Glioma tropism of lentivirally transduced hematopoietic progenitor cells

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Abstract. Experimental gliomas attract hematopoietic progenitor cells (HPC) *in vivo*. HPC are therefore promising candidates for a cell-based delivery of therapeutic molecules to experimental gliomas. A therapeutic application requires efficient genetic manipulation of the cellular vector and a lack of tumorigenicity. Here, we studied the impact of lentiviral transduction on the glioma tropism of human or murine HPC. Transduction of human or murine HPC with a GFP lentivirus (lenti-GFP) did not interfere with the glioma-mediated attraction of HPC. Bone marrow reconstitution of C57Bl/6 mice with syngeneic GFP-transgenic lineage-depleted bone marrow cells (lin$^-$ BM) was as efficient as reconstitution with syngeneic lin$^-$ BM transduced *ex vivo* with lenti-GFP. SMA-560 gliomas growing orthotopically in lenti-GFP-reconstituted VM/Dk mice recruited GFP-positive bone marrow-derived cells. Thus, lentiviral transduction did not interfere with the attraction of exogenously injected HPC or endogenous bone marrow-derived cells by experimental gliomas. Lenti-GFP-HPC implanted directly into tumor-free brains were not tumorigenic. The intravenous injection of lenti-GFP-HPC in glioma-bearing mice did not alter the survival of otherwise untreated animals and had no impact on the survival benefit conferred by cerebral irradiation. Taken together, genetic manipulation of HPC with lenti-GFP neither made these cells tumorigenic nor interfered with their glioma tropism.

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Abbreviations: CXCL12, CXC chemokine ligand 12; CD, cluster of differentiation; DAPI, 4',6-diamidino-2-phenylindole; FCS, fetal calf serum; GFP, green fluorescent protein; H&E, hematoxylin and eosin; HPC, hematopoietic progenitor and stem cells; IL, interleukin; lenti-GFP-HPC, hematopoietic progenitor cells transduced with GFP lentivirus; i.v., intravenous; lin$^-$ BM, murine lineage-depleted bone marrow cells; LSK, lineage-depleted Sca1$^+$ Kit$^+$ murine HPC; MAPC, multipotent adult progenitor cells; MSC, mesenchymal stem cells; PBS, phosphate-buffered saline; Sca1, stem cell antigen 1; SCF, stem cell factor; SDF, stromal cell-derived factor; SN, supernatant; SN-G, SN of glioma cell lines LNT-229, LN-308 and primary glioma cultures T113, T132, T159; SN-FHAS, SN of SV-FHAS cells; SFM, serum-free medium; SV-FHAS, human astrocytic cell line; TGF-ß, transforming growth factor beta; VEGF, vascular endothelial growth factor; VEGF-R, VEGF receptor

Key words: cell-based therapy, glioma, adult hematopoietic progenitor cell, lentivirus

Introduction

Intravenously (i.v.) injected human CD34$^+$ hematopoietic progenitor and stem cells (HPC), murine lineage-depleted bone marrow cells (lin$^-$ BM) or murine lineage-depleted Sca1$^+$ c-kit$^+$ cells (LSK) home into experimental intracerebral gliomas (1). The key mediators in this process include glioma-derived stromal cell-derived factor (SDF)-1α/CXC chemokine ligand 12 (CXCL12) and soluble Kit ligand (sKitL), also known as stem cell factor (SCF), which interact with their corresponding receptors, CXCR4 and CD117, on the surface of HPC. Irradiation, hypoxia or chemotherapy promote the glioma tropism of HPC by a hypoxia-inducible factor-1α (HIF-1α)-mediated increase in CXCL12 promoter activity in glioma cells, resulting in higher CXCL12 release (2). Further, E-selectin expression is induced by the exposure of human cerebral endothelial cells, human microvascular endothelial cells or brain tumor endothelial cells derived from human glioblastomas to supernatants (SN) of glioma cell lines and primary glioma cells (SN-G). Tissue microarrays sampling normal brain tissue and astrocytomas of WHO grades II-IV confirm the expression of CD62E on endothelial cells of tumor vessels. Importantly, LSK homing to experimental gliomas *in vivo* is reduced by the application of neutralizing CD62E antibodies, demonstrating that adhesion to CD62E might be a pivotal first step in the glioma tropism of HPC (3).

HPC represent an attractive source for gene therapy of inherited hematopoietic disorders because these cells are capable of regenerating the complete hematopoietic system. Preclinical and clinical applications of genetically modified HPC have been described e.g., in Fanconi anemia (4-6). The only curative approach in the treatment of patients with Fanconi anemia is allogeneic stem cell transplantation. This approach, however, is limited by the unavailability of matched unaffected siblings and inefficient alternative donor transplantation (7,8). As an alternative approach, *ex vivo* genetically restored autologous HPC have been applied. Retroviral and lentiviral vectors have been used to achieve genetic correction of patient-derived autologous HPC (9,10). In this regard, lentiviral vectors provided advantages because of their ability to stably transduce nondividing cells and thus enabling short *ex vivo* culture durations (11,12).

The most important prerequisites for the design of a HPC-based cellular therapy that targets experimental gliomas include: i) the efficient and stable genetic manipulation of the cellular carriers without interfering with the glioma tropism, as well as: ii) the lack of tumorigenicity of the cellular carrier *per se*. Based on these considerations, we used a lentivirus for *ex vivo* genetically modifying human or murine HPC. We introduced a GFP cassette into these cells and analyzed: i) the glioma tropism of i.v. injected exogenous human or murine lenti-GFP-HPC and grafted endogenous bone marrow-derived cells *in vivo*, ii) the tumorigenicity of human or murine lenti-GFP-HPC, and iii) their effect on survival when combined in a therapeutic model with cerebral radiotherapy in two rodent glioma models *in vivo*.

