Gene therapy of malignant glioma with retroviral vectors and tumor-infiltrating progenitor cells

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1. Abbreviations

BM-TICs	- Bone-marrow derived tumor-infiltrating cells
BM-TIPCs	- Bone-marrow derived tumor-infiltrating packaging cells
CD	- Cluster of differentiation / Cytosine deaminase
CDK	- Cyclin dependent kinase
CT	- Computer tomography
[^{11}C]MET	- methyl-[^{11}C]L-methionine
EGFR	- Epidermal growth factor receptor
FCS	- Fetal calf serum
GC/GCV	- Ganciclovir
GFP	- Green fluorescent protein
GP	- Glycoprotein
HSV-1	- Herpes simplex virus type1
[^{124}I]FIAU	- [124I]-29-fluoro-29- deoxy-1-*b*-D-arabinofuranosyl-5-iodo-uracil
IL	- Interleukin
LCMV	- Lymphocytic choriomeningitis virus
LOH	- Loss of heterozygosity
LTR	- Long terminal repeats
MAPC	- Multipotent adult progenitor cells
MLV	- Murine leukemia virus
MRI	- Magnetic resonance imaging
PD	- Population doublings
PET	- Positron emission tomography
PTEN	- Phosphatase and tensin homology gene
PC	- Packaging cell
RCR	- Replication competent retrovirus
TGF	- Transforming growth factor
tk	- Thymidine kinase
TU	- transducing units
VSV	- Vesicular stomatitis virus
WHO	- World Health Organisation

2. Summary (English)

Gene therapy as a therapeutic strategy in the treatment of human gliomas is limited by the efficacy of gene transfer and intratumoral distribution of viral vectors. The major goals of this study were to enhance the gene transfer to glioma cells *in vivo* by using lentiviral vectors and to improve intratumoral distribution by selecting migratory progenitor cells that could function as packaging cells for the viral vectors. Therefore, the final goal was to establish tumor infiltrating packaging cells that release viral vectors within glioma *in vivo*.

Lentiviral vectors were chosen to deliver genes into glioma cells. In contrast to currently used retroviral vectors, they transduce quiescent as well as mitotic cells. This is of major importance as within a defined treatment window, the majority of tumor cells are not mitotically active. However, lentiviral vectors can also infect normal brain cells. To define vectors with a specific tropism for glioma cells, lentiviral vectors pseudotyped with two different glycoproteins were used. Vectors pseudotyped with glycoproteins of the lymphocytic choriomeningitis virus (LCMV-GP) mediated efficient and specific transduction of rat glioma cells *in vitro* and *in vivo*, whereas vectors pseudotyped with the glycoproteins of the vesicular stomatatitis virus (VSV-G) preferentially transduced normal brain cells [Miletic et al., 2004].

Bone marrow derived progenitor cells were isolated to establish the tumor-infiltrating cells (BM-TICs) that could also serve as packaging cells. BM-TICs have a high passaging capacity *in vitro*, which is necessary for genetic modification and large scale production in the clinic. They were also found to show specific migration towards and into malignant glioma *in vivo*. In a therapeutic approach using BM-TICs stably expressing a suicide gene, an efficient therapeutic effect was demonstrated. The modified cells were also detected *in vivo* by non-invasive positron emission tomography (PET) and therapeutic outcome was followed-up by imaging methods and correlated with histopathology [Miletic et al., 2007].

To test packaging capabilities of BMTICs, the cells were modified with packaging constructs for retroviral LCMV-GP pseudotypes (BM-TIPCs). BM-TIPCs continuously produced retroviral vector particles for several weeks. Upon injection into experimental rat glioma, these cells migrated and were widely distributed within the tumor. Furthermore, released vector particles transduced glioma cells in solid as well as border areas [Fischer, Miletic et al., 2007].

In conclusion, the presented packaging system is highly attractive for future therapeutic applications in human glioblastoma especially in conjunction with an imaging-guided approach.

3. Summary (German)

Die Wirksamkeit der Gentherapie maligner Gliome war in bisherigen klinischen Studien durch einen ineffizienten Gentransfer und eine geringen Ausbreitung von viralen Vektoren innerhalb des Tumorgewebes limitiert. Die Zielsetzung dieser Arbeit war sowohl die Tranduktionseffizienz maligner Gliomzellen durch Verwendung von lentiviralen Vektoren zu erhöhen als auch die Vektorverteilung innerhalb des Tumorgewebes durch Einsatz von migratorischen Vorläuferzellen aus dem Knochenmark zu optimieren. Diese Zellen sollten dabei als Verpackungszellen für die viralen Vektoren dienen.

In der vorliegenden Arbeit wurden lentivirale Vektoren für die Transduktion maligner Gliome untersucht, weil diese im Gegensatz zu den bisher häufig verwendeten retroviralen Vektoren neben teilungsaktiven auch ruhende Zellen infizieren. Dies ist von herausragender Bedeutung, da sich die meisten Tumorzellen innerhalb eines definierten therapeutischen Fensters nicht in Mitose befinden. Ein Nachteil lentiviraler Vektoren ist jedoch, dass sie auch normale Hirnzellen infizieren. Um Vektoren mit einem spezifischen Tropismus für Gliomzellen zu identifizieren, wurden zwei verschiedene pseudotypisierte lentivirale Vektoren verwendet. Dabei zeigten Vektoren, pseudotypisiert mit dem Glykoprotein des Lymphozytären Choriomeningitis Virus (LCMV-GP), *in vivo* und *in vitro* einen hohen Tropismus für maligne Gliomzellen, während Vektoren, pseudotypisiert mit dem Glykoprotein des Vesikulären Stomatitis Virus (VSV-G), bevorzugt normale Hirnzellen transduzierten [Miletic et al., 2004].

Ein weiteres Ziel war nun tumorinfiltrierende Zellen zu generieren, welche als Verpackungszellen für die LCMV-GP Pseudotypen dienen können und das Potential haben, durch intratumorale Migration die Vektorverteilung zu erhöhen. Dazu wurden multipotente adulte Vorläuferzellen (BM-TICs) nach einem etablierten Protokoll aus dem Knochenmark isoliert. BM-TICs sind *in vitro* stark expandierbar, was die Grundlage für die Etablierung einer stabilen Verpackungslinie ist. Darüber hinaus zeigten die Zellen eine ausgeprägte Migrationsfähigkeit innerhalb von experimentellen Rattengliomen *in vivo*. In einem therapeutischen Ansatz konnten wir demonstrieren, dass BM-TICs, stabil transduziert mit einem Suizidgen, einen effizienten therapeutischen Effekt aufwiesen. Weiterhin konnten die modifizierten Zellen mittels Positronen-Emissions-Tomographie (PET) detektiert werden. Auch der

therapeutische Effekt konnte mittels nicht-invasiver Bildgebung verfolgt werden und korrelierte mit der histopathologischen Auswertung [Miletic et al., 2007].
Als „proof of principle“ wurde eine stabile Verpackungslinie für retrovirale LCMV-GP Pseudotypen auf Basis von BM-TICs etabliert (BM-TIPCs). Nach genetischer Modifikation mit den Verpackungskonstrukten produzierten die Zellen kontinuierlich, über einen Zeitraum von mehreren Wochen, retrovirale Vektorpartikel. Bei Injektion in experimentelle Rattengliome, zeigten BM-TIPCs keinen Verlust der Migrationsfähigkeit. Von den Zellen freigesetzte Vektorpartikel transduzierten benachbarte Gliomzellen in soliden Tumorarealen als auch im Randbereich [Fischer, Miletic et al., 2007].
Somit hat das hier vorgestellte System ein hohes Potential für die therapeutische Applikation bei humanen Glioblastomen insbesondere in Verbindung mit nicht-invasiver Bildgebung als Kontrolle der therapeutischen Effizienz.

4. List of publications

I.

Miletic H*+, Fischer Y*, Neumann H, Hans V, Hermann M, Stenzel W, Giroglou T, Deckert M and von Laer D (2004). Selective transduction of malignant glioma by lentiviral vectors pseudotyped with LCMV glycoproteins. *Hum Gene Ther* 15:1091-100.

