Bone Marrow–Derived Mesenchymal Stem Cell–Mediated Dual-Gene Therapy for Glioblastoma

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Bone-marrow mesenchymal stem cells (BMSCs) have been used for systemic delivery of therapeutic genes to solid tumors. However, the optimal treatment time post-BMSC implantation and the assessment of the long-term fate of therapeutic BMSCs post-tumor treatment are critical if such promising therapies are to be translated into clinical practice. An efficient BMSC-based therapeutic strategy has been developed that simultaneously allows killing of tumor cells, inhibiting of tumor angiogenesis, and assessment and eradication of implanted BMSCs after treatment of glioblastoma. BMSCs were engineered to co-express the angiogenesis inhibitor krigle 5 (*K5*) of human plasminogen, under the control of the cytomegalovirus promoter (*CMV*) and the human sodium–iodide symporter (*NIS*), involved in uptake of radioisotopes, under the control of early growth response factor 1 (*Egr1*), a radiation-activated promoter. A significant decrease in tumor growth and tumor angiogenesis and a subsequent increase in survival were observed when mice bearing glioblastoma were treated with ^{188}Re post-therapeutic intravenous BMSC implantation. Furthermore, the systemic administration of ^{188}Re post-tumor treatment selectively eliminated therapeutic BMSCs expressing *NIS*, which was monitored in real time by ^{125}I micro single photon emission computed tomography/computed tomography imaging. Meanwhile, the *Egr1* promoter induced a ^{188}Re radiation positive feedback effect absorbed by *NIS*. After intravenous BMSC implantation, BMSCs levels in the tumor and lung both peaked on day 10 and decreased to the lowest levels on days 24 and 17, respectively. These findings suggest that day 17 post-BMSC implantation could be an optimal time for ^{188}Re treatment. These results provide a new adjuvant therapy mediated by BMSCs for glioblastoma treatment.

Keywords: bone-marrow mesenchymal stem cells, gene therapy, glioma, lentiviral virus, radiation therapy

INTRODUCTION

GLIOBLASTOMA IS THE MOST AGGRESSIVE subtype of high-grade glioma, with a median survival time of <15 months.¹ Despite the recent advances in neuro-oncology, the majority of patients ultimately experience local or locoregional failure, and no significant improvements in patient survival rates have been observed.² Novel treatments are urgently needed. Virus-mediated gene therapy has become a hot topic in adjuvant therapy for cancer. However, challenges such as the immunogenicity of certain viral vectors, virus tropism that is inconsistent with the desired target, the blood–brain barrier, and the presence of a blood–tumor barrier^{3,4} have limited the success of clinical trials to date. Therefore, the key to gene therapy is the se-

lection of a suitable gene carrier to achieve specific transfer and efficient target gene expression in tumors.

Cell-mediated antitumor gene therapy, in which cells transport the therapeutic gene to the solid tumor, is a new approach for solid tumor targeting therapy. Bone-marrow mesenchymal stem cells (BMSCs) have unique advantages in local and disseminated malignant tumor targeting^{5,6} and the ability to cross the blood–brain barrier and the blood–tumor barrier in glioblastoma.⁷ Additionally, BMSCs have a reliable source, as they can be isolated and expanded with relative ease from bone marrow, are easy to obtain, lack immunogenicity, and can be used for autologous implantation with no ethical issues, suggesting that BMSCs may be an

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ideal cell carrier for gene therapy of glioblastoma.⁸ In addition to these properties, the increase in vascular permeability and the release of various cytokines after tumor irradiation can also enhance BMSC migration to the tumor site.⁹

However, in order to avoid continuous access of antitumor agents to normal tissue and to circumvent any malignant transformation of BMSCs, it is critical to develop and test BMSCs that simultaneously allow killing of tumor cells, follow the fate of therapeutic BMSCs with a clinically relevant noninvasive imaging method, and ultimately selectively eradicate BMSCs post-tumor treatment. The sodium-iodide symporter (NIS) is an intrinsic membrane glycoprotein responsible for iodide transport, which is expressed in thyroid follicular cells and others cells containing a lactoperoxidase system.^{10,11} NIS is involved in the uptake of several isotopes, including ¹²⁵I, ^{99m}Tc, ¹⁸⁸Re, and ¹³¹I.¹² ¹³¹I radioiodine therapy mediated by NIS has been shown to be a safe and effective approach for the imaging and treatment of thyroid disease.¹³ The authors and other researchers have successfully introduced ectopic expression of *NIS* by virus for imaging and therapy in nonthyroidal carcinoma.^{14–18}

To reduce immunogenicity and increase tumor tropism, virus intratumoral injection or stable therapeutic gene expression tumor cell lines were used in these studies, both of which, however, limit the clinical application, especially for patients with a disseminated malignant tumor or with tumors in deep tissues. Some studies^{19,20} have reported that the early growth response factor 1 (*Egr1*), a radiation-sensitive promoter that can be activated by radiation, can initiate expression of downstream target genes efficiently and specifically. Based on this property, the *Egr1* promoter is inserted upstream of the *NIS* gene. NIS absorbs radioisotopes, and the radiation emitted by the isotopes also activates the *Egr1* promoter, inducing more expression of NIS. This forms a positive feedback loop to ensure tumor tissues have an adequate uptake of radionuclide. Among various radioisotopes, ¹⁸⁸Re²¹ can emit high energy γ and β rays, exerts strong biological effects, has a short half-life (16.9 h), and is easy to control, making it an ideal radionuclide for imaging and therapy.

