A Novel Approach for Image-Guided $^{131}$I Therapy of Pancreatic Ductal Adenocarcinoma Using Mesenchymal Stem Cell-Mediated NIS Gene Delivery

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Abstract

The sodium iodide symporter (SLC5A5/NIS) as theranostic gene would allow for non-invasive imaging of functional NIS expression and therapeutic radioiodine application. Genetically engineered mesenchymal stem cells (MSC), based on their tumor-homing abilities, show great promise as tumor-selective NIS gene delivery vehicles for non-thyroidal tumors. As a next step towards clinical application, tumor specificity and efficacy of MSCs were investigated in an advanced genetically engineered mouse model of pancreatic ductal adenocarcinoma (PDAC). Syngeneic murine MSCs were stably transfected with a NIS-expressing plasmid driven by the CMV-promoter (NIS-MSC). In vivo $^{123}$I-scintigraphy and $^{124}$I-PET revealed significant perchlorate-sensitive NIS-mediated radioiodide accumulation in PDAC after systemic injection of NIS-MSCs. Active MSC recruitment into the tumor stroma was confirmed using NIS immunohistochemistry (IHC). A therapeutic strategy, consisting of three cycles of systemic MSC-mediated NIS delivery, followed by $^{131}$I application, resulted in a significant delay and reduction in tumor growth as compared to controls. Furthermore, IHC analysis of α-SMA and Ki67 revealed differences in the amount and behavior of activated fibroblasts in tumors of mice injected with NIS-MSCs as compared with saline-treated mice. Taken together, MSCs as NIS gene delivery vehicles in this advanced endogenous PDAC mouse model demonstrated high stromal targeting of NIS by selective recruitment of NIS-MSCs after systemic application resulting in an impressive $^{131}$I therapeutic effect.

Implications: These data expand the prospect of MSC-mediated radioiodide imaging-guided therapy of pancreatic cancer using the sodium iodide symporter as a theranostic gene in a clinical setting.

Introduction

The sodium iodide symporter (NIS) is an intrinsic transmembrane glycoprotein that is responsible for the active transport of iodide into the thyroid gland (1). As NIS is also expressed in follicular cell-derived differentiated thyroid cancer cells, its expression provides the molecular basis for diagnostic and therapeutic application of radioiodine in patients with thyroid cancer (1, 2). The extensive clinical experience of using NIS as theranostic gene in the management of patients with thyroid cancer has provided the basis for the development of NIS gene-based therapy approaches in nonthyroidal tumors (3, 4). The NIS transgene has been successfully transferred selectively into extrathyroidal tumor cells or cells of the tumor environment using various gene delivery systems where diagnostic use of NIS has allowed the direct monitoring and detailed characterization of vector biodistribution, localization, and duration of transgene expression within tumors using $^{123}$I-scintigraphy and $^{124}$I-PET imaging (4-19). The dosimetric calculations derived from the imaging studies allowed the application of an optimized therapeutic dose of radioiodine ($^{131}$I). Different approaches for systemic NIS gene delivery [i.e., polyplexes, mesenchymal stem cells (MSC), viral vectors] are currently under evaluation in several experimental settings and in tumor mouse models (5-17). One promising approach has been the use of bone-marrow derived MSCs as tumor therapy vehicles based on their excellent intrinsic tumor-homing capacity (20-22). Their active recruitment into growing tumor stroma is mediated by mechanisms that are thought to be similar to those that occur in the context of wound healing (20, 21). Once MSCs enter the tumor environment, they differentiate into various tumor stroma-associated cell types (23). These include cells