II.

Miletic H*+, Fischer YH*, Litwak S, Giroglou T, Waerzeggers Y, Winkeler S, Li H, Himmelreich U, Stenzel W, Deckert M, Lange C, Neumann H, Jacobs AH, and von Laer D (2007). Bystander killing of malignant glioma by bone marrow-derived tumor infiltrating progenitor cells expressing a suicide gene. *Mol Ther* 15:1373-81

III.

Fischer YH*, Miletic H*, Litwak S, Giroglou T, Stenzel W, Neumann H, and von Laer D+ (2007). A retroviral packaging cell line for pseudotype vectors based on glioma-infiltrating progenitor cells. *J Gene Med* 9:335-44.

* both authors contributed equally to these studies.

+ corresponding author

Contributions:

I. Hrvoje Miletic performed and analyzed the transduction studies on normal brain cells *in vitro* and *in vivo* and the transduction study on experimental glioma *in vivo.* Further, he acted as corresponding author. Yvonne Fischer constructed the vectors, produced the viral supernatants, and performed the transduction study on tumor cells *in vitro*. Harald Neumann supervised the transduction study on cultured normal brain cells. Volkmar Hans and Werner Stenzel supervised the animal experiments. Werner Stenzel and Manuel Hermann participated in counting transduced cells and in analyzing the statistical data. Tsanan Giroglou helped with the construction of vectors and production of viral supernatants. Martina Deckert supervised manuscript preparation. Dorothee von Laer supervised experimental design and manuscript preparation.

II. Hrvoje Miletic performed and analyzed all *in vivo* studies and acted as corresponding author. Yvonne Fischer constructed the vectors, gene modified the progenitor cells, and performed *in vitro* studies. Sara Litwak isolated the progenitor cells in the laboratory of Harald Neumann (supervisor). Tsanan Giroglou characterized the progenitor cells. Yannic Waerzeggers and Alexandra Winkeler helped with the analysis of PET experiments. Huongfeng Li produced the PET tracers. Uwe Himmelreich performed and analyzed MRI. Wernel Stenzel supervised the animal experiments. Martina Deckert supervised manuscript preparation. Andreas Jacobs supervised the experimental design of the PET and MRI studies. Dorothee von Laer supervised experimental design and manuscript preparation.

III. Hrvoje Miletic tested the packaging cell line *in vivo* for migration and transduction of glioma cells. Yvonne Fischer established the packaging cell line and performed *in vitro* studies. Sara Litwak isolated the progenitor cells in the laboratory of Harald Neumann (supervisor). Tsanan Giroglou participated in the establishment of the packaging cell line and the *in vitro* studies. Werner Stenzel supervised the animal experiments. Dorothee von Laer supervised experimental design and preparation of the manuscript. Further, she acted as corresponding author.

Parts of these data are published in Yvonne Fischer´s PhD thesis (“Gentherapie von malignen Gliomen mit retroviralen Pseudotypvektoren”, Frankfurt am Main, 2005).

5. Introduction

5.1. General aspects of gliomas

Gliomas, the most common primary intracranial neoplasms, are histologically classified as astrocytomas, oligodendrogliomas, ependymomas and mixed gliomas. Grading is performed according to the World Health Organization [1] taking into account the presence of polymorphic nuclei, mitotic activity, endothelial cell proliferation, and necrosis. Glioblastoma, corresponding to WHO grade IV, is the most fatal and most common primary brain neoplasm with an incidence of 3-6 new cases per 100.000 population [1]. Approximately 50 % of all gliomas and 12-15 % of all primary intracranial tumors are glioblastomas.

The current standard treatment for patients with glioblastoma consists of surgical resection followed by radio- and chemotherapy [2]. If surgical access of the lesion is not possible, stereotactic needle biopsy is performed for diagnostic purpose and ^{125}I-seeds can be implanted for interstitial radiation therapy [3]. Despite improvement in these therapeutic fields, the median survival of patients with glioblastoma is still less than one year [1].

5.2. Gene therapy of malignant glioma

Due to the poor prognosis of glioblastoma patients today, many alternative therapeutic strategies are under development. Gene therapy is a promising therapeutic strategy aiming to deliver therapeutic genes into the tumor. Of the gene delivery systems that have been developed, liposomes, viral vectors, and stem cells are the most important ones.

5.2.1. Retroviral versus lentiviral vectors

The most common retroviral vectors used in gene therapy of glioma are derived from the murine leukemia virus (MLV). The genome of the vector contains a marker or a

therapeutic gene and the cis-active elements, which are necessary for packaging, reverse transcription, integration and expression. Major components of these elements are the Long terminal repeats (LTR) and the packaging signal (Ψ) [4] (Figure 1). The genes encoding the viral proteins are deleted, thereby minimizing the risk of replication competent retrovirus (RCR).

Figure 1: retroviral vector after reverse transcription. The long terminal repeats consist of U3, R and U5 regions. The transcription of the transgene is controlled by the enhancer/promoter of the U3 region of the 5´LTR. The packaging signal Ψ mediates the interaction of the vector RNA with gag proteins to package the RNA into viral particles. Modified from Fischer [5].

MLV derived vectors selectively infect mitotic cells [6]. Since the normal brain cells are in a post-mitotic state, these vectors have a specific tropism for glioma cells. In clinical studies, however, MLV-vectors have shown very low transduction efficiencies with no significant therapeutic effects [7]. One major reason is the low percentage of mitotic tumor cells in a given treatment window. In this regard, lentiviral vectors, which also belong to the group of retroviral vectors, are an attractive alternative as these vectors also transduce quiescent cells [8]. However, a major concern is the loss of tumor specificity and the transduction of normal brain cells including neurons. A strategy to improve specificity of these vectors is the introduction of surface glycoproteins from a different virus.

5.2.2. Modification of viral tropism: Pseudotyped retroviral vectors

The tropism of a retroviral vector is determined by its envelope protein (Env), which interacts with a specific receptor on the cell surface, thereby mediating the entry of the viral particle into the cytoplasm [9]. MLV vectors with the amphotropic envelope protein (A-MLV) have been widely used in gene therapy of malignant glioma [10, 11]. The exchange of the retroviral envelope protein by an envelope protein of a different

virus is called pseudotyping and changes the tropism of the viral particle. Pseudotyping is an excellent tool for gene therapy, since viral vectors can be modified to infect certain cell populations. Retroviral and lentiviral vectors have been successfully pseudotyped with glycoproteins from the Vesicular stomatitis virus (VSV-G) [12] and the Lymphocytic Choriomeningitis Virus (LCMV-GP) [13, 14]. The cellular receptor for LCMV-GP has been identified as α-Dystroglycan [15], whereas the receptor for VSV-G has not been determined precisely [16]. Still, the tropism can be influenced by other factors, for example, the virus itself: Lentiviral VSV-G pseudotypes infect neurons and astrocytes in the normal brain [8], whereas retroviral VSV-G pseudotypes have shown specific tropism for malignant glioma cells [17].

5.2.3. Packaging cell lines for retroviral vectors

Retroviral vectors are produced in packaging cell lines, which contain the genes for the viral proteins and enzymes. In these gene segments, the packaging signal has been deleted to avoid packaging into the viral particle. In safe packaging systems the viral genes and the vector are separated on three different plasmids for retroviral vectors [18] and on four different plasmids for lentiviral vectors [19] (Figure 2). Thus, the development of RCR is not expected.

