Selective sodium iodide symporter (NIS) gene therapy of glioblastoma mediated by EGFR-targeted lipopolyplexes

Rebekka Spellerberg,1 Teoman Benli-Hoppe,2 Carolin Kitzberger,1 Simone Berger,2 Kathrin A. Schmohl,1 Nathalie Schwenk,1 Hsi-Yu Yen,3 Christian Zach,4 Franz Schilling,5 Wolfgang A. Weber,5 Roland E. Kälin,6,7 Rainer Glass,6,7,8 Peter J. Nelson,1 Ernst Wagner,2 and Christine Spitzweg1,9

1Department of Internal Medicine IV, University Hospital, LMU Munich, 81377 Munich, Germany; 2Pharmaceutical Biotechnology, Department of Pharmacy, LMU Munich, 81377 Munich, Germany; 3Institute of Pathology, School of Medicine, Technical University of Munich, 81675 Munich, Germany; 4Department of Nuclear Medicine, University Hospital, LMU Munich, 81377 Munich, Germany; 5Department of Nuclear Medicine, School of Medicine, Klinikum rechts der Isar, Technical University Munich, 81675 Munich, Germany; 6Neurosurgical Research, Department of Neurosurgery, University Hospital, LMU Munich, 81377 Munich, Germany; 7Walter Brendel Center of Experimental Medicine, Faculty of Medicine, LMU Munich, 81377 Munich, Germany; 8German Cancer Consortium (DKTK), partner site 80336 Munich and German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany; 9Division of Endocrinology, Diabetes, Metabolism and Nutrition, Mayo Clinic, Rochester, MN 55905, USA

Lipo-oligomers, post-functionalized with ligands to enhance targeting, represent promising new vehicles for the tumor-specific delivery of therapeutic genes such as the sodium iodide symporter (NIS). Due to its iodide trapping activity, NIS is a powerful theranostic tool for diagnostic imaging and the application of therapeutic radionuclides.124I PET imaging allows non-invasive monitoring of the in vivo biodistribution of functional NIS expression, and application of 131I enables cytoreduction. In our experimental design, we used epidural growth factor receptor (EGFR)-targeted polyplexes (GE11) initially characterized in vitro using 125I uptake assays. Mice bearing an orthotopic glioblastoma were treated subsequently with mono-dibenzocyclooctyne (DBCO)-PEG24-GE11/NIS or bisDBCO-PEG24-GE11/NIS, and 24–48 h later, 124I uptake was assessed by positron emission tomography (PET) imaging. The best-performing polyplex in the imaging studies was then selected for 131I therapy studies. The in vitro studies showed EGFR-dependent and NIS-specific transfection efficiency of the polyplexes. The injection of monoDBCO-PEG24-GE11/NIS polyplexes 48 h before 124I application was characterized to be the optimal regime in the imaging studies and was therefore used for an 131I therapy study, showing a significant decrease in tumor growth and a significant extension of survival in the therapy group. These studies demonstrate the potential of EGFR-targeted polyplex-mediated NIS gene therapy as a new strategy for the therapy of glioblastoma.

INTRODUCTION

Glioblastoma (GBM) is a highly aggressive tumor with very limited therapeutic options. It is the most common type of malignant primary brain tumors. Currently, the median survival time after diagnosis is 12–15 months, and fewer than 5% of patients survive more than 5 years.1–3 The current clinical treatment involves surgical resection followed by external beam radiotherapy with concurrent chemotherapy.2 Due to the infiltrating nature of GBM, local therapies or a complete resection are rarely possible, and the clinical relapse of the tumor is usually unavoidable. Therefore, new post-operative therapeutic strategies are seen as the key for novel curative GBM treatments.3,4

Targeted gene therapy is a promising approach for novel therapeutics. Research in this field has been progressing for the past few decades, with the majority of clinical trials focusing on cancer gene therapy.5 The sodium iodide symporter (NIS) gene is a promising, efficient, and safe therapy gene for systemic application. The NIS protein is an intrinsic plasma membrane glycoprotein, localized at the basolateral membrane of thyroid follicular cells that mediates the active uptake of iodide for thyroid hormone synthesis. Due to its iodide trapping activity, NIS is a powerful dual-function tool, with diagnostic and therapeutic applications.5 The functional expression of NIS can be visualized by 123I scintigraphy or 124I or 18F-tetrafluoroborate (TFB) positron emission tomography (PET) imaging.6,8 The application of 131I or 188Re and their NIS-mediated accumulation in tumor tissue allow therapeutic cytoreduction through the β emission of these radionuclides. This therapeutic concept is approved, well understood and safe, and has been in routine clinical use for more than 80 years for the treatment of thyroid cancer.6–11 In a pioneering
| Core formation | Cationic lipo-oligomer |
|----------------|-----------------------|
| **A**          | **C**                 |
| Lipo-oligomer   | H₂N                   |
| NIS pDNA        | C                    |
| 30 min at RT    | Y₂                  |
|                 | CO₂H                 |

| Post-functionalization | Monovalent DBCO agent | Bivalent DBCO agent |
|------------------------|-----------------------|---------------------|
| **B**                  | **D**                 | **E**               |
| monoDBCO agent         | DBCO-PEG24            | bisDBCO-PEG24-GE11  |
| 4 h at RT              | COOH                  | bisDBCO-PEG24-GE11  |
|                       |                       | Targeting           |
|                       |                       | Non-targeting       |

**F**

| Particle size (nm) | PDI     |
|--------------------|---------|
| monoDBCO-PEG24/NIS | 120     |
| monoDBCO-PEG24-GE11/NIS | 110     |
| bisDBCO-PEG24/NIS  | 140     |
| bisDBCO-PEG24-GE11/NIS | 150     |

**G**

| Zeta potential (mV) |
|---------------------|
| monoDBCO-PEG24/NIS  | 10      |
| monoDBCO-PEG24-GE11/NIS | 15      |
| bisDBCO-PEG24/NIS   | 5       |
| bisDBCO-PEG24-GE11/NIS | 10      |

(legend on next page)
preclinical study in prostate cancer, Spitzweg et al. took the initial step toward human NIS gene transfer to non-thyroidal cancer. In the subsequent years, multiple groups, including our own, have established new strategies and refined diverse approaches for NIS gene transfer into various tumor models. To this end, non-viral gene delivery represents a promising technology for the transfer of genetic material into malignant primary tumors, offering the advantages of safety, easy modification, and enhanced biocompatibility after systemic application. In addition to NIS-engineered versions of mesenchymal stem cells with tumor-tissue-specific promoters for selective NIS gene expression, the potential of using targeted polyplexes for the delivery of NIS transgenes into tumor environments has been demonstrated by several studies by our group. These include the use of polycationic molecules based on linear polyethyleneimine (LPEI) that make use of the enhanced permeability and retention effect caused by the leaky vasculature found in the tumor stroma. A PEGylated (PEG: polyethylene glycol) and epidermal growth factor receptor (EGFR)-targeted LPEI molecule (LPEI-PEG-GE11) was demonstrated to enhance tumor-specific accumulation and could be optimized by attaching targeting domains. To test a broader platform of ligands targeting different tumor tissue surface receptors, ligands selectively targeting the receptor tyrosine kinase cMET and the transferrin receptor were developed.32,34

In the current study, we combined the theranostic NIS gene therapy approach with novel sequence-defined synthetic polyplexes to create an optimized, individual, and powerful treatment concept for GBM. This new generation of nanosized polyplexes is based on sequence-defined cationic lipo-oligominoamides (OAAs) synthesized by solid-phase assisted peptide synthesis (SPPS). In addition to complexing plasmid DNA (pDNA) through electrostatic interactions, the OAA are azido-functionalized as a new feature that enables post-modification of the surface with targeting domains, to elicit an enhanced tumor-specific gene delivery. The functional azido group reacts with the dibenzocyclooctyne (DBCO) unit of potential ligands via copper-free click reaction. PEGylation of the ligands lowers the surface charge to avoid non-specific aggregation or interaction with biomacromolecules, allowing an improved blood circulation and reducing undesired potential immune responses. A monodisperse PEG24 (24 ethylene oxide units) was selected, which was already found to be suitable for the in vivo targeting of related OAA-PEG-peptide conjugates. The previously established decapetide GE11, a highly specific allosteric EGFR ligand, conjugated to DBCO was used. EGFR is an attractive candidate for GBM targeting as its overexpression is a histopathological hallmark of GBM. In GBM development, EGFR is the most frequently amplified receptor tyrosine kinase and the receptor expression occurs early in the tumorigeness. The peptide GE11 was selected for EGFR targeting based on its convincingly demonstrated capacity to provide EGFR-specific transfection efficiency in nanoparticle delivery both in vitro and in vivo in our previous studies. In the present study, we monitored vector biodistribution and transfection efficiency by non-invasive imaging in an orthotopic GBM mouse model and subsequently demonstrated the potential therapeutically efficacious of our novel GE11-targeted NIS polyplexes after application.