Materials and methods

Human and murine HPC. Human adult HPC were isolated by anti-CD34 immunomagnetic microbeads (13). After informed consent, peripheral blood cells were obtained from healthy donors or patients with non-hematological malignancies during granulocyte colony stimulating factor-induced stem cell mobilization, according to the guidelines of the ethics committee of the University of Tübingen. We used this easily accessible cell population because a putative clinical application of a cell-based therapy requires feasible availability of the cellular vector. We are aware of the fact that this cell population is highly enriched but by no means a pure stem cell fraction. Therefore, we designate them human hematopoietic progenitor and stem cells (HPC). The human CD34 antigen is a reliable marker for identifying a small fraction of human bone marrow and peripheral blood mono-nuclear cells that contains primitive uncommitted and pluripotent HPC (14-18).

Lin$^-$ BM and LSK were isolated from VM/Dk mice (1). Briefly, femora and tibiae of VM/Dk mice were flushed with 2% FCS-containing PBS and erythrocytes were lysed. Bone marrow cells were incubated for 60 min at 4˚C with the lineage marker antibodies rat anti-mouse CD4, CD8a, CD45R/B220, Gr-1, CD11b and TER119 (BD Biosciences Pharmingen, San Diego, CA, USA), and then washed with PBS prior to the addition of prewashed sheep anti-rat IgG magnetic beads (Dynabeads, Dynal, Biotech ASA, Oslo, Norway) at 4 beads per cell. After 45 min, beads and attached cells were removed magnetically to obtain lin$^-$ BM. For further purifying the lineage-depleted Sca1$^+$ Kit$^+$ (LSK) for our *in vitro* studies, we further sorted the lin$^-$ BM cells with anti-Sca1$^+$ and anti-CD117 (c-kit) beads by MACS (Miltenyi Biotech, Bergisch Gladbach, Germany). Compared with lin$^-$ BM cells, LSK represent the purified hematopoietic stem cell fraction.

For our *in vitro* studies, we used the purified LSK fraction. For our *in vivo* studies, however, the application of the pure LSK is not feasible, because only ~2x10^5 LSK can be harvested per mouse and this would require too many animals. Therefore, we used lin$^-$ BM and designated them murine HPC in the *in vivo* studies. The lin$^-$ BM cell fraction is enriched for murine committed or uncommitted hematopoietic progenitor and hematopoietic stem cells. We are aware that this cell fraction is not a pure hematopoietic stem cell fraction. Murine HPC were cultured with murine IL-3 (20 ng/ml), IL-6 (50 ng/ml) and SCF (50 ng/ml) (R&D Systems) overnight and transduced with GFP lentivirus. For the assessment of viability as summarized in Table I, cells were stained with trypan blue. The percentages of unstained (viable) and blue (permeable, non-viable) cells was determined (19).

Cell lines. All glioma cell lines were kindly provided by Professor N. De Tribolet (Lausanne, Switzerland). The following primary glioma cultures were used: T113, T159, derived from glioblastomas, and T132, derived from an anaplastic astrocytoma. The primary human glioma cultures were established from samples with high tumor content (20). The source and the generation of the astrocytic cell line SV-FHAS have been described (21). SN of glioma cells (SN-G) were generated in serum-free medium (SFM): 1.5x10^6 glioma cells were first seeded in serum-containing medium in T75 cell culture flasks. The medium was removed the next day and the cells were washed three times with PBS. SFM (7 ml) was added. SN were harvested 48 h later. Concentration of SN was performed with the Centriplus centrifugal filter device YM-3 (3 kDa cut-off, Millipore, Eschborn, Germany). Protein quantification was performed by Bradford assay (Bio-Rad, Munich, Germany).

HPC migration. Migration assays were performed in transwell plates (Costar, Cambridge, MA, USA) of 6.5 mm diameter, with 5 μm pore filters. CD34$^+$ HPC (10^5), lin$^-$ BM or LSK in 200 μl SFM were added to the upper compartment. SFM, recombinant CXCL12 diluted in SFM or SN-G (100 μg) were added to the lower compartment. Anti-human CXCR4 or anti-human/mouse CXCL12 (R&D Systems, Wiesbaden-Nordenstadt, Germany) or appropriate control antibodies were used for the neutralization experiments. Migration was assessed at 37˚C, 5% CO_2 for 4 h. Cells that had migrated to the lower compartment were quantified with the CyQuant Assay (Invitrogen, Karlsruhe, Germany).

Lentiviral production, titration and transduction. A lentivirus containing a GFP cassette driven by an internal spleen focus forming virus promoter was generated by cotransfecting 293T cells with the vector and the packaging constructs (22). SN containing the lentivirus were collected 48 and 72 h after co-transfection. SN were concentrated by ultracentrifugation and used directly to transduce human or murine HPC or stored at -80˚C. For titration, serial dilutions of virus SN were prepared, 500 μl of each dilution was added to 10^4 cells seeded in a well of a 24-well plate and GFP expression was

Table I. Culturing HPC in supernatants of glioma cells.

	7 days (%)	14 days (%)	28 days (%)
$CD34^+$ HPC, SFM	2±3	0	0
$CD34^+$ HPC, cytokines	83±7	70±6	77±9
$CD34^+$ HPC, SN SV-FHAS	20±6	17±4	19±4
$CD34^+$ HPC, SN LNT-229	79±9	71±3	65±8
$CD34^+$ HPC, SN LN-308	72±15	68±10	62±8
$CD34^+$ HPC, SN TU113	69±7	65±6	60±4
$CD34^+$ HPC, SN TU132	73±13	69±7	64±6
$CD34^+$ HPC, SN TU159	76±7	75±6	68±8
$CD34^+$ HPC, boiled SN-G	0	0	0
Lin^- BM, SFM	0	0	0
Lin^- BM, cytokines	78±8	69±9	59±16
Lin^- BM, SN SMA-560	76±7	70±8	73±6
Lin^- BM, boiled SN SMA-560	0	0	0
LSK, SFM	0	0	0
LSK, SFM, cytokines	87±6	71±14	52±15
LSK, SN SMA-560	78±8	73±11	57±5
LSK, boiled SN SMA-560	0	0	0

HPC, lin^- BM or LSK were either cultured in SFM, in the presence of cytokines (IL-3, IL-6, SCF), in SN of SV-FHAS, in SN-G of the indicated cells or in boiled SN-G. The cells were stained with trypan blue. The percentage of viable cells was determined after 7, 14 or 28 days (mean, n=5).

assessed by flow cytometry (Becton-Dickinson, Heidelberg, Germany). For transduction, 100 transducing units per ml (TdU/ml) were used in 24-wells pre-coated with Retronektin® (Takara, Lonza, Vervier, Belgium).