Recent preclinical studies have suggested that radiotherapy in combination with antiangiogenic/vasculature targeting agents enhances the therapeutic ratio of ionizing radiation alone.²² Previous work has shown that kringle 5 (*K5*) of human plasminogen or *K5* combined with ¹³¹I absorbed by NIS can inhibit tumor angiogenesis.^{15,16,23,24} However, these gene therapies were mediated by

virus. In this study, an efficient BMSC-based therapeutic strategy was developed that simultaneously allows killing of tumor cells, inhibiting of tumor angiogenesis, and assessment and eradication of BMSCs after treatment of glioblastoma. BMSCs were engineered to co-express *K5* under the control of the *CMV* promoter and *NIS* under the control of *Egr1* promoter.

Then, ¹⁸⁸Re combined with *K5* expression was investigated for the treatment of glioblastoma, BMSC tumor targeting after intravenous (i.v.) implantation by dynamic micro single photon emission computed tomography/computed tomography (micro-SPECT/CT) imaging. The findings identify a novel therapeutic approach for glioblastoma.

MATERIALS AND METHODS

Cell lines and animals

The U87 human glioblastoma cell line (ATCC, Manassas, VA), human umbilical vein endothelial cells (HUVECs), and the 293T cell line (Cell Bank of the Chinese Academy of Science, Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin. All cell lines were maintained at 37°C in a humidified 5% CO₂ incubator.

BALB/c nude mice and Sprague–Dawley rats were purchased from Shanghai Slaccas Experiment Animal Corp. (Shanghai Institute for Biological Science, Shanghai, China). Animal studies were approved by the local Ethics Committee (Shanghai Jiaotong University School of Medicine) and performed according to ethical principles of animal experimentation.

Plasmid construction

and lentiviral preparation

The *NIS*, *K5* (His-tagged), and *Egr1* genes were present in the laboratory.¹⁵ The *pLVX-CMV-Puro* (puromycin) expression vector was purchased from Clontech (Takara, Dalian, China). The *NIS*, *Egr1*, and *K5* genes were polymerase chain reaction amplified separately and then cloned into the *pLVX-CMV-Puro* vector to construct *pLVX-CMV-K5-Egr1-NIS*, *pLVX-CMV-K5-Egr1-GFP*, and *pLVX-CMV-GFP-Egr1-NIS* plasmids. The lentiviral particles were produced and amplified in 293T cells.

Stable transfection of BMSCs and U87 cells

BMSCs were isolated from bone-marrow aspirates of Sprague–Dawley rats (5 weeks old), propagated and characterized, as previously described,²⁵ and then infected with the above lentivirus. The

stably transfected cell lines were termed BMSC-K5-NIS, BMSC-K5-GFP, and BMSC-GFP-NIS after puromycin selection.

To monitor the established glioma tumor by an *in vivo* optical imaging system, U87 cells were infected with GV260 lentivirus containing a luciferase reporter gene. The stably transfected cell lines were termed U87-Luc after puromycin selection.

Western blot analysis

BMSC-K5-NIS, BMSC-K5-GFP, BMSC-GFP-NIS, and BMSC cell lysates were prepared using standard methods.²⁵ To detect *NIS* and *K5* expression, Western blot was performed using antibodies against the following proteins: NIS (Millipore, Boston, MA), His-tag (Abgent, Suzhou, China), and β -actin (Abgent).

¹²⁵I uptake assay

Following transfection, iodide uptake of BMSC-K5-NIS, BMSC-K5-GFP, BMSC-GFP-NIS, and BMSC cells was determined under steady-state conditions, as described previously.¹⁶ The day before the experiment, BMSC-K5-NIS, BMSC-K5-GFP, BMSC-GFP-NIS, and BMSC cells were plated (2×10^5 cells/well) onto 24-well plates. After 24 h, 500 μ L of Hanks' balanced salt solution (HBSS) containing 3.7 kBq of ¹²⁵I and 10 μ mol/L of sodium iodide (NaI) was added. The cells were incubated at 37°C for 5–180 min, washed twice with ice-cold HBSS, and lysed using 0.5 mol/L of NaOH. The radioactivity (counts/min, cpm) of the cell lysates was measured using an automatic γ -counter (Shanghai Rihuan Company, Shanghai, China).

Cell irradiation

To study the influence of ¹⁸⁸Re irradiation on the *NIS* expression (*Egr1* promoted), BMSC-K5-NIS cells were irradiated with ¹⁸⁸Re (Shanghai Kexing Pharmaceutical Company, Shanghai, China) at varying doses (0.4625–7.4 MBq/mL) for different time periods (0–48 h). *NIS* expression was detected by Western blot.

In vitro migration assay

To examine the effect of K5 on HUVECs, a Boyden Chamber-based assay was performed. HUVECs (5×10^3) were plated into the upper chamber of a 24-well transwell (Corning, Corning, NY) in DMEM without FBS and incubated for 6 h. The medium was removed, and the medium from different stable BMSC cell lines was loaded into the upper chambers. DMEM containing 10% FBS was added into the lower chambers. Following incubation for 12 h, the upper chamber was dismantled.

The non-migrated cells were scraped from the upper surface of the membrane with cotton swabs. Then, the membrane was stained with crystal violet (Beyotime Institute of Biotechnology, Shanghai, China). Each sample was tested in quadruplicate, and a representative field in each well was counted at 10 $\times$ magnification to determine the number of migrated cells.