RESULTS

Polyplex characterization

NIS polyplexes (Figures 1A–1E) were formed with 200 μg/mL pDNA (in vivo conditions) and particle sizes were measured by dynamic light scattering (DLS). We aimed at a size of <200 nm to ensure unhindered blood circulation after intravenous (i.v.) injection and a sufficient cellular uptake. The approximate dimensions were 120–140 nm and the polydispersity indexes (PDIs), an indicator of the heterogeneity of particle sizes in a mixture, were all below 0.2, which reflects a uniform and narrow size distribution (Figure 1F). The particle sizes did not significantly differ between targeted (monoDBCO-PEG24-GE11/NIS, bisDBCO-PEG24-GE11/NIS) and their corresponding non-targeted polyplexes (monoDBCO-PEG24/NIS, bisDBCO-PEG24/NIS). Zeta potential measurements were performed to determine the surface charge of the polyplexes. A positive surface charge is achieved through the good nucleic acid compaction of the OAAs and is desirable to ensure sufficient interaction with negatively charged cell membranes and subsequent internalization. However, at the same time, it is a balancing act to prevent undesired aggregation with negatively charged macromolecules in the bioenvironment. Taken together, a slightly positive surface charge is optimal. The zeta potentials of both PEGylated polyplexes (monoDBCO-PEG24/NIS and bisDBCO-PEG24/NIS) differed from those of GE11-targeted polyplexes (monoDBCO-PEG24-GE11/NIS and bisDBCO-PEG24-GE11/NIS). Using DBCO agents containing just the shielding domain (PEG24) is more efficient in lowering the surface charge compared with using ligands with a shielding and a targeting domain (PEG24 + GE11). No formulation exceeded a zeta potential of 20 mV (Figure 1G).

In vitro NIS gene transfer mediated by EGFR-targeted polyplexes

Cell-surface EGFR expression levels were determined on human breast cancer cells MCF-7, human follicular thyroid carcinoma cells FTC-133,
and the human GBM cell lines GBM14, U87, and LN229 by flow cytometry. GBM14 cells showed no EGFR expression, MCF-7 a very low expression level, FTC-133 a low level of EGFR expression, and LN229 the highest EGFR density on their surface. U87 expressed an intermediate level of EGFR (Figure 2A). The results indicate that the EGFR expression levels on the cells correlated with transfection efficiency after transfection with bisDBCO-PEG24-GE11/NIS polyplexes. LN229 cells showed significantly higher 125I uptake than U87 cells, while no EGFR-expressing GBM14, very low EGFR-expressing MCF-7, and low EGFR-expressing FTC-133 cells exhibited a significantly lower 125I uptake (Figure 2B). The transfection efficiency was higher in U87 and LN229 cells using the targeting ligand GE11: transfection of U87 with monoDBCO-PEG24-GE11/NIS polyplexes led to a 2.5-fold increase in 125I uptake compared with non-targeting monoDBCO-PEG24/NIS polyplexes (Figure 2C). The transfection of U87 with monoDBCO-PEG24-GE11/NIS polyplexes resulted in higher iodide uptake levels compared with the transfection with bisDBCO-PEG24-GE11/NIS, indicating a higher efficiency of the monoDBCO-PEG24-GE11/NIS polyplexes (Figures 2B and 2C). In all cell lines, the addition of the NIS-specific inhibitor perchlorate blocked 125I uptake in NIS-transfected cells, and no iodide uptake above background was seen using luciferase (LUC)-coding polyplexes (Figures 2B and 2C). To further validate the EGFR-dependent transfection efficiency, U87 cells were treated simultaneously with increasing concentrations of the selective EGFR inhibitor cetuximab and monoDBCO-PEG24-GE11/NIS polyplexes. A decrease in radiiodide uptake was shown at 2.5 mg/mL cetuximab with a complete inhibition of radiiodide uptake activity at 3.5 mg/mL cetuximab (Figure 2D). All results were normalized to cell survival and U87 cell viability was not affected by polyplex treatment (Figure 2E).

**Tumoral iodide uptake in vivo after systemic NIS gene transfer**

To determine EGFR expression levels, tissue samples from an orthotopic U87 GBM xenograft mouse model (Figure 3A) were stained...
using specific antibodies. All tumors (n = 7) were EGFR positive, with up to 40% positive cells per tumor (Figure 3B). GBM-bearing mice received EGFR-targeted polyplexes systemically and were evaluated for functional NIS expression in the tumor tissue. Polyplex injection was scheduled 24–28 days after intracranial (i.c.) tumor cell inoculation and 24 or 48 h later high-resolution 124I PET imaging was performed to quantify tumoral radioiodide uptake. The contrast between high radioiodide uptake in the tumors of mice treated with EGFR-targeted polyplexes (Figures 3C, 3D, and 3G) and low tumoral radionuclide uptake in mice injected with non-targeted polyplexes (Figures 3E and 3H) is indicated by the differences in signal strength. No tumoral iodide uptake above background was measured in mice that received LUC-coding polyplexes (Figures 3F and 3I). Due to physiological NIS expression, the thyroid, salivary glands, and stomach normally accumulate radioiodide. The bladder also contains radioiodide due to renal elimination (Figure 3C–3J).

In the quantitative analysis, tumors of mice that received mono-DBCO-PEG24-GE11/NIS showed a significantly higher 124I uptake of 4.36 ± 0.65% ID/mL (48 h) and 2.86 ± 0.24% ID/mL (24 h) compared with tumors from mice that received non-targeted mono-DBCO-PEG24/NIS polyplexes, which exhibited an uptake of 1.96 ± 0.52% ID/mL.

Measurements in mice that received bisDBCO-PEG24-GE11/NIS confirmed the advantageous effect of EGFR-targeted compared with non-targeted polyplexes. With a tumoral iodide uptake of 3.74 ± 0.83% ID/mL, the cohort pretreated with bisDBCO-PEG24-GE11/NIS showed a higher signal amplification than the group injected with bisDBCO-PEG24/NIS (1.44 ± 0.42% ID/mL) (Figure 3K).

Considering a tumor mass of 0.1 g, dosimetric calculations revealed the highest tumor-absorbed dose of 58.0 ± 18.3 mGy/MBq 131I with an effective half-life of 9.6 h in the mice treated with mono-DBCO-PEG24-GE11/NIS followed by radioiodide 48 h later. For mice treated with non-targeted polyplexes (mono-DBCO-PEG24/NIS), a dose of 8.2 ± 1.0 mGy/MBq and an effective half-life of 2.9 h for 131I were calculated. A dose of 35.0 ± 14.2 mGy/MBq 131I and an effective half-life of 5.4 h were determined for the bisDBCO-PEG24-GE11/NIS group. Matching the in vitro data, mono-DBCO-PEG24-GE11/NIS polyplexes resulted in a higher tumoral iodide uptake and a higher tumor-absorbed dose compared with the bisDBCO-PEG24-GE11/NIS polyplexes, corroborating the greater transfection efficiency of the monoDBCO-PEG24-GE11/NIS polyplexes, as a basis for their application in the in vivo therapy study.