LNT-229 xenograft glioma model. All animal studies were performed in accordance with the permissions of the local authorities. For the experiments with nude mice, 75,000 LNT-229 cells were implanted into the striatum of nude mice on day 0. In the first experiment, we i.v. injected human lenti-GFP-HPC (10^6) on day 21 in tumor-bearing mice. Forty-eight hours later, we sacrificed the mice and analyzed the brains by histology. In the second set of experiments, we analyzed 4 experimental groups of glioma-bearing mice (n=6): The mice in group 1 served as controls (LNT-229 only). In group 2, lenti-GFP-HPC (10^6) were injected into the tail vein on day 21 (LNT-229, lenti-GFP-HPC d21). In group 3, the mice were irradiated cerebrally on day 23 (LNT-229, 6 Gy d23). In group 4, lenti-GFP-HPC were injected on day 21 and the mice were irradiated on day 23 (LNT-229, lenti-GFP-HPC d21, 6 Gy d23). Cerebral irradiation on day 23 was performed with a single 6 Gy dose using a lead/plastic device that allows the exact application of the radiation with a 90% isodose to the targeted brain section, sparing the throat of the mice (23). For survival analysis, mice were examined daily and euthanized at the onset of neurological symptoms. In the third set of experiments, we injected human lenti-GFP-HPC in the right striatum of tumor-free brains (10^6 cells per brain) of 9 nude mice. Three mice were sacrificed 8 months after the injection and the brains were analyzed by histology (see below). The other mice were monitored for survival.

Syngeneic SMA-560 glioma model. On day 0, 5,000 SMA-560 cells were stereotactically implanted into the right striatum of VM/Dk mice. In the first experiment, we injected lenti-GFP-HPC (10^6) i.v. on day 10. Forty-eight hours later, we sacrificed the mice and analyzed the brains by histology. In the second experiment, we analyzed 6 experimental groups of glioma-bearing mice (n=8): Group 1 served as a control group (SMA-560). In group 2, murine lenti-GFP-HPC (10^6) were injected i.v. on day 7 (SMA-560, lenti-GFP-HPC d7). In group 3, cerebral irradiation was performed on day 7 (SMA-560, 6 Gy d7). In group 4, cerebral irradiation was performed on day 7 and murine lenti-GFP-HPC (10^6) were i.v. injected on day 10 (SMA-560, 6 Gy d7 + lenti-GFP-HPC d10). In group 5, cerebral irradiation was performed on day 10 (SMA-560, 6 Gy d10). In group 6, murine lenti-GFP-HPC (10^6) were i.v. injected on day 7 and cerebral irradiation was performed on day 10 (SMA-560, lenti-GFP-HPC d7 + 6 Gy d10). Cerebral irradiation was performed with a single 6 Gy dose (23). For survival analysis, mice were examined daily and euthanized at the onset of neurological symptoms. In the third experiment, we injected murine lenti-GFP-HPC (10^6) in the tumor-free brain of 11 VM/Dk mice. Three mice were sacrificed after 8 months. The brains were analyzed by histology. The remaining 8 mice were monitored for survival.

Bone marrow reconstitution of C57Bl/6 or VM/Dk mice and glioma implantation. We used two mouse models for these experiments. In the first model, we used C57Bl/6 wild-type mice as recipients. We reconstituted these recipients either with murine HPC from syngeneic GFP-transgenic mice or with murine HPC from syngeneic wild-type mice that had been transduced with lenti-GFP. In the second model, we used VM/Dk mice as recipients. These mice were reconstituted with syngeneic lenti-GFP-transduced murine HPC. The experiment in both models was performed in the following way: on day 0, C57Bl/6 or VM/Dk mice were irradiated for bone marrow depletion (VM/Dk with 9 Gy, C57Bl/6 with 12 Gy) and treated with tetracycline to prevent infection after myeloablative irradiation. One hour after irradiation, 10^6 HPC were injected i.v. On day 42, peripheral blood was analyzed by flow cytometry for the presence of GFP-positive cells. On day 49, 5,000 SMA-560 or 20,000 GL261 glioma cells were implanted into lenti-GFP-reconstituted VM/Dk or C57Bl/6 mice. Mice were euthanized at the onset of neurological symptoms and the brains were removed for histological analysis. Bone marrow-derived cells in the brain were detected by GFP fluorescence.

Histology. Cryostat sections (8 μm) were stained with hematoxylin and eosin (H&E). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (Vectashield with DAPI, Vector Laboratories, Axxora GmbH, Loerrach, Germany).

GFP-positive human or murine HPC in the histologies were quantified (2). Briefly, for each brain the rostrocaudal section with the maximal tumor area, designated as central tumor section, and three additional sections cranially and caudally of the central tumor sections were identified and GFP-positive HPC counted on these sections.

Statistical analysis. The *in vitro* experiments were performed at least three times in triplicate. Quantitative data were obtained as indicated. Data are expressed as mean and SEM. Statistical significance was assessed by One-way ANOVA followed by the Tukey *post hoc* test (Excel, Microsoft, Seattle, WA, USA).

