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Targeted delivery of the theranostic sodium iodide symporter (NIS) for cancer gene therapy

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Erklärung

Diese Dissertation wurde im Sinne von § 7 der Promotionsordnung vom 28. November 2011 von Frau Professor Dr. C. Spitzweg betreut und von Herrn Professor Dr. E. Wagner vor der Fakultät für Chemie und Pharmazie vertreten.

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Meiner Familie

Though much is taken, much abides; and though

We are not now that strength which in old days

Moved earth and heaven; that which we are, we are;

One equal temper of heroic hearts,

Made weak by time and fate, but strong in will

To strive, to seek, to find, and not to yield.

Ulysses - Alfred, Lord Tennyson

Table of contents

1. Introduction

1.1 Cancer and cancer therapy

1.1.1 Cancer

"Cancer" is a generic term that covers all malignant neoplasms in general that all have certain hallmarks in common. The continuing progress achieved by medical science in recent years allowed to increasingly clarify the picture of these very complex diseases. In 2000, Hanahan and Weinberg (Hanahan and Weinberg, 2000) proposed six hallmarks of cancer as biological capabilities that are acquired during the development of these neoplastic diseases: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. More than ten years later, this list has been extended by the two emerging hallmarks of deregulating cellular energetics and avoiding immune destruction as well as by the two enabling characteristics of genome instability and mutation and tumor-promoting inflammation (Hanahan and Weinberg, 2011).

The insights into cancer research gained so far elucidated only parts of the very complex biology of cancer. As a result, it is very unlikely that it will be possible to find the one universal drug to cure every specific cancer entity, but it becomes increasingly obvious that the fight against these diseases is a process that requires the development of customized cancer therapies that are tailor made and individualized for each patient. Along this way, numerous approaches have been investigated, one of which is gene therapy.

1.1.2 Gene therapy

In a pharmaceutical view, genes can be considered as drugs that are able to prevent, detect, alleviate, or cure diseases. Gene therapy can therefore be defined as an experimental method that uses the genetic information of deoxyribonucleic acids (DNA) as a pharmaceutical agent. DNA is used in order to supplement or to alter genetic information of an individual's cells in an attempt to restore, to correct, or to influence physiological functions by a pharmacological, immunological, or metabolic action.

Over the last two decades, one particular gene has gained remarkable attention throughout the gene therapy society since its cloning in 1996 (Dai *et al.*, 1996; Smanik *et al.*, 1996) and has evolved as a highly promising potential candidate gene for gene therapy approaches as it combines both, diagnostic and therapeutic properties, in one single gene.

1.2 The sodium iodide symporter (NIS)

1.2.1 The physiological role of NIS

The sodium iodide symporter (NIS or SLC5A5) is an intrinsic transmembrane glycoprotein with thirteen transmembrane domains that mediates the cellular uptake of iodine from the bloodstream (Dai *et al.*, 1996; Smanik *et al.*, 1997). Most endogenous NIS expression can be found in thyroid follicular cells or in cells of the lactating breast (Dohan *et al.*, 2003). Thyroidal NIS expression is required to provide the thyroid gland with sufficient iodide for thyroid hormone synthesis and lactating mammary glands express NIS in order to supply infants with iodine-enriched breast milk (Semba and Delange, 2001). In thyroid cells, iodide is organified after NIS-mediated uptake. That means, iodide is oxidized by the thyroid peroxidase (TPO) in the presence of H_2O_2 and covalently bound to the tyrosyl residues of thyroglobulin (Tg). Thyroid hormones tri-iodothyronine (T_3) and tetra-iodothyronine (T_4) are synthesized by coupling of iodinated tyrosyl residues and stored within the colloid space until Tg is taken up by thyroid follicular cells and hormones are released into the blood stream (Carrasco, 1993). Thyroid hormones play an important role in metabolism, growth and maturation of a variety of organ systems, particularly the nervous system.

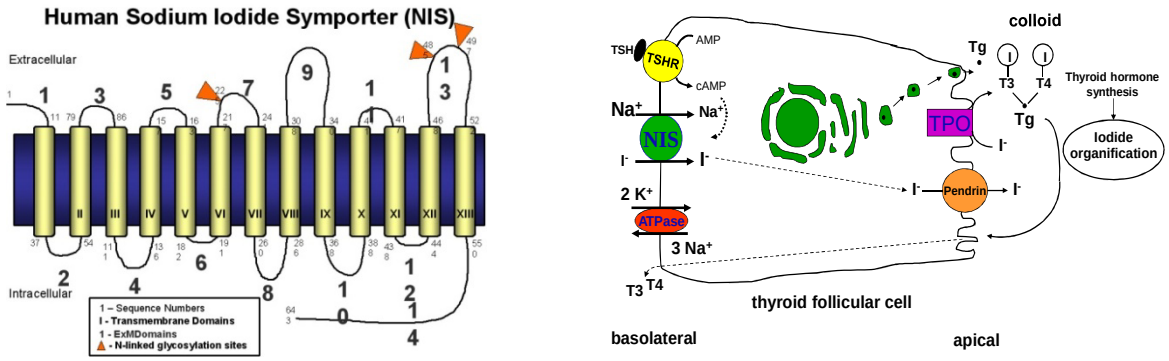

Fig. 1: Schematic model of the protein structure of the human sodium iodide symporter (NIS) protein (left) and its role in iodine transport and thyroid hormone synthesis in the thyroid gland (right).
With permission reproduced from Spitzweg *et al.*, J Clin Endocrinol Metab, 2001.

1.2.2 NIS-mediated radioiodine therapy

The ability of NIS to concentrate iodine intracellularly with high efficiency provides the possibility of diagnostic (^{123}I, ^{124}I, ^{125}I, $^{99m}TcO_4^-$, [^{18}F]-TFB) and therapeutic (^{131}I, ^{188}Re, ^{211}At) application of radioiodine or other radioactive substrates of NIS (Dohan *et al.*, 2003; Jauregui-Osoro *et al.*, 2010; Spitzweg and Morris, 2002; Van Sande *et al.*, 2003; Willhauck *et al.*, 2008a; Willhauck *et al.*, 2007; Zuckier *et al.*, 2004). This has successfully been used for more than 70 years in the management of thyroid cancer patients based on the expression of NIS - although at a usually lower level - in follicular cell-derived thyroid cancer cells. Due to organification of therapeutic radioiodine in follicular thyroid cancer cells the tumoral iodide retention time is substantially prolonged leading to sufficiently high tumor absorbed doses of ^{131}I thereby providing clinicians with the most effective form of systemic anticancer radiotherapy available today. In the routine management of patients with differentiated thyroid cancer, radioiodide whole body imaging is able to visualize local and metastatic residual or recurrent disease and quantification of tracer uptake allows for exact dosimetric calculations of tumor absorbed doses for ^{131}I before therapeutic ^{131}I application, thereby aiming at maximal therapeutic efficacy at minimal toxicity in a personalized manner. NIS-based thyroid cancer radioiodine therapy is a clinically already approved anticancer therapy with a well-understood therapeutic window and safety profile. High doses of radiation are able to destroy cells by damaging cellular proteins and induction of DNA double strand breaks leading to subsequent apoptosis (Magnander and Elmroth, 2012). β-emitting ^{131}I (maximum energy 0.61 MeV) was one of the first radionuclides used for therapy in clinical oncology (Beierwaltes, 1979) and is nowadays routinely used for therapeutic ablation of residual NIS-expressing thyroidal cancer cells after total thyroidectomy.

In order to extend the numerous advantages of NIS-mediated radioiodine therapy also to the treatment of non-thyroidal cancers, a promising cytoreductive gene therapy strategy based on targeted delivery of the theranostic NIS gene in extrathyroidal tumors followed by radioiodine application was developed.

1.2.3 NIS and its role as reporter gene

Recent technological advances in nuclear medicine, magnetic resonance spectroscopy, optical and bioluminescence imaging have resulted in a variety of new,

exciting developments in the field of molecular imaging, aiming at non-invasive imaging of endogenous and exogenous gene expression and intracellular signal transduction pathways (Ahn, 2012; Alberti, 2012; Brader *et al.*, 2013; Thorek *et al.*, 2012). In addition, the field of gene therapy has made considerable strides in the last decade by the development of new vectors and an increasing repertoire of therapeutic genes. Non-invasive monitoring of the *in vivo* distribution of viral and non-viral vectors, as well as monitoring of the biodistribution, level and duration of transgene expression have been recognized as critical elements in the design of clinical gene therapy trials. The need for this technology is further highlighted by the advent of replication-competent viruses for cancer gene therapy where it is critically important to monitor biodistribution, replication and elimination *in vivo*.

Cloning of NIS has provided us with one of the most promising reporter genes available today. NIS has many characteristics of an ideal reporter gene, as 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, ^{99m}Tc, ^{188}Re, or ^{211}At. Therefore, the experimental use of NIS as reporter gene can employ various imaging techniques in order to visualize radionuclide uptake.

Besides 2-dimensional gamma camera scintigraphy, 3-dimensional images can be acquired using emerging imaging techniques like single-photon emission computed tomography (SPECT) or positron emission tomography (PET) that have been shown to provide significant advantages for exact localization and quantitative analysis of NIS-mediated radioiodine accumulation due to enhanced resolution and sensitivity (Baril *et al.*, 2010; Dingli *et al.*, 2006; Groot-Wassink *et al.*, 2004; Merron *et al.*, 2007; Penheiter *et al.*, 2012; Richard-Fiardo *et al.*, 2011). These images can further be correlated with computed tomography (CT) or magnetic resonance tomography (MRT) scans for exact anatomical identification of regions of tracer uptake. Recently, [^{18}F]-tetrafluoroborate ([^{18}F]-TFB), a known alternative substrate of NIS, has been evaluated as new PET imaging agent in preclinical models, demonstrating high sensitivity and significantly improved resolution as compared to ^{124}I (Jauregui-Osoro et al., 2010; Weeks et al., 2011). This is particularly important for systemic NIS gene delivery approaches in orthotopic and metastatic tumor models with low volume disease and/or overlap with organs that physiologically accumulate iodide, in particular stomach.

In addition to non-invasive imaging of radioiodine accumulation after NIS gene transfer by the different imaging techniques described above, several investigators including the group of Christine Spitzweg have studied the potential of NIS as reporter gene in various applications, demonstrating that *in vivo* imaging of radioiodine accumulation correlates well with the results of *ex vivo* gamma counter measurements as well as NIS mRNA and protein analysis (Baril *et al.*, 2010; Blechacz *et al.*, 2006; Goel *et al.*, 2007; Merron *et al.*, 2007).

In several studies, NIS was successfully used as a reporter gene to monitor *in vivo* biodistribution of replication-competent viral vectors, including oncolytic measles virus in liver cancer and myeloma xenograft models, oncolytic vesicular stomatitis virus in a myeloma xenograft model, as well as oncolytic adenovirus in peritoneal tumors or colon and prostate cancer xenograft models using conventional ^{123}I- or ^{99m}Tc-gamma camera imaging or ^{99m}Tc-SPECT/CT fusion imaging (Blechacz *et al.*, 2006; Goel *et al.*, 2007; Merron *et al.*, 2010; Merron *et al.*, 2007; Peerlinck *et al.*, 2009; Penheiter *et al.*, 2011; Touchefeu *et al.*, 2011; Trujillo *et al.*, 2010). Non-invasive imaging is further able to provide information about the *in vivo* biodistribution of several target cells, such as tumor cells, immune cells, or stem cells.

In particular, mesenchymal stem cells (MSCs) have been the object of recent research activity in the fields of both gene and cellular therapies. However, to date, there is insufficient information about the exact *in vivo* biodistribution, survival and biological compartment of these cells in targeted tissues. In this context, NIS reporter gene imaging also provides a means for non-invasive, repeated and quantitative tracking of stem cell implant or transplant from initial deposition to survival, migration and differentiation of stem cells, which has been successfully demonstrated by several groups including our own (Hwang *et al.*, 2008; Knoop *et al.*, 2011; Knoop *et al.*, 2013; Lee *et al.*, 2008).

These studies convincingly demonstrate that NIS represents one of the most promising reporter genes for molecular imaging offering a broad range of application possibilities, in particular in the context of innovative molecular therapies.

1.2.4 NIS as novel therapeutic gene

Based on its well characterized dual function as reporter and therapy gene, in a first step, NIS can be used for direct, non-invasive multimodal imaging of vector biodistribution and functional NIS expression by ^{123}I-scintigraphy/SPECT imaging and

^{124}I- or [^{18}F]-TFB-PET imaging, as described above. The data acquired by imaging analysis allow for exact dosimetric calculations before proceeding to therapeutic application of ^{131}I, followed by the *in vivo* and *ex vivo* monitoring of the therapy response after NIS-targeted radionuclide therapy in a multimodal concept.

As the molecular basis of ^{131}I therapy of benign and malignant thyroid diseases, NIS provides several advantages as therapy gene. In clinical routine, the individualized radioiodine therapy shows overall good clinical tolerability due to relatively mild side effects as known from the extensive experience with radioiodine therapy in thyroid cancer. Moreover, NIS gene therapy is associated with a substantial bystander effect based on the crossfire effect of the β-particles emitted by ^{131}I with a path length of up to 2.4 mm (Dingli *et al.*, 2003a). This bystander effect allows that not only transduced but also neighbouring non-transduced tumor cells can be reached by the radiation, which reduces the level of transduction efficiency required for a therapeutic response. Another advantage is, that NIS expression outside the thyroid gland is very low and therefore does not cause significant toxicity to non-target organs after therapeutic application of radioiodine. To prevent damage to the thyroid gland, thyroidal uptake of therapeutic radioiodine can be reduced by thyroid hormone pretreatment thereby effectively downregulating thyroidal NIS expression (Wapnir *et al.*, 2004). Further, NIS is a normal human gene and protein and causes no toxicity or diminished efficacy by immune responses as it is often observed after the use of other protein and gene therapeutics (Duffy *et al.*, 2012).

Several studies have demonstrated, that even if the iodine organifying cellular machinery is absent after NIS gene transfer in non-thyroidal tumor cells, the accumulated dose can be high enough to reach a therapeutic effect of radioiodine, clearly demonstrating that iodide organification is not a mandatory requirement for a therapeutic effect of ^{131}I (Dadachova *et al.*, 2005; Klutz *et al.*, 2009; Klutz *et al.*, 2011a; Klutz *et al.*, 2011b; Klutz *et al.*, 2011c; Knoop *et al.*, 2011; Knoop *et al.*, 2013; Petrich *et al.*, 2006; Spitzweg *et al.*, 2001b; Spitzweg *et al.*, 2000; Spitzweg *et al.*, 1999; Willhauck *et al.*, 2008a; Willhauck *et al.*, 2007).

1.3 Gene delivery concepts

1.3.1 Gene transfer

The greatest barrier for the evaluation of any gene therapy approach is the efficient, safe, and selective transduction of target cells inside the patient's body. Once inside these cells, the DNA is translated into the therapeutic protein. In case of cancer metastases target cells might be spread throughout the whole patient's body and therefore, intravenous vector administration is undeniably the only possible and promising route of administration. However, anionic charge, large size, and degradability of nucleic acids by nucleases preclude systemic administration of naked nucleic acids and subsequent location at the target site (Krebs and Alsberg, 2011; Mintzer and Simanek, 2009). To make systemic gene delivery feasible, special DNA carriers, called vectors, have been developed to facilitate the delivery of genetic material to target cells. These vectors can be divided into two main subgroups, the viral and the non-viral systems.

1.3.2 Non-viral gene delivery vectors

In order to overcome the major bottlenecks for efficient delivery of nucleic acid therapeutics to target cells, synthetic carriers for the different types of nucleic acids have been designed and steadily evolved over the past decades. Particular progress in this area had been achieved by the pioneering work of Ernst Wagner and Manfred Ogris (Felgner *et al.*, 1997; Ogris and Wagner, 2002a; Ogris and Wagner, 2002b; Ogris and Wagner, 2011; Russ and Wagner, 2007; Scholz and Wagner, 2012; Troiber and Wagner, 2011; Wagner, 2004; Wagner, 2007). It is important that the synthetic delivery vectors, which are usually cationic liposomes, peptides, or polymers, build a stable complex with the negatively charged therapeutic nucleic acid based on electrostatic interaction to avoid enzymatic degradation by nucleases in the blood stream and destabilization by electrostatic interactions with serum proteins (Burke and Pun, 2008).

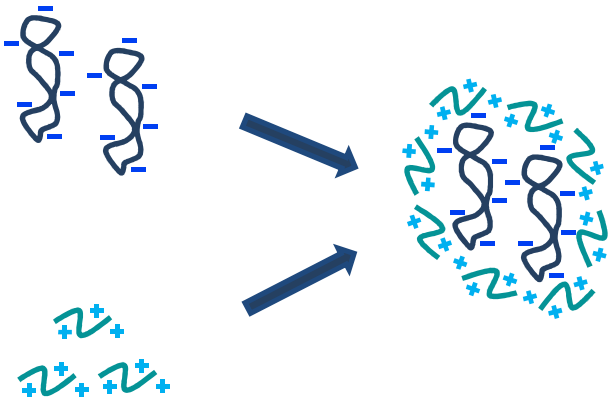

Fig. 2: Polycationic polymer binds to negatively charged DNA based on electrostatic interactions. This subsequently leads to a hydrophobic collapse resulting in particle formation in the nanometer range (polyplexes).

Further barriers for successful DNA delivery are the need of efficient endocytosis, endolysosomal escape, cytoplasmatic trafficking, vector unpacking, and nuclear import (Chen *et al.*, 2008; Grosse *et al.*, 2006; Midoux *et al.*, 2008; Nishiyama *et al.*, 2006). Most synthetic gene delivery vectors have been developed for tumor-targeted nucleic acid delivery and therefore utilize the specific properties of tumor tissues. The DNA-carriers with a size of about ≤ 300 nm show elongated plasma circulation times and passive accumulation at tumor sites due to leaky tumor vasculature combined with an inadequate lymphatic drainage, resulting in the enhanced permeability and retention (EPR) effect (Maeda, 2001).

Synthetic vectors offer certain advantages, such as a promising safety profile with low immunogenic potential, enhanced biocompatibility, low mutagenic risk, and easy handling and manufacturing, although their major drawback is the relatively low efficiency of transgene expression (Gao and Huang, 2009; Lin *et al.*, 2008; Schaffert and Wagner, 2008; Wolff and Rozema, 2008). For polymers, the stability and transfection efficiency of polyplexes formed with plasmid DNA depend on the chemical type, molecular weight, and topology of the cationic polymer, as well as the ratio of conjugate to plasmid (c/p ratio) (Christie *et al.*, 2010; Itaka *et al.*, 2004; Kunath *et al.*, 2003).

Polyamine structures, like polyamidoamine (PAMAM) dendrimers, are a commonly used class of synthetic vector system and also able to form stable complexes with plasmid DNA (Bielinska *et al.*, 1996; Haensler and Szoka, 1993; Tang *et al.*, 1996). Dendritic structures are highly ordered and built from a series of branches extending outward from an inner core with positively charged primary

amino groups on their surface at physiological pH. The structure comprises three individual parts: a core, branching units, and branches that can be chemically modified. Each iteration leads to a higher generation material and subsequently to dendrimers with larger molecular diameter and higher molecular weight (Ravina *et al.*, 2010).

1.3.3 Non-viral NIS gene delivery

For the first pioneering proof-of-principle studies of systemic non-viral NIS gene transfer in a syngeneic neuroblastoma mouse model, Klutz *et al.* in Christine Spitzweg's laboratory used novel biodegradable and highly efficient polycations as gene delivery vehicles that are based on oligoethylenimine (OEI)-grafted polypropylenimine dendrimers (G2-HD-OEI) and were developed in the laboratory of Ernst Wagner (Klutz *et al.*, 2009). This study clearly showed the high potential of branched polycations based on OEI-grafted polypropylenimine dendrimers for tumor-specific delivery of the NIS gene after systemic application. NIS in its well characterized function as reporter gene allowed for non-invasive imaging of functional NIS expression by ^{123}I-scintigraphy. Tumor-specific iodide accumulation was further shown to be sufficiently high for a significant delay of tumor growth associated with increased survival in syngeneic mice bearing neuroblastoma tumors after two cycles of NIS-polyplex application followed by ^{131}I therapy. Data of a subsequent study using the same polyplexes for systemic delivery in a murine xenograft model of human hepatocellular carcinoma correlated well with the previous findings and demonstrated that the application of these synthetic nanoparticles is not restricted to a specific tumor model, but is suitable for many cancers with hypervascularized tumors (Klutz *et al.*, 2011b). These studies demonstrated therapeutic efficacy of the NIS gene therapy concept using non-viral gene delivery systems.

With the aim of optimizing tumor selectivity polyplexes can be actively targeted to tumor cells by the attachment of receptor-specific active targeting ligands, which in addition to passive tumoral accumulation facilitate receptor-mediated endocytosis. In this regard, a further study by Klutz *et al*. demonstrated the feasibility of novel synthetic nanoparticle vectors based on linear polyethylenimine (LPEI), shielded by polyethylenglycol (PEG), and coupled with the synthetic peptide GE11 as an epidermal growth factor receptor (EGFR)-specific ligand for targeting the NIS gene to

human hepatocellular carcinoma overexpressing EGFR (Klutz *et al.*, 2011a). These novel polymers were developed in Manfred Ogris' group. LPEI as the "gold standard" of PEI-based gene carriers represents an alternative polymeric backbone as compared to those gene carriers based on dendrimers. LPEI is the linear form of PEI, and used with a molecular weight of 22 kDa. The major drawback of LPEI is its significant toxicity after systemic application due to acute and long-term toxic effects, especially to the lungs (Chollet *et al.*, 2002). Shielding of LPEI-based polyplexes by hydrophilic polymer, like PEG, can reduce unspecific toxicity and at the same time prolong blood circulation times (Zintchenko *et al.*, 2009). The PEGylation of the DNA complexes can block the interaction with several plasma components and erythrocytes and strongly changes the *in vivo* characteristics of particles leading to gene expression in distant tumor tissue after systemic vector administration (Ogris *et al.*, 1999). However, PEGylation also results in decreased cell-binding capacity and subsequently reduced efficacy. Adding of receptor-specific targeting ligands to these shielded polyplexes was shown to provide the vector with active tumor-targeting thereby enhancing transfection efficiency and tumor selectivity (de Bruin *et al.*, 2007; Klutz *et al.*, 2011a).

In conclusion, non-viral gene delivery systems provide the feasibility of a combination of both, passively and actively, targeted systemic NIS gene delivery and show high transduction efficiencies *in vivo* as well as a promising safety profile. This class of synthetic polymer-based gene delivery vehicles is often referred to as "synthetic viruses" implicating that there is another highly effective class of gene delivery vehicles based on infectious virus particles.

1.3.4 Viral gene delivery vectors

Viruses themselves cannot replicate in a self-sustaining manner and are therefore dependent on an individual's cellular machinery. The viruses transfer their genes into the cells of an individual organism thereby driving them to express their viral proteins in order to produce progeny virions. This process generally proceeds in a pathogenic way and the human immune system has several antiviral immune response strategies. Viruses have therefore evolved over countless years of evolution as transfer vehicles for their own genes in order to overcome the hurdles of their host's immune response. This virus' ability for gene transfer is being used in

medical science as the viral genome can be easily genetically manipulated in order to exchange disease-causing genes with therapeutic genes.

However, one has to be aware of the fact, that in particular viral gene therapy holds some potential risks. Viruses may be recognized and attacked by the patient‘s immune system. This may cause inflammation and, in severe cases, organ failure (Raper *et al.*, 2003). Since many viruses are able to infect more than only one single cell type there is a certain risk of infection of non-target cells and this may result in undesirable side-effects of anti-cancer therapy. Another scenario that has to be taken into consideration regarding the vector‘s safety profile is the possibility that the virus recovers its original ability to cause disease. Furthermore, depending on the type of virus used for gene delivery, there is a possible risk of insertional mutagenesis. If the carried genes are inserted directly into the DNA, insertion in the wrong location owns the risk to result in tumorigenesis (Gabriel *et al.*, 2012).

Despite these potential drawbacks, due to a number of positive characteristics various virus strains have been analyzed to date for their potential use in cancer gene therapy (Cattaneo *et al.*, 2008). Every strain has its particular advantages and disadvantages and the choice of the most suitable vector highly depends on the specific type of disease to be treated and the therapeutic setup. After basic research and proof-of-principle studies one has to have in mind the potential methodological transfer into the clinic. It is noticeable that at the Mayo Clinic (Rochester, MN, USA) the first NIS-expressing adenovirus is currently Food and Drug Administration approved for a human clinical trial in patients with locally recurrent prostate cancer (U.S. National Institute of Health, 2012).

1.3.5 Adenovirus-mediated gene transfer

Currently, adenoviruses are the most widely used vectors in clinical trials (Duffy *et al.*, 2012). Adenoviruses are particularly suitable for gene therapy of cancer because they have a rather harmless pathology, a stable genome that is relatively easy to manipulate, low risk of genomic integration, and with little effort can also be produced clinically under GMP conditions in large quantities and to high titers. In particular, gene therapy strategies that have no need for long-term transfection but whose goal is to maximize short-term transfection combined with minimum toxicity benefit from the use of adenoviruses. Genetically engineered oncolytic adenovirus vectors bear the potential to kill cancer cells by their viral replication cycle and

improve the therapeutic effect by production of progeny virions. These, in turn, are able to infect neighbouring tumor cells and at the same time amplify the therapeutic transgene. Previous clinical trials with oncolytic adenoviruses have shown promising safety with low toxicity and no major adverse side effects (Toth *et al.*, 2010).

Therefore, the future challenges for researchers are the integration of oncolytic virotherapy in existing therapy regimens and to further improve the efficiency and safety of these vectors, in particular because wild-type adenovirus vectors are not well suited for systemic vector administration.

1.4 Adenovirus-mediated NIS gene therapy

1.4.1 Intratumoral adenoviral NIS gene delivery

Local intratumoral application of adenovirus gene delivery vectors allows for treatment of accessible neoplastic lesions. Furthermore, this type of application is basically needed to prove in a first step the feasibility of a newly developed viral gene delivery construct. In 2001, Spitzweg *et al.* injected prostate cancer xenografts in nude mice with a replication-deficient adenovirus carrying the NIS gene linked to the cytomegalovirus (CMV) promoter, which led to highly active uptake of radioiodine (Spitzweg *et al.*, 2001a). These results showed for the first time that *in vivo* NIS gene delivery into non-thyroidal tumors is capable of inducing accumulation of therapeutically effective doses of radioiodine.