**Immunohistochemical ex vivo analysis of NIS protein expression**

After tissue preparation, sections were stained immunohistochemically using an anti-NIS monoclonal antibody. Tumor sections derived from mice that received monoDBCO-PEG24-GE11/NIS polyplexes 48 h (Figure 4A) before sacrifice showed a higher number of NIS-positive cells (red) than tumor sections from the 24h group (Figure 4B). Immunohistochemical staining of tumor sections from control animals that received non-targeted monoDBCO-PEG24/NIS (Figure 4C) or LUC-coding monoDBCO-PEG24-GE11/LUC (Figure 4D) polyplexes showed no NIS-specific immunoreactivity that was comparable to untreated (Figure 4E) tumor tissue.

Immunohistochemical NIS staining of tumor sections derived from mice treated with bisDBCO-PEG24-GE11/NIS (Figure 4F) polyplexes demonstrated an analogous outcome. The experimental group revealed clusters of NIS-positive cells, in contrast with control groups bisDBCO-PEG24/NIS (Figure 4G) and bisDBCO-PEG24-GE11/LUC (Figure 4H), which showed no NIS detection.

In tissue sections of control organs (liver [Figure 4I], lung [Figure 4J], kidney [Figure 4K], and spleen [Figure 4L]), no NIS expression was detected.

**131I therapy studies after polyplex-mediated NIS gene transfer in vivo**

Based on the results of the imaging studies, GBM bearing mice were then treated with monoDBCO-PEG24-GE11/NIS followed by 131I application 48 h later (therapy group). This application cycle was repeated three times. Control groups concurrently received non-targeting monoDBCO-PEG24/NIS polyplexes followed by 131I or monoDBCO-PEG24-GE11/NIS polyplexes and NaCl or NaCl only as a negative control. Tumor growth was monitored by high-resolution magnetic resonance imaging (MRI) twice a week. The therapy group (Figure 5A) showed a significant delay in tumor growth compared with the control groups. The tumor growth was only mildly decreased in the group monoDBCO-PEG24/NIS followed by 131I (Figure 5B) and an aggressive tumor growth was observed in the two control groups, namely, monoDBCO-PEG24-GE11/NIS plus NaCl (Figure 5C) and NaCl only (Figure 5D). The enhanced therapy effect seen in tumor growth (Figure 5E) resulted in a significant extension of survival of the therapy group (Figure 5F).
On day 26, the last control mouse was sacrificed based on the animal welfare protocol, while 60% of the therapy mice were still alive. The mean survival times were 26.6 days for the therapy group, 22.6 days for the monoDBCO-PEG24/NIS group, 20.4 days for the monoDBCO-PEG24-GE11/NIS + NaCl group, and 20 days for the NaCl-only group.

The results were further validated by staining of the blood vessel density and proliferation status (Figures 6A–6D). The therapy group showed a trend toward the lowest number of Ki67-positive cells (Figure 6E) and a significantly smaller area of CD31 positivity (Figure 6F) compared with the control groups.

**DISCUSSION**

As the most common malignant primary brain tumor, GBM has an incidence of 3.19 cases per 100,000 person years. The remarkably poor prognosis of 15 months median survival results from very limited treatment options and the diffuse-invasive nature of GBM with a remaining poor understanding of tumor pathophysiology.

The cloning of NIS in 1996 opened up the opportunity of using this theranostic gene for non-invasive imaging and therapy purposes. Due to its origin in thyroid follicular cells, it is a self-protein with no immunogenic potential and no cell toxicity. In addition to scintigraphic imaging, NIS facilitates tumor monitoring by PET using $^{124}$I or $^{18}$F-TFB as tracers. PET allows for the quantitative analysis of tumoral iodide uptake mediated by functional NIS expression with a high resolution and sensitivity and allows a three-dimensional reconstruction of tumors. NIS imaging allows a precise estimation of radiation dose for radioablation of the individual tumor based on dosimetric calculations. Applying $^{131}$I leads to radionuclide trapping within the NIS-positive cells and cell death induced by beta decay. The cross-fire effect further boosts the impact of $^{131}$I, as neighboring cells also suffer cytotoxic destruction. Off-target toxicity affects mainly the thyroid and salivary glands due to their physiologic NIS expression. Pretreatment with LT4 causes a downregulation of thyroidal iodide uptake due to the TSH dependency of NIS expression. Should hypothyroidism nonetheless arise after therapy, it can be treated by thyroid hormone substitution. The efficacy of radioiodide therapy is well established in thyroid cancer treatment, even in advanced metastatic disease.

**Figure 4. Analysis of NIS protein expression in U87 tumors ex vivo**

Immunohistochemical staining of NIS protein in GBM xenografts embedded in paraffin revealed a higher NIS expression (red) in mice treated with targeted polyplexes (monoDBCO-PEG24-GE11/NIS [A] and bisDBCO-PEG24-GE11/NIS [F]) 48 h before sacrifice compared with the 24 h time point (B). No positive NIS staining in tumors of mice that received control polyplexes (C, D, G, and H) or untreated (E) mice was observed. Liver (I), lung (J), kidney (K), and spleen (L) did not show any NIS expression. One representative image with 20× original magnification is shown for each group (scale bar: 100 μm). A 40× original magnification was chosen for the close up (scale bar: 50 μm).
This therapeutic effectiveness empowers the potential translation of NIS-mediated radioiodide therapy to other tumor diseases such as GBM. In the past, Cho et al. showed functional NIS expression in subcutaneous glioma tumors after intratumoral injection of NIS-expressing recombinant adenoviruses. In a further step, Opyrchal et al. have shown a prolonged survival of orthotopic GBM-bearing mice treated with intratumoral injection of measles viruses engineered to express NIS followed by the intraperitoneal (i.p.) injection of 131I compared with the MV-NIS-only group. In the present study, we used sequence-defined polyplexes as artificial virus-like carrier systems. These synthetic carriers may have distinct advantages over viral vectors, as they overcome limitations in virus gene therapies such as immunogenicity, limited cargo capacity, and difficulties in production. But, at the same time, this technology is inspired by virus biology in that they allow targeted, dynamic, and potent nucleic acid delivery. Importantly, in our study, we injected polyplexes systematically instead of intratumorally, highlighting the flexibility of this approach in clinical applicability. Critical parameters for polyplexes are size, charge, and surface characteristics. The T-shaped lipo- OAA 1252 packages the NIS pDNA and is responsible for the balance between stability and endosomal release. Very small polyplexes (<6 nm) are rapidly eliminated by the kidney, while very big polyplexes (>400 nm) need extensive vascularization for their accumulation in solid tumors. Our polyplexes have been designed for a size between 120 and 140 nm for optimal biodistribution and pharmacokinetics. Modification with the PEGylated ligands resulted in surface charges below 20 mV. This might be advantageous in view of avoiding self-aggregation and aggregation with biomacromolecules and to provide longer blood circulation (Figure 1). Size and charge influence non-specific accumulation in the liver, lungs, and kidneys, which can create toxicity issues. In ex vivo immunohistochemical stainings, we found no NIS expression in these healthy organs (Figures 6I–6L). To increase the internalization of our synthetic vectors to tumor stroma, we used GE11 ligands for specific tumor targeting. We further showed EGFR-dependent transfection efficiency in vitro and in vivo. The comparison of targeted polyplexes (monoDBCO-PEG24-GE11/NIS and bisDBCO-PEG24-GE11/NIS) with their corresponding non-targeted polyplexes (monoDBCO-PEG24/NIS and bisDBCO-PEG24/NIS) demonstrated the advantageous effect of using GE11 targeting ligands. In in vivo cell transfection (Figure 2) and in vivo PET imaging experiments (Figure 3), the use of GE11 ligands led to a significantly higher transfection efficiency compared with the PEG24 ligands alone. Transfection resulted in background levels when using LUC-coding polyplexes (monoDBCO-PEG24-GE11/LUC and bisDBCO-PEG24-GE11/LUC), thus demonstrating that iodide uptake is indeed NIS mediated. The outcome of our therapy study matched closely the results of the PET imaging study. The effective therapeutic cytoreduction achieved after treatment with targeted monoDBCO-PEG24-GE11/NIS polyplexes followed by 131I application resulted in a significant decrease in tumor growth compared with the two control groups monoDBCO-PEG24-GE11/NIS plus NaCl and NaCl only. The non-targeted polyplexes (monoDBCO-PEG24/NIS) showed an uptake of 131I only slightly above background levels in the imaging study. Accordingly, mice treated with these non-targeted polyplexes followed by 131I in the therapy study showed a mild delay in tumor growth compared with the other two control groups. These observations in tumor growth behavior during therapy were mirrored by animal survival (Figure 5). The ex vivo analysis of NIS protein expression showed a heterogeneous, patchy transgene expression pattern in vivo after polyplex-mediated transfection (Figures 4A and 4F). Nevertheless, the 131I therapy resulted in a significant therapeutic effect. This outcome is attributed to the bystander effect of the beta-emitter 131I, which is able to compensate heterogeneous tumor NIS expression due to the range of approximately 2.4 mm of the beta particles. This is one of the major advantages of NIS as a therapy gene and makes the approach highly effective. Ex vivo staining for blood vessel density demonstrated a long-term antiangiogenic therapeutic effect of 131I treatment. The vascularization status of a tumor influences the growth rate. A highly vascularized tumor grows more rapidly, whereas low vascularization decelerates tumor growth. The tumors of the therapy group showed the lowest blood vessel density, delayed growth, and a trend toward lower cell proliferation as determined by Ki67 staining (Figure 6).