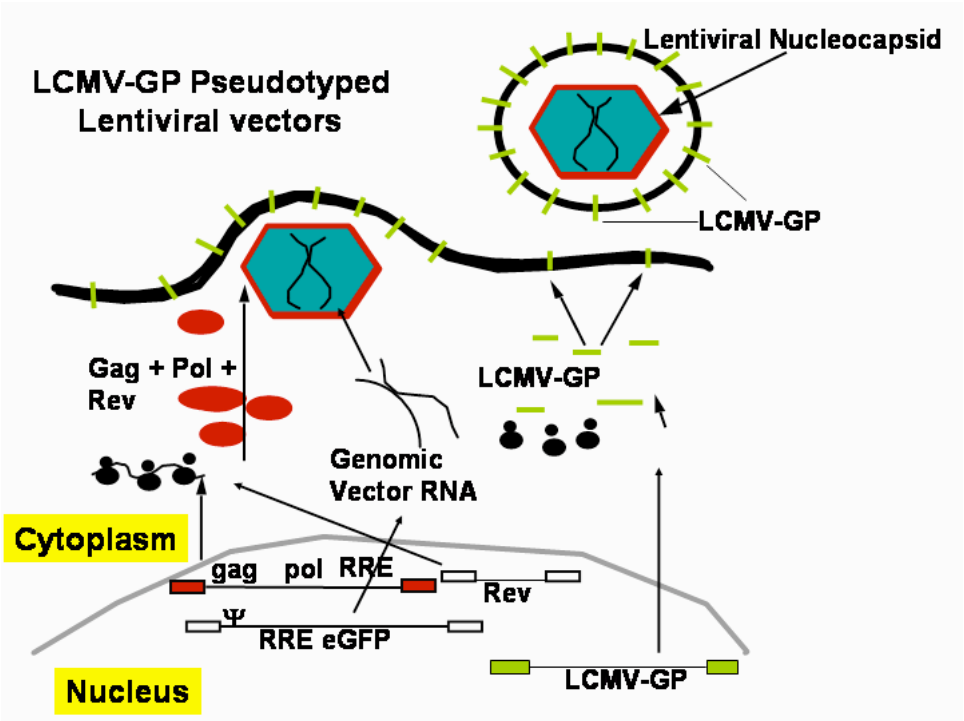

Figure 2: A packaging cell line for LCMV-GP pseudotyped lentiviral vectors.
The genes encoding virus proteins are separated on three plasmids. The packaging signal has been deleted to prevent packging into the viral particle. The vector is separated and contains the marker gene GFP and a packaging signal.

For transient production of retroviral vectors, the plasmids are co-transfected into mammalian cells. For establishment of stable packaging cells, co-transfected plasmids contain selection genes, which allow selection of high-titer producer cells. Stable packaging cells are suitable for clinical use as they can be characterized and tested for RCR, which is an important safety aspect. Furthermore, the packaging cells can be directly implanted into a tumor, which might be more efficient than injecting the viral supernatants. Packaging cells used in clinical studies for malignant glioma have been derived from the murine fibroblast cell line NIH3T3 [18].

5.2.4. Migrating stem cells with glioma tropism

A major goal in therapy of malignant glioma is the elimination of infiltrating tumor cells, which migrate into healthy brain tissue and therefore are responsible for tumor recurrence. To target these infiltrating cells, stem cells with migratory abilities are attractive as vehicles for the gene. It has been shown that neural and mesenchymal stem cells migrate towards and into malignant glioma (Figure 3) upon injection into

the contralateral brain hemisphere, the vicinity of the tumor and upon intravenous or intra-arterial injections [20-22].

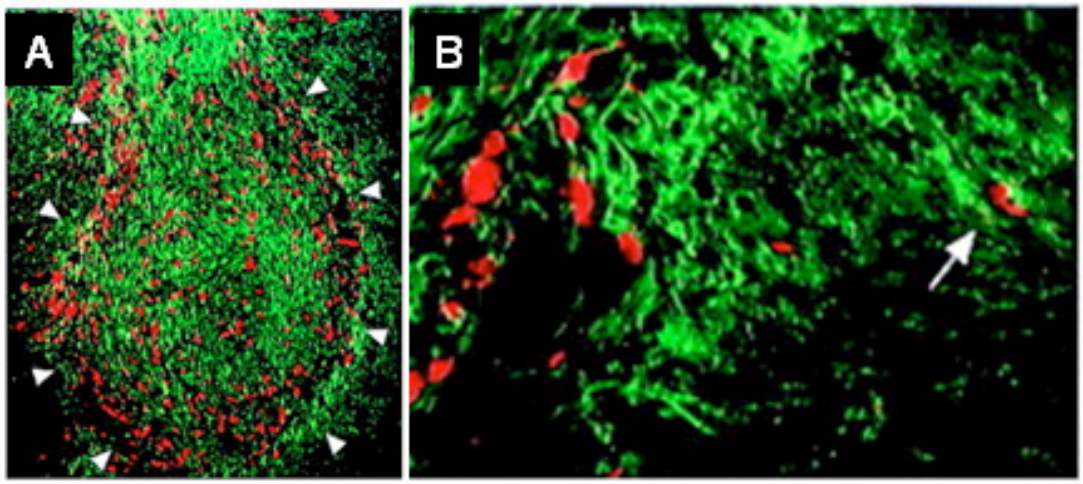

Figure 3: Neural stem cells display tropism for intracranial malignant glioma.
(A) Neural stem cells (red) infiltrate malignant glioma (green) in vivo. **(B)** Single neural stem cells "track" invasive glioma cells. Modified, from Aboody et al. [20].

Neural and mesenchymal stem cells expressing therapeutic genes mediated significant therapeutic effects in experimental animal models [21-23]. However, the use of these cells as packaging cells for retroviral vectors is limited as they have a low passaging capacity. A high passaging capacity is necessary for genetic modification, selection of high titre clones, and clinical application. In previous animal and clinical studies only highly proliferating fibroblasts such as NIH3T3 have been used to establish retroviral packaging cells. However, these cells stay at the injection site, thereby limiting the intratumoral vector distribution and therapeutic effects. In this regard, multipotent adult progenitor cells (MAPC), a subpopulation of mesenchymal stem cells, that have a high passaging capacity with more than 100 population doublings (PD) [24], would be ideal candidates to enhance the distribution of viral vectors and target infiltrating tumor cells.

5.2.5. Suicide genes are effective mediators in gene therapy

Therapeutic genes are important tools in gene therapy as they mediate the therapeutic effect on the target tissue. They can be delivered by viral vectors directly into the target cells or can be expressed by therapeutic cells injected into the target lesion. Suicide genes are widely used in gene therapy of many different tumor types. They encode an enzyme which catalyzes the conversion of a non-toxic substrate into a toxic product thus eliminating the target cell. One of the first and also currently used suicide genes is the Herpes simples virus type 1 (HSV-1) thymidine kinase (tk)-gene in combination with the nucleoside analogue Ganciclovir (GC) as a substrate [25]. HSV-1-tk phosphorylates GC resulting in GC-monophosphate (GC-P). GC-P is converted to GC-triphosphate (GC-PPP) by cellular kinases and integrated into the DNA during replication, causing damage to the newly synthethised DNA and thereby apoptosis of the cell (Figure 4). The elimination of neighboring, non-transduced tumor cells is mediated through the bystander effect. The toxic GC-P is transported through gap junctions from the transduced to the nontransduced cells (Figure 4).

