward the tumor. Neuroblasts expressed a marker (Pbx) for developing striatal neurons but no evidence of neuroblast differentiation into NeuN+ neurons was seen in our experimental paradigm.

We found that the number of cell nuclei immunopositive for BrdU and Ki-67 within the ipsilateral SVZ *decreases* when the tumor has grown to a considerable size. The decrease in BrdU and Ki-67 labeling is likely to reflect an actual decrease in the rate of stem cell and/or precursor cell proliferation within the SVZ at a late stage in tumor growth. Such an antiproliferative effect on the SVZ could, potentially, be due to several factors including an inflammatory reaction surrounding the growing tumor (Morris and Esiri, 1991; Monje et al., 2003) or mechanical compression of the SVZ. Interestingly, and perhaps seemingly paradoxically, we found a concomitant *increase* in the area immunoreactive for neuroblasts (DCx and PSA-NCAM) in the SVZ ipsilateral to the tumor. The latter finding reflects either an increased number of neuroblasts within the SVZ or neuroblast hypertrophy including increased length of fibers. The total number of cell nuclei (visualized by Hoechst nuclear staining) within the SVZ was not different between tumor and control group, suggesting that the increase in neuroblast immunoreactivity in the SVZ was due to hypertrophy rather than increased number of cells

within this region. We observed no difference in the number of apoptotic cells within the SVZ or in the area between the tumor and SVZ, speaking against alterations in the rate of cell death as the major cause for the decrease in proliferation marker labeling. Expression of DCx occurs in post-mitotic neuroblasts (Couillard-Despres et al., 2005) and previous data have shown that increased neuroblast immunoreactivity in the SVZ is not necessarily coupled to increased SVZ cell proliferation. In mice, cortical lesions were followed by a similar increase in PSA-NCAM immunoreactivity within the ipsilateral SVZ, although the total numbers of SVZ cells were unchanged, and a biphasic decrease in the number of proliferating, BrdU+ cells (Goings et al., 2002).

The source of neuroblasts in our study could, in principle, be the SVZ/RMS or the parenchyma surrounding the tumor. Despite the fact that there is a decrease in proliferating cell markers within the SVZ at a late stage of tumor growth, it is most likely that the neuroblasts observed in the zone between the SVZ and the tumor emanate from the SVZ. The SVZ has previously been implicated in the generation of cells in response to the growth of a malignant tumor (Glass et al., 2005) and the distribution pattern of the neuroblasts seen in the present paper clearly points to the SVZ as the source of the migrating neuroblasts. DCx expressing cells were found at highest density in proximity to the SVZ and clusters of neuroblasts with their bipolar processes arranged in parallel were seen in the area between the SVZ and the tumor. Following ischemic stroke, neuroblasts born in the SVZ have been shown to migrate in the striatum toward the lesion (Ohab et al., 2006; Yamashita et al., 2006) and tracing experiments revealed the SVZ, not the striatal parenchyma, to be the principal source of neuroblasts following stroke. The fact that only a minority of the DCx+ neuroblasts outside of the SVZ was co-labeled with the proliferation marker BrdU in the present study suggests that the majority of the migrating DCx+ cells were post-mitotic already at the time of tumor inoculation.

Nestin is expressed in proliferating glial and neuronal precursor populations and in reactive astrocytes but not in migrating neuroblasts (Lin et al., 1995; Couillard-Despres et al., 2005). In contrast to previous data (Duntsch et al., 2005; Glass et al., 2005), we found only single nestin+ cells between the SVZ and the tumor, except for a dense peritumoral rim of nestin+ cells with astrocyte like morphology. Thus, no substantial signs of migration of nestin+ cells from the SVZ toward the tumor were found in our study. The peritumoral rim of nestin+/GFAP+ cells most likely represents reactive astrocytes residing in the brain parenchyma without any certain origin in the SVZ.

Signals governing the migration of neural stem and precursor cells toward gliomas have started to be elucidated. Soluble factors expressed at high levels by brain tumor cells, tumor endothelium, infiltrating inflammatory cells or by the surrounding reactive brain parenchyma might attract neural precursor cells specifically toward the site surrounding the tumor. A correlation was found between the levels of brain tumor production of vascular endothelial growth factor (VEGF) and the capacity of human neural stem cells to migrate toward tumor cells *in vitro* (Schmidt et al., 2005). Local intracerebral infusion of VEGF induced a long-range attraction of transplanted neural stem cells from distant sites in the adult brain. Scatter factor/hepatocyte growth factor presumably produced by tumor microvasculature, was also found to be a powerful chemoattractant for neural stem cells (Heese et al., 2005; Schmidt et al., 2005). Stromal cell–derived factor 1, which is upregulated on tumor vasculature, has also been reported to specifically attract stem cells (Ehtesham et al., 2004). In addition to factors produced by tumor cells and tumor endothelium, soluble chemoattractants produced by inflammatory cells surrounding and infiltrating the tumor might direct the specific migration of neuroblasts toward the site of tumor. Activated microglial cells infiltrate human gliomas (Morris and Esiri, 1991) and surround and infiltrate experimental N32 brain tumors (our unpublished observations) and these cells have the capacity to attract neural precursor cells (Aarum et al., 2003). Chemokines such as monocyte chemoattractant protein-1, produced by activated microglia, have recently been shown to play an important role in directing neural progenitors to sites of neuroinflammation (Belmadani et al., 2006). It is conceivable that similar mechanisms to those described above, at least to some extent, are responsible for redirecting and attracting endogenous neuroblasts from their normal migratory route in the RMS toward the site of a tumor.

We show here that migrating neuroblasts adopt an immature striatal phenotype, suggesting that these cells are on their way to differentiate into striatal mature neurons; i.e. the phenotype of the neurons that have died. We did not, however, find any signs of newly formed, fully differentiated neurons. Whether this is due to a permanent halt of differentiation or simply due to insufficient time for maturation is not clear. In addition, we cannot exclude the possibility that some BrdU neuroblasts mature fully. Consistent with our findings, aspiration lesions of the sensorimotor cortex in adult rats induced a marked increase in immunoreactivity to PSA-NCAM, expressed by the majority of cells of the SVZ during development (Goings et al., 2004). In contrast, immunolabeling for molecules found in mature neurons and glia did not increase in the SVZ after this lesion, and immunoreactivity for factors inducing differentiation of SVZ cells *in vitro* decreased or remained undetectable, suggesting that a lack of appropriate maturation signals may contribute to the halt in differentiation seen *in vivo* in this experimental model (Goings et al., 2004). It is also possible that the tumor environment in our study is nonpermissive for the further maturation of the neuroblasts.

The function of neuroblast recruitment to the site of tumor remains speculative at this stage. Co-culture experiments showed that SVZ neural precursors suppressed the increase in tumor cell number and induced glioma cell apoptosis. Furthermore, co-injection of mouse glioma cells with SVZ precursors prolonged survival, although only in old mice (Glass et al., 2005). However, direct evidence demonstrating a neuroblast-mediated antitumor effect *in*

vivo remains to be shown. Another potential function of the migrating neuroblasts is that they may constitute the source for neuronal replacement in the striatum following tumor- or tumor treatment–induced neuronal cell death.

Our findings point to a marked reactive response from the neurogenic SVZ region in the adult rat ipsilateral to a tumor located in the striatum. Factors within the tumor microenvironment promoting and limiting the migration and differentiation of cells from the SVZ need to be determined before strategies to enhance this potentially beneficial neurogenic response can be developed.

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COMPARATIVE ANALYSIS OF RAT BONE MARROW-DERIVED AND SUBVENTRICULAR ZONE-DERIVED PRECURSOR CELLS AS CELLULAR VECTORS IN GLIOMA GENE THERAPY

III

Comparative analysis of rat bone marrow-derived and subventricular zone-derived precursor cells as cellular vectors in glioma gene therapy

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Objective: To study migratory and proliferative properties of adult neural precursor cells (NPCs) and multipotent mesenchymal stromal cells (MSCs) following grafting to cerebral experimental gliomas in order to choose the most effective vector system for delivery of therapeutic transgenes to gliomas.

Methods: Adult rat subventricular zone-derived NPCs and bone marrow-derived MSCs, transduced to express enhanced green fluorescent protein (eGFP), were grafted into or at a distance from established syngeneic N32 rat intracerebral gliomas in Fischer344 rats. NPC and MSC migratory patterns were analyzed at three time-points and the amount of MSCs relative to NPCs within tumors was quantified. Proliferation of grafted NPCs and MSCs within tumors was assessed using confocal microscopy to determine co-expression of eGFP and Ki67.

Results: Intratumorally grafted NPCs and MSCs migrated specifically within N32 gliomas and avoided normal brain tissue. NPCs and MSCs were distributed preferentially in the tumor periphery and at later time-points in the tumor core. eGFP+ MSCs displayed approximately 11-fold higher survival compared to eGFP+ NPCs following grafting. NPCs and MSCs grafted at a distant site in the normal brain parenchyma displayed no substantial migration toward tumors. Grafted NPCs and MSCs did not proliferate within tumors.

Conclusion: MSCs display higher survival and better intratumoral migratory capacity, compared to NPCs, following grafting to gliomas. The absence of proliferation of grafted NPCs and MSCs within tumors indicates a low risk of secondary malignancies and might facilitate clinical development of this vector system. Taken together, our data indicate that adult MSCs are better suited than adult NPCs as a vector system for gene therapy of malignant brain tumors.

Keywords: glioma, rat, multipotent mesenchymal stromal cell, neural precursor cell

INTRODUCTION

Malignant gliomas have been the target for clinical gene therapy trails using viral vectors with a limited success in the past[1]. Because of inefficient spread of viral vectors *in vivo*, gene delivery into brain tumors still presents a major obstacle. As an alternative to gene delivery by means of viral vectors, grafted stem- and precursor cells have emerged. Malignant gliomas have been shown to attract grafted neural precursor cells (NPCs), multipotent mesenchymal stromal cells (MSCs), as well as endothelial and hematopoietic stem and progenitor cells[2-10]. The ability of these cells to track and home to malignant brain tumors makes them potentially more efficient than viral vectors for local delivery of therapeutic tumoricidal substances. Primary or immortalized murine and human NPCs

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have been allo- or xenografted into immunocompetent mice and rats and into immunodeficient nude mice in order to track and attack experimental brain tumors[2-5, 11-15]. Intracerebral injection of immortalized neural stem cell (NSC) lines at a distance from tumors or in the contralateral hemisphere results in infiltration of tumors [2, 14]. As an alternative to NPCs, bone marrow-derived MSCs have been used. MSCs are precursors to adipocytes, chondrocytes and osteoblasts that form the bone marrow stroma. Bone marrow-derived MSCs have been demonstrated to infiltrate gliomas following intratumoral, intracerebral and intraarterial administration[8, 9, 16]. The use of skin-derived, endothelial and hematopoietic stem and progenitor cells has also been explored and these cells might constitute useful sources of cellular vehicles for glioma therapy[7, 10, 17].