In a next step, tumor-specific promoters can be used for targeted radioiodine therapy of non-thyroidal cancers (Cengic *et al.*, 2005; Kakinuma *et al.*, 2003; Spitzweg *et al.*, 2007; Willhauck *et al.*, 2008c). In this way, adenovirus transgene expression can be restricted to specific cell types. Kakinuma *et al*. demonstrated probasin promoter (ARR2PB)-driven, prostate-specific expression of the NIS gene for targeted radioiodine therapy of prostate cancer after *in vitro* application of a genetically engineered replication-deficient adenovirus (Kakinuma *et al.*, 2003). In a further study, Spitzweg *et al.* demonstrated a therapeutic effect of ^{131}I *in vivo* in medullary thyroid cancer cell xenografts after replication-deficient adenovirus-mediated induction of tumor-specific iodide accumulation using the carcinoembryonic antigen (CEA) promoter to drive NIS expression (Spitzweg *et al.*, 2007). Furthermore, Klutz *et al*. reported on NIS-mediated radionuclide (^{131}I, ^{188}Re) therapy of liver cancer

xenografts after transcriptionally targeted, alpha fetoprotein (AFP)-driven intratumoral *in vivo* NIS gene delivery using a replication-deficient adenovirus (Klutz *et al.*, 2011c).

Trujillo *et al*. extended this approach to NIS-mediated radiovirotherapy of prostate cancer using a conditionally replicating adenovirus under control of the prostate-specific probasin promoter, in order to combine adenovirus-mediated oncolysis with NIS-mediated radioiodine therapy (Trujillo *et al.*, 2010). In its well characterized dual function as reporter gene as well as therapy gene associated with a significant bystander effect, NIS represents an ideal candidate gene for replication-selective adenovirus-mediated gene-virotherapy, providing the potential to monitor *in vivo* biodistribution of virus replication in addition to stimulation of therapeutic efficacy of oncolytic virotherapy by additional radionuclide therapy.

1.4.2 Radiovirotherapy

Oncolytic viruses are able to selectively kill human tumor cells during their replication cycle. At the same time oncolytic viruses can be genetically engineered in order to deliver therapeutic transgenes selectively into tumor cells. The term “radiovirotherapy” describes the combined use of oncolytic viral vectors that are able to induce tumoral uptake of therapeutic radioisotopes.

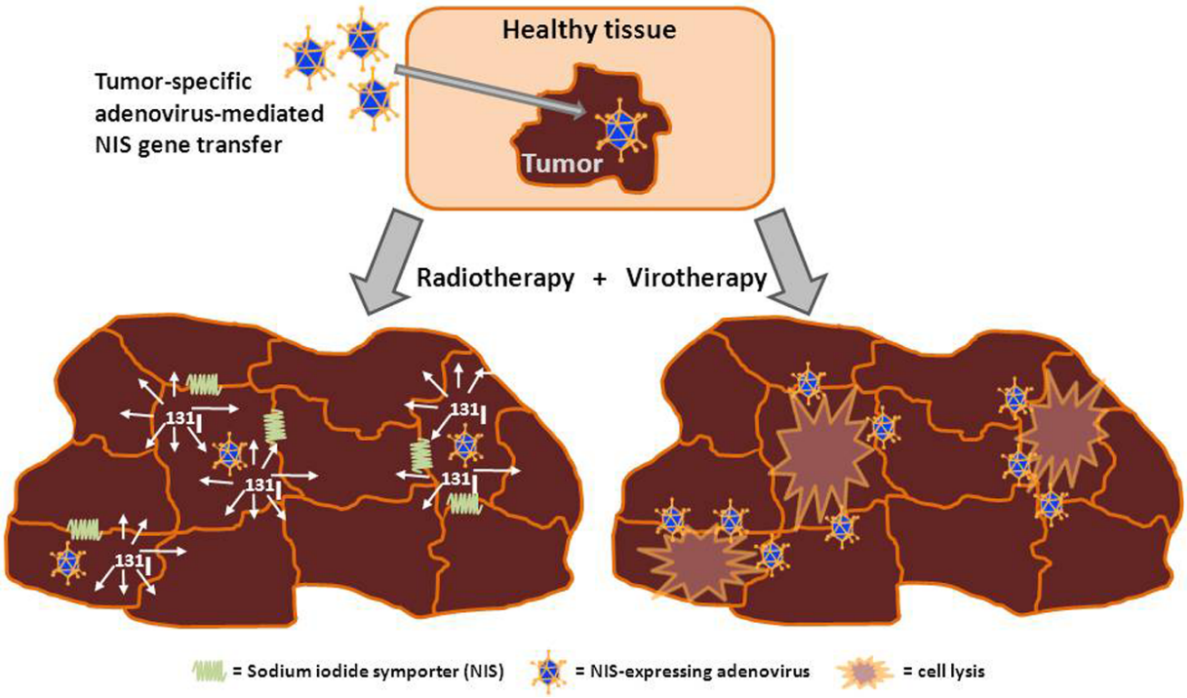

Fig. 3: Replication-selective adenovirus vectors replicating and expressing NIS under control of tumor-specific promoters are able to selectively infect cancer cells. Tumor cells are killed by oncolytic virus replication during the production of progeny virions (virotherapy) and this treatment can be additionally combined with NIS-mediated ^{131}I radiotherapy (radiovirotherapy).

These two therapeutic strategies are particularly suitable for combination because they can have synergistic antitumor effects. Viral infection may sensitize tumor cells for ionizing radiation and in turn radiation can enhance viral replication and oncolysis (Advani *et al.*, 2006; Hart *et al.*, 2007; Hart *et al.*, 2005; Hingorani *et al.*, 2008a; Hingorani *et al.*, 2008b). Trujillo *et al*. demonstrated that local *in vivo* NIS gene transfer using a replication-selective oncolytic adenovirus is able to induce a significant therapeutic effect, which can be further enhanced by additional ^{131}I application (Trujillo *et al.*, 2012a; Trujillo *et al.*, 2010; Trujillo *et al.*, 2012b). Moreover, the feasibility of combined radiovirotherapy using NIS-encoding adenovirus has been studied and proven by multiple groups in several tumor models (Barton *et al.*, 2011; Hakkarainen *et al.*, 2009; Huang *et al.*, 2011; Oneal *et al.*, 2012; Peerlinck *et al.*, 2009; Trujillo *et al.*, 2012a; Trujillo *et al.*, 2010; Trujillo *et al.*, 2012b).

1.4.3 Systemic adenoviral gene delivery

The greatest barrier for systemic adenoviral gene delivery is the interaction with several blood components like platelets, erythrocytes, and coagulation factors. In particular, binding of hexon protein on the adenovirus' surface to coagulation factor X leads to hepatic sequestration and profound liver transduction (Waddington *et al.*, 2008). Moreover, due to its negative surface charge the adenovirus is recognized by the Kupffer cells' scavenger receptor, which leads to uptake by these resident liver macrophages, subsequent virus inactivation, and contributes to the host inflammatory response (Lieber *et al.*, 1997). Furthermore, due to the widespread anti-adenovirus immunity in humans (Molnar-Kimber *et al.*, 1998; Yang *et al.*, 1995), pre-existing anti-adenovirus antibodies clear the adenovirus rapidly from the bloodstream and strong immune responses are triggered, most likely by interaction with antigen-presenting cells such as macrophages and dendritic cells, resulting in the release of proinflammatory cytokines/chemokines such as interleukin-6 (IL-6), tumor necrosis factor-α, interferon γ inducible protein-10, and RANTES, resulting in unwanted toxic side effects as well as low antitumor efficacy (Lieber *et al.*, 1997; Liu *et al.*, 2003; Muruve *et al.*, 1999; Schnell *et al.*, 2001; Zaiss *et al.*, 2002; Zhang *et al.*, 2001). Additionally, high promiscuity due to widespread expression of the coxsackie-adenovirus receptor (CAR), and on the other hand potential lack of CAR on tumor cells strongly limit its clinical application. As all these drawbacks are initially mediated

by interactions with adenovirus capsid proteins masking of the adenovirus' surface represents a promising approach to overcome these hurdles. Therefore, different strategies have been developed in order to blind the vector for off-targets by combining viral and synthetic vectors into a hybrid vector, including covalent conjugation of reactive polymers based on polyethylene glycol (PEG) or N-[2-hydroxypropyl]methacrylamide (HPMA) (Laga *et al.*, 2012) and non-covalent modification of the negatively charged adenovirus' surface by electrostatic interaction with cationic polymers (Yao *et al.*, 2011a). These new technologies demonstrated first evidence to provide the vector with the ability to overcome neutralizing anti-vector antibodies, to escape liver tropism and to reduce innate and adaptive immune responses and liver toxicity even after systemic vector application, while maintaining its natural biological activity (Laga *et al.*, 2012).

1.4.4 Dendrimer coating of adenovirus vectors for systemic NIS gene delivery

Vetter *et al.* recently reported on a novel strategy developed in Manfred Ogris' laboratory in order to modify the adenovirus' tropism and evade the patient's immune system, and proved that improvement of adenoviral vectors for gene delivery can be achieved by surface modification using the same class of synthetic polymer as previously established for non-viral gene delivery (as described under 1.3.2 and 1.3.3) (Vetter *et al.*, 2013). With the goal of developing an adenovirus-based vector suitable for systemic vector application, chemically well-defined dendritic PAMAM (poly(amidoamine)) dendrimers bearing positively charged terminal amines were utilized in order to coat the negatively charged adenoviral capsid based on electrostatic interaction. By attachment of the positively charged polymer, the surface charge of the virus is inverted, thereby allowing it to bind to the cell surface (Davis *et al.*, 2004). This modification allowed efficient internalization and transduction of tumor cells *in vitro* otherwise refractory towards adenoviral transduction.

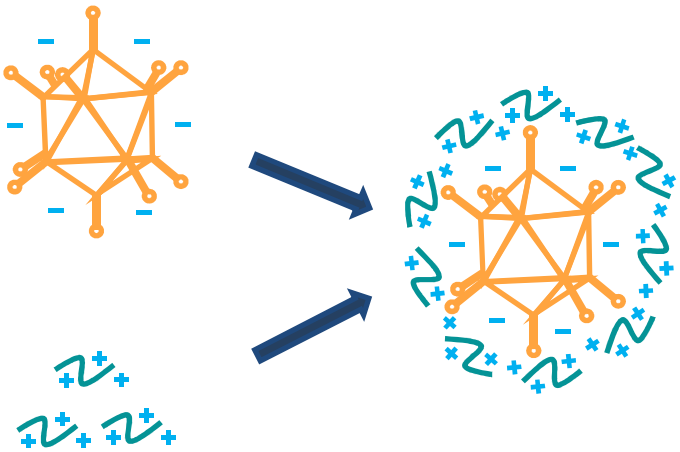

Fig. 4: The negatively charged adenovirus capsid can be shielded by coating with polycationic polymers in order to form a complex based on electrostatic interaction.

Besides the capacity of dendrimer-coated adenovirus to infect tumor cells with high efficiency through CAR-independent uptake mechanisms, dendrimer coating was further shown to form stable complexes in the presence of serum, and to protect the adenovirus, at least in part, from neutralizing antibodies. Protection from neutralizing antibodies raises hope for a prolonged blood circulation time, as it was shown by Green *et al*. (Green *et al.*, 2004). An evasion from neutralizing antibodies may further make a second systemic vector injection feasible without the need to suppress the patients' immune system.

After the proof-of-principle of dendrimer coating of the adenoviral surface *in vitro*, shielding and targeting can be further improved by coating of adenoviruses with a conjugate consisting of cationic PAMAM dendrimer linked to the peptidic, EGFR-specific ligand GE11 in order to redirect the virus tropism (Vetter *et al.*, 2013).

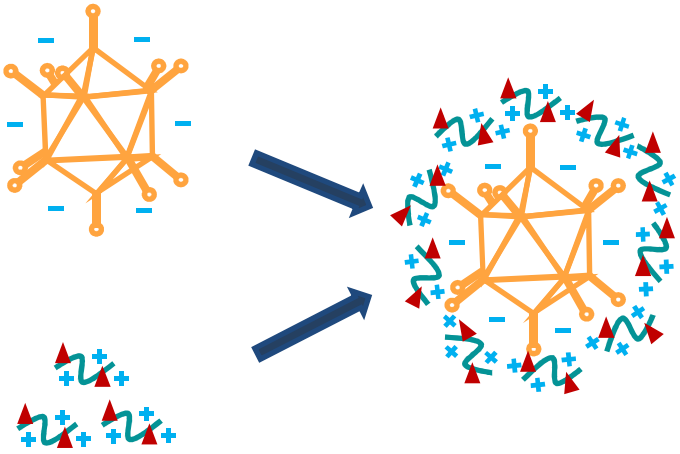

Fig. 5: The synthetic dendrimer used for adenovirus' surface modification can be coupled to tumor-specific targeting ligands enabling receptor-mediated cellular vector uptake.

In vitro experiments demonstrated CAR-independent but EGFR-specific transduction efficiency. The specificity for tumor cell infection was increased by targeting the coated adenovirus to the EGFR and selectivity for EGFR has been demonstrated.

1.5 Aim of the thesis

After the proof-of-principle of the diagnostic and therapeutic potential of the NIS gene therapy concept in the previous work of Christine Spitzweg and her group, the major challenge on the way to future clinical application now is to develop and characterize new gene delivery constructs that allow for safe application in humans and that are able to selectively transduce tumor cells with high efficiency after systemic application in order to detect and to treat neoplastic malignancies also in the metastatic stage.

Therefore, the first aim of this thesis was the basic characterization of a newly developed, conditionally replicating adenovirus construct that expresses the NIS gene. We studied the *in vitro* and *in vivo* efficacy of NIS gene transfer and the therapeutic potential of oncolytic virotherapy combined with radioiodine therapy after intratumoral vector application in a xenograft mouse model of hepatocellular carcinoma (HCC) with respect to selectivity and efficacy of replication and transgene expression.

The next logical step was the evaluation of the newly developed adenovirus construct for systemic vector application. Currently, major limitations for clinical application of adenovirus-mediated gene therapy are high prevalence of neutralizing antibodies, widespread expression of the coxsackie-adenovirus receptor (CAR) and adenovirus sequestration by the liver after systemic application. Therefore, based on the previous findings of Alexandra Vetter in Manfred Ogris' laboratory, we used the theranostic NIS gene to investigate whether coating of adenovirus vectors with synthetic dendrimers can be useful to overcome these hurdles in order to develop adenoviral vectors for combination of systemic oncolytic virotherapy and NIS-mediated radiotherapy of mice bearing HCC xenografts. Based on the dual function of the NIS gene encoded by our adenovirus as reporter and therapy gene, at first we investigated its potential for non-invasive imaging of vector biodistribution and transgene expression of our targeted and shielded adenovirus by molecular imaging. Furthermore, the potential of stimulation of therapeutic efficacy of adenovirus-mediated oncolysis was investigated by subsequent combination with systemic NIS-mediated radiotherapy (radiovirotherapy).

To further improve safety, shielding and targeting of the surface-modified adenovirus vectors, we physically coated replication-selective adenoviruses carrying

the NIS gene with a conjugate consisting of cationic poly(amidoamine) (PAMAM) dendrimer linked to the peptidic, epidermal growth factor receptor-specific ligand GE11. We investigated the potential improvement of safety and transduction efficacy *in vitro* and subsequently analyzed the specificity and biodistribution of functional NIS expression as well as the therapeutic efficacy of oncolytic virotherapy in combination with ^{131}I after systemic, EGFR-targeted NIS gene delivery.

Another critical issue that has to be taken into consideration during preclinical evaluation of therapeutic approaches is the possibility of limited validity and clinical transferability of data gained in xenograft animal models. Despite outstanding effectiveness of a series of compounds *in vitro* and in xenograft models *in vivo*, the results of clinical trials are sometimes conflicting. Therefore, we used recently characterized non-viral gene delivery vehicles developed in Manfred Ogris' laboratory for NIS gene delivery in a genetically engineered mouse model of pancreatic cancer, which may be better suited to adequately reflect the clinical situation.

2. Chapter 1

Sodium iodide symporter (NIS)-mediated radiovirotherapy of hepatocellular cancer using a conditionally replicating adenovirus

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2.1 Abstract

In the present study we determined the in vitro and in vivo efficacy of sodium iodide symporter (NIS) gene transfer and the therapeutic potential of oncolytic virotherapy combined with radioiodine therapy using a conditionally replicating oncolytic adenovirus. For this purpose we used a replication-selective adenovirus in which the E1a gene is driven by the mouse alpha-fetoprotein promoter and the human NIS gene is inserted in the E3 region (Ad5-E1/AFP-E3/NIS). Human hepatocellular carcinoma cells (HuH7) infected with Ad5-E1/AFP-E3/NIS concentrated radioiodine at a level that was sufficiently high for a therapeutic effect in vitro. In vivo experiments demonstrated that 3 days after intratumoral injection of Ad5-E1/AFP-E3/NIS HuH7 xenograft tumors accumulated approximately 25% ID/g ^{123}I as shown by ^{123}I gamma camera-imaging. A single intratumoral injection of Ad5-E1/AFP-E3/NIS (virotherapy) resulted in a significant reduction of tumor growth and prolonged survival, as compared to injection of saline. Combination of oncolytic virotherapy with radioiodine treatment (radiovirotherapy) led to an additional reduction of tumor growth that resulted in markedly improved survival as compared to virotherapy alone. In conclusion local in vivo NIS gene transfer using a replication-selective oncolytic adenovirus is able to induce a significant therapeutic effect, which can be enhanced by additional ^{131}I application.

2.2 Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer worldwide and the third leading cause of cancer deaths in the world with an increasing incidence in the western world (Forner *et al.*, 2012; Siegel *et al.*, 2012). Due to limited response to conventional radio- or chemotherapy, surgery including partial hepatectomy or liver transplantation is the only potentially curative therapy that is currently available. Despite the development of various alternative therapeutic options, such as kinase inhibitors, the prognosis of patients suffering from advanced HCC is still poor (Cao *et al.*, 2012). Consequently the development of novel therapeutic strategies is indispensable.

The sodium iodide symporter (NIS) is an intrinsic transmembrane glycoprotein that mediates the uptake of iodide into thyroid follicular cells (Carrasco, 1993; Jhiang *et al.*, 1998). Due to its expression in differentiated thyroid cancer cells, NIS represents the molecular basis for the diagnostic and therapeutic application of radioiodine, which has been successfully used for over 70 years in the treatment of thyroid cancer patients. In the last 15 years NIS has been identified as a novel promising therapeutic gene for the treatment of extrathyroidal tumors by directed NIS gene transfer into tumor cells followed by diagnostic and therapeutic application of radioiodine. The capacity of the NIS gene to induce radioiodine accumulation in non-thyroidal tumors has been investigated in a variety of tumor models by several groups including our own (Hingorani *et al.*, 2010; Klutz *et al.*, 2009; Klutz *et al.*, 2011a; Richard-Fiardo *et al.*, 2011; Scholz *et al.*, 2005; Spitzweg *et al.*, 2000; Willhauck *et al.*, 2008b). These data clearly demonstrate the potential of NIS as a novel reporter and therapy gene for the treatment of extrathyroidal tumors.

To ensure tumor specificity of radiation exposure, the application of tumor-specific promoters offers the ability to transcriptionally target NIS gene expression exclusively to tumor cells. Alpha-fetoprotein (AFP) is only expressed in the yolk sac and liver of mammals during embryonic development and nearly disappears after birth. Reinitiation of AFP expression during neoplastic transformation in HCCs and teratocarcinomas provides us with a well characterized and frequently used tumor marker (Chan *et al.*, 1986; Johnson, 1999). Due to its high specificity, the AFP promoter is an ideal tool for transcriptional targeting of gene delivery for the treatment of HCCs (Watanabe *et al.*, 1987).

The current study was developed as a result of the successful induction of

tumor-specific iodide uptake activity in a xenograft mouse model of hepatocellular carcinoma (HepG2) after local infection with a replication-deficient adenovirus carrying the human NIS gene linked to the mouse AFP promoter (Klutz *et al.*, 2011c). With the goal of a possible future systemic application, as a next step we aimed at extending this promising technique to a replication-selective oncolytic adenovirus using the same AFP promoter construct for transcriptional targeting of NIS expression. The use of genetically engineered replication-competent adenoviruses has emerged as a powerful approach for increasing transduction efficiency and therapeutic efficacy by an additional oncolytic effect due to cancer-selective virus replication. We therefore examined the feasibility of oncolytic virotherapy following replication-selective adenovirus-mediated human NIS gene transfer in a HCC xenograft mouse model. In the newly developed Ad5-E1/AFP-E3/NIS construct the E1a gene, which is essential for viral replication, is driven by the HCC-specific AFP promoter resulting in tumor-specific replication. Since the NIS gene is inserted in the E3 region under control of the replication-dependent E3 promoter, NIS expression only occurs in HCC cells, where adenoviral replication takes place. We further evaluated, if tumor-specific NIS expression allows for enhancement of the therapeutic effect of virus-mediated oncolysis through additional radioiodine therapy (radiovirotherapy).

2.3 Materials and Methods

Cell culture

The human HCC cell line HuH7 (JCRB 0403) was cultured in DMEM/F12 medium (Invitrogen Life Technologies Inc., Karlsruhe, Germany) supplemented with 10% fetal bovine serum (v/v) (PAA, Colbe, Germany), 5% L-glutamine (Invitrogen Life Technologies Inc.) and 1% penicillin/streptomycin (v/v) (Invitrogen Life Technologies Inc.) and the human melanoma cell line 1205 Lu (kindly provided by Meenhard Herlyn, The Wistar Institute, Philadelphia, PA, USA) was grown in MCDB 153 medium (Invitrogen Life Technologies Inc.) supplemented with 20% Leibovitz´s L-15 medium (v/v) (Invitrogen Life Technologies Inc.), 10% fetal bovine serum (v/v), 5 µg/ml insulin (Sigma, Munich, Germany) and 1% penicillin/streptomycin (v/v). The human follicular thyroid carcinoma cell line FTC-133 (kindly provided by Björn E. Wenzel, University of Lübeck, Lübeck, Germany) was grown in DMEM/F12 medium supplemented with 10% fetal bovine serum (v/v) and 1% penicillin/streptomycin (v/v). Cells were maintained at 37°C and 5% CO_2 in an incubator with 95% humidity. Cell culture medium was replaced every second day and cells were passaged at 85% confluency.

Recombinant replication-selective adenovirus production

The human NIS cDNA (kindly provided by Sissy M Jhiang, Ohio State University, Columbus, OH, USA) was cloned into the shuttle vector (pVQAd-AscI-NpA) and linked to the mouse AFP promoter/enhancer I fragment (kindly provided by M. Geissler, Esslingen, Germany) using *Kpn I* and *Xho I*. A replication-competent human recombinant type 5 adenovirus (Ad5-E3) carrying the human NIS gene linked to the *m*AFP basal promoter/enhancer element I (Ad5-E1/AFP-E3/NIS) was developed by ViraQuest Inc. (North Liberty, IA, USA). As control, a replication-deficient adenovirus carrying the NIS cDNA under the control of the tumor specific AFP promoter (Ad5-AFP/NIS), generated as described previously, was used (Klutz *et al.*, 2011c). As further control an empty adenovirus (Ad5-control) was used.

Adenovirus-mediated NIS gene delivery in vitro

For *in vitro* infection experiments, HuH7, 1205 Lu and FTC-133 cells ($1.5x10^5$ cells/well in 12-well plates) were washed and incubated with 25 MOI (multiplicity of infection)/well of Ad5-E1/AFP-E3/NIS or with control virus in OptiMEM medium

(Invitrogen Life Technologies Inc.) for 45 min at 37°C and 5% CO_2. Plates were slightly shaken every 15 min to ensure an equal distribution of the virus. Medium was replaced by fresh culture medium and virus-infected cells were further maintained for up to 4 days, before iodide accumulation was measured (see below) to determine levels of functional NIS protein expression.

^{125}I uptake studies in vitro

Following infection with Ad5-E1/AFP-E3/NIS or Ad5-control, iodide uptake of HuH7 or control cells was determined at steady-state conditions as described previously (Spitzweg *et al.*, 1999; Weiss *et al.*, 1984). Results were normalized to cell viability and expressed as cpm/A 490 nm.

Cytopathic effect (CPE) assay

HuH7 or FTC-133 cells were seeded in 12-well plates ($1.5x10^5$ per well). Cells were infected with increasing doses (0, 1, 5, 10, 25, 50 MOI) for 45 min with replication-selective Ad5-E1/AFP-E3/NIS or replication-deficient Ad5-AFP/NIS. Cells were fixed after 4 days with 10% TCA over night at 4°C and stained with 0.5% sulforhodamine B (SRB, Sigma-Aldrich, Darmstadt, Germany) in 1% acetic acid. Quantification was done by photometric measurement at 590 nm after dissolving dried SRB with 10 mM tris buffer at pH 8.

Cell viability assay

Cell viability was measured using the commercially available MTS-assay (Promega Corp., Mannheim, Germany) according to the manufacturer's recommendations as described previously (Unterholzner *et al.*, 2006).

Analysis of NIS mRNA expression and fiber DNA level using quantitative real-time PCR

After infection with 25 MOI Ad5-E1/AFP-E3/NIS or Ad5-control total RNA was isolated from HuH7 xenografts using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The level of NIS mRNA was analyzed via quantitative real-time PCR (qPCR) as described previously (Klutz *et al.*, 2009).

After infection with Ad5-E1/AFP-E3/NIS or Ad5-AFP/NIS total DNA was

isolated from HuH7 and FTC-133 cells using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's recommendations. Quantitative real-time PCR was performed with 100 ng DNA using the SYBR green PCR master mix (Qiagen) in a Rotor Gene 6000 (Corbett Research, Mortlake, New South Wales, Australia). Following primers were used: fiber-fw (5`-AAGCTAGCCCTGCAAACATCA-3`) and fiber-rev (5`-CCCAAGCTACCAGTGGCAGTA-3`). Cycling conditions started with initial enzyme activation at 95°C for 15 min, followed by 40 cycles of 15 sec denaturation at 95°C, 15 sec annealing at 55°C, and 15 sec elongation at 72°C.