Results

Lentiviral transduction does not modify the glioma tropism of HPC in vitro. Adult human HPC or murine lin$^-$ BM or LSK from VM/Dk mice were transduced with a GFP lentivirus. Transduction efficacy was assessed 24, 48 and 72 h after transduction by fluorescence microscopy (Fig. 1A) or flow cytometry (Fig. 1B). The efficacy was in the range of 68-89% as quantified by GFP expression on flow cytometry. To characterize the influence of lenti-GFP transduction on the glioma tropism of HPC *in vitro*, we compared the migration of non-transduced with transduced human HPC. The basal migration of non-transduced and transduced HPC, lin$^-$ BM or LSK towards SFM did not differ. The induced migration towards recombinant CXCL12 and SN-G was also unaltered. Pretreatment of human HPC with antibodies neutralizing CXCR4 resulted in a strong reduction of SN-G-mediated attraction of non-transduced and transduced human HPC. We used neutralizing antibodies to the ligand of CXCR4, CXCL12 for the parallel experiments with murine lin$^-$ BM or LSK. These antibodies strongly reduced the attraction of non-transduced and transduced lin$^-$ BM or LSK (Fig. 1C). In parallel, we assessed the viability of HPC, lin$^-$ BM or LSK in SN-G aiming at modeling the microenvironment of experimental gliomas *in vitro*. We cultured them in SN-G and quantified viable cells by trypan blue exclusion (Table I). In the presence of cytokines, HPC, lin$^-$ BM or LSK or were viable after 7, 14 or 28 days of culture. In the presence of SFM, however, the viability decreased rapidly. Culturing in SN of LNT-229 or LN-308 glioma cells or SN of the primary glioma cultures TU113, TU132 or TU159 yielded survival rates similar to culturing in cytokines. The viability of murine lin$^-$ BM or LSK in SN of syngeneic SMA-560 glioma cells or medium containing cytokines was also comparable. In contrast, HPC, lin$^-$ BM or LSK did not survive in boiled SN-G of any glioma cells. A small percentage of HPC also survived in the SN of the astrocytic cell line SV-FHAS.

Lentiviral transduction does not modify the glioma tropism of HPC or bone marrow-derived cells in vivo. We next assessed the glioma tropism of *ex vivo* genetically modified human or murine HPC *in vivo*. Human LNT-229 or murine SMA-560 glioma cells were orthotopically implanted into the right striatum of nude mice or VM/Dk mice. Lenti-GFP-HPC were injected i.v. on day 21 in nude mice and on day 10 in VM/Dk mice. The mice were sacrificed 48 h later and the brains were assessed by fluorescence microscopy. GFP-positive cells were detected in the LNT-229 (Fig. 2) and in the SMA-560 gliomas (data not shown), but not in the normal brain parenchyma. Of note, quantification of GFP-positive cells in the experimental gliomas showed that the tropism of lenti-GFP-HPC was unaltered compared to our previous studies (2). Thus, lentiviral transduction did not interfere with the glioma tropism of exogeneously injected human or murine HPC.

To characterize the influence of lentiviral transduction on the glioma-mediated attraction of bone marrow-derived cells, we next analyzed whether lentivirally transduced grafted bone marrow-derived cells are still attracted by intracerebral gliomas. For this purpose, we performed bone marrow reconstitutions with GFP-expressing HPC in myeloablatively irradiated recipient mice to obtain reconstituted mice with GFP-expressing bone marrow-derived cells. We then orthotopically implanted experimental gliomas.

First, we ensured that lentiviral transduction *per se* did not interfere with the bone marrow engraftment in reconstituted mice. For this purpose, we used syngeneic GFP-transgenic C57Bl/6 mice. This enabled us to compare the engraftment efficacy of HPC from syngeneic GFP-transgenic mice with the engraftment efficacy of lenti-GFP-HPC from wild-type C57Bl/6 mice that had been *ex vivo* transduced with lenti-GFP. Six weeks after reconstitution with GFP-transgenic HPC, we detected 75-89% GFP-positive cells in the peripheral blood of recipient mice, indicating successful reconstitution (Fig. 3A). After the reconstitution with *ex vivo* lenti-GFP-transduced HPC, we detected 80-92% GFP-positive cells in the peripheral blood (Fig. 3B) indicating that the reconstitution of GFP-transgenic HPC and lenti-GFP-HPC was similarly effective.

Then we proceeded with our studies in VM/Dk mice. Six weeks after the reconstitution with syngeneic lenti-GFP-HPC, 55-60% GFP-positive cells were detected in the peripheral blood (Fig. 3C). We then implanted SMA-560 gliomas into reconstituted VM/Dk mice and sacrificed them at the onset of neurological symptoms. The brains were analyzed by histology for the presence of GFP-positive bone marrow-derived cells. We detected GFP-positive cells in the experimental gliomas of reconstituted VM/Dk mice (Fig. 4B), demonstrating attraction of lentivirally transduced bone marrow-derived cells by intracerebral experimental gliomas.

Human or murine lenti-GFP-HPC are not tumorigenic in the brains of nude or VM/Dk mice. The use of genetically modified HPC as cellular delivery vehicles in the treatment of experimental gliomas requires that the cellular vehicle itself does not undergo oncogenesis in the brain. Therefore, we implanted 10^6 human or murine lenti-GFP-HPC in the striatum of 9 nude mice or 11 VM/Dk mice. Three mice were sacrificed after 8 months to analyze the brains by histology. We did not detect any tumor formation on H&E staining (data not shown). We analyzed the survival in the remaining 6 nude mice or 8 VM/Dk mice. The mice are still alive after 18 months.

Lenti-GFP-HPC do not alter the growth of human LNT-229 or murine SMA-560 experimental gliomas in vivo. We next assessed the influence of i.v. injected lenti-GFP-HPC on