To treat glioma patients with tumor-tracking stem and progenitor cells, several criteria have to be met by the cellular vehicles: 1) easy access, 2) efficient and specific migration within tumors, 3) immunologic compatibility with host, and 4) non-tumorigenicity *in vivo*[18]. Previous studies have demonstrated promising tumor-tropic capacities of MSCs and NPCs [2, 3, 8, 9] but quantitative studies evaluating the capacity and limitations of these cells are lacking and highly warranted in order to choose the best possible system for therapeutic gene delivery to brain tumors.

In the present study, we therefore grafted rat bone marrow-derived MSCs and subventricular zone-derived adult NPCs into established syngeneic N32 experimental gliomas. Rat N32 gliomas are fast proliferative tumors which show characteristics of anaplastic astrocytomas[19]. The aims were to semiquantitatively quantify and compare survival, migration and proliferation of MSCs and NPCs following grafting to experimental malignant brain tumors.

MATERIALS AND METHODS

Rat N32 glioma cell line

The rat glioma cell line N32, syngeneic with the Fischer344 rat was originally induced by transplacental injection of ethyl-N-nitrosourea to pregnant rats whose offsprings developed malignant brain tumors[20]. Tumor cells were maintained in R10 medium, consisting of: RPMI 1640 medium (1X) with L-glutamine supplemented with 10% fetal calf serum (FCS) (VWR, West Grove, PA), 10 mM HEPES buffer solution, 1 mM sodium pyruvate and 50 μg/ml gentamicin (all chemicals except FCS from GIBCO, Invitrogen, Carlsbad, CA).

R10 medium was changed every 2-3 days and cells were passaged about twice a week and detached using trypsin-EDTA (0.25% trypsin with EDTA 4Na) 1X (Invitrogen). Cells were incubated at 37°C in a humidified atmosphere containing 6.0% CO_2. Cells were kept in culture for a maximum of 6 weeks. Before inoculation *in vivo*, cells were washed and resuspended in medium without FCS and gentamicin (referred to as R0 medium). Cells were counted and resuspended in a volume corresponding to the target concentration.

Establishment and culture of SVZ-derived rat adult NPCs

Brains of male Fischer344 rats (8 weeks old) were dissected, placed into ice-cold Pipes-buffer and kept on ice. The SVZ was dissected under sterile conditions and pieces collected in ice-cold Pipes-buffer. Tissue pieces were enzymatically digested in papain solution for 30 min at 37° C. The supernatant was carefully removed and the tissue resuspended in 1 ml control media containing DMEM/F12/N2 (Gibco), 30% glucose, 1 M HEPES, and penicillin/streptomycin and 1 mg/ml DNAse, mechanically triturated and spun for 10 min at 1000 rpm and then resuspended in 1 ml proliferation media (control media with bovine serum albumin, heparin solution and 20 ng/ml EGF and 10 ng/ml bFGF). Cells were plated at a density of $1x10^5$ cells /ml in uncoated T25 flasks in proliferation medium at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown as neurospheres and fresh growth factors were added every other day. For passaging, spheres were collected into a Falcon tube, allowed to settle, the supernatant aspirated and 1 ml fresh medium added. Cells were mechanically triturated, counted and plated at desired density in T25 flasks.

Establishment and culture of bone marrow-derived rat MSCs

MSC cultures were derived from Fischer344 male rat bone marrow (8 weeks old). The animal was deeply anaesthetized with Isoflurane (2.5% in O_2, Forene, Abbott Scandinavia AB, Solna,

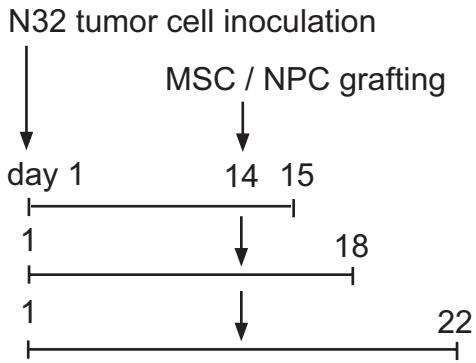


Fig. 1 Experimental design. On day 1, 3000 N32wt tumor cells were inoculated into the striatum of male rats. Either 2.5 x 10^5 adult bone marrow-derived eGFP+ multipotent mesenchymal stromal cells (MSCs, n = 14) or 2.5 x 10^5 adult subventricular zone-derived eGFP+ neural precursor cells (NPCs, n = 14) were grafted into tumors on day 14. Animals were sacrificed on day 15, 18 and 22, corresponding to day 1, 4 and 8 following MSC/NPC grafting (n = 4-5 for each time-point).

Sweden), sacrificed and femur was dissected and adherent tissue removed. The marrow cavity was flushed with PBS supplemented with 2% FCS (GIBCO). 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-alpha supplemented with 10% fetal bovine serum and 1% Antibiotic-Antimycotic-Solution (Sigma). Non-adherent cells were removed after three days and culture medium was changed weekly thereafter. Cells were passaged at 80-90% confluency.

eGFP retroviral production and transduction of MSCs and NPCs

The Moloney leukemia retroviral vector pC-MMP-IRES2eEGFP-WPRE used in this study has been described elsewhere [21]. The viral particles were produced using the producer cell line 293VSVG[22]. Concentrated particles were resuspended into 0.5 ml of DMEM medium (Sigma-Aldrich, St. Louis, MO). The titer was measured by FACSCalibur analysis, based on eGFP reporter gene expression, 3 days after infection of the HT1080 cells and varied from 0.7x10^9 to 1.2x10^9 TU/ml depending on the batches. When at 60-70% confluency, MSCs and NPCs 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 1μg/ml (Sigma).

Animal procedure and experimental design

Adult male Fischer344 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, Sweden. Animals were anaesthetized with Isoflurane (2.5% in O_2, Forene, Abbott Scandinavia AB, Solna, Sweden) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, USA). The following coordinates were used for tumor cell inoculation (relative to bregma): anterior-posterior (AP): +1.7, medial-lateral (ML) –2.5 and dorso-ventral (DV) –5.0. The tooth bar was set at –3.3 mm.

For the experiment comparing MSC and NPC migratory capacity, 3000 N32wt tumor cells were inoculated into the right striatum of male rats (n = 28) on day 1. eGFP+ MSCs (2.5 x 10^5 cells) or NPCs (2.5 x 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. Tumor cells were inoculated at 1 μl/min, using a 10 μl Hamilton syringe. MSCs and NPCs were grafted at 0.5 μl/min, using 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 slowly retracted. Animals were sacrificed on day 1, 4 and 8 following stem cell grafting (n = 4-5 for each time-point, fig. 1 for experimental design).

In the experiment studying MSC and NPC migration throughout the normal brain parenchyma toward a distant malignant brain tumor, we established N32wt tumors (3000 cells) in the right striatum. 14 days later, eGFP+ MSCs or NPCs (2.5 x 10^5 cells, n = 5 in each group) were grafted 2.5 mm caudally and 2.0 mm lateral of the inoculation coordinates of the tumor. Animals were sacrificed 8 days following MSC/NPC grafting.

In another experiment, proliferation rate of grafted MSCs and NPCs was determined. eGFP+ cells were grafted intratumorally into established N32wt intracerebral tumors (n = 3 for MSCs, n = 3 for NPCs). Animals were sacrificed on day seven or

around day 30 following MSC and NPC grafting.

Immunohistochemistry

The rats were deeply anaesthetized and perfused through the ascending aorta with PBS, pH 7.4, followed by cold 4% paraformaldehyde (PFA) in PBS. The brains were removed and postfixed in cold 4% PFA 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 anti-freeze solution. Free-floating sections were rinsed three times in KPBS (Potassium Phosphate Buffer Solution). Sections were blocked with 5% Normal Goat Serum and 5% Normal Donkey Serum in 0.25% Triton X-100 solution. Sections were incubated with the primary antibodies chicken anti-EGFP (1:1500, Chemicon, Temecula, CA), mouse anti- Ki67 (1:50, Novocastra), mouse anti-NG2 (1:500, Chemicon), rabbit anti-α-sma (1:400, Abcam), mouse anti-rat nestin (1:1000, Chemicon), rabbit anti-GFAP (1:1000, DAKO, Glostrup, Denmark), goat anti-doublecortin (1:150, Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C over night. The next day the sections were rinsed three times in appropriate sera in KPBS and incubated for 1 hour with one or two of the following secondary antibodies: Alexa488 goat anti-chicken (1:400, Molecular Probes, Eugene, OR), Alexa594 goat anti-rabbit (1:400, Probes), Alexa568 donkey anti-goat (1:400, Probes), Cy3 donkey anti-mouse (1:400, Jackson Immunoresearch, West Grove, PA). The sections were counterstained with Hoechst nuclear staining to visualize tumors, mounted onto glass slides and cover-slipped with DABCO mounting medium.

For quantitative analysis of eGFP within tumors, sections were rinsed three times in KPBS and incubated in 3% H_2O_2 and 10% methanol in KPBS for 10 minutes to quench endogenous peroxidase activity. Sections were incubated in 5% NGS containing 0.25% Triton X-100 solution for 1 h and with the primary antibody chicken anti-GFP (1:1500) over night. The next day, sections were rinsed in KPBS and incubated in biotinylated goat anti-chicken (1:200, Vector Laboratories, Burlingame, CA) followed by avidin-biotin-peroxidase solution (ABC Elite, Vector) for 1 h and 3,3'-diaminobenzidine (DAB) solution for 10 minutes. Sections were mounted onto glass slides, dehydrated in alcohol solutions, cleared in Xylene and finally cover-slipped with Pertex mounting media (Histolab, Göteborg, Sweden).

Image analysis

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

For calculation of eGFP area within tumors, three tissue sections including tumor periphery and tumor core were analyzed for each animal. Sections were visualized using a light microscope and captured by a Color View II digital camera (Olympus) in 10X magnification. Analysis-Pro™ software (Olympus) was used to distinguish the area of eGFP-labeled cells within tumors from tumor area with no eGFP-labeled cells and, subsequently, for quantification of eGFP-labeled area within tumors.

Statistical analysis

Student´s unpaired t-test was used for comparison between groups. Data are presented as means ± standard error of the mean (SEM) and data are considered significant at $p<0.05$.

RESULTS

Bone marrow-derived MSC characteristics in vitro

Bone marrow-derived MSCs used in our study have previously been characterized[23]. Briefly, adherently growing spindle-shaped cells were isolated from the bone marrow. These cells were positive for the mesenchymal markers CD73 and C90, negative for the hematopoietic marker CD45, as assessed by flow cytometry, and displayed capacity to differentiate into osteoblasts and adipocytes upon exposure to conditions promoting differentiation[23].