In vitro clonogenic assay

HuH7 cells were infected with 25 MOI of Ad5-E1/AFP-E3/NIS as described above. After 48 h the cells were incubated for 7 h with increasing doses (7.4 MBq, 14.8 MBq and 29.6 MBq) of ^{131}I in HBSS (Invitrogen Life Technologies Inc.) supplemented with 10 µmol/l NaI and 10 mmol/l HEPES (pH 7.3) at 37°C. After incubation with ^{131}I, the HuH7 cells were detached by incubation with 0.05% trypsin/0.02% EDTA in PBS for 10 min at 37°C. The HCC cells were then plated at cell densities of 250, 500 and 1000 cells/well in 12-well plates. Two weeks later, after colony development, cells were fixed with methanol, stained with crystal violet, and HCC colonies containing more than 50 cells were counted. Parallel experiments were performed using HBSS without ^{131}I or with uninfected cells, respectively and all values were adjusted for plating efficiency. The percentage of survival represents the percentage of cell colonies after ^{131}I treatment, compared with mock treatment with HBSS.

Establishment of HuH7 xenografts

HuH7, FTC-133 and 1205 Lu xenografts were established in 5 weeks old female CD-1 nu/nu mice (Charles River, Sulzfeld, Germany) by subcutaneous injection of 5 x 10^6 HuH7, 1.5 x 10^6 FTC-133 or 5 x 10^6 1205 Lu cells suspended in 100 µl PBS into the flank region. Animals were maintained under specific pathogen-free conditions with access to mouse chow and water *ad libitum*. The experimental protocol was approved by the regional governmental commission for animals (Regierung von Oberbayern, Munich, Germany).

In vivo NIS gene transfer in HuH7 xenografts

Experiments started when tumors had reached a size of 5-6 mm. After a 10-day pretreatment with L-T4 (L-thyroxin Henning, Sanofi-Aventis, Frankfurt, Germany) (5 mg/l) in their drinking water to reduce radioiodide uptake by the thyroid gland, animals were anesthetized with ketamin (Hameln pharmaceuticals, Hameln, Germany) (100 µg/g) and xylazine 2% (v/v) (Bayer, Leverkusen, Germany) (10 µg/g). Thereafter, 5 x 10^8 PFU (1.83 x 10^{10} vp), diluted with PBS to a total volume of 100 µl) of the recombinant Ad5-E1/AFP-E3/NIS were injected at different injection sites directly into the tumor using tuberculin syringes with a 30-gauge x 0.5-inch needle. The needle was moved to various sites within the tumor during injection to maximize the area of virus exposure.

Radioiodine uptake studies in vivo after local NIS gene transfer

On days 3, 4 and 7 after intratumoral injection of Ad5-E1/AFP-E3/NIS or Ad5-control mice received 18.5 MBq ^{123}I intraperitoneally (i.p.) and radioiodine distribution was monitored by serial imaging on a gamma camera (Forte, ADAC Laboratories, Milpitas, CA, USA) equipped with a VXHR (Ultra High Resolution) collimator as described previously (Willhauck *et al.*, 2007). The control mice were injected i.p. with 2 mg of the competitive NIS-inhibitor sodium perchlorate 30 min before ^{123}I administration. Regions of interest were quantified and expressed as a fraction of the total amount of applied radionuclide per gram tumor tissue. The retention time within the tumor was determined by serial scanning after radioiodine injection, and dosimetric calculations were performed according to the concept of MIRD, with the dosis factor of RADAR-group (www.doseinfo-radar.com).

Radionuclide therapy study in vivo

Subcutaneous HuH7 xenografts were established in four groups of mice as described above. When tumors reached about 5-6 mm in diameter, one group of mice was used as saline-injected control (NaCl-control, n=12), a second group received a single intratumoral dose of Ad5-E1/AFP-E3/NIS at 5 x 10^8 PFU (virotherapy, n=14), the third group received a single intratumoral dose of Ad5-E1/AFP-E3/NIS at 5 x 10^8 PFU and 3 days later a single intraperitoneal dose of 55.5 MBq (1.5 mCi) ^{131}I (radiovirotherapy 1, n=16), and the fourth group received a single intratumoral dose of Ad5-E1/AFP-E3/NIS at 5 x 10^8 PFU and two intraperitoneal doses of 55.5 MBq (1.5 mCi) ^{131}I each on day 3 and 5 after virus administration

(radiovirotherapy 2, n=12). Tumor measurements started at the day of virus administration and were performed twice weekly thereafter, tumor volume was estimated using the equation: tumor volume = length × width × height × 0.52. Mice were followed for a total of 100 days or until tumor burden was such that animals had to be killed. The end point event was set at tumor burden ≥ 1500 mm^3.

Immunohistochemical analysis of NIS protein expression

Immunohistochemical staining of paraffin embedded tissue sections derived from HuH7 tumors after adenovirus-mediated gene delivery was performed using a mouse monoclonal antibody directed against amino acid residues 468–643 of human NIS (kindly provided by John C. Morris, Mayo Clinic, Rochester, MN, USA) as described previously (Spitzweg *et al.*, 2007). For histological examination parallel slides were also routinely stained with hematoxylin and eosin.

Indirect immunofluorescence assay

Indirect immunofluorescence staining was performed on frozen tissues using an antibody against human Ki67 (Abcam, Cambridge, UK) and an antibody against mouse CD31 (BD Pharmingen, Heidelberg, Germany) as described previously (Willhauck *et al.*, 2007).

Statistical methods

All *in vitro* experiments were carried out in triplicates. Results are represented as means +/- SD of triplicates. Statistical significance was tested using Student's t-test. Statistical significance of survival curves was tested using logrank test.

2.4 Results

Iodide uptake studies in vitro

Transduction conditions using Ad5-E1/AFP-E3/NIS were optimized in HuH7 cells by measurement of perchlorate-sensitive iodide uptake activity. At a dose of 25 MOI we achieved highest transduction efficiency at low cytotoxicity, which was used for all subsequent *in vitro* experiments (data not shown). The perchlorate-sensitive iodide uptake activity was measured at various time points after Ad5-E1/AFP-E3/NIS infection (Fig. 1A). Maximum iodide uptake activity was observed 3 days following infection, when cells showed a 79-fold increase in perchlorate-sensitive ^{125}I accumulation as compared to HuH7 cells infected with the control virus (Ad5-control) (***P < 0.001; Fig. 1A). Tumor specificity of Ad5-E1/AFP-E3/NIS was confirmed by infection of control cancer cell lines (FTC-133, 1205 Lu) not expressing AFP showing lack of perchlorate-sensitive iodide uptake activity (Fig. 1B).

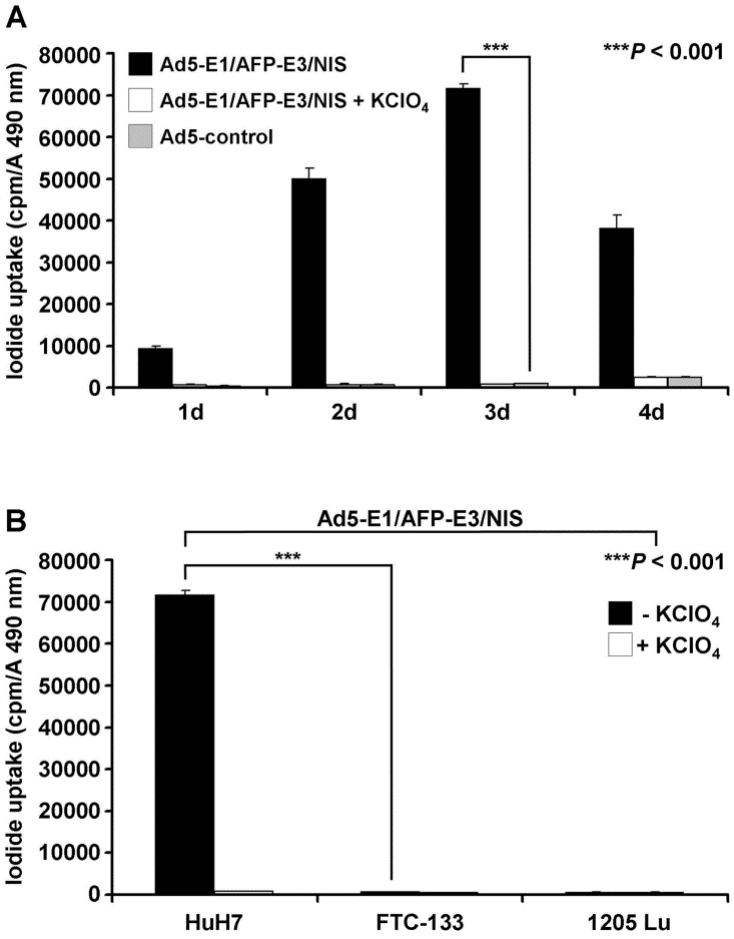

Fig. 1: (**A**) Kinetics of NIS-mediated ^{125}I uptake. HuH7 cells were infected with 25 MOI Ad5-E1/AFP-E3/NIS or Ad5-control and ^{125}I uptake was measured on days 1, 2, 3 and 4. Maximum iodide uptake with low toxicity was observed 3 days following infection with Ad5-E1/AFP-E3/NIS, when cells showed an eightfold increase in perchlorate-sensitive uptake activity as compared with day 1 after infection

(***P<0.001). (**B**) Specificity of NIS-mediated ^{125}I uptake. HuH7 cells infected with Ad5-E1/AFP-E3/NIS (25 MOI) showed a 79-fold increase in perchlorate-sensitive ^{125}I accumulation. In contrast, no iodide uptake above background level was observed in HuH7 cells infected with Ad5-control or in control cancer cells (FTC-133 and 1205 Lu) infected with Ad5-E1/AFP-E3/NIS (***P<0.001). cpm, counts per minute; d, day.

Analysis of tissue-selective viral replication in vitro

Quantitative real-time PCR (qPCR) analysis revealed a strong nearly 50,000-fold increase of fiber DNA level in HuH7 cells 3 days after infection with 25 MOI Ad5-E1/AFP-E3/NIS as compared to infection of AFP-negative control cells or HuH7 cells infected with the replication-deficient Ad5-AFP/NIS, which showed no significant increase of fiber DNA level (****P* < 0.001; Fig. 2A).

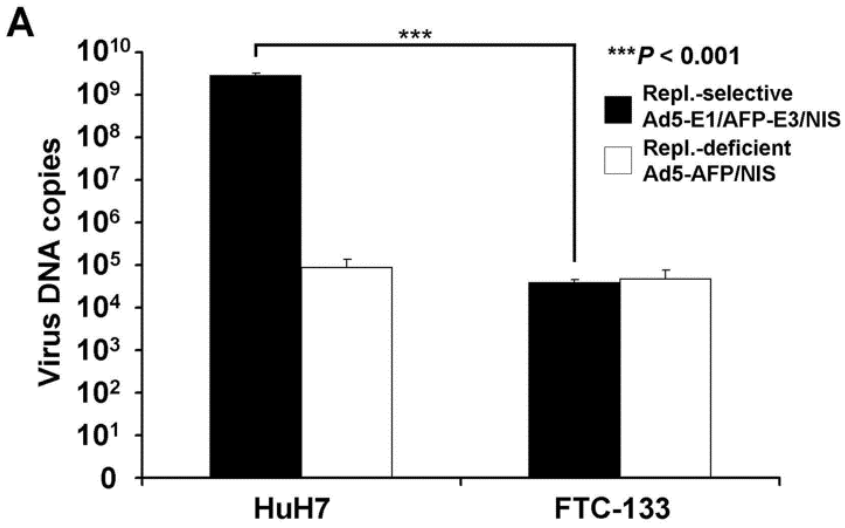

Fig. 2: (**A**) Tissue-specific replication of Ad5-E1/AFP-E3/NIS. Analysis of fiber protein DNA level in HuH7 and FTC-133 cells 3 days after infection with replication-selective Ad5-E1/AFP-E3/NIS or replication-deficient Ad5-AFP/NIS. HuH7 cells showed a 10 000-fold increase in fiber protein DNA level, as compared with AFP-negative FTC-133 cells. In contrast, replication-deficient adenovirus showed no increase in fiber protein DNA level (***P<0.001).

Induction of cytopathic effect (CPE)

To investigate whether Ad5-E1/AFP-E3/NIS and Ad5-AFP/NIS induce CPE in HCC and control cell lines, a CPE assay was performed. As shown in Figure 2B, Ad5-E1/AFP-E3/NIS caused strong cytolysis of infected HuH7 cells at day 4 in a dose dependent manner (upper panel). In contrast, no CPE could be detected in Ad5-E1/AFP-E3/NIS-infected AFP-negative FTC-133 control cells (lower panel) or in HuH7 cells after infection with the replication-deficient Ad5-AFP/NIS virus.

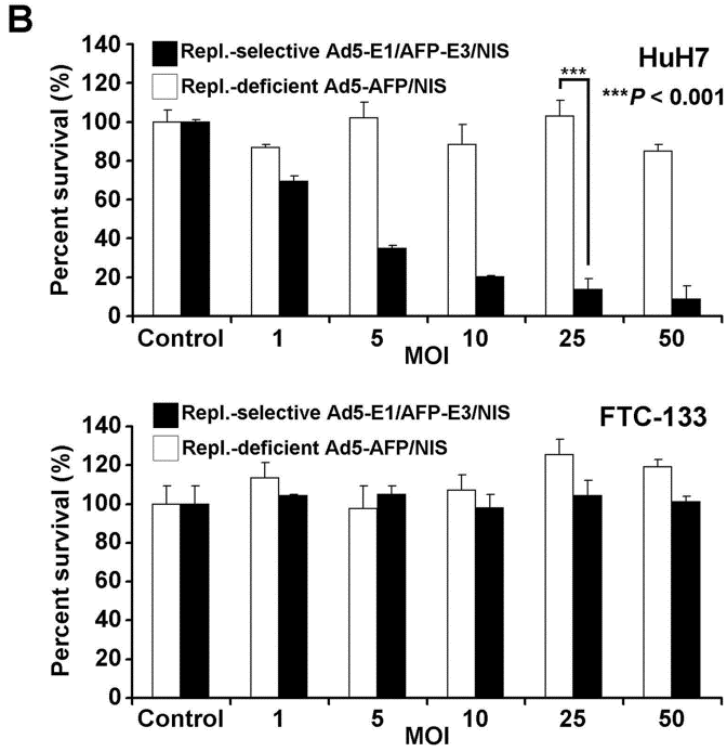

Fig. 2: (**B**) Tissue-specific CPE of Ad5-E1/AFP-E3/NIS. Assessment of AFP-dependent, virus-mediated cell killing, 4 days after infection with different MOI of Ad5-E1/AFP-E3/NIS using a CPE assay. Strong cytolysis was observed in HuH7 cells (upper panel), as opposed to AFP-negative FTC-133 cells (lower panel) or HuH7 cells infected with the replication-deficient adenovirus, thereby demonstrating replication selectivity of the vector (***P<0.001).

In vitro clonogenic assay

To evaluate the therapeutic potential of ^{131}I in hepatoma cells (HuH7) after Ad5-E1/AFP-E3/NIS-mediated NIS gene transfer *in vitro*, a clonogenic assay was performed at increasing doses (7.4 MBq, 14.8 MBq and 29.6 MBq) of ^{131}I (Fig. 3). 100% of uninfected hepatoma cells survived the exposure to 29.6 MBq ^{131}I, while up to 90% of Ad5-E1/AFP-E3/NIS-infected hepatoma cells were killed by the treatment with ^{131}I in a dose-dependent manner (***P < 0.001). Without radioiodine treatment, uninfected hepatoma cells showed survival rates comparable to those of Ad5-E1/AFP-E3/NIS-infected cells 48 h after infection. This indicates that at this time point no major cell lysis has occurred and therefore survival of the cells was not dependent on viral infection in this experimental setup.

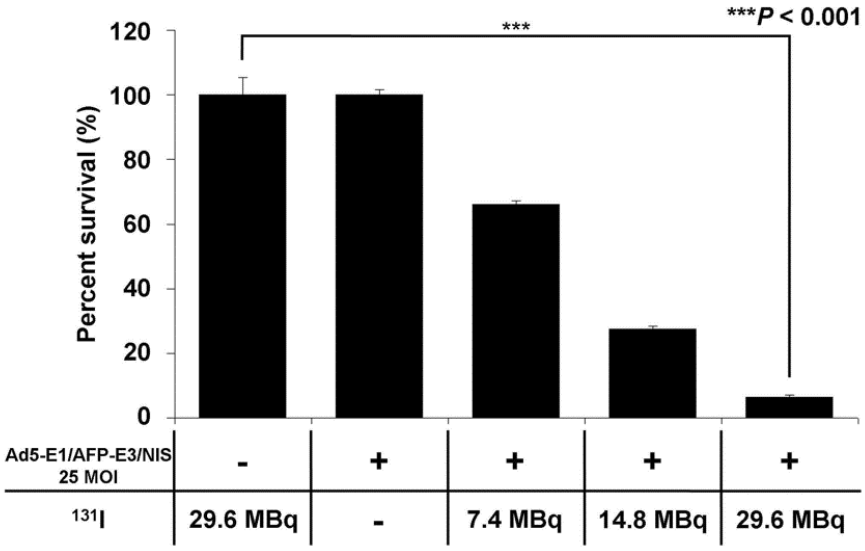

Fig. 3: *In vitro* clonogenic assay. For evaluation of the therapeutic effect of ^{131}I *in vitro* HuH7 cells infected with Ad5-E1/AFP-E3/NIS were exposed to 7.4, 14.8 or 29.6 MBq ^{131}I. While 100% of the cells incubated with 29.6 MBq ^{131}I only or cells treated with the adenovirus only survived, up to 90% of Ad5-E1/AFP-E3/NIS-infected cells were killed by radioiodine in a dose-dependent manner (***P<0.001).

Radioiodine uptake studies after local in vivo NIS gene transfer

Radioiodine biodistribution was monitored in tumor bearing mice on days 3, 4 and 7 after intratumoral (i.t.) injection of 5 x 10^8 PFU (1.83 x 10^{10} virus particles (vp)) Ad5-E1/AFP-E3/NIS using a gamma camera to determine propagation of the virus and the peak of adenoviral spread by *in vivo* imaging of NIS expression. Highest transduction efficiency was observed 3 days after single intratumoral injection of the replication-selective adenovirus (Fig. 4A). Thus, all following imaging experiments were carried out 3 days after adenoviral infection.

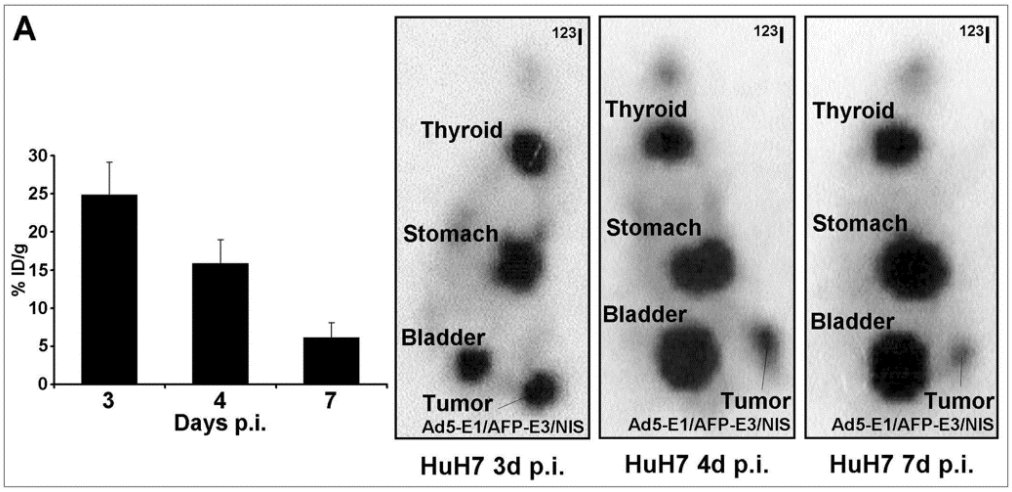

Fig. 4: ^{123}I uptake studies *in vivo*. Scans of nude mice bearing xenografts, show the kinetics of NIS expression 3, 4 and 7 days following i.t. injection of 5x10^8 PFU Ad5-E1/AFP-E3/NIS (**A**).

Although no specific radioiodide accumulation was detected in HuH7 tumors after infection with Ad5-control (Fig. 4B), Ad5-E1/AFP-E3/NIS-infected HuH7 tumors (Figure 4A) showed a significant time-dependent uptake of ^{123}I with a maximum at 3 days after virus injection. As determined by serial scanning, 25% ID/g (percentage of the injected dose per gram tumor tissue) ^{123}I were accumulated 1 h post injectionem (p.i.) in NIS-transduced xenograft tumors with an effective half-life of 5.5 h (Fig. 4A). Considering a tumor mass of 1g and an effective half-life of 3.6 h for ^{131}I, a tumor absorbed dose of 126 mGy/MBq was calculated. In addition to tumoral uptake, significant radioiodide accumulation was observed in tissues physiologically expressing NIS, including stomach and thyroid, and tissues involved in iodide elimination (bladder).

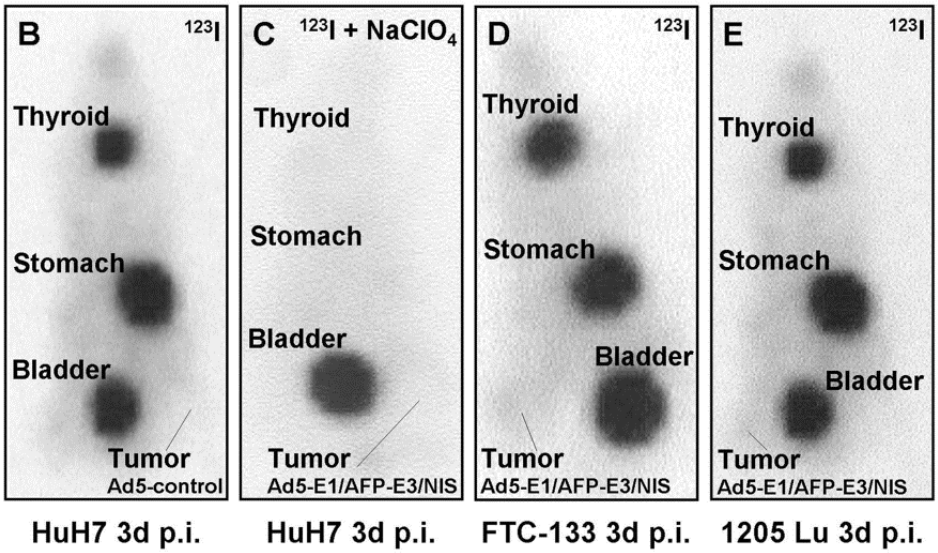

Fig. 4: Three days after injection with Ad5-E1/AFP-E3/NIS (**A**, **C**–**E**) or Ad5-control (**B**), Ad5-E1/AFP-E3/NIS-infected HuH7 tumors showed an ^{123}I uptake of 25% ID g^{-1}, which was completely abolished on pre-treatment with $NaClO_4$ (**C**), whereas Ad5-control-infected HuH7 tumors showed no significant ^{123}I uptake (**B**). In contrast, FTC-133 (**D**) and 1205 Lu (**E**) control xenografts infected with Ad5-E1/AFP-E3/NIS showed no tumoral iodide accumulation. d, day, p.i., post injection.

To confirm that tumoral iodide uptake was indeed NIS-mediated, Ad5-E1/AFP-E3/NIS-injected mice were additionally treated with the competitive NIS-inhibitor sodium perchlorate ($NaClO_4$) 30 min before ^{123}I administration, which completely blocked tumoral iodide accumulation in addition to the physiological NIS-mediated iodide uptake in stomach and thyroid gland (Fig. 4C). Furthermore, hepatoma-specificity of Ad5-E1/AFP-E3/NIS was confirmed by infection of control tumor

xenografts (FTC-133, 1205 Lu), which did not result in tumoral iodide uptake activity (Fig. 4D, E).

Analysis of NIS mRNA expression in HuH7 xenografts

The mRNA of tumors was analyzed for the level of NIS mRNA expression after intratumoral Ad5-E1/AFP-E3/NIS-mediated NIS gene transfer *in vivo* by quantitative real-time PCR (qPCR) (Fig. 5). A 29-fold increase in NIS mRNA expression of HuH7 xenografts was detected 72 h after intratumoral injection of Ad5-E1/AFP-E3/NIS (***P < 0.001). In contrast, no significant NIS mRNA expression above background level was observed in untreated tumors or in tumors treated with the control adenovirus (Ad5-control).

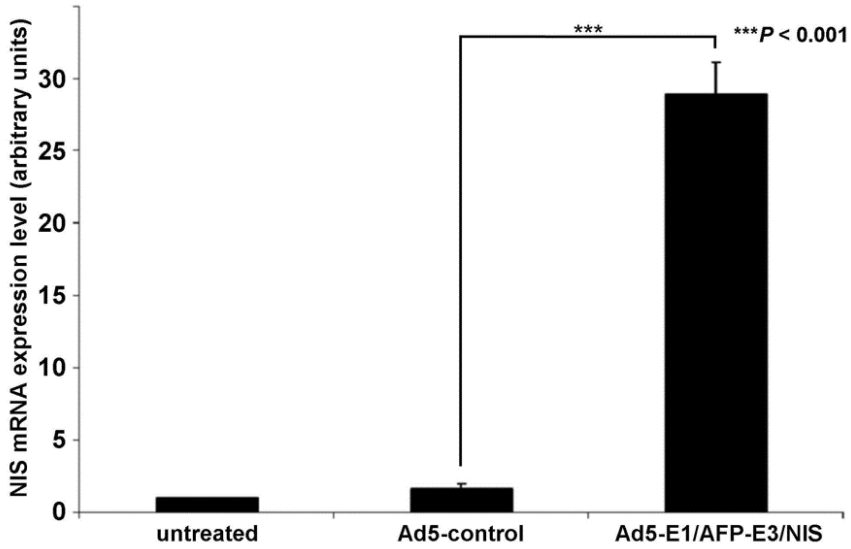

Fig. 5: Analysis of NIS mRNA expression in HuH7 xenografts. A significant increase in the NIS mRNA level was observed after i.t. injection of Ad5-E1/AFP-E3/NIS. In contrast, no significant NIS expression above background level was found in tumors after infection with Ad5-control or in untreated tumors (***P<0.001).

