THEMATIC REVIEW

The sodium iodide symporter (NIS): novel applications for radionuclide imaging and treatment

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Abstract

Cloning of the sodium iodide symporter (NIS) 25 years ago has opened an exciting chapter in molecular thyroidology with the characterization of NIS as one of the most powerful theranostic genes and the development of a promising gene therapy strategy based on image-guided selective NIS gene transfer in non-thyroidal tumors followed by application of ¹³¹I or alternative radionuclides, such as ¹⁸⁸Re and ²¹¹At. Over the past two decades, significant progress has been made in the development of the NIS gene therapy concept, from local NIS gene delivery towards promising new applications in disseminated disease, in particular through the use of oncolytic viruses, non-viral polyplexes, and genetically engineered MSCs as highly effective, highly selective and flexible gene delivery vehicles. In addition to allowing the robust therapeutic application of radioiodine in non-thyroid cancer settings, these studies have also been able to take advantage of NIS as a sensitive reporter gene that allows temporal and spatial monitoring of vector biodistribution, replication, and elimination – critically important issues for preclinical development and clinical translation.

Key Words

- ▶ sodium iodide symporter
- ▶ radioiodine therapy
- ▶ gene therapy
- ▶ oncolytic virus
- ▶ mesenchymal stem cells
- ▶ polyplexes
- ▶ reporter gene
- ▶ therapy gene

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Introduction

Based on its well-characterized role in radioiodine imaging and therapy in differentiated thyroid cancer, the sodium iodide symporter (NIS) represents one of the oldest targets for molecular imaging and targeted radionuclide therapy. The cloning of NIS cDNA by N Carrasco's team in 1996 has not only been a milestone in thyroid physiology but has also provided a powerful new reporter and therapy gene allowing the development of a promising antitumor gene

therapy strategy based on image-guided selective NIS gene transfer into non-thyroidal tumors followed by therapeutic application of radioiodine that – based on its crossfire effect – offers a significant advantage in the gene therapy setting (Dai *et al.* 1996, Smanik *et al.* 1996, Spitzweg *et al.* 2001b, Spitzweg & Morris 2002, Hingorani *et al.* 2010, Ravera *et al.* 2017) (Fig. 1).

Shimura *et al.* were the first to report successful stable transfection of transformed rat thyroid cells (FRTL-Tc) lacking endogenous iodide transport activity with rat

Figure 1

Overview of the image-guided sodium iodide symporter (NIS) gene therapy concept using different gene delivery methods. Cloning of the NIS has allowed the characterization of NIS as one of the most powerful theranostic genes and the development of a promising gene therapy strategy based on image-guided selective NIS gene transfer in non-thyroidal tumors followed by application of ^{131}I or alternative radionuclides, such as ^{188}Re and ^{211}At . Significant progress has been made towards promising new applications in disseminated disease through the use of oncolytic viruses, non-viral polymers alone or in combination with adenovirus-based vectors, and genetically engineered MSCs.

NIS cDNA using electroporation resulting in restoration of radioiodine accumulation *in vitro* as well as *in vivo* in xenografts derived from the stable transfectants in Fisher 344 rats. A therapeutic effect, however, could not be achieved after administration of 1 mCi ^{131}I (Shimura *et al.* 1997).

With the cloning of NIS in 1996 and this early study, the idea of targeted exogenous NIS gene delivery into non-thyroidal tumors as a potential antitumor treatment strategy was born and has been pursued by a number of groups by exploiting the expanding repertoire of tumor-selective gene delivery techniques. In addition to allowing the robust therapeutic application of radioiodine in non-thyroid cancer settings, these studies have also been able to take advantage of NIS as a sensitive reporter gene that allows temporal and spatial monitoring of vector biodistribution, replication, and elimination – critically important issues for preclinical development and clinical translation in gene therapy (Fig. 1).

NIS gene delivery: from *ex vivo* stable transfection to the first clinical trial for NIS gene delivery in prostate cancer

In one of the earliest proof-of-principle studies for exogenous NIS expression, the prostate-specific antigen (PSA) promoter was used to selectively express NIS in the prostate cancer cells LNCaP resulting in tissue-specific androgen-dependent iodide uptake activity and selective killing by accumulated ^{131}I *in vitro* (Spitzweg *et al.* 1999, 2000). Subcutaneous xenografts derived from these stably NIS-transfected LNCaP cells in athymic nude mice were sensitive to radioiodine accumulation leading to a pronounced therapeutic response with an average tumor volume reduction of more than 90% after application of a single therapeutic ^{131}I dose of 3 mCi (Spitzweg *et al.* 2000). Glucocorticoids and all-*trans*-retinoic acid were demonstrated to stimulate PSA promoter-directed NIS expression in LNCaP cells, thereby enhancing

NIS-mediated iodide uptake and therapeutic efficacy of ^{131}I (Spitzweg *et al.* 2003, Scholz *et al.* 2004). As a next important step in the preclinical evaluation of the NIS gene radioiodine therapy concept in prostate cancer, a replication-deficient human adenovirus carrying the human NIS gene linked to the CMV promoter (Ad5-CMV-NIS) was used for local *in vivo* NIS gene delivery into LNCaP cell xenografts in nude mice. Following i.p. injection of a single therapeutic dose of 3 mCi ^{131}I 4 days after Ad5-CMV-NIS-mediated intratumoral NIS gene delivery, a significant therapeutic response with an average volume reduction of more than 80% was achieved (Spitzweg *et al.* 2001a). These early studies clearly demonstrated for the first time that NIS gene delivery into non-thyroidal and non-organifying tumor cells allows the accumulation of therapeutically effective radioiodine doses. In preparation of a first phase 1 clinical trial of replication-deficient adenovirus-mediated gene therapy in patients with locally recurrent prostate cancer who have failed external beam radiotherapy at the Mayo Clinic (Ahmed *et al.* 2012), which is currently being completed, a toxicity study was performed using adult male beagle dogs, as a clinically relevant preclinical large animal model (Dwyer *et al.* 2005). Direct intraprostatic injection of Ad5-CMV-NIS was followed by serial ^{123}I SPECT/CT fusion imaging that allowed dosimetric measurements that demonstrated an absorbed dose of 23 ± 42 cGy/mCi ^{131}I to the prostate, suggesting that a 85 mCi dose of ^{131}I would be sufficient to obtain a target dose of 2000 cGy to the prostate. After application of a therapeutic dose of 116 mCi/m 2 ^{131}I to the dogs (equivalent to 200 mCi in adult humans), no major toxicity to other organs was observed (Dwyer *et al.* 2005). This first NIS gene delivery study in a large animal model provided further evidence of the safety and efficacy of NIS as a therapy and reporter gene.

These logistically demanding studies performed in the early years of NIS gene delivery in the J C Morris' laboratory at the Mayo Clinic (Rochester, MN) were only possible due to the available extensive network of experts in endocrinology, urology, nuclear medicine, molecular medicine and radiation safety. These pioneering studies convincingly demonstrated to the oncology, nuclear medicine and molecular medicine communities the enormous potential of NIS as a novel therapy/imaging gene.

As a further development towards clinical translation, the same group explored the use of oncolytic viruses carrying the NIS gene in the prostate cancer models. Oncolytic replication-competent viruses impart several advantages leading to enhanced therapeutic efficacy of

NIS-based gene therapy. These viral systems preferentially target and propagate in malignant tissues, thereby inducing an oncolytic therapy effect. Intratumoral replication and intratumoral spread of replicating virus can help amplify any antitumor effect. These viruses may also have a radiosensitizing effect, and their use could offer the possibility of multimodal therapy based on combination of virus-mediated oncolysis with NIS-mediated radioiodine therapy (radiovirotherapy). Importantly, the dual function of NIS as a therapy and reporter gene provides a well-established and convenient method to measure the kinetics of temporal and spatial viral gene expression as a critical basis for monitoring and optimizing dosing and administration schedules, thereby tailoring treatment protocols as a prerequisite of safe and effective clinical translation. A conditionally replicating adenovirus in which the E1A gene was driven by the prostate-specific probasin promoter (Kakinuma *et al.* 2003) (Ad5PB_RSV-NIS) was evaluated showing promising efficacy in the LNCaP xenograft mouse model after intratumoral virus application (Trujillo *et al.* 2010). In further *in vivo* NIS studies using an s.c. LNCaP xenograft mouse model, a linear correlation between adenovirus density and NIS expression was demonstrated and further showed that the outcome of radiovirotherapy was greatly dependent on viral spread within the tumor (Trujillo *et al.* 2013). Modifications of adenovirus subtype 5 vectors carrying NIS under control of the probasin promoter were made to improve transduction efficiency, tumor selectivity and the rate of viral spread (Oneal *et al.* 2012, 2013).