In summary, our work clearly shows the potential of post-function-alized targeted polyplexes for NIS gene therapy of GBM using the EGFR targeting ligand GE11. During the last decade, it has been shown that GBMs comprise a group of highly heterogeneous tumor types, including mutations, rearrangements, and genetic alterations of EGFR. EGFR amplification is acquired by GBM cells early in tumorigenesis and substantially contributes to the invasive process. In a study by van den Bent et al., approximately 84% of the evaluated GBMs were considered to retain their EGFR amplification at the time of tumor recurrence. As amplification of tumoral EGFR is essential for the success of our personalized therapy approach, EGFR expression is optimally assessed pre-therapeutically as part of the molecular tumor profiling. The therapeutic approach of the NIS gene therapy offers the major advantage of non-invasive monitoring of the efficacy of EGFR-targeted NIS gene delivery.
before a therapeutic dose of radioiodide is applied, as demonstrated in our preclinical in vivo studies (Figures 3C, 3D, and 3G). Furthermore, the use of DBCO click chemistry provides the opportunity to design polyplexes quickly, based on the genetic differentiation and receptor status of the individual tumor. After biopsy and analysis of the molecular tumor profile, the polyplex design can be tailored via the targeting domain to provide a personalized and individual therapy. The application of alternative targeting ligands suitable for DBCO click chemistry is the subject of ongoing work and can provide a broad spectrum of polyplexes for individualized therapy.

MATERIALS AND METHODS

Cell culture

The GBM cell line U87 (CLS 300367, Cell Line Service GmbH, Eppelheim, Germany) was cultured in Dulbecco’s modified Eagle’s medium (DMEM; 1 g/L glucose; Sigma Aldrich, St. Louis, MO) supplemented with 1% (v/v) MEM non-essential amino acids (Thermo Fisher Scientific, Waltham, MA). The GBM cell line LN229 (ATCC CRL-2611, American Type Culture Collection, Manassas, VA) was grown in Roswell Park Memorial Institute (RPMI)-1640 culture medium (Sigma Aldrich) supplemented with 1% (v/v) sodium pyruvate (Thermo Fisher Scientific). The follicular thyroid carcinoma cell line FTC-133 (94,060,902, Sigma Aldrich) was cultured in DMEM/F12 (Sigma Aldrich) supplemented with 1% (v/v) L-glutamine (Sigma Aldrich). The human breast cancer cell line MCF-7 (ATCC HTB-22) was grown in minimum essential Eagle’s medium (MEM; Sigma Aldrich) supplemented with 1% (v/v) L-glutamine (Sigma Aldrich), 1% (v/v) sodium pyruvate (Thermo Fisher Scientific), and 5 μg/mL insulin (Sigma Aldrich). We added 10% (v/v) fetal bovine serum (FBS Superior, Sigma Aldrich) and 1% (v/v) penicillin/streptomycin (Sigma Aldrich) to all media.

The patient-derived GBM cell line GBM14 was cultured in DMEM/F12 (Sigma Aldrich) supplemented with 1% (v/v) penicillin/streptomycin (Sigma Aldrich), B-27 supplement (Thermo Fisher Scientific), 10 ng/mL human EGF (PeproTech, Hamburg, Germany), and 10 ng/mL human FGF (PeproTech). All cells were passaged at 70% confluency and maintained at 37°C, 5% CO2, and a relative humidity of 95%. The culture medium was replaced every 48 h.

Synthesis of plasmids, carrier, and DBCO agents

The NiS cDNA was synthesized and optimized by GENEART (Regensburg, Germany) based on the plasmid pCpG-hCMV-Luc. The
establishment of the expression vector pcP-G-hCMV-NIS has been described in detail previously.29 The pNIS-DNA and pCMVLuc29 (encoding a Photinus pyralis LUC under control of the cytomegalovirus promoter) that were applied in all in vitro and in vivo experiments were produced and purified by Plasmid Factory GmbH (Bielefeld, Germany).

The T-shaped OAA 1252 was synthesized via standard Emoc SPPS as described previously.45,63

The shielding and EGFR targeting agents, bearing one or two DBCO units as attachment sites for orthogonal click reaction, were synthesized as described previously.45,47

Polyplex formation

The final pDNA concentration was 10 μg/mL for cell transfection experiments and 200 μg/mL for in vivo experiments. The pDNA and the calculated amount of OAA at N/P 12 (protonatable nitrogen/phosphate ratio) were diluted separately in the same volume. The solvent was 20 mM HEPES buffer with 5% (w/v) glucose (pH 7.4) (HBG buffer). The pDNA solution was mixed in OAA solution by pipetting rapidly 10 times, followed by an incubation period of 30 min at room temperature to form core polyplexes. Ligands for post-modification were diluted in HBG buffer with an equivalence of 0.25.36 The total volume of the diluted ligand was one-quarter of the volume of the OAA-pDNA mixture. The ligand was added to the core polyplex solution after the incubation period by pipetting rapidly 10 times, followed by further incubation for 4 h at room temperature.

Particle size and zeta potential measurement

DLS was performed on a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) to measure the particle size and zeta potential of the polyplexes. Polyplexes were formed in 100 μL HBG buffer with a final DNA concentration of 200 μg/mL (in vivo condition). For zeta potential measurement, 700 μL HBG was added.

EGFR expression levels in vitro

Flow cytometry was performed to screen for EGFR expression levels on cell surfaces. U87, LN229, MCF-7, and FTC-133 were trypsinized and GBM14 was treated with Accutase solution (Sigma Aldrich). We washed 8 × 10^5 cells of each cell line and resuspended them in 100 μL PBS supplemented with 10% (v/v) FBS (FACS buffer). An antibody for human EGFR detection (monoclonal mouse IgG1, clone H11; Dako, Glostrup, Denmark) or a negative isotype control antibody (Abcam, Cambridge, UK) was added at a dilution of 1:200 and the samples were incubated for 1 h on ice. Subsequently, the cells were washed with FACS buffer and stained with an Alexa Fluor 488 antibody at a dilution of 1:400 (Thermo Fisher Scientific) for 1 h on ice. Propidium iodide (Sigma Aldrich) was added at a dilution of 1:100 to exclude dead cells. An analysis was performed on a BD Accuri C6 flow cytometer (BD Bioscience, Franklin Lakes, NJ). Cell aggregates or fractions were excluded by appropriate gating.

