TGFB1-driven mesenchymal stem cell-mediated NIS gene transfer

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Short title: MSC-mediated NIS gene delivery

Keywords: Sodium iodide symporter, mesenchymal stem cells, hepatocellular carcinoma, gene therapy, TGFB signaling

Word count: 5219
ABSTRACT

Based on their excellent tumor-homing capacity, genetically engineered mesenchymal stem cells (MSCs) are under investigation as tumor-selective gene delivery vehicles. Transgenic expression of the sodium iodide symporter (NIS) in genetically engineered MSCs allows noninvasive tracking of MSC homing by imaging of functional NIS expression as well as therapeutic application of $^{131}$I. The use of tumor-stroma activated promoters can improve tumor-specific MSC-mediated transgene delivery. The essential role of transforming growth factor B1 (TGFβ1) and the SMAD downstream target in the signaling between tumor and the surrounding stroma makes the biology of this pathway a potential option to better control NIS expression within the tumor milieu. Bone marrow-derived MSCs were stably transfected with a NIS-expressing plasmid driven by a synthetic SMAD-responsive promoter (SMAD-NIS-MSCs). Radioiodide uptake assays revealed a 4.9-fold increase in NIS-mediated perchlorate-sensitive iodide uptake in SMAD-NIS-MSCs after TGFβ1 stimulation compared to unstimulated cells demonstrating the successful establishment of MSCs which induce NIS expression in response to activation of TGFβ1 signaling using a SMAD-responsive promoter. $^{131}$I-scintigraphy revealed significant tumor-specific radioiodide accumulation and thus NIS expression after systemic application of SMAD-NIS-MSCs into mice harboring subcutaneous tumors derived from the human hepatocellular carcinoma (HCC) cell line HuH7, which express TGFβ1. $^{131}$I therapy in SMAD-NIS-MSCs-treated mice demonstrated a significant delay in tumor growth and prolonged survival. Making use of the tumoral TGFβ1 signaling network in the context of MSC-mediated NIS gene delivery is a promising approach to foster tumor stroma-selectivity of NIS transgene expression and tailor NIS-based gene therapy to TGFβ1-rich tumor environments.
INTRODUCTION

Therapeutic options for hepatocellular carcinoma (HCC) are limited and novel targets and therapeutic strategies are urgently needed considering the increasing incidence of HCC worldwide (Siegel, et al. 2016; Stewart 2014). Solid tumors are thought to be seen by the body as “wounds that never heal” driving tissue repair and remodeling with active recruitment of mesenchymal stem cells (MSCs). This biology has been used to generate engineered versions of adoptively applied MSCs as a tool to deliver therapeutic genes, such as the sodium iodide symporter (NIS), deep into tumor microenvironments (Hagenhoff, et al. 2016; Melzer, et al. 2016). NIS is an intrinsic transmembrane glycoprotein with 13 putative transmembrane domains that mediates the active transport of iodide into the thyroid. When used in the context of a reporter gene, NIS can be used for noninvasive imaging of NIS transgene biodistribution by well-established multimodal nuclear medicine techniques, such as $^{123}$I-scintigraphy and $^{124}$I-PET imaging. At the same time, it can be used as an effective therapy gene after application of radioiodine $^{131}$I (Baril, et al. 2010; Hingorani, et al. 2010; Penheiter, et al. 2012; Spitzweg and Morris 2002). A diverse series of studies have demonstrated the efficacy of NIS-mediated radioiodide accumulation in a variety of non-thyroidal tumors using different gene delivery vehicles for NIS transgene expression (Ahn, et al. 2010; Dwyer, et al. 2006; Dwyer, et al. 2011; Grunwald, et al. 2013a; Grunwald, et al. 2013b, c; Huang, et al. 2011; Klutz, et al. 2009; Klutz, et al. 2011a; Klutz, et al. 2011b; Klutz, et al. 2011c; Knoop, et al. 2011; Knoop, et al. 2013; Knoop, et al. 2015; Mansfield, et al. 2016; Muller, et al. 2016; Niu, et al. 2004; Peerlinck, et al. 2009; Urnauer, et al. 2016). The use of genetically engineered MSCs to deliver NIS into various types of tumors has been demonstrated in many studies (Dwyer et al. 2011; Knoop et al. 2011; Knoop et al. 2013; Knoop et al. 2015; Muller et al. 2016). Although these results have shown comparably high levels of NIS transgene expression in the tumor microenvironment followed by a therapeutic effect of $^{131}$I, with a delay in tumor growth and prolonged survival of treated animals, the use of specific gene promoters for NIS expression that are activated by tumor micromilieu-derived signals has been shown to enhance selectivity and effectiveness and limit potential off-target effects following MSC recruitment to tissues as part of normal tissue homeostasis (Grunwald et al. 2013a; Grunwald et al. 2013b, c; Klutz et al. 2011).
We have studied various gene promoters for the delivery of MSC-transgene expression in tumor stroma. These include a RANTES (Regulated on Activation, normal T-cell Expressed and presumably Secreted)/CCL5 promoter activated by proinflammatory signals in tumor settings and a synthetic hypoxia-inducible factor (HIF)1-responsive promoter that selectively activates NIS transgene expression in tumors by targeting hypoxia, a central feature of solid tumors (Knoop et al. 2013; Knoop et al. 2015; Muller et al. 2016). The use of gene promoters activated by micromilieu-derived signals also offers the possibility of tailoring the NIS-therapy approach to the individual tumor micromilieu. The transforming growth factor B (TGFB) signaling pathway is strongly linked to the biology of tumor cells and their microenvironment. The TGFB signaling pathway is involved in cell growth, apoptosis, invasion, angiogenesis and immune response regulation (Neuzillet, et al. 2015). The isoforms of TGFB (TGFB1, -B2 and -B3) bind to heteromeric complexes of type I and II transmembrane Ser/Thr kinase receptors leading to transphosphorylation of the GS domain of the type I kinase through the type II receptor kinase resulting in phosphorylation of downstream target proteins such as SMADs (Derynck, et al. 2001; Massague 1998; Wrana, et al. 1994). Activated SMAD2 and SMAD3 form heterotrimeric complexes with the signal mediator SMAD4. The complex translocates to the nucleus to modulate transcription of target genes. Inhibitory SMADs, such as SMAD6 and SMAD7, interfere with the type I receptors, thereby blocking phosphorylation of other SMADs (Breuhahn, et al. 2006). SMAD7 expression is also driven by TGFB signaling thus inducing a TGFB-induced negative feedback loop (Breuhahn et al. 2006). Depending on early or late steps of carcinogenesis, some tumors are able to shut down the tumor-suppressive part of this signaling pathway thereby restricting it to pro-tumoral effects (Lopez-Novoa and Nieto 2009). TGFB is present in the micromilieu of most tumors (Massague 2008). In addition to the tumor cells, cancer-associated fibroblasts (CAFs) and stellate cells of the surrounding tumor stroma also secrete TGFB to control inflammatory response (Lopez-Novoa and Nieto 2009; Neuzillet et al. 2015). CAFs and stellate cells can promote tumor growth and metastasis among others (Lopez-Novoa and Nieto 2009). The TGFB pathway is strongly associated with HCC as it is well
known to be dysregulated in these tumors (Breuhahn et al. 2006). Several studies demonstrated that in
almost 50% of HCCs TGFB signaling is significantly upregulated (Abou-Shady, et al. 1999; Bedossa, et
al. 1995). TGFB plays an important role in tumor angiogenesis and its regulation in HCC (Neuzillet et al.
2015). From the three different isoforms of TGFB (TGFB1, -B2 and -B3), TGFB1 is most commonly
upregulated isoform in cancer cells (Derynck et al. 2001). The critical role of TGFB1 in HCC tumor
biology makes the TGFB signaling pathway a promising tool for targeting NIS transgene expression by
engineered MSCs in experimental HCC, with the aim to enhance tumor stroma selectivity and improve
therapeutic effectiveness.