Radionuclide therapy study in vivo

All saline treated tumors continued their extensive growth throughout the observation period (Fig. 6A). In contrast, a single intratumoral injection of 5 x 10^8 PFU (1.83 x 10^{10} vp) Ad5-E1/AFP-E3/NIS (virotherapy) resulted in a significant reduction of tumor growth with prolonged survival of virus-injected mice (Fig. 6A, B). A single i.p. injection of 55.5 MBq ^{131}I 72 h after i.t. injection of Ad5-E1/AFP-E3/NIS (radiovirotherapy 1) led to an additional slowdown of tumor growth (Fig. 6A), that resulted in markedly improved survival as compared to virotherapy alone (Fig. 6B).

Two i.p. injections of 55.5 MBq ^{131}I on days 3 and 5 after Ad5-E1/AFP-E3/NIS treatment (radiovirotherapy 2) were able to further reduce tumor growth of HuH7 xenografts (Fig. 6A) with further extension of survival of mice (Fig. 6B). While all mice in the control group had to be killed within the first 3 weeks after onset of the experiments due to excessive tumor growth, 92% of mice treated twice with ^{131}I after local *in vivo* NIS gene transfer survived approx. 7-8 weeks (Fig. 6B). Taken together, these results indicate that the combination of radiotherapy and cytolytic virotherapy was significantly superior to virotherapy alone (***P<0.001). No mouse showed major adverse effects after virus or radionuclide administration in terms of body weight loss, lethargy or respiratory failure.

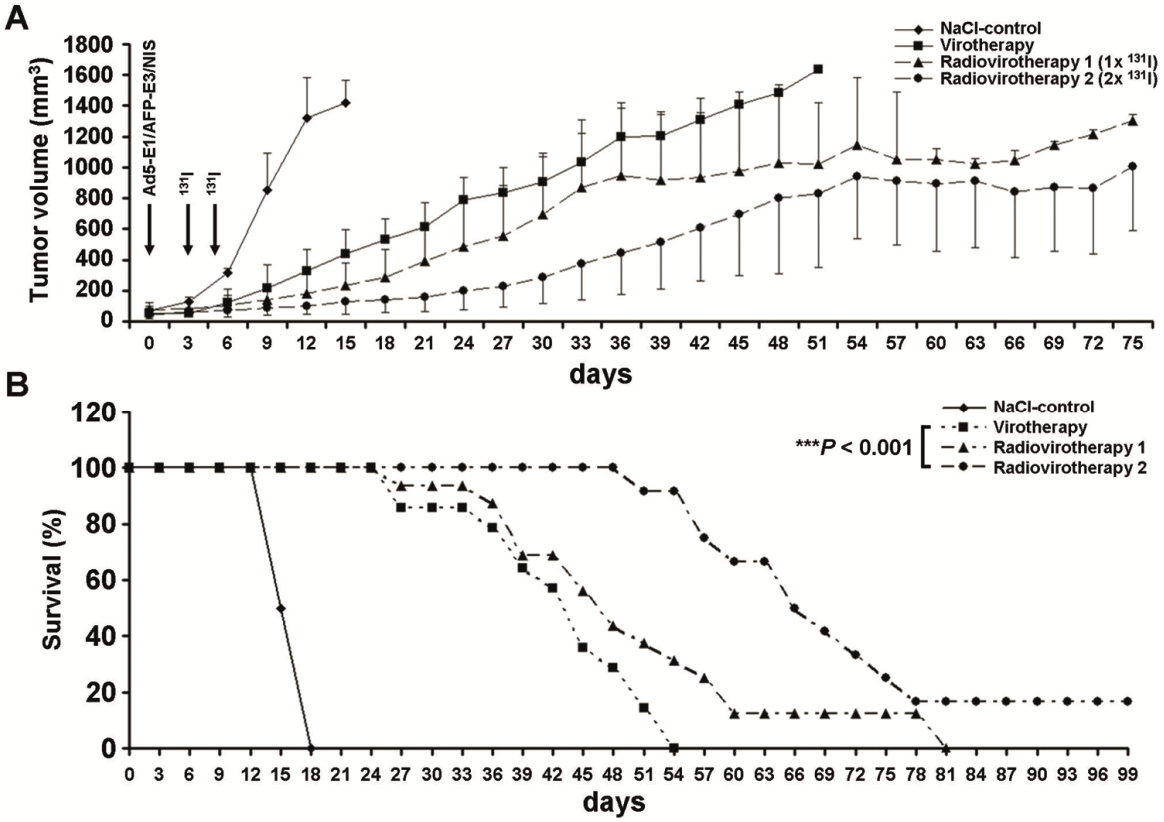

Fig. 6: (**A**, **B**) Therapy studies of mice bearing HuH7 tumor xenografts. Injection of a single i.t. dose of Ad5-E1/AFP-E3/NIS (virotherapy) resulted in a significant delay in tumor growth (**A**) that was associated with markedly improved survival (**B**) as compared with the control group (NaCl-control) that was injected with saline only. A single i.t. dose of Ad5-E1/AFP-E3/NIS followed by either a single i.p. dose of 55.5 MBq ^{131}I 3 days later (radiovirotherapy 1) or by two i.p. doses of 55.5 MBq ^{131}I on days 3 and 5 post injectionem (radiovirotherapy 2) further significantly decreased tumor growth (**A**) and enhanced overall survival (**B**).

Immunohistochemical analysis of NIS protein expression in HuH7 xenografts

Three days after the start of treatment mice were sacrificed and HuH7 xenografts were dissected and processed for immunohistochemical analysis using a *h*NIS specific antibody (red). Analysis revealed a patchy staining pattern with areas of NIS-specific immunoreactivity in tumors after intratumoral application of Ad5-E1/AFP-E3/NIS (Fig. 6C). In contrast, tumors treated with saline only (NaCl-control) showed no NIS-specific immunoreactivity (Fig. 6D). Parallel control slides with the primary and secondary antibodies replaced in turn by PBS and isotype-matched non immune immunoglobulin were negative (data not shown).

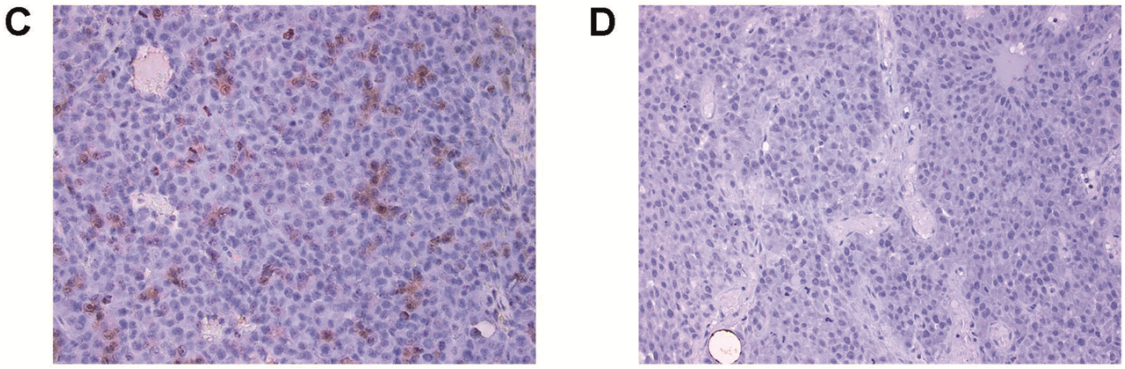

Fig. 6: (**C**, **D**) Immunohistochemical staining of HuH7 tumors 3 days after (**C**) Ad5-E1/AFP-E3/NIS application using a *h*NIS-specific antibody showed clusters of NIS-specific immunoreactivity. In contrast, HuH7 tumors treated with saline only (**D**) did not reveal NIS-specific immunoreactivity. Magnification: x20.

Immunofluorescence analysis

Eight days after the start of treatment mice were sacrificed and tumors were dissected and processed for immunofluorescence analysis using a Ki67-specific antibody (green) and an antibody against CD31 (red, labeling blood vessels) (Fig. 6E, F). Ad5-E1/AFP-E3/NIS-treated tumors (Fig. 6E) exhibited a significantly lower intratumoral blood vessel density and proliferation index after ^{131}I therapy when compared to saline-treated tumors (Fig. 6F).

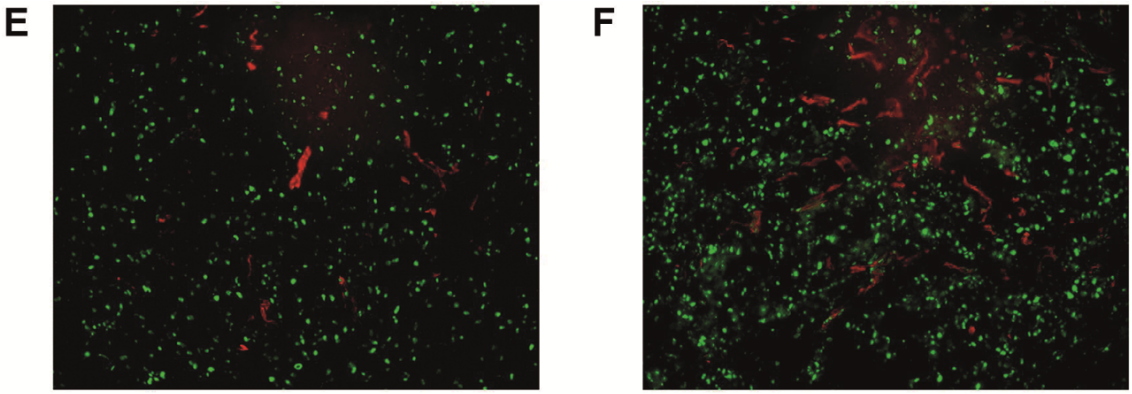

Fig. 6: (**E**, **F**) Immunofluorescence analysis using a Ki67-specific antibody (green) and an antibody against CD31 (red, labeling blood vessels) showed significantly decreased proliferation and blood vessel density in (**E**) NIS-transduced tumors following radiovirotherapy (Ad5-E1/AFP-E3/NIS + ^{131}I treatment) as compared with (**F**) saline-treated tumors. Magnification: x100.

2.5 Discussion

Our previous work in various tumor models has convincingly demonstrated the high efficacy of radionuclide therapy after tumor-selective NIS gene delivery (Kakinuma *et al.*, 2003; Klutz *et al.*, 2009; Klutz *et al.*, 2011a; Klutz *et al.*, 2011b; Klutz *et al.*, 2011c; Spitzweg *et al.*, 2007; Spitzweg *et al.*, 2001a). In its dual role as reporter and therapy gene NIS allows direct, non-invasive imaging of functional NIS expression by ^{123}I-scintigraphy, ^{123}I-SPECT-imaging or ^{124}I-PET-imaging, as well as exact dosimetric calculations before proceeding to therapeutic application of ^{131}I or alternative radionuclides (Dingli *et al.*, 2003b; Hingorani *et al.*, 2010; Spitzweg and Morris, 2002).

Tissue-specific promoters may serve as targeting mechanisms for gene expression and have been described to mediate tissue-specific expression, also in the context of NIS gene therapy of different types of cancer, thereby introducing cancer specificity for improved safety and efficacy (Cengic *et al.*, 2005; Klutz *et al.*, 2011c; Scholz *et al.*, 2005; Spitzweg *et al.*, 2007; Spitzweg *et al.*, 2001a; Spitzweg *et al.*, 2000; Spitzweg *et al.*, 1999; Trujillo *et al.*, 2009; Trujillo *et al.*, 2010; Willhauck *et al.*, 2008c). In previous studies we already reported hepatoma-specific NIS expression after application of the alpha-fetoprotein (AFP) promoter (Klutz *et al.*, 2011c; Willhauck *et al.*, 2008c). To target NIS gene expression to HCC cells, we applied a 2666 bp mouse AFP promoter construct consisting of the basal promoter and enhancer I element that already proved maximal tissue specificity and promoter activity (Willhauck *et al.*, 2008c; Zhang *et al.*, 1992).

Species C adenoviruses are some of the best-studied viruses and most frequently used for oncolytic vectors (Russell, 2009). The anti-tumor efficacy described for oncolytic adenoviruses is promising and they have been reported to be remarkably safe in animals in efficacy studies, with no significant toxicity reported (Shirakawa, 2008; Toth *et al.*, 2010). A replication-deficient adenovirus vector carrying the NIS gene under control of the hepatoma-specific AFP promoter was able to induce tumor-specific functional NIS expression in AFP-positive hepatocellular carcinoma cells, which was sufficiently high to allow a cytoreductive response to accumulated ^{131}I *in vitro* as well as *in vivo* after local application (Klutz *et al.*, 2011c).

As a next crucial step towards clinical application we have now explored the combination of the two approaches of targeted oncolytic virotherapy and NIS-mediated radiotherapy to further increase transduction efficiency and therapeutic

efficacy. In the current study we therefore designed a replication-selective adenovirus Ad5-E1/AFP-E3/NIS carrying the NIS gene under the control of the same AFP promoter fragment that already proved high activity and tumor specificity in our former studies (Klutz *et al.*, 2011c; Willhauck *et al.*, 2008c). A prerequisite for functional activity of the NIS protein is its proper targeting to the cell membrane (Kaminsky *et al.*, 1993). This functional membrane-associated NIS protein expression after replication-selective adenoviral NIS gene transfer was confirmed by measurement of *in vitro* radioiodine uptake, which showed high transduction efficiency and tumor selectivity of Ad5-E1/AFP-E3/NIS with maximal transduction efficiency 72 h after virus application. The ability to concentrate high levels of radioiodine resulted in a significant therapeutic effect of ^{131}I in hepatoma cells *in vitro* which was confirmed by a clonogenic assay, performed with increasing doses of ^{131}I. Studies by Ma *et al.* and Klutz *et al.* previously reported efficient and specific cell killing of hepatoma cells *in vitro* using a NIS-expressing replication-deficient adenovirus under control of the AFP promoter combined with ^{131}I treatment (Klutz *et al.*, 2011c; Ma *et al.*, 2009).

We have further investigated application of the Ad5-E1/AFP-E3/NIS virus construct for *in vivo* delivery of the NIS gene in hepatocellular carcinoma xenografts. NIS as novel reporter and suicide gene offers the possibility of non-invasive monitoring of NIS expression by radioiodine imaging and thereby allows exact planning of the NIS gene therapy approach (Spitzweg and Morris, 2002). The timing of administration of the therapeutic radionuclide after oncolytic virus-mediated NIS gene transfer is crucial to improve therapeutic efficacy (Msaouel *et al.*, 2009; Peerlinck *et al.*, 2009; Penheiter *et al.*, 2010). In this context, molecular imaging of the level of Ad5-E1/AFP-E3/NIS-mediated NIS expression over time prior to administration of the therapeutic radionuclide provides essential information on the time point of maximal NIS expression and is a prerequisite for an optimized combination therapy strategy (Penheiter *et al.*, 2010). In these *in viv*o imaging experiments, HuH7 cell xenografts showed highest NIS expression 72 h after oncolytic virus administration. Other studies that used NIS-expressing replication-selective adenoviral vectors revealed comparable results and showed the peak of tumoral NIS expression to be 3 to 4 days after intratumoral injection of the adenovirus (Merron *et al.*, 2007; Trujillo *et al.*, 2010).

72 h following intratumoral injection of the replication-selective Ad5-E1/AFP-

E3/NIS, tumor-specific ^{123}I accumulation of approximately 25% ID/g with an effective half-life of 5.5 h was observed resulting in a tumor-absorbed dose of 126 mGy/MBq ^{131}I. In comparison, in our former study using a replication-deficient adenovirus carrying the NIS gene under control of the AFP promoter (Ad5-AFP/NIS) HepG2 xenografts accumulated approximately 14.5% ID/g ^{123}I with an effective half-life of 13 h and a tumor absorbed dose of 318 mGy/MBq ^{131}I was calculated (Klutz *et al.*, 2011c). Although we accumulated a higher amount of radioiodine the resulting tumor absorbed dose in our current study is lower, which is mainly due to the shorter half-life of the tumoral ^{123}I accumulation. A possible reason might be differences in virus dissemination and NIS expression patterns due to the oncolytic activity of the replication-selective adenovirus used in the current study. A further explanation might be differences in tumor biology as different HCC cell lines were used in both studies.

Therapy studies after intratumoral injection of Ad5-E1/AFP-E3/NIS in HuH7 xenografts resulted in distinct oncolytic activity leading to a significant delay of tumor growth associated with significantly prolonged survival as compared to the saline-treated control group. Despite the distinct effect of Ad5-E1/AFP-E3/NIS for oncolytic virotherapy and promising results from other studies, selectively-replicating viruses, when used as single agents, may have limited efficacy primarily due to limited viral spread in the tumor (Toth *et al.*, 2010). The oncolytic effect can be further enhanced by simultaneous expression of therapeutic genes, e.g. the NIS gene allowing for additional radionuclide therapy of tumor tissue. Radiation therapy causes damage to cellular proteins and nucleic acids leading to cell death (Singh and Kostarelos, 2009). Adenoviruses are particularly attractive as carrier of the NIS gene for radiotherapy because adenoviruses themselves act as radiosensitizers through their natural functions (Hart *et al.*, 2007; Hart *et al.*, 2005). Conversely, delivery of ionizing radiation to the tumor site has been shown to create an environment that is more sensitive to adenoviral transduction and replication, which might enhance transduction efficiency and oncolytic activity (Advani *et al.*, 2006; Hingorani *et al.*, 2008a; Hingorani *et al.*, 2008b). In fact, clinical trials have shown improved results when combining virus vectors with radiotherapy (Kumar *et al.*, 2008). Moreover, NIS-mediated radionuclide therapy is associated with a substantial bystander effect, based on the crossfire-effect of the beta-emitter ^{131}I with a path length of up to 2.4 mm, thereby compensating limited viral spread in the tumor and reducing the level of transduction efficiency required for a therapeutic response (Dingli *et al.*, 2003a). In its

well characterized dual function as reporter gene as well as therapy gene associated with a significant bystander effect, NIS represents an ideal candidate gene for replication-selective adenovirus-mediated gene-virotherapy, providing the potential of non-invasive whole-body monitoring of *in vivo* biodistribution and kinetics of virus replication in addition to image-guided radiotherapy combined with oncolytic virotherapy.

In the current study, intratumoral injection of the replication-selective Ad5-E1/AFP-E3/NIS led to a significant delay in tumor growth due to a distinct oncolytic effect. Moreover, local Ad5-E1/AFP-E3/NIS-mediated NIS gene transfer resulted in tumor-specific iodide uptake activity, which was sufficiently high for a significant therapeutic effect of ^{131}I additional to oncolysis. Combination of oncolytic virotherapy with radioiodine treatment resulted in a further decrease of tumor growth as well as prolongation of survival. As discussed above, monitoring of tumoral radioiodine accumulation displayed highest uptake activity 3 days after local virus administration with slowly decreasing levels of NIS expression within time. Therefore, we tested the feasibility and the impact of repeated administration of ^{131}I on days 3 and 5 after a single intratumoral injection of the replication-selective adenovirus for combination therapy. Two injections of radioiodine after the single administration of Ad5-E1/AFP-E3/NIS further decelerated tumor growth and improved survival. These data are consistent with previous radiovirotherapy studies in different tumor models showing higher therapeutic efficacy of oncolytic virotherapy when combined with NIS-mediated radiotherapy (Goel *et al.*, 2007; Msaouel *et al.*, 2009; Peerlinck *et al.*, 2009; Trujillo *et al.*, 2010). Penheiter *et al.* observed a trend towards decreased tumor volume and increased mouse survival, but no complete eradication, with no significant benefit of ^{131}I radiovirotherapy over virotherapy alone using a NIS-encoding measles virus (Penheiter *et al.*, 2010). This study demonstrated that appropriate timing of ^{131}I administration after viral infection and the best possible intratumoral vector distribution are critical factors for efficient radiovirotherapy of tumor xenografts.

In the current study we used a therapeutic dose of 55.5 MBq ^{131}I to be able to compare the results with our earlier studies after *in vivo* NIS gene transfer in xenograft mouse models (Klutz *et al.*, 2009; Klutz *et al.*, 2011a; Klutz *et al.*, 2011b; Klutz *et al.*, 2011c; Knoop *et al.*, 2011; Willhauck *et al.*, 2007; Willhauck *et al.*, 2008c). Initially, this dose was empirically tested in stably NIS expressing xenograft

tumor models and chosen in consideration of radiation safety, tolerability as well as accordance with animal protection laws. Based on our dosimetric calculations, a total tumor absorbed dose of approximately 7 Gy was achieved, which is less than the generally assumed target dose of 80 Gy needed for a therapeutic effect in lymph node metastases according to Maxon *et al*. (Maxon *et al.*, 1997), but it is in the range of target doses reported for ^{131}I therapy in thyroid cancer patients (0.5-288 Gy/lesion)(Chiesa *et al.*, 2009; Lassmann *et al.*, 2010) and was clearly high enough for a significant therapeutic effect of ^{131}I in addition to the oncolytic effect in our presented study. In an attempt to transfer these dosimetric calculations to humans, we would reach a tumor-absorbed dose of approximately 93.3 Gy after therapeutic application of 7.4 GBq (200mCi) ^{131}I, a dose generally applied to patients with thyroid cancer metastases. In comparison, a recent clinical trial quantified the volume and magnitude of *h*NIS gene expression in human prostate cancers following local injection of a high dose of a NIS expressing oncolytic adenovirus, and estimated the radiation dose that would be delivered to the prostate after ^{131}I administration with curative intent (Barton *et al.*, 2011). Assuming a standard radiation dose of 7.4 GBq (200 mCi) ^{131}I, the mean absorbed dose to the prostate was calculated to be only 7.2 ± 4.8 Gy (range 2.1–13.3 Gy). Differences in the cancer entity, the experimental approach as well as adenovirus engineering might explain this pronounced discrepancy.

Inefficient intratumoral viral spread limits the therapeutic effect by reduction of oncolytic cell killing as well as hampered effectiveness of radioiodine treatment. Although this is partially compensated by the crossfire effect of the β-emitting ^{131}I, it could be the therapy-limiting factor in our current study, in which therapy with Ad5-E1/AFP-E3/NIS slowed the progression of HuH7 xenografts, but did not lead to complete ablation of the tumors except in two mice in group radiovirotherapy 2 that had complete tumor regression up to 100 days after onset of the experiment. In addition, immunofluorescence analysis showed markedly reduced proliferation associated with decreased tumoral blood vessel density after local adenovirus-mediated NIS gene transfer followed by ^{131}I application, suggesting oncolytic efficacy and radiation-induced tumor stromal cell damage in addition to tumor cell death. The crossfire effect of ^{131}I might be responsible for stromal cell damage leading to reduced angiogenesis and secretion of growth-stimulatory factors, thereby enhancing therapeutic efficacy. However, it has been proven, that virotherapeutics not only

show anticancer effect by their direct oncolytic effect, but are also able to elicit a tumor-specific immune response, thereby breaking tumor tolerance (Woller *et al.*, 2011). To address this aspect, we are currently planning future studies using NIS-expressing oncolytic adenoviruses in an immunocompetent mouse model.

In conclusion, our data clearly demonstrate that tumor-specific NIS gene transfer using a replication-selective adenoviral gene delivery vector allows for targeted NIS-mediated, imaging-guided radionuclide therapy of extrathyroidal tumors, which enhances the therapeutic effect of oncolytic virotherapy and proves its potential use for clinical application.

2.6 Acknowledgments

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The authors declare no conflict of interest.

3. Chapter 2

Systemic image-guided liver cancer radiovirotherapy using dendrimer-coated adenovirus encoding the sodium iodide symporter (NIS) as theranostic gene

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3.1 Abstract

Currently, major limitations for clinical application of adenovirus-mediated gene therapy are high prevalence of neutralizing antibodies, widespread expression of the coxsackie-adenovirus receptor (CAR) and adenovirus sequestration by the liver. In the current study we used the sodium iodide symporter (NIS) as theranostic gene to investigate whether coating of adenovirus with synthetic dendrimers can be useful to overcome these hurdles in order to develop adenoviral vectors for combination of systemic oncolytic virotherapy and NIS-mediated radiotherapy.

Methods: We coated replication-deficient (Ad5-CMV/NIS) and replication-selective (Ad5-E1/AFP-E3/NIS) adenovirus serotype 5 carrying the *h*NIS gene with poly(amidoamine) dendrimers generation 5 (PAMAM-G5) in order to investigate transduction efficacy and altered tropism of these coated virus particles by ^{123}I scintigraphy and to evaluate their therapeutic potential for systemic radiovirotherapy in a liver cancer xenograft mouse model.

Results: After dendrimer coating Ad5-CMV/NIS demonstrated partial protection from neutralizing antibodies and enhanced transduction efficacy in CAR-negative cells *in vitro*. *In vivo* ^{123}I-scintigraphy of nude mice revealed significantly reduced levels of hepatic transgene expression after intravenous (i.v.) injection of dendrimer-coated Ad5-CMV/NIS (dcAd5-CMV/NIS). Evasion from liver accumulation resulted in significantly reduced liver toxicity and increased transduction efficiency of dcAd5-CMV/NIS in hepatoma xenografts. After PAMAM-G5 coating of the replication-selective Ad5-E1/AFP-E3/NIS a significantly enhanced oncolytic effect was observed following i.v. application (virotherapy) that was further increased by additional treatment with a therapeutic dose of ^{131}I (radiovirotherapy) and was associated with markedly improved survival.

Conclusion: These results demonstrate efficient liver detargeting and tumor retargeting of adenoviral vectors by coating with synthetic dendrimers thereby representing a promising innovative strategy for systemic NIS gene therapy. Moreover, based on its function as theranostic gene allowing non-invasive imaging of NIS expression by ^{123}I-scintigraphy, our study provides detailed characterization of *in vivo* vector biodistribution as well as localization, level and duration of 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.