Transfection studies and 125I uptake assay

Cells (U87, LN229, GBM14, MCF-7, and FTC-133) were seeded in six-well plates and grown to 60%-70% confluency. Medium was replaced by 400 μL well serum- and antibiotic-free medium. We added 200 μL well monoDBCO-PEG24-GEl1/NIS or bisDBCO-PEG24-GEl1/NIS polyplex solutions with a DNA concentration of 10 μg/mL and the cells were incubated for 4 h at 37°C before the medium was changed to normal growth medium. As negative controls, ligands without the targeting domain (monoDBCO-PEG24/NIS or bisDBCO-PEG24/NIS) or LUC-coding polyplexes (monoDBCO-PEG24-GEl1/LUC or bisDBCO-PEG24-GEl1/LUC) were applied. The EGFR-specific antibody cetuximab (Erbitux, Merck, Darmstadt, Germany) was added in different concentrations (0.5, 1.5, 2.5, and 3.5 mg/mL) 15 min before the cell treatment with monoDBCO-PEG24-GEl1/NIS polyplexes. Furthermore, the NIS-specific inhibitor perchlorate (1 mM potassium perchlorate; Merck) was added as an additional control. At 24 h after transfection, NIS-mediated 125I uptake was examined as described previously.10,14 Results are normalized to cell survival and specified as counts per minute (cpm)/A620 (for cell viability assay, see below).

Cell viability assay

A commercially available 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Sigma Aldrich) was added 24 h after transfection and cells were incubated for 1 h at 37°C. For cell lysis, 10% dimethyl sulfoxide in isopropanol with an incubation time of 15 min at room temperature was used. The measurement was performed on a Sunrise microplate absorbance reader (Tecan, Männedorf, Switzerland) at a wavelength of 620 nm.

Establishment of intracranial U87 tumors in vivo

Six- to 7-week-old female CD-1 nu/nu mice (Charles River, Sulzfeld, Germany) were anesthetized and immobilized, and a skin incision was made on the top of the skull. Mice were mounted onto a stereotactic head holder (David Kopf Instruments, Tujunga, CA) in the flat-skull position. A hole was carefully drilled into the skullcap 1.0 mm anterior and 1.5 mm lateral to the bregma with a 21G cannula. A blunt syringe with an injection volume of 1 μL (22G Hamilton syringe; Hamilton, Reno, NV) was inserted 4 mm deep and retracted to 3 mm depth. We injected 1 μL U87 cell suspension (1 × 10^5 cells/μL PBS) slowly (over 2 min) into the brain before the syringe was removed carefully within 2 further minutes. The area of injection was the right caudate putamen. The skin incision was stitched with surgical thread (Johnson & Johnson, New Brunswick, NJ) and the mice were kept warm while awakening. Mice were treated with Metacam (0.5 mg/kg) pre- and post-operatively to reduce pain and the risk of inflammation. Animals were maintained with access to mouse chow and water ad libitum and under specific-pathogen-free conditions. More than 15% weight loss or signs of ill health (impairment of breathing, drinking, eating, or cleaning behavior) led to sacrifice. All experimental protocols were authorized by the regional governmental commission for animals (Regierung von Oberbayern) and meet the requirements of the German Animal Welfare Act.
**In vivo PET imaging studies after systemic NIS gene transfer**

At 3.5–4 weeks after i.c. tumor cell inoculation, polyplexes (monoDBCO-PEG24-GE11/NIS and bisDBCO-PEG24-GE11/NIS polyplexes for EGFR targeting, non-targeted monoDBCO-PEG24/LUC and bisDBCO-PEG24/LUC polyplexes, monoDBCO-PEG24-GE11/LUC and bisDBCO-PEG24-GE11/LUC containing pCMVLuc as additional negative control) with a DNA dose of 2.5 mg/kg (for a 20-g mouse, 50 μg DNA in a total volume of 250 μL; solvent, HBG) were applied systemically via the tail vein. After 24 or 48 h, the mice received 10 MBq of 124I (PerkinElmer, Waltham, MA, or DSD HBG) were applied systemically via the tail vein. After 24 or 48 h, the mice were pretreated with L-thyroxine (LT4; 5 mg/mL, Sigma Aldrich) and bisDBCO-PEG 24/NIS polyplexes, monoDBCO-PEG 24-GE11/NIS-mediated iodide accumulation in tumor areas was determined by small-animal PET (Inveon, SIEMENS Preclinical Solutions, Erlangen, Germany). Serial scanning took place 1, 3, and 5 h after 124I application. Results were assessed with the software Inveon Acquisition Workplace (Siemens, Munich, Germany), were analyzed using Inveon Research Workplace (Siemens), and are represented as a percentage of the injected dose per milliliter tumor (% ID/mL). Mice were pretreated with L-thyroxine (LT4; 5 mg/mL, Sigma Aldrich) in their drinking water 10 days before imaging to reduce thyroid iodide uptake, and at the same time the mouse chow was changed to a low-iodine diet (ssniff Spezialdiäten GmbH, Soest, Germany).

**Mouse brain tissue preparation**

After anesthesia and thorax incision, mice were perfused transcardially with 1× PBS followed by a 4% formaldehyde solution. The brain was explanted and fixed in 4% formaldehyde solution for 48 h at room temperature and stored in 1× PBS at 4°C for further preparation. The liver, spleen, kidney, and lungs were collected as control organs under the same procedure.

**Immunohistochemical EGFR staining**

Immunohistochemistry of tumor tissues derived from mice used for the imaging study was performed using a Bond RXm system (Leica, Wetzlar, Germany; all reagents from Leica) with an EGFR antibody (clone E235, 1:100, ab32077; Abcam). Brieﬂy, slides were deparaffinized and pretreated with Epitope retrieval solution 1 (EDTA buffer [pH 6]) before the diluted primary antibody was applied for 15 min. Antibody binding was detected with a polymer reﬁne detection kit without a post primary agent and visualized with diaminobenzidine as a dark brown precipitate. Counterstaining was done with hematoxylin. A positive control was included in each run. The stained slides were scanned with an automated slide scanner (Leica Biosystems; AT-2), and the Aperio Imagescope software (version 12.3; Leica Biosystems) was used to take representative images. The receptor expression level was evaluated by a veterinary pathologist.

**Tumor volume estimation ex vivo**

Tumors were cut in axial sections with a microtome. Twenty transverse layers with deﬁned anatomical characteristics were selected with the help of a mouse brain atlas. The interval between selected brain sections was 0.32–0.52 mm. Hematoxylin and eosin (H&E) staining was performed according to standard protocol, slides were scanned, all sections containing tumor were taken into consideration, and the tumor area (A) was determined by encircling the tumor (Aperio Imagescope software). The average area was calculated ($A_{average} = A_{total}/N$ [number of selected sections]) and the height of the tumor (H) was considered as the interval between the first and the last section containing tumor. The final tumor volume (in cubic millimeters) is the multiplication of $A_{average}$ (in square millimeters) and H (in millimeters).

Only mice bearing a GBM with a size of >30 mm³ were considered in the in vivo PET imaging studies. There was no signiﬁcant difference in the mean tumor sizes between control and experimental groups: the groups that received targeted polyplexes had a mean tumor size of 44.6 ± 5.1 mm³ (monoDBCO-PEG24-GE11/NIS 48 h), 51.2 ± 6.0 mm³ (monoDBCO-PEG24-GE11/NIS 24 h), and 57.6 ± 8.6 mm³ (bisDBCO-PEG24-GE11/NIS), while those treated with non-targeted polyplexes developed tumors 43.9 ± 2.9 mm³ (monoDBCO-PEG24/NIS) and 43.9 ± 6.2 mm³ (bisDBCO-PEG24/NIS) in size. Animals treated with LUC-coding polyplexes had tumors with a mean size of 71.5 ± 10.0 mm³ (monoDBCO-PEG24-GE11/LUC) and 42.7 ± 8.9 mm³ (bisDBCO-PEG24-GE11/LUC).

**Immunohistochemical staining of NIS protein**

Paraffin-embedded tumor and control organ samples were immunohistochemically stained as described previously. A primary mouse monoclonal NIS-speciﬁc antibody (Merck Millipore; dilution 1:500) was incubated on tissue samples for 60 min at room temperature, followed by a biotin-SP-conjugated goat antimouse IgG antibody (Jackson ImmunoResearch, West Grove, PA; dilution 1:200) for 20 min and peroxidase-conjugated streptavidin (Jackson ImmunoResearch; dilution 1:300) for a further 20 min. Scanning was performed as described above.