In the current study, we used a TGFB1-inducible SMAD-responsive promoter to control NIS transgene
expression in genetically engineered human MSCs. After adoptively applying MSCs in HCC xenografts,
MSC biodistribution and biological targeting of NIS expression to the tumor stroma through NIS-mediated
accumulation of radioiodide was examined by \(^{123}\)I-scintigraphy. Therapeutic efficacy was determined by
\(^{131}\)I application after systemic MSC-mediated NIS transgene delivery.

MATERIALS AND METHODS

Cell culture

The human hepatocellular carcinoma cell line HuH7 was authenticated and purchased from JCRB Cell
Bank (JCRB 0403, Osaka, Japan). Cells were cultured in DMEM (Sigma-Aldrich, St. Louis, Missouri,
USA) supplemented with 10% FBS (FBS Superior, Biochrom/Merck Millipore, Berlin, Germany) and 100
U/ml penicillin/100 µg/ml streptomycin (Sigma-Aldrich). The human breast adenocarcinoma cell line
MDA-MB 231 was cultured in RPMI (Sigma-Aldrich), supplemented with 10% FBS and
100 U/ml penicillin/100 µg/ml streptomycin. The human bone marrow-derived, SV40 large T antigen
immortalized MSC cell line used here was established as described previously (Thalmeier and Huss 2001)
and cultured in RPMI (Sigma-Aldrich), supplemented with 10% FBS and 100 U/ml penicillin/100 µg/ml
streptomycin. The easily engineered and expandable cells were previously shown to demonstrate similar
homing and activation characteristics as seen in primary human MSCs (Von Luttichau, et al. 2005). All cells were maintained in an incubator at 37°C and 5% CO₂.

**Plasmid constructs**

The expression vector pcDNA6-2ITRNEO-SMAD-NIS was established using the Multiside Gateway Pro Plus Kit (Invitrogen Thermo Scientific, Waltham, Massachusetts, USA) following the manufacturer’s instructions. The promoterless Gateway destination vector (pcDNA6) contains a Sleeping Beauty transposon system as described previously (Jackel, et al. 2016). The used SMAD-responsive promoter consists of a 5× multimer of the SMAD binding site AGCCAGACAGT. The vector pSBTR.TGF/SMAD2 containing the promoter was established as described previously (Jackel et al. 2016). Full length NIS cDNA was removed from the pcDNA3 expression vector and was cloned into the vectors provided in the Multiside Gateway Pro Plus Kit. After a 2-fragment recombination the pcDNA6-2ITRNEO-SMAD-NIS plasmid was obtained. The resulting plasmid contains the NIS gene driven by a SMAD-responsive promoter, two sleeping beauty transposition sites and a geneticin resistance gene. The vectors pSB.H.CMV/TO.SMAD3 (expressing SMAD3) and pSB.H.CMV/TO.SMAD4 (expressing SMAD4) were constructed as described previously (Jackel et al. 2016).