Disappointing results were reported from a phase 1 clinical trial of replication-competent adenovirus-mediated NIS gene transfer in patients with localized prostate cancer at Henry Ford Health System in Detroit, Michigan, reaching NIS expression in approximately 45% of the prostate volume. Limited virus spread within the tumor prohibited sufficiently high levels of NIS expression achieving an average tumor-absorbed dose of only 7.2 ± 4.8 Gy to the prostate after administration of 200 mCi ^{131}I (Barton *et al.* 2011).

Other oncolytic viruses evaluated as vectors for therapeutic NIS gene therapy in prostate cancer include an oncolytic herpes simplex virus type 1 encoding human NIS (HSV-NIS) and an oncolytic vaccinia virus (VV-NIS). HSV-NIS showed promising efficacy in the s.c. LNCaP mouse model, where systemic administration of HSV-NIS was found to prolong survival of tumor-bearing mice. Therapeutic efficacy was significantly enhanced by ^{131}I administration after the intratumoral spread of the virus

had peaked that was monitored by non-invasive $^{99m}\text{TcO}_4$ SPECT/CT imaging based on the NIS reporter gene function (Li *et al.* 2013).

VV-NIS was administered intratumorally in an s.c. PC3 xenograft nude mouse model and systemically in the immunocompetent TRAMP prostate cancer mouse model. An enhanced therapeutic effect of the combined treatment of radioiodine and VV-mediated oncolysis (radiovirotherapy) was found to be superior to that seen for each individual therapy (Mansfield *et al.* 2016).

The attenuated Edmonston measles virus (MV) vaccine strain (MV-Edm) was subsequently explored as a NIS gene delivery agent for imaging and therapy purposes in various tumor models (see below), including prostate cancer. MV-Edm enters cells via the complement regulatory protein CD46 thus preferentially infecting cells showing overexpression of the surface protein, in particular tumor cells, which serves as a tumor-selective mechanism of MV-Edm (Msaouel *et al.* 2009a,b,c). A recombinant MV-Edm strain expressing NIS (MV-NIS) was evaluated for prostate cancer-selective radiovirotherapy in the s.c. LNCaP mouse model. After significant *in vitro* and *in vivo* oncolytic activity of MV-NIS was demonstrated against prostate cancer, the *in vivo* oncolytic effect of MV-NIS was further enhanced by combination with ^{131}I following intratumoral as well as i.v. virus application (Msaouel *et al.* 2009b).

Oncolytic measles virus (MV) encoding NIS for radiovirotherapy of various tumor types

Replication-competent oncolytic viruses (Fig. 1) are promising tools for cancer gene therapy approaches due to their ability to specifically target and replicate in cancer cells, thereby inducing an oncolytic therapy effect. They can elicit a tumor-selective oncolytic antitumor effect, and effectively amplify the expression of vector-associated genes, which can not only be used for monitoring of viral replication and biodistribution but also for enhancing the therapy effect through combination therapy. One of the most extensively studied oncolytic viruses in the context of NIS gene transfer is MV. Evidence for anticancer activity of MV goes back to 1949 when regression of Hodgkin's lymphoma was reported in a patient following a measles infection (Msaouel *et al.* 2018). As outlined above, MV virotherapy is based on tumor-selective oncolytic properties of an attenuated MV vaccine strain derived from the Edmonston-B (MV-Edm) vaccine lineage characterized by an exceptional safety profile based on millions of

vaccine doses safely administered over the past 50 years with minimal toxicity. Tumor selectivity is provided by the CD46 receptor cell entry mechanism (Msaouel *et al.* 2018). In addition, MV-Edm infection is associated with a significant bystander effect by generating syncytia by cell-cell fusion, which can destroy adjacent cells (Msaouel *et al.* 2009a).

These characteristics have prompted the development of MV-Edm-based oncolytic therapy approaches using NIS as a reporter and/or therapy gene for various cancers (MV-NIS) – work primarily performed by the team of the Molecular Medicine Program at the Mayo Clinic. The general approach has been widely evaluated for the treatment of multiple myeloma starting with D Dingli's preclinical study using multiple myeloma xenograft mouse models. Intratumoral spread of MV-NIS was non-invasively monitored by serial ^{123}I gamma camera imaging that demonstrated iodide uptake both in MV-sensitive KAS-6/1 myeloma xenografts that regressed completely after a single i.v. dose of MV-NIS, as well as in MM1 myeloma xenografts, which were unresponsive to MV-NIS oncolytic therapy. However, the MV-resistant MM1 tumors also completely regressed when NIS was additionally used as a therapy gene, and ^{131}I was administered 9 days after a single i.v. injection of MV-NIS (radiovirotherapy) (Dingli *et al.* 2004). Successful preclinical pharmacology and toxicology studies (Myers *et al.* 2007) led to the first phase 1 trial of i.v. oncolytic treatment with MV-NIS with or without concomitant cyclophosphamide administration in patients with recurrent or refractory multiple myeloma using NIS as a reporter gene to monitor virus activity. A preliminary report presented encouraging data on two heavily pretreated patients with refractory multiple myeloma treated with a single i.v. injection of MV-NIS. Both patients responded with the resolution of bone marrow plasmacytosis. One patient experienced durable complete remission. SPECT/CT imaging showed radiotracer uptake in MV-NIS-infected plasmacytomas indicating viral replication. NIS-mediated imaging allowed the assessment of the extent and duration of MV-NIS infection and also demonstrated its specificity to tumor mass (Russell *et al.* 2014). In the phase 1 trial, ^{123}I SPECT/CT scans were positive in 8 out of 32 patients, uptake was not seen in all lesions and was modest (Dispenzieri *et al.* 2017). A phase 2 study that was conducted at the University of Arkansas has just been completed but data are not yet published (NCT02192775).

Additional promising preclinical data have been obtained in head and neck cancer s.c. xenografts, s.c. anaplastic thyroid cancer xenografts, s.c. and orthotopic

glioma xenografts and s.c. pancreatic cancer xenografts after intratumoral MV-NIS injection that allowed monitoring of virus replication, intratumoral spread and levels of achieved NIS-mediated radioiodine accumulation. In each of these studies, an additional application of a therapeutic dose of ^{131}I resulted in an increase of antitumor activity (Penheiter *et al.* 2010, Li *et al.* 2012, Opyrchal *et al.* 2012, Reddi *et al.* 2012).

MV-NIS has also been explored in ovarian cancer where it has shown significant antitumor activity in s.c and orthotopic SKOV3 ovarian cancer xenograft mouse models after intratumoral or i.p. MV-NIS application, respectively. Viral NIS gene expression was localized by $^{99\text{m}}\text{Tc}$ gamma camera imaging and demonstrated a robust uptake in both s.c. and peritoneal tumors (Hasegawa *et al.* 2006). A phase 1/2 trial was subsequently launched at the Mayo Clinic in women with treatment-resistant ovarian cancer (Galanis *et al.* 2015). The study evaluated the general safety of MV-NIS, the utility of NIS-based imaging as an effective monitoring strategy, and the applicability of future radiovirotherapy modalities aimed at increasing treatment efficacy and the potential ability to enhance an antitumor immune response. ^{123}I SPECT/CT imaging revealed NIS-mediated radiotracer uptake in 3 out of 13 patients treated at the highest virus dose and was associated with long progression-free survival (Msaouel *et al.* 2018). A second phase 2 trial of MV-NIS therapy in patients with platinum-resistant ovarian cancer is currently ongoing (NCT02364713).

Additional clinical trials of MV-NIS in malignant pleural mesothelioma, malignant peripheral nerve sheath tumors, advanced recurrent head and neck and breast cancer have been initiated at the Mayo Clinic following a series of promising preclinical studies (NCT01503177 (completed), NCT02700230, NCT01846091).

Other oncolytic viruses engineered to express NIS

As outlined above, replication-competent adenovirus-based vectors have been explored using NIS as a reporter and therapy gene in various tumor settings, in particular prostate cancer. Recently, it was shown that Ad death protein (ADP) expression negatively affected NIS membrane localization and inhibited radiotracer uptake. ADP deletion significantly improved NIS-based imaging in pancreatic cancer models *in vivo* and resulted in an enhanced therapeutic effect in combination with ^{131}I . This study perfectly demonstrates that improved oncolysis may

hinder the therapeutic efficacy of NIS radiovirotherapy and highlights the importance of imaging-based optimal timing of ^{131}I administration (Robertson *et al.* 2021).