3.2 Introduction

Genetically engineered replication-selective adenoviruses represent very efficient gene transfer vehicles with the advantage of potentiating therapeutic efficacy of gene therapy by its own oncolytic activity (Duffy *et al.*, 2012). Due to limited virus spread in the tumor, virotherapy is ideally combined with therapeutic genes that are associated with a bystander effect. In its well characterized dual function as reporter and therapy gene associated with a significant physical bystander effect, the sodium iodide symporter (NIS) represents an ideal candidate gene for replication-selective adenovirus-mediated gene-virotherapy. It provides the possibility of detailed non-invasive monitoring of biodistribution of virus infection and replication in addition to stimulation of therapeutic efficacy of oncolytic virotherapy by additional NIS-mediated radionuclide therapy (Baril *et al.*, 2010; Grünwald *et al.*, 2012).

However, recombinant adenoviruses still face hurdles that strongly limit efficient and safe application, in particular for systemic gene delivery, including induction of immune and inflammatory responses, elimination by neutralizing antibodies, high promiscuity due to widespread expression of the coxsackie-adenovirus receptor (CAR), and significant pooling in the liver (Kreppel and Kochanek, 2008). Besides genetic engineering (Yao *et al.*, 2011a), chemical modification of the adenovirus' surface represents a convenient method to shield the virus from undesired interactions with blood components and allows its re-targeting to tumor cells lacking adenoviral receptors (Kreppel and Kochanek, 2008). Therefore, different strategies have been developed in order to combine viral and synthetic vectors into a hybrid vector, including covalent conjugation of reactive polymers based on polyethylene glycol (PEG) or N-[2-hydroxypropyl]methacrylamide (HPMA) (Laga *et al.*, 2012) and non-covalent modification of the negatively charged adenovirus surface by electrostatic interaction with cationic polymers (Yao *et al.*, 2011a). These new technologies demonstrated first evidence to provide the vector with the ability to overcome neutralizing anti-vector antibodies, to escape liver tropism and to reduce innate and adaptive immune responses and liver toxicity even after systemic vector application, while maintaining its natural biological activity (Laga *et al.*, 2012).

We have recently utilized chemically well-defined dendritic PAMAM (poly(amidoamine)) dendrimers bearing positively charged terminal amines to coat the negatively charged adenoviral capsid by virtue of electrostatic interaction (Vetter

et al., 2013). This modification allowed efficient internalization and transduction of tumor cells *in vitro* otherwise refractory towards adenoviral transduction.

In the present study, we used this technology for coating of adenovirus vectors carrying the *h*NIS gene followed by the analysis of altered transduction efficiency, biodistribution, vector-related toxicity and therapeutic potential after systemic adenovirus-mediated NIS gene delivery. Based on its function as theranostic gene, NIS was used for non-invasive imaging of biodistribution and transduction efficiency by ^{123}I-scintigraphy. Evaluation of tumor-specific oncolytic efficacy (virotherapy) was followed by the assessment of NIS-mediated therapy response after the application of an additional therapeutic dose of ^{131}I (radiovirotherapy).

3.3 Materials and Methods

Cell culture

The human cell lines HuH7 (HCC, JCRB 0403), SKOV-3 (ovarian carcinoma, ATCC, HTB-77), and U87 MG (glioblastoma, ATCC, HTB-14) were cultured as described previously (Vetter *et al.*, 2013). Analysis of cellular CAR receptor levels by flow cytometry was carried out as described previously (Vetter *et al.*, 2013).

Production and dendrimer coating of recombinant adenovirus

The replication-deficient adenovirus Ad5-CMV/NIS ($1.1x10^{12}$ particles = $5.0x10^{10}$ PFU) carrying the *h*NIS cDNA under the control of the unspecific cytomegalovirus (CMV) promoter (Spitzweg *et al.*, 2001a) and the replication-selective adenovirus Ad5-E1/AFP-E3/NIS ($1.1x10^{12}$ particles = $3.0x10^{10}$ PFU) carrying the human NIS gene linked to the mouse alpha-fetoprotein promoter (Grünwald *et al.*, 2012) were developed as described previously. Amine-terminated generation 5 PAMAM dendrimer was used as described previously (Vetter *et al.*, 2013). For surface modification, the adenovirus was dispensed with serum-free OPTI-MEM (Invitrogen) to a volume of 50 µL (*in vitro* experiments) or 125 µL (*in vivo* experiments) and gently mixed with an equal volume of PAMAM-G5 (10 ng or 300 ng) diluted in HBG. The mixture was incubated at room temperature for 30 min before use. Dendrimer coating of the virus with 10 ng PAMAM-G5 is indicated in writing by the prefix dc_{10} and with 300 ng PAMAM-G5 by the prefix dc_{300}.

In vitro NIS gene delivery

24 h after seeding ($1.5x10^5$ cells/well in 12-well plates) cells were incubated with increasing MOI (multiplicity of infection) of Ad5-CMV/NIS, dc_{10}Ad5-CMV/NIS or dc_{300}Ad5-CMV/NIS per well in serum-free OPTI-MEM for 45 min at 37°C. Medium was replaced by fresh culture medium and virus-infected cells were further maintained for 4 days, before iodide uptake was measured at steady-state conditions as described previously (Spitzweg *et al.*, 1999). Results were normalized to cell viability as described previously (Unterholzner *et al.*, 2006) and expressed as cpm/A 490 nm.

For *in vitro* neutralization experiments, Ad5-CMV/NIS or dc_{300}Ad5-CMV/NIS were incubated for 30 min with increasing amounts of polyvalent adenovirus-neutralizing IgG (Privigen, CSL Behring, Marburg, Germany) at room temperature.

The final concentration of IgG was between 0.1-3.0 mg/mL as indicated. HuH7 cells were infected with virus at 100 MOI per well and analyzed for iodide uptake activity.

In vivo NIS gene transfer and biodistribution imaging studies

For proof of principle of systemic NIS gene transfer using a dendrimer-coated adenovirus the HuH7 xenograft mouse model was chosen that had already been used in our previous studies and that was established as outlined previously (Grünwald *et al.*, 2012). The experimental protocol was approved by the regional governmental commission for animals (Regierung von Oberbayern, Munich, Germany). Experiments started when tumors had reached a size of 7-9 mm (^{123}I biodistribution studies) or 4-5 mm (^{131}I radiovirotherapy studies). After a 10-day pretreatment with L-T4 (l-thyroxine, Henning, Sanofi-Aventis, Germany) (5 mg/L) in their drinking water to reduce iodide uptake by the thyroid gland and maximize radioiodine uptake in the tumor animals were injected intravenously (i.v.) via the tail vein with 1x10^9 PFU of the respective adenovirus.

Four days after systemic adenovirus injection mice received 18.5 MBq ^{123}I intraperitoneally (i.p.) and radioiodine biodistribution was monitored by serial gamma camera imaging as described previously (Willhauck *et al.*, 2007). Regions of interest in the liver were quantified and expressed as a fraction of the amount of accumulated radionuclide in the livers of mice injected with the unmodified Ad5-CMV/NIS. Regions of interest in the tumor were quantified and expressed as a fraction of the total amount of applied radioiodine per gram tumor tissue (%ID/g). The retention time within the tumor was determined by serial scanning after radioiodine injection, and dosimetric calculations were performed according to the concept of MIRD, with the dosis factor of RADAR-group (www.doseinfo-radar.com).

For serial iodide uptake studies mice were imaged for ^{123}I biodistribution on the indicated days after i.v. administration of Ad5-E1/AFP-E3/NIS or dc$_{300}$Ad5-E1/AFP-E3/NIS. Regions of interest in the tumor were quantified and expressed as percent of the injected dose per 100 mm^3 tumor tissue (%ID/100 mm^3).

Ex vivo analysis

After systemic adenovirus injection, NIS mRNA expression levels of liver, lung, spleen, kidney, and tumors were analyzed via quantitative real-time PCR as described previously (Klutz *et al.*, 2011a). Immunofluorescence staining of paraffin

embedded tissue sections derived from livers and HuH7 xenografts was performed using a *h*NIS-specific antibody directed against amino acid residues 625-643 of human NIS (Millipore) at a dilution of 1:750. Four days after systemic adenovirus injection mice were sacrificed and blood serum samples were collected to assess alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (measured at the Department of Clinical Biochemistry and Pathobiochemistry, Klinikum rechts der Isar, Munich, Germany). Subsequently, liver tissues were harvested and embedded in paraffin for hematoxylin and eosin staining.

Radiovirotherapy study

HuH7 xenografts were established in 5 groups of mice. The first group was injected i.v. with saline only (NaCl-control). A second group received a single i.v. injection of $1x10^9$ PFU of the conditionally replicating Ad5-E1/AFP-E3/NIS, and a third group received $1x10^9$ PFU of dc_{300}Ad5-E1/AFP-E3/NIS. The fourth group received $1x10^9$ PFU of Ad5-E1/AFP-E3/NIS and 3 days later a single i.p. dose of 55.5 MBq ^{131}I, and the fifth group received $1x10^9$ PFU of dc_{300}Ad5-E1/AFP-E3/NIS and 3 days later 55.5 MBq ^{131}I. Tumor measurements were performed twice weekly thereafter. Mice were followed for a total of 70 days or until tumor burden was such that animals had to be killed (≥ 1500 mm^3).

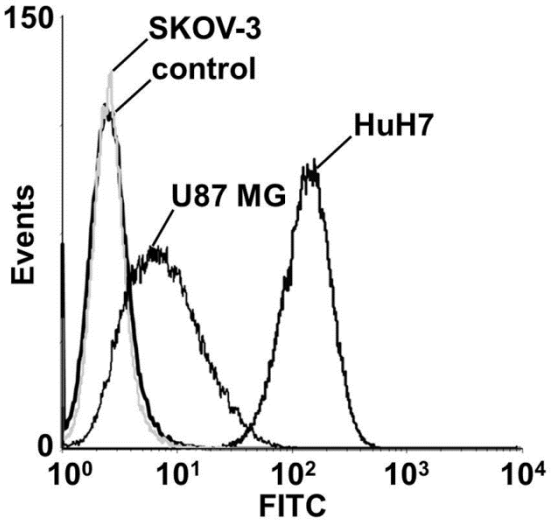
Statistical methods

All *in vitro* experiments were carried out in triplicates. Results are represented as means +/- SD of triplicates. Statistical significance was tested using Student's t-test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Statistical significance of *in vivo* experiments has been calculated using Mann–Whitney U test (two-tailed).

3.4 Results

Influence of dendrimer coating in vitro

Transfection efficacy of uncoated Ad5-CMV/NIS correlated well with levels of CAR expression (Figs. 1A-C; Supplemental Fig. 1).



Suppl. Fig. 1: Fluorescence-activated cell scanning (FACS) analysis of CAR expression. FACS analysis revealed high levels of CAR expression on the cell surface of HuH7, low CAR levels on U87 MG cells, and confirmed SKOV-3 cells to be CAR-negative.

CAR-positive HuH7 cells showed a MOI-dependent increase in perchlorate-sensitive ^{125}I accumulation of up to 70-fold as compared to uninfected cells, which was significantly increased after infection with dc_{10}Ad5-CMV/NIS and further retained after infection with dc_{300}Ad5-CMV/NIS (Fig. 1A). The low CAR level cell line U87 MG showed very low transduction efficacy when incubated with Ad5-CMV/NIS. In contrast, U87 MG cells treated with dc_{10}Ad5-CMV/NIS or dc_{300}Ad5-CMV/NIS (Fig. 1B) showed an up to 5.5-fold increase in iodide uptake activity. The CAR-negative SKOV-3 cells showed no iodide accumulation above background level, even when incubated with high MOI of Ad5-CMV/NIS. Coating of Ad5-CMV/NIS led to an up to 22-fold increase in iodide uptake activity (Fig. 1C). NIS gene transfer did not alter cell viability (data not shown).

Ad5-CMV/NIS was rapidly neutralized by increasing amounts of human IgG solution. In contrast, dc_{300}Ad5-CMV/NIS showed partial protection from neutralizing antibodies as demonstrated by a decelerated decrease in iodide uptake activity (Fig. 1D).

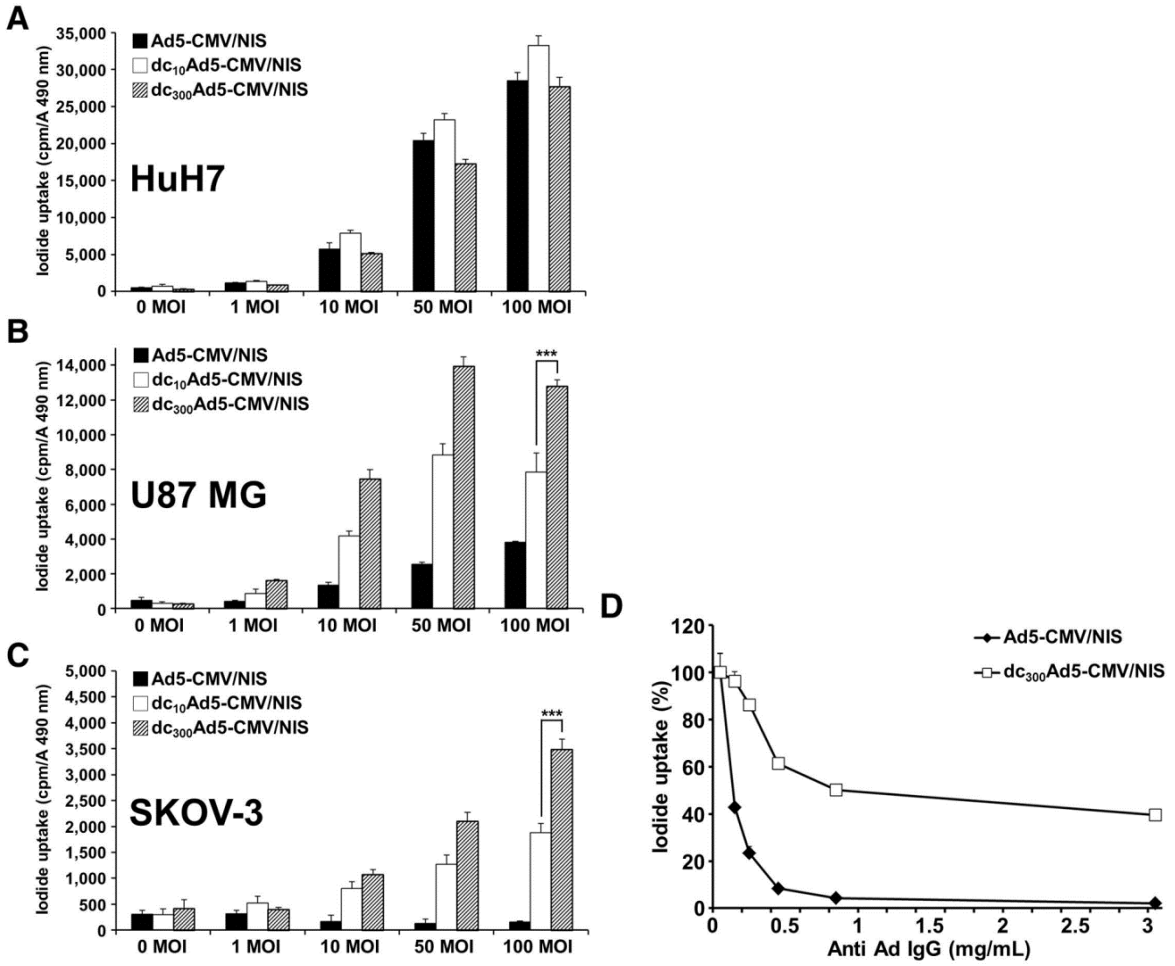


Fig. 1: *In vitro* iodide uptake studies and neutralization assay. *In vitro* iodide uptake experiments with dc_{10}Ad5-CMV/NIS or dc_{300}Ad5-CMV/NIS demonstrated retained transduction efficacy in CAR-positive cells (HuH7; **A**) and significantly enhanced transduction efficacy in low CAR level cells (U87 MG; **B**) or CAR-negative cells (SKOV-3; **C**), thereby indicating CAR-independent uptake mechanisms of dendrimer-coated adenovirus. Iodide uptake activity of cells infected with Ad5-CMV/NIS was progressively abolished by preincubation of the virus with increasing amounts of anti-Ad5 antiserum, whereas infection with dc_{300}Ad5-CMV/NIS showed a reduced decrease in iodide uptake activity, suggesting effective antibody protection (**D**). MOI = multiplicity of infection.

Virus biodistribution and toxicity studies in vivo

In vivo experiments showed high levels of radioiodine accumulation in the liver of tumor-free mice after systemic injection of Ad5-CMV/NIS (n=5) due to hepatic pooling of the vector as shown by ^{123}I γ-camera imaging 6 h p.i. (Fig. 2A, left), which was significantly (up to 70%) lower after systemic injection of virus particles coated with increasing amounts (10 ng, n=5; 300 ng, n=5) of PAMAM-G5 (Fig. 2A, right; Supplemental Fig. 2A).

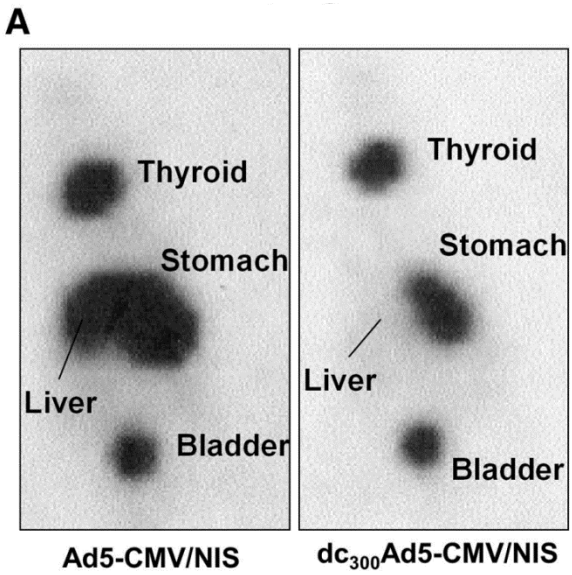


Fig. 2: *In vivo* iodide uptake studies and analysis of liver toxicity. Livers of mice accumulated high levels of radioiodine after i.v. injection of uncoated Ad5-CMV/NIS as shown by ^{123}I γ-camera imaging (**A**, left), which was markedly reduced after systemic injection of coated adenovirus (**A**, right).

Significant radioiodine accumulation was also observed in tissues physiologically expressing NIS, including stomach and thyroid, as well as in the urinary bladder due to renal elimination of the radionuclide. The results were confirmed by quantification of hepatic iodide uptake *ex vivo* (Supplemental Fig. 2A) and qPCR analysis of hepatic NIS mRNA expression (Supplemental Fig. 2B), which revealed a 16-fold lower hepatic NIS mRNA expression after injection of dc_{300}Ad5-CMV/NIS as compared to Ad5-CMV/NIS. Analysis of liver enzymes after injection of Ad5-CMV/NIS (n=5) revealed an ALT increase of 120% and a strong AST increase of approx. 400%, which was significantly decreased after coating of the adenovirus prior to systemic administration (n=5) (Fig. 2B).

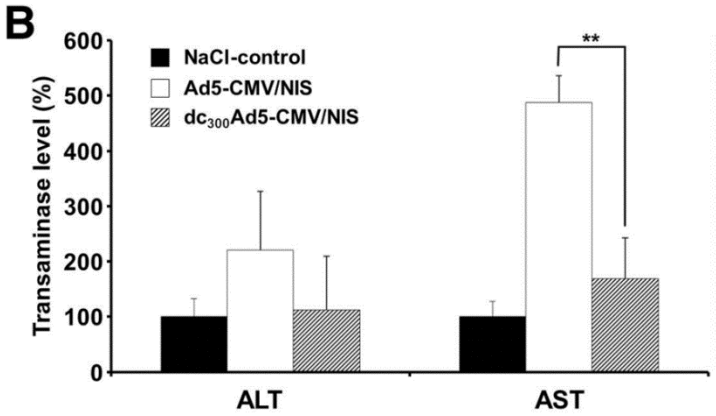
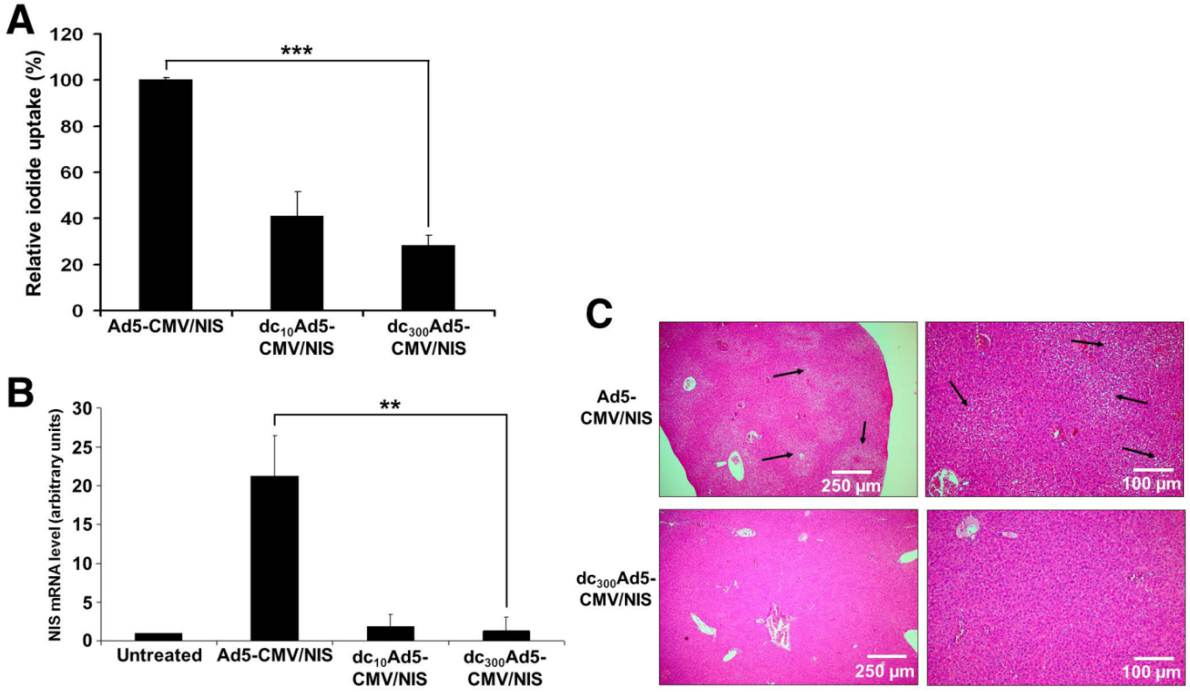


Fig. 2: Injection of Ad5-CMV/NIS led to increased levels of serum ALT and AST as compared to mice treated with saline only, which was mostly avoided by coating of the adenovirus prior to systemic administration (**B**).

Liver tissue correspondingly showed significantly increased fatty degeneration after injection of Ad5-CMV/NIS which was not observed after injection of dc_{300}Ad5-CMV/NIS (Supplemental Fig. 2C).



Suppl. Fig. 2: Adenoviral NIS gene transfer and toxicity. Hepatic accumulation of ^{123}I after i.v. injection of Ad5-CMV/NIS was significantly reduced (up to 70%) by coating of the virus (**A**). These results were further confirmed by analysis of hepatic NIS mRNA expression (**B**) and correlated well with a simultaneous reduction of liver toxicity as seen by H/E staining of liver tissue (**C**).

After i.v. injection of dc_{300}Ad5-CMV/NIS (n=9) into mice bearing subcutaneous HuH7 xenografts, tumoral radioiodine accumulation was approximately 13% ID/g ^{123}I (biological half-life 3.5 h) (Fig. 3A, right, 3B), while NIS-mediated radioiodine uptake in the liver was significantly lower after dendrimer coating (Fig. 3A, right) as compared to injection with the uncoated vector (Fig. 3A, left). Considering a tumor mass of 1 g and an effective half-life of 3 hours for ^{131}I, a tumor absorbed dose of approximately 91 mGy/MBq ^{131}I was calculated. In contrast, mice injected with the uncoated vector (n=10) showed only very low tumoral iodide accumulation above background level (approx. 3.5% ID/g, Fig. 3B). No additional uptake was observed in other non-target organs like lung, spleen or kidney.

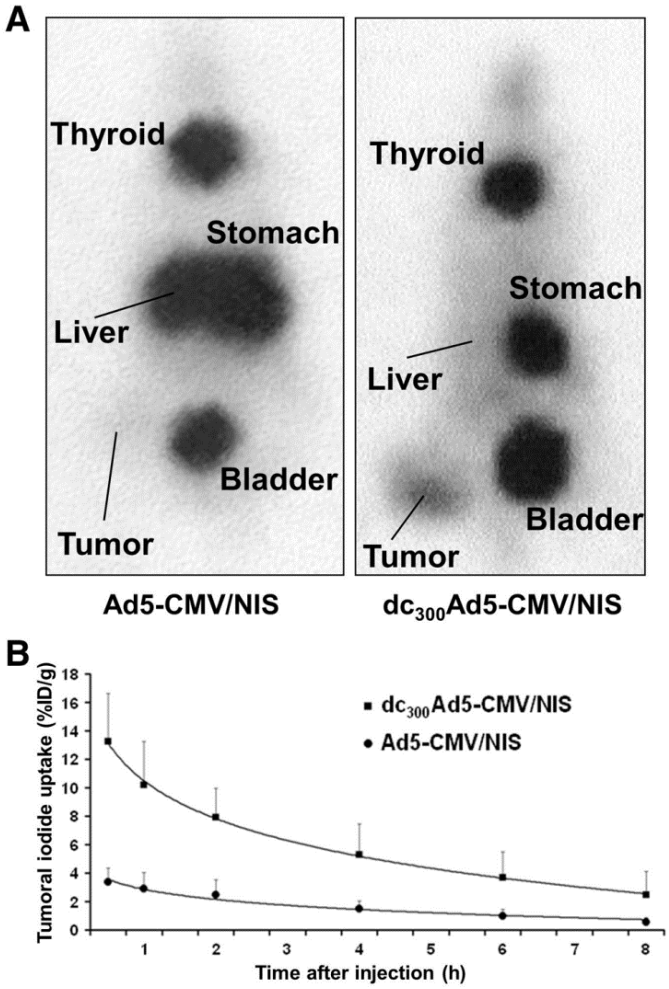


Fig. 3: *In vivo* iodide uptake studies in tumor bearing mice. *In vivo* experiments showed high levels of radioiodine accumulation in the liver of mice bearing HuH7 xenografts after i.v. injection of uncoated Ad5-CMV/NIS as shown by γ-camera imaging (**A**, left), which was significantly reduced after systemic injection of coated virus particles (**A**, right). Evasion from liver pooling of the adenovirus resulted in increased tumoral transduction as seen by significantly higher iodide uptake activity of HCC xenografts (**A**, right; **B**).