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associated with the tumor vasculature and stromal fibroblast-like cells. A series of studies have demonstrated the potential of using adoptively applied MSCs to deliver therapeutic genes into primary tumors as well as to tumor metastases (9–12). MSC-mediated NIS gene delivery in xenograft tumor mouse models has shown successful selective NIS-expression in tumors and metastases as well as a robust therapeutic response after 131I application (9–12). Although these results are very promising, the studies with implanted xenograft models often suffer from limited correlation to the human situation and are not ideal for clinical translation due to the immune deficient state of tumor carrying animals and a less than optimal tumor environment (27). By contrast, genetically engineered mouse models (GEMM) with endogenous tumor development represent a better model system for the evaluation of diagnostic and therapeutic tumor studies due to their heterogeneity on a genetic and morphological level, and their more complex tumor environment that better reflect that seen in patients with cancer (27, 28). Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths in developed countries, and while surgical intervention may be effective in very limited cases, no effective long-term therapeutic strategies are currently available (28, 29). PDAC development and progression is known to involve genetic and morphological changes such as the activation of the KRAS oncogene and inactivation of TP53, a tumor suppressor also known as “guardian of the genome.” When these genetic changes occur in concert with the activation and malfunction of diverse growth factor receptors and others, the process eventually manifests as aggressive PDAC, which leads to quick fatality. The tumors are characterized by strong desmoplasia as well as a dynamic communication between tumor cells and its environment and a complex microarchitecture (32, 37). Furthermore, PDAC has an important step towards studying this therapy approach in a clinically more relevant preclinical setting. NIS was used for noninvasive 123I-scintigraphy and 124I-PET imaging to determine MSC localization as well as level and duration of transgene expression. The efficacy of a NIS gene 131I therapy approach was further evaluated in this advanced endogenous PDAC mouse model.

Materials and Methods
Mesenchymal stem cells
The MSC cell line used in this study was isolated from the bone marrow of a female p53+/− mouse with Balb/c background (in the following referred to as wild type MSCs) as described previously (38). MSCs were cultured in RPMI (Sigma-Aldrich) supplemented with 10% FBS and 100 U/mL penicillin/100 µg/mL streptomycin. Cells were maintained at 37°C and 5% CO2 in an incubator.

Wild type MSCs (WT-MSC) were stably transfected with the expression vector CMV-NIS-pcDNA3, wherein the full-length NIS cDNA is coupled to the cytomegalovirus (CMV) promoter. The transfection and isolation of clones as well as the screening for iodide uptake levels was performed as described previously (9). The resulting stably transfected cell line for the following experiments was referred to as NIS-MSCs.

123I uptake assay
Radioiodide uptake of MSCs was determined at steady-state conditions as described previously (13).

Quantitative real-time PCR
Total RNA from MSCs was extracted using the RNeasy Mini Kit with QIAshredder (Qiagen). Reverse transcription and quantitative real-time PCR (qRT-PCR) were conducted using a Masterycycler ep gradientS PCR cycler as described previously (Eppendorf; ref. 10). Relative expression levels were calculated from ΔΔC, values normalized to internal β-actin and results are expressed as fold change relative to controls.

Animals
Establishment of the Kras;p53+ (Ptf1a+/-;Kras+/LSS-G12D, Tp53+loxp/loxp) (Kras;p53) model used in the present study (30, 32, 34–36). These mice develop extremely aggressive PDAC, which leads to quick fatality. The tumors are characterized by strong desmoplasia as well as a dynamic communication between tumor cells and its environment and a complex microarchitecture (32, 37). Furthermore, PDAC has an extensive tumor stroma consisting of fibroblasts, inflammatory cells and vasculature girded by high amounts of extracellular matrix. These tumors are also able to respond to treatments by remodeling and rearranging the tumor stroma (37).

We investigated the efficacy of adoptively applied murine MSCs as gene delivery vehicles for tumor-selective NIS gene transfer in the Kras;p53 PDAC mouse model, a model that provides an important step towards studying this therapy approach in a clinically more relevant preclinical setting. NIS was used for noninvasive 123I-scintigraphy and 124I-PET imaging to determine MSC localization as well as level and duration of transgene expression. The efficacy of a NIS gene 131I therapy approach was further evaluated in this advanced endogenous PDAC mouse model.

MSC application and 123I-scintigraphy
Experiments started when mice were about 6 to 8 weeks of age and tumors were developed. To suppress thyroidal iodide uptake for the imaging study, mice were given 5 mg/mL L-T4 (Sigma-Aldrich) in their drinking water. The first experimental group received NIS-MSCs (n = 5) or WT-MSCs (n = 2) three times on every second day via the tail vein at a concentration of 5 x 10^5 cells/500 µL PBS. As an additional control, 30 minutes before radioidioide administration, a subset of mice (n = 2) was pretreated with 2 mg of the competitive NIS inhibitor sodium perchlorate (Sigma-Aldrich). Seventy-two hours after the last MSC application, mice received 18.5 MBq (0.5 mCi) 123I (GE Healthcare, Braunschweig, Germany) intraperitoneal and radioidioide accumulation was monitored using a gamma camera provided with a low-energy high resolution collimator (e.cam, Siemens, Munich, Germany).

