Radiation-Induced Amplification of TGFB1-Induced Mesenchymal Stem Cell-Mediated Sodium Iodide Symporter (NIS) Gene 131I Therapy

Christina Schug1, Carolin Kitzberger1, Wolfgang Sievert2, Rebekka Spellerberg1, Mariella Tutter1, Kathrin A. Schmohl1, Bernadette Eberlein3, Tilo Biedermann3, Katja Steiger4, Christian Zach5, Markus Schweiger6, Gabriele Multhoff2, Ernst Wagner7, Peter J. Nelson8, and Christine Spitzweg1

Abstract

Purpose: The innate tumor homing potential of mesenchymal stem cells (MSCs) has been used for a targeted delivery of the theranostic sodium iodide symporter (NIS) transgene into solid tumors. We have previously shown that external beam radiotherapy (EBRT) results in the enhanced recruitment of NIS-expressing MSCs into human hepatocellular carcinoma (HuH7). In parallel, the tumor-associated cytokine TGFβ1 becomes strongly upregulated in HuH7 tumors in response to EBRT. Experimental Design: We therefore evaluated the effects of combining focused EBRT (5 Gy) with MSC-mediated systemic delivery of the theranostic NIS transgene under control of a synthetic TGFβ1-inducible SMAD-responsive promoter (SMAD-NIS-MSCs) using 123I-scintigraphy followed by 131I therapy in CD1 nu/nu mice harboring subcutaneous human hepatocellular carcinoma (HuH7).

Results: Following tumor irradiation and SMAD-NIS-MSC application, tumor iodide uptake monitored in vivo by 123I-scintigraphy was enhanced as compared with non-irradiated tumors. Combination of EBRT and SMAD-NIS-MSC–mediated 131I therapy resulted in a significantly improved delay in tumor growth and prolonged survival in therapy mice as compared with the combined therapy using CMV-NIS-MSCs or to control groups receiving EBRT or saline only, or EBRT together with SMAD-NIS-MSCs and saline applications.

Conclusions: MSC-based NIS-mediated 131I therapy after EBRT treatment dramatically enhanced therapeutic efficacy when a TGFβ1-inducible SMAD-responsive promoter was used to drive NIS expression in adoptively applied MSCs. The remarkable therapeutic effect seen is thought to be linked in large part to the enhanced TGFβ1 produced in this context, which leads to a highly selective and focused amplification of MSC-based NIS expression within the tumor milieu.

Introduction

The sodium iodide symporter (NIS) is an intrinsic transmembrane glycoprotein that actively transports iodide into the thyroid gland. NIS biology forms the basis for the efficient management of patients with thyroid cancer through the use of radioiodine. NIS functions as a theranostic protein, allowing noninvasive imaging, such as 123I-scintigraphy and 124I-PET imaging, as well as treatment by application of therapeutic radioiodine (131I; refs. 1–4). Diverse approaches are currently underway to investigate NIS-based therapy in nonthyroidal tumors using various delivery systems (1–3, 5–17). Mesenchymal stem cells (MSC) are attractive vehicles for the potential delivery of therapeutic genes into solid tumors. Adoptively applied, engineered versions of MSCs have been shown to allow the expression of therapeutic genes such as the NIS transgene deep within tumor environments (14–16). The ability of MSCs to be recruited to tumor environments is due, in large part, to the release of various factors produced in response to the inflammation and hypoxic damage found in solid tumors (18–20). The basic mechanisms underlying this recruitment are thought to parallel those seen in the recruitment of endogenous MSC during tissue repair (18, 19).

While radiation, in general, represents one of the most common therapies for the treatment of patients with carcinoma, external beam radiotherapy (EBRT) now allows the accurate and...
TGFB1 is expressed by tumor cells and also by the endothelial cells, mesenchymal and myeloid precursor cells, and has been shown to be expressed by most solid tumors (28). It plays important and diverse roles in the biology of tumor growth and proliferation. Increased levels of the theranostic indium-111-labeled MSCs to the tumor stroma that in turn led to increased levels of the theranostic indium-111-labeled MSCs to the tumor stroma that in turn led to increased recruitment of MSCs to the tumor site. This increased recruitment of MSCs to the tumor site led to increased production of various inflammatory cytokines and chemokines, which in turn increased the production of various inflammatory cytokines and chemokines, which in turn increased the recruitment of MSCs to the tumor site. These increased MSC recruitment to the tumor site further provide data showing potential radiation-induced amplification of TGFB1-inducible MSC-based NIS cancer gene therapy in solid tumors.

**Translational Relevance**

The use of radioiodine for the effective management of thyroid cancer is dependent on the biology of the sodium-iodide symporter (NIS). Expanded gene therapy strategies based on the targeted expression of NIS in nonthyroid cancer cells followed by therapeutic application of 131I are currently under development. It has been previously demonstrated the enormous potential of using NIS as a therapeutic tool for the treatment of thyroid cancer. The results show a remarkably enhanced therapeutic efficacy in a human hepatocellular carcinoma (HCC) cell line. The increased expression of the chemokine (C-X-C motif) ligand 12/stromal cell-derived factor 1 (CXCL12/SDF-1) and fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), thrombospondin 1 (TSP-1), VEGF, as well as TGFB1, was linked to the enhanced migration of genetically engineered MSCs to the tumor stroma that in turn led to increased levels of the therapeutic NIS transgene under control of the constitutively active cytomegalovirus (CMV)-promoter (26). Using radiation pretreatment to enhance the MSC tumor homing capability opens the exciting prospect of combining EBRT with MSC-mediated gene delivery strategies to improve gene delivery to tumor environments and thus improved tumor therapy.