SVZ-derived NPC characteristics in vitro

SVZ-derived NPCs used in our study were previously characterized[24]. Briefly, NPCs were grown as free-floating neurospheres. The cells within the spheres displayed capacity to differentiate into βIII-tubulin+ neuronal cells, GFAP+ astrocytes and CNPase+ oligodendrocytes[24].

eGFP expression of MSCs and NPCs

In order to visualize the MSCs and NPCs, we

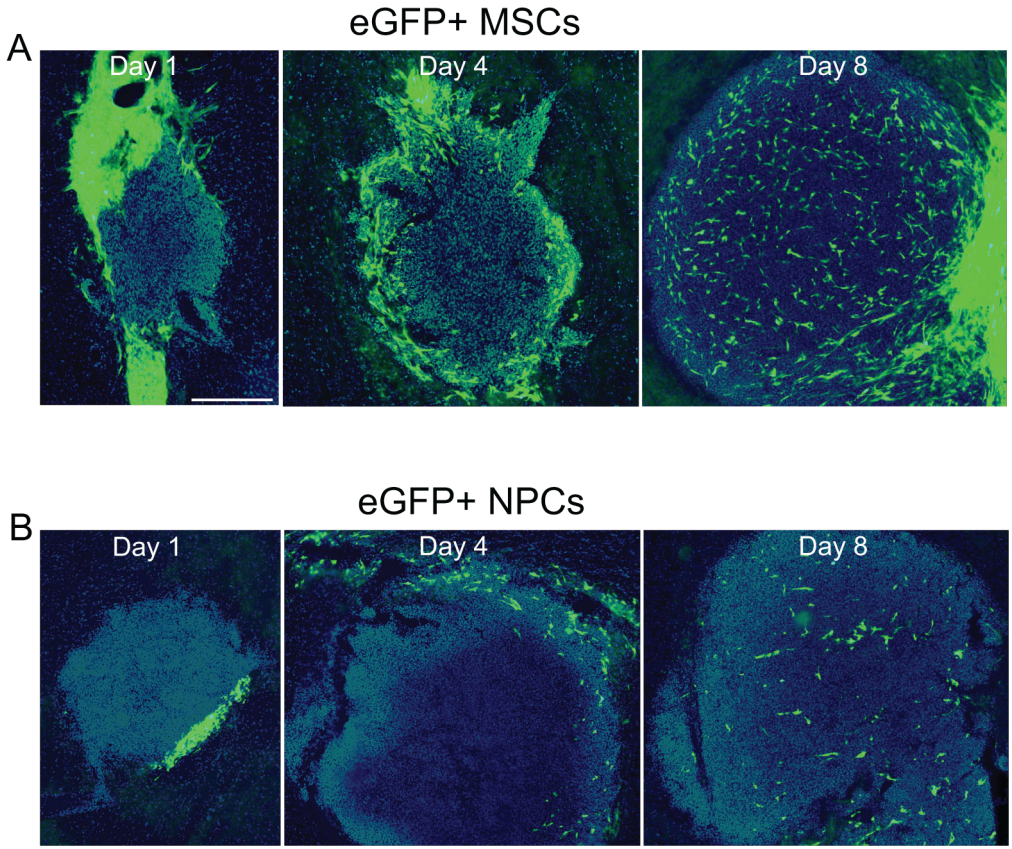


Fig. 2 Migration patterns of adult syngeneic bone marrow-derived MSCs and adult subventricular zone-derived NPCs following grafting into established N32 malignant brain tumors (depicted by Hoechst nuclear staining, light blue) in the striatum. (A) eGFP+ MSCs (green) grafted into malignant brain tumors. One day following grafting, eGFP+ MSCs are mainly located within the elongated graft. On day 4, MSCs are predominantly found 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. (B) eGFP+ NPCs (green) are found within the graft on day 1. NPCs are spread out in tumor peripheral zone on day 4. On day 8, NPCs are located within tumor periphery and tumor core. Note the relative high numbers of MSCs compared to the numbers of NPCs. Scale bar, 200 μm.

genetically modified them to express eGFP. Both MSCs and NPCs were transduced with a Moloney leukemia based-retroviral vector, which has the characteristic of infecting dividing cells. More than 90% of the cells expressed eGFP as assessed in an inverted microscope 4 days following transduction.

MSC and NPC migration following intratumoral grafting

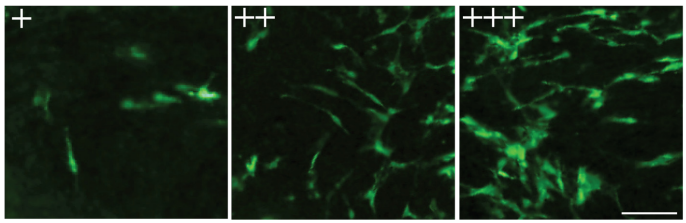
N32wt brain tumors were established in the striatum of all animals (n = 28). MSCs (n = 14) or NPCs (n = 14), transduced to express eGFP, were grafted into tumors and migration pattern was assessed at three time-points (n = 4-5 for each time-point) following grafting. On day 1 postgrafting, eGFP+ MSCs 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

Table 1 (A) The table shows the relative grading of the numbers of eGFP+ MSCs and NPCs within N32 tumor periphery and tumor core at three time-points following grafting (+++, high numbers; ++, moderate numbers; +, low numbers; –, no cells). (B) Representative examples illustrating grading of cell numbers within tumors. Scale bar is 50 μm.

A

Animal No	Days after MSC grafting	MSCs in tumor periphery	MSCs in tumor core	Animal No	Days after NPC grafting	NPCs in tumor periphery	NPCs in tumor core
1	1	+	-	15	1	+	-
2	1	+(+)	-	16	1	+	-
3	1	+	-	17	1	-	-
4	1	+	-	18	1	(+)	-
5	1	+	-	19	1	(+)	-
6	4	+	-	20	4	+	-
7	4	(+)	-	21	4	++	(+)
8	4	++	-	22	4	-	-
9	4	++	+	23	4	+	(+)
10	8	+(+)	(+)	24	8	+	-
11	8	++	+	25	8	(+)	(+)
12	8	++	++	26	8	++	(+)
13	8	+++	++	27	8	(+)	-
14	8	++	++	28	8	+	+

B



(Fig. 2A). Already on day 4 postgrafting, numerous MSCs were found within the peripheral zone of the tumor (Fig. 2A). Single MSCs were found in the core of the tumor. On day 8 postgrafting, abundant numbers of MSCs were located in both tumor periphery and tumor core (Fig. 2A). The vast majority of MSCs were located within the graft or within the tumor, and only very low numbers of MSCs were found in the normal brain parenchyma at any time-point.

SVZ-derived NPCs displayed similar tumor tropism and distribution pattern following intratumoral grafting as shown by the MSCs. One day following grafting, eGFP+ NPCs were located within a coherent graft at the inoculation site. On day 4, NPCs were predominantly found in the peripheral part of the tumor, and on day 8, NPCs were spread out both in tumor periphery and tumor core (Fig. 2B). Only minimal numbers of NPCs were found outside the tumor in the normal brain parenchyma.

A semiquantitative analysis of the numbers of eGFP+ MSCs and eGFP+ NPCs within N32wt tumors is presented in table 1. Quantitative analysis of eGFP area within tumors on day 8 following grafting of eGFP+ MSCs or eGFP+ NPCs was performed to give an indication of the numbers of eGFP+ MSCs relative to the numbers of eGFP+ NPCs within tumors. Tumors receiving eGFP+ MSCs displayed an approximately 11-fold larger area of eGFP compared to tumors that received eGFP+ NPCs ($7.6x10^4 \pm 2.5x10^4$ μm^2 eGFP area for MSCs and $6{,}8x10^3 \pm 3.7x10^3$ μm^2 eGFP area for NPCs, $p < 0.05$).

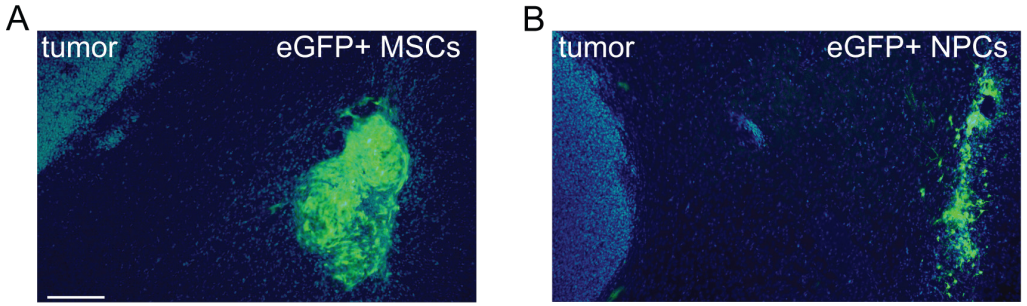


Fig. 3 Minimal MSC and NPC migration toward tumors following grafting at a distant site. eGFP+ MSCs and NPCs (green) grafted 2.5 mm behind and 2.0 mm lateral of established malignant N32 brain tumor (Hoechst nuclear staining, blue) in the striatum. (A) Eight days following grafting, MSCs are found within a coherent cluster of cells at the inoculation site. (B) NPCs are also mainly located at the inoculation site. There is no evidence of eGFP+ cells migrating from the graft toward tumors. Scale bar, 200 μm.

No substantial MSC and NPC migration toward tumors following grafting into the normal brain parenchyma

We investigated whether adult syngeneic MSCs and NPCs, grafted at a distant site to established N32wt tumors, migrated throughout the normal brain parenchyma toward tumors. eGFP+ MSCs and eGFP+ NPCs were grafted 2.5 mm behind and 2.0 mm lateral of established N32wt tumors in the striatum. Migration capacity was assessed 8 days following MSC and NPC grafting. In contrast to the intratumoral MSC and NPC distribution pattern seen following intratumoral grafting, grafting into the normal brain parenchyma did not result in any substantial MSC and NPC migration toward tumors. MSCs were seen in a coherent cluster of cells at the inoculation site and NPCs were spread out around the inoculation site (Fig.3). NPCs were found to migrate along the corpus callosum and this was also seen in animals without tumors.

In order to evaluate potential eGFP transgene silencing of MSCs and NPCs upon intracranial grafting, expression of endogenous markers (NG2 and α -sma for MSCs, and nestin, doublecortin and GFAP for NPCs) was used to assess potential migration of non-eGFP-expressing MSCs and NPCs in normal brain tissue toward tumors. These endogenous markers are expressed by MSCs and NPCs *in vitro* and by grafted eGFP+ cells within tumors ([23], own unpublished observations). We found no evidence of spindle-shaped cells expressing NG2 or a-sma that were migrating from the MSC graft toward tumors. We found no nestin, GFAP or doublecortin expressing cells with migratory morphology between the NPC graft and tumors (data not shown).

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 sacrificed 118 days following 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 the deep cervical lymph nodes were analyzed for presence of eGFP+ MSCs. We did not find any eGFP+ MSCs in sections from the liver, spleen or the cervical lymph nodes.