The second group received only one MSC application via the tail vein of 5 x 10^5 cells/500 µL PBS NIS-MSCs (n = 5) or WT-MSCs (n = 2) followed 48 hours later by 18.5 MBq (0.5 mCi) 123I intraperitoneal application and monitoring of radioidioide biodistribution as described above. Also, a subset of mice (n = 2) were treated with perchlorate as well 30 minutes before radioidioide application.

Analysis and Quantification of regions of interest were done using HERMES GOLD (Hermes Medical Solutions, Stockholm, Sweden). Results are expressed as a fraction of the total amount of applied radionuclide per gram tumor tissue (after post mortem
weighing; % ID/g). Radionuclide retention time was examined by serial scanning within the tumors. Dosimetric calculations for 131I were done according to the concept of medical internal radiation dose using the dosis factor of RADARgroup (http://www.doseinfo-radar.com/).

**MSC application and 124I-PET imaging**

To achieve a better discrimination between uptake in the tumor and the adjacent stomach, a 124I-PET imaging was performed. NIS-MSCs (n = 5) or WT-MSCs (n = 2) were applied three times for every second day as described above and mice received 10 MBq 124I (PerkinElmer) intraperitoneally 72 hours later. Thirty minutes before radioidoide administration, a mouse (n = 1) was pretreated with 2 mg of the competitive NIS inhibitor sodium perchlorate. Using a micro PET system (Inveon, Siemens Preclinical Solutions, Erlangen, Germany) radioidoide biodistribution was monitored by static acquisition 3 hours post injection.

**Radionuclide therapy study**

For inclusion of mice harboring PDAC, a 7T dedicated animal MR scanner was used for monitoring. Therapy started as soon as they fulfilled the inclusion criteria (tumor volume of 100–500 mm³). To monitor tumor growth, the 7T-MR imaging was done on a weekly basis. Following a L-T4 pretreatment as described above, three groups of mice were established receiving only one systemic NIS-MSCs application followed 48 h later by a therapeutic dose of 55.5 MBq 131I intraperitoneally (NIS-MSCs + 131I, n = 10) or, as control, received NaCl (saline) instead of radioidoide (NIS-MSCs + NaCl, n = 9). The therapy cycle consisting of systemic MSC-mediated NIS gene transfer followed by radioidoide was repeated for a total of three times on days 0/2, 4/6 and 7/9. The body conditions of the mice were closely monitored for the whole time of treatment. Mice were sacrificed after reaching one or more endpoint criteria (tumor volume >1,000 mm³, body weight loss >15%, abnormalities in physical or behavioral criteria).

**Immunohistochemical staining**

Immunohistochemical NIS staining of paraffin-embedded tissue sections derived from PDAC or non-target organs (liver, lung, and spleen) after systemic NIS-MSC or WT-MSC administration was performed as described previously (42). Quantification of NIS immunohistochemical staining was performed by a highly experienced pathologist. Areas (1 mm²) of high NIS protein expression were defined as hot spots and the number of NIS-expressing MSCs within a hot spot was quantified. Immunohistochemistry for all other markers was performed using a Bond RxM system (Leica, Wetzlar, Germany, all reagents from Leica) with primary antibodies against Ki67 (ab16667, Abcam) and α-SMA (ab124964, Abcam). Briefly, slides were deparaffinized using deparaffinization solution, pretreated with Epitope retrieval solution 1 (corresponding to citrate buffer pH6) for 20 minutes. For single stainings, antibody binding was detected with a polymer refine detection kit without post primary reagent and visualized with DAB as a dark brown precipitate. For double stainings, after DAB visualization as described above, a second primary antibody was applied, and detected and visualized with a polymer refine red kit without post primary reagent. Counterstaining was, in all protocols, done with hematoxylin.

Stromal contents were determined by MOVAT pentachrome staining as described previously (37).

**Statistical analysis**

Results are expressed as mean ± SEM, mean-fold change ± SEM or, for survival plots, percent. Statistical significance was tested by two-tailed Student t test or, for tumor volumes, using one-way ANOVA followed by Tukey’s Honestly Significant Difference test. For Kaplan–Meier plots statistical significance was analyzed by log-rank test. For all tests, P values <0.05 were considered statistically significant (*, P < 0.05; **, P < 0.01; ***, P < 0.001; n/s not significant).