**Stable transfection of MSCs**

Wild type MSCs (WT-MSC) were stably transfected with the pcDNA6-2ITRNEO-SMAD-NIS expression vector in combination with the pCMV(CAT)T7-SB100X plasmid (provided from Z Ivics, Max Delbrück Center for Molecular Medicine, Berlin, Germany). The pCMV(CAT)T7-SB100X vector contains a Sleeping Beauty transposase system for transgene insertion into the host cell genome. 0.5 × 10⁶ MSCs and a total amount of 3 µg plasmid were electroporated at 1300 V, 30 ms width and 1 pulse using the Neon® transfection system (Invitrogen, Karlsbad, California, USA). Selection medium was given 24 h after electroporation and contained 0.5 mg/ml geneticin (Invitrogen) in RPMI medium supplemented with 10% fetal bovine serum and 100 U/ml penicillin/100 µg/ml streptomycin. The resulting clones were isolated.
and analyzed for NIS-mediated radioiodide uptake activity using an iodide uptake assay (see below). Among ~40 screened colonies, the stably transfected cell clone with the highest levels of iodide accumulation was termed SMAD-NIS-MSC and used for further experiments.

For a more precise investigation of the SMAD-responsive promoter, SMAD-NIS-MSCs were further stably transfected with pSB.H.CMV/TO.SMAD3 (SMAD-NIS-MSCs+SMAD3), pSB.H.CMV/TO.SMAD4 (SMAD-NIS-MSCs+SMAD4), pSB.H.CMV/TO.SMAD3 and pSB.H.CMV/TO.SMAD4 (SMAD-NIS-MSCs+SMAD3+SMAD4) vector. For cells transfected with expression vectors for SMAD3 and/or SMAD4, 0.2 mg/ml Hygromycin was added to the cell culture medium described above. This stably transfected cell lines were maintained and tested as bulk cultures.

Tumor cell conditioned medium

1×10^6 HuH7 or MDA-MB 231 cells were seeded on 100 mm³ surface cell culture plates and starved for 12 h. Supernatant from the tumor cells (HuH7 conditioned medium (HuH7-CM) or MDA-MB 231-CM) was removed after 48 h, centrifuged and stored at -80°C.

^125^I uptake assay

Inducibility of the promoter was determined by starving cells overnight (medium without FBS) followed by stimulation of SMAD-NIS-MSCs and variants of those (see above) with 0-25 ng/ml recombinant TGFB1 for 24h in normal medium as described above. NIS-mediated radioiodide uptake was determined at steady-state conditions as described previously by Spitzweg et al. (Spitzweg, et al. 1999). Results were normalized to cell viability.

Cell viability assay

Cell viability was measured using the commercially available MTT assay (Sigma-Aldrich, ratio 1:100) according to the manufacturer’s recommendations. For the measurement of the absorbance of the formazan product at a wavelength of 620 nm, a Sunrise Microplate Absorbance Reader (Tecan,
Männedorf, Switzerland) was used.

**Animals**

Female 5-week-old CD1 nu/nu mice from Charles River (Sulzfeld, Germany) were 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).

**HuH7 xenograft tumors**

For HuH7 xenograft tumor establishment, $5 \times 10^6$ HuH7 cells in 100 µl PBS were injected subcutaneously into the right flank region as described previously (Knoop et al. 2011). Tumor volumes were regularly measured and estimated using the equation: length $\times$ width $\times$ height $\times$ 0.52. Mice were sacrificed when tumors exceeded a size of 1500 mm$^3$.

**$^{123}$I-scintigraphy**

To suppress thyroidal iodide uptake the drinking water of the mice was supplemented with 5 mg/ml L-T4 (Sigma-Aldrich). SMAD-NIS-MSCs were applied three times every second day *via* the tail vein at a concentration of $5 \times 10^5$ cells in 500µl PBS. 72 h after the last MSC application mice were injected intraperitoneally with 18.5 MBq (0.5 mCi) $^{123}$I. The gamma camera was equipped with a low-energy high resolution collimator (e.cam, Siemens, Munich, Germany) and was used to measure radioiodide biodistribution. For the analysis of regions of interests (ROIs) HERMES GOLD (Hermes Medical Solutions, Stockholm, Sweden) software was used. Results are expressed as % ID/g. The radionuclide retention time was determined by serial scanning within the tumors. Dosimetric calculations were performed according to the concept of medical internal radiation dose using the dose factor of RADARgroup (www.dosisinfo-radar.com).