Grafted MSCs and NPCs are essentially non-cycling in vivo

Confocal microscopy analysis of eGFP and Ki67 expression was used to investigate cell-cycle state of MSCs and NPCs grafted into N32 malignant brain tumors. Ki67 is a marker of cells in G1, S, M and G2 cell-cycle phases, i.e. dividing cells. MSCs and NPCs were grafted into established N29 and N32 tumors and analyzed seven days following 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 as well as migratory eGFP+ cells located far away

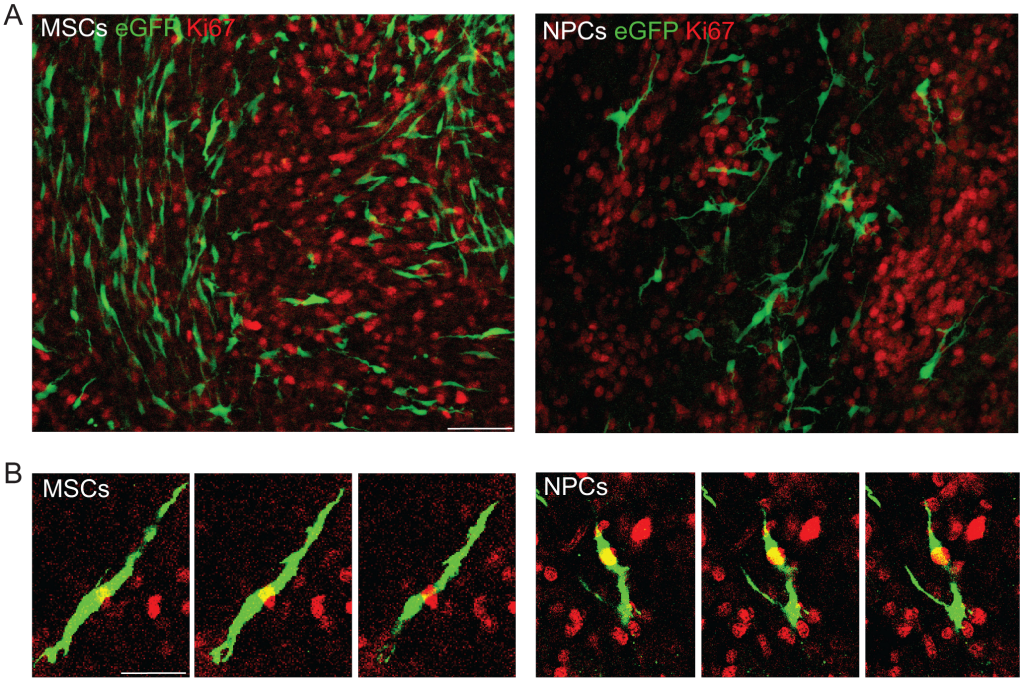


Fig. 4 MSCs and NPCs are essentially non-dividing seven days following grafting into established N32wt malignant brain tumors. (A) eGFP+ MSCs (green) and eGFP+ NPCs (green) grafted into the highly proliferative N32 tumor. No co-expression of eGFP and Ki67 (red) can be seen. (B) Confocal microscopy analysis demonstrating single eGFP+ MSCs and eGFP+ NPCs not co-expressing Ki67 but located in close association to Ki67+ cells. Scale bar is 60 μm in A and 30 μm in B.

from the graft were analyzed. We did not find a single eGFP+ MSC or NPC that expressed Ki67. Fig. 4 shows representative examples of non-Ki67 expressing eGFP+ MSCs and NPCs within highly proliferative tumors. We conclude that the absolute majority of intratumorally grafted MSCs and NPCs are in a non-cycling state seven days following grafting.

DISCUSSION

Neural and bone marrow-derived stem- and progenitor cells, engineered to produce anti-tumor substances, have shown promise as cellular delivery vehicles to experimental gliomas [2, 3, 8, 9]. Here, we quantified and compared the migratory and proliferative pattern of adult rat bone marrow-derived MSCs and SVZ-derived NPCs grafted to rat N32 malignant brain tumors. Grafting was performed into or at a distance from established syngeneic tumors in order to simulate a clinical scenario where cells might be grafted into tumor remnants. Following intratumoral grafting, both MSCs and NPCs migrated specifically within tumors, however MSCs showed much better survival and displayed higher migratory capacity. When MSCs and NPCs were grafted at a distant site to tumors, no substantial tropism toward tumors was seen. Intratumorally grafted MSCs and NPCs showed no signs of proliferative activity within highly proliferative tumors.

Our findings indicate that grafted MSCs possess higher survival and better intratumoral migratory capacity than grafted NPCs within gliomas. The quantitative analysis performed in the present study gives an estimation of the relative amounts of eGFP within tumors. The results are affected by other parameters than cell numbers, such as the size of MSCs and NPCs. Nevertheless, the magnitude of the difference in GFP amounts clearly suggests that grafted eGFP+ MSCs persist in much higher numbers within tumors as compared to eGFP+ NPCs.

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[25], but the factors that mediate the higher survival and intratumoral migratory capacity displayed by MSCs compared to NPCs remain to be elucidated. The substantial intratumoral MSC migration and virtual absence of MSC migration in normal brain tissue suggest that the tumor microenvironment, e.g. tumor vasculature and inflammatory cells, is permissive for survival and migration of grafted MSCs. Systemic administration of bone marrow-derived cells into tumor-bearing animals results in homing to tumors and incorporation into tumor vessels[8, 26, 27]. MSCs also home to sites of wounds[28] where they contribute to wound healing[29] and tumors, considered as "wounds that never heal"[30] provide a site with high numbers of inflammatory cells and cytokines which can attract MSCs[31]. Thus, our findings that grafted MSCs show better survival and display higher migratory capacity compared to NPCs within gliomas might be explained by the attraction and participation of MSCs to tumor stroma. In addition, the much higher numbers of grafted MSCs, compared to NPCs, found already at day one following grafting could also, at least in part, be explained by differences in cell survival during the process of MSC/NPC intracranial grafting.

Initial findings of stem cell tropism toward tumors reported wide-spread migration of the immortalized mouse NSC line C17.2, implanted in the contralateral hemisphere, across the corpus callosum, toward human or rat malignant brain tumors[2]. Human and rat MSCs have also been shown to migrate along the corpus callosum toward brain tumors in the contralateral hemisphere[8, 9]. Furthermore, mouse C17.2 NSCs and rat embryonic NPC lines Hib5 and ST14A have been reported to migrate throughout normal brain tissue toward tumors following injection at a distance to tumors with no connection to the corpus callosum[2, 32]. We investigated whether adult rat MSCs and NPCs, syngeneic to both tumor and host, migrate toward tumors when grafted at a distant site in the same hemisphere as tumors and with no connections to the corpus callosum. In this experimental setting, we found no substantial MSC and NPC migration toward tumors which may imply that MSC/NPC migration toward tumors in normal brain tissue is restricted to when the grafted cells and tumors are located adjacent to the corpus callosum or possibly to other white matter tracts. The discrepant findings between our results and previous studies using immortalized NSC/NPC lines[2, 32] might be due to species-specific interactions (i.e. between mouse NSCs lines and human or rat gliomas), to tumor-specific capacities in attracting grafted stem/precursor cells, and/or to different tumor-tropic migratory properties between immortalized NSC/NPC lines and adult primary MSCs/NPCs.

A major safety issue in the development of stem cell therapies for neurological disorders is the risk of tumor formation of the grafted stem cells[33]. Approximately 2% of immortalized human fetal NSCs continue to proliferate following grafting into glioma-bearing immune-compromised mice[34]. We therefore investigated the proliferative pattern of adult non-immortalized MSCs and NPCs grafted directly into gliomas and we found no indication of MSC or NPC proliferation *in vivo* seven and 30 days following grafting into the highly proliferative N32 tumor. These results point to a low risk of development of secondary malignancies of grafted MSCs and NPCs within gliomas. This favors adult MSCs and NPCs, compared to immortalized NSC lines, as the choice of cellular vector system for development of therapy of patients with gliomas.

Furthermore, grafting of immortalized NSCs into the brain faces the problem of immunorejection. HLA-matched allogeneic stem and precursor cells, as used in this study, are immunologically compatible with the host, which may favor their survival and migration within tumors. HLA-matched allogeneic MSCs are already used clinically for the treatment of severe graft-versus-host disease[35].

Conflicting data exists on the effects of MSCs on tumor growth. Nakamura et al. reported that grafted rat MSCs prolong survival of glioma-bearing rats[9]. In contrast, MSCs have been reported to favor tumor growth through production of immunosuppressive factors and inihibition of lymphocyte proliferation[36]. Our previous findings indicate that grafting of unmodified MSCs into gliomas does not affect the survival of glioma-bearing animals[23]. This implies that grafted MSCs need to be genetically modified in order to achieve a therapeutic benefit.

Human SVZ-derived NPCs can be harvested during endoscopic surgery of the ventricular system[37] and NPCs hold the potential of neuronal replacement in the damaged brain. Endogenous NPCs migrate specifically from the SVZ toward malignant brain tumors in the striatum[38, 39]. The SVZ-derived NPCs are associated with anti-tumor effects[39] and a high proportion of the migrating NPCs display a region-specific phenotype of immature striatal neurons indicating a possible attempt toward endogenous repair of damaged neurons[38]. An intriguing, but yet speculative, use of NPCs in glioma therapy thus includes both therapeutic and regenerative functions.

Taken together, this study provides novel data on the behavior of adult MSCs and NPCs as delivery vehicles in experimental gliomas and points to MSCs as the best suited vector system for delivery of therapeutic transgenes. In addition, we describe differences in tumor-tropic migratory capacities between adult MSCs/NPCs and commonly used immortalized NSC lines. While the migratory capacities possibly may favor the use of NSC lines as vector system, the absence of proliferation of grafted MSCs and NPCs speaks for adult non-immortalized precursor cells as a safer vector system in glioma therapy.

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Bone Marrow Multipotent Mesenchymal Stroma Cells Act as Pericyte-like Migratory Vehicles in Experimental Gliomas

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Bone marrow–derived multipotent mesenchymal stroma cells (MSCs) have emerged as cellular vectors for gene therapy of solid cancers. We implanted enhanced green fluorescent protein–expressing rat MSCs directly into rat malignant gliomas to address their migratory capacity, phenotype, and effects on tumor neovascularization and animal survival. A single intratumoral injection of MSCs infiltrated the majority of invasive glioma extensions (72 ± 14%) and a substantial fraction of distant tumor microsatellites (32 ± 6%). MSC migration was highly specific for tumor tissue. Grafted MSCs integrated into tumor vessel walls and expressed pericyte markers α-smooth muscle actin, neuron-glia 2, and platelet-derived growth factor receptor-β but not endothelial cell markers. The pericyte marker expression profile and perivascular location of grafted MSCs indicate that these cells act as pericytes within tumors. MSC grafting did not influence tumor microvessel density or survival of tumor-bearing animals. The antiangiogenic drug Sunitinib markedly reduced the numbers of grafted MSCs migrating within tumors. We found no MSCs within gliomas following intravenous (i.v.) injections. Thus, MSCs should be administered by intratumoral implantations rather than by i.v. injections. Intratumorally grafted pericyte-like MSCs might represent a particularly well-suited vector system for delivering molecules to affect tumor angiogenesis and for targeting cancer stem cells within the perivascular niche.