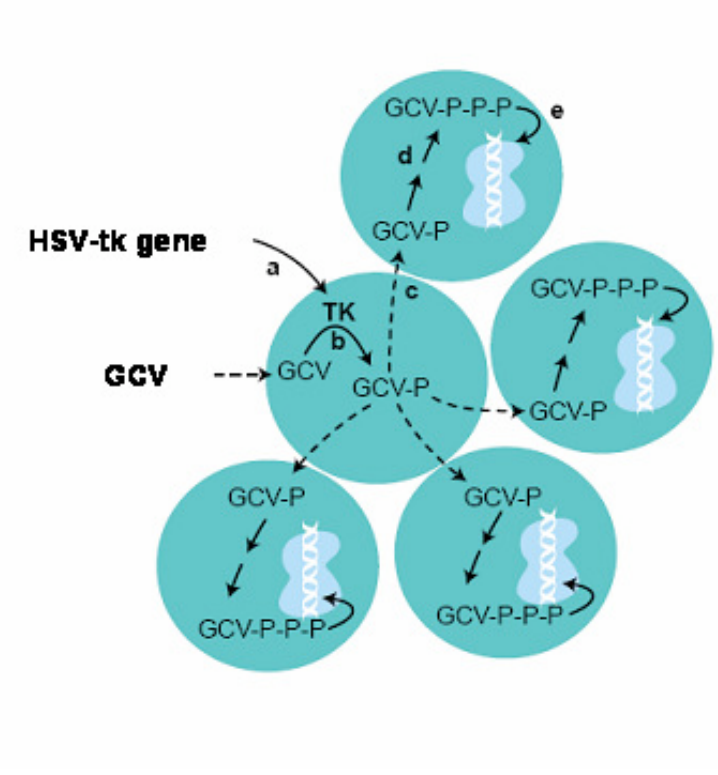

Figure 4: Mechanism of HSV-1-tk/GCV cell killing. a) After viral gene transfer, HSV-1-tk is expressed in the target cell. b) GCV, given systemically, is taken up by the target cell and is phosphorylated by HSV-1-tk (GCV-P). c) GCV-P is taken up by neighboring cells. d) Cellular kinases katalyze the phosphorylation of GCV-P, which results in GCV-PPP. e) GCV-PPP is integrated into the newly synthesized DNA, which causes apoptosis of the target cell. Modified from Hurwitz et al. [26]

5.3. Imaging of malignant glioma

In clinical practice, the most commonly used methods to image malignant gliomas are magnetic resonance imaging (MRI) and computed tomography (CT). In general, MRI and CT are applied to define the tumor margins. Contrast enhancement reflects regions of blood-brain barrier breakdown and serves as a marker to delineate tumor tissue towards the brain. However, contrast enhancement is an imprecise indicator as it may not appear in viable tumor parts that do not show blood-brain barrier breakdown. In addition, it may be falsely positive in necrotic or inflamed areas with blood-brain barrier disruption. Importantly, the tumor volumes are often underestimated with CT and MRI-techniques [27, 28]. In conclusion, therapeutic strategies need a more precise definition of tumor size for target volume planning than provided by standard MRI or CT.

An attractive alternative is positron emission tomography (PET) using amino acid tracers such as methyl-[^{11}C]L-methionine ([^{11}C]MET). The tumor margins defined by this technique are frequently wider than those assessed by MRI or CT [29]. [^{11}C]MET is incorporated into most brain tumors, even into low-grade gliomas, and is being used in the imaging of patients with gliomas [30]. [^{11}C]MET is a sensitive tracer in tumor detection, and it differentiates benign from malignant lesions with high sensitivity and specificity with relatively low background activity in normal brain tissue [29, 31]. [^{11}C]MET uptake correlates with cell proliferation in cell culture [32], Ki-67 expression [33], and microvessel density [34], indicating its role as a marker for tumor proliferation activity and angiogenesis. In a recent study using [^{11}C]MET, both, solid and infiltrating areas of human brain tumors were detected with high sensitivity and specificity [35].

5.4. Imaging of gene transfer

One major issue in clinical gene therapy studies is to non-invasively assess the transduced tissue dose of a therapeutic gene before treatment initiation, which can serve as a quality control and can be correlated to therapeutic outcome. This issue has been addressed by several groups using noninvasive clinical imaging systems such as positron emission tomography (PET) [36-41] and magnetic resonance

imaging (MRI) [42-44]. In particular, the HSV-1-*tk* suicide gene is a suitable marker for PET imaging, as the HSV-1-tk protein selectively incorporates certain radiolabeled nucleoside analogues into DNA [45-47]. Tjuvajev *et al*. [47] could demonstrate by use of an enzymatic assay that the accumulation rate of the specific HSV-1-tk marker substrate [124I]-29-fluoro-29- deoxy-1-*b*-D-arabinofuranosyl-5-iodo-uracil (FIAU) in tk-expressing tumors correlated with the level of tk-mRNA expression. Most importantly, various levels of tk-expression could be non-invasively distinguished *in vivo* by PET after retroviral [38, 48-50], adenoviral [37, 45], and herpes viral [39, 41, 51] vector-mediated *tk* gene transfer.

6. Present investigation – Gene therapy of malignant glioma with retroviral vectors and progenitor cells

In the present investigation, we aimed at improving several aspects in gene therapy of malignant glioma:
1. A specific and more efficient transduction of glioma cells using lentiviral pseudotyped vectors (6.1.)
2. Isolation of a glioma-infiltrating progenitor cell line with a high passaging capacity that can be non-invasively imaged in vivo (6.2.).
3. Establishment of a retroviral packaging cell line based on the glioma infiltrating progenitor cells as a proof of principle (6.3.).

For future applications, the presented single therapeutic strategies (1. and 2.) should result in the development of a combined therapeutic approach representing glioma-infiltrating progenitor cells as packaging cells for lentiviral vectors. Thereby the distribution of viral vectors and the transduction efficiency of glioma could be enhanced. The proof of principle of such a packaging cell line is described in 3. for retroviral vectors.

6.1. Selective and efficient transduction of malignant glioma [Miletic et al. 2004]

In the present study, we defined the tropism of two different lentiviral pseudotypes, LCMV-GP and VSV-G, for normal brain cells and glioma cells using the marker gene eGFP.

VSV-G pseudotypes transduce neurons and astrocytes more efficiently than do LCMV-GP pseudotypes
Cultured neurons and astrocytes derived from embryonic Wistar rats and the human glioma cell line G62 were infected by endpoint dilutions. VSV-G pseudotypes transduced GFAP-positive astrocytes and beta-tubulin-III-positive neurons at a higher level than LCMV-GP pseudotypes (Table 1). In particular, the transduction efficiency for neurons was markedly different: VSV-G pseudotypes transduced 62.5% whereas LCMV-GP pseudotypes infected only 2.2% of counted neurons (Table 1). In addition,

LCMV-GP pseudotypes showed a higher transduction efficiency for the human glioma cell line G62 (89.5%) than for cultured astrocytes (71.7%) and neurons (2.2%). In contrast, VSV-G pseudotypes transduced astrocytes (85.5%) and neurons (62.5%) at a higher level than G62 cells (56.9%).

Table 1: Tropism of lentiviral pseudotyped vectors for cultured brain cells

cultured cells	Lentiviral pseudotyped vector (Titer on TE 671 cells)	Transduced cells / high power field (± SD)	Total cell number / high power field (± SD)	Ratio (%) (± SD)
Astrocytes	LCMV-GP ($8x10^4$)	35.7 (± 12.6)	49.0 (± 13.3)	71.7 (± 10.3)
	VSV-G ($3x10^4$)	43.5 (± 18.0)	49.9 (± 16.4)	85.5 (± 12.4)
Neurons	LCMV-GP ($8x10^4$)	0.4 (± 0.5)	17.9 (± 5.9)	2.2 (± 3.0)
	VSV-G ($3x10^4$)	8.2 (± 3.3)	13.8 (± 6.1)	62.5 (± 16.9)
G62	LCMV-GP ($8x10^4$)	67.3 (± 18.3)	75.5 (± 20.3)	89.5 (± 6.7)
	VSV-G ($3x10^4$)	41.1 (± 14.5)	72.3 (± 12.2)	56.9 (± 17.0)

Cultured rat astrocytes or neurons were transduced with 3 x 10^4 to 8 x 10^4 eGFP TU of LCMV-GP or VSV-G pseudotyped lentiviral vectors. Cells were analyzed by fluorescence microscopy after immunostaining with monoclonal anti-GFAP antibodies for astrocytes or anti-beta-tubulin-III antibodies for neurons. Results are the mean cell numbers and standard deviations (± SD) from 10 random camera fields.

The *in vivo* tropism for normal brain cells was investigated by injection of lentiviral pseudoytypes either into the striatum or into the hippocampus of Fischer rats. LCMV-GP pseudotypes transduced almost exclusively astrocytes in both brain regions, even in areas with high neuron density (Figure 5A). Transduction of neurons was scarce in the striatum and the hippocampus. In contrast, VSV-G pseudotypes infected neurons with a high efficiency (Figure 5B). The estimated ratio of cells transduced by VSV-G pseudotypes was 3:1 for neurons : astrocytes in both brain regions.