Orthotopic glioblastoma tumor model

BALB/c nude mice, aged 5–6 weeks, were used for orthotopic glioblastoma experiments, as previously described.²⁶ To establish glioblastoma xenografts, 5×10^5 U87-Luc cells were stereotactically injected in a 1.5 μ L volume into the left striatum over a 10 min period based on the following coordinates: 1 mm anterior, 1 mm lateral from the bregma, and 3 mm deep from the cortical surface. Mice were injected intraperitoneally with D-luciferin (150 mg/kg body weight; Sciencelight, Shanghai, China) on day 14 after implantation, and the average luminescence (photons/s/cm²) of tumors was monitored by optical imaging (Xenogen IVIS, Alameda, CA). The tumor take rate was 100%.

Tumor tropism of BMSCs and ¹⁸⁸Re irradiation

To examine tumor tropism, survival time, and distribution of BMSCs after i.v. implantation, 5×10^5 BMSC-GFP-NIS were injected i.v. into orthotopic U87-Luc tumor-bearing nude mice ($n = 3$) every 3 days a total of three times. ¹²⁵I micro-SPECT/CT imaging was performed to monitor the migration and survival time of BMSC-GFP-NIS. Three-dimensional lung structures were constructed using CT images, and three-dimensional structures of U87-Luc tumors were constructed using ¹²⁵I tumor uptake. U87-Luc tumor-bearing nude mice injected with phosphate-buffered saline (PBS) were used as controls ($n = 3$).

To detect iodide uptake by *NIS* promoted by the radiosensitive *Egr1* promoter after ¹⁸⁸Re irradiation, U87-Luc tumor-bearing mice were injected i.v. with 7.4 MBq of ¹²⁵I 3 days after the last round of BMSC-GFP-NIS implantation, and 30 min later, micro-SPECT/CT imaging was performed. Twenty-four hours after micro-SPECT/CT imaging, 18.5 MBq ¹⁸⁸Re was injected i.v. into the nude mice for *Egr1* irradiation. Two days after ¹⁸⁸Re injection, 7.4 MBq ¹²⁵I micro-SPECT/CT imaging was performed again.

All of the images were processed and reconstructed using Nuclear v1.02 (Bioscan, Poway, CA). HiSPECT v1.4.2 (Bioscan) was used for image acquisition, and InVivoScope v1.44 (Bioscan) was used

for image analysis. Regions of interest (ROIs) were drawn around the U87-Luc tumor and lung, the radioactivity per volume unit (Conc) in the ROIs was measured using InVivoScope v1.44 (Bioscan).

BMSC-mediated dual gene therapy *in vivo*

After establishing orthotopic U87-Luc tumors, the average luminescence (photons/s/cm²) of the tumors was measured by optical imaging. The mice were randomly assigned to five groups ($n=6$ in each group), and the average tumor radiance in each group was roughly equivalent.

BMSC-K5-NIS, BMSC-GFP-NIS, BMSC-K5-GFP, BMSCs, or PBS (5×10^5) were injected i.v. into nude mice (three times every 3 days). Seventeen days after the final injection, 37 MBq of ¹⁸⁸Re (twice every other day) was applied for radiation therapy. Tumor growth monitored by optical imaging and survival time of the animals was used to assess the therapeutic effects.

Survival of BMSC-GFP-NIS after ¹⁸⁸Re radiation therapy

BMSC-GFP-NIS cells were implanted i.v. into U87-Luc tumor-bearing mice ($n=3$) as above. ¹²⁵I micro-SPECT/CT imaging and immunofluorescence were used to monitor the survival of BMSC-GFP-NIS cells in the orthotopic U87-Luc tumor before and after ¹⁸⁸Re treatment according to the method above. The tumors from mice without ¹⁸⁸Re radiation therapy were used as controls ($n=3$).

Histology and immunofluorescence

Three days after the final round of BMSC-GFP-NIS implantation, U87-Luc tumor-bearing nude mice were sacrificed. Serial coronal cryosections (6 μ m) from frozen brains were processed. The entire brain sections were subjected to hematoxylin and eosin (H&E) staining for visualization of the tumor mass and immunohistochemical analysis using mouse anti-NIS (Millipore) for visualization of the implanted BMSC-GFP-NIS cells. U87-Luc tumor sections were subjected to immunofluorescence analysis using mouse anti-NIS (Millipore) and mouse anti-CD31 (Dako, Glostrup, Denmark) combined with DyLight594 rabbit anti-mouse IgG (MultiSciences Biotech Co., Shanghai, China). Nuclei were labeled with 4',6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology), and images were acquired with a fluorescence microscope (Olympus, Tokyo, Japan).

Capillary density was defined as the number of CD31+ ECs/high-power field (hpf; 200 $\times$). Five high-power fields were counted per section from six sections/tumor tissue in three animals/group.

Statistical analysis

Data were analyzed by Student's *t*-test when comparing two groups and by analysis of variance when comparing more than two groups. Data are expressed as mean $\pm$ SD. *p*-Values <0.05 were considered significant. Survival times of the five mouse groups were compared using the log-rank test. All analyses were performed using GraphPad Prism v5 (GraphPad Software, La Jolla, CA).