**Radioiodide therapy study in vivo**

Starting 5 days after i.c. tumor cell inoculation, tumor growth was assessed twice a week by high-resolution MRI. A visible tumor in one slice with a diameter between 0.8 and 1.3 mm was used as inclusion parameter (day 0). Therapy trials were started the day after. To this end, therapy mice were treated systemically with monoDBCO-PEG24-GE11/NIS followed by an i.p. injection of 55.5 MBq 131I (GE Healthcare, Braunschweig, Germany) 48 h later. The therapy trial was repeated three times; thus, i.v. polyplex injection took place on days 1, 5, and 9 and i.p. 131I injections were performed on days 3, 7, and 11. Accordingly, control mice received monoDBCO-PEG24-GE11/NIS followed by 131I or monoDBCO-PEG24-GE11/NIS followed by saline (NaCl), or NaCl i.v. followed by NaCl i.p., respectively. Once at least one endpoint criterion was met (>15% weight loss; impairment of breathing, drinking, eating, or cleaning behavior; self-isolation from the group), as monitored by independent animal care personnel blind to treatment and hypothesis, the mice were sacrificed.

MRI was acquired with a small animal 7T preclinical scanner (Agilent Discovery MR901 magnet and gradient system, Bruker AVANCE III HD electronics running ParaVision software release 6.0.1). A
birdcage quadrature volume resonator (inner diameter 72 mm; RAPID Biomedical, Rimpar, Germany) was used for 300 MHz RF transmission, and a rigid-housing two-channel surface receiver coil array (RAPID Biomedical) was placed over the mouse’s head. Animals were screened for tumor growth with a T2-weighted rapid acquisition with relaxation enhancement (RARE) sequence, with a repetition time of 2.5 s, an effective echo time of 40 ms, 8 echoes per excitation, an acquisition matrix 192 × 192, an in-plane resolution of 0.104 × 0.104 mm², 1 average, and 7 slices with a thickness of 1 mm. The oblique coronal (horizontal) slices were tilted to be parallel with the brain anterior-posterior axis, which was tilted anterior-down due to mouse positioning under the coil. Images were exported in a Digital Imaging and Communications in Medicine (DICOM) format for analysis with the DICOM viewer RadiAnt (Medixant, Poznan, Poland). The tumor area of each slice was encircled and RadiAnt provided the size in square millimeters. The tumor volume was calculated using the same formula as for ex vivo tumor volume estimation (see above).

**Ex vivo immunofluorescence assay**

The U87 GBMs from therapy mice were prepared as described above. Two days after post-fixation in paraformaldehyde (PFA), the brains were left in 30% sucrose for at least 24 h at 4°C. Freezing was performed by embedding the tissue in Cryomatrix (Leica). Frozen tumor sections were stained with an antibody against Ki67 (Abcam; dilution 1:100) for blood vessel density as described previously. The stained tumor sections were scanned with the Panorma MIDI digital slide scanner and pictures were taken using Caseviewer software (3DHISTECH Ltd., Budapest, Hungary). For quantification, four visual fields (20× magnification) per tumor were chosen and analyzed with ImageJ software (NIH, Bethesda, MD).

**Statistical methods**

All in vitro experiments were performed at least in triplicate and results are shown as mean ± SEM, mean fold change ±SEM, and percentage for survival plots. Two-tailed Student’s t test was used to prove statistical significance.

For therapy studies, differences in tumor growth were tested by one-way ANOVA followed by post hoc Fisher’s LSD or Games Howell. Mouse survival is presented in a Kaplan-Meier-plot and statistical significance was determined by log rank. Statistical significance was defined as a p value of <0.05 (*p < 0.05; **p < 0.01; ***p < 0.001).

**ACKNOWLEDGEMENTS**

The authors want to thank Sissy M. Jhiang (Ohio State University, Columbus, OH, USA) for supplying the full-length human NIS cDNA. We owe special thanks to Sybille Reder, Markus Mittelhäuser, Hannes Rolbieski, Sandra Sühnel, Dr Geoffrey Topping, and Jakob Allmann (Department of Nuclear Medicine, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany) for their assistance and support in performing the imaging and therapy studies. We are grateful to Olga Seelbach and her team (Institute of Pathology, School of Medicine, Technical University of Munich, Munich, Germany) for the preparation of paraffin-embedded slides and the H&E and EGFR staining. We appreciate the help from Prof. Dr. Gabriele Multhoff and Dr. Stefan Stangl (Center for Translational Cancer Research, Klinikum rechts der Isar, Technical University of Munich, Munich, Germany) for establishing the orthotopic glioblastoma mouse model in our group. Furthermore, we thank Prof. Dr. Julia Mayerle, Dr. Ivonne Regel, and Dr. Ujjwal Mahajan for allowing us to use their laboratory equipment.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft within the Collaborative Research Center SFB 824 to C.S. (project C8) and to F.S. (project Z3), and SFB 1032 to E.W. (project B4), as well as within the Priority Program SPP1629 to C.S. and P.J.N., and by a grant form the Wilhelm Sander-Stiftung to C.S. and P.J.N. (2014.129.1). R.G. and R.E.K. gratefully acknowledge funding by the DFG (GR691/2; SFB824-B2) and the Anni-Hofmann Stiftung.

This work was performed as partial fulfillment of the doctoral thesis of R.S. at the Faculty for Chemistry and Pharmacy of the LMU Munich.

**AUTHOR CONTRIBUTIONS**

Conceptualization: R.S., K.A.S., S.B., R.G., E.W., P.J.N., and C.S.; methodology: R.S., T.B.-H., S.B., R.E.K., E.W., P.J.N., and C.S.; investigation: R.S., T.B.-H., C.K., N.S., and H.Y.; formal analysis: R.S., H.Y., and C.Z.; resources: F.S., W.A.W., and R.G.; writing – original manuscript: R.S.; writing – review & editing: T.B.-H., S.B., K.A.S., R.E.K., E.W., P.J.N., and C.S.; funding acquisition: E.W., P.J.N., and C.S.; supervision: E.W. and C.S.

**DECLARATION OF INTERESTS**

The authors have declared no conflict of interest.

**REFERENCES**

1. Aum, D.J., Kim, D.H., Beaumont, T.L., Leuthardt, E.C., Dunn, G.P., and Kim, A.H. (2014). Molecular and cellular heterogeneity: the hallmark of glioblastoma. Neurosurg. Focus 37, E11.
2. Louis, D.N., Perry, A., Reifenberger, G., von Deimling, A., Figarella-Branger, D., Cavenee, W.K., Ohgaki, H., Wiestler, O.D., Kleihues, P., and Ellison, D.W. (2016). The 2016 world health organization classification of tumors of the central nervous system: a summary. Acta Neuropathol. 131, 803–820.
3. Bastien, J.I.L., McNeill, K.A., and Fine, H.A. (2015). Molecular characterizations of glioblastoma, targeted therapy, and clinical results to date. Cancer 121, 502–516.
4. Aldape, K., Brindle, K.M., Chesler, L., Chopra, R., Gajjar, A., Gilbert, M.R., Gottardo, N., Gutmann, D.H., Hargrave, D., Holland, E.C., et al. (2019). Challenges to curing primary brain tumours. Nat. Rev. Clin. Oncol. 16, 509–520.
5. Ginn, S.L., Amaya, A.K., Alexander, I.E., Edelstein, M., and Abedi, M.R. (2018). Gene therapy clinical trials worldwide to 2017: an update. J. Gene Med. 20, e3015.
6. Spitzweg, C., and Morris, J.C. (2002). The sodium iodide symporter: its pathophysiological and therapeutic implications. Clin. Endocrinol. 57, 559–574.
7. Penheiter, A.R., Russell, S.J., and Carlsson, S.K. (2012). The sodium iodide symporter (NIS) as an imaging reporter for gene, viral, and cell-based therapies. Curr. Gene Ther. 12, 33–47.
8. Urmauer, S., Müller, A.M., Schug, C., Schmohl, K.A., Tutter, M., Schwenk, N., Rödl, W., Morys, S., Ingrisch, M., Bertram, J., et al. (2017). EGFR-targeted nonviral NIS
gene transfer for bioimaging and therapy of disseminated colon cancer metastases. Oncotarget 8, 92195–92208.