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INTRODUCTION

Solid tumors are dependent of neovascularization to grow beyond a certain size.[1] Several antiangiogenic drugs have therefore been developed to target the tumor vasculature.[2] The tumor vasculature consists of endothelial cells which form the inner lining of vessels, and pericytes (perivascular cells) which provide structural support to endothelial cells. Pericytes also play an important role for tumor vessel function and survival by providing, *e.g.*, vascular endothelial growth factor (VEGF) to endothelial cells.[3,4] It has been suggested that bone marrow–derived endothelial cells and pericytes are recruited to contribute to the neovasculature of tumors,[4–8] although recent data question the contribution of bone marrow circulating endothelial precursors to tumor vascular endothelium.[9] Impaired recruitment of endothelial cells from the bone marrow leads to inhibition of tumor angiogenesis and decreased tumor growth.[6] Depletion of pericytes causes hyperdilation of tumor vessels and increased apoptosis of tumor cell endothelium.[4] Therapeutic targeting of tumor vasculature may thus involve targeting of both endothelial cells and pericytes.[10]

Glioblastoma multiformae (GBM) is a highly malignant brain tumor which displays extensive neovascularization. The growth pattern of GBM is characterized by tumor extensions and tumor microsatellites growing far away from the main tumor mass into the normal brain. Conventional therapy is not curative and the shortcomings of GBM treatment have, in part, been attributed to the failure to target the infiltrating tumor cells.[11] Antiangiogenic treatment has been explored in malignant gliomas.[12] Systemic administration of angiogenic inhibitors as well as local delivery of antiangiogenic factors by means of viral-vector mediated gene delivery and encapsulated producer cells have been utilized. Although these modes of delivery have shown some promising results against gliomas there are serious shortcomings, such as short half-life of systemically administered inhibitors, host immune response to viral vectors and inefficient intratumoral spread of viral vectors and encapsulated cells.[12]

Neural, mesenchymal, and endothelial stem or progenitor cells have, because of their tumor-tropic migratory capacity, emerged as promising delivery vehicles of antitumoral substances in therapy of malignant tumors, including GBM.[13–17] Culture-derived mesenchymal stroma cells (MSCs), also called mesenchymal stem cells or marrow stroma cells, possess tropism for experimental tumors, including gliomas, following intraarterial and intracranial injections.[16,17] Moreover, MSCs genetically modified to produce antitumoral substances have been shown to prolong survival in experimental brain tumor therapy.[16,17] Because of the infiltrative

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growth pattern of GBM, the success of MSCs as delivery vehicles in human GBM therapy will likely depend on their capacity to migrate to invasive tumor extensions and to distant tumor microsatellites. A quantitative assessment of the capacity and specificity of intratumorally grafted MSCs to infiltrate tumor tissue is highly warranted. Furthermore, their phenotype and relation to tumor microvessels need to be clarified.

In this study, we grafted bone marrow–derived rat MSCs into established orthotopic N29 and N32 rat malignant brain tumors. These glioma models are syngeneic when implanted in Fischer344 rats and exhibit characteristics of human high-grade malignant brain tumors. From a clinical perspective, grafting of MSCs into tumor remnants following surgical tumor resection is a potentially efficient route of delivery of therapeutic transgenes. MSCs can be isolated from the bone marrow, expanded in culture, and genetically modified to produce antitumoral substances before grafting. The aims of this study were to explore the capacity of intratumorally grafted and intravenously (i.v.) injected MSCs to migrate to invasive tumor cells, and to investigate the association between MSCs and tumor vasculature as well as the phenotype of MSCs in brain tumors.

RESULTS

MSCs characteristics *in vitro*

Following seeding in mesenchymal culture medium, typical fibroblastoid colonies could be detected after a few days rapidly forming a monolayer of adherent cells. MSC cultures contained a homogenous population of spindle-shaped cells (**Figure 1a**). To assay the differentiation potential of the MSCs, second passage cells were grown to near confluency and assayed for differentiation into osteoblasts or adipocytes. When MSC cultures were incubated in the adipogenic medium, lipid vacuoles could be clearly detected by Oil-red-O staining (**Figure 1b**). When cells were grown in the osteogenic medium, a change in cell morphology was found from spindle shaped to cuboidal shaped and calcium mineral deposits could be observed (**Figure 1c**). MSCs grown in control medium, on the other hand, showed no signs of differentiation.

Flow cytometric analysis revealed that MSCs were positive for the typical mesenchymal markers CD73 and CD90 but negative for the hematopoietic marker CD45. While CD73 expression was homogenous in all MSCs, CD90 was heterogeneously expressed in one high (60% of total cells)- and one low (40%)-expressing subpopulation (**Figure 1d**).

Enhanced green fluorescent protein expression of MSCs

To visualize the MSCs following grafting *in vivo*, we genetically modified the cells to express enhanced green fluorescent protein (eGFP). MSCs were transduced with a Moloney leukemia–based retroviral vector, which has the characteristic of infecting dividing cells. Flow cytometry revealed that >98% of MSCs expressed eGFP *in vitro*.

MSC migration to tumor extensions and tumor microsatellites

The growth pattern of the malignant N29 tumor resembles the invasive growth pattern of human GBM, which means that the tumor grows with infiltrative tumor extensions reaching far into the normal brain and with distant tumor microsatellites separated

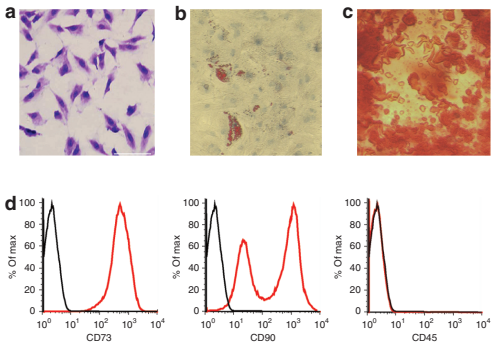
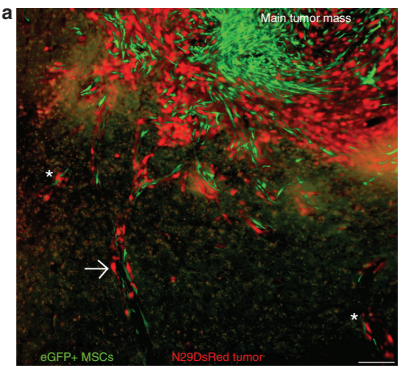


Figure 1 ***In vitro*** **characteristics of bone marrow–derived rat multipotent mesenchymal stroma cells (MSCs).** (**a**) Spindle-shaped morphology of MSCs generated from adult bone marrow. Cultures were stained with crystal violet. (**b**) Differentiation capacity of MSCs into adipocytes and (**c**) osteoblasts was assessed for passage 2 cells. (**b**) Lipid vacuoles were stained with Oil-red-O. (**c**) Osteogenic differentiation was detected by staining with Alizarin Red. Bar = 60 µm. (**d**) Flow cytometric analysis of MSCs using antibodies against CD73 (left panel), CD90 (middle panel), and the hematopoietic marker CD45 (right panel).



b

Days following MSC grafting	Tumor extensions infiltrated by MSCs	Distant tumor microsatellites infiltrated by MSCs
5	58 ± 16%	19 ± 9%
10	71 ± 6%	25 ± 8%
15	72 ± 14%	32 ± 6%

Figure 2 eGFP+ MSCs grafted into established N29DsRed tumor migrate along infiltrative tumor extensions and to distant microsatellites. The invasive growth pattern of the N29 tumor resembles that of glioblastoma multiformae. (**a**) eGFP+ MSCs (green) are mainly located within the main tumor mass but several mesenchymal stroma cells (MSCs) are also found along tumor extensions (arrow) and at distant tumor microsatellites (asterisks) infiltrating into the normal brain parenchyma. Only minimal numbers of grafted eGFP+ MSCs can be found in normal brain tissue adjacent to tumor. Bar = 100 µm. (**b**) Number of tumor extensions and distant tumor microsatellites infiltrated by eGFP+ MSCs at various time points following MSC grafting into established N29DsRed tumor. Data are shown as mean ± SEM, n = 4–5 animals for each time point. eGFP, enhanced green fluorescent protein.

from the main tumor mass. The numbers of tumor extensions and distant tumor microsatellites were 7.2 ± 1.4 and 4.6 ± 1.0 per section on day 15 following tumor cell inoculation, 9.9 ± 0.7 and 9.4 ± 0.6 on day 20, and 10.1 ± 1.2 and 12.7 ± 2.2 on day 25. We estimated the capacity of intratumorally grafted eGFP+ MSCs to migrate to tumor extensions and to distant tumor microsatellites. The majority of tumor extensions contained at least one, but most often several, eGFP+ MSCs and MSCs were also found in a substantial fraction of distant tumor microsatellites 5, 10, and 15 days following MSC grafting (**Figure 2a,b**). The capacity of MSCs to migrate to distant tumor extensions and microsatellites was persistent in parallel with the infiltrative growth of the N29 tumor (**Figure 2b**). Only minimal numbers of intratumorally grafted MSCs were found in normal brain parenchyma adjacent to tumor (**Figure 2a**). The numbers of grafted eGFP+ MSCs were, 15 days following MSC grafting, 0.5 ± 0.1 per section in normal brain tissue adjacent to tumor, 41.5 ± 5.0 in tumor extensions ($P < 0.05$, compared to MSCs numbers in normal brain tissue) and 7.1 ± 2.7 in tumor microsatellites ($P < 0.05$).

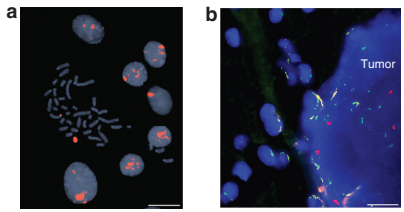


Figure 3 Whole-chromosome fluorescent *in situ* hybridization (FISH) painting for the Y chromosome in mesenchymal stroma cells (MSCs) derived from a male rat. (**a**) A metaphase spread with a Y chromosome (large red signal) and several interphase nuclei with a corresponding signal pattern. (**b**) Tissue section showing parts of normal brain parenchyma and N29 tumor. Male-derived MSCs are grafted into N29 tumors (female-derived tumor) established in female hosts ($n = 4$). Cell nuclei are depicted by 4′,6-diamidino-2-phenylindole staining (blue). The intratumoral localization of male-derived MSCs is confirmed by Y chromosome signals (red). Enhanced green fluorescent protein expression was lost because of heavy enzymatic pretreatment. No Y carrying cells can be seen in the normal brain. FISH for chromosome 12 (green) is used as positive control. Bar = 10 µm in **a** and 15 µm in **b**.

Fluorescent *in situ* hybridization

In addition to analysis of eGFP+ cells, fluorescent *in situ* hybridization (FISH) was used to independently assess MSC migration *in vivo*. MSCs, derived from a male rat, were grafted into female animals, carrying the N29wt tumor developed in a female animal. MSC migration pattern was analyzed on day 15 following intratumoral grafting. FISH for the Y chromosome (red signal) was performed to specifically label MSCs (*in vitro*, **Figure 3a**). eGFP expression of the MSCs was lost in this experiment because of heavy enzymatic pretreatment of the tissue sections. However, cells carrying the Y chromosome were clearly visualized, locating within the N29wt tumor. No Y chromosome–positive cells were seen in the normal brain. FISH for rat chromosome 12 (green signal) was performed as positive control (**Figure 3b**).