Oncolytic vaccinia virus (VV) is a recognized oncolytic virus originally evaluated in prostate cancer, as outlined above (Mansfield *et al.* 2016), but has now been expanded to a large series of other cancers in combination with NIS reporter/therapy gene approaches. VV carrying human NIS has been evaluated for possible treatment of endometrial cancer (Liu *et al.* 2014), pancreatic cancer (Haddad *et al.* 2011, Haddad *et al.* 2012a), malignant pleural mesothelioma (Belin *et al.* 2013), gastric cancer (Jun *et al.* 2014), breast cancer (Gholami *et al.* 2014), peritoneal colorectal cancer metastases (Eveno *et al.* 2015) as well as anaplastic thyroid carcinoma (Gholami *et al.* 2011) in preclinical studies *in vitro* as well as *in vivo* after intratumoral and i.v. virus injection. Much of this work has been performed by Y Fong's group at the Memorial Sloan-Kettering Cancer Center in New York. The GLV-1h153 vector was modified from the parental VV GLV-1h68 to carry human NIS, which did not alter the replication or oncolytic capability of the virus. NIS was used as a reporter gene to monitor levels of viral replication and biodistribution as well as timing dynamics between viral infection, uptake of radioiodine and oncolysis. In pancreatic cancer xenografts in nude mice, i.v. injection of GLV-1hNIS followed by 37 MBq or 92.5 MBq ^{131}I resulted in retardation and even regression of tumor volume, while virus alone had no antitumor effect (Haddad *et al.* 2012b). NIS was used as a therapeutic gene in triple-negative breast cancer xenografts in nude mice, where intratumoral injection of GLV-1h153 followed by application of ^{131}I a week later resulted in a six-fold increase in tumor regression as compared to virus alone (Gholami *et al.* 2014). In a very interesting study, VV GLV-1h153 was used as an imaging tool to detect and potentially effectively control positive surgical margins of breast cancer in a murine model (Gholami *et al.* 2013). Administration of GLV-1h153 into the surgical wound after surgical resection of mammary fat pad breast cancer xenografts in nude mice allowed positive surgical margins to be identified via ^{124}I PET scanning and offer GLV-1h153 as a promising therapeutic agent to achieve local control of positive surgical margins by oncolytic virotherapy plus/minus ^{131}I (Gholami *et al.* 2013).

A novel chimeric poxvirus as another promising oncolytic virus was developed encoding human NIS at a redundant tk locus. Early expression of hNIS by infected cells made viral replication reliably imageable via ^{124}I PET, the intensity of ^{124}I uptake mirrored viral replication and tumor regression. Finally, an additional application of ^{131}I

enhanced and sustained tumor regression in an HCT116 colon cancer xenograft mouse model (Warner *et al.* 2019).

Vesicular stomatitis virus (VSV) engineered to express NIS is a replication-competent tumor-selective oncolytic virus that has shown promising activity against hematologic malignancies. This work performed by the Mayo Molecular Medicine group is designed to facilitate non-invasive imaging of virus spread and to enhance therapeutic efficacy with concurrent ^{131}I therapy (Goel *et al.* 2007, Shen *et al.* 2016, Zhang *et al.* 2016, Velazquez-Salinas *et al.* 2017). Immunocompetent mice with syngeneic 5TGM1 myeloma tumors (s.c. and orthotopic) showed significant enhancement of tumor regression and survival when the VSV (intratumoral and i.v.) was combined with ^{131}I . Intratumoral spread of the VSV was non-invasively and serially imaged by planar radioiodine scintigraphy that allowed dosimetric calculations that pointed to the feasibility of combination radiovirotherapy (Goel *et al.* 2007). Based on these studies, VSV engineered to express interferon β and NIS have been further developed for the treatment of acute myeloid leukemia. This work has also explored the potential of potentiating the local host immune response after viral infection by combination with immune checkpoint blockade, thereby potentially increasing antileukemia activity (Shen *et al.* 2016). Clinical translation of VSV-IFN β -NIS is underway in patients with relapsed hematological malignancies, endometrial cancer, refractory non-small cell lung cancer and head and neck squamous cell carcinoma (NCT03647163, NCT03017820, NCT03120624).

Systemic NIS gene transfer using non-viral gene delivery vectors

A crucial step towards the clinical application of NIS gene therapy is the evaluation of gene transfer methods that have the potential to achieve sufficient tumor-selective transgene expression levels not only after local but also after systemic vector application. Of central importance is the ability to reach tumor metastases. In a subset of the studies outlined above using oncolytic virus-mediated NIS gene delivery, i.v. virus application has shown promise for systemic NIS gene delivery in various tumors. A series of non-viral approaches for systemic NIS gene delivery, primarily developed in the group of C Spitzweg in collaboration with P Nelson and E Wagner at the Ludwig-Maximilians-Universität in Munich, are summarized in the following section.

Polyplexes as tumor-selective non-viral gene delivery vehicles

As an alternative to virus-based methods, synthetic vectors have emerged as promising delivery vehicles for systemic gene delivery based on their superior immunogenic profile, good nucleic acid binding capacity, and ease of synthesis and upscaling, including flexibility in chemical properties to increase efficacy and biocompatibility. In collaborative efforts of the Munich researchers at LMU, several generations of synthetic gene delivery systems based on polycationic polymers that condensate DNA forming a positively charged complex have been evaluated for systemic NIS gene delivery (Peng & Wagner 2019, Freitag & Wagner 2021) (Figs 1 and 2).

A novel class of biodegradable branched polycations based on low molecular weight oligoethylenimine (OEI) grafted onto a polypropylenimine core via degradable di-ester bonds (G2-HD-OEI and G3-HD-OEI) was generated and characterized (Russ *et al.* 2008a,b). These agents form stable polyplexes with plasmid DNA and show a high level of intrinsic tumor affinity due to their unique, soft positive surface charge ('chemical tumor targeting') (Luo *et al.* 2021) supported by a leaky tumor vasculature combined with inadequate lymphatic drainage. Due to their biodegradability, no toxic side effects were observed in non-target organs. The tumor selectivity and transduction efficiency of these polyplexes following systemic administration was evaluated using NIS-based imaging of vector biodistribution and level and duration of transgene expression. In an early study, G2-HD-OEI was used for systemic NIS gene delivery in syngeneic neuroblastoma (Neuro2A) mouse model. Tumor-selective NIS gene expression was demonstrated using ^{123}I scintigraphy and *ex vivo* gamma counting (accumulation of 8–13% ID/g ^{123}I , tumor-absorbed dose 247 mGy/MBq) after i.v. polyplex application. The *in vivo* ^{123}I scintigraphic imaging studies were confirmed by *ex vivo* biodistribution analysis also demonstrating tumor-selective radioiodine accumulation. No iodide uptake was detected in non-target organs, including lung, liver, spleen or kidneys. NIS-specific expression in the tumor was mainly membrane-associated and occurred in clusters. Taking advantage of NIS as a therapy gene, two cycles of systemic NIS gene transfer followed by ^{131}I application resulted in a significant delay in tumor growth and improved survival (Klutznick *et al.* 2009). The efficacy of G2-HD-OEI polymers for systemic NIS gene delivery was confirmed in hepatocellular cancer cell (HuH7) xenografts (Klutznick *et al.* 2011b). These initial studies demonstrated the potential of polymer-based

Figure 2

Tumor targeting of non-viral pDNA/polymer complexes (polyplexes). Cationic polymers serve for compaction of the negatively charged plasmid DNA (pDNA) into small nanoparticles. The soft cationic charges of biodegradable G2-HD-OEI also trigger specific tumor attachment (chemical tumor targeting). Alternatively, the surface charge of sequence defined polymer or LPEI polyplexes is shielded by PEG molecules, and cell receptor targeting is mediated by terminal EGFR or c-Met binding peptides.

synthetic vectors for tumor-selective NIS gene delivery after systemic application.