RESULTS

Construction of lentiviral vector and BMSC stable cell lines

The *pLVX-CMV-K5-Egr1-NIS*, *pLVX-CMV-K5-Egr1-GFP*, and *pLVX-CMV-GFP-Egr1-NIS* constructs were successfully generated and packaged. BMSCs were successfully isolated and cultured as per a previous study.²⁵ After lentivirus infection and puromycin selection, the stable cell lines BMSC-K5-NIS, BMSC-GFP-NIS, and BMSC-K5-GFP were generated. BMSCs were transfected with *NIS* linked to *Egr1* promoter for positive radioisotope uptake of the construct. The treatment regimen consisted of systemic stem cell (carrying therapeutic gene *K5*) administration followed by systemic ¹⁸⁸Re injections (Fig. 1a). Western blot analysis showed *K5* protein expressed in BMSC-K5-NIS and BMSC-K5-GFP cells but not BMSC-GFP-NIS and BMSCs, and *NIS* protein expressed in BMSC-K5-NIS and BMSC-GFP-NIS cells but not BMSC-K5-GFP and BMSC cells (Fig. 1b). BMSC-K5-NIS and BMSC-GFP-NIS cells showed a significant increase in ¹²⁵I uptake *in vitro* that peaked at 120 min and decreased only slightly at 180 min. There was no ¹²⁵I uptake observed in BMSC-K5-GFP cells or BMSCs (Fig. 1c). These results revealed that BMSCs could coexpress *K5* and *NIS* simultaneously and absorb radioisotope by *NIS*.

Migration assay *in vitro*, specific function of *Egr1* promoter *in vitro*

Migration assays showed that using medium from BMSC-K5-NIS and BMSC-K5-GFP to culture HUVECs for 12 h resulted in approximately 50% of HUVECs migrating to the opposite membrane (Fig. 2a and b). In contrast, BMSC-GFP-NIS and BMSCs culture medium had no influence on HUVECs migration, with approximately 100% of the cells migrating to the opposite membrane (Fig. 2a and b). These results show that culture medium from BMSC-K5-NIS and BMSC-K5-GFP could significantly inhibit the migration of HUVECs.

After culturing with medium containing 1.85 MBq/mL of ¹⁸⁸Re, Western blot analysis showed

Figure 1. Schematic depiction of the *CMV-K5-Egr1-NIS* bone marrow mesenchymal stem cell (BMSC) construct, krigle 5 (K5) and sodium-iodide symporter (NIS) expression, and iodide uptake *in vitro*. **(a)** BMSC/gene construct. BMSCs were transfected with K5 under control of the cytomegalovirus promoter (*CMV*) promoter, NIS under control of the radio-sensitive early growth response factor 1 (*Egr1*) promoter. Alternatively, a reporter gene replaced K5 or NIS. All vectors contained a puromycin resistance gene under the control of the phosphoglyceric kinase (*PGK*) promoter. **(b)** K5 and NIS expression in BMSC-K5-NIS, BMSC-K5-GFP, BMSC-GFP-NIS, and BMSCs. **(c)** Iodide uptake in BMSC-K5-NIS, BMSC-K5-GFP, BMSC-GFP-NIS, and BMSCs *in vitro* at different time points (5–180 min; $n=3$).

that NIS protein expression in BMSC-K5-NIS increased gradually with time, with the highest levels expressed at 36 h, and levels did not decrease by 48 h (Fig. 2c). The 36 h time point was selected as the optimum time for ^{188}Re radiation to activate the *Egr1* promoter. BMSC-K5-NIS cells were cultured with medium containing different concentrations of ^{188}Re (0.4625–7.4 MBq/mL) for 36 h. Western blot showed that the highest expression of NIS protein was induced by 0.925 MBq/mL of ^{188}Re (Fig. 2d).

Micro-SPECT/CT imaging *in vivo* and immunofluorescence analysis

The U87-Luc stable cell line was established successfully after infection with GV260 lentivirus (Fig. 3a). The orthotopic U87-Luc tumor in the brain could be monitored by optical imaging after D-luciferin administration (Fig. 3b). To detect the function of radiosensitive *Egr1* promoter *in vivo*, ^{125}I micro-SPECT/CT imaging was performed before and after 18.5 MBq of ^{188}Re irradiation.

Increased radionuclide uptake was observed after ^{188}Re radiation with $0.4212 \pm 0.0102 \times 10^{-3}$ MBq/mm³ to $0.9863 \pm 0.0093 \times 10^{-3}$ MBq/mm³ (Fig. 3c and d; c: before ^{188}Re irradiation; d: after ^{188}Re irradiation; $n=3$). H&E staining showed the U87-Luc tumor in the brain (Fig. 3e). Immunohistochemical analysis showed NIS-expressed BMSC-GFP-NIS cells existed in the U87-Luc tumor but not in normal brain tissues (Fig. 3f). Fluorescence confocal microscopy pictures confirmed the existence of BMSC-GFP-NIS cells in the orthotopic U87-Luc tumor after their i.v. implantation (Fig. 3g).

BMSC-GFP-NIS cells' survival time after implantation

To follow BMSCs fate after implantation, micro-SPECT/CT imaging was performed using ^{125}I , an optimal radiotracer to monitor NIS activity that has high uptake rate and low background activity. Micro-SPECT/CT imaging allowed specific detection of i.v. implanted BMSC-GFP-NIS cells on day

Figure 2. Migration assay *in vitro*, the function of *Egr1* promoter *in vitro*. **(a and b)** *In vitro* migration assay showing that BMSC-K5-NIS ($p < 0.01$ vs. BMSC-GFP-NIS medium; $p < 0.01$ vs. BMSC medium) and BMSC-K5-GFP medium ($p < 0.01$ vs. BMSC-GFP-NIS medium; $p < 0.01$ vs. BMSC medium) could significantly inhibit the migration of human umbilical vein endothelial cells (HUVECs; bar = 100 μ m; $n = 3$; $**p < 0.01$). **(c)** Specific function of the *Egr1* promoter *in vitro*. After culturing in medium containing 1.85 MBq/mL of ^{188}Re , Western blotting showed that NIS protein expression in BMSC-K5-NIS cells increased gradually with time, and reached the highest levels at 36 h. **(d)** Optimal concentration of ^{188}Re for *Egr1* promoter. BMSC-K5-NIS cells cultured with medium containing different concentrations (0.4625–7.4 MBq/mL) of ^{188}Re for 36 h. Western blotting showed that the highest expression of NIS protein was induced by 0.925 MBq/mL of ^{188}Re . Color images available online at www.liebertpub.com/hum