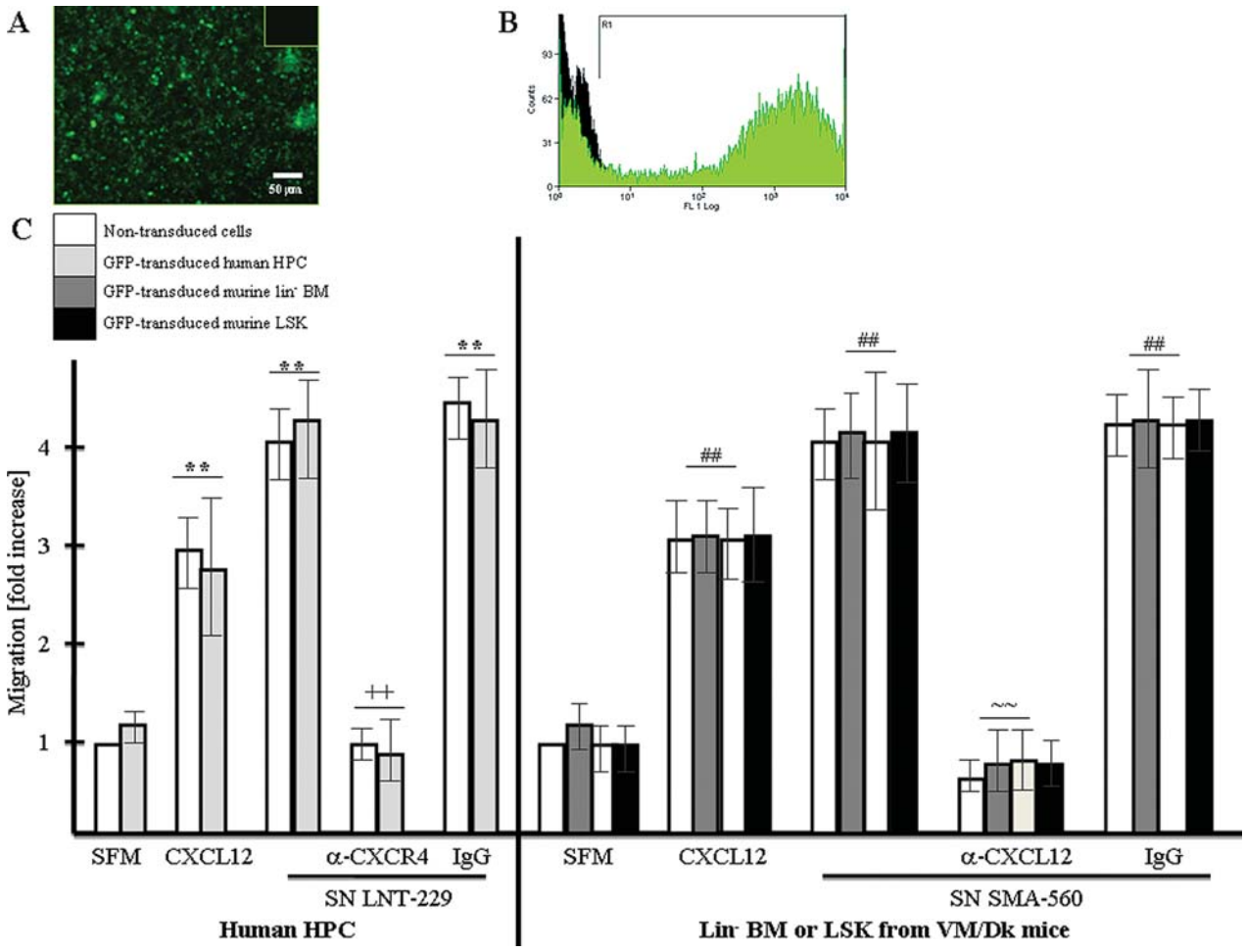

Figure 1. Glioma tropism of lenti-GFP-HPC *in vitro*. (A) Fluorescence microscopy of murine lenti-GFP-HPC at 72 h after lentiviral transduction (scale bar, 50 μm bottom right) is demonstrated on the left side. The insert displays control cells. (B) Quantification of the transduction efficacy of murine lenti-GFP-HPC (from A) by flow cytometry (control cells, black curve; transduced cells, green curve). (C) Human HPC or murine lin$^-$ BM or LSK (10^5 each), either non-transduced or lenti-GFP-transduced, were added to the upper compartment of a chemotaxis chamber. SFM, CXCL12 or LNT-229 or SMA-560 SN were added to the bottom compartment. Migration was assessed 4 h later. The bars indicate mean migration relative to non-transduced cells towards SFM (n=5, **p<0.01 compared with non-transduced HPC in upper well/SFM in bottom well; $^{++}$p<0.01 compared with migration of HPC in upper well/IgG in bottom-well; $^{\#\#}$p<0.01 compared with non-transduced lin$^-$ BM or LSK in upper well/SFM in bottom well; $^{\sim\sim}$p<0.01 compared with migration of lin$^-$ BM or LSK in upper well/IgG in bottom well).

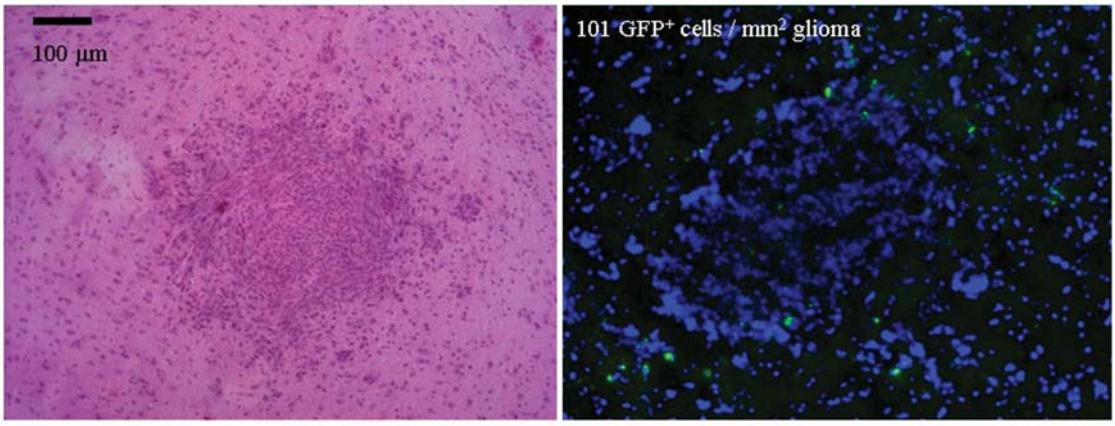

Figure 2. Glioma tropism of lenti-GFP-HPC *in vivo*. The morphology of LNT-229 gliomas was assessed by H&E staining (left). Fluorescence microscopy after nuclear counterstaining with DAPI detected glioma cells (blue) and lenti-GFP-HPC (green). Size bar, 100 μm.