9. Smanik, P.A., Liu, Q., Furminger, T.L., Ryu, K., Xing, S., Mazzaferrri, E.L., and Jiang, S.M. (1996). Cloning of the human sodium iodide symporter. Biochim. Biophys. Res. Commun. 226, 339–345.

10. Willhaus, M.J., Sharif Samani, B.R., Gïldâe, F.I., Woll, I., Senekowitsch-Schmidtknecht, R., Stark, H.I., Gïke, M., Morris, J.C., and Spitzweg, C. (2007). Application of 188rhenium as an alternative radionuclide for treatment of prostate cancer after tumor-specific sodium iodide symporter gene expression. J. Clin. Endocrinol. Metab. 92, 4451–4458.

11. Spitzweg, C. (2009). Gene therapy in thyroid cancer. Horm. Metab. Res. 41, 500–509.

12. Spitzweg, C., Dietz, A.B., O’Connor, M.K., Bergert, E.R., Tindall, D.J., Young, C.Y., and Morris, J.C. (2001). In vivo sodium iodide symporter gene therapy of prostate cancer. Gene Ther. 8, 1524–1531.

13. Spitzweg, C., O’Connor, M.K., Bergert, E.R., Tindall, D.J., Young, C.Y., and Morris, J.C. (2000). Treatment of prostate cancer by radiodine therapy after tissue-specific expression of the sodium iodide symporter. Cancer Res. 60, 6526–6530.

14. Spitzweg, C., Zhang, S., Bergert, E.R., Castro, M.R., McKer, B., Heufer, A.E., Tindall, D.J., Young, C.Y., and Morris, J.C. (1999). Prostate-specific antigen (PSA) promoter-driven androgen-inducible expression of sodium iodide symporter in prostate cancer cell lines. Cancer Res. 59, 2136–2141.

15. Ader, M.H. (2014). Gene therapy for cancer: present status and future perspective. Mol. Cell. Ther. 2, 27.

16. Knoop, K., Kolokithas, M., Klutz, K., Willhaus, M.J., Wunderlich, N., Draganovic, D., Zach, C., Gïldâe, F.I., Bïning, G., Bartenstein, P., et al. (2015). Mesenchymal stem cell-mediated, EGFR heterogeneity and implications for therapeutic intervention in glioblastoma tumors following systemic nonviral delivery of the sodium iodide symporter gene. Clin. Cancer Res. 21, 1534–1543.

17. Knoop, K., Schwenk, N., Dolp, P., Willhaus, M.J., Grïnwald, G.K., Haase, R., Wunderlich, N., Zach, C., Gïldâe, F.I., Senekowitsch-Schmidtknecht, R., Gïke, M., et al. (2011). Image-guided tumor-selective radioiodine therapy of liver cancer after systemic nonviral delivery of the sodium iodide symporter gene. Hum. Gene Ther. 22, 1563–1574.

18. Schmohl, K.A., Dolp, P., Schug, C., Knoop, K., Klutz, K., Schwenk, N., Bartenstein, P., Nelson, P.J., Ogris, M., Wagner, E., et al. (2017). Reintroducing the sodium-iodide symporter to anaplastic thyroid carcinoma. Thyroid 27, 1534–1543.

19. Knoop, K., Schwenk, N., Grïnwald, G.K., Haase, R., Wunderlich, N., Zach, C., Gïldâe, F.I., Senekowitsch-Schmidtknecht, R., Gïke, M., et al. (2011). Epidermal growth factor receptor-targeted (131)I-therapy of liver cancer following systemic delivery of the sodium iodide symporter gene. Mol. Ther. 19, 676–685.

20. Schmohl, K.A., Gupta, A., Grïnwald, G.K., Trajkovic-Arsic, M., Klutz, K., Bartenstein, P., Schwenk, N., Ogris, M., Wagner, E., et al. (2017). Imaging and targeted therapy of pancreatic ductal adenocarcinoma using the theranostic sodium iodide symporter (NIS) gene. Oncotarget 8, 33393–33404.

21. Schïfer, A., Pahmk, A., Schaffert, D., van Weerden, W.M., de Riddler, C.M.A., Rödl, W., et al. (2011). Disconnecting the yin and yang relation of epidermal growth factor receptor (EGFR)-mediated delivery: a fully synthetic, EGFR-targeted gene transfer system avoiding receptor activation. Hum. Gene Ther. 22, 1463–1473.

22. Schmohl, K.A., Gupta, A., Grïnwald, G.K., Trajkovic-Arsic, M., Klutz, K., Bartenstein, P., Schwenk, N., Ogris, M., Wagner, E., et al. (2017). Systemic tumor-targeted sodium iodide symporter (NIS) gene therapy of hepatocellular carcinoma mediated by 8 peptide polyplexes. J. Gene Med. 19, e2957.

23. Schmohl, K.A., Schwenk, N., Krïlavac, A., Müller, A.M., Schug, C., Schmohl, K.A., Schwenk, N., Zach, C., Carlens, J., Bartenstein, P., et al. (2016). Sequence-defined cMET/EGFR-targeted polymers as gene delivery vehicles for the theranostic sodium iodide symporter (NIS) gene. Mol. Ther. 24, 1395–1404.

24. Urnauer, S., Schmohl, K.A., Tutter, M., Schug, C., Schwenk, N., Morsy, S., Ziegler, S., Bartenstein, P., Clevert, D.A., Wagner, E., et al. (2019). Dual-targeted NIS polyplexes—a theranostic strategy toward tumors with heterogeneous receptor expression. Gene Ther. 26, 93–108.

25. Lïchelt, U., and Wagner, E. (2015). Nucleic acid therapeutics using polyplexes: a journey of 50 years (and beyond). Chem. Rev. 115, 11043–11078.

26. Kleïn, P.M., Kern, S., Lee, D.J., Schmaus, J., Höhn, M., Gorges, J., Kazmaier, U., and Wagner, E. (2018). Folate receptor-directed orthogonal click-functionalization of siRNA lipopolyplexes for tumor cell killing in vivo. Biomaterials 178, 630–642.

27. Hager, S., and Wagner, E. (2018). Bioresponsive polyplexes—chemically programmed for nucleic acid delivery. Expert Opin. Drug Deliv. 15, 1007–1083.

28. Ogris, M., Brunner, S., Schïiller, S., Kircheis, R., and Wagner, E. (1999). PEGylated DNA/transferin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery. Gene Ther. 6, 595–605.

29. Wang, S., Reinhard, S., Li, C., Qïan, M., Jiang, H., Du, Y., Lïchelt, U., Lu, W., Wagner, E., and Huang, R. (2017). Antimural cascade-targeting ligand for IL-6 receptor-mediated gene delivery to glioma. Mol. Ther. 25, 1556–1566.

30. Kos, P., Lïchelt, U., He, D., Nie, Y., Gu, Z., and Wagner, E. (2015). Dual-targeted polyplexes based on sequence-defined peptide-PEG-oligoamino amides. J. Pharm. Sci. 104, 464–475.

31. Li, Z., Zhao, R., Wu, X., Sun, Y., Yao, M., Li, J., Xu, Y., and Gu, J. (2005). Identification and characterization of a novel peptide ligand of epidermal growth factor receptor for targeted delivery of therapeutics. FASEB J. 19, 1978–1985.

32. Rao, S.A., Arikampamagan, A., Pandey, P., Santosh, V., Hegde, A.S., Chandramouli, B.A., and Somasundaram, K. (2013). miR-219–5p inhibits receptor tyrosine kinase pathway by targeting EGFR in glioblastoma. PLoS one 8, e63164.