10, 17, and 24 in tumor-bearing nude mice (Fig. 4a). As shown in representative images, ^{125}I uptake in the tumor and lung reached the highest levels on day 10 (conc: $0.401 \pm 0.011 \times 10^{-3}$ MBq/mm 3 and $0.312 \pm 0.026 \times 10^{-3}$ MBq/mm 3 , respectively), then decreased over time, reaching the lowest levels on day 24 (tumor conc: $0.230 \pm 0.017 \times 10^{-3}$ MBq/mm 3) and day 17 (lung conc: $0.227 \pm 0.022 \times 10^{-3}$ MBq/mm 3), respectively. However, levels in the tumor on day 17 (conc: $0.303 \pm 0.014 \times 10^{-3}$ MBq/mm 3) were higher than those in the lung. Concentrations in the lung on day 17 were similar to the control group (i.v. injection of PBS; conc: $0.247 \pm 0.048 \times 10^{-3}$ MBq/mm 3 ; Fig. 4a and c). There was almost no change in ^{125}I uptake in the tumor and lung in the control group over time (Fig. 4b and c). Together, these results showed that BMSCs fate can be detected by clinically relevant micro-SPECT/CT imaging, and day 17 post implantation may be an optimal time for radiation treatment, while

day 10 treatment could possibly lead to severe lung injury.

Dual gene therapy *in vivo*

Mice were randomly assigned to one of five groups ($n = 6$ in each group) based on average luminescence ($1\text{--}2 \times 10^7$ photons/s/cm 2). After 37 MBq of ^{188}Re treatment (twice every other day), the tumor growth in the BMSCs group and sham group increased quickly (monitored by optical imaging), and mice died in a short time (Fig. 5a and b). However, tumor growth in the BMSC-K5-NIS, BMSC-GFP-NIS, and BMSC-K5-GFP groups was inhibited, and mice survived longer, especially those in the BMSC-K5-NIS group (Fig. 5a and b). Kaplan–Meier survival curves showed that the median survival time for the BMSC-K5-NIS, BMSC-GFP-NIS, BMSC-K5-GFP, BMSC, and sham group was 54.5, 42, 41.5, 31.5, and 31.5 days, respectively (Fig. 5c).

Figure 3. Optical and micro single photon emission computed tomography/computed tomography (micro-SPECT/CT) imaging *in vivo*, immunohistochemical and immunofluorescence analysis. **(a)** The plasmid map of GV260 containing a luciferase gene. **(b)** Optical imaging of the orthotopic U87-Luc tumor in the brain. **(c and d)** ^{125}I radionuclide uptake before and after 18.5 MBq of ^{188}Re radiation monitored by micro-SPECT/CT imaging. Increased radionuclide uptake is observed after ^{188}Re radiation with $0.4212 \pm 0.0102 \times 10^{-3} \text{ MBq/mm}^3$ (c: before ^{188}Re radiation; $n=3$) to $0.9863 \pm 0.0093 \times 10^{-3} \text{ MBq/mm}^3$ (d: after ^{188}Re radiation; $n=3$; yellow arrow: ^{125}I radiouptake in orthotopic U87-Luc tumor; radionuclide uptake are represented as the mean $\pm$ standard deviation [SD]). **(e)** Hematoxylin and eosin (H&E) staining reveals tumor in the brain (bar = 1 mm). **(f)** Immunohistochemical analysis reveals NIS-expressed BMSC-GFP-NIS in the brain and U87-Luc tumor. **(g)** BMSC-GFP-NIS detection by immunofluorescence analysis (green: GFP; red: NIS; blue: cell nucleus). Color images available online at www.liebertpub.com/hum

Survival of implanted BMSC-GFP-NIS cells after ^{188}Re radiation therapy

Post BMSC-GFP-NIS i.v. implantation, ^{125}I micro-SPECT/CT imaging was performed. Radionuclide uptake was observed before ^{188}Re treatment (Fig. 6a1 and b1) but not 5 days after ^{188}Re treatment (Fig. 6a2) in the orthotopic U87-Luc tumor. However, ^{125}I uptake was observed by micro-SPECT/CT imaging on day 5 in the absence of ^{188}Re treatment (Fig. 6b2). BMSC-GFP-NIS cells were found in the U87-Luc tumor before ^{188}Re treatment but not after treatment, as determined by immunofluorescence (Fig. 6c1, 2). These results confirmed that the implanted BMSCs expressing NIS can be eliminated selectively with ^{188}Re *in vivo*.

Immunofluorescence analysis

Figure 7 depicts representative images of the indirect tumor xenograft immunofluorescence staining. Immunofluorescence revealed fewer CD31⁺ capillaries in the BMSC-K5-NIS group compared to the BMSC-K5-GFP ($p < 0.05$), BMSC-GFP-NIS ($p < 0.01$), BMSC ($p < 0.01$), and PBS ($p < 0.001$) groups. There were fewer CD31⁺ capillaries in the BMSC-K5-GFP group compared to the BMSC-GFP-NIS ($p < 0.05$), BMSC ($p < 0.05$), and PBS ($p < 0.01$) groups (Fig. 7a–f).