the survival of glioma-bearing mice in two rodent glioma models. We performed the experiments in nude mice, using orthotopic LNT-229 experimental gliomas and human lenti-GFP-HPC (Fig. 5), as well as SMA-560 experimental

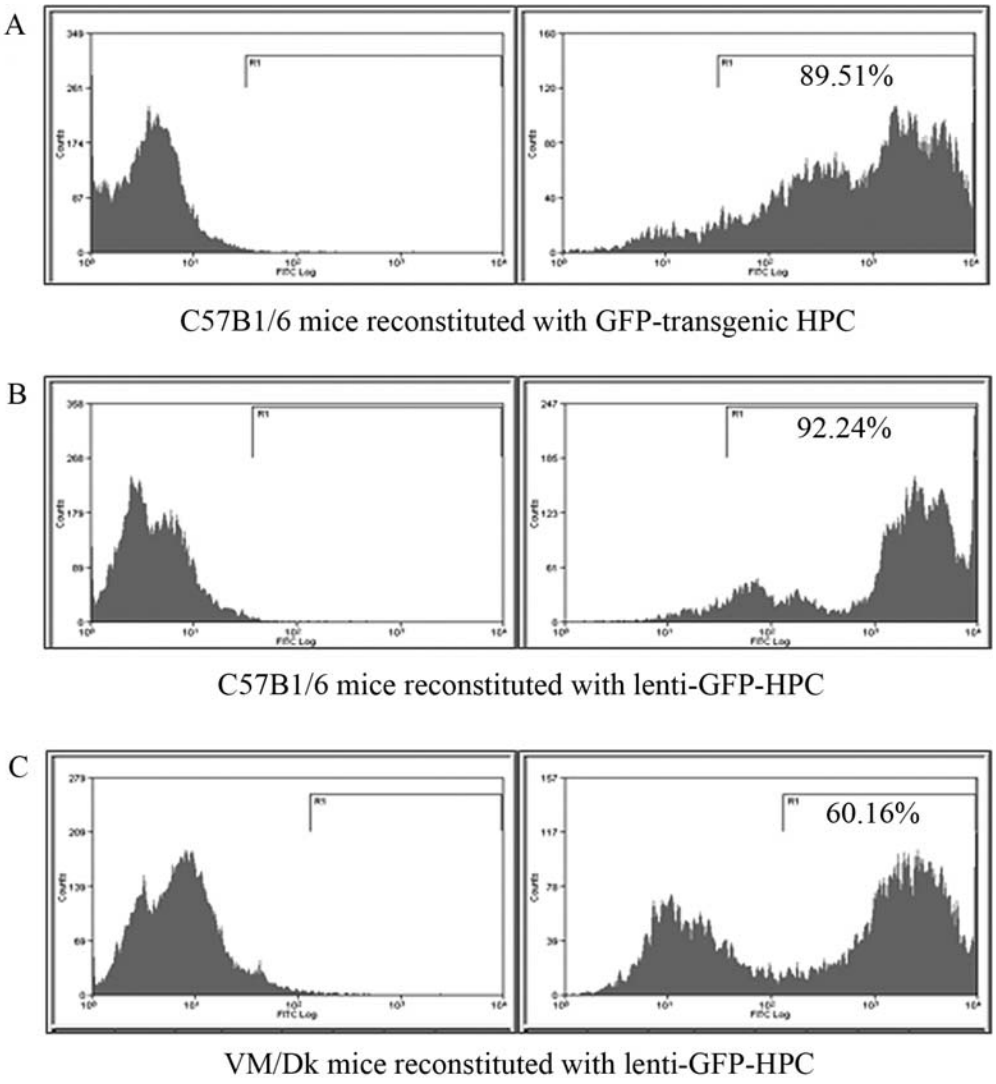

Figure 3. Efficient bone marrow reconstitution with *ex vivo* transduced lenti-HPC. (A and B) GFP expression was quantified in peripheral blood of C57Bl/6 recipient mice 6 weeks after bone marrow reconstitution. The numbers under the bar represent the percentage of GFP-positive cells in the peripheral blood. (A) Peripheral blood of C57Bl/6 reconstituted with GFP-transgenic HPC; wild-type C57Bl/6 control animal (left). (B) C57Bl/6 reconstituted with *ex vivo* lenti-GFP-transduced HPC (right). Control C57Bl/6 animal (left). (C) Peripheral blood of VM/Dk mice after reconstitution with *ex vivo* lenti-GFP-transduced HPC was assessed by flow cytometry for GFP expression (right). Control VM/Dk animal (left).

gliomas grown in syngeneic VM/Dk mice and murine lenti-GFP-HPC (Fig. 6). The i.v. injection of lenti-GFP-HPC into glioma-bearing mice did not alter the survival in either model (Figs. 5 and 6).

Lenti-GFP-HPC do not alter the survival benefit conferred by cerebral irradiation in human LNT-229 or murine SMA-560 glioma-bearing mice. Radiotherapy is an essential part in the treatment of glioblastoma patients. We have previously shown that cerebral irradiation of glioma-bearing mice enhanced the attraction of HPC 2-fold. Human and murine HPC are enriched for stem cell populations with the capacity of self renewal and multipotency. The genotoxic effects of irradiation could lead to the activation of regeneration capacities of HPC. Therefore, we analyzed the impact of combining cerebral irradiation with i.v. injection of lenti-GFP-HPC on the survival of the glioma-bearing animals. Cerebral irradiation with a single fraction of 6 Gy prolonged the median survival from 69 to 91 days in LNT-229-bearing nude mice (Fig. 5) and from 68 to 88 days in SMA-560-glioma-bearing VM/Dk mice mice (Fig. 6). The i.v. injection of human lenti-GFP-HPC did not alter the survival benefit of radiotherapy in the LNT-229 glioma-bearing mice (Fig. 5). Moreover, i.v. injection of murine lenti-GFP-HPC before or after irradiation did not modify the survival of SMA-560 glioma-bearing mice either (Fig. 6).