We have previously shown that the use of tissue or signal-specific gene promoters, whose activation is linked to tumor-derived signals, allows a more selective and focused activation of MSC-based transgenes such as NIS and can thereby improve the specificity of tumor treatment (14–16, 25). The cytokine TGFB plays important and diverse roles in the biology of tumor growth and has been shown to be expressed by most solid tumors (28). TGFB1 is expressed by tumor cells and also by the fibroblasts, endothelial cells, mesenchymal and myeloid precursor cells, leucocytes, and other cells found within the tumor setting where it can act in a paracrine or autocrine manner on tumor tissues (28).

On the basis of this biology, the potential efficacy of a synthetic TGFB1-inducible SMAD-responsive promoter was recently evaluated in engineered MSCs as a means to control and focus NIS transgene expression (SMAD-NIS-MSCs) within tumors. We reported that the systemic application of SMAD-NIS-MSCs to mice harboring a subcutaneous human HCC derived from the TGFB1-expressing HuH7 cell line resulted in a significant delay in tumor growth and prolonged survival of animals upon treatment with radioiodine (25). This proof-of-principle of using a TGFB1-inducible SMAD-responsive promoter as a tumor signal-responsive promoter in NIS gene-based therapy, together with the findings from our previous study demonstrating an enhanced recruitment of MSCs in response to EBRT tumor pretreatment with the identification of increased TGFB1 following EBRT treatment, suggested that EBRT may not only enhance the migratory behavior of MSCs but may also act to amplify promoter activation in SMAD-NIS-MSCs due to increased TGFB1 expression in irradiated tumors (25, 26). In this regard, we and others have previously speculated that gene promoters that are directly or indirectly responsive to radiation-induced signals could potentially allow an amplification of therapy transgene expression when used in the setting of NIS-mediated radioiodine therapy. The radioiodine-induced tumor damage in combination with an inflammatory response could further drive activation of the radiation-responsive promoter leading to higher NIS expression and thus therapy effect. On the basis of this hypothesis and the data described above, in this study we combined EBRT treatment with MSC-mediated SMAD-promoter-driven NIS gene transfer to evaluate potential additive effects. The therapeutic efficacy of engineered MSCs was tested by combining radiation pretreatment of tumors with the application of either SMAD- or CMV-NIS-MSC-mediated NIS gene delivery in mice harboring subcutaneous HuH7 tumors. MSCs were systemically injected 24 hours after low-dose radiation pretreatment of the tumors. The subsequent effects on the tumoral TGFB1-induced, NIS-mediated radioiodide accumulation were examined using 123I-scintigraphy. Therapeutic efficacy was then determined after pretreating tumors with radiation followed by SMAD- or CMV-NIS-MSC-mediated NIS transgene delivery and 131I application. Pretreatment with low-dose external beam radiation of subcutaneous human HuH7 tumors, following systemic application of SMAD-NIS-MSCs resulted in a pronounced reduction in tumor growth up to complete remission of tumors in a subset of animals and dramatically prolonged survival of animals as compared with animals receiving CMV-NIS-MSC treatment or controls.

**Materials and Methods**

**Cell culture**

The human HCC cell line HuH7 used in the experiments were authenticated and purchased from JCRB Cell Bank (JCRB 0403) and passaged up to six times. DMEM (Sigma-Aldrich) supplemented with 10% FBS (FBS Superior, Biochrom/Merck Millipore) and 100 U/mL penicillin/100 µg/mL streptomycin (Sigma-Aldrich) was used for cell culture. The human bone marrow-derived, SV40 large T-antigen–immortalized MSC cell line was described and cultured as described previously (29). An incubator at 37°C and 5% CO2 was used for all cells.
CMV-NIS-MSCs and SMAD-NIS-MSCs
The design of the expression vectors and the stable transfection of MSCs were performed as described previously (13, 25). CMV-NIS-MSCs and SMAD-NIS-MSCs were cultured in RPMI medium containing 0.5 mg/mL geneticin (Invitrogen/Thermo Fisher Scientific). 10% FBS, and 100 U/mL penicillin/100 μg/mL streptomycin and passaged up to three times until use.

125I uptake assay
To validate SMAD-based promoter inducibility, an in vitro 125I uptake assay was used. The SMAD-NIS-MSCs were first stimulated by various agents. Prior to stimulation, SMAD-NIS-MSCs were serum-starved overnight (using medium lacking FBS). Control stimulation was performed for 24 hours by adding 10 ng/mL recombinant TGFβ1 to the SMAD-NIS-MSCs. For stimulation with HuH7-CM (conditioned medium), cells were treated with HuH7-CM from either irradiated or nonirradiated HuH7 cells for 24 hours or simultaneously treated with TGFβ1 and irradiated or nonirradiated HuH7-CM for 24 hours, respectively. NIS-mediated radiiodide uptake of SMAD-NIS-MSCs was determined at steady-state conditions as described previously (30). For control experiments, the selective TGFβ1 receptor ALK4/ALK5 inhibitor EW-7197 (vactosertib; Selleckchem) was used. The SMAD-NIS-MSCs were treated either with 10 ng/mL TGFβ1 alone or together with different concentrations of EW-7197 (0, 0.05, 0.1, 0.2, 0.5, or 1 μmol/L) for 24 hours followed by radiiodide uptake assay. Results were normalized to cell viability.