**Immunohistochemical staining**
After systemic SMAD-NIS-MSC administration and imaging studies, HCC xenografts or other organs (liver, lung and spleen) were dissected from all mice of the $^{125}$I-scintigraphy study and tissues embedded in paraffin. Immunohistochemical NIS staining of paraffin-embedded tissue sections was performed as described previously (Spitzweg, et al. 2007). Immunohistochemical staining of TGFB1 was performed on HuH7 tumors on a BenchMark XT automated stainer (Ventana, Tucson, AZ) with an antibody against TGFB (ab92486, abcam, Cambridge, UK) using the ultraVIEW DAB Detection Kit (all reagents from Ventana, Tucson, AZ). The tumor sections were deparaffinized with EZ Prep at 75°C and 76°C, heat pretreated in Cell Conditioning 1 (CC1) for antigen retrieval at 76°C – 100°C and then incubated with the primary antibody diluted in antibody diluent 1:150 for 32 min at 37°C after inactivation of the endogenous peroxidase using UV-inhibitor for 4 min at 37°C. The slides were incubated with a HRP Universal Multimer for 8 min. Antibody binding was detected using DAB as chromogen and counterstained with hematoxylin for 10 min with subsequent bluing in bluing reagent for 10 min. Afterwards, slides were manually dehydrated by washing with alcohol of increasing concentration (70%, 96%, 100%) and xylene, and coverslipped using Pertex® mounting medium (Histolab, Goeteborg, Sweden, 00801).

**Radioiodine therapy study**

Mice were treated 10-days before therapeutic application of radioiodide with 5 mg/ml L-T4 in their drinking water as described above. The therapy group received three SMAD-NIS-MSC injections on every second day (each $5 \times 10^5$ cells/500 µl PBS) followed by 55.5 MBq $^{131}$I 48 hours after the last SMAD-NIS-MSC injection (n=6; SMAD-NIS-MSCs + $^{131}$I). As a control, a subset of mice received saline (NaCl) instead of radioiodine (n=6; SMAD-NIS-MSCs + NaCl). 24 h after the $^{131}$I or NaCl application the treatment cycle was repeated. One additional MSC application followed by a third $^{131}$I (55.5 MBq) or NaCl injection 48 hours later was done for therapy completion. A further control group was injected with NaCl only (n=5; NaCl + NaCl). Tumor volume was measured regularly and estimated as described above.
Mice were euthanized when the tumor volume exceeded 1500 mm$^3$.

**Indirect immunofluorescence assay**

Frozen tissue sections of tumors from the radioiodine therapy study were used for indirect immunofluorescence analysis of Ki67 (cellular proliferation) and CD31 (blood vessel density) as described previously (Muller *et al.* 2016). Identical conditions as for illumination, exposure time and system settings for digital image processing were used for capturing regions of the slides. The percentage of positive cells for Ki67 and areas for CD31 in the tumors was quantified by evaluation of 6 high-power fields per tumor using ImageJ software (NIH, Bethesda, MD). Results are presented as means ± SEM.

**Statistical methods**

Results are expressed as mean ± SEM, mean-fold change ± SEM or percentage. Statistical significance for *in vitro* experiments was tested by two-tailed Student’s *t*-test. Statistical significance for *in vivo* experiments was performed by 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**

**SMAD-NIS-MSCs show enhanced NIS-mediated radioiodide accumulation following TGFB1 stimulation**

Radioiodide uptake assays revealed a 3.4 to 4.9 fold increase in NIS-mediated radioiodide uptake activity in MSCs stably transfected with the NIS gene under control of a SMAD-responsive promoter (SMAD-NIS-MSCs) after stimulation with TGFB1 (5-25 ng/ml) (Fig. 1A). Stimulation with 5-15 ng/ml TGFB1 resulted in a dose dependent increase of radioiodide accumulation, which reached a plateau at higher doses of TGFB1 (20 or 25 ng/ml). NIS-specificity was confirmed using the NIS-specific inhibitor perchlorate, which blocked radioiodide uptake in all experiments (Fig. 1A).
SMAD-NIS-MSCs were additionally stimulated by adding HCC (HuH7)-conditioned medium (CM) (Fig. 1B). Stimulation of SMAD-NIS-MSCs with both TGFB1 (10 ng/ml) and HuH7-CM resulted in a robust increase in radioiodide accumulation as compared to stimulation with TGFB1 alone. No radioiodide uptake activity was observed when SMAD-NIS-MSCs were exposed to HuH7-CM only (Fig. 1B). TGFB1 protein levels in HuH7-CM were analyzed by enzyme-linked immunosorbent assay (ELISA), which confirmed TGFB1 protein expression in HuH7 cells, but showed that HuH7-CM contains only inactive TGFB1 (data not shown). These findings correlate with the results shown above, where no radioiodide uptake is seen in SMAD-NIS-MSCs stimulated with HuH7-CM only. Additionally, to determine this effect is also seen with other tumor cells, SMAD-NIS-MSCs were treated with CM from MDA-MB 231 cells (human breast carcinoma cells) (Fig. 1B). Again, stimulation of SMAD-NIS-MSCs with MDA-MB 231-CM alone resulted in no radioiodide uptake activity. Further, only a slight increase of radioiodide uptake was observed after combined treatment with MDA-MB 231-CM and TGFB1 which was not statistically significant.