Immunofluorescence and qPCR analysis

QPCR analysis of hepatic NIS mRNA expression revealed a 28-fold decrease after i.v. injection of dc_{300}Ad5-CMV/NIS (n=9) as compared to injection of Ad5-CMV/NIS (n=10) (Fig. 4A, left). Moreover, a significant 10-fold increase of NIS mRNA expression above background level was induced in HuH7 tumors after systemic injection of dc_{300}Ad5-CMV/NIS (Fig. 4A, right). In contrast, tumoral NIS mRNA expression of mice injected systemically with Ad5-CMV/NIS was only 2.5 times higher than in untreated tumors. The NIS mRNA expression in lung, spleen and kidney of mice showed no significant increase comparable to saline-treated mice (data not shown).

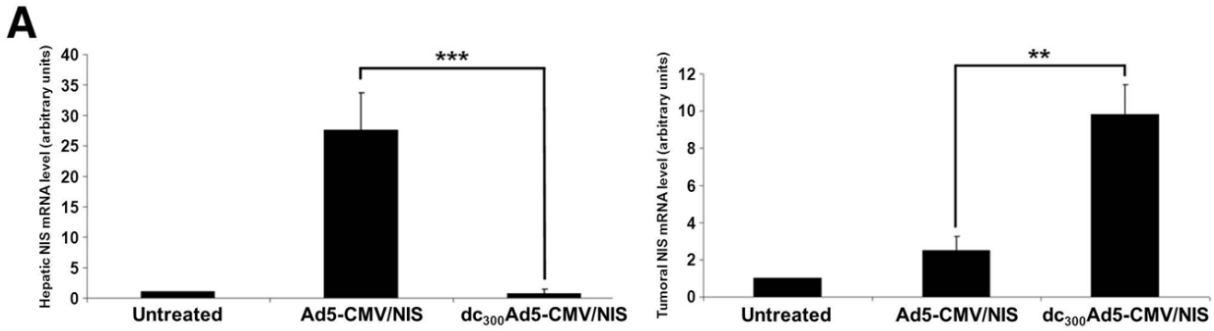


Fig. 4: qPCR analysis. Analysis of hepatic NIS mRNA expression revealed a 28-fold decrease after systemic injection of coated virus particles as compared to uncoated adenoviruses (**A**, left). Significantly increased levels of NIS mRNA expression were induced in HuH7 tumors after systemic NIS gene transfer when dc_{300}Ad5-CMV/NIS was used as compared to injection of Ad5-CMV/NIS (**A**, right).

Immunofluorescence staining showed high levels of NIS-specific immunoreactivity in livers of mice after systemic injection of Ad5-CMV/NIS (Fig. 4B, left), whereas livers of mice injected with dc_{300}Ad5-CMV/NIS showed only very low levels of hepatic immunoreactivity (Fig. 4B, left). Staining of HuH7 tumors revealed pronounced NIS-specific immunoreactivity after systemic injection of dc_{300}Ad5-CMV/NIS (Fig. 4B, right), which was significantly reduced after injection of Ad5-CMV/NIS (Fig. 4B, right).

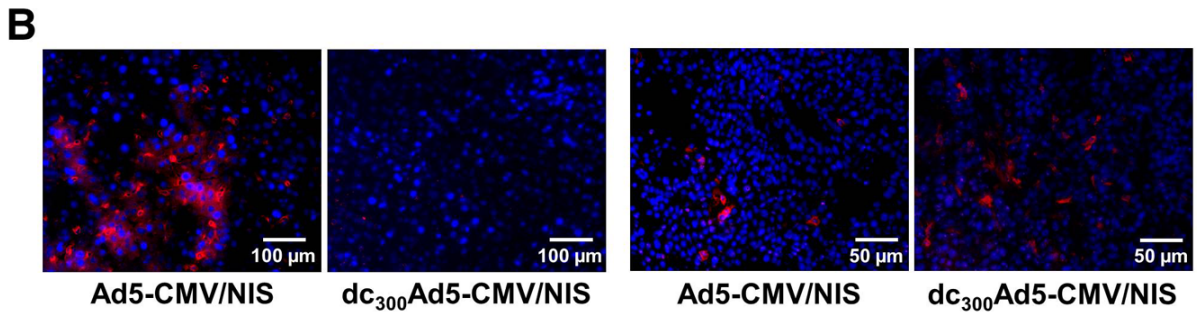


Fig. 4: Immunofluorescence staining. Immunofluorescence staining showed high levels of primarily membrane-associated NIS-specific immunoreactivity in livers of mice, systemically injected with Ad5-CMV/NIS (**B**, left). In contrast, livers of mice injected with dc_{300}Ad5-CMV/NIS showed only very low levels of immunoreactivity (**B**, left). After systemic injection of dendrimer-coated adenoviruses, tumors revealed high NIS-specific immunoreactivity as compared to low levels after injection of uncoated adenoviruses (**B**, right).

Serial imaging

Pronounced tumoral iodide uptake was detected up to 15-18 days after a single i.v. injection of the conditionally replicating dc_{300}Ad5-E1/AFP-E3/NIS (n=7) (Fig. 5A). In contrast, only low tumoral iodide accumulation even after several days

was observed in mice injected with Ad5-E1/AFP-E3/NIS (n=6) (Fig. 5B).

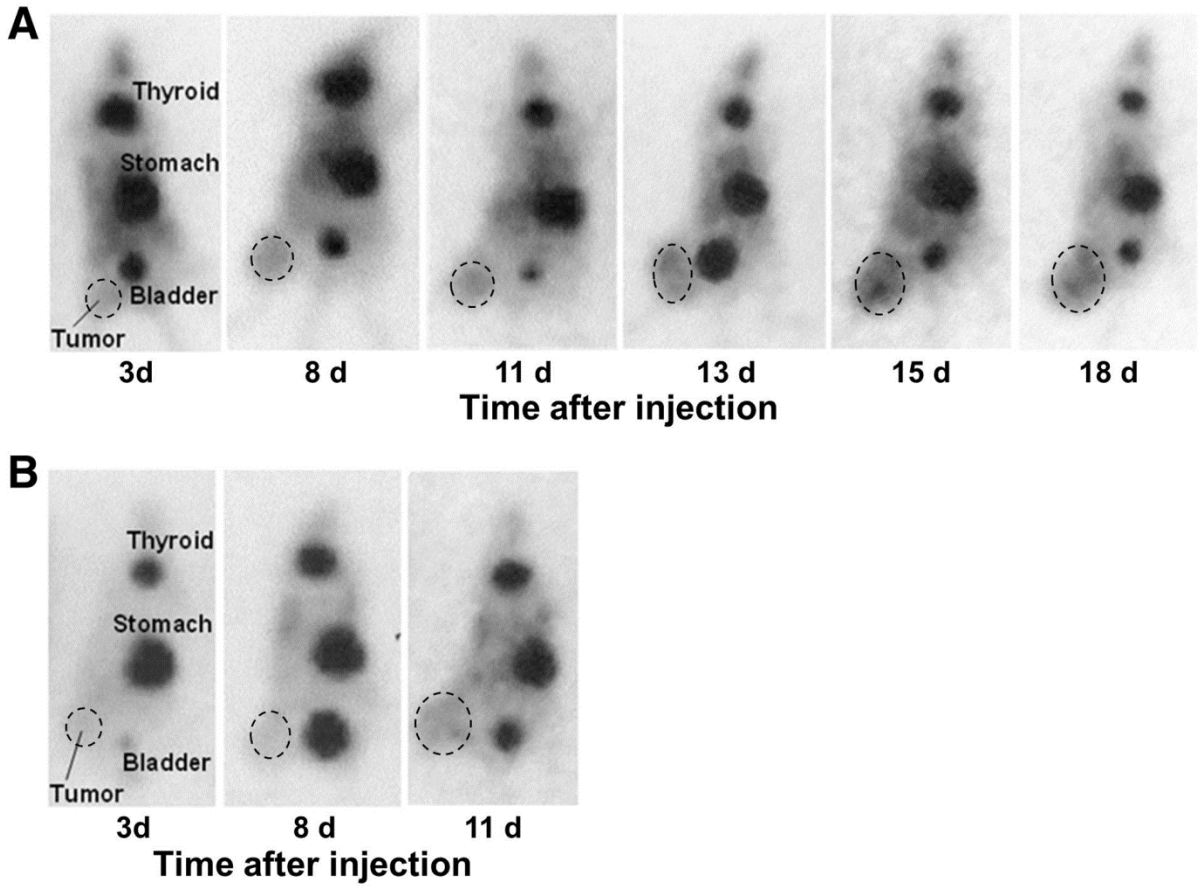
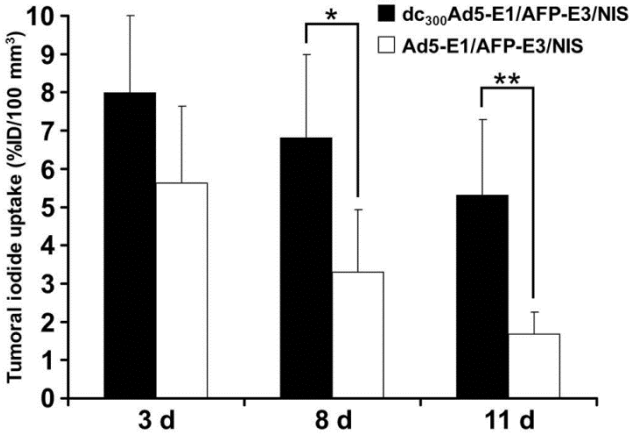


Fig. 5: Serial iodide uptake studies. Serial ^{123}I scintigraphy of mice bearing hepatoma xenografts after i.v. injection of Ad5-E1/AFP-E3/NIS with (**A**) or without (**B**) surface modification. Analysis of iodide uptake activity confirmed significantly higher levels of tumor-specific iodide accumulation after application of coated adenovirus (**A**) as compared to injection of uncoated virus (**B**). Pronounced tumoral iodide uptake was detected up to 15-18 days after infection with dendrimer-coated adenovirus.

After injection of uncoated virus, serial scanning had to be stopped at day 11 due to excessive tumor growth (Fig. 5B). In contrast, tumors of mice injected with the coated adenovirus grew slower due to viral replication (Fig. 5A) and therefore allowed measurement beyond day 11. Regions of interest were quantified and confirmed significantly higher levels of tumor-specific iodide accumulation after application of coated adenovirus with a maximum at day 3 as compared to injection of uncoated virus (Supplemental Fig. 3).



Suppl. Fig. 3: Quantification of tumoral iodine uptake. Serial quantification of tumoral iodide uptake activity over several days confirmed significantly higher levels of tumor-specific, NIS-mediated iodide accumulation after i.v. injection of dc_{300}Ad5-E1/AFP-E3/NIS as compared to injection of Ad5-E1/AFP-E3/NIS.

The most pronounced difference was seen at day 11, when HuH7 xenografts of mice injected with Ad5-E1/AFP-E3/NIS accumulated only 1-2% ID/100 mm^3 2 hours after injection of ^{123}I. In contrast, mice injected with dc_{300}Ad5-E1/AFP-E3/NIS still showed distinct tumoral iodide uptake activity of 5-6% ID/100 mm^3.

Radionuclide therapy

Mice injected with saline only (NaCl-control, n=8) showed an exponential tumor growth and had to be killed within 2-3 weeks after onset of the experiments (Figs. 6A, B). After a single i.v. injection of the conditionally replicating Ad5-E1/AFP-E3/NIS (n=10), a significant delay in tumor growth was observed when dendrimer-coated adenovirus was used (dc_{300}Ad5-E1/AFP-E3/NIS, n=10) as compared to injection of uncoated adenovirus (Ad5-E1/AFP-E3/NIS, n=10) which showed no therapeutic effect, thereby indicating higher levels of viable virus reaching the tumor after dendrimer coating (Fig. 6A). One mouse treated with dc_{300}Ad5-E1/AFP-E3/NIS showed complete tumor regression after 36 days until the end of the observation period (Fig. 6B).

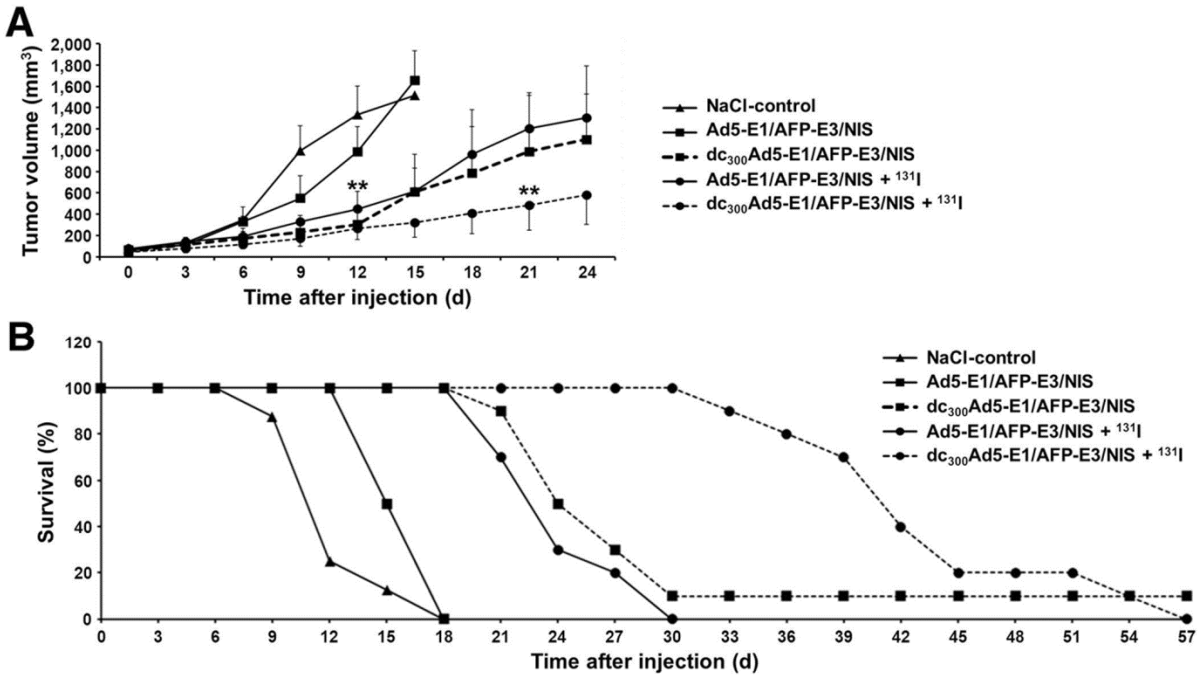


Fig. 6: Therapeutic efficacy. Radiovirotherapy studies in mice bearing HCC xenografts showed excessive tumor growth of the control group treated with saline only (NaCl-control, **A**). After a single i.v. injection of the conditionally replicating Ad5-E1/AFP-E3/NIS (without surface modification) no therapeutic effect was observed (**A**). In contrast, i.v. injection of dc_{300}Ad5-E1/AFP-E3/NIS showed improved oncolytic efficacy (**A**). I.v. application of Ad5-E1/AFP-E3/NIS followed by a therapeutic dose of ^{131}I revealed a comparable delay in tumor growth (**A**). I.v. injection of dc_{300}Ad5-E1/AFP-E3/NIS followed by the additional application of ^{131}I resulted in a strongly enhanced therapeutic effect, as seen by significantly delayed tumor growth (**A**) and prolonged survival (**B**).

To further evaluate the therapeutic efficacy of combined radiovirotherapy treatment, in two additional therapy groups Ad5-E1/AFP-E3/NIS and dc_{300}Ad5-E1/AFP-E3/NIS were administered i.v. followed by injection of a therapeutic dose ^{131}I. Mice treated with Ad5-E1/AFP-E3/NIS and ^{131}I (Ad5-E1/AFP-E3/NIS + ^{131}I, n=10) showed a significant delay in tumor growth and prolonged survival as compared to saline treatment or virus treatment alone (Figs. 6A, B). Remarkably, radiovirotherapy of mice injected with dc_{300}Ad5-E1/AFP-E3/NIS (dc_{300}Ad5-E1/AFP-E3/NIS + ^{131}I, n=10) showed a strongly enhanced therapeutic effect, as seen by significantly delayed tumor growth and extensively prolonged survival (Figs. 6A, B).

3.5 Discussion

As one of the oldest targets of molecular imaging and targeted radionuclide therapy, characterization of NIS as a novel reporter and suicide gene offers the possibility of NIS gene transfer in non-thyroidal tumors followed by diagnostic and therapeutic application of radioiodine (Spitzweg and Morris, 2002). Our previous work convincingly demonstrated the proof of principle of the NIS gene therapy concept (Grünwald *et al.*, 2012; Klutz *et al.*, 2009; Klutz *et al.*, 2011a; Klutz *et al.*, 2011b; Spitzweg *et al.*, 2001a; Spitzweg *et al.*, 1999; Willhauck *et al.*, 2007). The next step towards clinical application has to be the development of gene transfer vehicles that are able to promote targeted and efficient systemic NIS gene transfer with the potential to reach tumor metastases.

Species C adenoviruses have been shown to own high potential as vectors for gene transfer as well as for oncolytic virotherapy. However, following systemic application, wild-type adenovirus particles are rapidly cleared from the blood stream into the liver tissue by specific molecular mechanisms, including virus-coagulation factor interaction and Kupffer cell trapping (Duffy *et al.*, 2012). Moreover, adenovirus infection relies on the widely expressed coxsackie-adenovirus receptor (CAR) on the cell surface, resulting in reduced tumor-selectivity (Vetter *et al.*, 2013). Further obstacles for successful systemic application of adenoviruses are elimination by neutralizing antibodies as well as the induction of immune and inflammatory responses (Kang and Yun, 2010). With the goal of systemic adenovirus-mediated NIS gene delivery, in the current study we have explored the use of lower generation PAMAM dendrimers (Navarro *et al.*, 2010) bearing terminal primary amino groups able to promote non-covalent electrostatic interaction with negatively charged amino acids on the external surface of the hexon protein of adenovirus serotype 5 in order to form a complex and provide efficient coverage.

To overcome the limited therapeutic potential of oncolytic virotherapy alone, adenoviruses may be armed with therapeutic genes, such as the NIS gene that allows for enhanced radionuclide therapy of tumor tissue in addition to oncolysis. Combination of adenovirus-mediated virotherapy and NIS-mediated radiotherapy is particularly attractive as adenoviruses are known to have a radiosensitizing effect (Hart *et al.*, 2005) and at the same time ionizing radiation has been shown to generate an environment that is more susceptible to adenoviral transduction and replication (Advani *et al.*, 2006; Hingorani *et al.*, 2008b). Moreover, NIS gene therapy

is associated with a substantial bystander effect based on the crossfire effect of the β-emitter ^{131}I with a path length of up to 2.4 mm that provides a powerful means to compensate for the limited tumor spread of viral vectors (Dingli *et al.*, 2003a), thus enhancing the efficacy of virotherapy. A potential limitation of the NIS gene therapy concept is the endogenous NIS expression in the thyroid gland that, however, can effectively be downregulated by thyroid hormone pretreatment due to the exquisite regulation of thyroidal NIS by TSH (thyroid stimulating hormone) as demonstrated in humans by Wapnir *et al*.(Wapnir *et al.*, 2004). Another major argument that is frequently raised against the feasibility of using radioiodine therapy following NIS gene transfer into non-thyroidal tumours is the general assumption that organification of trapped radioiodine is a crucial prerequisite for effective radioiodine treatment due to increased retention of accumulated radioiodine. However, it has been shown that even thyroid carcinomas and their metastases often reveal a reduced capacity for iodide organification and thyroid hormone synthesis due to disrupted follicular architecture and function and lack of thyroglobulin expression (Mandell *et al.*, 1999; Valenta, 1966). Because accumulated radioiodine is not organified in HuH7 carcinoma cells, the data obtained using HuH7 cell xenografts and presented in the manuscript clearly indicate that iodide organification is not required to achieve a therapeutic effect of radioiodine in tumor tissue. In order to overcome the limitation of therapeutic efficacy by the lack of iodide organification, in the present study we are aiming at combining NIS-mediated radioiodine therapy with virus-mediated oncolysis. Synergies between oncolytic virotherapy and NIS-mediated radioiodine treatment have been reported previously (Dingli *et al.*, 2004; Goel *et al.*, 2007; Hakkarainen *et al.*, 2009; Peerlinck *et al.*, 2009; Trujillo *et al.*, 2010). In a recent study, Haddad *et al.* (Haddad *et al.*, 2012) used an oncolytic vaccinia virus encoding the human NIS gene for local and systemic therapy of pancreatic carcinoma in a murine xenograft model. They demonstrated the feasibility of long-term serial imaging of tumoral NIS expression as well as enhanced therapeutic response after combination of oncolytic virotherapy and NIS-mediated radiotherapy treatment. We previously illustrated the advantages of using oncolytic adenoviruses for successful realization of the NIS gene therapy approach (Grünwald *et al.*, 2012). As a next step towards clinical application, here we are the first to show the feasibility of NIS-mediated radiovirotherapy using dendrimer-coated replication-selective adenovirus vectors for systemic administration.

In the first steps of dendrimer-coated vector analysis, we have modified our replication-deficient adenovirus (Ad5-CMV/NIS) by PAMAM-G5 coating, and have used NIS in its well characterized function as reporter gene to monitor transduction efficiency and CAR selectivity (Spitzweg and Morris, 2002).

We have been able to demonstrate the capacity of dendrimer-coated Ad5-CMV/NIS to infect tumor cells with high efficiency through CAR-independent uptake mechanisms, to form stable complexes in the presence of serum, and to protect the adenovirus, at least in part, from neutralizing antibodies (Vetter *et al.*, 2013). Our results are consistent with a study from Kasman *et al.,* who reported polymer-enhanced adenoviral transduction of CAR-negative cancer cells (Kasman *et al.*, 2009). By attachment of the positively charged polymer, the surface charge of the virus is inverted, thereby allowing it to bind to the cell surface (Davis *et al.*, 2004). Subsequently, cellular transduction in our study was significantly enhanced, especially in the low CAR or CAR-deficient cell lines. Protection from neutralizing antibodies raises hope for a prolonged blood circulation time, as it was shown by Green *et al*. (Green *et al.*, 2004), with potential feasibility of repetitive applications *in vivo*. Based on our *in vitro* and *in vivo* data and the fact that both components of the complex were already applied in humans, we believe that clinical application of dendrimer-coated adenovirus is feasible and complexes will be stable in the blood stream.

In vivo PAMAM-G5 coating of Ad5-CMV/NIS resulted in significantly lower hepatic accumulation of ^{123}I after systemic application. Evasion from liver pooling facilitated significantly enhanced radioiodine accumulation in hepatoma xenografts based on enhanced functional NIS expression. Hence, a tumor absorbed dose of 91 mGy/MBq for therapeutic ^{131}I was calculated. In comparison, a study of Dingli *et al.* reported a comparable tumor absorbed dose of 108 mGy/MBq ^{131}I after systemic injection of a NIS encoding measles virus which facilitated to eliminate tumors resistant to the virus alone (Dingli *et al.*, 2004). Serial ^{123}I γ-camera-imaging confirmed significantly higher levels of tumor-specific, NIS-mediated iodide accumulation after application of the coated replication-selective dc_{300}Ad5-E1/AFP-E3/NIS as compared to uncoated virus. Pronounced tumoral iodide uptake even after 15-18 days demonstrates high transduction efficiency and efficient replication of Ad5-E1/AFP-E3/NIS in the tumor. Evasion of the hybrid vector from scavenging by Kupffer cells could be the possible mechanism behind the observed liver detargeting

of transgene expression as it was suggested by Prill *et al.* (Prill *et al.*, 2011).

The enhanced permeability and retention (EPR) effect is a phenomenon, which describes the passive accumulation of macromolecules within the tumor stroma due to leaky tumor vasculature combined with inadequate lymphatic drainage (Klutz *et al.*, 2011b), and is the basis for the observed passive tumor-targeting of dendrimer-coated adenovirus vectors after systemic delivery. Our dual targeting strategy was further enhanced by an active transcriptional tumor targeting using the AFP promoter for tumor-selective replication and NIS expression. Effective liver detargeting furthermore resulted in significantly lower extent of adenovirus-related liver toxicity in mice injected i.v. with dendrimer-coated adenovirus as shown by analysis of serum liver enzymes and liver histology, which is in agreement with previous studies (Green *et al.*, 2004; Kim *et al.*, 2011).

Therapy studies demonstrated that coating of the replication-selective Ad5-E1/AFP-E3/NIS prior to systemic administration significantly delayed tumor growth and extended survival as compared to injection of the uncoated adenovirus. Most importantly, the combined radiovirotherapy treatment using the dendrimer-coated Ad5-E1/AFP-E3/NIS followed by a single application of a therapeutic dose of ^{131}I resulted in a strong further stimulation of the therapeutic effect, as seen by extensively delayed tumor growth and prolonged survival as compared to virotherapy alone or to control groups that used the uncoated vector. Our data suggest that dendrimer coating of adenoviral vectors increases the level of viable virus reaching peripheral tumor tissues.

Taken together, our results indicate that non-covalent coating of adenoviral vectors with synthetic dendrimers shows considerable promise for effective adenovirus liver detargeting and tumor retargeting taking advantage of the merge of non-viral and viral vector technology, and therefore has the potential to improve current systemic gene delivery and tumor targeting strategies. It represents an innovative strategy to optimize efficacy and safety of systemic NIS gene delivery that allows imaging and radiovirotherapy of nonthyroidal cancers exploiting synergies between oncolytic virotherapy and NIS-mediated radionuclide therapy.