Cell viability assay
Cell viability was determined using the commercially available MTT assay (Sigma-Aldrich) according to the manufacturer’s recommendations as described previously (16).

ELISA
HuH7-CM was assayed for TGFβ1 protein using a DuoSet ELISA Kit (R&D Systems). To assess the amount of active versus latent TGFβ1, samples were run with or without prior activation reaction (to assess total vs. active TGFβ1, respectively) according to the manufacturer’s instructions.

Irradiation of HuH7 cells
Irradiation of HuH7 cells using 5 Gy was performed as described previously (26). Supernatants of irradiated and nonirradiated HuH7 cells were removed 48 hours after irradiation, centrifuged, and stored at −80°C.

IHC TGFβ1 staining
IHC TGFβ1 staining was conducted as described previously (using the antibody ab92486, Abcam; ref. 25). A semiquantitative evaluation of TGFβ1 expression of HuH7 tumors was performed. An immunoreactive score (range, 0–3), reflecting expression intensity (range, 0–3) and frequency (percentage of TGFβ1-positive tumor cells) was applied.

Animals
Five-week-old female CD1 nu/nu mice were purchased from Charles River Laboratories and housed under specific pathogen-free conditions. Mice had access to mouse chow and water ad libitum. Experiments were approved by the regional governmental commission for animals (Regierung von Oberbayern) and performed in accordance with institutional guidelines of the Klinikum rechts der Isar, Technische Universität München (Munich, Germany).

Establishment of HuH7 xenograft tumors
To establish HuH7 xenograft tumors in CD1 nu/nu mice, 5 × 106 HuH7 cells/100-μL PBS was injected subcutaneously into the right flank region as described previously (13). Regular measurement and estimation of the tumor volume were done using the equation: length × width × height × 0.52. Exceeding a tumor size of 1,500 mm3 led to sacrifice of mice.

Irradiation of HuH7 xenograft tumors
Precise irradiation using 5 Gy was performed as described previously (26) or by using the Dermopan 2 (Siemens) device after subcutaneous HuH7 tumors reached a diameter of approximately 5–10 mm for 123I-scintigraphy or 1–5 mm for therapy.

Systemic SMAD-NIS-MSC application and in vivo 123I-scintigraphy
L-T4 (5 mg/mL; Sigma-Aldrich) was supplemented to the drinking water of mice to suppress thyroidal iodide uptake. Twenty-four hours after tumor irradiation, 5 × 106 SMAD-NIS-MSCs in 500-μL PBS were systemically applied. Seventy-two hours after SMAD-NIS-MSC injection, 18.5 MBq (0.5 mCi) of 123I were applied intraperitoneally. Radiodiodide biodistribution was assessed using 123I-scintigraphy on a gamma camera equipped with a low-energy high resolution collimator (e.cam, Siemens). Analysis of regions of interest (ROI) was done with the HERMES GOLD (Hermes Medical Solutions) program. Quantified results were expressed as a fraction of the total amount of applied radionuclide per gram tumor tissue (% ID/g). The radioiodide retention time was determined by serial scanning within the tumors and dosimetric calculations were done as described previously (16).

IHC staining for NIS expression
Following in vivo application of SMAD-NIS-MSCs, tumors and nontarget organs (liver, lung, and spleen) were dissected. Paraffin-embedded tissues were immunohistochemically stained for NIS expression as described previously [antibody MAB3564 (anti NIS a.a. 625-642 clone F5SA), Merck Millipore; ref. 31)]. Quantification of NIS IHC staining was performed by an experienced pathologist. The percentage of tissue covered by NIS-positive MSCs was evaluated in 10 randomly selected low-power field equivalents (on digitalized slides with 0.5-μm/pixel resolution) per tumor, followed by a comparison of both treatment regimens.

Radioiodine therapy study
Ten days before therapy start, mice received drinking water supplemented with 5 mg/mL L-T4. For therapy, tumors were irradiated (5 Gy) followed by a single SMAD-NIS-MSC application 24 hours later. Seventy-two hours after the SMAD-NIS-MSC injection, mice received 55.5 MBq 131I (n = 7; 5 Gy + SMAD-NIS-MSCs + 131I). Twenty-four hours after 131I application, the treatment cycle of radiation pretreatment and MSC application was repeated. For therapy completion, a last cycle as described was done without radiation pretreatment of tumors. As controls, a subset of mice received NaCl instead of radiiodide (n = 7; SMAD-NIS-MSCs + NaCl) or saline only after radiation treatment (n = 6; 5 Gy + NaCl). For exact evaluation of the therapeutic effect, a subset of mice received no radiotherapy and only saline.
applications \((n = 7; \text{NaCl} + \text{NaCl})\). To illustrate the improved therapeutic effect of SMAD-NIS-MSCs, CMV-NIS-MSCs were applied instead for therapy \((n = 6; 5 \text{ Gy} + \text{SMAD-NIS-MSCs} + {\text{NaCl})}\). Tumor volume was measured regularly and mice were euthanized when a tumor volume of 1,500 mm\(^3\) was exceeded.