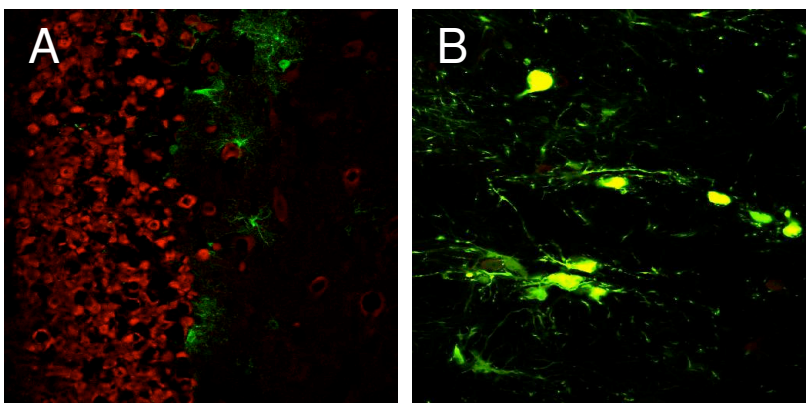

Figure 5: Transduction of normal brain cells in vivo.
Normal rat brain was infected with LCMV-GP or VSV-G pseudotyped vectors expressing eGFP. Transduction of neurons and astrocytes was analyzed after staining with antibodies

against NeuN and GFAP by confocal laser scanning microscopy on day 14. **(A)** In regions with high neuron density (hippocampus) neurons were not transduced by LCMV-GP pseudotypes, but single astrocytes. **(B)** Transduction of striatal neurons by VSV G- pseudotyped vectors. Merge of green (eGFP) and red (NeuN) fluorescence. Original magnification 400x.

LCMV-GP pseudotypes show specific and efficient transduction of glial tumor cells in vivo

The tropism of both pseudotypes for glioma cells was analyzed by injection into solid and infiltrating areas of syngneic 9L rat glioma, expressing the marker gene DsRed. The transduction efficiencies in infected tumor areas were determined quantitatively. LCMV-GP pseudotypes showed a very effective transduction of the solid tumor: 69.3% ± 10.6 of tumor cells were GFP-positive in tumor areas injected with vector supernatants (Table 2). In the infiltrating areas of 9LDsRed tumors, LCMV-GP pseudotypes specifically transduced the glioma cells: while 71.5% ± 10.6 of tumor cells were transduced, only 4.2% ± 4.7 of normal brain cells were GFP positive (Table 2, Figure 6A). Only few reactive astrocytes around the tumor bed were GFP positive, as revealed by staining with antibodies against GFAP. Neurons were not infected in infiltrating tumor areas.

Table 2: Transduction efficiency of lentiviral pseudotyped vectors for 9LDsRed cells *in vivo*

Lentiviral Pseudotyped vector	Tumor area	Cell type	Transduced cells / power field (±SD)	Total cells / power field (±SD)	Ratio (%) (± SD)
LCMV-GP	solid	9LDsRed	191.3 (± 44.4)	276.5 (± 51.1)	69.3 (± 10.6)
VSV-G	solid	9LDsRed	19.3 (± 6.5)	279.9 (± 35.6)	6.8 (± 1.9)
LCMV-GP	infiltrating	9LDsRed	98.4 (± 25)	137.9 (± 27.4)	71.5 (± 10.6)
VSV-G	infiltrating	9LDsRed	2.6 (± 2.1)	125.4 (± 31.2)	2.2 (± 1.9)
LCMV-GP	infiltrating	Brain cells	3.7 (± 3.4)	95 (± 19.4)	4.2 (± 4.7)
VSV-G	infiltrating	Brain cells	35.2 (± 11.4)	81.6 (± 12.7)	42.9 (± 11.4)

9LDsRed tumors were transduced with 2 x 10^6 to 1 x 10^7 eGFP TU/ml of LCMV-GP or VSV-G pseudotyped lentiviral vectors. Cells were counted using a fluorescence microscope. Data represent the mean cell numbers and standard deviations from 10 microscopic fields (magnification x 200) of solid and infiltrating tumor areas, respectively.

VSV-G pseudotypes showed a different transduction pattern: solid tumor was transduced to a much lower extent (6.8% ± 1.9) than with the LCMV-GP pseudotypes (Table 2). In contrast, many normal brain cells including neurons and reactive astrocytes in the infiltration area and surrounding brain parenchyma were GFP positive (42.9% ± 11.4, Figure 6B), while only rarely tumor cells (2.2% ± 1.9) were transduced (Table 2).

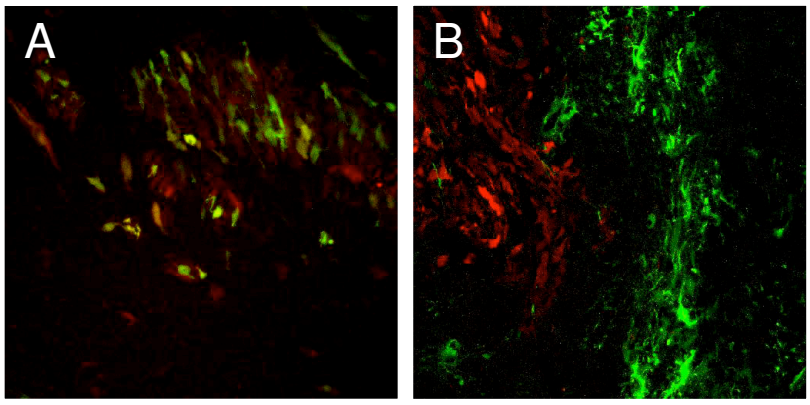

Figure 6: Transduction pattern in infiltrating areas of rat glioma.
Intracranial 9LDsRed gliomas were infected with LCMV-GP or VSV-G pseudotyped lentiviral vector expressing eGFP 7 days after tumor implantation and analyzed by confocal laser scanning microscopy at day 14. **(A)** Infiltrating glioma parts transduced by LCMV GP-pseudotyped vectors. **(B)** VSV G-pseudotyped vectors transduce normal brain cells at the tumor border. Merge of green and red fluorescence. Original magnification x20.

Concluding remarks

In summary, the present study demonstrates that lentiviral LCMV-GP pseudotypes specifically and efficiently transduce rat glioma in vivo and therefore are attractive candidates for glioma gene therapy. In previous studies [13, 14, 52], we characterized the major features of both lentiviral and gammaretroviral LCMV-GP pseudotypes: The glycoprotein is stable, allowing concentration by ultracentrifugation for high vector titers. The vectors can be frozen and thawed without significant loss of virus titer. In addition, stable packaging cell lines producing LCMV-GP pseudotyped retroviral vectors have been established. This is an important advantage over VSV-G pseudotyped vectors, because cytotoxicity of the VSV glycoprotein impedes generation of packaging cell lines constitutively expressing VSV-G. Furthermore, VSV-G pseudotyped lentiviral vectors infected normal brain cells at the tumor border more efficiently than tumor cells. Also within solid tumor, transduction of tumor cells was not as efficient as with LCMV pseudotypes. *In vitro* and *in vivo* we could demonstrate a strong transduction of neurons and astrocytes with VSV-G pseudotypes. Thus, LCMV-GP pseudotypes are excellent candidates for the

establishment of packaging cell lines based on glioma-infiltrating stem or progenitor cells to enhance vector distribution and tumor cell transduction.
In further experiments we demonstrated therapeutic efficacy of lentiviral LCMV-GP pseudotypes expressing the suicide gene HSV-1-tk using the 9L glioma model (Miletic et al., Clin. Cancer Res., under revision). However, the transduction and therapeutic efficacy of this vector has to be evaluated in human glioblastoma xenograft models before clinical translation. This is a critical step as the animal model used for this study is a cell-line based glioma model which does not mimic the highly invasive behavior of human glioblastoma.