**Results**

Characterization of MSCs stably expressing NIS

After stable transfection of bone marrow–derived murine MSCs with a NIS-expressing plasmid (CMV-NIS-pcDNA3; NIS-MSC), NIS-MSCs showed a 38-fold increase in NIS-mediated iodide uptake activity as compared with non-transfected wild type MSCs (WT-MSCs; Fig. 1A). Addition of the NIS-specific inhibitor perchlorate significantly decreased levels of iodide uptake in NIS-MSCs to background levels. No perchlorate-sensitive iodide uptake above background levels could be observed in WT-MSCs. A time course of iodide uptake in NIS-MSCs (and WT-MSCs controls) identified half-maximal levels of radioiodide accumulation within 15 to 25 minutes, and a saturation of uptake at approximately 50 minutes in the NIS-MSCs whereas no NIS-specific iodide uptake was observed when cells were treated with perchlorate (Fig. 1B).

The expression levels of steady state NIS mRNA in the NIS-MSC and WT-MSCs were validated using qRT-PCR. NIS was significantly higher expressed at relative mRNA levels (approximately 6500-fold) in NIS-MSCs as compared to WT-MSCs (Fig. 1C).

In vivo imaging studies reveal high NIS-mediated radioiodide accumulation in PDAC

To compare the general efficacy of MSC-mediated NIS gene delivery and radioiodide uptake activity using 123I-scintigraphy in mice harboring endogenous PDAC with the results of earlier studies in xenograft mouse models, a group of mice received three applications at 2-day intervals of NIS-MSCs (5 × 10⁶ cells, intravenously via the tail vein) or WT-MSCs, followed by a single radioiodide application (18.5 MBq 123I, intraperitoneally) 72 hours later—the application regimen that we had applied in our previous studies. Although no radioiodide accumulation above background levels was detected in tumors of mice receiving WT-MSCs (Fig. 2C), significant iodide accumulation was observed in tumors of mice which had received NIS-MSCs (Fig. 2A). Physiologic iodide accumulation was observed in the thyroid and salivary glands (SG), stomach and bladder (Fig. 2A and C). As determined by serial scanning, a maximum of approximately 16.2% ± 2.9% injected 123I dose per gram (ID/g) tumor was accumulated after three cycles of NIS-MSCs application which showed a biological half-life of 7 h, and a calculated tumor absorbed dose of 136.9 mGy/MBq 131I (Fig. 21). To confirm that tumoral iodide uptake was NIS-mediated, a subset of mice treated with NIS-MSCs received perchlorate 30 minutes before 131I administration. Perchlorate treatment completely blocked tumoral iodide accumulation as well as iodide uptake in stomach and thyroid gland (Fig. 2B). To assess an optimized, less time intense treatment schedule more applicable in the rapidly growing tumor model, an additional 123I-scintigraphy experiment was performed with only one MSC application (Fig. 2G and H). PDAC harboring...
mice received only one NIS- or WT-MSC application followed by an injection of 18.5 MBq $^{123}$I 48 hours later. Radioidide distribution revealed significant radioidide accumulation in the tumors (Fig. 2G), whereas no iodide accumulation was detected in tumors of mice receiving perchlorate 30 minutes before $^{123}$I administration (Fig. 2H). As determined by serial scanning, a maximum of 16.3 ± 2.3% ID/g $^{123}$I was shown to accumulate after a single NIS-MSC application, with a biological half-life of 4 hours, and a calculated tumor absorbed dose of 100.7 mGy/MBq $^{131}$I (Fig. 2I). Although the maximum radioidide uptake obtained in this experiment was approximately the same as that seen in the first experimental setting, radioidide efflux was slightly more rapid and biological half-life was shorter; however, the overall tumor absorbed dose of $^{131}$I was only mildly reduced. $^{123}$I-PET imaging allowed a detailed 3D-analysis of tumoral iodide uptake. 3 h after injection of 10 MBq $^{123}$I in a subset of mice that had received three NIS-MSC applications every second day (Fig. 2D–F), PET-imaging confirmed a significant tumor-selective iodide accumulation, which was blocked upon perchlorate treatment (Fig. 2D and E). No iodide uptake in tumors above background levels was observed when mice were injected with WT-MSCs (Fig. 2F).