**Indirect immunofluorescence assay**

Frozen tissue sections of tumors from the radioiodine therapy study were used for indirect immunofluorescence analysis of Ki67 (cellular proliferation; antibody ab15580, Abcam) and CD31 (blood vessel density; antibody 550274, BD Pharmingen) as described previously \((16)\). The percentage of positive cells for Ki67 and CD31-positive areas within the tumors were quantified by evaluation of five high-power fields per tumor \((n = 3, 5 \text{ Gy} + \text{SMAD-NIS-MSCs} + \text{NaCl}; n = 4, 5 \text{ Gy} + \text{CMV-NIS-MSCs} + \text{NaCl}; n = 6, 5 \text{ Gy} + \text{SMAD-NIS-MSCs} + \text{NaCl}; n = 5, 5 \text{ Gy} + \text{NaCl}; n = 7, \text{NaCl} + \text{NaCl})\) using Imaged software (NIH, Bethesda, MD). Results are presented as means ± SEM.

**Statistical methods**

Results are expressed as mean ± SEM or percentage, and statistical significance was tested by two-tailed Student \(t\) test or one-way ANOVA for tumor volumes and log-rank test for survival plots. \(P\) values of \(< 0.05\) were considered significant \((*, P < 0.05; **, P < 0.01; ***, P < 0.001)\).

**Results**

**In vitro NIS-mediated radioiodide uptake in SMAD-NIS-MSCs**

To verify the selective response of the SMAD-based synthetic gene promotor, SMAD-NIS-MSCs were stimulated \(\text{in vitro}\) with TGF\(\beta\) \((10 \text{ ng/mL})\), which led to a 5.4-fold increase in NIS-mediated radioiodide \(123^\text{I}\) accumulation (Fig. 1A). No accumulation of \(123^\text{I}\) was observed above background levels without TGF\(\beta\) stimulation. To further characterize the potential activation of this transgene response to tumor-radiation signals, SMAD-NIS-MSCs were additionally treated with irradiated and nonirradiated HuH7-CM. Whereas no accumulation of \(123^\text{I}\) was observed after subjecting MSCs to HuH7-CM only, treatment with HuH7-CM together with TGF\(\beta\) resulted in a significant increase of radioiodide uptake of approximately 59% in MSCs as compared with stimulation with TGF\(\beta\) only (Fig. 1A). Subsequent ELISA analysis showed that the TGF\(\beta\) produced by HuH7 cells was primarily in the latent form (Fig. 1B) and thus could not activate the TGF\(\beta\) receptors on the MSCs without prior proteolytic processing. Radioiodide uptake activity in SMAD-NIS-MSCs stimulated with HuH7-CM from 5-Gy-irradiated HuH7 cells (SMAD-NIS-MSCs + 5-Gy-treated HuH7-CM) and TGF\(\beta\) resulted in an additional increase of about 12% as compared with the stimulation with normal HuH7-CM and TGF\(\beta\) (Fig. 1A).

To further validate the specificity of TGF\(\beta\)-induced NIS expression in SMAD-NIS-MSCs, SMAD-NIS-MSCs were additionally treated with the selective TGF\(\beta\) receptor ALK4/ALK5 inhibitor EW-7197 (Fig. 1C). SMAD-NIS-MSCs without TGF\(\beta\) stimulation with or without EW-7197 did not show radioiodide uptake activity above background levels. Simultaneous treatment of SMAD-NIS-MSCs with TGF\(\beta\) with increasing concentrations of EW-7197 for 24 hours showed a decrease of radioiodide uptake already at 0.05 \(\mu\text{mol/L EW-7197}\). Using concentrations from 0.1 to 1 \(\mu\text{mol/L of the TGF\(\beta\) receptor inhibitor demonstrated a complete inhibition of the radioiodide uptake activity in TGF\(\beta\)-stimulated SMAD-NIS-MSCs (Fig. 1C). The concentrations of the inhibitor used in this study showed no toxicity to the cells (Supplementary Fig. S1).**

**TGF\(\beta\) expression in nonirradiated and irradiated HuH7 tumors**

TGF\(\beta\) protein expression in subcutaneous HuH7 tumors was analyzed using IHC (Fig. 2). All HuH7 tumors were found to express TGF\(\beta\) (Fig. 2). The TGF\(\beta\) expression intensity in nonirradiated tumors consistently showed some expression (score \(1\) with a mean of 29% positive cells and an immunoreactive score of 0.29; Fig. 2A), while after irradiation with 5 Gy, the tumors increased TGF\(\beta\) expression (Fig. 2B), which was visible as moderate to strong expression in IHC (score \(1–3\) with a mean of 48% positive cells and an immunoreactive score of 0.84).