Building on these studies, conjugates were subsequently designed in a modular way allowing the incorporation of polyethylene glycol (PEG) to improve the peripheral circulation of the vectors, protect them from immune recognition and improve accumulation in tumor tissue. This generation of polyplexes also included the incorporation of cell targeting and internalizing ligands, thereby considerably improving their efficiency and specificity. Synthetic peptides were attached to linear polyethylenimine (LPEI), the 'gold standard' for non-viral transfection, via a PEG molecule allowing ligand-mediated tumor targeting into receptor overexpressing tumor cells. For example, the EGF receptor (EGFR) is upregulated in > 60% of solid tumors and represents a validated surface marker for targeted delivery (Schäfer *et al.* 2011). To avoid potential EGFR activation, the EGFR-binding peptide GE11 was utilized that does not lead to activation of the receptor tyrosine kinase. GE11 was coupled via a PEG

linker molecule to LPEI (LPEI-PEG-GE11) (Schäfer *et al.* 2011). LPEI-PEG-GE11-based polyplexes were then used for systemic NIS gene delivery in an s.c. EGFR overexpressing liver cancer (Huh7) xenograft mouse model. After i.v. application, LPEI-PEG-GE11/NIS effective tumor targeting was demonstrated (tumor-selective ^{123}I accumulation 6.5–9% ID/g, tumor-absorbed dose 47 mGy/MBq) without iodide uptake in non-target tissues. After pretreatment with cetuximab, an EGFR-specific antibody, tumoral iodide uptake was markedly reduced confirming the specificity of EGFR-targeted polyplexes. Three to four cycles of polyplex/ ^{131}I application significantly delayed tumor growth and prolonged survival (Klut *et al.* 2011a). This EGFR-targeted approach was then evaluated in an anaplastic thyroid cancer (ATC) model characterized by high levels of EGFR expression. High transduction efficiency and EGFR-specificity were demonstrated *in vitro* and *in vivo* and correlated with EGFR expression levels in ATC cell lines. The highest NIS expression levels were achieved in the SW1736 ATC cell line that also showed the

highest EGFR expression levels. The therapeutic efficacy of EGFR-targeted systemic NIS gene delivery was confirmed in an s.c. SW1736 xenograft mouse model showing significant tumor growth inhibition and prolonged survival (Schmohl *et al.* 2017a). Tumor specificity and transfection efficiency of EGFR-targeted LPEI-PEG-GE11 were next investigated in an advanced genetically engineered mouse model of pancreatic ductal adenocarcinoma (*Ptf1a⁺/Cre*; *Kras⁺/LSL-G12D*; *Tp53^{lox/lox}* [Kras;p53]). ¹²³I gamma camera and ¹²⁴I PET imaging were used to demonstrate significant tumor-specific radioiodide accumulation after i.v. injection of LPEI-PEG-GE11/NIS (14.2 ± 1.4% ID/g, tumor-absorbed dose 96.5 mGy/MBq/g). Reduced tumor growth was seen after administration of ¹³¹I in this aggressive tumor model (Schmohl *et al.* 2017b). EGFR-targeted LPEI-PEG-GE11 polymers were then evaluated in a disseminated colon cancer liver metastasis model. ¹⁸F-TFB-PET imaging again showed high tumoral levels of NIS-mediated radionuclide uptake (4.8 ± 0.6% ID/g) in contrast to mice that received untargeted control vectors (LPEI-PEG-Cys/NIS). Three cycles of polymer/¹³¹I application resulted in a marked delay in tumor growth and improved survival (Urnauer *et al.* 2017).

Improvement of the polyplex/NIS gene therapy concept has focused on the generation of more precise sequence-defined polymer shuttle systems leading to enhanced efficiency, specificity and reduced cytotoxicity. In this context, small, well-defined biocompatible polymer backbones have been generated with precisely integrated functional groups, including cationic (oligoethanamine) amide cores for nucleic acid binding, PEG linkers for surface shielding, targeting ligands for cell binding and protonatable amino acids with buffering function for a higher rate of endosomal escape, resulting in optimized DNA carrier-improved systems for systemic administration (He & Wagner 2015). For active ligand-mediated tumor targeting of hepatocellular carcinoma, a c-MET-binding peptide (cMBP2) was coupled to a small cationic oligo(ethanamine)amide Stp core for nucleic acid binding via a polyethylene glycol (PEG₂₄) linker for surface shielding (cMBP2-PEG-Stp). When complexed with NIS DNA, these polyplexes demonstrated high transduction efficiency and c-MET-specificity *in vitro* and *in vivo* in an s.c. Huh7 xenograft mouse model. A significant delay in tumor growth with prolonged survival was seen after polyplex/¹³¹I application (Urnauer *et al.* 2016). To help address tumor heterogeneity, a dual-targeting approach was then investigated integrating both the EGFR and c-MET binding peptides. The results demonstrated high levels of tumor-selective transduction efficacy in the HuH7

xenograft mouse model as monitored by ¹²⁴I PET NIS imaging (Urnauer *et al.* 2019).

Polymer coating of adenoviral vectors for improved systemic delivery

Recombinant adenoviruses still face hurdles that may limit their application *in vivo*. These issues include induction of immune and inflammatory responses, their elimination by neutralizing antibodies, high promiscuity due to widespread expression of the coxsackie and adenovirus receptor (CAR), and significant pooling in the liver after systemic application. Genetic engineering approaches to modify their tropism and reduce immunogenic properties have been an extensive field of research in adenoviral vector studies. Surface modification of adenoviral vectors using synthetic polymers promises to aid significantly in overcoming most of the hurdles as listed above (Fig. 1). The advantages of polymer coating of adenoviruses include greater flexibility, and as it is performed after production and purification of the vectors, it still allows the use of conventional producer cells for vector production to obtain high virus titers. Polymer coating of adenoviral vectors represents a very effective technology to generate adenoviral vectors with reduced immunogenic and inflammatory potential associated with an improved tumor-specific tropism and safety profile (Kreppel & Kochanek 2008).

Chemically defined dendritic PAMAM (poly(amidoamine)) dendrimers (repetitively branched molecules, which constitute a core and a tree-like spherical 3D morphology) bearing positively charged terminal amines that coat the negatively charged adenoviral capsid by virtue of electrostatic interaction (Vetter *et al.* 2013) were evaluated. This resulted in efficient internalization and transduction of tumor cells otherwise refractory toward adenoviral transduction. To increase specificity for tumor cells, receptor targeting peptides were chemically coupled to PAMAM via a PEG spacer in a similar way as described for LPEI-PEG-GE11. Using these novel PAMAM-PEG-peptide conjugates, enhanced transduction levels of EGFR overexpressing cancer cell lines were achieved via the EGFR targeting ligand GE11.

The viral coating technology was then evaluated in a liver cancer-specific replication-selective oncolytic adenovirus, in which E1A was driven by the AFP promoter and NIS was inserted in the E3 region driven by the replication-dependent E3 promoter (Ad5-E1/AFP-E3/NIS). High tumor selectivity of virus replication and transduction efficacy of uncoated Ad5-E1/AFP-E3/NIS was confirmed after intratumoral injection. This also resulted in a significant

reduction in tumor growth and improved survival due to strong oncolytic activity in Huh7 xenografts (virotherapy) that was further increased by application of ^{131}I (55.5 MBq) radiovirotherapy) (Grünwald *et al.* 2013a). PAMAM coating of replication-deficient virus (Ad5-CMV-NIS) led to protection from neutralizing antibodies and resulting in significantly reduced levels of ‘non-specific’ hepatic transgene expression after i.v. injection with a 70% reduction of hepatic iodide accumulation. Evasion from liver pooling resulted in reduced liver toxicity and increased transduction efficiency of the coated Ad5-CMV-NIS in Huh7 xenografts. After PAMAM coating of the replication-selective Ad5-E1/AFP-E3/NIS, serial ^{123}I gamma camera imaging showed significantly higher levels of tumor-specific iodide accumulation, which resulted in a significantly enhanced oncolytic effect following i.v. application (virotherapy), that was further increased following a therapeutic dose of 55.5 MBq ^{131}I (radiovirotherapy). These data demonstrate that polymer coating is effective in increasing the load of viable virus reaching peripheral tumors by decreasing liver pooling (Grünwald *et al.* 2013c). Coating with PAMAM-PEG-GE11 for additional EGFR targeting was found to reduce liver pooling and a significantly increased oncolytic effect of Ad5-E1/AFP-E3/NIS, which was enhanced by ^{131}I application (Grünwald *et al.* 2013b).