DISCUSSION

This study evaluated the role of engineered BMSCs as a therapeutic gene vehicle for the se-

Figure 4. BMSC-GFP-NIS cell survival after implantation. (a and b) ^{125}I uptake in the tumor and lung on days 10, 17, and 24 after BMSC-GFP-NIS cell implantation in the BMSC-GFP-NIS group and the control group (i.v. injected PBS), respectively, monitored by micro-SPECT/CT. (a: BMSC-GFP-NIS group; b: control group; imaging figures contain a one-dimensional structure of the tumor-bearing nude mice and three-dimensional structures of the tumor and the lung; red: tumor; green: lung; yellow arrow: ^{125}I uptake in the U87-Luc tumor). (c) Analysis of ^{125}I uptake in the tumor and lung in the implanted experimental group and the control group ($n=3$ in each group; values are mean $\pm$ SD). Color images available online at www.liebertpub.com/hum

lective delivery of an antiangiogenesis gene combined with a gene involved in absorbing radio-nuclides in the context of the glioblastoma microenvironment. ^{125}I micro-SPECT/CT imaging was used to monitor the migration of intravenous implanted BMSCs.

The ability of mesenchymal stem cells (MSCs) to migrate specifically to tumors has led to the use of these cells as a delivery vector for anticancer agents in different tumor models. They have been engineered to express and provide targeted delivery of immunomodulatory cytokine interleukin-12,²⁷ interferons (IFN) such as IFN- α and IFN- β ,^{28–30} the prodrug converting enzyme such as herpes simplex virus thymidine kinase,³¹ tumor necrosis factor apoptosis-inducing ligand,³² or NIS³³ to many types of tumors. The systemic MSC-mediated NIS gene delivery followed by ^{131}I application resulted in a significant delay in hepatocellular tumor growth.³³ In this study, K5 and NIS were chosen as the therapeutic agent because of their potent

synergistic antitumor effect. In addition, NIS has been shown to be a safe and effective approach not only for the treatment of tumors but also for following the fate of therapeutic MSCs with a non-invasive imaging method.

The approach showed efficacy in an orthotopic glioblastoma tumor model. The antiangiogenesis K5 protein combined with ^{188}Re uptake mediated by NIS gene inhibited tumor angiogenesis and growth prolonged the survival time of tumor-bearing nude mice. K5 is an antiangiogenesis factor that targets endothelial cells and is cytotoxic. The *in vitro* results showed that K5 inhibited migration of HUVEC cells and treatment study *in vivo* showed fewer CD31 $^{+}$ capillaries in the BMSC-K5-NIS group compared to the BMSC-K5-GFP ($p<0.05$), BMSC-GFP-NIS ($p<0.01$), BMSC ($p<0.01$), and PBS ($p<0.001$) groups. As angiogenesis is largely absent in healthy adult cells and present in solid tumors, especially in glioblastoma, which is one of the most vascularized cancers,³⁴

Figure 5. Dual gene therapy *in vivo*. **(a and b)** Tumor growth in the BMSC-K5-NIS, BMSC-K5-GFP, BMSC-GFP-NIS, BMSC, and sham groups ($n=6$ in each group) monitored by optical imaging after treatment. **(c)** Survival of the animals. Survival of animals receiving PBS (black), BMSCs (blue), BMSC-K5-GFP cells (purple), BMSC-GFP-NIS cells (green), or BMSC-K5-NIS cells (red) after ^{188}Re irradiation therapy. Survival was significantly prolonged in the BMSC-K5-NIS group ($p < 0.01$). Kaplan–Meier curves indicate the median survival time for the BMSC-K5-NIS, BMSC-GFP-NIS, BMSC-K5-GFP, BMSC, and sham groups were 54.5, 42, 41.5, 31.5, and 31.5 days, respectively. Color images available online at www.liebertpub.com/hum

Figure 6. ^{125}I micro-SPECT/CT imaging and immunofluorescence. **(a)** ^{125}I micro-SPECT/CT imaging before and 5 days after ^{188}Re treatment following BMSC-GFP-NIS implantation. **(b)** ^{125}I micro-SPECT/CT imaging before and 5 days after BMSC-GFP-NIS implantation in the absence of ^{188}Re treatment (yellow triangle: ^{125}I uptake in orthotopic U87-Luc tumor; 1: before ^{188}Re treatment; 2: after ^{188}Re treatment). **(c)** Immunofluorescence analysis before and 5 days after ^{188}Re treatment following BMSC-GFP-NIS implantation (green: GFP protein; red: NIS protein; yellow: the combined color of GFP and NIS; blue: cell nucleus, DAPI; 1: before ^{188}Re treatment; 2: after ^{188}Re treatment; bar = 100 μm ; $n=3$ in each group). Color images available online at www.liebertpub.com/hum

Figure 7. Immunofluorescence analysis. (a–e) Expression of CD31 in xenograft tumors of different groups treated with ^{188}Re (magnification: 200 $\times$; a–e: xenograft tumor tissues of BMSC-K5-NIS, BMSC-K5-GFP, BMSC-GFP-NIS, BMSC, and PBS groups). (f) The capillary density of vessels positive for CD31 (BMSC-K5-NIS group: * $p < 0.05$ vs. BMSC-K5-GFP group; # $p < 0.01$ vs. BMSC-GFP-NIS group; % $p < 0.01$ vs. BMSC group; & $p < 0.001$ vs. PBS group; BMSC-K5-GFP group: \$ $p < 0.05$ vs. BMSC-GFP-NIS group; ^ $p < 0.05$ vs. BMSC group; @ $p < 0.01$ vs. PBS group). Density was defined as the number of CD31+ capillaries per high-power field (200 $\times$). Data are presented as means $\pm$ SD. $n = 6$ in each group. Color images available online at www.liebertpub.com/hum

only the tumor environment is targeted using this approach. BMSCs can be obtained directly from glioblastoma patients. Therefore, this therapy would not only reduce the side effects of residual implanted BMSCs in the tumor after treatment, but also permit the specific delivery of the therapeutic gene through easy i.v. administration without the risk of allogeneic implantation rejection.