**In vivo \(123^\text{I}\)-scintigraphy**

Nude mice with subcutaneous HCC xenograft tumors received a single SMAD-NIS-MSC \((0.5 \times 10^7)\) injection via the tail vein 72 hours prior to \(123^\text{I}\)-scintigraphy (Fig. 3A and B). To monitor radioiodide biodistribution, 18.5 MBq \(123^\text{I}\) were administered on January 8, 2021. © 2019 American Association for Cancer Research. clincancerres.aacrjournals.org Downloaded from clincancerres.aacrjournals.org on January 8, 2021. © 2019 American Association for Cancer Research.

**Ex vivo NIS protein expression in HuH7 tumors**

NIS-IHC was performed on nonirradiated as well as irradiated tumors after application of SMAD-NIS-MSCs. The results showed high NIS-specific immunoreactivity throughout the tumor stroma (Fig. 4A and E). Quantification of NIS IHC staining revealed an average of 5.8% ± 0.8% area covered in NIS-positive MSCs in nonirradiated tumors (Fig. 4A), whereas 11.4 ± 3.4% of the tumor area was covered with NIS-expressing MSCs in tumors preirradiated with 5 Gy (Fig. 4E). Thus, NIS protein expression was shown to be increased in the tumors receiving radiation pretreatment confirming an enhanced recruitment and activation of SMAD-NIS-MSCs in irradiated tumors (Fig. 4E). No NIS protein expression was detected in nontarget organs such as liver, lung, and spleen (Fig. 4B–D, F–H).

**In vivo radioiodine therapy studies**

The therapeutic efficacy of the SMAD-NIS-MSC-based NIS-mediated \(131^\text{I}\) therapy was then evaluated by systemically applying SMAD-NIS-MSCs to mice with subcutaneous HuH7 tumors, which had been pretreated with 5 Gy (5 Gy + SMAD-NIS-MSCs +
SMAD-NIS-MSCs stably expressing NIS under control of a SMAD-responsive promoter. Iodide uptake studies demonstrated a 5.4-fold higher NIS-specific, perchlorate-sensitive $^{125}$I uptake in SMAD-NIS-MSCs stimulated with TGFB1 (10 ng/mL) as compared with unstimulated cells, where no radioiodide uptake above background levels was observed (A). Combined treatment of SMAD-NIS-MSCs with TGFB1 (10 ng/mL) and HuH7-CM led to further increased radioiodide uptake as compared with stimulated cells without HuH7-CM. This effect was even enhanced adding HuH7-CM from 5-Gy-irradiated HuH7 cells, which were irradiated with 5 Gy (A). No radioiodide accumulation above background levels was observed treating SMAD-NIS-MSCs with HuH7-CM (nonirradiated or irradiated) alone. Data are represented as means of three independent experiments ± SEM (n = 3; two-tailed Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001; A). HuH7-CM from nonirradiated and 5-Gy-irradiated HuH7 cells contains mainly latent TGFB1, as measured by ELISA. Data are represented as means of three independent experiments ± SEM (n = 3; B). Treatment with the highly potent and selective TGFB1 receptor ALK4/ALK5 inhibitor EW-7197 resulted in dose-dependent inhibition of radioiodide uptake in TGFB1-treated SMAD-NIS-MSCs demonstrating the selective stimulation of the SMAD-responsive promoter in SMAD-NIS-MSCs through TGFB1 (C). Data are represented as means of four independent experiments ± SEM (n = 4; two-tailed Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.001; C).
I-131; Fig. 5). As controls, mice received radiation pretreatment followed by only saline (5 Gy + NaCl) or SMAD-NIS-MSCs followed by saline applications (5 Gy + SMAD-NIS-MSCs + NaCl). A further control group received saline only (NaCl + NaCl). To evaluate whether an improved therapeutic effect was seen due to the use of a tumor signal–responsive promoter enhanced by radiation, a group of mice with irradiated tumors were treated with MSCs that had been engineered using the constitutive active CMV promoter to control NIS transgene expression followed by radioiodine treatment (5 Gy + CMV-NIS-MSCs + I-131). Mice receiving NaCl only showed exponential tumor growth and had to be sacrificed within 35 days (Fig. 5A and B). Radiation-pretreated tumors of control mice (5 Gy + NaCl and 5 Gy + SMAD-NIS-MSCs + NaCl) led to significantly slower tumor growth as compared with the saline group (Fig. 5A) and prolonged survival for up to 69 days (Fig. 5B). The therapy mice (5 Gy + SMAD-NIS-MSCs + I-131 and 5 Gy + CMV-NIS-MSCs + I-131) demonstrated significantly reduced tumor growth over that seen with radiation alone up to 20 days (Fig. 5A). After 20 days, tumors of the 5 Gy + CMV-NIS-MSCs + I-131 group started growing and nearly no difference in tumor growth was observed as compared with irradiated control groups (Fig. 5A). In contrast, 5 Gy +
SMAD-NIS-MSCs + I-131 mice showed dramatically slower tumor progression after 20 days (Fig. 5A). Tumors in two of the mice within this group showed complete remission after approximately 40–50 days with no tumor regrowth until mice had to be sacrificed at day 89 and 197 as both mice suffered from severe bloating of unknown cause. Until the end of the observation period of 225 days, two mice of the SMAD-NIS-MSCs–treated group (5 Gy + SMAD-NIS-MSCs + I-131) were still alive demonstrating small tumors with little growth during this period. Dissected tumors were stained for the proliferation marker Ki67 (green) and the blood vessel marker CD31 (red) using immunofluorescence analysis (Supplementary Fig. S2). In this instance, no difference was seen between the therapy groups (5 Gy + SMAD-NIS-MSCs + I-131 and 5 Gy + CMV NIS-MSCs + I-131) and mice of the controls (NaCl + NaCl, 5 Gy + NaCl or 5 Gy + SMAD-NIS-MSCs + NaCl; Fig 6A–C).