Mesenchymal stem cells and their role as NIS gene delivery vehicles

Mesenchymal stem cells (MSC) are pluripotent progenitor cells with high proliferative and self-renewal capacity

that play a key role in the maintenance and regeneration of diverse tissues and are linked to tumor biology (Dwyer & Kerin 2010, Cheng *et al.* 2019). Tumors are complex tissues composed of malignant tumor cells within the ‘benign’ tumor stromal compartment comprised of distinct cell types, including endothelial cells, infiltrating inflammatory cells, extracellular matrix, smooth muscle cells, pericytes and carcinoma-associated fibroblasts. The tumor stroma plays a key role in tumor growth, tumor angiogenesis and in the metastatic potential of a tumor and has, therefore, become an important target for tumor therapy (Dwyer & Kerin 2010, Hagenhoff *et al.* 2016, Cheng *et al.* 2019). The process of tumor stroma formation has been compared to that seen in ‘wounds that do not heal’, driving tissue remodeling with recruitment and high proliferation of mesenchymal cells (Dvorak 1986). It has been convincingly demonstrated that MSCs are actively recruited to growing tumor stroma where they differentiate into diverse tumor stroma-associated cell types, including cells that comprise the tumor vasculature and stromal fibroblast-like cells (Hagenhoff *et al.* 2016) (Fig. 3). The mechanisms underlying MSC recruitment to tumors have not been fully elucidated but are thought to mirror those used by leukocytes during their recruitment to inflamed tissues, utilizing a cascade of events driven by cytokines, chemokines, integrins and selectins. A group of factors, including PDGFB, VEGF, FGF2, chemokines/cytokines, such as CXCL8 and CXCL12/SDF-1 and the pleiotropic growth factor TGF- β 1/3 among others, have been linked to MSC migration (Spaeth *et al.* 2008). The general tropism of MSCs for tumors forms the basis for a ‘Trojan horse’ therapy

Figure 3

Mesenchymal stem cells as tumor-selective NIS gene delivery vehicles. MSCs are actively recruited to growing tumor stroma where they differentiate into diverse tumor stroma-associated cell types, including cells that comprise the tumor vasculature and stromal fibroblast-like cells. The mechanisms underlying MSC recruitment to tumors have not been fully elucidated but are thought to involve a cascade of events driven by cytokines, chemokines, integrins and selectins, induced by the proinflammatory and proangiogenic milieu in the tumor stroma. The general tropism of MSCs for tumors forms the basis for a ‘Trojan horse’ therapy approach, where MSCs are genetically engineered to be used as shuttle vectors for the delivery of therapeutic genes deep into critical microenvironments of growing tumors. Gene promoters that become activated in response to the signaling pathways associated with MSC activation/differentiation within tumor microenvironments are used to enhance tumor specificity of MSC-mediated transgene expression.

approach, where MSCs are genetically engineered to be used as shuttle vectors for the delivery of therapeutic genes deep into critical microenvironments of growing tumors (Conrad *et al.* 2007, Dwyer & Kerin 2010, Hagenhoff *et al.* 2016, Cheng *et al.* 2019). Potential off-target tissue damage represents a potential side effect of MSC-directed tumor gene therapy, as adoptively applied MSCs may also home to normal tissues as part of normal homeostasis. This issue has been addressed by using gene promoters that become activated in response to the signaling pathways associated with MSC activation/differentiation within tumor microenvironments, thereby enhancing tumor specificity of MSC-mediated transgene expression. 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. For example, tumor angiogenesis signals were used to selectively activate MSC-transgenes. MSCs engineered to express either reporter genes or a suicide gene (herpes simplex virus thymidine kinase (HSV-TK) gene) under control of the Tie2 promoter/enhancer show strong transgene expression within the tumor milieu. Expression of the Tie2 gene is largely restricted to angiogenic ‘hot spots’ in tumors. MSCs recruited to the vasculature of spontaneous breast tumors or orthotopic pancreatic tumors induced transgene expression as the MSCs responded to angiogenic signals (Zischek *et al.* 2009, Conrad *et al.* 2011). A proportion of MSCs recruited to tumors also act as progenitor cells for carcinoma-associated fibroblasts (CAFs). The cytokine RANTES/CCL5 is induced by MSCs in the process of tumor homing and differentiation into CAFs. MSCs were engineered to express either TK or red fluorescent protein (RFP) under control of the RANTES/CCL5 promoter (Nelson *et al.* 1993). The homing and activation of RANTES/CCL5 promoter-engineered MSCs was verified by the expression of the RFP reporter gene *ex vivo*. In parallel studies using CCL5-TK engineered MSCs, treatment with GCV led not only to a significant reduction in the growth of primary pancreatic tumors but also dramatically reduced the incidence of metastases (Zischek *et al.* 2009).

The application of NIS in this context represents a next-generation approach for MSC-based therapy vehicles (Fig. 1). MSCs genetically engineered to express NIS as a therapy gene can deliver therapeutically effective levels of radionuclides deep into tumor environments. At the same time, when used as a reporter gene, NIS allows efficient evaluation of the *in vivo* biodistribution of MSCs. In initial experiments, human bone marrow-derived CD34⁺ MSCs were stably transfected with NIS cDNA under the control of the CMV promoter (CMV-NIS-MSC). After

i.v. injection of CMV-NIS-MSCs in the Huh7 xenograft mouse model, MSC body distribution was investigated by ¹²³I scintigraphy and ¹²⁴I PET imaging showing active MSC recruitment into the tumor stroma. NIS-specific immunoreactivity was detected throughout the tumor stroma with the most prominent staining seen in areas neighboring blood vessels, while lungs, liver, and kidneys showed no detectable immunoreactivity. Three cycles of systemic MSC-mediated NIS gene delivery, each followed by ¹³¹I application resulted in a significant delay in tumor growth (Knoop *et al.* 2011). Dwyer *et al.* explored a similar approach using human bone-marrow-derived MSCs infected with NIS under control of the CMV promoter using Ad5-CMV-NIS (see above; Spitzweg *et al.* 2001a) that were injected intratumorally and intravenously in mice carrying s.c. breast cancer (MDA-MB-231) xenograft flank tumors. Effective tumoral MSC homing and engraftment were visualized by ^{99m}Tc-pertechnetate SPECT imaging followed by effective ¹³¹I therapy with a significant reduction in tumor growth (Dwyer *et al.* 2011).

In further studies, the CCL5 promoter, as outlined above, was used to target NIS expression to the tumor stroma and reduce potential side effects linked to MSC recruitment to non-tumor tissues (RANTES-NIS-MSC). After systemic RANTES-NIS-MSC injection in the Huh7 xenograft mouse model, ¹²³I scintigraphy revealed active MSC recruitment into the tumor stroma and CCL5 promoter activation as shown by tumor-selective iodide accumulation (6.5% ID/g, tumor-absorbed dose 44.3 mGy/MBq). In comparison, 7% ID/g ¹⁸⁸Re was accumulated in tumors (tumor-absorbed dose 128.7 mGy/MBq), whereas non-target organs showed no functional NIS expression. Administration of a therapeutic dose of ¹³¹I or ¹⁸⁸Re (55.5 MBq) in RANTES-NIS-MSC-injected mice resulted in a significant delay in tumor growth and improved survival. Immunofluorescence analysis using Ki67- and CD31-specific antibodies showed decreased proliferation (Ki67) and reduced blood vessel density (CD31) in tumors of mice treated with RANTES-NIS-MSC followed by ¹⁸⁸Re or ¹³¹I treatment as compared to control groups (Knoop *et al.* 2013).

The essential role of TGF- β in tumor biology, in particular in tumor micromilieu-associated signaling, makes the TGF- β /SMAD signaling pathway an attractive option to potentially control NIS expression within the tumor milieu. A synthetic TGF- β 1/SMAD-responsive promoter was evaluated as a means to control and focus NIS transgene expression in engineered MSCs within tumors (SMAD-NIS-MSC). *In vivo* studies using the HuH7 liver cancer model demonstrated a robust tumor-selective

radioiodine accumulation ($6.8\% \pm 0.8\%$ ID/g) as visualized by ^{123}I scintigraphy after three rounds of systemic SMAD-NIS-MSC application, thus demonstrating effective MSC recruitment to the tumor and pronounced TGF- β -induced NIS expression. Although the tumor-absorbed dose was lower than that observed in previous studies, the ^{131}I therapy study revealed a strong therapeutic effect when compared to previous studies leading to a significant delay in tumor growth and prolonged survival (Schug *et al.* 2019c).