6.2. Bystander killing of malignant glioma with migrating progenitor cells [Miletic et al, 2007]

In this study, mesenchymal progenitor cells derived from rat bone marrow (BM-TICs) with a high passaging capacity *in vitro*, were tested for their glioma infiltrating capacity and therapeutic efficacy *in vivo* using a suicide gene therapy approach.

Migratory capacity of bone marrow-derived tumor infiltrating cells (BM-TICs) in 9L glioma in vivo.
Cell lines with a high passaging capacity, which are suitable for genetic modification and large-scale production, were tested for migration in a rat glioma model. BM-TICs were used at population doublings (PD) 15 to 70. No major influence of the passage number on the migratory behavior described below was observed. The mouse neural stem cell line C17.2, for which migration has been shown previously, was used as positive control and mouse 3T3 fibroblasts as negative control. To rule out that an immune response against allogeneic cell lines interferes with their migration ability, syngeneic Fischer rat fibroblasts Rat-1 were used as a second negative control. Glioma cells (9L glioma cell line) transduced with the red fluorescence gene DsRed (9LDsRed) were implanted into the striatum of Fischer rats and 5 days thereafter eGFP-transduced cell lines were injected into established tumors or at the vicinity of tumors. Within 3 days after injection, BM-TICs had migrated from the injection site to the tumor border as well as into the solid tumor areas. At day 7, BM-TICs had infiltrated the majority of the solid tumor mass (Figure 7A) and were also found at the

tumor border, even tracking tumor cells infiltrating the normal tissue. Importantly, BM-TICs did not migrate into normal brain tissue without having been triggered by a tumor cell. The neural stem cell line C17.2 showed a similar migratory behavior (Figure 7B). In contrast, 3T3 cells remained at the injection site and were well demarcated from the tumor tissue (Figure 7C). 3T3 cells did not migrate towards the border of the tumor or infiltrating tumor cells. The cell line Rat-1 too, did not migrate into the tumor mass, but stayed in the brain adjacent to the tumor mass.

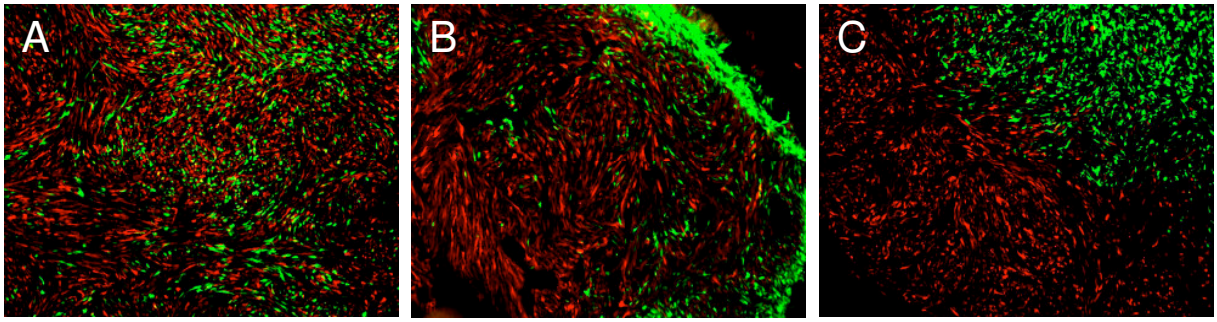

Figure 7: Migration of BM-TICs in DsRed-transduced 9L glioma.

Distinct cell lines transduced with eGFP were tested for migration in established 9LDsRed glioma. Cell lines were injected into glioma or in the vicinity 5 days after tumor implantation. Histological sections were analyzed by fluorescence microscopy 3 and 7 days after implantation of cell lines. Overlays of green (eGFP cell line signal) and red (DsRed glioma signal) fluorescence are shown. **(A)** Distribution of BM-TICs in the tumor after 7 days. **(B)** C17.2 neural stem cells showed migration from the border to solid tumor parts at 3 days after implantation. **(C)** 3T3 cells stayed sharply demarcated from the tumor at 3 days after injection. Magnification 50x.

In vivo bystander killing of 9L glioma by BM-TIC-tk-GFP

To analyze the therapeutic impact of BM-TICs *in vivo*, BM-TICs transduced with HSV-tk and GFP (BM-TIC-tk-GFP) were injected into 9L tumors 5 days after tumor implantation. As a control non-migrating Rat-1 transduced with HSV-tk and GFP (Rat-1-tk-GFP) were implanted. On day 4 after cell injection, one group of BM-TICs implanted rats (n =9), one group of Rat-1 implanted rats (n=8), and one control group implanted with 9L only (n=8), received daily i.p. injections of 30 mg/kg GC for 10 days. Two control groups of either BM-TIC-tk-GFP (n=8) or Rat-1-tk-GFP implanted rats (n=8) were not treated with GC. Kaplan Meier survival analysis showed over 60% long-term survivors in the BM-TIC-tk-GFP plus GC treated group surviving 110 days (Figure 8). All animals in the control groups died within the observation period. Thus, treated animals showed a prolonged survival compared to both control groups, which was statistically highly significant ($p<0.001$). In contrast, Rat-1-tk-GFP and GC

treated animals showed a significantly reduced survival compared to BM-TIC-tk-GFP and GC treated animals ($p<0.01$), but also a significantly prolonged survival compared to control groups ($p<0.05$).

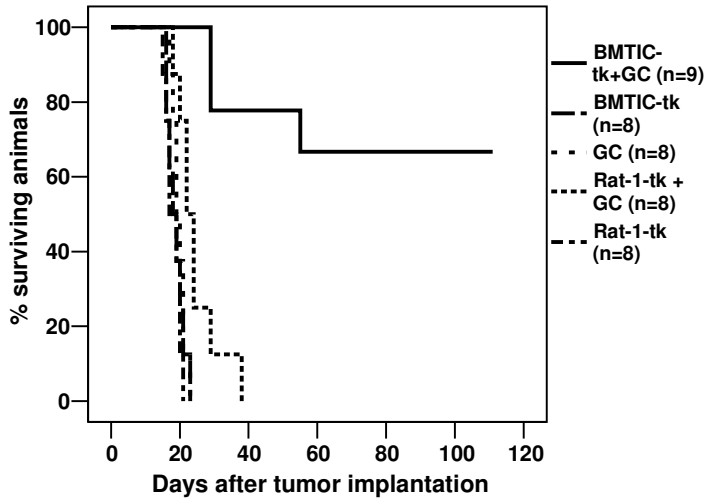

Figure 8: Bystander effect of BM-TIC-tk-GFP on 9L glioma *in vivo*.
The graph shows the Kaplan-Meier survival curve. In total 66.67 % of rats survived 110 days in the BM-TIC-tk-GFP plus GC treated group. The difference in survival between the treated group and control groups was statistically significant ($P<0.001$; log-rank test). The Rat-1-tk-GFP + GC treated group survived significantly shorter compared to the BMTIC-tk-GFP+GC treated group ($p<0.01$; log rank test).

Detection of HSV-1-tk-GFP transduced BM-TICs and therapeutic follow-up by PET and MR imaging

For clinical application of therapeutic cells, it is essential to locate these cells by noninvasive methods. For this purpose, BM-TIC-tk-GFP were injected into 9LDsRed tumors and [^{18}F]FHBG PET scans for detection of BM-TIC-tk-GFP were applied 6-7 days later. [^{18}F]FHBG is the specific substrate for HSV-tk and therefore confirms HSV-tk expression in vivo. In addition, [^{11}C]MET PET scans and MRI scans for detection of the 9LDsRed tumor were performed. BM-TIC-tk-GFP were detected in the tumor (Figure 9A,B) and fluorescence microscopy of histological sections confirmed eGFP positive cells at the border and in solid tumor parts (Figure 9E). The relative expression level of HSV-tk was 0.113 ± 0.025 % of the injected dose/g tissue (Figure 9F). In contrast, animals, which had received glioma cells only (control group), had a significantly lower [^{18}F]FHBG uptake in the tumor (0.053 ± 0.006 % of injected dose/g; $p<0.05$; Figure 9C,D,F).