Several studies have reported that BMSCs could increase tumor growth and metastasis.^{35–37} However, these effects were not observed in this study. No difference in tumor growth were observed between the sham group (i.v. injected PBS) and BMSC-implanted group (without therapeutic gene). Bexell *et al.*³⁸ also reported that BMSC grafting had no effect on tumor microvessel density or survival of glioma-bearing animals. It is possible that the effects of BMSCs on tumor growth and metastasis *in vivo* are not universal in solid tumors, especially glioblastoma. In addition, no BMSC-GFP-NIS cells were observed in the tumor by ^{125}I micro-SPECT/CT imaging or immunofluorescence 5 days after ^{188}Re radiation therapy in this study, indicating that NIS may act as a safety mechanism in BMSC implantation. Bexell *et al.*³⁹ found no BMSCs in gliomas at 2 and 7 days following BMSCs injection. The authors suggested that BMSCs should be administered by

intratumoral implantations rather than by i.v. injection. However, in this study, after three rounds of i.v. BMSC implantation, ^{125}I micro-SPECT/CT imaging showed that there were BMSCs within the glioblastoma tumor on days 10 and 17 but not on day 24. The quantity of grafting BMSCs in the tumor tissue peaked on day 10, decreased by day 17, reaching the lowest levels on day 24, as determined by ^{125}I micro-SPECT/CT imaging. The quantity of BMSCs in the lung also peaked on day 10, and then decreased almost to the same levels as the control group on day 17. These results demonstrate that BMSCs could be administered by i.v. injection. However, the migration time is longer than by intratumoral implantation, so ^{188}Re or other therapies relying on BMSCs should be used only once the BMSCs have had sufficient time to migrate to the tumor. Furthermore, ^{188}Re or other treatment drug may lead to severe lung injury on day 10 after BMSC i.v. implantation due to the high levels of BMSCs in lung tissue on at that time. Therefore, day 17 may be the most suitable time for treatment.

Using a glioma surgical treatment model, Bexell *et al.*³⁹ found that intratumorally grafted BMSCs could migrate efficiently within glioma remnants after partial surgical resection. Klopp *et al.*⁹ also showed that tumor irradiation increased the recruitment of circulating MSCs into the tumor mi-

microenvironment. In glioblastoma clinical treatment, the majority of patients have undergone tumor resection and irradiation, and most of them experience high tumor recurrence. Therefore, BMSC-mediated gene therapy is more suitable for these patients.

Future studies should focus on BMSCs-mediated gene therapy for glioma remnants and the tumor after irradiation.

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AUTHOR DISCLOSURE

The authors declare that they have no competing interests.