After proof-of-principle of MSC-based NIS gene delivery in s.c. xenograft mouse models (see above), the next step was to address the question of how effectively the MSC-based approach would work when targeting tumor metastases. The RANTES/CCL5 promoter was used again to control NIS expression in the MSCs. An LS174T colon cancer liver metastasis mouse model showed remarkable metastatic selectivity of RANTES-NIS-MSC recruitment and tumor-specific promoter activation as demonstrated by NIS reporter gene imaging. While ^{123}I scintigraphic imaging showed a diffusely elevated radioiodine uptake in the hepatic area, ^{124}I PET imaging allowed a 3D analysis of NIS-mediated radioiodine accumulation with higher resolution showing iodide uptake confined to metastatic nodules. *Ex vivo* analysis of radioiodine biodistribution confirmed NIS-specific iodine uptake confined to the liver, which was further validated by quantitative PCR analysis and immunohistology demonstrating strong NIS immunoreactivity strictly confined to metastatic tissue without expression in normal liver tissue or non-target organs. In addition, a significant reduction in the growth of metastases as assessed by serial MRI imaging was observed after ^{131}I application associated with improved survival (Knoop *et al.* 2015).

The efficient targeting of hypoxic regions has become an important issue in cancer therapy as they are more resistant to conventional treatment options such as chemo- or radiotherapy. Hypoxia-inducible factor-1 (HIF-1) is a key mediator of the cellular response to hypoxia (Vaupel & Multhoff 2018). MSCs were engineered to express the NIS transgene under the control of a synthetic HIF-responsive promoter (HIF-NIS-MSCs). HIF-NIS-MSCs showed robust transgene induction under hypoxia as demonstrated in tumor cell spheroid models and *in vivo* in the orthotopic HuH7 xenograft mouse models that revealed significant levels of tumor-selective NIS-mediated radioiodide accumulation after effective tumor recruitment of HIF-NIS-MSCs as shown by ^{123}I scintigraphy and ^{124}I PET imaging. Interestingly, levels of radioiodine accumulation were significantly higher in orthotopic xenografts as compared

to s.c. HuH7 xenografts based on more effective MSC tumor homing – very likely due to the more ‘physiologic’ and permissive tumor microenvironment in the orthotopic models for the efficient engraftment of exogenously applied MSCs in contrast to the more artificial tumor milieu in the s.c. model. In line with these pretherapy *in vivo* imaging studies, no significant reduction in tumor growth or survival was observed in the s.c. HCC xenograft model after application of ^{131}I , while intrahepatic tumor-bearing mice showed a strong response to HIF-NIS-MSC/ ^{131}I treatment with significantly reduced tumor growth, associated with reduced tumor cell proliferation and blood vessel density as well as reduced tumor perfusion assessed by contrast-enhanced ultrasound. This ultimately resulted in the prolonged survival of treated animals. The data on reduced blood vessel density and tumor perfusion following NIS-mediated radioiodine therapy also point towards a self-energizing effect of this therapy strategy, as the resultant hypoxia continues to drive HIF-responsive promoter-driven NIS expression and NIS-induced radioiodine accumulation (Muller *et al.* 2016).

As a further step in the preclinical development of MSC-based NIS gene therapy, tumor selectivity and level of NIS transgene expression were characterized after MSC-based NIS gene delivery in the advanced genetically engineered pancreatic cancer mouse model *Ptf1a⁺/Cre;Kras⁺/LSL-G12D;Trp53^{loxP/loxP} (Kras;p53)*. This model is a well-characterized preclinical model of pancreatic ductal adenocarcinoma (PDAC) that closely resembles the human disease (Mazur & Siveke 2012). These mice spontaneously develop extremely aggressive PDAC. The tumors are characterized by strong desmoplasia and an extensive tumor stroma consisting of fibroblasts, inflammatory cells and vasculature girded by high amounts of extracellular matrix (Schug *et al.* 2019a). Syngeneic mouse MSCs were engineered to express NIS driven by the CMV promoter (CMV-NIS-mMSC). The cells were injected i.v. followed by ^{123}I scintigraphy and ^{124}I PET imaging. The results showed active MSC recruitment into the tumor stroma of the pancreatic tumors with impressive levels of NIS-mediated radioiodine accumulation (up to 16.3% ID/g; tumor-absorbed dose up to 136.9 mGy/MBq) that was higher than the level achieved after LPEI-PEG-GE11/NIS-mediated NIS gene delivery (as outlined above). Three cycles of systemic CMV-NIS-MSC applications followed by ^{131}I application resulted in a significant delay and reduction in tumor growth (Schug *et al.* 2019a).

As a next step, several strategies were explored to enhance the effectiveness of MSC-based NIS gene therapy by enhancing tumor tropism and engraftment of MSCs as

well as taking advantage of potential synergistic/additive therapeutic effects (Karp & Leng Teo 2009, De Becker & Riet 2016).

Tumor external beam radiation (EBRT) represents an important pillar of conventional cancer therapy. Due to the extensive tissue damage that occurs during treatment, it may also represent a means of enhancing MSC homing to tumor microenvironments. The tissue damage and resultant inflammatory response induced by irradiation lead to increased secretion of inflammatory chemokines and growth factors known to be linked to MSC migration (Klopp *et al.* 2007, Zielske *et al.* 2009, Schug *et al.* 2018). EBRT pretreatment on MSC migration was characterized *in vitro* and *in vivo* using HuH7 xenograft models and NIS as a reporter gene. The irradiation of HuH7 cells *in vitro* lead to a strong dose-dependent increase in steady-state mRNA levels of CXCL8, CXCL12, FGF2, PDGF β , thrombospondin-1 (THBS1), VEGF, as well as TGF β 1, which was confirmed on protein level using ELISA. MSC migration was analyzed using a live cell tracking migration assay monitored by time-lapse microscopy for 24 h. MSCs under the influence of gradients generated from untreated HuH7 supernatant vs supernatant from EBRT-treated HuH7 showed enhanced migration of MSCs towards supernatants from irradiated HuH7 cells. In s.c. HuH7 xenograft tumors, tumoral iodide uptake monitored by ^{123}I scintigraphy showed a significant dose-dependent stimulation after i.v. application of CMV-NIS-MSCs following low dose irradiation with 5 Gy (9.2% ID/g) or 2 Gy (7.9% ID/g) as compared to non-irradiated tumors (5.3% ID/g) (Schug *et al.* 2018).

These data suggested that EBRT may not only enhance the migratory behavior of MSCs but may also act to potentially amplify activation of the TGF β 1-inducible SMAD-responsive promoter in SMAD-NIS-MSCs (see above) due to the increased levels of TGF β 1 expression seen in irradiated tumors. In this regard, it has been speculated that gene promoters that are directly or indirectly responsive to radiation-induced signals could potentially allow amplification of transgene expression when used in the setting of NIS-mediated radioiodine therapy. This is based on the radioiodine-induced tumor damage that results in an inflammatory response that could further drive activation of the radiation-responsive promoter. In a study that sought to combine these two approaches, the results demonstrated a pronounced increase in tumor-selective radioiodine accumulation in HuH7 xenograft tumors after tumor irradiation followed by SMAD-NIS-MSC application. Further, EBRT pretreatment of s.c. HuH7 tumors following systemic application of SMAD-NIS-MSCs resulted in a pronounced reduction in tumor growth after ^{131}I application leading to

a complete remission of tumors in a subset of animals and dramatically prolonged survival of animals as compared to animals receiving CMV-NIS-MSC treatment or untreated controls. The TGF β 1-inducible SMAD-responsive promoter used in the study responded to the enhanced TGF β 1 present in the tumor stimulated by EBRT and can thus be seen as an indirect but powerful radiation-responsive promoter. Subsequent tissue damage induced by SMAD-NIS-MSC-based radioiodine therapy is thought to result in further enhancement of the inflammatory response thus promoting additional MSC recruitment and tumoral TGF β 1 levels. This is thought to support a self-energizing therapy cycle that is likely responsible for the pronounced therapeutic effect seen. The therapeutic effects went well beyond what was seen in all previous studies by this group using MSCs or polymers as non-viral NIS transgene delivery vehicles (Schug *et al.* 2019b).