NIS protein expression in PDAC

To correlate the in vivo imaging data with NIS protein expression within the tumors and control organs (liver, spleen, lung), tissues were dissected and immunohistochemically stained. NIS-specific immunoreactivity was detected in tumors of mice that received NIS-MSCs applications demonstrating efficient MSC-mediated NIS transgene expression in PDAC after systemic application (Fig. 3A and F). MSCs genetically engineered to express NIS were localized within the tumor stroma based on detection of NIS-specific immunostaining (Fig. 3A and F, arrows). NIS-specific immunoreactivity was detected at the membrane and in the cytoplasm of MSCs in tumors of mice that received NIS-MSCs applications demonstrating efficient MSC-mediated NIS transgene expression in PDAC after systemic application (Fig. 3A and F). The cytoplasmic staining results from NIS protein that is not properly targeted to the membrane after NIS transduction of MSCs. While there was no clear visual difference detectable in NIS expression in mice receiving just a single (Fig. 3F) or three NIS-MSCs applications (Fig. 3A), a pathologist-based quantification of the amount of NIS-positive MSCs within PDAC showed following results: the analysis of hot spots (1 mm$^2$) revealed an average of 4.3 hot spots with a total of 18.3 NIS-positive MSCs within tumors of mice receiving a total of three MSC applications and an average of 10.3 hot spots with a total of 32 NIS-positive MSCs within the group receiving only MSCs once. Lung, liver and spleen showed no detectable NIS protein expression (Fig. 3B-D and G-I). Mice, which received three applications with WT-MSCs showed no NIS protein expression in tumors (Fig. 3E).

Therapeutic application of radioidine $^{131}$I

A relatively short therapy cycle after imaging-guided standardized detection of advanced local tumor growth was chosen given the aggressive nature of tumor growth in this model. On the basis of the NIS imaging results after only one NIS-MSC application (Fig. 2G-I), the therapy study was performed with three cycles of one NIS-MSC application, followed by $^{131}$I injection 48 hours later (Fig. 4). Mice were then monitored on a 7T-dedicated animal MR scanner as soon as they fulfilled the inclusion criteria. Treatment with NIS-MSCs started on the day of the inclusion scan. The MR imaging was done on a weekly basis to closely monitor PDAC growth kinetics (Fig. 4A). Tumor analysis of different groups revealed a significant delay and reduction of tumor burden of the animals in the therapy group (NIS-MSCs + $^{131}$I; Fig. 4A, C, and D) as compared with control groups (NIS-MSCs + NaCl; Fig. 4A, E and F; NaCl + NaCl; Fig. 4A, ref. 41). After an initial exponential growth in all groups, which was significantly decreased in therapy mice, a plateau occurred in the therapy group with almost complete stop of tumor growth (Fig. 4A). However, no significant difference in survival was detected (Fig. 4B).

Histological and immunohistochemical analysis

Morphologically, there were only slight differences between the pancreatic neoplasia of all groups. All tumors were moderately to
poorly differentiated and showed predominantly ductal growth patterns. No tumor cell necrosis or apoptosis as signs of tumor regression were observed after treatment. Interestingly, in animals receiving NIS-MSCs, stroma content (consisting of cancer-associated fibroblasts (CAF) and extracellular matrix (glyco-) proteins) was more pronounced.

To further analyze the changes within the tumor microenvironment due to the presence of MSCs, further immunohistochemical analysis of tumors was performed. Movat staining revealed a significant increase of collagen fibers within tumors of mice receiving NIS-MSCs + NaCl, as well as a more modest increase (not reaching statistical significance) in therapy mice (NIS-MSCs + 131I) as compared with tumors of control mice that received no MSCs (NaCl + NaCl; Fig. 5A–C and G). No change in extracellular interstitial or intracellular mucin was seen between the different groups (Fig. 5A–C and G). Quantification of proliferating cells (Ki67) within the tumor and stroma cells showed a higher number of Ki67-positive cells within the tumor stroma of mice receiving NIS-MSCs as compared with mice receiving saline only (NaCl + NaCl; Fig. 5D–F). Also, no tumoral radioiodide accumulation was observed after applications of WT-MSCs (n = 2; F). One representative image is shown each. Data are represented as mean values ± SEM.