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4. Chapter 3

EGFR-targeted adenovirus dendrimer coating for improved systemic delivery of the theranostic NIS gene

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4.1 Abstract

We recently demonstrated tumor-selective iodide uptake and therapeutic efficacy of combined radiovirotherapy after systemic delivery of the theranostic sodium iodide symporter (NIS) gene using a dendrimer-coated adenovirus. To further improve shielding and targeting we physically coated replication-selective adenoviruses carrying the *h*NIS gene with a conjugate consisting of cationic poly(amidoamine) (PAMAM) dendrimer linked to the peptidic, epidermal growth factor receptor (EGFR)-specific ligand GE11. *In vitro* experiments demonstrated coxsackie-adenovirus receptor-independent but EGFR-specific transduction efficiency. Systemic injection of the uncoated adenovirus in a liver cancer xenograft mouse model led to high levels of NIS expression in the liver due to hepatic sequestration, which were significantly reduced after coating as demonstrated by ^{123}I-scintigraphy. Evasion from liver pooling resulted in decreased hepatotoxicity and increased transduction efficiency in peripheral xenograft tumors. ^{124}I-PET-imaging confirmed EGFR specificity by significantly lower tumoral radioiodine accumulation after pretreatment with the EGFR-specific antibody cetuximab. A significantly enhanced oncolytic effect was observed following systemic application of dendrimer-coated adenovirus that was further increased by additional treatment with a therapeutic dose of ^{131}I. These results demonstrate restricted virus tropism and tumor-selective retargeting after systemic application of coated, EGFR-targeted adenoviruses therefore representing a promising strategy for improved systemic adenoviral NIS gene therapy.

4.2 Introduction

We recently reported on the feasibility of non-covalent adenovirus surface modification using synthetic polycationic dendrimers resulting in partial protection from neutralizing antibodies, coxsackie-adenovirus receptor (CAR)-independent infectivity and efficient liver detargeting after systemic vector administration, leading to reduced toxicity as well as enhanced tumoral transduction and therapeutic efficacy (Grünwald *et al.*, 2013; Vetter *et al.*, 2013).

Once a viral gene transfer vehicle has been developed that allows for systemic application and provides sufficiently high transgene expression in the target tissue, a key task is to further increase levels of oncolysis and tumoral transgene expression with optimal specificity and lowest possible toxicity in non-target organs (Choi *et al.*, 2012; Kim *et al.*, 2011). A variety of different methods have been tested in recent times to make viral gene transfer even more secure and successful in terms of development of targeted and shielded vectors for future clinical applications in humans (Campos and Barry, 2007; Duffy *et al.*, 2012). Among others, targeting ligands that have been tested recently to optimize tumor-selectivity of viral vectors include ligands of the epidermal growth factor receptor (EGFR), the fibroblast growth factor receptor 2, CGKRK motifs and alpha-v integrins on the cell surface (Rojas *et al.*, 2012; Yao *et al.*, 2011a; Yao *et al.*, 2011b). Targeting the EGFR is of particular interest since it has been shown that EGFR triggers tumor growth and progression and is significantly upregulated in a large number of epithelial tumors (Harari, 2004). Therefore, the EGFR has been evaluated as a promising target structure for viral and non-viral gene transfer (de Bruin *et al.*, 2007; Harvey *et al.*, 2010; Kawashima *et al.*, 2011; Klutz *et al.*, 2011a). In a recent study, we reported on systemic non-viral sodium iodide symporter (NIS) gene transfer using polyplexes coupled to the synthetic peptide GE11 as an EGFR-targeting ligand with high receptor affinity that does not activate the receptor tyrosine kinase (Li *et al.*, 2005), capable of inducing high levels of tumor-specific transgene expression (Klutz *et al.*, 2011a). NIS represents one of the oldest targets for molecular imaging and therapy. Due to its ability to concentrate iodine in the thyroid gland it provides the molecular basis for thyroid scintigraphy and radioiodine whole body scanning as well as therapeutic application of radioiodine in thyroid cancer - the most effective form of systemic anticancer radiotherapy available today (Spitzweg and Morris, 2002). Transduction of cancer cells with the theranostic NIS gene therefore gives us the possibility of non-

invasive monitoring of NIS biodistribution before application of a therapeutic dose of radioiodine, which is of particular importance after systemic vector application (Hingorani *et al.*, 2010; Spitzweg and Morris, 2004).

In a further study we have previously reported on the feasibility of systemic NIS gene transfer using a dendrimer-coated replication-selective adenovirus. To further improve safety, tumor selectivity, and therapeutic efficacy of the dendrimer-coated adenovirus vector, in the current study we added another level of tumor specificity by combining the two approaches through attachment of the EGFR-specific peptide GE11 to the virus coating polymer. Thereby NIS transgene expression is not only detargeted from the liver after systemic virus administration and passively accumulated in the tumor by the enhanced permeability and retention (EPR) effect (Maeda, 2001), but also actively targeted to the EGFR expressing tumor cells.

Based on the dual function of the NIS gene encoded by our adenovirus as reporter and therapy gene, at first we investigated its potential for non-invasive imaging of vector biodistribution and transgene expression of our targeted and shielded adenovirus by 2-dimensional ^{123}I-scintigraphy as well as 3-dimensional high resolution ^{124}I PET imaging. Furthermore, the potential of further stimulation of therapeutic efficacy of adenovirus-mediated oncolysis was investigated by subsequent combination with systemic NIS-mediated radiotherapy (radiovirotherapy).

4.3 Materials and Methods

Cell culture

The human HCC cell line HuH7 (JCRB 0403), the human ovarian carcinoma cell line SKOV-3 (ATCC, HTB-77), and the human HCC cell line HepG2 (ATCC, HB-8065) were cultured as described previously.(Vetter *et al.*, 2013) Flow cytometry analysis of EGFR levels was carried out as described previously.(Vetter *et al.*, 2013)

Recombinant adenovirus production and coating with EGFR-specific dendrimer

The replication-deficient adenovirus Ad5-CMV/NIS carrying the *h*NIS gene under the control of the unspecific cytomegalovirus (CMV) promoter (Spitzweg *et al.*, 2001a) and the replication-selective adenovirus Ad5-E1/AFP-E3/NIS were generated as described previously. In Ad5-E1/AFP-E3/NIS replication is controlled by cloning the E1A region under control of the liver cancer-specific mouse alpha-fetoprotein (AFP) promoter and the *h*NIS gene is inserted in the E3 region under control of the replication-dependent E3 promoter (Grünwald *et al.*, 2012). As a control, a replication-deficient adenovirus carrying the *h*NIS gene under the control of the AFP promoter Ad5-AFP/NIS was used as described previously (Klutz *et al.*, 2011c). The replication-selective human recombinant type 5 adenovirus Ad5-E1/AFP-RSV/NIS ($1.1x10^{12}$ particles = $1.0x10^{10}$ plaque forming units (PFU)) replicating under control of the mouse AFP promoter and expressing the human NIS gene under control of the unspecific RSV promoter was developed by ViraQuest Inc. (North Liberty, IA, USA).

Synthesis of dendrimers PAMAM-G2-PEG-GE11, PAMAM-G2-PEG-Cys (Vetter *et al.*, 2013), and adenoviral surface modification (Grünwald *et al.*, 2013) were carried out as described previously. Dendrimer coating of the virus with 10 ng PAMAM-G2-PEG-GE11 is indicated in writing by the prefix $dc_{10/GE11}$, with 300 ng PAMAM-G2-PEG-GE11 by the prefix $dc_{300/GE11}$, and with 300 ng PAMAM-G2-PEG-Cys by the prefix $dc_{300/Cys}$.

Adenovirus-mediated NIS gene delivery in vitro

In vitro infection, iodide uptake experiments, and measurement of cell viability were carried out as described previously (Grünwald *et al.*, 2013).

In vivo NIS gene transfer and radioiodine biodistribution studies

Establishment of HuH7 xenografts (Grünwald *et al.*, 2012) and *in vivo* NIS gene transfer (Grünwald *et al.*, 2013) were carried out as described previously. The experimental protocol was approved by the regional governmental commission for animals (Regierung von Oberbayern, Munich, Germany).

Four days after systemic adenovirus injection mice received 18.5 MBq ^{123}I intraperitoneally (i.p.) and radioiodine biodistribution was monitored by serial gamma camera imaging as described previously (Willhauck *et al.*, 2007). Quantification of regions of interest and dosimetric calculations were carried out as described previously (Grünwald *et al.*, 2013).

Three days after systemic injection of Ad5-E1/AFP-RSV/NIS or $dc_{300/GE11}$Ad5-E1/AFP-RSV/NIS mice received 10 MBq ^{124}I i.p. and radioiodine biodistribution was monitored by a 15 min static acquisition 3 hours post injection using a micro PET system (Inveon, SIEMENS Preclinical Solutions, Erlangen, Germany). A subset of mice was pretreated i.p. with 0.25 mg of the EGFR-specific antibody cetuximab (Erbitux; Merck, Darmstadt, Germany) 24 hours prior to adenovirus administration. Mean tumoral radioiodine uptake was calculated in megabequerel per milliliter (MBq/mL) by manually placing 3D regions of interest in the tumor.

Ex vivo analysis

NIS mRNA expression levels of livers and tumors were analyzed *ex vivo* via quantitative real-time PCR as described previously (Klutz *et al.*, 2011a). For analysis of hepatotoxicity, uncoated Ad5-E1/AFP-RSV/NIS or dendrimer-coated $dc_{300/GE11}$Ad5-E1/AFP-RSV/NIS were injected i.v. and 3 days thereafter, mice were sacrificed and blood serum samples were collected to assess alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (measured at the Department of Clinical Biochemistry and Pathobiochemistry, Klinikum rechts der Isar, Munich, Germany). Subsequently, liver tissues were harvested and embedded in paraffin for hematoxylin and eosin (H/E) staining.

Radiovirotherapy study in vivo

HuH7 xenografts were established in 4 groups of mice. The first group was used as control and injected i.v. with saline only (NaCl-control, n=8). A second group received a single i.v. injection of $1x10^9$ PFU of the dendrimer-coated replication-

selective $dc_{300/GE11}$Ad5-E1/AFP-E3/NIS ($dc_{300/GE11}$Ad5-E1/AFP-E3/NIS, virotherapy, n=8). The third group received a single i.v. injection of $1x10^9$ PFU of the dendrimer-coated replication-deficient $dc_{300/GE11}$Ad5-AFP/NIS and 3 days later a single i.p. dose of 55.5 MBq ^{131}I ($dc_{300/GE11}$Ad5-AFP/NIS + ^{131}I, radiotherapy, n=7). The fourth group received a single i.v. injection of $1x10^9$ PFU of the dendrimer-coated replication-selective $dc_{300/GE11}$Ad5-E1/AFP-E3/NIS and 3 days later a single i.p. dose of 55.5 MBq ^{131}I ($dc_{300/GE11}$Ad5-E1/AFP-E3/NIS + ^{131}I, radiovirotherapy, n=10). Tumor measurements were performed twice weekly thereafter. Tumor volume was estimated using the equation: tumor volume = length × width × height × 0.52. Mice were followed for a total of 100 days or until tumor burden was such that animals had to be killed (≥ 1500 mm^3).

Statistical methods

All *in vitro* experiments were carried out in triplicates. Results are represented as means +/- SD of triplicates. Statistical significance was tested using Student's t-test (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Statistical significance of *in vivo* experiments has been calculated using Mann–Whitney U test (two-tailed).

4.4 Results

Influence of EGFR-targeted adenoviral surface modification in vitro

Three cell lines with different levels of CAR and EGFR expression (HuH7: high CAR, high EGFR; SKOV-3: CAR-negative, high EGFR; HepG2: High CAR, low EGFR) as determined by flow cytometry (data not shown) were used. After infection with uncoated Ad5-CMV/NIS the CAR-positive cell lines HuH7 and HepG2 showed a dose-dependent increase in perchlorate-sensitive ^{125}I uptake activity of up to 80-fold, which was fully retained after EGFR-targeted coating of the adenovirus with increasing amounts of dendrimer (Figs. 1A, C). The CAR-negative cell line SKOV-3 showed no iodide accumulation above background level, even when incubated with high multiplicity of infection (MOI) of the uncoated Ad5-CMV/NIS. Adenovirus coating with increasing amounts of EGFR-targeted dendrimer led to an increase in perchlorate-sensitive iodide uptake activity of up to 6 orders of magnitude (Fig. 1B), thereby indicating CAR-independent uptake mechanisms of dendrimer-coated adenovirus. Replacement of the targeting ligand GE11 by a cysteine residue (Cys) significantly lowered transduction efficiency in EGFR-positive HuH7 and SKOV-3 cells (Figs. 1A, B) whereas transduction efficiency in the low EGFR expressing HepG2 cells remained unchanged (Fig. 1C), thereby demonstrating targeting specificity and increased transduction efficiency by the use of the EGFR-specific targeting ligand. Viral NIS gene transfer using uncoated or dendrimer-coated Ad5-CMV/NIS did not alter cell viability as measured by MTS assay (data not shown).

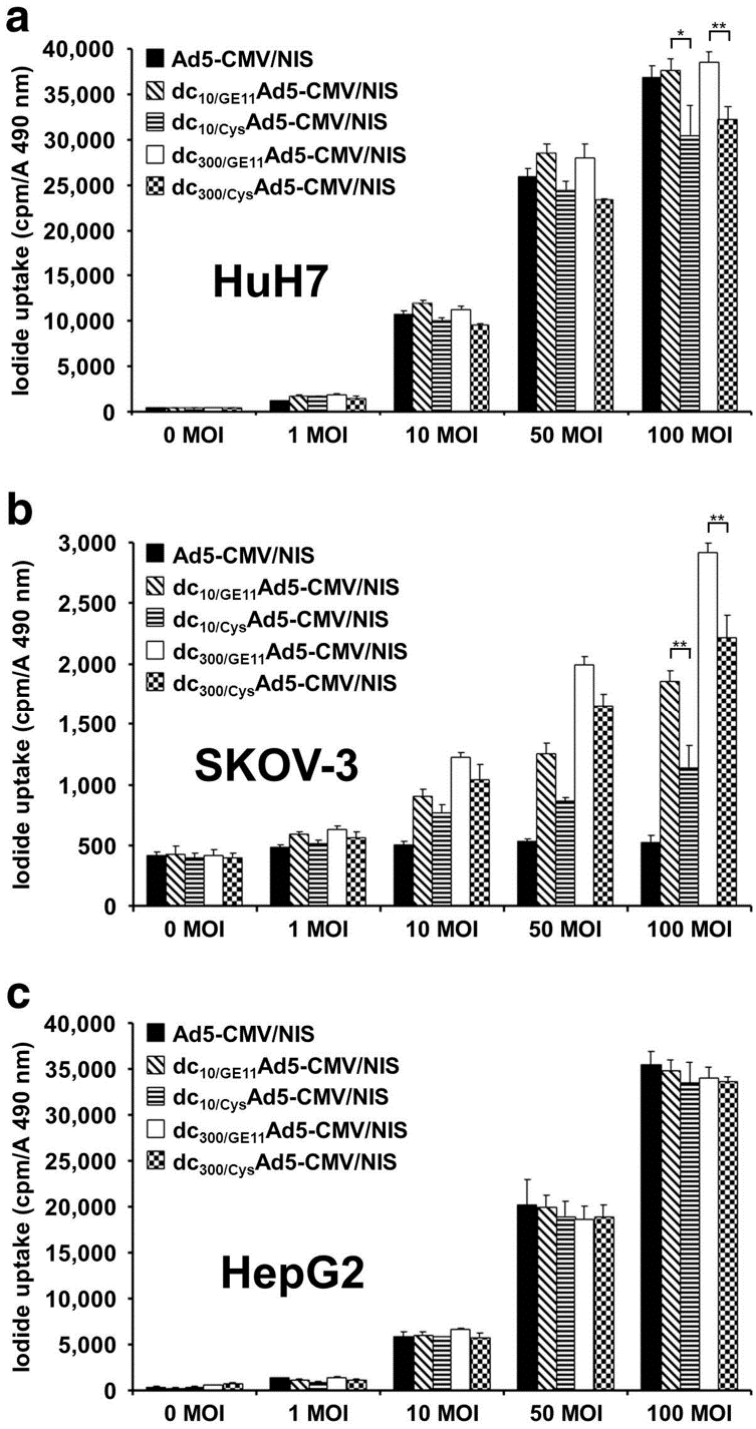

Fig. 1: *In vitro* iodide uptake studies of EGFR-targeted adenovirus. *In vitro* transduction experiments with uncoated Ad5-CMV/NIS showed dose-dependent transduction efficiency in CAR-positive cells (HuH7, HepG2), which was fully retained after EGFR-targeted coating of the adenovirus with increasing amounts of dendrimer (**A**, **C**). The CAR-negative cell line SKOV-3 showed no iodide accumulation above background level, even when incubated with high MOI of the uncoated Ad5-CMV/NIS but adenoviral coating with increasing amounts of EGFR-targeted dendrimer caused a significant increase in perchlorate-sensitive iodide uptake activity (**B**). Replacement of the targeting ligand GE11 by a cysteine residue (Cys) lowered transduction efficiency in EGFR-positive HuH7 and SKOV-3 cells (**A**, **B**) whereas transfection efficiency in the low EGFR expressing HepG2 cells remained unchanged (**C**).

Non-invasive imaging of EGFR-targeted NIS gene delivery

Mice bearing high EGFR-expressing HuH7 xenograft tumors were imaged after intravenous vector administration for functional NIS expression via whole body ^{123}I-scintigraphy. *In vivo* imaging of vector biodistribution demonstrated high levels of NIS-mediated radionuclide accumulation in the livers of mice after systemic injection of uncoated Ad5-CMV/NIS due to hepatic sequestration of the vector. As a consequence of hepatic vector trapping only very low ^{123}I accumulation above background level was observed in peripheral xenograft tumors (Fig. 2A).

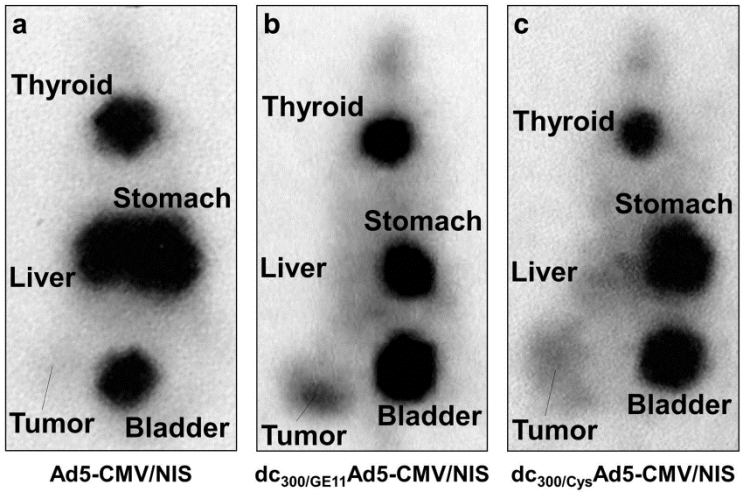

Fig. 2: *In vivo* iodide uptake studies of EGFR-targeted adenovirus. ^{123}I-scintigraphy of mice bearing high EGFR-expressing HuH7 xenografts demonstrated high hepatic and low tumoral NIS-mediated radionuclide accumulation after systemic injection of uncoated Ad5-CMV/NIS (**A**). Coating of Ad5-CMV/NIS with EGFR-targeted PAMAM-G2-PEG-GE11 ($dc_{300/GE11}$Ad5-CMV/NIS) prior to systemic administration strongly reduced liver transduction resulting in significantly increased transduction efficiency of xenograft tumors (**B**). Replacement of the dendrimer-coupled targeting ligand by a cysteine residue ($dc_{300/Cys}$Ad5-CMV/NIS) still prevented liver pooling of the vector but significantly reduced tumor-specific radionuclide accumulation (**C**).

By coating of Ad5-CMV/NIS with EGFR-targeted PAMAM-G2-PEG-GE11 ($dc_{300/GE11}$Ad5-CMV/NIS) prior to systemic administration liver transduction was strongly reduced by over 80% (Figs. 2B, 3A) resulting in significantly increased transduction efficiency of xenograft tumors. Serial scanning of mice revealed an accumulated dose of approximately 15% of the injected dose per gram tumor tissue (%ID/g) with an average biological half-life of 4.5 h (Figs. 2B, 3B), resulting in a calculated tumor-absorbed dose of 103 mGy for ^{131}I. Replacement of the dendrimer-coupled targeting ligand by a cysteine residue ($dc_{300/Cys}$Ad5-CMV/NIS) still prevented

liver pooling of the vector but significantly reduced tumor-specific radionuclide accumulation nearly by half (Figs. 2C, 3A, B). In addition to ^{123}I uptake in liver and tumor, radioiodine accumulation was also observed in stomach and thyroid that physiologically express NIS and in the urinary bladder due to radionuclide elimination but in no case in other non-target organs (Figs. 2A-C). *Ex vivo* analysis of NIS mRNA expression in livers and tumors correlated well with the radionuclide biodistribution observed and therefore confirmed the findings of ^{123}I scintigraphy (Fig. 3C).

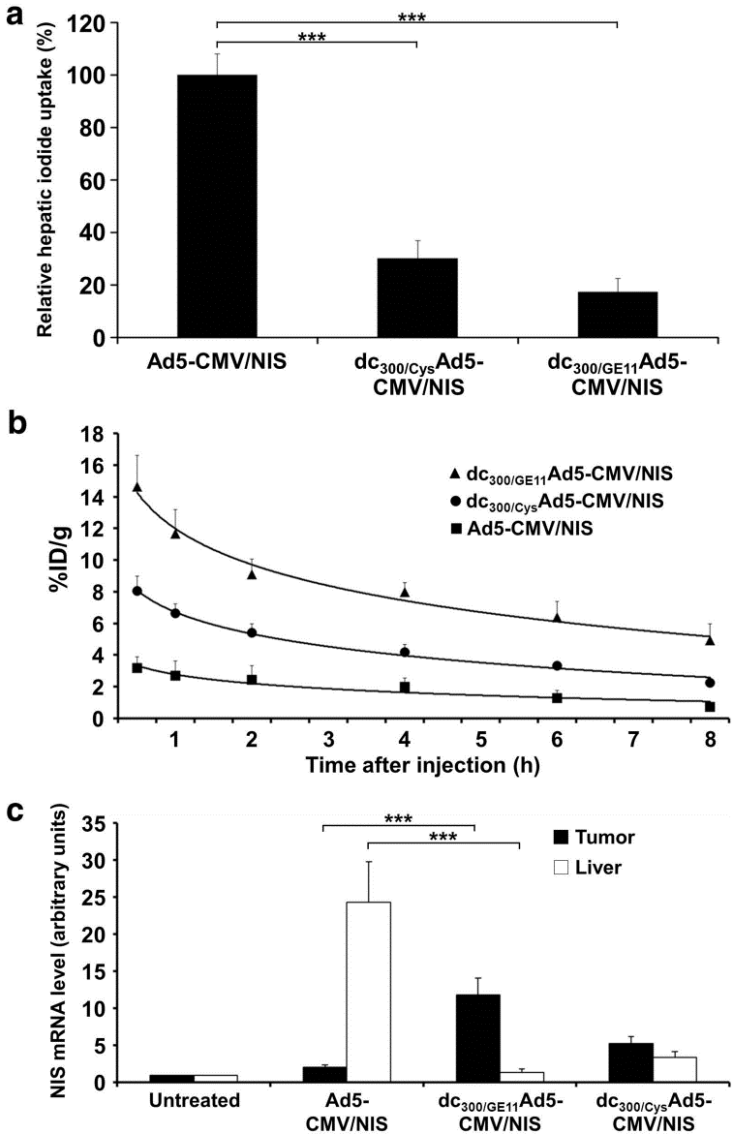

Fig. 3: Biodistribution of NIS transgene expression. Quantification of hepatic transgene expression revealed over 80% reduction after intravenous injection of dendrimer-coated $dc_{300/GE11}$Ad5-CMV/NIS as compared to injection of uncoated Ad5-CMV/NIS (**A**). Detargeting of hepatic transgene expression resulted in significantly increased transduction efficiency of xenograft tumors (**B**). Replacement of the dendrimer-coupled targeting ligand by a cysteine residue ($dc_{300/Cys}$Ad5-CMV/NIS) still prevented liver pooling of the vector but reduced tumor-specific radionuclide accumulation nearly by half (**A**, **B**). *Ex vivo* analysis of NIS mRNA expression in livers and tumors correlated well with the observed radionuclide biodistribution and confirmed the findings of ^{123}I scintigraphy (**C**).

Influence of vector modification on hepatotoxicity

Assessment of hepatotoxicity after intravenous injection of uncoated Ad5-E1/AFP-RSV/NIS demonstrated a small 1.75-fold increase in ALT and a strong 128-fold increase in AST (Fig. 4C) as well as a significant increase in fatty degeneration of liver tissue (Fig. 4A). Coating of the adenovirus before intravenous injection abrogated hepatotoxic effects almost completely as seen by a reduction of increase in ALT by half (45.6%) and in AST by 98.6 % (Fig. 4C) as well as liver histology without pathological findings (Fig. 4B).