To further investigate activation of the promoter through TGFB1 (SMAD) signaling, variants of SMAD-NIS-MSCs where established by stably transfecting SMAD-NIS-MSCs with SMAD3 or SMAD4 expression constructs (Fig. 1C). In SMAD-NIS-MSCs transfected with the SMAD3 expression vector (SMAD-NIS-MSCs + SMAD3) TGFB1 stimulation (10 ng/ml) led to significantly increased NIS-mediated perchlorate-sensitive radioiodide uptake as compared to stimulated SMAD-NIS-MSCs. Transfection of SMAD-NIS-MSCs with a SMAD4 expression vector (SMAD-NIS-MSCs + SMAD4) resulted in no increase of radioiodide uptake activity upon TGFB1 stimulation as compared to stimulated SMAD-NIS-MSCs. Combination of SMAD3 and SMAD4 expression in SMAD-NIS-MSCs (SMAD-NIS-MSCs + SMAD3 + SMAD4) revealed an increase of radioiodide uptake after TGFB1 stimulation as compared to stimulated SMAD-NIS-MSCs and as compared to SMAD-NIS-MSCs + SMAD4, but a decrease as compared to TGFB1 stimulated SMAD-NIS-MSCs + SMAD3 (Fig. 1C). No radioiodide uptake activity above background levels was observed without TGFB1 stimulation or upon treatment with perchlorate (Fig. 1C).
Radioiodide biodistribution in vivo

SMAD-NIS-MSCs ($0.5 \times 10^6$) where injected intravenously via the tail vein into nude mice harboring subcutaneous HCC xenograft tumors, three times in 48 h intervals. 72 h after the last MSC application, 18.5 MBq $^{123}$I were administered intraperitoneally. Radioiodide biodistribution monitored by $^{123}$I-scintigraphy (Fig. 2) revealed approximately 6.8 ± 0.8 % of the injected dose per gram (ID/g) radioiodide accumulation in tumors after SMAD-NIS-MSC application (Fig. 2A, C). A biological half-life of 2.2 h and a tumor absorbed dose of 28.2 mGy/MBy was calculated for $^{131}$I. Endogenous NIS-mediated radioiodide uptake was observed in the stomach, salivary glands and thyroid gland. Radioiodide uptake in the urinary bladder is due to renal excretion of $^{123}$I (Fig. 2A). Injection of perchlorate 30 min prior to $^{123}$I administration resulted in blockage of radioiodide accumulation in tumors, stomach, salivary glands and thyroid gland (Fig. 2B).

NIS and TGFB1 protein expression in HuH7 tumors ex vivo

To evaluate biodistribution of SMAD-NIS-MSCs ex vivo, NIS protein expression in resected tumors and non-target organs (liver, lung, spleen) was investigated by NIS-immunohistochemistry (Fig. 3). Throughout the tumor stroma, NIS-specific immunoreactivity was detected in mice that were injected with SMAD-NIS-MSCs (Fig. 3A), whereas no NIS protein expression was observed in non-target organs (Fig. 3B-D). These data show efficient MSC recruitment into the tumor stroma after systemic application and activation of the SMAD-responsive promoter resulting in NIS protein expression. To confirm TGFB1 expression in subcutaneous HuH7 tumors, tumors were stained for TGFB1 protein expression, showing TGFB1 expression within tumor and its stroma (Fig. 3E).

In vivo radioiodine therapy studies

Therapeutic efficacy of $^{131}$I was evaluated using SMAD-NIS-MSCs in HuH7 tumors (Fig. 4). Animals of the therapy group received two cycles of three SMAD-NIS-MSC applications in 48 h intervals followed
by a single $^{131}$I injection (55.5 MBq each) after the third MSC application. For a third and last therapy round, a single SMAD-NIS-MSC application was given followed by a therapeutic dose of $^{131}$I. A significant delay in tumor growth was seen in therapy mice treated with SMAD-NIS-MSCs followed by $^{131}$I application (SMAD-NIS-MSCs + $^{131}$I) as compared to controls, which received either SMAD-NIS-MSCs followed by application of saline (NaCl) instead of $^{131}$I (SMAD-NIS-MSCs + NaCl) or NaCl only (NaCl + NaCl) (Fig. 4A). Both controls showed continuous exponential tumor growth (Fig. 4A).

Analysis of survival revealed a maximum of 21-24 days for mice in control groups, whereas mice in therapy showed prolonged survival of up to 63 days (Fig. 4B).

For a more detailed analysis, dissected tumors were stained for Ki67 (green; proliferation marker) and CD31 (red; marker for blood vessels) immunofluorescence (Fig. 5). Striking differences were seen between mice in therapy (SMAD-NIS-MSCs + $^{131}$I) and mice in control groups (SMAD-NIS-MSCs + NaCl; NaCl + NaCl) (Fig. 5A). Tumors after $^{131}$I-therapy revealed a lower Ki67-index of 35.3 ± 5% and a mean blood vessel density of 1.4 ± 0.4%, whereas tumors of control groups showed a higher proliferation index of 52.9 ± 2.8% (SMAD-NIS-MSCs + NaCl) and 64.4 ± 4.2% (NaCl + NaCl) and blood vessel density of 2.7 ± 0.5% (SMAD-NIS-MSCs + NaCl) and 2.2 ± 0.2% (NaCl + NaCl) (Fig. 5B, C).