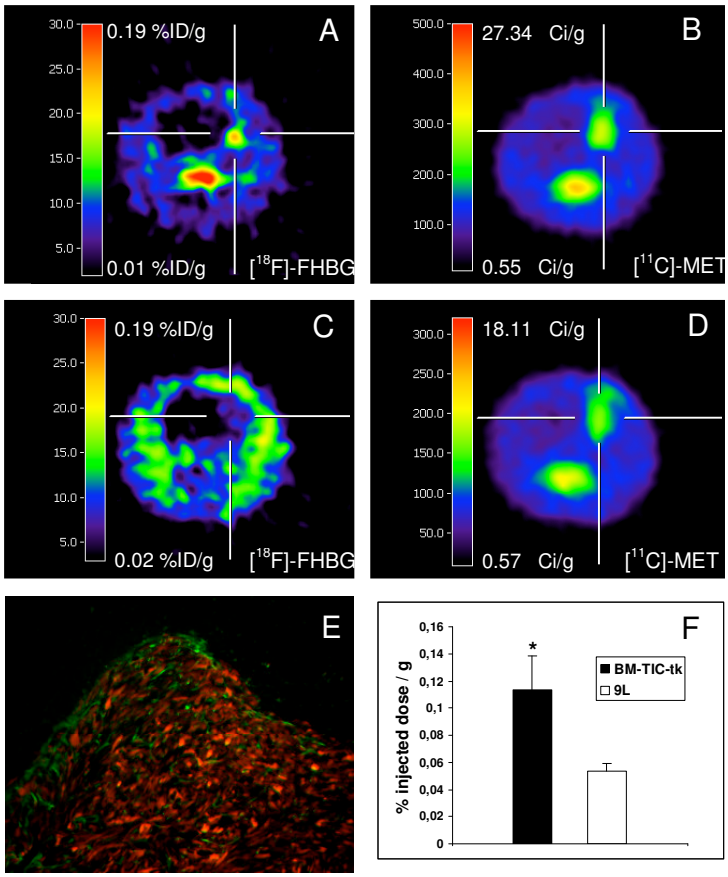

Figure 9: Detection of BM-TIC-tk-GFP by PET imaging.

(A) [^{18}F]FHBG PET scan of a BM-TIC-tk injected animal showing HSV-tk expression in the tumor area with a maximum of 0.14 % of injected dose/g. **(B)** [^{11}C]MET PET scan of the same animal detecting 9LDsRed tumor 7 days after tumor implantation. **(C)** [^{18}F]FHBG PET scan of a control animal (9LDsRed only) showing low [^{18}F]FHBG uptake in the tumor area with a maximum of 0.05 % of injected dose/g. **(D)** [^{11}C]MET PET scan of the same animal detecting 9LDsRed tumor 7 days after tumor implantation. **(E)** eGFP- positive BM-TIC-tk-GFP are detected at the tumor border and the solid tumor part. Overlay of green (BM-TIC-tk-GFP) and red (9LDsRed tumor) fluorescence. Magnification 50x. **(F)** Histogram showing relative HSV-tk expression level of BM-TIC-tk-GFP injected animals (n=3) and the control group (n=3). Shown are the mean values of each group. *P* refers to levels of statistical significance between BM-TIC-tk-GFP vs 9LDsRed only. Student´s *t*-test for nondiscrete grade values. ∗ $P<0.05$

In further experiments, the therapeutic effect of BM-TIC-tk-GFP was monitored successfully during and after termination of GC treatment by MRI and [^{11}C]MET PET imaging. In general, tumors of animals in the therapeutic group were reduced in size compared to control animals (Figure 10A-C). Histopathology revealed only residual tumor cells in the treatment group (Figure 10D) and large tumor masses in both control groups (Figure 10E,F) showing that imaging data was strongly correlated with histology.

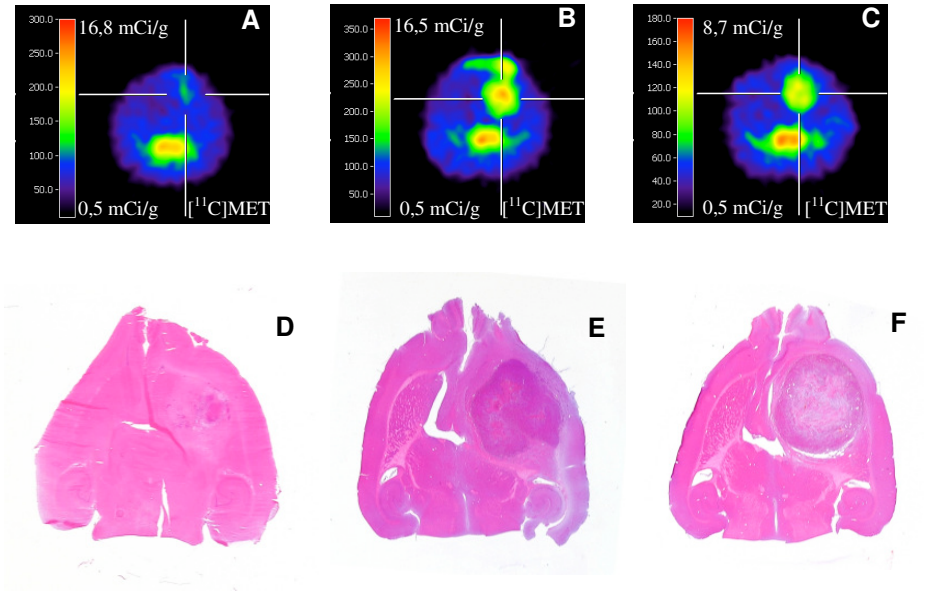

Figure 10: Correlation of imaging with histology.

Animals were euthanized and perfused when they became terminally ill or in the therapeutic group 4-8 days after end of GC treatment. (A-C) [^{18}C]MET PET scans. **(A)** BMTIC-tk-GFP plus GC injected animal after GC treatment (22 days after tumor implantation). **(B)** BMTIC-tk-GFP injected animal (14 days after tumor implantation) **(C)** 9LDsRed only plus GC injected animal during GC treatment (14 days after tumor implantation) **(D-F)** Macroscopic photographs of transversal brain sections, H&E staining. **(D)** BM-TIC-tk-GFP plus GC treated animal with a small lesion. Control animals show large tumor masses: **(E)** BM-TIC injected animal. **(F)** Control animal with 9LDsred only and GC treatment.

Concluding remarks

The present study demonstrates that a subpopulation of bone marrow-derived mesenchymal stem cells can be used as tumor infiltrating therapeutic cells against malignant glioma. *In vivo*, more than 60% of BM-TIC-tk-GFP plus GC-treated animals were long-term survivors. In contrast, animals treated with non-migrating, syngeneic fibroblasts Rat-1-tk-GFP plus GC survived significantly shorter without long-term survival. The HSV-tk/ganciclovir system is considered to be highly effective for cancer therapy [25, 53-55]. The role of gap junctions in the transport of phosphorylated GC to neighbouring cells has been verified by co-culture experiments of connexin expressing and connexin deficient cells [56]. This communication is the basis for the strong therapeutic bystander effect of the HSV-tk/GC system, which can compensate for low transduction efficiencies. Besides its therapeutic effect, HSV-tk has gained increasing interest as a marker for noninvasive imaging. In our study, this technique was applied to localize therapeutic cells in vivo. The visualization of therapeutic cells within a tumor is an important clinical issue as the intratumoral

distribution of these cells is the prerequisite for an efficient therapeutic effect. Using the HSV-tk specific tracer [^{18}F]FHBG, BM-TIC-tk-GFP were detected intratumorally in vivo by PET imaging 6 to 7 days after implantation. The uptake of [^{18}F]FHBG in BM-TIC-tk-GFP-injected animals was significantly (two-fold) increased compared to the control group. The intratumoral localization of BM-TIC-tk-GFP was confirmed by fluorescence microscopy of histological sections. Furthermore, BMTIC-tk-GFP-injected and GC-treated animals showed a lower [^{18}F]FHBG uptake in the tumor area than BMTIC-tk-GFP-injected and non GC-treated animals, indicating loss of HSV-tk activity due to the treatment. In fact, to our knowledge, this is the first demonstration that therapeutic cells can be non-invasively imaged intracerebrally by PET.