Figure 2.
123I-scintigraphy and 124I-PET imaging revealed high pancreatic tumoral radioiodide uptake. One (A) or three (G) systemic injections of NIS-MSCs in mice harboring PDAC resulted in a maximum of approximately 16.2% to 16.3% of injected dose per gram (ID/g) tumor (n = 5; I). Tumor radioiodide uptake was NIS-specific as shown by perchlorate-sensitivity (n = 2; B, H). Treatment with WT-MSCs showed no NIS-specific tumoral radioiodide uptake (n = 2; C). Furthermore, application of three intravenous injections of NIS-MSCs on every second day confirmed high perchlorate-sensitive NIS-specific tumoral iodide uptake using 124I-PET (n = 1–5; D and E). Also, no tumoral radioiodide accumulation was observed after applications of WT-MSCs (n = 2; F). One representative image is shown each. Data are represented as mean values ± SEM.
Stainings for CD45, CD11b, F4/80, CD206 and cleaved Caspase-3 revealed no differences in intratumoral cell numbers between the groups.

**Discussion**

Because of the increasing incidence and lack of effective therapeutic options, pancreatic cancer may become the second leading cause of cancer-related deaths by 2030, illustrating the urgent need for new therapeutic strategies (43). Various combinations of chemotherapies have shown some degree of therapeutic efficacy and slightly increased overall survival, but are accompanied by high toxicity (28, 44, 45). Our increasing understanding of the central molecular targets and the pivotal role of the microenvironment and its regulation in PDAC, has suggested new directions for the development of novel therapeutic strategies. GEMMs provide a far better platform for the evaluation of these novel therapy strategies, and for the prediction of a therapy response, with a better perspective for subsequent translation to the clinic. We evaluated the effects of an MSC-mediated NIS-based radio-nuclide therapy approach in a GEMM showing a complex and prominent desmoplastic component. Our previous studies of NIS-MSCs have shown excellent tumor homing and expression of the NIS-transgene in MSCs, as well as significant therapeutic effects after $^{131}$I treatment in subcutaneous and orthotopic xenograft models of PDAC.

**Figure 3.**

High NIS protein expression in PDAC tumors. NIS-specific immunoreactivity (red) was detected in PDAC after systemic application of NIS-MSCs (black arrows; A and F). No NIS protein expression was seen in nontarget organs (B-D and G-I) or tumors of mice, which received WT-MSCs (E). One representative image is shown each using $\times 20$ magnification or also $\times 40$ magnification for tumors showing NIS-specific immunoreactivity.
human hepatocellular carcinoma and colon metastases. In the present study, we have built on these studies to further evaluate the MSC-mediated NIS gene therapy approach in a more challenging and clinically highly relevant PDAC mouse model (9–12). An important advantage of the NIS therapy gene is its additional role as a reporter gene, allowing noninvasive monitoring by $^{123}$I-scintigraphy and/or $^{124}$I-PET-imaging. $^{123}$I-scintigraphy of previous studies revealed a radioiodide uptake of 7 to 9 % ID/g tumor using the same CMV-NIS construct introduced into human MSCs, which were adoptively applied in nude mice harboring subcutaneous tumors from a human hepatocellular carcinoma cell line (HuH7; ref. 9). In comparison, the $^{123}$I-scintigraphy data shown here demonstrate with both MSC application schemes an impressive level of tumor-selective MSC recruitment and NIS transgene expression in endogenous PDAC with an almost 100% increase in radioiodide uptake activity per gram tumor. However, the iodide efflux was slightly faster in the group with only one MSC application as compared with the group receiving a total of three MSC applications. As the tumoral iodide uptake and calculated tumor absorbed dose of 100.7 mGy/MBq for $^{131}$I was expected to be high enough for a therapy effect, based on past experience, we conducted the therapy study using the single NIS-MSC application regimen. We observed a significant delay in tumor growth in the therapy group that received NIS-MSCs followed by $^{131}$I in addition to a plateau in tumor growth between the first and second week as compared with control groups, which showed continued growth over the entire observation period (41). Interestingly, a slight plateau in tumor growth between week one and two was also seen in the control group receiving NIS-MSCs and NaCl only (A). However, no significantly improved survival was observed (B).