Discussion
MSCs represent a promising tool for the delivery of therapeutic genes into tumor environments based on their strong tumor-homing properties. The mechanisms underlying MSC migration to the tumor are thought to be driven by the inflammatory response of the tumor (19). Key mediators in this scenario include inflammatory chemokines and growth factors such as the EGF, PDGF, VEGF, CXCL12/SDF-1, and TGFB1/3 among others, which have been shown to influence MSC migration (20, 32). The irradiation of tumors has been previously shown to lead to an enhanced inflammatory response resulting in the increased secretion of growth factors and chemokines, which in turn is associated with enhanced recruitment of MSCs (23, 33). In a recent proof-of-principle study, our group has shown that the recruitment of MSCs expressing NIS under control of the CMV promoter was strongly enhanced toward subcutaneous HuH7 tumors after tumor pretreatment with low-dose radiation (26). Radiation treatment (1–10 Gy) of HuH7 cells was found to lead to a dose-dependent increase in expression of CXCL8, CXCL12/SDF-1, FGF2, PDGFB, VEGF, TSP-1, as well as TGFB1 (26). In addition, the combination of tumor radiation pretreatment and MSC-mediated NIS transgene delivery under the control of the constitutive CMV gene promoter resulted in enhanced NIS-mediated tumor-specific radiodiode accumulation as compared with nonirradiated tumors (26). A major focus of our group has been the evaluation of tumor-derived signal-specific promoters for NIS transgene activation to enhance tumor specificity and address potential variability regarding tumor heterogeneity. NIS transgene expression driven by the inflammation-responsive RANTES/CCL5-promoter was evaluated in a series of tumor mouse models that demonstrated improved survival of the animals as compared with the use of the unspecific CMV promoter (13–15). In a recent report, we showed that tumor hypoxia could also be used for inducing NIS transgene expression leading to a prolonged survival of animals and a significant delay in tumor growth in an orthotopic HCC mouse model (16). Our most recent approach of using individually designed synthetic promoters that can be selectively activated by signals from the tumor micromilieu, was the use of a TGFB1-inducible SMAD-responsive promoter (25). In most solid tumors, including HCC, the pleiotropic factor TGFB1 is upregulated by cells of the tumor stroma as well as the tumor cells themselves, and has been found to be an important tumor micromilieu-associated signaling factor (34–36). This biology was used to selectively activate NIS transgene expression using the TGFB1-inducible synthetic SMAD-responsive promoter in engineered MSCs (SMAD-NIS-MSCs). This approach led to a robust tumor-specific therapy response in HCC (25). On the basis of our data outlined above describing radiation-induced stimulation of MSC tumor homing, at least in part, due to enhanced tumoral TGFB1 expression (26), we
speculated that the combination of enhancing MSC-mediated NIS gene delivery and amplifying TGFβ1-inducible NIS expression by radiation pretreatment of tumors offers great potential to improve tumoral NIS-mediated radioiodide accumulation by taking advantage of these additive effects. In vitro, SMAD-NIS-MSCs demonstrated a robust and significant radioiodine uptake activity upon TGFβ1 stimulation, which was significantly enhanced upon concomitant treatment with conditioned

Figure 5.