33. Eskalisson, E., Resland, G.V., Solecki, G., Wang, Q., Harter, P.N., Graziani, G., et al. (2018). EGFR heterogeneity and implications for therapeutic intervention in glioblastoma. Neuro Oncol. 20, 743–752.
Mickler, F.M., Möckl, L., Ruthardt, N., Ogris, M., Wagner, E., and Bräuchle, C. (2012). Tuning nanoparticle uptake: live-cell imaging reveals two distinct endocytosis mechanisms mediated by natural and artificial EGFR targeting ligand. Nano Lett. 12, 3417–3423.

Wang, Y., Luo, J., Truubenbach, I., Reinhard, S., Klein, P.M., Hohn, M., Kern, S., Morys, S., Loy, D.M., Wagner, E., et al. (2020). Double click-functionalized siRNA polyplexes for gene silencing in epidermal growth factor receptor-positive tumor cells. ACS Biomater. Sci. Eng. 6, 1074–1089.

Morys, S., Urnauer, S., Spitzweg, C., and Wagner, E. (2018). EGFR targeting and shielding of pDNA lipopolyplexes via bivalent attachment of a sequence-defined PEG agent. Macromol. Biosci. 18, 1700203.

Truubenbach, I., Zhang, W., Wang, Y., Kern, S., Hohn, M., Reinhard, S., Gorges, J., Karmarier, U., and Wagner, E. (2019). Co-delivery of pretubulin and siEGF to EGFR overexpressing carcinoma cells. Int. J. Pharm. 569, 118570.

Steinborn, B., Truubenbach, I., Morys, S., Lächelt, U., Wagner, E., and Zhang, W. (2018). Epidermal growth factor receptor targeted methotrexate and small interfering RNA co-delivery. J. Gene Med. 20, e3041.

Jo, D.H., Kim, J.H., Lee, T.G., and Kim, J.H. (2015). Size, surface charge, and shape treatment of brain tumors. The Neurol. next generation blueprint. Acta Pharmacol. Sin.

Mickler, F.M., Möckl, L., Chakraborty, S., Borkar, S.A., Dey, G., Mukherjee, A., and Ogris, M. (2004). Puriﬁcation of polyethylenimine polyplexes highlights the role of free polyacrylates in gene transfer. J. Gene Med. 6, 1102–1111.

Batach, R., Asna, N., Schaffer, P., Francis, N., and Schaffer, M. (2017). Glioblastoma multiforme, diagnosis and treatment; recent literature review. Curr. Med. Chem. 24, 3002–3009.

Samec, N., Zottel, A., Videtic Paska, A., and Jovicvskva, I. (2020). Nanomedicine and immunotherapy: a step further towards precision medicine for glioblastoma. Molecules 25, 490.

Rajesh, Y., Pal, I., Banik, P., Chakraborty, S., Borkar, S.A., Dey, G., Mukherjee, A., and Mandal, M. (2017). Insights into molecular biology of glioma: current challenges and next generation blueprint. Acta Pharmacol. Sin. 38, 591–613.

Pruitt, A.A., and Rosenfeld, M.R. (2005). 10 questions about temozolomide and the treatment of brain tumors. The Neurol. 51, 362–365.

Zhoa, M., van Straten, D., Broekman, M.L.D., Préat, V., and Schifflers, R.M. (2020). Nanocarrier-based drug combination therapy for glioblastoma. Theranostics 10, 1355–1372.

Dai, G., Levy, O., and Caras, N. (1996). Cloning and characterization of the thyroid iodide transporter. Nature 379, 458–460.

Baril, P., Martin-Duque, P., and Vassaux, G. (2010). Visualization of gene expression in the live subject using the Na+/I symporter as a reporter gene: applications in biotherapy. Br. J. Pharmacol. 159, 761–771.

Li, H., Nakashima, H., Decklever, T.D., Nace, R.A., and Russell, S.J. (2013). HSV-NIS, an oncolytic herpes simplex virus type 1 encoding human sodium iodide symporter for preclinical prostate cancer radiotherapy. Cancer Gene Ther. 20, 478–485.

Spitzweg, C., Harrington, K.J., Pinke, L.A., Vile, R.G., and Morris, J.C. (2001). Clinical review 132: the sodium iodide symporter and its potential role in cancer therapy. J. Clin. Endocrinol. Metab. 86, 3327–3335.

Cho, J.Y., Xing, S., Liu, X., Buckwalter, T.L., Hwa, L., Sferra, T.J., Chiu, I.M., and Jhiang, S.M. (2000). Expression and activity of human Na+/I- symporter in human glioma cells by adeno virus-mediated gene delivery. Gene Ther. 7, 740–749.

Özpiychal, M., Allen, C., Iankov, I., Adrea, I., Schroeder, M., Sarkaria, J., and Galanis, E. (2012). Effective radiotherapy for malignant gliomas by using oncolytic measles virus strains encoding the sodium iodide symporter (MV-NIS). Hum. Gene Ther. 23, 419–427.

Zhang, P., and Wagner, E. (2017). History of polymeric gene delivery systems. Top. Curr. Chem. 375, 26.

Berger, S., Kriac Levačić, A., Hörterer, E., Wilk, U., Benli-Hoppe, T., Wang, Y., Oztürk, Ö., Luo, J., and Wagner, E. (2021). Optimizing pDNA pico-polypelexes: a balancing act between stability and cargo release. Biomacromolecules 22, 1282–1296.

Klutz, K., Willhauck, M.J., Wunderlich, N., Zach, C., Anton, M., Senekowitsch-Schmidtk, R., Göke, B., and Spitzweg, C. (2011). Sodium iodide symporter (NIS)-mediated radionuclide ([131I]) ([188Re]) therapy of liver cancer after transcriptionally targeted intratumoral in vivo NIS gene delivery. Hum. Gene Ther. 22, 1403–1412.

Willhauck, M.J., Sharif-Samani, B., Senekowitsch-Schmidtk, R., Wunderlich, N., Göke, B., Morris, J.C., and Spitzweg, C. (2008). Functional sodium iodide symporter expression in breast cancer xenografts in vivo after systemic treatment with retinoic acid and dexamethasone. Breast Cancer Res. Treat. 109, 263–272.

Spitzweg, C., Nelson, P.J., Wagner, E., Bartenstein, P., Weber, W.A., Schwager, M., and Morris, J.C. (2021). The sodium iodide symporter (NIS): novel applications for radionuclide imaging and treatment. Endocr. Relat. Cancer 28, T193–T213.

Frisch, A., Kälín, S., Monk, R., Radke, J., Heppner, F.L., and Kälín, R.E. (2020). Apelin controls angiogenesis-dependent glioblastoma growth. Int. J. Mol. Sci. 21, 4179.

van den Bent, M.J., Gao, Y., Kerkhof, M., Kros, J.M., Gorlia, T., van Zwieten, K., Prince, J., van Duinen, S., Silveira Smitt, P.A., Taphoorn, M., et al. (2015). Changes in the EGFR amplification and EGFRIVIII expression between paired primary and recurrent glioblastomas. Neuro Oncol. 17, 935–941.

Plank, C., Zatloukal, K., Cotten, M., Mechtler, K., and Wagner, E. (1992). Gene transfer into hepatocytes using asialoglycoprotein receptor mediated endocytosis of DNA complexed with an artiﬁcial tetra-antennary galactose ligand. Bioconjug. Chem. 3, 533–539.

Franklin, K.B., and Paxinos, G. (2019). Paxinos and Franklin’s the Mouse Brain in Stereotaxic Coordinates, Compact: The Coronal Plates and Diagrams (Academic press).

Kälín, R.E., Cai, L., Li, Y., Zhao, D., Zhang, H., Cheng, J., et al. (2021). TAMEP are effective radiotherapy for malignant gliomas by using oncolytic measles virus strains encoding the sodium iodide symporter (MV-NIS). Hum. Gene Ther. 23, 419–427.

Spitzweg, C., Baker, C.H., Bergert, E.R., O’Connor, M.K., and Morris, J.C. (2007). Image-guided radioiodide therapy of medullary thyroid cancer after carcinoembryonic antigen promoter-targeted sodium iodide symporter expression. Hum. Gene Ther. 18, 916–924.

Molecular Therapy: Oncolytics Vol. 23 December 2021