**Figure 4.** $^{131}$I therapy study led to a delay in tumor growth. For in vivo radionuclide therapy studies, therapy mice received a single NIS-MSC application followed by 55.5 MBq $^{131}$I 48 hours later and this cycle was repeated to a total of three ($n = 10$). Therapy mice harboring PDAC resulted in a significant delay in tumor burden (A, C, and D) as compared to controls receiving NIS-MSCs and NaCl instead ($n = 9$; A, E, and F) or NaCl only (A). However, no significantly improved survival was observed (B).
aspect worth looking at is the tumor stroma. Previous studies point out the tumor-suppressive, rather than supportive, role of the tumor stroma and its compartments in PDAC (50, 51). Rhim and colleagues (51) suggest that specific components of the tumor stroma, such as myofibroblasts among others, play a tumor-suppressive role. Furthermore, fibrosis associated with myofibroblasts and type-1 collagen seems to have rather a protective role for the host than a supportive role for pancreatic cancer (50). In the present study we were able to demonstrate a change in the content of collagen fibers in tumors of mice that were injected with NIS-MSCs, as compared to tumors without MSCs (NaCl + NaCl group). These findings go along with the observation that some types of collagens are more likely to be tumor-suppressive (50). Furthermore, a major difference in the number of proliferating cells was observed in tumors containing NIS-MSCs as compared with tumors without. In contrast with those findings, former studies of our group, using subcutaneous or orthotopic liver carcinoma as well as colon cancer liver metastasis mouse

Figure 5.
Immunohistochemical analysis. In tumors of mice receiving NIS-MSCs and saline, movat staining demonstrated a significant increase of collagen fibers as compared to mice receiving saline only (A–C and G). Also, in tumors of mice receiving NIS-MSCs + 131I a more modest increase (not reaching statistical significance) of collagen fibers was observed. No difference in extracellular interstitial or intracellular mucin was observed (A–C and G). Ki67 staining detected more proliferating cells within the tumor and stroma of mice receiving NIS-MSCs as compared with mice receiving saline only (D–F and H). A double stain for Ki67 and the activated fibroblast marker α-SMA (I–K) revealed α-SMA-positive fibroblasts within the neoplasia of all mice, regardless of the treatment, but differed in the arrangement within the tumor and stroma (black arrows; I–K). In the control group, which received saline only, no Ki67-positive fibroblasts were detected (I). Single α-SMA-positive proliferating fibroblasts were visible within the tumor stroma of mice receiving NIS-MSCs (J and K). One representative image is shown each using ×5 (Movat staining), ×10 (Ki67 staining), or ×20 as well as ×40 (Ki67 and α-SMA double staining) magnification. Data are represented as mean values ± SEM (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
models, demonstrated a significant decrease of proliferating tumor cells of therapy tumors as compared with control groups (9, 10, 12). Completely unexpected in the current study, we observed a significant increase of Ki67 in tumors containing NIS-MSCs (therapy and control group) as compared with tumors without MSCs (saline only). As it seemed that in both groups receiving NIS-MSCs proliferating cells were rather cells of the stromal compartment than tumor cells, we performed a Ki67 and α-SMA–double staining to examine the content and proliferating potential of fibroblasts within these groups. Results demonstrated a higher content of activated fibroblasts within the tumor stroma of tumors containing NIS-MSCs as compared with the saline only control group. Furthermore, it was observed that fibroblasts were located immediately around tumor cell nests and infiltrative ducts, which was not seen in the absence of NIS-MSCs. These findings resemble observations in human pancreatic tumors, where CAF are also densely arranged around the tumorous structures (52). How these CAFs affect tumor development and growth is not fully understood yet. Some studies suggest that they promote tumor growth and that an increased number of α-SMA-positive myofibroblasts is associated with a poor prognosis, whereas other studies, as already discussed and which corroborate our findings, allot fibroblasts a rather protective role for the host (53). Altogether the data presented here suggest that the systemically applied NIS-MSCs are actively recruited to the growing pancreatic carcinoma stroma, where they seem to influence the proliferation of various tumor associated cells through their secretion of growth factors resulting in increased Ki67 stain and altered arrangement of α-SMA–positive cells. As NIS-MSCs should be destroyed in the context of 131I treatment, the described processes would already take place before radiiodine treatment. Further investigations of changes in CD45, CD11b, F4/80, CD206, and cleaved Caspase-3 did not reveal differences between therapy and control groups and showed rather a heterogeneous staining within the same group. However, a focus on secondary effects of MSC delivery on intratumoral immune cell regulation is a key aim in future studies albeit beyond the scope of this current work. Although our findings demonstrated changes in the composition of the tumor stroma and the tumor micromilieu, the exact mechanisms underlying the reduced tumor growth in this mouse model under our experimental conditions have to be further investigated. A more intensive investigation, taking the tissue complexity of PDAC into consideration as well as the ability of MSCs themselves to modulate the tumor microenvironment, might provide the possibility to use MSCs not only for targeted therapy but also in combination with chemotherapy or immune approaches.