$^{131}$I therapy of subcutaneous HuH7 tumors led to a significant delay in tumor growth and improved survival. For an in vivo radionuclide therapy study, mice received radiation pretreatment followed by a single SMAD-NIS-MSC application and 55.5 MBq $^{131}$I 48 hours afterward. This cycle was repeated, followed by a final cycle of a single SMAD-NIS-MSC application and $^{131}$I (5 Gy + SMAD-NIS-MSC + $^{131}$I; A). Therapy of mice harboring HuH7 tumors resulted in a significant delay in tumor growth as compared with irradiated controls [5 Gy + NaCl (days 19–35, **, P < 0.01) and 5 Gy + SMAD-NIS-MSC + NaCl (days 25–38, **, P < 0.01 and days 39–49, **, P < 0.05)] or mice receiving NaCl only [NaCl + NaCl (days 2–17, **, P < 0.01 and days 18–21, **, P < 0.01; A)]. Another therapy group received CMV-NIS-MSCs instead of SMAD-NIS-MSCs (5 Gy + CMV-NIS-MSCs + $^{131}$I) to evaluate the therapeutic efficacy of the tumor signal-responsive promoter. Both therapy groups demonstrated halted tumor growth at first, but CMV-NIS–treated mice developed fast tumor progression after 20 days with nearly no difference in tumor growth as compared with irradiated controls (A). Therapy using radiation treatment in combination with SMAD-NIS-MSCs illustrated only slow tumor progression including 2 mice with complete tumor regression. Furthermore, therapy mice (5 Gy + SMAD-NIS-MSC + $^{131}$I) demonstrated significantly improved overall survival after an observation period of 225 days as compared with all other groups (B).
medium from HuH7 cells. A further stimulating effect was seen when conditioned medium from radiation-pretreated (5 Gy) HuH7 cells was used. As in vitro HuH7-CM contains an inactive latent form of TGFB1, no radioiodide uptake activity was observed in SMAD-NIS-MSCs treated with CM alone. Latent TGFB1 is activated by proteolytic cleavage in vivo by tumor tissue–associated proteases. The increase in radioiodine accumulation seen after the cells were stimulated with CM and TGFB1 suggested the presence of factors within the CM that can enhance SMAD activation within the target cells, potentially through noncanonical signaling pathways. The mechanisms at work in this context require further evaluation. Immunohistology showed that nonirradiated HuH7 tumors showed TGFB1 protein expression throughout the tumor with a pronounced increase of TGFB1-specific immunoreactivity after low-dose irradiation (5 Gy) of HuH7 tumors. These findings correlate with data presented in our recent report that showed an enhanced and dose-dependent increase in TGFB1 expression found on the mRNA and protein level in irradiated HuH7 cells in vitro (26). On the basis of these results, the biodistribution of functional TGFB1 was investigated and quantified in vivo using SMAD-NIS-MSCs with induced functional NIS as a reporter gene. External beam radiation enhanced TGFB1 expression and thus increased promoter activity in the SMAD-NIS-MSCs in addition to enhancing their migratory potential. Tumors pretreated with 5-Gy radiation followed by a single application of SMAD-NIS-MSCs showed enhanced tumor-selective, TGFB1-driven induction of functional NIS expression as demonstrated by high levels of radioiodide accumulation of approximately 9.8% ID/g tumor as compared with nonirradiated tumors (7.0% ID/g tumor) using 123I-scintigraphy. Parallel studies using the constitutively active CMV promoter in combination with tumor irradiation revealed similar results with approximately 9.2% ID/g tumor using 5 Gy and 5.3% ID/g tumor in nonirradiated tumors (26). As a next step toward clinical application, the potential therapeutic efficacy of combining EBRT with MSC-based, NIS-mediated radiiodine therapy was evaluated. Radiation pretreatment itself demonstrated therapeutic efficacy in control mice (5 Gy + NaCl; 5 Gy + SMAD-NIS-MSCs + NaCl)
as compared with the saline only group ([NaCl + NaCl]). EBRT in combination with the biologically targeted, NIS-mediated therapy (5 Gy + SMAD-NIS-MSCs + I-131) illustrated a robust significant therapeutic effect. Therapy mice showed a halt in tumor growth for 20–30 days with some tumors growing very slowly afterward. Tumors in two of the mice within this group showed complete remission after approximately 40–50 days with no tumor regrowth until mice had to be sacrificed at day 89 and 197 as both mice suffered from severe bloating of unknown cause. Until the end of the observation period of 225 days, two mice of the SMAD-NIS-MSCs-treated group (5 Gy + SMAD-NIS-MSCs + I-131) were still alive demonstrating small tumors with little growth during this period. We had previously shown the general efficacy of SMAD-NIS-MSC–based radiiodine therapy of experimental HCC (25). The robust level of therapeutic effectiveness achieved in this study went well beyond that seen in the previous report, that even included more rounds of SMAD-NIS-MSCs treatment, but the therapeutic effect seen was still far below that seen in this study (25).

Thus, the combination of EBRT and SMAD-NIS-MSC–mediated, NIS-induced 131I therapy demonstrated significantly reduced tumor growth in therapy mice as well as significantly improved survival as compared with controls or therapy using CMV-NIS-MSCs, which at first demonstrated halted tumor growth for 3 to 4 weeks but showed tumor growth afterward. The therapeutic effect seen was also dramatically improved as compared with all former studies by our group using MSCs as NIS transgene delivery vehicles (13–16, 25). No difference was found between the groups in staining proliferating cells (Ki67) or blood vessels (CD31) potentially because the time point at which the tumors were resected and stained. The respective tumors were dissected and stained at the time of sacrifice after the tumor reached a critical volume based on the animal welfare protocol. In this study, mice from the therapy group as well as radiation control groups lived much longer and the time between treatment and tumor resection may have been too long, allowing some recovery of the tumors and reestablishment of tumor vasculature. To more accurately investigate the direct effects of therapy on the proliferative behavior of tumor cells and vascularization of the tumors, tumors will also be evaluated at earlier time points in future studies.

A critical molecular feature underlying the functional differences seen between the SMAD- and CMV-NIS-MSC–based therapies appears to lie in tumor-focused promoter activation as opposed to constitutive promoter expression. The CMV promoter is constitutively active, and application of radiiodine presumably effectively eliminates all NIS-expressing MSCs present in the animal. However, SMAD-NIS-MSCs upregulate NIS expression based on TGFβ tumor signals within the micromilieu. In this way, only a subset of SMAD-NIS-MSCs may become activated by TGFβ1, while other MSCs remain inactivated, thus surviving the 131I therapy round and may then be available for the next round of EBRT or radiiodine application. A second hypothesis that may help explain the pronounced therapy effect seen is based on the use of an inducible promoter activated in response to tissue radiation, and the potential for an amplification of effects in the context of subsequent radiiodine treatment.