In summary, BMTICs cover all relevant features for the translation of a cell-based therapy into the clinic: In addition to their migratory ability, they show a high passaging capacity, which allows genetic modification and large-scale production. Furthermore, they can be non-invasively imaged in vivo and exhibit a bystander-mediated therapeutic effect by expression of the suicide gene HSV-1-*tk*.

6.3. A retroviral packaging cell line based on progenitor cells [Fischer, Miletic et al., 2007]

The present work describes the generation of a packaging cell line for LCMV-GP pseudotyped retroviral vectors based on BMTICs and their analysis for migration and release of vector particles in the 9LDsRed glioma model.

Generation of a BM-TIC packaging cell line (BM-TIPC)

The packaging cell line based on BMTICs was established by Yvonne Fischer and is described in detail in her thesis ("Gentherapie von malignen Gliomen mit retroviralen Pseudotypvektoren", Frankfurt am Main, 2005).

Briefly, BM-TICs were genetically modified to express MLV gag-pol and LCMV-GP. A lentiviral self-inactivating vector encoding LCMV-GP was created and used to transduce bulk cultures of BM-TICs. This led to stable and prolonged LCMV-GP expression in BM-TICs over more than 2 month in culture. Titers achieved on human glioma cells reached $1x10^4$ TU/ml and are therefore comparable to vector titers of PC used in previous clinical studies [57]. Titers remained stable over one month in culture and after several cycles of freezing/thawing. The presence of replication-competent retrovirus in BM-TIPCs supernatants was tested after prolonged culturing and was found to be negative.

Glioma-specific migration of BM-TIPCs and transduction of glioma cells in vivo

The ability of these genetically modified BM-TIPCs to still specifically infiltrate glioma tissue *in vivo* was analyzed. BM-TIPCs were injected directly into established 9L-DsRed gliomas in Fischer rats. After seven days, examination of brain sections revealed an efficient infiltration of gliomas by BM-TIPCs (Figure 11). BM-TIPCs migrated away from the injection site (arrows) and infiltrated large proportions of the tumor.

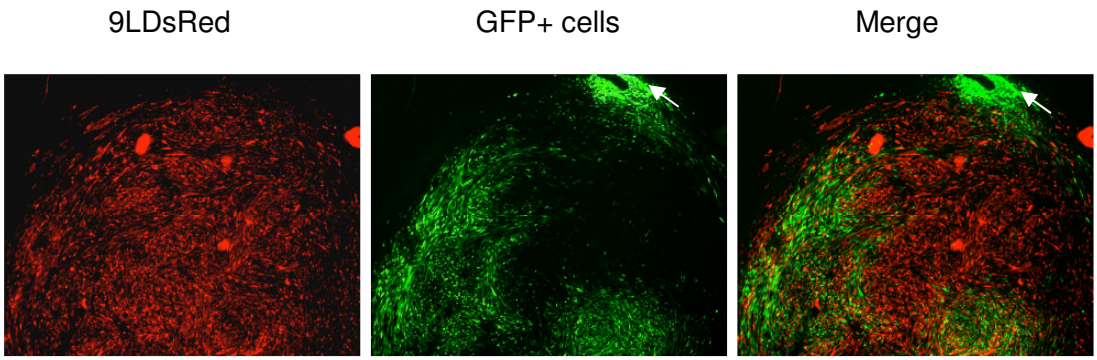

Figure 11: BM-TIPCs infiltrate gliomas in vivo with high specificity and efficacy.

BM-TIPCs were injected intratumorally into established 9L-DsRed gliomas in Fischer rats. After 7 days, rats were killed, brain sections were prepared and analyzed by fluorescence microscopy. Magnification 2,5x, white arrows indicate the site of injection of BM-TIPC.

Transduction of glioma cells in vivo was examined by confocal microscopy on the same brain sections. Co-localization of green and red fluorescence, indicative of transduced glioma cells, were detected in both solid (Figure 12) and infiltrating tumor areas. The percentage of transduced glioma cells reached up to 33 percent in different tumor parts. This shows that BM-TIPCs continue to produce retroviral vectors in vivo and facilitate glioma cell transduction.

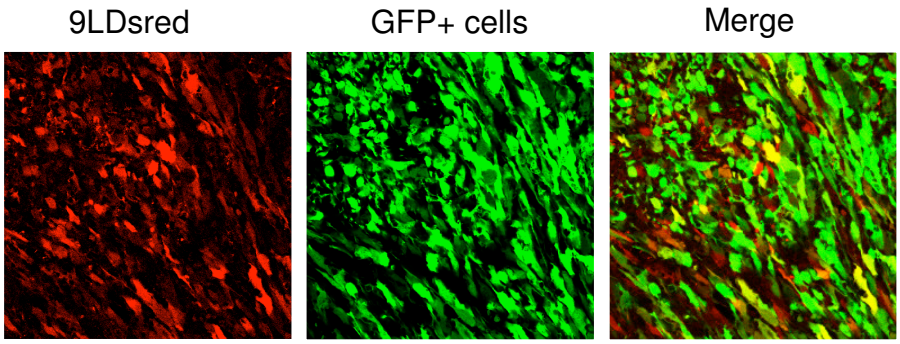

Figure 12: BM-TIPC mediate *in vivo* transduction of rat glioma cells.

BM-TIPCs were injected intratumorally into established 9L-DsRed gliomas in Fischer rats. After 7 days, rats were killed, brain sections were prepared and analyzed by confocal microscopy. Colocalization of green and red fluorescence demonstrates tumor cell transduction. Magnification 400x.

Concluding remarks

The present study demonstrates that adult progenitor cells derived from the bone marrow can be modified to stably produce retroviral LCMV-GP pseudotyped vectors while maintaining their high expansion capacity and their specific tropism for gliomas.

When tested for glioma-specific migration in vivo, BM-TIPCs behaved similarly to the parental BM-TIC cell line: after a single intratumoral injection, BM-TIPCs were detected in most tumor areas, but never in normal brain parenchyma. This shows that BM-TIPCs retain their glioma-specific migratory capacity even after extensive culturing, genetic modification and subcloning. One important option for clinical application of BM-TIPCs may be their injection into gliomas during stereotactic biopsy. The very efficient tumor infiltration by BM-TIPCs may then lead to widespread distribution of viral vectors within human gliomas and ensure the targeting of tumor cells distant from the injection site. This targeting of distant tumor cells can currently not be achieved by injection of replication-incompetent vectors due to only limited vector diffusion [7]. However, this specific targeting of infiltrating tumor cells by BM-TIPCs has to be proven in a human glioblastoma xenograft model with highly invasive properties.

In the rat glioma model, tranduction was detected in both solid and infiltrating tumor regions, showing that BM-TIPC application is a suitable method to achieve gene transfer into glioma in vivo. Interestingly, BM-TIPCs colocalized with single invasive glioma cells and mediated transduction of these cells. Transduction efficiencies achieved by BM-TIPC injection varied, ranging from undetectable to up to 33 percent of transduced glioma cells in different areas of the same glioma.

In future studies, the transduction efficiency in vivo may be enhanced by using lentiviral vectors instead of Mo-MLV-based gammaretroviral vectors. In particular, the transduction of tumor cells with low proliferation rate may be improved by employing lentiviral LCMV-GP pseudotypes. Thus, the main goal will be the establishment of a lentiviral packaging cell line based on tumor infiltrating cells.

7. References

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9. Appendix: Publications I-III

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen -, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie – abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von (Prof. Dagmar Mörsdorf / Prof. Rolf-Ingo Ernestus) betreut worden.