**DISCUSSION**

The theranostic NIS gene allows noninvasive imaging of functional NIS expression by $^{123}$I-scintigraphy, thereby enabling tracking of gene delivery vehicles by radioiodide imaging, as well as application of $^{131}$I for a therapeutic purpose. Our laboratory and others have extensively investigated the capacity and efficacy of NIS gene transfer to induce radioiodide accumulation in non-thyroidal tumors using MSCs as NIS transgene delivery vehicles (Dwyer et al. 2011; Knoop et al. 2011; Knoop et al. 2013; Knoop et al. 2015; Muller et al. 2016). Earlier studies of active MSC engraftment in a HCC xenograft model using the constitutively active cytomegalovirus (CMV) promoter to express NIS in MSCs showed the proof-of-principle of active NIS-MSC tumor homing, followed by effective NIS expression in the tumor stroma and a significant delay in tumor growth after application of $^{131}$I (Knoop et al. 2011). Although these results
were highly promising for a MSC-based NIS-mediated radionuclide therapy approach in non-thyroidal tumors, using the non-selectively activated CMV-promoter involves the risk of potential side effects due to MSC recruitment to normal tissues in the process of normal tissue homeostasis. To reduce toxicity by undesired effects in non-target organs, further studies focused on restricting NIS expression to the tumor environment. Growing HCC tumors require an active tumor stroma consisting of various tumor stroma-specific cells such as cancer-associated fibroblasts (CAFs) and inflammatory cells, but are also marked by high angiogenesis. The inflammatory response within the tumor leads to increased levels of various cytokines and chemokines (Capece, et al. 2013; Knoop et al. 2013; Knoop et al. 2015; Niess, et al. 2011).

In previous studies from our group a RANTES/CCL5 promoter was used to drive NIS expression in MSCs thus enhancing tumor stroma-specificity in subcutaneous HCC xenografts as well as colon cancer liver metastases mouse models (Knoop et al. 2013; Knoop et al. 2015). The NIS-mediated therapy approach resulted in an improved therapeutic response in animals harboring subcutaneous HCC tumors, namely a significant delay in tumor growth and prolonged animal survival, as compared to the former study using a CMV-promoter for NIS transgene expression (Knoop et al. 2011; Knoop et al. 2013). The clinically more relevant question whether this therapy approach could also be used in metastatic disease was examined using a colon cancer liver metastases model, where similarly high therapeutic efficacy and improved survival was observed (Knoop et al. 2015). Further studies to limit activation of NIS transgene expression to the tumor environment of subcutaneous and orthotopic HCC xenografts have included targeting the tumor hypoxia response (Muller et al. 2016). A synthetic HIF1A-responsive promoter was designed to activate NIS transgene expression as soon as MSCs encounter hypoxic regions of solid tumors. Interestingly, systemic application of these HIF-NIS-MSCs followed by $^{131}$I injection resulted in a significant delay in tumor growth and prolonged animal survival only an orthotopic HCC model (Muller et al. 2016). Although these approaches revealed promising therapeutic responses, our aim in the present study was to expand the individualized design of genetically engineered MSCs for NIS expression under control of promoters activated by micromileu-derived signals to enhance selectivity, effectiveness and flexibility of MSC-based NIS gene delivery, and in parallel, potentially addressing issues related to tumor
heterogeneity. As interindividually and intraindividual tumor heterogeneity is an enormous challenge for successful cancer therapy, the search for tools for individualization of cancer treatment is critical for future personalized care of cancer patients. In this context, the growing knowledge of tumor stroma biology offers interesting strategies to tailor tumor stroma targeted therapy approaches. TGFB1 is known to be a central player in tumor biology, in particular tumor micromilieu-associated signaling, and has also been characterized to be significantly upregulated in HCC to promote tumorigenesis and exert immunosuppressive effects (Yang, et al. 2011). While at first TGFB1 is expressed in the microenvironment of pre-malignant tumors to control tumor progression, its local expression is later required to promote tumor growth as well as metastasis (Papageorgis and Stylianopoulos 2015). Source of this cytokine is not only its expression in tumor cells but also in endothelial cells, fibroblasts, leucocytes, mesenchymal and myeloid precursor cells, among others, where it can act in a paracrine or autocrine manner (Papageorgis and Stylianopoulos 2015).

TGFB plays an important role for HCC treatment as higher levels of TGFB lead to resistance to anticancer treatments. As radiotherapy and chemotherapy can increase TGFB expression and activity, a combination with TGFB inhibitors is thought to enhance tumor sensitivity to those treatment strategies (Neuzillet et al. 2015). Further, TGFB is linked to hypoxia in terms of epithelial-mesenchymal transition (EMT) induction and VEGF signaling and a combination of TGFB inhibitors with anti-angiogenic or hypoxia-induced treatment strategies might be an option as well (Neuzillet et al. 2015). Other than that, the high expression levels of TGFB1 in tumor cells and the tumor microenvironment makes TGFB1 signaling an interesting candidate to create novel promoters for tumor stroma targeted NIS expression in the context of NIS gene therapy. In the present study a TGFB1-inducible SMAD-responsive promoter, consisting of a 5× multimer of the SMAD binding site AGCCAGACAGT, was designed to drive transgene MSCs (referred to as SMAD-NIS-MSCs). In vitro analysis of SMAD-NIS-MSCs showed a robust dose-dependent NIS-mediated accumulation of radioiodide upon TGFB1 stimulation. Accumulation of radioiodide in SMAD-NIS-MSCs was further enhanced through combined stimulation with TGFB1 and HuH7-CM. This additive increase in radioiodide uptake activity appears to be dependent on the tumor line used, as...
treatment of SMAD-NIS-MSCs with CM from MDA-MB 231 cells did not result in a significant increase in promoter activity. An explanation might be the presence of co-factors in CM, which trigger SMAD expression in MSCs and thus altering sensitivity for TGFB1. As HuH7-CM only contained inactive TGFB1 protein, the exact mechanisms of the increased activity are not known yet and have to be further investigated in future studies.