Radiation-inducible promoters are currently under investigation as a means to more efficiently combine gene therapy with radiation treatment. These promoters regulate gene expression as a response to the stress seen during radiation treatment and contain what can be thought of as radio-responsive elements (37). The TGFβ1-inducible SMAD-responsive promoter used here responds to the TGFβ1 present in the tumor, further stimulated by radiation treatment and can thus be seen as an indirect but powerful radiation-inducible promoter. The tissue damage induced following the application of radiiodine may also result in an enhanced inflammatory response. Thus, NIS-based radiiodine therapy itself promotes enhanced MSC recruitment and increased levels of TGFβ1. Subsequent TGFβ1-induced SMAD-promoter activity in MSCs leads to prolonged and enhanced NIS transgene expression in response to repeated EBRT and radiiodine applications, leading to a self-energizing therapy cycle (Fig. 6).

Improved biologically targeted and tumor-selective radiiodine accumulation demonstrated a dramatic therapeutic response by combining radiation pretreatment and NIS-mediated 131I therapy. The robust therapeutic effect of this novel combination underscores the potential for more than additive effects of radiation-induced tumor signals and tumor signal-responsive promoter activation allowing an amplification of tumor-specific NIS transgene expression.

The results presented here demonstrate the enormous potential of using a TGFβ1-inducible promoter response to drive tumor-specific and radiation-inducible responses in the context of NIS cancer gene therapy to selectively control therapeutic transgene expression within the tumor environment and to amplify therapeutic efficacy. These data provide exciting preclinical evidence and open the prospect of clinical translation of this highly promising combination therapy approach for highly effective TGFβ1-inducible NIS cancer gene therapy taking advantage of the increased understanding of the tumor-homing capacity of MSCs as one of the most promising and flexible systemic gene delivery approaches available to date.

Disclosure of Potential Conflicts of Interest
M. Schwaiger is a consultant/advisory board member for GE Healthcare. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: C. Schug, K.A. Schmohl, M. Schwaiger, P.J. Nelson, C. Spitzweg
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Kitzberger, W. Sievert, R. Spellerberg, M. Tutter, B. Eberlein, K. Steiger, G. Multhoff, C. Spitzweg
Writing, review, and/or revision of the manuscript: C. Schug, W. Sievert, M. Tutter, K.A. Schmohl, B. Eberlein, T. Biedermann, K. Steiger, G. Multhoff, E. Wagner, P.J. Nelson, C. Spitzweg
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Sievert, M. Schwaiger, P.J. Nelson, C. Spitzweg
Study supervision: E. Wagner, P.J. Nelson, C. Spitzweg

Acknowledgments
We are grateful to Dr. S.M. Jiang, Ohio State University (Columbus, OH) for supplying the full-length human NIS cDNA. We also thank Prof. Dr. K. Schiedhauer and J. Allmann, Department of Nuclear Medicine, Klinikum rechts der Isar der Technischen Universität München (Munich, Germany), for their assistance with the imaging studies. We thank D. Mayr (Department of Pathology, Ludwig-Maximilians-Universität Munich, Munich, Germany) for
preparation of paraffin-embedded slides and M. Mielke and O. Seidlach (Department of Pathology and Comparative Experimental Pathology, Klinikum rechts der Isar der Technischen Universität München, Munich, Germany) and N. Schwenk (Medizinische Klinik und Poliklinik IV - Campus Grosshadern, University Hospital of Munich, Ludwig-Maximilians-University Munich, Munich, Germany) for performing the IHC. This work was supported by grants from the Deutsche Forschungsgemeinschaft within the Collaborative Research Center SFB 824 to C. Spitzweg (project C8), T. Biedermann (project B10), G. Multhoff (project B4), and K. Steiger (project Z2) as well as SFB 1335 to T. Biedermann (project P17) and K. Steiger (project Z01) and grant DFGINST411/37-1HUC to G. Multhoff and within the Priority Program SPP1629 to C. Spitzweg and P.J. Nelson (SP 581/6-1, SP 581/6-2, NE 648/5-2) as well as a grant from the Wilhelm-Sander-Stiftung to C. Spitzweg (2014.129.1). This work was performed as partial fulfillment of the doctoral thesis of C. Schug at the Faculty for Chemistry and Pharmacy of the LMU Munich.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 14, 2018; revised April 10, 2019; accepted June 10, 2019; published first June 13, 2019.

References


32. Droujinine IA, Eckert MA, Zhao W. To grab the stroma by the horns: from biology to cancer therapy with mesenchymal stem cells. Oncotarget 2013;4:651–64.
Radiation-Induced Amplification of TGFB1-Induced Mesenchymal Stem Cell–Mediated Sodium Iodide Symporter (NIS) Gene ¹³¹I Therapy

Christina Schug, Carolin Kitzberger, Wolfgang Sievert, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-18-4092

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2019/06/13/1078-0432.CCR-18-4092.DC1

Cited articles
This article cites 37 articles, 6 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/25/19/5997.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://clincancerres.aacrjournals.org/content/25/19/5997.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.