REFERENCES

- Stupp R, Mason WP, Van Den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005; 352:987–996.
- Mrugala MM. Advances and challenges in the treatment of glioblastoma: a clinician's perspective. *Discov Med* 2013;15:221–230.
- Tyler MA, Sonabend AM, Ulasov IV, et al. Vector therapies for malignant glioma: shifting the clinical paradigm. *Expert Opin Drug Deliv* 2008;5: 445–458.
- Mohit E, Rafati S. Biological delivery approaches for gene therapy: strategies to potentiate efficacy and enhance specificity. *Mol Immunol* 2013;56: 599–611.
- Horton JA, Hudak KE, Chung EJ, et al. Mesenchymal stem cells inhibit cutaneous radiation-induced fibrosis by suppressing chronic inflammation. *Stem Cells* 2013;31:2231–2241.
- Zhao M, Sachs PC, Wang X, et al. Mesenchymal stem cells in mammary adipose tissue stimulate progression of breast cancer resembling the basal-type. *Cancer Biol Ther* 2012;13:782–792.
- Bexell D, Svensson A, Bengzon J. Stem cell-based therapy for malignant glioma. *Cancer Treat Rev* 2013;39:358–365.
- Nakamizo A, Marini F, Amano T, et al. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005;65: 3307–3318.
- Klopp AH, Spaeth EL, Dembinski JL, et al. Tumor irradiation increases the recruitment of circulating mesenchymal stem cells into the tumor microenvironment. *Cancer Res* 2007;67:11687–11695.
- Dai G, Levy O, Carrasco N. Cloning and characterization of the thyroid iodide transporter. *Nature* 1996;379:458–460.
- Bosch EH, Van Doorne H, De Vries S. The lactoperoxidase system: the influence of iodide and the chemical and antimicrobial stability over the period of about 18 months. *J Appl Microbiol* 2000; 89:215–224.
- Shi S, Zhang M, Guo R, et al. *In vivo* molecular imaging and radionuclide (131I) therapy of human nasopharyngeal carcinoma cells transfected with a lentivirus expressing sodium iodide symporter. *PLoS One* 2015;10:e0116531.
- Mazzaferri EL, Kloos RT. Clinical review 128: Current approaches to primary therapy for papillary and follicular thyroid cancer. *J Clin Endocrinol Metab* 2001;86:1447–1463.
- Guo R, Zhang M, Xi Y, et al. Theranostic studies of human sodium iodide symporter imaging and therapy using 188Re: a human glioma study in mice. *PLoS One* 2014;9:e102011.
- Zhang M, Guo R, Shi S, et al. Baculovirus vector-mediated transfer of sodium iodide symporter and plasminogen kringle 5 genes for tumor radioiodide therapy. *PLoS One* 2014;9:e92326.
- Shi S, Zhang M, Guo R, et al. 131I therapy mediated by sodium/iodide symporter combined with kringle 5 has a synergistic therapeutic effect on glioma. *Oncol Rep* 2016;35:691–698.
- Riesco-Eizaguirre G, Santisteban P. A perspective view of sodium iodide symporter research and its clinical implications. *Eur J Endocrinol* 2006;155: 495–512.
- Hingorani M, Spitzweg C, Vassaux G, et al. The biology of the sodium iodide symporter and its potential for targeted gene delivery. *Curr Cancer Drug Targets* 2010;10:242–267.
- Seiwert TY, Darga T, Haraf D, et al. A Phase I dose escalation study of Ad GV.EGR.TNF.11D (TNFerade Biologic) with concurrent chemoradiotherapy in patients with recurrent head and neck cancer undergoing reirradiation. *Ann Oncol* 2013;24:769–776.
- Chang KJ, Reid T, Senzer N, et al. Phase I evaluation of TNFerade biologic plus chemoradiotherapy before esophagectomy for locally advanced resectable esophageal cancer. *Gastrointest Endosc* 2012;75:1139–1146.
- Klut Z, Willhauck MJ, Wunderlich N, et al. Sodium iodide symporter (NIS)-mediated radionuclide ((131)I), ((188)Re) therapy of liver cancer after transcriptionally targeted intratumoral *in vivo* NIS gene delivery. *Hum Gene Ther* 2011;22:1403–1412.
- Wachsberger P, Burd R, Dicker AP. Tumor response to ionizing radiation combined with anti-angiogenesis or vascular targeting agents: exploring mechanisms of interaction. *Clin Cancer Res* 2003;9:1957–1971.
- Guo R, Ma Y, Zhang R, et al. Rhenium-188 labeled recombinant human plasminogen kringle5 (rhk5) and preliminary biodistribution. Evaluation in mice bearing A549 tumours. *Nuklearmedizin* 2011;50: 234–239.
- Guo R, Liang S, Ma Y, et al. Evaluation of biodistribution and antitumor effects of (188)Re-rhk5 in a mouse model of lung cancer. *Oncol Lett* 2011; 2:865–870.
- Shi S, Zhang M, Guo R, et al. Molecular imaging to monitor repair of myocardial infarction using genetically engineered bone marrow-derived mesenchymal stem cells. *Curr Gene Ther* 2015;15: 460–471.
- Zeng J, See AP, Phallen J, et al. Anti-PD-1 blockade and stereotactic radiation produce long-term survival in mice with intracranial gliomas. *Int J Radiat Oncol Biol Phys* 2013;86:343–349.
- Chen X, Lin X, Zhao J, et al. A tumor-selective biotherapy with prolonged impact on established

- metastases based on cytokine gene-engineered MSCs. *Mol Ther* 2008;16:749–756.
28. Nakamizo A, Marini F, Amano T, et al. Human bone marrow–derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005;65:3307–3318.
29. Studeny M, Marini FC, Champlin RE, et al. Bone marrow–derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* 2002;62:3603–3608.
30. Studeny M, Marini FC, Dembinski JL, et al. Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J Natl Cancer Inst* 2004;96:1593–1603.
31. Miletic H, Fischer Y, Litwak S, et al. Bystander killing of malignant glioma by bone marrow–derived tumor-infiltrating progenitor cells expressing a suicide gene. *Mol Ther* 2007;15:1373–1381.
32. Martinez-Quintanilla J, Bhore D, Heidari P, et al. Therapeutic efficacy and fate of bimodal engineered stem cells in malignant brain tumors. *Stem cells* 2013;31:1706–1714.
33. Knoop K, Kolokythas M, Klutz K, et al. Image-guided, tumor stroma-targeted ¹³¹I therapy of hepatocellular cancer after systemic mesenchymal stem cell-mediated NIS gene delivery. *Mol Ther* 2011;19:1704–1713.
34. Brem S, Cotran R, Folkman J. Tumor angiogenesis: a quantitative method for histologic grading. *J Natl Cancer Inst* 1972;48:347–356.
35. Houghton J, Stoicov C, Nomura S, et al. Gastric cancer originating from bone marrow–derived cells. *Science* 2004;306:1568–1571.
36. Zhu W, Xu W, Jiang R, et al. Mesenchymal stem cells derived from bone marrow favor tumor cell growth *in vivo*. *Exp Mol Pathol* 2006;80:267–274.
37. Karnoub AE, Dash AB, Vo AP, et al. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;449:557–563.
38. Bexell D, Gunnarsson S, Tormin A, et al. Bone marrow multipotent mesenchymal stroma cells act as pericyte-like migratory vehicles in experimental gliomas. *Mol Ther* 2009;17:183–190.
39. Bexell D, Gunnarsson S, Svensson A, et al. Rat multipotent mesenchymal stromal cells lack long-distance tropism to 3 different rat glioma models. *Neurosurgery* 2012; 70:731–739.

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