Grafted MSCs migrate to tumor endothelium and do not express endothelial markers or affect tumor microvessel density

Colabeling of eGFP and rat endothelial cell antigen (RECA) was performed to assess the association between grafted MSCs and tumor endothelium. There was a strong tropism of grafted MSCs to RECA+ tumor vessels (**Figure 4a**), but not to RECA+ blood vessels in normal brain tissue adjacent to tumor (**Figure 4b**). Quantitative analysis revealed that 84 ± 4% of the migratory MSCs closely associated to RECA+ tumor vessels and a significant fraction of MSCs also integrated into the vessel wall (**Figure 4a**). Highly vascularized tumor extensions attracted higher numbers

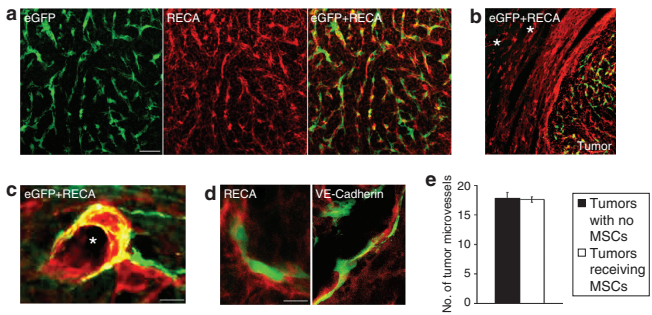


Figure 4 Grafted eGFP+ MSCs closely associate to tumor endothelium and do not express endothelial cell markers. (**a**) eGFP+ MSCs (green) 8 days following grafting into established N32wt brain tumor. Tumor endothelium is delineated by rat endothelial cell antigen (RECA; red). The majority of mesenchymal stroma cells (MSCs) are closely associated to tumor endothelium. (**b**) Intratumorally grafted MSCs migrate along RECA+ tumor vessels and do not associate with RECA+ blood vessels in normal brain tissue adjacent to tumor. Asterisks indicate major blood vessels in normal brain. (**c**) Grafted eGFP+ MSCs closely attached to a major RECA+ tumor vessel. Asterisk indicates tumor vessel lumen. (**d**) Confocal microscopy analysis was used to determine coexpression of grafted eGFP+ MSCs with endothelial markers RECA or VE-Cadherin within tumors. Grafted eGFP+ MSCs attached to tumor endothelium (RECA and VE-Cadherin, red) and without coexpression of RECA or VE-Cadherin. Bar = 60 µm in **a**, 100 µm in **b**, 20 µm in **c**, and 10 µm in **d**. (**e**) Quantification of RECA+ tumor microvessels revealed no difference in tumor microvessel density in tumors receiving eGFP+ MSCs compared to tumors with no MSCs. Data are shown as mean ± SEM, $n = 4$ in each group. eGFP, enhanced green fluorescent protein; VE-Cadherin, vascular endothelial-Cadherin.

of MSCs compared to tumor extensions with a lower vascular density. Migratory MSCs, clearly separated from the core of the graft, could be found closely attached to major tumor vessels (**Figure 4c**), whereas minimal numbers of MSCs were seen in the surrounding nonvascularized tumor area.

We determined whether MSCs themselves are differentiating into endothelial cells and directly contribute to tumor endothelium. There was no MSC expression of the endothelial cell markers RECA or vascular endothelial-Cadherin (VE-Cadherin) *in vitro* (data not shown). Confocal microscopy analysis was used to determine coexpression of grafted eGFP+ MSCs, integrated to tumor vessels, with RECA or VE-Cadherin. We found no evidence of grafted eGFP+ MSCs that coexpressed RECA or VE-Cadherin (**Figure 4d**). We therefore conclude that MSCs are nonendothelial cells which become closely associated and integrate to tumor neovasculature following intratumoral grafting.

To investigate the effect of MSC grafting on tumor microvessel density, we quantified the numbers of RECA+ tumor vessels in tumors receiving eGFP+ MSCs and in tumors with no eGFP+ MSCs. Results indicate that there was no difference in tumor microvessel density between the two groups (17.8 ± 0.9 for tumors with no MSCs and 17.6 ± 0.5 for tumors with MSCs, **Figure 4e**).

MSCs express pericyte markers *in vitro* and *in vivo*

In light of the tropism and close association of grafted MSCs to tumor vasculature, we analyzed the MSC expression pattern of perivascular cell (pericyte) markers α-smooth muscle actin (α-sma),[4,18] neuron-glia 2 (NG2),[19,20] and platelet-derived growth factor receptor-β (PDGF-receptor-β).[4,21] *In vitro*, the absolute majority (>99%) of MSCs expressed pericyte markers α-sma, NG2, and PDGF-receptor-β (**Figure 5a**). A high fraction of eGFP+ MSCs grafted into established tumors continued to express pericyte markers α-sma (~60%, **Figure 5b**) and NG2 (~80%, **Figure 5c**). We found PDGF receptor-β–expressing cells with migratory morphology within tumors receiving MSCs; however eGFP expression was lost in the tissue processing and we were therefore unable to colocalize eGFP with PDGF-receptor-β *in vivo* (**Figure 5d**). Thus, MSCs utilized in our experiments display a pericyte-like phenotype *in vitro* and *in vivo*.

Sunitinib treatment inhibits MSC migration into tumors

N32wt glioma-bearing animals were treated with either vehicle or the antiangiogenic drug Sunitinib which inhibits multiple tyrosine kinase receptors including PDGF-receptor-α and -β, VEGF receptor-1 and -2, KIT (stem cell factor receptor), and Fms-like tyrosine kinase-3 receptor.[22] The numbers of tumor microvessels and grafted MSCs within tumors were quantified 4 days following MSC grafting. As expected, the numbers of tumor microvessels decreased in animals receiving Sunitinib treatment. The numbers of RECA+ microvessels within tumors were, after vehicle treatment, 20 ± 1.8, compared to 8 ± 0.7 following treatment with Sunitinib (**Figure 6a**, $P < 0.05$, unpaired *t*-test). We counted the numbers of eGFP+ MSCs that migrated from the injection site, within or outside tumor, to tumor periphery. Treatment with Sunitinib effectively reduced the numbers of eGFP+ MSCs migrating to tumor periphery compared to the numbers of migrating MSCs following

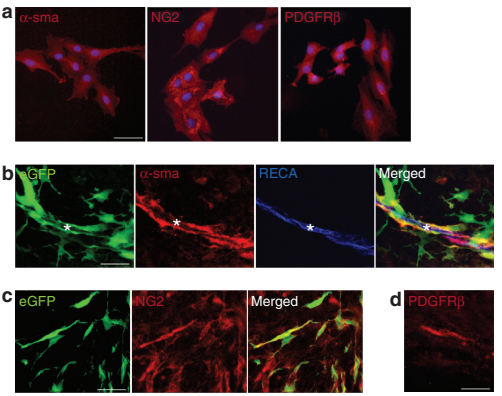


Figure 5 Mesenchymal stroma cells (MSCs) express pericyte markers *in vitro* and *in vivo*. (**a**) The absolute majority (>99%) of MSCs express pericyte markers α-smooth muscle actin (α-sma), neuron-glia 2 (NG2) and platelet-derived growth factor receptor-β (PDGF-receptor-β) *in vitro*. Cells are counted-stained with 4′,6-diamidino-2-phenylindole nuclear staining (blue). (**b**) eGFP+ MSCs grafted into established tumors continue to express pericyte marker α-sma (red) and localize closely to a tumor vessel [rat endothelial cell antigen (RECA), blue)]. Other eGFP+ MSCs display no or very weak α-sma expression. Asterisks indicate tumor vessel lumen. (**c**) eGFP+ MSCs expressing NG2 within tumor. (**d**) Fresh-frozen tissue section showing a representative PDGF receptor-β–expressing migratory cell with typical MSC morphology found within tumors containing grafted MSCs. The expression of enhanced green fluorescent protein (eGFP) was lost in tissue processing. Bar = 50 µm in **a** and 30 µm in **b–d**.

administration of vehicle. The numbers of eGFP+ MSCs within tumor periphery were, after vehicle treatment, 40 ± 2.2, compared to 10 ± 1.9 following treatment with Sunitinib (**Figure 6b**, $P < 0.05$, unpaired *t*-test). High numbers of grafted MSCs were found migrating into the tumor core in vehicle-treated animals while grafted MSCs were found mainly in the outer tumor periphery in Sunitinib-treated animals.

Grafted MSCs do not prolong survival of tumor-bearing animals

To investigate the effect of MSC grafting on survival of glioma-bearing animals, we grafted MSCs intratumorally on day 2 into N29 or N32 tumor–bearing animals. Animals were killed when symptoms of tumor growth emerged. There was no significant difference in survival of tumor-bearing animals that received eGFP+ MSCs intratumorally compared to animals that received control medium. The mean survival for N32 tumor–bearing animals receiving MSCs was 24 (range: 21–27) days and for tumor-bearing animals receiving control medium 23 (range: 21–25) days. N29 tumor–bearing animals that received MSCs survived 44 (range: 32–56) days and animals that received control medium 49 (range: 40–54) days.

No evidence of MSC homing to established gliomas following i.v. injections

N29 or N32 glioma cells were inoculated intracerebrally and eGFP+ MSCs were administered systemically by i.v. injections

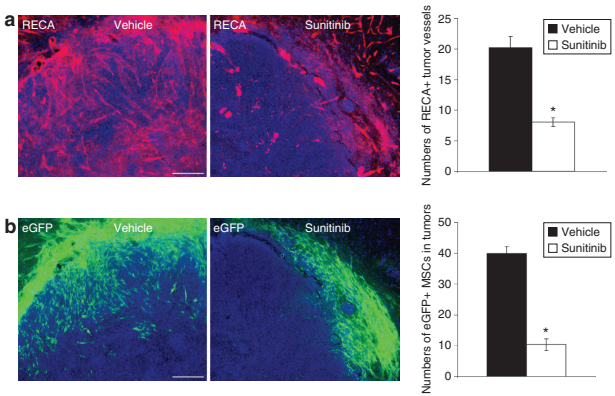


Figure 6 The antiangiogenic drug Sunitinib decreases tumor vascularization and the numbers of grafted mesenchymal stroma cells (MSCs) migrating to the periphery of N32 gliomas. (**a**) Tumor microvessels [rat endothelial cell antigen (RECA), red] in N32wt tumors (Hoechst nuclear staining, blue) following administration of either vehicle or Sunitinib. Treatment with Sunitinib significantly decreased the numbers of RECA+ tumor microvessels. (**b**) High numbers of grafted eGFP+ MSCs (green) were found migrating from the injection site to tumor periphery (Hoechst, blue) following administration of vehicle. Treatment with Sunitinib effectively reduced the numbers of grafted MSCs found within tumor periphery. Data are presented as mean ± SEM. *$P < 0.05$, $n = 4$ in each group. Bar = 170 µm in **a** and **b**. eGFP, enhanced green fluorescent protein.

14 days later. Animals were killed 2 and 7 days following MSC injections. At both time points, we found vascularized tumors in the striatum of all animals but careful microscopic analysis of serial sections revealed no evidence of eGFP+ MSCs within tumors. Neither did we detect NG2 or α-sma expressing cells with spindle-shaped morphology within tumors.