Using the early growth response factor 1 (Egr1) promoter as another radiation-activated promoter to drive NIS expression in bone marrow-derived MSCs also resulted in a ^{188}Re and ^{131}I radiation positive feedback effect in s.c. and orthotopic human glioma xenografts (U87) in mice (Shi *et al.* 2016, 2019). In the orthotopic model, MSCs were engineered to co-express the angiogenesis inhibitor kringle 5 of human plasminogen and NIS, which enhanced the therapeutic efficacy of ^{188}Re therapy (Shi *et al.* 2019).

Hyperthermia is an emerging therapeutic modality for the treatment of cancer. Its use as an adjuvant in multimodal treatment approaches enhances therapeutic efficacy in a variety of tumor types (Hildebrandt *et al.* 2002, Repasky *et al.* 2013). Hyperthermia shows pleiotropic effects on malignant cells, such as reduction of DNA repair, production of heat shock proteins, modulation of inflammatory cytokines. These transient changes within the tumor microenvironment are thought to help trigger an antitumor immune response. In addition, hyperthermia is capable of increasing the amount of neutrophils, natural killer cells and lymphocytes in the tumor microenvironment, primarily by the enhanced expression of chemokines, integrins and chemokine receptors linked to leukocyte migration (Skitzki *et al.* 2009).

Regional hyperthermia was tested as a potential strategy to enhance the efficacy of MSC-based NIS gene therapy. Hyperthermia of human hepatocellular carcinoma cells (HuH7) led to increased production of immunomodulatory factors resulting in enhanced migration of CMV-NIS-MSCs to the tumor as demonstrated by significantly enhanced ^{123}I uptake in heat-treated HuH7 tumors. Therapeutic efficacy was significantly enhanced by combining CMV-NIS-MSC-mediated ^{131}I therapy with regional hyperthermia

(Tutter *et al.* 2020). In a parallel study, MSCs were engineered to express NIS under control of a heat-inducible HSP70B promoter (HSP70B-NIS-MSC) allowing an increased tumor-specific, temperature- and time-dependent NIS-mediated accumulation of radioiodine in heat-treated tumors. The highest tumoral iodine accumulation monitored by non-invasive ^{123}I scintigraphic imaging was seen 12 h after heat application. HSP70B-NIS-MSC-mediated ^{131}I therapy combined with hyperthermia resulted in a significantly reduced tumor growth with prolonged survival in the HuH7 xenograft tumor model (Tutter *et al.* 2021).

Taking advantage of their excellent tumor homing properties, MSCs were also explored as oncolytic virus carriers. Using passively immunized mice through i.p. injection of antiviral antibodies, it was demonstrated that adipose tissue-derived MSCs can be infected by MV, home efficiently to ovarian tumor xenografts in nude mice and induce a superior therapeutic outcome compared to virus alone (Mader *et al.* 2009). After further optimization using patient-derived MSCs as virus carriers (Mader *et al.* 2013), MV-NIS infected MSCs are currently being evaluated in a phase 1/2 clinical trial in patients with recurrent ovarian, primary peritoneal or fallopian tube cancer (NCT02068794).

Lack of iodide organification: an obstacle to effective NIS gene therapy?

In the thyroid gland thyroid peroxidase (TPO)-catalyzed oxidation, and the incorporation of iodide into tyrosyl residues along the thyroglobulin (Tg) backbone, is a process called iodide organification. This action increases the effective half-life of iodine and enhances its accumulation. In the treatment of thyroid cancer, it is thought to increase the therapeutic efficacy of radioiodine (Spitzweg & Morris 2002). Organification does not generally occur in non-thyroid tumors and for many years this was thought to limit the applicability of NIS in non-thyroid settings. Following NIS transduction in tumors, the radiation dose responsible for a therapeutic effect of ^{131}I treatment is dependent on a variety of factors, including the rate of iodide uptake and efflux, which together with the level of iodide recirculation between NIS-expressing and non-expressing cells determines the iodide retention time.

The results outlined in the extensive preclinical studies summarized above, in various non-organifying tumor entities, conclusively demonstrate that high tumoral levels of NIS expression when combined with the associated iodide recirculation effect can effectively compensate for a lack of organification. In this context,

it is also important to point out that in many thyroid cancers the iodide organification apparatus is also diminished following damage to the follicular structure, as reflected by a decrease in Tg and TPO production. While it is widely accepted that iodide organification is helpful in enhancing iodide retention time and the tumor-absorbed dose, detailed preclinical and clinical ^{124}I -PET dosimetry data have demonstrated that it is not a 'conditio sine qua non' for effective radioiodine therapy (Jentzen *et al.* 2014, Nagarajah *et al.* 2016). In fact, due to loss of the colloidal organization of thyrocytes, differentiated thyroid cancer cells do not metabolize iodine effectively (with the exception of the rare finding of thyroid hormone-producing thyroid cancers) (Chakravarty *et al.* 2011, Ejeh & Adedapo 2011), thereby significantly reducing the residence time of radioiodine within the tumor cells as compared to benign thyrocytes. In spite of the potentially reduced residence time, the majority of thyroid cancer cells still respond well to radioiodine and achieve tumoricidal radiation doses. These observations further support the contention that critical radiation doses delivered to the tumor cells can be driven by efficient NIS-mediated radioiodine uptake. This has been confirmed in a number of dosimetry preclinical and clinical studies that have shown that an increase in iodide uptake without an increase in radioiodine residence time is sufficient to increase the radiation dose with accompanying therapeutic response (Jentzen *et al.* 2014, Nagarajah *et al.* 2016).

In addition, alternative radionuclides, such as the beta-emitter ^{188}Re or the alpha-emitter ^{211}At , that are also transported by NIS offer the possibility of higher dose rates due to their higher energy and shorter half-life (^{188}Re : physical half-life 16.7 h, average beta-energy = 0.764 MeV, mean path length 3.5 mm; ^{211}At : physical half-life 7.2 h, average alpha energy 6.8 MeV, mean path length 65 nm) as compared to ^{131}I (physical half-life 8 days, average beta-energy 0.192 MeV, mean path length 0.8 mm). Due to the enhanced crossfire effect resulting from its longer path length, ^{188}Re appears to be a promising alternative to ^{131}I , in particular in larger tumors in the context of NIS-targeted radionuclide therapy. Due to its high linear energy transfer with short path length, ^{211}At may also represent a potent alternative radionuclide in the context of NIS gene therapy, in particular in the treatment of small tumors and micrometastases (Dadachova *et al.* 2002, Shen *et al.* 2004, Zuckier *et al.* 2004, Dadachova *et al.* 2005, Petrich *et al.* 2006, Willhauck *et al.* 2007, 2008, Hingorani *et al.* 2010, Klutz *et al.* 2011c, Knoop *et al.* 2013).

These observations point to a further advantage of NIS-mediated radionuclide therapy in non-thyroid tumors,

which is the broad ‘bystander effect’ resulting from the crossfire effect of the applied radionuclide (maximum path length of 2.4 mm for ^{131}I and 32 mm for ^{188}Re), as well as a radiation-induced biologic bystander effect, that appears central in viral and non-viral gene delivery often resulting in a heterogeneous transgene expression pattern in the tumor that can limit therapeutic efficacy in the absence of a compensating bystander effect.

Taken together, as summarized above, the extensive preclinical studies now available have convincingly demonstrated that the level of radionuclide accumulation (radioiodide or alternative radionuclides such as ^{188}Re and ^{211}At) achieved in the tumor, the duration of radionuclide retention, and the distribution of NIS transgene expression was sufficient to reach a tumor dose within the range considered sufficient for therapeutic response in thyroid cancer (Ho *et al.* 2013, Jentzen *et al.* 2014). More importantly, these levels have been sufficient to elicit a significant therapeutic effect of ^{131}I or alternative radionuclides in a variety of tumor models, including clinically highly relevant aggressive tumor models, such as a genetically induced pancreatic cancer model that is widely accepted as a model that closely reflects human disease, making it highly suitable to predict the clinical effectiveness of novel treatment strategies.

An additional issue in the wider translation of NIS-based methodologies into the clinic is whether the dose of radioiodine used in animal studies can be scalable to humans. While the most common empirical ^{131}I dose for advanced thyroid cancer ranges from 150 to 300 mCi, dosimetrically determined doses can be considerably higher ranging from 300 to 600 mCi. The Food and Drug Administration of the United States (FDA) has formulated a table of dose conversion factors that allow for allometric adaptation from preclinical animals to humans based on body surface area (BSA). After adjusting the general BSA formula to a 75 kg human, the administered dose of 1–2 mCi given to a mouse as performed in most preclinical studies translates to a value between 248 and 496 mCi for a human being – a level that lies well within the dose range administered to patients with advanced metastasized differentiated thyroid cancer (Trujillo *et al.* 2012).