The canonical TGFB signaling pathway (SMAD dependent) is regulated by different SMAD proteins. After binding of active TGFB1 to the respective receptor, SMAD1, 2, 3, 5 and 8 are phosphorylated and form complexes with the common mediator SMAD4 (Papageorgis and Stylianopoulos 2015). Translocation of the SMAD complex into the cell nucleus leads to gene expression regulation. To understand the exact mechanisms of promoter activation of SMAD-NIS-MSCs in our study, SMAD-NIS-MSCs were additionally transfected with vectors expressing SMAD3 or SMAD4. These vectors are driven by the CMV-promoter and are thus permanently expressing SMAD3 or SMAD4. To investigate regulation of NIS expression, the additionally transfected SMAD-NIS-MSCs were tested by iodide uptake assay with or without TGFB1 stimulation. As TGFB signaling requires phosphorylated SMADs to become active, no radioiodide activity was observed in SMAD-NIS-MSCs, containing expression vectors for SMAD3 or SMAD4, in the absence of TGFB1 stimulation. Upon TGFB1 stimulation an enhanced NIS-mediated radioiodide uptake activity in the presence of SMAD3 was observed, whereas this effect was not observed by SMAD4, showing the influence of different SMADs on the SMAD-responsive promoter used to control NIS expression. The subsequent in vivo \(^{123}\)I-scintigraphy studies showed the recruitment of SMAD-NIS-MSCs into the tumor environment of experimental HuH7 tumors and led to a robust tumor-selective TGFB1-driven induction of the NIS transgene as shown by tumor-specific radioiodide accumulation.

NIS expression within the tumors was further confirmed by NIS immunoreactivity, which demonstrated high NIS protein expression within tumors but not in non-target organs such as liver, lung or spleen. Further, TGFB1 immunohistochemistry confirmed expression of TGFB1 within the experimental HuH7 tumors. SMAD-NIS-MSCs were then applied followed by therapeutic application of \(^{131}\)I which resulted in
a significant reduction in tumor growth and prolonged survival. Although the calculated biological half-life of 2.2 h and tumor-absorbed dose of 28.2 mGy/MBy/g tumor was lower than those observed in previous studies, the therapy study revealed as strong a therapeutic effect comparable to our previous studies using the specific RANTES/CCL5 promoter (Knoop et al. 2013).

The use of traditional anticancer therapies, such as radiation treatment or chemotherapy, demonstrated an induction of TGFB1 expression. Biswas et al. illustrated enhanced levels of circulating TGFB1 in plasma after thoracic irradiation or chemotherapy (doxorubicin) in mice with mammary carcinoma (Biswas, et al. 2007). Further, as shown by our group (unpublished data) and others, external beam radiation therapy of tumors is able to induce an enhanced inflammatory response resulting in increased secretion of diverse growth factors and chemokines, including TGFB1, which in turn also led to an enhanced recruitment of MSCs (Kim, et al. 2010; Klopp, et al. 2007). Therefore, the approach outlined in the present study based on NIS gene expression driven by a TGFB1-inducible SMAD-responsive promoter opens the prospect of combination with conventional therapies, such as external beam radiation or chemotherapy, to take advantage of synergistic effects thereby fostering TGFB1-induced tumoral NIS expression and improving therapeutic efficacy of MSC-based NIS-induced radioiodide therapy. The results presented here build on previous studies, including the initial clinical trials of engineered MSCs for tumor therapy, by providing new targeting approaches that could expand targeting options in the next generation of engineered anti-tumor MSCs.

Taken together, our study demonstrates high tumor stromal recruitment of SMAD-NIS-MSCs and a robust, biologically targeted NIS transgene expression in subcutaneous HuH7 tumors through TGFB1-induced SMAD promoter activity. After systemic application of SMAD-NIS-MSCs followed by $^{131}$I injection, a significant therapeutic effect was observed, resulting in reduction of tumor growth and improved survival. The proof-of-principle in this study opens the exciting prospect for future studies, where we will build on the presented results to investigate the enormous potential of TGFB1-induced promoters for NIS transgene expression for an improved and novel theranostic NIS gene approach. As native gene promoters are generally more efficient than the synthetic promoters used in this study as a
proof of concept, in future studies it will be important to identify efficient TGFB1-regulated native
promoters that may allow a more robust level of transgene induction in the context of the tumor
environment.

DECLARATION OF INTEREST

The authors declare that they have no conflict of interest. This work was performed as partial fulfillment
in the doctoral thesis of Christina Schug within the LMU Medical Faculty.

FUNDING

This work was supported by grants from the Deutsche Forschungsgemeinschaft within the Collaborative
Research Center SFB 824 to C Spitzweg (project C8) and within the Priority Program SPP1629 to C
Spitzweg and PJ Nelson (SP 581/6-1, SP 581/6-2, NE 648/5-2) as well as within a grant from the

AUTHOR CONTRIBUTIONS

K.A.S. and N.S.; Resources, M.S.; Writing – Original Draft: C.Sc., C.Sp., P.J.N and E.W.; Supervision,
C.Sp., P.J.N. and E.W.; Funding Acquisition, C.Sp., P.J.N. and M.S.