Discussion

Adult stem cells are promising candidates for a targeted cell-based delivery of therapeutic molecules to experimental gliomas. The investigation of such cellular carriers is motivated by the urgent necessity to establish novel tumor-selective therapeutic approaches for glioblastoma patients who, despite advances in the fields of neurosurgery, radiation oncology and neurooncology, still experience a poor prognosis (24). One of the major reasons for the failure of the established therapeutic modalities or the experimental approaches including

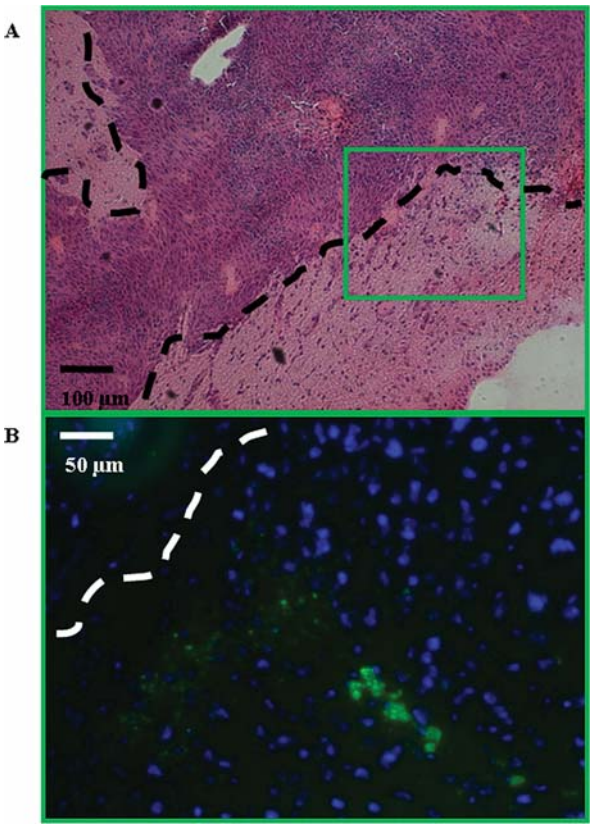

Figure 4. Glioma-mediated attraction of bone marrow-derived cells. (A) The morphology of SMA-560 gliomas was assessed by H&E staining. Black dotted lines indicate tumor margins. Size bar bottom left, 100 μm. The indicated area is shown. (B) Fluorescence microscopy after nuclear counterstaining with DAPI for the detection of glioma cells (blue) and GFP-expressing bone marrow-derived cells (green). White dotted lines indicate tumor margin. Size bar, 50 μm.

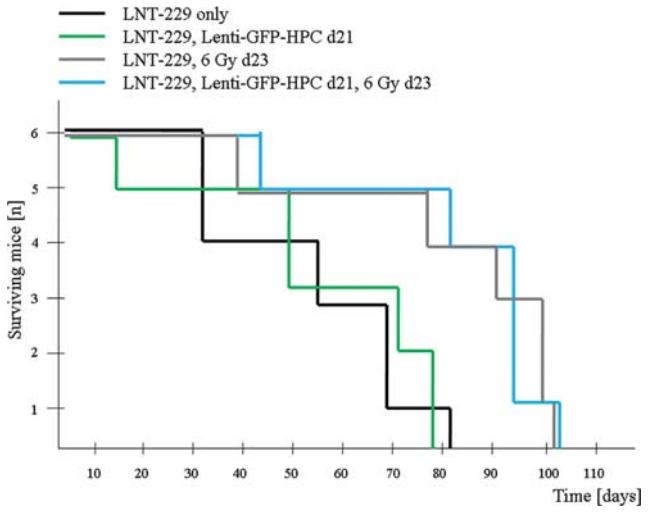

Figure 5. Human lenti-GFP-HPC do not modify the growth of LNT-229 gliomas. LNT-229 glioma cells (75,000) were implanted into the right striatum of nude mice. Lenti-GFP-HPC (10^6) were injected i.v. either with or without cerebral irradiation on day 10. The mice were examined daily and euthanized at the onset of neurological symptoms.

gene therapy is the failure to target deeply infiltrating tumor cells and the intrinsic resistance of glioma cells to various apoptotic stimuli.

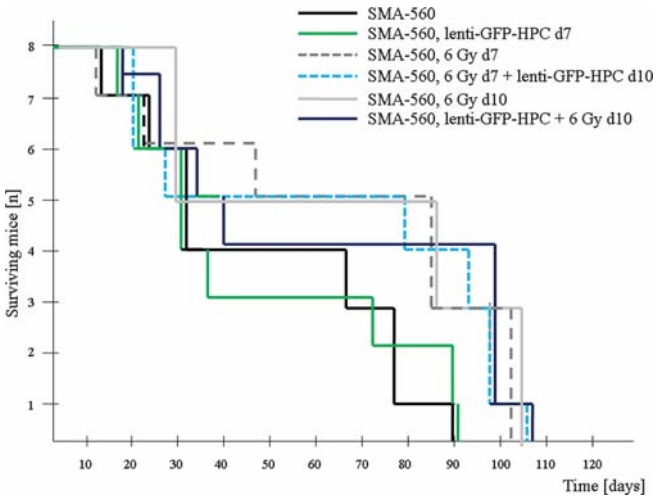

Figure 6. Murine lenti-GFP-HPC do not modify the growth of SMA-560 gliomas. SMA-560 glioma cells (5,000) were implanted into the right striatum of VM/Dk mice. Syngeneic lenti-GFP-HPC (10^6) were injected i.v. either without, before or after cerebral irradiation. The mice were examined daily and euthanized at the onset of neurological symptoms.