Recognition of NIS as an important imaging reporter for molecular therapies

As outlined above, the cloning of NIS cDNA has provided us not only with a powerful therapeutic gene but – as one of the oldest examples of an imaging reporter gene

in thyroid disease – NIS has also emerged as one of the most promising reporter genes available today for the monitoring of molecular therapies in preclinical and translational research (Fig. 1). NIS has many characteristics of an ideal reporter gene, it is highly homologous between human, rat and mouse, it reports only viable cells, it has not been found to impart toxicity upon ectopic expression in non-thyroidal cells, and it represents a non-immunogenic protein with a well-defined body biodistribution and expression that mediates the transport of readily available radionuclides, such as ^{131}I , ^{123}I , ^{125}I , ^{124}I , $^{99\text{m}}\text{Tc}$, ^{188}Re or ^{211}At (Penheiter *et al.* 2012). The NIS reporter gene approach can be limited by background signal due to radiotracer uptake by endogenously NIS-expressing tissues, such as salivary glands, thyroid gland and stomach. Interestingly, it has recently been reported that NIS from minke whale owns a relative resistance to the competitive NIS inhibitor perchlorate and may, therefore, serve as the superior candidate reporter gene, as background radiotracer uptake by endogenous NIS can be blocked by perchlorate (Concilio *et al.* 2021).

Radionuclide-based reporter systems, such as the NIS reporter system, allow non-invasive imaging by standard nuclear medicine imaging modalities, PET or single-photon emission CT (SPECT), thereby offering high sensitivity, resolution and greatest promise for deep tissue imaging in humans. The field of gene and cell therapy has made considerable strides in the last decades with the development of new vectors for gene, viral and cell-based therapies. Non-invasive monitoring of the *in vivo* distribution of these viral and non-viral vectors, as well as monitoring of the biodistribution, level and duration of transgene expression has been recognized as important biologic criteria in the design of clinical trials. The need for this technology is further highlighted by the increasing repertoire of replication-competent viruses for cancer gene therapy where for maximal patient safety it is critically important to monitor biodistribution, replication and elimination in the individual patient.

In an extensive series of studies, including the ones summarized above, the role of NIS as a reporter gene allowed non-invasive multimodal imaging of functional NIS expression by $^{99\text{m}}\text{Tc}$ or ^{123}I scintigraphy as well as SPECT and ^{124}I PET imaging correlating well with the results of *ex vivo* gamma counter measurements as well as NIS mRNA and protein analysis. This provided the possibility of detailed characterization of *in vivo* biodistribution of viral and non-viral vectors especially after systemic application as well as localization, level and duration of NIS transgene expression – an essential prerequisite for exact planning

and monitoring of clinical gene therapy trials with the aim of individualization of the NIS gene therapy concept in the clinical setting. The utility of the NIS reporter for monitoring of the delivery and intratumoral propagation of oncolytic viruses has been demonstrated in a series of studies summarized above using different oncolytic viruses, such as adenovirus, MV, VSV, VV. Some of these studies have now reached the clinical stage with multiple ongoing clinical trials utilizing NIS as a reporter gene (see above). In addition, NIS reporter imaging has been used to monitor the delivery and fate of adoptively transferred cells, such as MSCs (as outlined above), dendritic cells (Ahn *et al.* 2017) and immune cells (Moroz *et al.* 2015, Ashmore-Harris *et al.* 2020), such as chimeric antigen receptor T cells (Emami-Shahri *et al.* 2018, Volpe *et al.* 2020) and regulatory T cells (Jacob *et al.* 2020). Most cell therapy trials are still performed without data detailing the *in vivo* biodistribution and fate of administered therapeutic cells. This can be seen as a general limitation in their preclinical and clinical development. NIS reporter gene imaging can provide an efficient means of non-invasive quantitative tracking of their *in vivo* biodistribution and survival at on-target tissues as well as off-target tissues as a source of potential toxicity.

For the application of NIS as a reporter gene, PET imaging using ^{124}I provides advantages for determining localization and quantitative analysis of NIS-mediated radioiodine accumulation. It offers the highest imaging sensitivity for the detection of low levels of NIS reporter gene expression that is particularly important when systemic NIS gene delivery approaches are used in orthotopic and metastatic tumor models with low volume disease and/or overlap with organs that physiologically accumulate iodide, particularly in stomach. It is also suitable for whole-body cell tracking applications. However, ^{124}I does have several disadvantages that limit its routine diagnostic use. It has a relatively long half-life of 4.2 days, a low positron yield (23%), high positron energy and abundant high energy gamma emissions that result in high radiation exposure and less-than-ideal imaging properties. In addition, the production of ^{124}I is relatively complex limiting its availability (Jiang & DeGrado 2018). In addition, the use of radioiodine in the context of NIS reporter imaging can be hampered by the organification of iodide in the thyroid gland. While this metabolic trapping is an advantage in imaging normal thyroid function or radioiodine accumulation in thyroid cancer, it complicates reporter gene imaging due to a 'thyroid sink' effect making the tracer less available for uptake in non-thyroidal target cells.

In many respects, ^{18}F represents a better potential radionuclide in this context. It is the most widely used radionuclide for PET, as it is readily available and has a half-life (110 min) that is compatible with typical imaging times and a low energy ($E_{\text{max}}=0.634$ MeV), high yield (97%) positron emission. Philip J Blower from King's College London was the first to report a simple synthesis of ^{18}F -tetrafluoroborate (^{18}F -TFB) and its application as a new PET imaging agent for NIS imaging (Jauregui-Osoro *et al.* 2010, Weeks *et al.* 2011). TFB is a fluorine-containing ion with a similar size and shape to pertechnetate that has been shown to be a substrate for NIS by electrochemical studies (Jiang & DeGrado 2018). From historical studies in rats, TFB is known to accumulate in the thyroid reaching a high thyroid to blood concentration ratio (Jauregui-Osoro *et al.* 2010). ^{18}F -TFB has subsequently emerged as a promising iodide analog radiotracer for NIS imaging. ^{18}F -TFB has been evaluated *in vivo* in several preclinical animal models, including mice, pigs and non-human primates (Jauregui-Osoro *et al.* 2010). ^{18}F -TFB has also been applied in several human studies in healthy individuals as well as in thyroid cancer patients. The results show ^{18}F -TFB kinetics and biodistribution that is similar to $^{99\text{m}}\text{TcO}_4^-$ and shows superior sensitivity and accuracy than that seen with ^{131}I whole-body scan and SPECT-CT in detecting recurrent DTC (O'Doherty *et al.* 2017, Jiang & DeGrado 2018, Samnick *et al.* 2018, Dittmann *et al.* 2020). This novel ^{18}F -labeled PET imaging agent for functional NIS expression allows for much improved, high-quality PET imaging, potentially allowing expanded development and application of the NIS reporter gene imaging.

Conclusions and perspectives

NIS represents one of the oldest and most successful targets for molecular imaging and targeted radionuclide therapy, providing the molecular basis for the diagnostic and therapeutic application of radioiodine in the last 80 years. Cloning of NIS 25 years ago has opened an exciting chapter in molecular thyroidology with the characterization of NIS as one of the most powerful theranostic genes and the development of a promising gene therapy strategy based on image-guided selective NIS gene transfer in non-thyroidal tumors followed by application of ^{131}I or alternative radionuclides, such as ^{188}Re and ^{211}At . These novel applications build upon the 80 years of clinical experience in the application of NIS-based radioiodine imaging and therapy in the treatment of thyroid patients and take advantage of the bystander effect

of NIS-mediated radionuclide therapy compensating for heterogeneity of transgene expression, the availability of several therapeutic radionuclides that can be individually applied, and the fact that NIS represents a non-toxic and non-immunogenic protein – all important prerequisites for successful future clinical application. Over the past two decades, significant progress has been made in the development of the NIS gene therapy concept, from local NIS gene delivery towards promising new applications in disseminated disease, in particular through the use of oncolytic viruses, non-viral polyplexes, and genetically engineered MSCs as highly effective, highly selective and flexible gene delivery vehicles. The further development of these approaches will allow for expanded individualization of the NIS gene therapy concept. The preclinical data as summarized in this review provide a solid basis for clinical translation, as evidenced by the series of clinical trials currently underway.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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