Although tumor growth was significantly reduced in the present study, no prolonged survival was observed in mice receiving treatment likely due to the overall highly aggressive course of disease in this model. Mice in the control group receiving NIS-MSCs and NaCl had to be sacrificed as early as 9 days after treatment start, whereas the first mice in the therapy group had to be sacrificed at day 16. Around that time, therapy mice showed rapid health deterioration and had all to be sacrificed within a few days. Despite smaller tumors, these mice showed adverse symptoms such as ascites or icterus, which also occurred in the control group and were reasons for sacrifice.

Recently, our group had also reported the application of the NIS gene therapy approach in the same PDAC GEMM using EGFRI-targeted polyplexes for NIS transgene delivery, which resulted in tumor reduction and prolongation of survival (41). Polyplexes based on linear polyethylenimine (LPEI) are accompanied by endogenous cytotoxic effects. Although these effects were not seen in former studies using subcutaneous xenograft mouse models, toxicity to the LPEI-based polyplexes was observed in Kras; p53 mice. In contrast with those findings, MSCs did not show toxic side effects in animals of former studies as well as of the current study using the same PDAC mouse model. For this mouse model, MSCs thus might be a better choice for NIS-mediated radiiodine therapy. Our results demonstrate the potential of genetically modified MSCs in PDAC to reduce tumor growth. We are aiming at further optimization of MSC-mediated NIS gene therapy approach to enhance therapeutic efficacy. For proof-of-principle of MSC-mediated NIS gene delivery in this PDAC model, the unspecific CAR-promoter was used to control NIS expression. More specific promoters with enhanced tumor-specificity specifically designed for the respective tumor environments are currently under evaluation. Recent studies using a tumor stroma-specific RANTES/CCL5 promoter in an orthotopic liver metastases mouse model led to reduced metastases growth and improved survival of animals (11). Furthermore, as hypoxia is a common feature in tumors driving angiogenesis and resistance to conventional therapies, a synthetic hypoxia inducible factor (HIF)–responsive promoter was designed to target NIS expression into hypoxic tumor cells in an orthotopic HCC xenograft mouse model by our group, resulting in reduced tumor growth and prolonged survival (12). Using a tumor-specific promoter designed for the tumor stroma of PDAC might enable increased NIS transgene expression and improved therapeutic efficacy of radionuclide therapy. In addition, application of the alternative radionuclide 188Re instead of 131I will be considered for therapy. 188Re is also transported by NIS and offers different advantages compared with 131I: it provides the possibility to enhance tumor absorbed doses due to higher energy and shorter half-life, and is associated with an increased crossfire effect due to a longer path length (up to 10.4 mm as compared with 1.24 mm; ref. 10). A further option for improvement would be the combination with radiosensitizing agents, such as gemcitabine—a commonly used chemotherapeutic drug in pancreatic cancer, to increase radiosensitivity of tumors, which in turn might lead to enhanced therapeutic effectiveness of MSC-based NIS-mediated radiiodine therapy.

Taken together, our results show great potential of MSC-mediated NIS gene delivery in PDAC. Adoptively applied NIS-MSCs were actively recruited to PDAC in a highly efficient manner resulting in high tumor-specific radioiodide uptake as confirmed by 123I-scintigraphy and 124I-PET imaging. The translation of these results into a therapy study showed significantly delayed and reduced tumor growth. Our data also demonstrate the high potential of the application of NIS reporter gene imaging for monitoring and planning of a NIS gene therapy approach in PDAC in a clinical setting.

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

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