DISCUSSION

The principal findings of this study are that bone marrow–derived MSCs, grafted into malignant brain tumors, associate closely and integrate to tumor vessels and express typical pericyte markers *in vitro* as well as *in vivo* within tumors. MSCs, identified by their adherent growth, marker expression profile, and mesenchymal differentiation capacity, have been used as cellular delivery vehicles in various types of cancers, including melanoma, breast cancer, Kaposi's sarcoma, lung cancer, and brain tumors.[16,17,23–26] Our findings suggest that the cells used in these previous studies might associate to tumor vessels and possibly act like pericytes within tumors. Furthermore, we demonstrate by quantitative analysis that intratumorally grafted bone marrow–derived MSCs migrate effectively to infiltrative tumor extensions and display capacity to migrate to distantly located tumor microsatellites. MSC grafting did not affect tumor microvessel density or survival of glioma-bearing animals. Our findings indicate that tumor angiogenic signaling factors, and possibly tumor vessels *per se*, are important in the recruitment of grafted MSCs within tumors. However, we found no evidence of MSC homing to established gliomas following i.v. injections.

Tumor neovasculature is critical for tumor growth[1] and the finding that grafted MSCs associates to and integrates into tumor vessels indicates that these cells may act as very potent vehicles for delivery of antiangiogenic substances to vascularized tumors. Antiangiogenic therapy of gliomas and of brain metastases have previously shown that tumors might adopt to decreased vessel density and hypoxia by increased migration along preexisting microvessels and by increased perivascular satellite formation, a phenomenon known as vascular co-option.[27,28] This needs to recognized when designing antiangiogenic trials for gliomas but it is possible that MSCs robust capacity to track tumor extensions and microsatellites might overcome this therapeutic challenge.

Human MSCs can be found in human glioma xenografts in immunocomprised mice following intracarotid injections.[16] We pursued to investigate whether i.v. injected rat MSCs home to established syngeneic orthotopic gliomas and we found no evidence of i.v. injected MSCs within tumors following single injections. Another study reported tropism of i.v. injected human MSCs to melanomas in immunocompromised mice.[26] MSC homing efficiency to subcutaneous tumors was, however, very low because five MSC injections during a 20-day period resulted in homing to only a fraction of melanoma-bearing animals. Furthermore, melanoma lung metastasis were established by i.v. melanoma cell inoculation, and i.v. administered MSCs were found randomly distributed in both the lung parenchyma and tumor at 1 day after injection.[26] This suggests a lack of tumor-specific homing of i.v. injected MSCs in the melanoma lung metastasis model. Although we performed systematic analysis of serial sections, we cannot exclude the possibility that very low numbers of MSCs home to glioma vasculature following i.v. injections. In contrast, a single intratumoral injection of MSCs resulted in infiltration of the majority of the invasive tumor extensions and to a significant fraction of distantly located tumor microsatellites in the invasive N29 glioma model. Thus, our results clearly show that the MSC cellular vector system in glioma therapy should be administered by intratumoral implantation rather than by i.v. injections.

Using two independent methods, retroviral eGFP labeling of MSCs and FISH to track male-derived MSCs grafted into female hosts, we showed that MSC migration was largely restricted to tumor and not to normal brain parenchyma. The tumor-specific migratory capacity of MSCs makes these cells promising cellular vehicles for delivery of cytotoxic substances that specifically can kill tumor cells without substantially damaging normal brain tissue. Moreover, our finding that grafted MSCs intermingled with tumor endothelial cells and tumor pericytes implies that an approach to locally target both endothelial cells and pericytes within tumors is possible. Combinatorial targeting of both tumor endothelium and tumor pericytes by systemic administration of VEGF receptor and PDGF receptor tyrosine kinase inhibitors indeed diminish tumor vascularization more than any of the respective inhibitors individually.[10] The therapeutic implication of the perivascular tropism of grafted MSCs within tumors is further strengthened by recent findings by Calabrese *et al*. demonstrating that cancer stem cells within brain tumors are maintained within a perivascular niche. This study suggests that tumor endothelial cells secrete factors that maintain brain cancer stem cells in a stem cell–like state.[29] Grafted MSCs are therefore strategically well located to deliver substances that can either directly attack cancer stem cells within the perivascular niche, or indirectly by eliminating tumor endothelial cells necessary for cancer stem cell growth. In cancer immunotherapy, blood-borne immune cells, *e.g.*, cytotoxic T cells and natural killer cells, cross the blood–brain barrier to reach the tumor. MSCs, genetically modified to produce proinflammatory substances such as interleukin-2, interferon-β, and interleukin-23, can decrease tumor growth and prolong survival of tumor-bearing animals.[16,17,30] The close association of grafted MSCs to tumor vessels found in the present study may thus explain their capacity to attract blood-borne immune cells to tumors.

Our findings indicate that grafted MSCs utilize tumor vessels as their migratory route. The tumor-specific migratory behavior of MSCs has previously shown to be associated to glioma-produced angiogenic cytokines. *In vitro* assays have revealed that specifically VEGF-A, interleukin-8, transforming growth factor-β1, and neurotrophin-3, all involved in tumor angiogenesis, mediate recruitment of MSCs to gliomas.[31,32] Findings from our study suggest that tumor angiogenic signaling factors regulate MSC migration intratumorally *in vivo*. Although we cannot exclude decreased MSC survival following Sunitinib treatment, the MSC migratory pattern was radically changed by Sunitinib, thus suggesting that tumor vascularization is important for substantial migration of grafted MSCs within tumors. The pericyte-like phenotype of MSCs indicates that endothelial interactions and specifically PDGF-β, sphingosine-1-phosphate, and angiopoietin-1 may mediate MSC recruitment to tumors as these factors contribute to recruit pericytes to tumor vessels.[33,34]

We could not detect an effect of grafted MSCs on tumor microvessel density. This is in contrast to previous findings showing that systemic administration of human MSCs can contribute to angiogenesis in the pathological brain.[35] No effect on survival of tumor-bearing animals was seen in the present study. However, the pericytic phenotype of MSCs gives an indication of their potential functions within tumors. Normal pericytes are essential for endothelial cell survival and function and tumor pericytes may regulate tumor vessel stability, maintenance, and function.[33,36] Recent findings suggest that pericyte maturation within tumors contributes to vascular normalization which in turn enhances the influx of immune effector cells leading to decreased tumor growth.[37] It would be interesting to see whether grafted pericyte-like MSCs can contribute to normalization of tumor vasculature and in this way facilitate immunotherapy of the tumor. In addition, the role of MSCs and pericytes in tumor cell metastasis has recently been addressed. Xian *et al.* demonstrated that pericytes within tumors limit tumor cell metastasis via neural cell adhesion molecule–dependent normalization of tumor vasculature.[38] On the other hand, Karnoub *et al.* showed that MSCs, when mixed with breast cancer cells, can increase the metastatic potency of the cancer via CCL5 signaling.[39] Further studies are needed to clarify the role of grafted pericyte-like MSCs in glioma cell invasion and metastasis.

Future studies should also aim at optimizing the infiltrative capacity of grafted MSCs because not all tumor extensions and microsatellites were infiltrated by MSCs. Grafting of MSCs genetically modified to overexpress receptors crucial for tumor-specific migration,[40] and identification of potential MSC subpopulations more prone to migrate, could in combination with multiple MSC injections, improve the MSC tumor infiltration.

In brief, our results indicate that bone marrow–derived MSCs act as pericyte-like cells following intratumoral grafting, efficiently migrating to and integrating into the tumor neovasculature to which they likely are recruited by factors involved in neoangiogenesis.

MATERIALS AND METHODS

Tumor cell lines and culture. The rat glioma cell lines N29 and N32 were originally induced by transplacental injection of ethyl-*N*-nitrosourea to pregnant Fischer344 rats.[41] Tumor cell culturing was performed as previously described (see **Supplementary Materials and Methods**).[42]

Flow cytometry. MSCs were detached using Trypsin/0.5% EDTA (GIBCO, Invitrogen, Carlsbad, CA) and washed in phosphate-buffered saline plus 1% bovine serum albumin. Primary antibodies were mouse anti-rat CD45 (1:25; BD Bioscience, Franklin Lakes, NJ), mouse anti-rat CD73 (1:400; BD Bioscience), and mouse anti-rat CD90 (1:400; BD Bioscience). Cells were blocked in 5% normal goat serum in phosphate-buffered saline and stained with secondary antibody goat anti-mouse-IgG $F(ab')_2$ APC (Jackson Immunoresearch Laboratories, West Grove, PA). Secondary antibody alone served as control for unspecific binding. Samples were measured on a FACSCalibur flow cytometer with CellQuest software (BD Bioscience) and data were analyzed using FlowJo software (Tree star, Ashland, OR).

Animal procedure and experimental design. Adult male and female 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, Sweden. Animals were anesthetized with isoflurane (2.5% in O_2, Forene; Abbott Scandinavia AB, Solna, Sweden) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). The following coordinates were used for tumor cell inoculation and stem cell grafting (relative to bregma): anterior–posterior: +1.7, medial–lateral: −2.5, and dorso–ventral: −5.0. Tooth bar was set at −3.3 mm. Tumor cells were inoculated at 1 µl/min, using a 10-µl Hamilton syringe. eGFP+ MSCs (2.5×10^6 cells) were grafted at 0.5 µl/min, using 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 slowly retracted. The above-mentioned parameters were used in all experiments unless otherwise mentioned.

To study MSC migration along tumor extensions and to distant tumor microsatellites, 3,000 N29DsRed tumor cells were inoculated on day 1 into the striatum of adult rats ($n = 15$). eGFP+ MSCs were grafted on day 10 and the animals were killed on day 5, 10, and 15 following MSC grafting ($n = 4$–5 animals for each group). For FISH, eGFP+ MSCs were grafted intratumorally into N29wt tumor–bearing female rats ($n = 4$) using the above-mentioned parameters.

Animals ($n = 4$ in each group) were treated p.o. once daily for 6 days with either vehicle solution (0.5% Polysorbate80; Sigma, St. Louis, MO, and 10% polyethylene glycol; Sigma, dissolved in water, pH adjusted to 3.5) or Sunitinib (50 mg/kg, kindly provided by Pfizer, New York, NY) dissolved in vehicle solution. Three thousand N32wt glioma cells were inoculated on day 1 and treatment with either vehicle or Sunitinib started on day 12. On day 14, eGFP+ MSCs were grafted into tumors and animals were killed on day 18. Animals were continuously observed for signs of illness.

We performed two survival studies to assess the effect of grafted MSCs on tumor growth. Three thousand N32 tumor cells were inoculated on day 1 and eGFP+ MSCs ($n = 8$) or control medium ($n = 8$) was inoculated intratumorally on day 2. In a similar experiment utilizing the invasive N29 tumor model, 5,000 N29 tumor cells were inoculated on day 1 and eGFP+ MSCs ($n = 10$) or medium ($n = 10$) was stereotactically injected intratumorally on the following day. Animals were continuously observed and immediately killed when symptoms of tumor growth (*e.g.*, reduced motor activity, reduced washing, weight loss) were seen.