ACKNOWLEDGEMENTS

We are grateful to Dr. S.M. Jhiang, Ohio State University, Columbus, OH, USA for supplying the full-
length human NIS cDNA. We also thank Prof. Dr. K. Scheidhauer and Jakob Allmann, Department of
Nuclear Medicine, Klinikum rechts der Isar der Technischen Universität München, Munich, Germany, for
their assistance with the imaging studies. We thank Doris Mayr (Department of Pathology, LMU Munich,
Munich, Germany) for preparation of paraffin-embedded slides and Marion Mielke (Department of
Pathology and Comparative Experimental Pathology, Klinikum rechts der Isar der Technischen Universitaet Muenchen) for performing the immunohistochemistry.

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FIGURE LEGENDS

FIGURE 1: Establishment of mesenchymal stem cells (MSCs) stably expressing NIS under control of a SMAD-responsive promoter. Iodide uptake studies revealed a 3.4 up to 4.9-fold high NIS-specific and perchlorate-sensitive $^{125}$I uptake in SMAD-NIS-MSCs stimulated with TGFβ1 (5-25 ng/ml) as compared to unstimulated cells, where no radioiodide uptake above background levels was observed (A). Combined treatment of SMAD-NIS-MSCs with TGFβ1 (10 ng/ml) and HuH7-CM led to further increased radioiodide uptake as compared to TGFβ1 stimulated cells without HuH7-CM (B). In contrast to that, a stimulation with MDA-MB 231-CM and TGFβ1 did not result in a significant increase in radioiodide uptake activity of SMAD-NIS-MSCs (B). SMAD-NIS-MSCs were additionally transfected with constructs expressing SMAD3 (SMAD-NIS-MSCs + SMAD3), SMAD4 (SMAD-NIS-MSCs + SMAD4) or transfected with both expression constructs (SMAD-NIS-MSCs + SMAD3 + SMAD4) (C). Higher radioiodide uptake activity after TGFβ1 (10 ng/ml) treatment was revealed in the presence of SMAD3, whereas it did not increase with higher expression levels of SMAD4. SMAD-NIS-MSCs expressing SMAD3 and SMAD4 showed a radioiodide uptake higher than SMAD-NIS-MSCs and SMAD-NIS-MSCs + SMAD4, but was lower than SMAD-NIS-MSCs + SMAD3 after TGFβ1 treatment (C). Data are represented as means of three independent experiments ± SEM (n=3; two-tailed Student’s t-test: *p<0.05; **p <0.01, ***p <0.001).

FIGURE 2: $^{123}$I-scintigraphy showed high NIS-specific tumoral radioiodide uptake. Three systemic injections of SMAD-NIS-MSCs on every second day in mice harboring subcutaneous HuH7 tumors resulted in a maximum of approximately 6.8 % ID/g tumor (n=5) (A, C). Tumoral radioiodide uptake was blocked upon treatment with the NIS-specific inhibitor perchlorate (n=1) (B). Data are represented as mean values ± SEM.

FIGURE 3: Tumor-specific NIS and TGFβ1 protein expression. NIS-specific immunoreactivity was detected in all tumors of mice that received systemically applied SMAD-NIS-MSCs (A) as compared to
non-target organs, where no NIS protein expression was detected (B-D). Further, TGFB1 protein expression was confirmed within subcutaneous HuH7 tumors and the tumor stroma (E). One representative image at 20× magnification is shown each.

**FIGURE 4**: 131I therapy of subcutaneous HuH7 tumors led to a delay in tumor growth and improved survival. For an in vivo radionuclide therapy study, mice received three SMAD-NIS-MSC applications on every second day followed by 55.5 MBq 131I 48 h after the last MSC injection. For therapy end, a final cycle was done consisting of a single SMAD-NIS-MSC application and 131I injection 48 h afterwards (n=6; SMAD-NIS-MSC + 131I) (A). Therapy of mice harboring HuH7 tumors resulted in a significant delay in tumor growth as compared to controls receiving SMAD-NIS-MSCs and NaCl (n=6; SMAD-NIS-MSC + NaCl) or NaCl only (n=5; NaCl + NaCl) (A). Further, therapy led to an improved overall survival in therapy animals (B).

**FIGURE 5**: Ki67 and CD31 immunofluorescence staining. Immunofluorescence analysis for Ki67 (green) and CD31 (red, labeling blood vessels) (A) showed significantly decreased proliferation (Ki67, 35.3 ± 5 %) as well as reduced blood vessel density (CD31, 1.4 ± 0.4%) in resected tumors of mice treated with SMAD-NIS-MSC followed by 131I treatment as compared to tumors of mice injected with SMAD-NIS-MSCs and NaCl (Ki67, 52.9 ± 2.8%; CD31, 2.7 ± 0.5%) or NaCl only (Ki67, 64.4 ± 4.2%; CD31, 2 ± 0.2%) (B, C). Slides of tumors were counterstained with Hoechst nuclear stain. One representative image at 20× magnification is shown each.
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174x70mm (250 x 250 DPI)
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174x76mm (250 x 250 DPI)
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