For the clinical perspective of cell-based therapies, HPC represent a very attractive adult stem cell population because they are the best characterized adult stem cell population (25,26) that displays a glioma tropism (1-3) and are accessible from bone marrow, cord blood or mobilized peripheral blood. The bone marrow harbours several other adult progenitor cell populations, e.g., multipotent adult progenitor cells (MAPC) or mesenchymal stem cells (MSC). Primary adult MAPC have been modified to stably produce retroviral vectors while maintaining their high expansion capacity and their specific tropism for gliomas (27). Intra-arterially injected MSC were also attracted by glioma-derived CXCL12 in a murine glioma model (28). After intratumoral injection in a rat glioma model, GFP-expressing MSC integrated into tumor vessel walls (29). Intravenously injected MSC, however, did not home into intracerebral gliomas. MSC were not only investigated as cellular carriers in glioma models, but were also applied in an experimental model of stroke. Since human MSC secrete VEGF, Chen and colleagues (30) aimed at exploiting the putative pro-angiogenic potential of MSC in a rat model of stroke. MSC injection 24 h after middle cerebry artery occlusion led to enhanced angiogenesis in the host brain. The MSC-induced angiogenesis was mediated by increased levels of endogenous VEGF and VEGF-R2. Of note, MAPC and MSC are a highly proliferative cell type. Thus, large scale production would be feasible, but the proliferative activity could lead to tumorigenicity. Further, while the induction of angiogenesis in a stroke model might be beneficial, it would be hazardous in malignant gliomas. The therapeutic application of HPC was not studied only in cancer models. Cord blood $CD34^+$ cells have been transduced with a lentivirus encoding a secretory monoclonal anti-HIV antibody. In the future, an HIV vaccine might be delivered by autologous transplantation of *ex vivo* modified HPC, which would develop into antibody-secreting B cells *in vivo* and provide a continuous supply of anti-HIV neutralizing antibodies (31).

The exploitation of HPC as delivery vehicles for glioma therapy requires an efficient genetic modification. First, we

transduced HPC, lin$^-$ BM or LSK with a GFP lentivirus. We performed a single round of exposure to a significant excess of viral particles (6,11,12). The transduction efficacy was >75% while the glioma-mediated attraction of the HPC *in vitro* or *in vivo* was unaltered (Figs. 1 and 2). Lentiviral transduction of peripherally mobilized CD34$^+$ HPC was also efficient, and did not interfere with their stem cell features (32). We next analyzed the impact of the glioma microenvironment on the survival of the cellular vectors *in vitro*. Trypan blue exclusion indicated that survival was promoted by SN-G (Table I). Thus, glioma tissue might provide a specialized microenvironment that, in addition to attracting exogenous and endogenous bone marrow-derived cells, promotes the maintenance and the survival of these cells comparable to a *niche*. Many different cell types and different factors may contribute to the formation of hematopoietic stem cell niches (33). Of note, some HPC survived in SN of the non-neoplastic SV-FHAS (Table I). Further studies will be important to identify key components mediating the survival of HPC within the glioma tissue. This might further contribute to the optimization of an HPC-based therapy against malignant gliomas.

Endogenous bone marrow-derived cells have been detected in experimental gliomas (34). To analyze the influence of lentiviral transduction on the glioma-mediated attraction of bone marrow-derived cells, we performed bone marrow reconstitutions. The percentage of GFP-positive cells in the peripheral blood of C57Bl/6 mice after reconstitution with GFP-transgenic HPC or lenti-GFP-HPC did not differ (Fig. 3A and B). Lentivirally GFP-transduced human cord blood CD34$^+$ HPC have previously been transplanted into NOD/SCID mice to examine gene transfer and expression in engrafting human cells. Fifteen weeks post-transplantation, 37±12% of engrafted human cells expressed GFP introduced by a lentivirus (35). For our further studies, we selected the syngeneic SMA-560-VM/Dk model. We reconstituted VM/Dk mice with syngeneic lenti-GFP-HPC (Fig. 3C) and orthotopically implanted SMA-560 gliomas into reconstituted VM/Dk mice. GFP-positive bone marrow-derived cells accumulated in the experimental gliomas (Fig. 4), underscoring that lentiviral transduction does not interfere with the migration characteristics of bone marrow-derived cells.

Radiotherapy is essential in the treatment of glioblastoma. We asked whether adult HPC that have homed to the gliomas before cerebral irradiation display any regenerative function for the restoration of the tumor and thus interfere with the effects of irradiation and modify the survival advantage of the animals conferred by radiotherapy. This was not the case either for lenti-GFP-HPC in LNT-229 or in SMA-560-glioma-bearing mice (Figs. 5 and 6). Of note, these data do not exclude the possibility that cerebral irradiation after the injection of lenti-GFP-HPC leads to their cell death and precludes a therapeutic effect from cell-based therapy. Finally, intracerebral implantation of human or murine lenti-GFP-HPC alone was not tumorigenic and the mice are still alive. Of course, our data do not completely exclude the potential of tumor formation by insertional mutagenesis, because insertional mutagensis is a very rare event, and due to the number of our animals per experimental group, we might not have picked up this rare event. The oncogenic potential of retrovirally and lentivirally transduced lin$^-$ BM has also been evaluated in the tumor-prone Cdkn2a$^{-/-}$ mice. These mice exhibit enhanced susceptibility towards cancer-triggering genetic lesions. Retroviral vectors triggered a dose-dependent acceleration of tumor onset. In contrast, tumorigenesis was unaffected by lentiviral vectors and did not enrich for specific integrants, despite the higher integration load and robust expression in all hematopoietic lineages (36). These results validate that prototypical lentiviral vectors have low oncogenic potential, underscoring the rationale for their application in cellular therapy for gliomas. This is particularly relevant in view of the recent report of brain tumor formation by therapeutically administered neural stem cells in a patient with ataxia telangiectasia (37).

In conclusion, lentiviral transduction did not interfere with the glioma tropism of exogenously injected HPC or grafted bone marrow-derived cells. Lenti-GFP-HPC were not tumorigenic, did not alter the growth of experimental gliomas and did not modulate the therapeutic effect of radiotherapy. Thus, these studies provide an important proof of principle for the feasibility and safety of an HPC-based cellular therapy for malignant gliomas.

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