MSCs were administered i.v. to glioma-bearing animals. N29 tumor cells (5,000 cells/5 µl) or N32 tumor cells (3,000 cells/5 µl) were inoculated intracerebrally as previously described and eGFP+ MSCs (2×10^6 cells in 500 µl) were administered during 5 minutes via the tail vein 14 days later. Animals were killed either 2 or 7 days following MSC injections ($n = 5$ animals for each tumor type and time point).

Immunohistochemistry. Processing of brains and immunohistochemistry on tissue sections were performed as previously described.[42] Alternatively, for PDGF-receptor-β immunohistochemistry, brains were removed and placed for 2 minutes in isopentane at a temperature of −50 °C. Brains were kept in −80 °C until sectioned (6 µm) on a freezing microtome. Fresh-frozen tissue sections were mounted onto glass slides and fixated with Acetone for 10 minutes. Primary antibodies were chicken anti-GFP (1:1,500; Chemicon, Temecula, CA), mouse anti-rat RECA (1:100; Serotec, Oxford, UK), mouse anti-VE-Cadherin (1:150; Abcam, Cambridge, UK), mouse anti-NG2 (1:500; Chemicon), rabbit anti-α-sma (1:400; Abcam), and rabbit anti-PDGF-receptor-β (1:100; Abcam). The following secondary antibodies were used: Alexa488 goat anti-chicken (1:500; Molecular Probes, Eugene, OR), Alexa594 goat anti-mouse (Molecular Probes), Alexa594 goat anti-rabbit (Molecular Probes), and Cy5 goat anti-mouse (Jackson Immunoresearch Laboratories). Free-floating sections were mounted onto glass slides. All glass slides were cover-slipped with 1,4-diazabicyclo[2.2.2] octane mounting medium.

FISH. FISH was performed on 25-µm thick tissue sections pretreated with 1 mg/ml proteinase K in phosphate-buffered saline for 1 hour, followed by 20 mg/ml pepsin in 0.01 mol/l HCl for 1 hour. After protease treatment, sections were washed in buffered saline, dehydrated in 70–85–96% ethanol, and codenatured for 10 minutes with the rat Y/12 Probe (Y Cy3; 12 FITC, CA-1631; Cambio, Cambridge, UK) for the rat Y chromosome and rat chromosome 12. Hybridization was performed overnight at 37 °C. Stringency washing was performed in 0.2× saline-sodium citrate for 5 minutes and sections were counterstained by 4′,6-diamidino-2-phenylindole.

Microscopic analysis. Sections were examined with an Olympus BX60 epifluorescense microscope (Olympus, Tokyo, Japan) using ×20 objective or by confocal laser scanning microscopy (Leica Microsystems, Mannheim, Germany).

The capacity of grafted eGFP+ MSCs to migrate within invasive N29DsRed tumors was estimated. A tumor extension was defined as an arm of the tumor, coherent with the main tumor mass, infiltratively growing into the normal brain parenchyma. A distant tumor microsatellite was defined as a cluster of tumor cells, clearly separated from the main tumor mass. Systematic histological analysis of tumor area of interest was performed to determine whether clusters of cells were cross-sections of tumor extensions or microsatellites. The number of tumor extensions and distant tumor microsatellites containing at least one eGFP+ MSC were counted and divided by the total number of tumor extensions and distant tumor microsatellites, respectively. For each animal, one section containing dorsal tumor periphery, one section containing tumor center, and one section containing ventral tumor periphery were quantified. However, 3–10 sections, depending on the size of the microsatellite, were analyzed (to distinguish cross-sections of tumor extensions from microsatellites) for every section that was quantified. Four to five animals at each time point were analyzed.

To assess eGFP+ cells in close association with tumor blood vessels, the number of eGFP+ cells located within the distance of one cell body from tumor vessels (delineated by RECA) were counted within a 0.2 mm × 0.2 mm grid, using a ×20 objective, and divided by the total number of eGFP+ cells within the area. Five animals from day 8 to 12 following MSC grafting were included and three sections per animal were quantified.

Tumor microvessel density was assessed by quantification of the numbers of RECA+ tumor vessels within a 0.2 mm × 0.2 mm grid, using a ×20 objective. The numbers of RECA+ tumor vessels were counted in tumors with high numbers of migratory eGFP+ MSC and in tumors with no eGFP+ MSCs. Four animals from each group were chosen and five fields of each tumor were analyzed.

In the Sunitinib experiment, tumor microvessel density was assessed by quantification of the numbers of RECA+ tumor vessels within a 0.2 mm × 0.2 mm grid, using a ×20 objective. The numbers of eGFP+ MSCs, found migrating from the injection site within or outside tumor to tumor periphery, were counted within a 0.2 mm × 0.2 mm grid, using a ×40 objective. Four fields of each tumor were analyzed.

Statistical analysis. Student's unpaired *t*-test was used for comparison between groups. Data are presented as means ± SEM and considered significant at $P < 0.05$. Survival curves were compared using a log rank test. Survival is presented as mean (range).

SUPPLEMENTARY MATERIAL

Materials and Methods.

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Bone marrow multipotent mesenchymal stroma cells act as pericyte-like migratory vehicles in experimental gliomas

Supplementary Information:
Supplementary Materials and Methods

Tumor cell lines and culture

The cells were maintained in R10 medium, consisting of: RPMI 1640 medium (1X) with L-glutamine supplemented with 10% fetal bovine serum (FBS) (VWR, West Grove, PA), 10 mM HEPES buffer solution, 1 mM sodium pyruvate and 50 µg/ml gentamicin (all chemicals except FCS from GIBCO, Invitrogen, Carlsbad, CA). R10 medium was changed every 2-3 days and cells were passaged about twice a week and detached using trypsin-EDTA (0.25% trypsin with EDTA 4Na) 1X (Invitrogen). Cells were incubated at 37°C in a humidified atmosphere containing 6.0% CO_2. Cells were kept in culture for a maximum of 6 weeks. Before inoculation *in vivo*, cells were washed and resuspended in medium without FBS and gentamicin (referred to as R0 medium). Cells were counted and resuspended in a volume corresponding to the target concentration.

DsRed2-labeling of N29 tumor cells

At 80% confluency, N29 tumor cells were transduced in R10 medium at different multiplicity of infection (MOI), from MOI=1 to 10, with a Moloney leukemia-derived viral vector encoding the Discosoma red fluorescent protein (DsRed2) under control of the viral promoter and a splicing acceptor sequence (pCMMP-IRES2Dsred2-WPRE). Protamine sulfate was added to the medium to increase the transduction efficiency (4 µg/ml, Sigma). Cells were incubated at 37°C in a humidified atmosphere containing 6.0% CO_2. The day after addition of viral vector, tumor cells started to express the fluorescent protein DsRed2. Transduced cells were then purified through single cell cloning. Cells were counted and diluted to obtain concentrations of 5 cells and 0.5 cells/200 µl R10 medium per well in 96-well plates. An incubation period for 10 days followed in order for colonies to form. DsRed2 positive colonies formed from single cells were washed with PBS, trypsinated, resuspended in R10 medium and collected in a 6-well plate. After a few days, when the cells had reached a higher confluence, cells were transferred to a T25 flask in 5 ml R10 medium. Single cell cloning resulted in a DsRed2 expression *in vitro* of 98% as analyzed by flow cytometry.

eGFP retroviral production and transduction of MSCs

The Moloney leukemia retroviral vector pCMMP-IRES2eGFP-WPRE used in this study has been described elsewhere[1, 2] The viral vector is replication incompetent due to both a deletion mutation in its 3' long term repeat region and a lack of genes that are vital for its replication: *gag*, *pol* and *env*. The retrovirus backbone contains the following elements: a 5' long terminal repeat (LTR) sequence, a downstream splicing acceptor sequence, which, together with the LTR drive the expression of the enhanced green fluorescent protein (eGFP) reporter gene, an internal ribosomal entry sequence, upstream to the eGFP reporter sequence and woodchuck hepatitis virus post-transcriptional regulatory sequences. The viral particles were produced as described using the producer cell line 293VSVG[3]. Concentrated particles were resuspended into 0.5 ml of DMEM medium (Sigma-Aldrich, St. Louis, MO). The titer was measured by FACSCalibur analysis, based on eGFP reporter gene expression, 3 days after infection of the HT1080 cells and varied from $0.7x10^9$ to $1.2x10^9$ TU/ml depending on the batches. When at 60-70% confluency, 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 1µg/ml (Sigma).

MSC isolation and culture

MSC cultures were derived from the Fischer344 male rat bone marrow (8 weeks old). The animal was deeply anaesthetized with Isoflurane (2,5% in O_2, Forene, Abbott Scandinavia AB, Solna, Sweden), sacrificed and femur was dissected and adherent tissue removed. The marrow cavity was flushed with PBS supplemented with 2% FBS (GIBCO). 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-alpha supplemented with 10% FBS and 1% Antibiotic-Antimycotic-Solution(Sigma). Non-adherent cells were removed after three days and culture medium was changed weekly thereafter. Cells were passaged at 80-90% confluency.

MSC differentiation potential was assessed using 2nd passage MSCs. Adipogenic differentiation was induced by supplementation of MSC cultures with 10% adipogenic stimulatory supplements (StemCell Technologies, Vancouver, Canada). The differentiation medium was changed every third day. After two weeks, the cells were fixed with 10% formalin, and stained with fresh Oil-red-O solution (Sigma)[4]. Osteogenic differentiation was induced by incubating the cells with medium supplemented with 0.1 μM dexamethasone (Sigma), 0.05 mM ascorbic acid-2-phosphate (Wako, Osaka, Japan), and 10 mM β-glycerophosphate (Sigma). Medium was changed every third day for two weeks. Cultures were washed with PBS, fixed in ice-cold 70% ethanol for 1h, and stained for 10 min with 40 mM Alizarin red (Sigma)[5].

Immunocytochemistry of MSCs

Cultured MSCs were harvested and transferred to multi-chamber culture slides (BD Bioscience, Franklin Lakes, NJ) four days before immunocytochemistry. Cells were fixed in 4% paraformaldehyde (PFA) for 30 min, and permeabilized using 0.3% Triton X-100 for 5 min. The cells were blocked with 5% NGS for 20 min and incubated with the primary antibodies; chicken anti-GFP (1:2000, Chemicon, Temecula, CA), mouse anti-rat endothelial cell antigen (RECA), (1:100, Serotec, Oxford, UK), mouse anti-neuron-glia 2 (NG2), (1:500, Chemicon), rabbit anti-α smooth muscle actin (α-sma), (1:400, Abcam, Cambridge, U.K) and rabbit anti-platelet derived growth factor (PDGF)- receptor-β, (1:100, Abcam) for 2.5 hours at 37°C. The cells were washed in PBS and incubated with the secondary antibodies Alexa488 goat anti-chicken (Molecular Probes, Eugene, Oregon) and either Alexa594 goat anti-rabbit (Probes) or Alexa594 goat anti-mouse (Probes) for 30 min at 37°C. The chamber-slides were mounted wet using Pro-Long Gold anti-fading reagent (Probes) with nuclear staining (DAPI). Omission of primary antibodies was used as negative control.

References for Supplementary Information

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