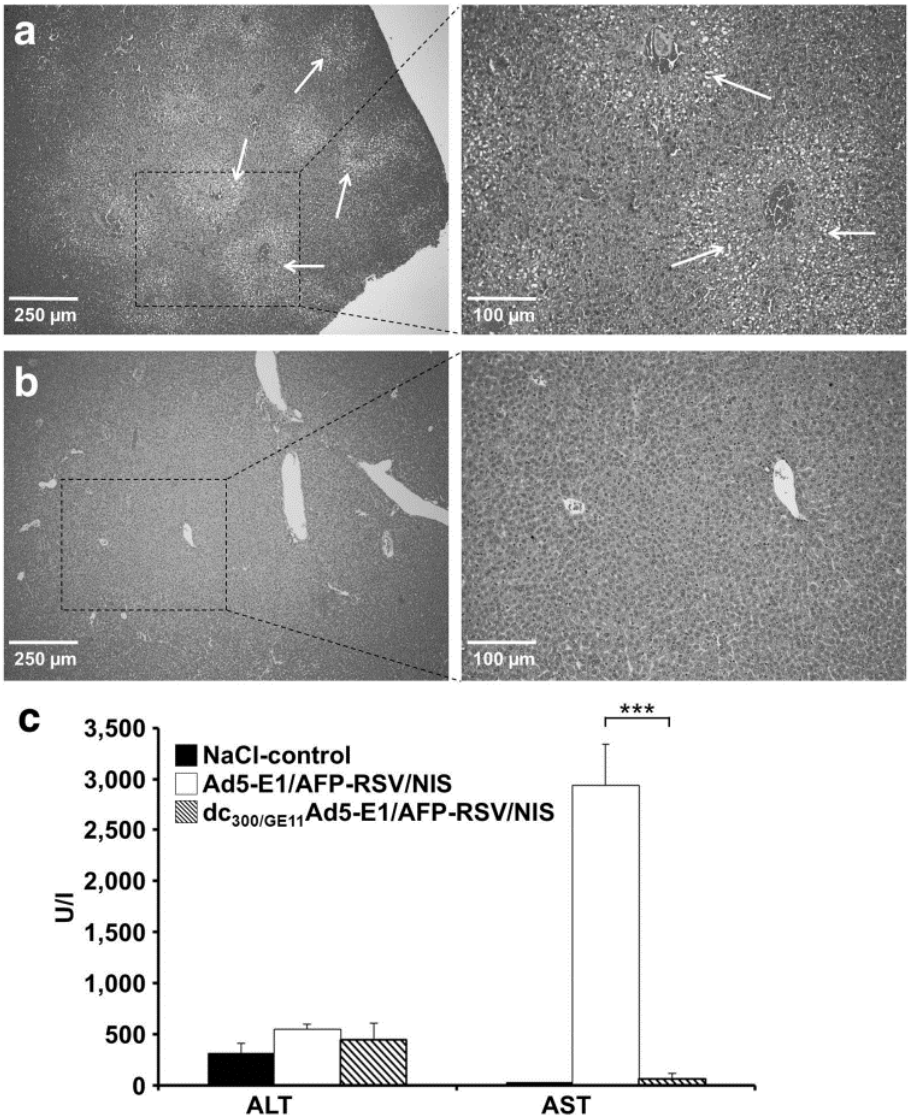

Fig. 4: Analysis of liver toxicity. H/E staining of liver sections of mice injected intravenously with Ad5-E1/AFP-RSV/NIS showed fatty degeneration of liver tissue (**A**), which was not observed in livers of mice treated with $dc_{300/GE11}$Ad5-E1/AFP-RSV/NIS (**B**). Injection of Ad5-CMV/NIS without surface modification led to a minor increase in AST level and a strong increase in ALT level as compared to mice treated with saline only, which was mostly avoided by coating of the adenovirus prior to systemic administration (**C**).

EGFR-specificity of vector targeting

3-dimensional high resolution ^{124}I-PET-imaging was used to investigate EGFR-specificity of NIS gene delivery after intravenous injection of the targeted replication-selective dc$_{300/GE11}$Ad5-E1/AFP-RSV/NIS. As shown before by ^{123}I-scintigraphy, i.v. injection of the uncoated vector (Ad5-E1/AFP-RSV/NIS) resulted in strong transduction of liver tissue that resulted in poor tumoral transduction (Figs. 5A, D). In contrast, coating of the adenovirus with PAMAM-G2-PEG-GE11 resulted in prevention of hepatic radioiodine accumulation and significantly enhanced transduction of tumor xenografts (Figs. 5B, D). By pretreatment of mice with the monoclonal anti-EGFR antibody cetuximab prior to systemic dc$_{300/GE11}$Ad5-E1/AFP-RSV/NIS administration tumoral radioiodine accumulation was significantly reduced while liver detargeting of NIS expression was not affected, thereby confirming EGFR-specificity of the targeted vector (Figs. 5C, D).

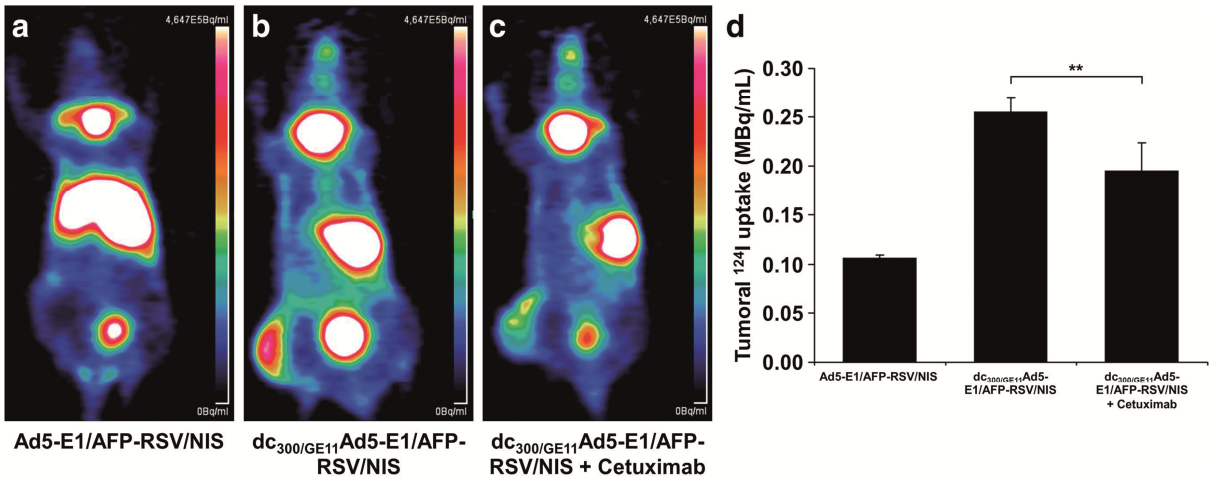

Fig. 5: *In vivo* analysis of EFGR-specificity. ^{124}I-PET-imaging demonstrated strong hepatic transduction after i.v. injection of the uncoated vector (Ad5-E1/AFP-RSV/NIS) (**A**) and quantification of radioiodine accumulation revealed only poor tumoral transduction (**A**, **D**). In contrast, coating of the adenovirus with PAMAM-G2-PEG-GE11 (dc$_{300/GE11}$Ad5-E1/AFP-RSV/NIS) prior to systemic injection resulted in prevention of hepatic radioiodine accumulation and distinct transduction of tumor xenografts (**B**, **D**). By pretreatment of mice with the monoclonal anti-EGFR antibody cetuximab prior to systemic dc$_{300/GE11}$Ad5-E1/AFP-RSV/NIS administration tumoral radioiodine accumulation was significantly reduced while liver detargeting of NIS expression was still effective (**C**, **D**).

Radionuclide therapy study in vivo

A single i.v. injection of the replication-deficient Ad5-AFP/NIS coated with PAMAM-G2-PEG-GE11 followed by a therapeutic dose of ^{131}I (dc$_{300/GE11}$Ad5-

AFP/NIS + ^{131}I, radiotherapy) showed a significant delay in tumor growth and improved survival (Figs. 6A, B) as compared to mice treated with saline only (NaCl-control, Figs. 6A, B). I.v. application of the oncolytic replication-selective Ad5-E1/AFP-E3/NIS with EGFR-targeted surface modification ($dc_{300/GE11}$Ad5-E1/AFP-E3/NIS, virotherapy) revealed a comparable delay in tumor growth and enhancement of survival due to the oncolytic activity of the adenovirus (Figs. 6A, B). Combined radiovirotherapy by i.v. injection of PAMAM-G2-PEG-GE11-coated replication-selective Ad5-E1/AFP-E3/NIS followed by application of ^{131}I ($dc_{300/GE11}$Ad5-E1/AFP-E3/NIS + ^{131}I, radiovirotherapy) resulted in a strongly enhanced therapeutic effect, as seen by significantly delayed tumor growth and further improvement of survival (Figs. 6A, B). While mice treated with saline only (NaCl-control) had to be killed within 2–3 weeks after onset of the experiment due to excessive tumor growth, 50% of mice treated with combined radiovirotherapy survived at least 9 weeks and 30% were even still alive at day 100, the endpoint of the observation period (Fig. 6B). None of the treated mice, even with combined radiovirotherapy, showed major adverse effects in terms of lethargy or respiratory failure due to oncolytic virotherapy or radionuclide treatment.

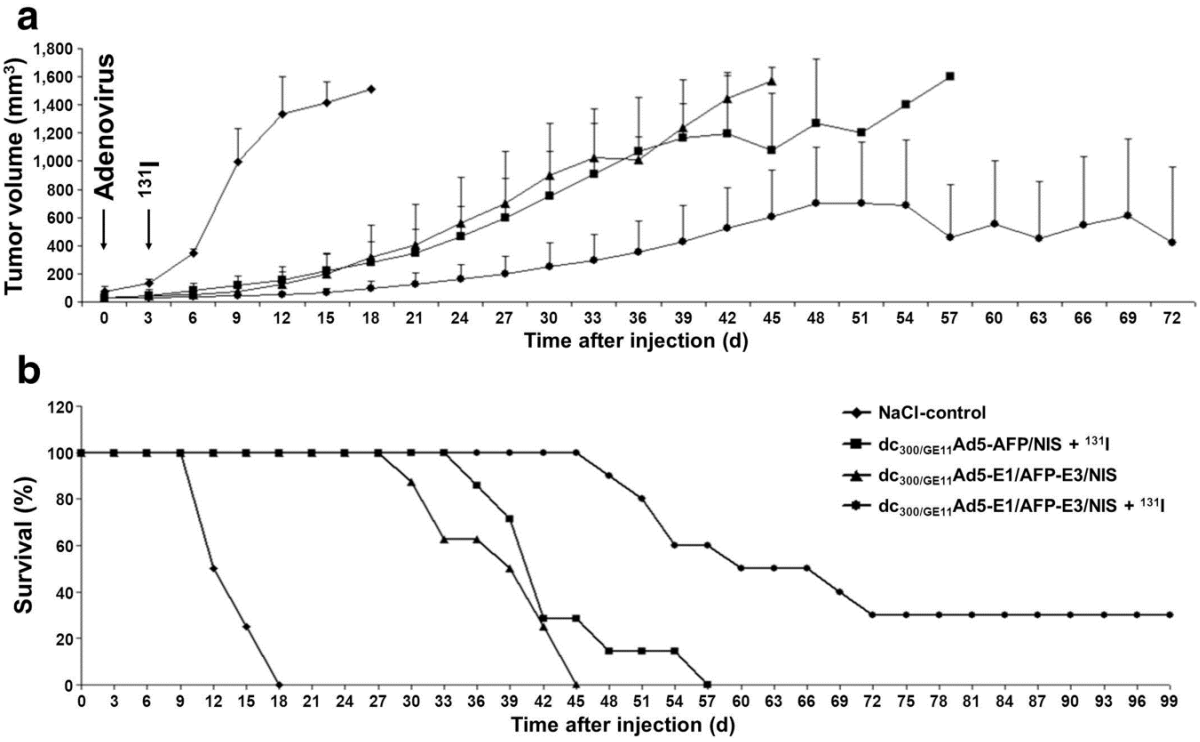

Fig. 6: *In vivo* therapeutic efficacy. A single i.v. injection of the replication-deficient Ad5-AFP/NIS coated with PAMAM-G2-PEG-GE11 followed by a therapeutic dose of ^{131}I ($dc_{300/GE11}$Ad5-AFP/NIS + ^{131}I, radiotherapy) showed a significant delay in tumor growth and improved survival (**A**, **B**) as compared to mice treated with saline only (NaCl-control, **A**, **B**). I.v. application of the oncolytic

replication-selective Ad5-E1/AFP-E3/NIS with EGFR-targeted surface modification ($dc_{300/GE11}$Ad5-E1/AFP-E3/NIS, virotherapy) revealed a comparable delay in tumor growth and enhancement of survival (**A**, **B**). Combined radiovirotherapy treatment ($dc_{300/GE11}$Ad5-E1/AFP-E3/NIS + ^{131}I, radiovirotherapy) resulted in a strongly enhanced therapeutic effect, as seen by significantly delayed tumor growth and further improved survival (**A**, **B**).

4.5 Discussion

The tropism of adenoviruses is greatly influenced by interaction with several blood components and the widespread expression of the coxsackie-adenovirus receptor (CAR) (Duffy *et al.*, 2012; Kalyuzhniy *et al.*, 2008; Vigant *et al.*, 2008; Waddington *et al.*, 2008). Aside from the inherent hepatic tropism after intravenous injection (Huard *et al.*, 1995), the CAR-dependent way of infection results in a broad cellular tropism with no intrinsic cancer specificity of wild type adenovirus (Green *et al.*, 2008). On the other hand, therapeutic efficacy of an adenovirus can be diminished by the lack of CAR on the tumor cell surface (Kanerva and Hemminki, 2005). Hence, a variety of methods have been developed, to alter the natural virus tropism and to detarget the vector away from its natural receptors. Through surface modification by chemical or genetical engineering, the adenovirus can be retargeted to cancer cell-specific targets in order to achieve sufficient transgene expression in cancerous tissues while expression in non-target organs and toxic side effects are minimized (Cattaneo *et al.*, 2008). In recent years, several studies provided evidence that adenoviruses can enter cells via cell surface molecules that are not natural viral receptors, for example the EGFR (Kim *et al.*, 2012). Cancer specificity of the ligand chosen for retargeting purposes is of great importance since unspecific infection of non-target cells can significantly reduce the availability of the therapeutic vector (Green *et al.*, 2008) as well as infection of non-target tissues may result in increased toxicity (Mizuguchi and Hayakawa, 2004), in particular regarding NIS-mediated radiotherapy. Moreover, targeted delivery of the NIS gene potentially allows for direct radiation treatment of tumors on-site and owns the advantage of achieving high radiation doses in the tumor while minimizing side effects to normal tissue. Although NIS as normal human protein is also endogenously expressed, in particular in the thyroid, the patient's thyroid gland can be protected by pretreatment with thyroid hormone L-T4 (levothyroxine), which effectively downregulates thyroidal NIS expression. In fact, NIS-mediated radiotherapy is well known to be remarkably safe in humans and has been routinely used as standard therapy in the management of thyroid cancer patients for over 70 years (Hingorani *et al.*, 2010). Moreover, NIS as normal human gene and protein causes no toxicity or diminished efficacy by immune responses as it is often observed after the use of other protein and gene therapeutics (Duffy *et al.*, 2012).

As a consequence of our recent characterization of a high-affinity, EGFR-

selective peptide (GE11) coupled to synthetic nanoparticles for systemic non-viral NIS gene delivery (Klutz *et al.*, 2011a), in this study we combined dendrimeric adenovirus surface modification with the EGFR-targeting strategy in order to generate a shielded, targeted and armed adenovirus for systemic radiovirotherapy of high EGFR-expressing hepatocellular carcinoma. In this way a triple cancer-specific adenovirus was developed that is transcriptionally targeted to hepatocellular carcinoma by the use of the alpha fetoprotein (AFP) promoter (Grünwald *et al.*, 2012) to control replication and NIS expression, that is actively targeted to the EGFR through attachment of the GE11 peptide (Klutz *et al.*, 2011a) and passively targeted to leaky tumor vasculature through the EPR effect (Iyer *et al.*, 2006). Here, we show the biodistribution and the retargeting capacity of adenovirus vectors coated with EGFR-specific dendrimer *in vitro* and *in vivo* using NIS in its dual function as reporter and therapy gene for non-invasive imaging of transgene expression and calculated radiotherapeutic treatment of hepatocellular carcinoma.

In vitro experiments using the EGFR-targeted adenovirus proved that transduction efficiency in CAR-positive cancer cells is barely hampered after dendrimer coating with mild but non-significant improvement if the cells additionally express the EGFR. In contrast, the CAR-negative ovarian cancer cells SKOV-3, that were shown to be refractory to infection with uncoated adenovirus, can be efficiently infected by dendrimer-coated adenoviral vectors with a significant additional increase in transduction efficiency by attachment of the targeting ligand GE11. These experiments suggest that adenovirus vectors coated with targeted dendrimer can transduce cells CAR-independently by employing a different receptor for cell entry and may be of great potential for therapy of EGFR-expressing neoplasms lacking CAR.

In the current study, ^{123}I scintigraphy after intravenous administration of the dendrimer-coated adenovirus revealed strong detargeting of hepatic transgene expression that is usually caused by i.v. administration of the uncoated vector. The reduction in hepatic NIS-mediated iodine accumulation is even stronger than observed in our former study (Grünwald *et al.*, 2013) (80 vs 70 %), which might be due to improved covering of the adenoviral surface epitopes by the smaller dendrimer used for surface modification (molecular weight PAMAM-G2 3,284 Da vs PAMAM-G5 28,854 Da). By improved liver detargeting the vector was able to infect peripheral hepatoma xenografts of mice upon systemic delivery even more efficiently than the

previous vector (15 vs 13 %ID/g) with an increased average biological half-life and tumor absorbed dose calculated for therapeutic ^{131}I (103 vs 91 mGy/MBq). This may be explained by active GE11-mediated tumor targeting combined with an extended blood circulation time of the vector enforcing the passive tumor targeting through the EPR effect as it was shown before by Yao *et al*. (Yao *et al.*, 2011b). Substitution of the targeting ligand by a cysteine residue led to a significant decrease of tumoral transgene expression, thereby confirming the targeting benefit. These results were further corroborated by analysis of hepatic and tumoral NIS mRNA expression. To ensure that the targeting ligand was indeed targeting the EGF receptor, we pretreated mice bearing high EGFR expressing HuH7 xenografts with the high affinity anti-EGFR antibody cetuximab prior to infection with the targeted adenovirus. Pretreatment with cetuximab lowered the tumoral transduction efficiency of $dc_{300/GE11}$Ad5-E1/AFP-RSV/NIS significantly as shown by high resolution ^{124}I-PET-imaging, while hepatic detargeting was not affected and remained stable.

For reliable quantitative analysis of such experiments highly sensitive imaging modalities are needed to display even small changes in biodistribution that, however, may have great biological impact. Recently, [^{18}F]-tetrafluoroborate, a known alternative substrate of NIS, was evaluated as new PET imaging agent in preclinical models, demonstrating high sensitivity and significantly improved resolution as compared to ^{124}I, which will improve NIS biodistribution analysis in orthotopic tumor models with overlap in radioiodine accumulation with organs endogenously expressing NIS (Jauregui-Osoro *et al.*, 2010; Weeks *et al.*, 2011).

Wild-type adenovirus is initially recognized by the scavenger receptors of Kupffer cells (KC) due to its negative surface charge leading to rapid clearance from the bloodstream as well as distinct liver pathology (Xu *et al.*, 2008) as seen in the current study by strong liver transduction, increased liver transaminase levels, as well as pathologic liver histology. In contrast, the electrostatically coated adenovirus complex is no longer negatively charged as described by Vetter *et al.* (Vetter *et al.*, 2013) and the avoidance of liver toxicity observed in our study may be explained by preventing activation of KCs and the induction of proinflammatory processes.

One approach to improve viral oncolytic therapy is its combination with standard anticancer therapies such as radiotherapy (Advani *et al.*, 2006) as we and others have shown before (Dilley *et al.*, 2005; Grünwald *et al.*, 2012; Grünwald *et al.*, 2013). Thus, in the next step we addressed the question whether the advantage in

tumoral transduction efficiency is able to also improve therapeutic radionuclide application in liver cancer xenografts, after it had previously been demonstrated after local administration of a NIS-expressing adenovirus that the outcome of combined radiovirotherapy is highly dependent upon the viral dose that is delivered to the tumor (Trujillo *et al.*, 2012a) possibly based on increased tumoral transduction subsequently leading to decelerated radioiodine efflux. In the current study, NIS-mediated radiotherapy using a replication-deficient dendrimer-coated adenovirus showed a significant delay in tumor growth that was associated with markedly improved survival as compared to control mice treated with saline only. A comparable delay in tumor growth has been reached by injection of the oncolytic replication-selective $dc_{300/GE11}$Ad5-E1/AFP-E3/NIS. Furthermore, the effects of oncolysis and radiation therapy have been used synergistically and tumor-specific oncolysis was shown to be further enhanced by combination with NIS-mediated radiotherapy. Potent and selective systemic anti-tumoral efficacy was demonstrated. Radiovirotherapy using PAMAM-G2-PEG-GE11 instead of PAMAM-G5 (Grünwald *et al.*, 2013) for coating of our replication-selective Ad5-E1/AFP-E3/NIS demonstrated further deceleration of tumor growth as well as improved survival of mice. The synergistic therapeutic effectiveness of the combination therapy may also allow a reduction of the doses usually applied in individual single therapies and thereby reduce potential toxic side effects as it was shown before (Lamfers *et al.*, 2002). Moreover, Trujillo *et al*. (Trujillo *et al.*, 2012b) recently showed after intratumoral application of a replication-selective adenovirus carrying the NIS gene that a minimal applied dose of 37 MBq ^{131}I is required for radiovirotherapy in a murine xenograft model in order to improve efficacy of oncolytic virotherapy alone and that the doses needed to achieve reduced tumor growth and extended survival in mice are scaled well within doses currently clinically used for the treatment of thyroid cancer patients. With regards to potential clinical application of adenovirus-based NIS gene therapy it is noticeable that at the Mayo Clinic (Rochester, MN, USA) the first NIS-expressing adenovirus is currently Food and Drug Administration approved for a human clinical trial in patients with locally recurrent prostate cancer.

In conclusion we developed a new adenovirus-based vector by EGFR-targeted dendrimeric surface modification that retained the superior characteristics of dendrimer coating described in our former study (Grünwald *et al.*, 2013) and additionally improved its biodistribution and selective transduction efficiency of

peripheral tumor tissues upon systemic vector administration by EGFR-specific targeting. The ability of the coated vector to improve NIS gene delivery to EGFR expressing tumor cells, combined with its reduced hepatic tropism and toxicity profile, which warrants further investigation in more advanced tumor models, highlights its potential as a prototype virus for future clinical investigation.

4.6 Acknowledgments

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5. Chapter 4

Systemic epidermal growth factor receptor-targeted sodium iodide symporter (NIS) gene therapy in a genetically engineered mouse model of pancreatic ductal adenocarcinoma

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5.1 Abstract

The pancreatic ductal adenocarcinoma (PDAC) belongs to the cancers with most unfavorable prognoses. Despite outstanding effectiveness of a series of compounds *in vitro* and in xenograft models, the results of clinical trials are predominantly disappointing and new treatment options are urgently needed.

In the mouse a PDAC can be genetically induced by activation of constitutively active $Kras^{G12D}$ in combination with a deletion of p53, which shows the typical changes of human disease. This development of endogenous mouse models, away from the usual transplant models represents an important step as these tumor models are more suitable to predict clinical effectiveness of a specific cancer treatment.

In previous studies using xenograft mouse models the sodium iodide symporter (NIS) as well characterized theranostic gene allowed detailed non-invasive analysis of vector biodistribution and transgene expression by ^{123}I-scintigraphy/SPECT and ^{124}I-PET imaging, as well as highly effective therapeutic application of radionuclides (^{131}I, ^{188}Re). As a logical consequence of these proof-of-principle studies and as a next step towards clinical application of the NIS gene therapy concept, in the current study we investigated tumor specificity and transduction efficiency of tumor-targeted polyplexes as systemic NIS gene delivery vehicles in an advanced genetically engineered mouse model (GEMM) of PDAC. Therefore, we used novel tumor-targeted polyplexes based on linear polyethylenimine (LPEI), polyethylene glycol (PEG), and the synthetic peptide GE11 as an epidermal growth factor receptor (EGFR)-specific ligand (LPEI-PEG-GE11) to target a NIS-expressing plasmid to the high EGFR-expressing PDAC.

In vitro iodide uptake studies with cell explants derived from murine EGFR-positive and EGFR-knockout PDAC lesions demonstrated high transduction efficiency and EGFR-specificity of LPEI-PEG-GE11/NIS. *In vivo* 2-dimensional ^{123}I γ-camera imaging and 3-dimensional high-resolution ^{124}I-PET imaging experiments were performed at different time points after systemic EGFR-targeted NIS gene transfer and showed significant tumor-specific accumulation of radioiodine in the PDAC of mice at a magnitude, that can be expected to result in a therapeutic effect of ^{131}I. These results were further confirmed by NIS-specific qPCR analysis and immunohistochemistry. A first series of therapy studies indicates that the tumoral

accumulation is indeed high enough for a therapeutic effect of ^{131}I as demonstrated by a reduction in tumor volume that was measured by magnetic resonance imaging.

In conclusion, our preclinical data in an advanced genetically engineered PDAC mouse model clearly demonstrate the high potential of systemic NIS gene therapy using EGFR-targeted synthetic gene transfer vehicles, opening the prospect of clinical application of targeted NIS-mediated radionuclide therapy in non-thyroidal cancers.

5.2 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth leading cause of cancer deaths in the Western world despite its comparably low incidence, which demonstrates the lack of efficient therapeutic strategies (Siegel *et al.*, 2013). The median survival time after diagnosis is less than 6 months, mostly due to late diagnosis at the stage of inoperability and due to the high resistance of tumor cells to external beam radiation and chemotherapy (Li *et al.*, 2004). Despite intensive scientific and industrial efforts, so far no significant extension of survival could be achieved by any of the numerous tested drugs (Mazur and Siveke, 2012).

The genetic and morphological changes in the carcinogenesis of the PDAC are well known and include the initiation and progression of premalignant lesions to the invasive and metastatic pancreatic cancer (Hingorani *et al.*, 2003; Izeradjene *et al.*, 2007; Mazur and Siveke, 2012; Siveke and Schmid, 2005). The most common genetic changes include activating mutations in the Kras gene, inactivation of the tumor suppressor $p16^{INK4a}$, p53 and SMAD4 genes, and activation of the EGF receptor and other receptors for growth factors such as MET (Ardito *et al.*, 2012; Mazur and Siveke, 2012).

In recent years, several complex genetically modified mouse models were generated (Bardeesy *et al.*, 2006; Hingorani *et al.*, 2003; Schneider *et al.*, 2005; Siveke *et al.*, 2007), in which pancreatic cancers arise from precursor lesions. In the mouse a PDAC can be induced by activation of constitutively active $Kras^{G12D}$ in combination with a conditional deletion of p53, which shows the typical changes of human disease (Bardeesy *et al.*, 2006). This development of endogenous mouse models, away from the usual transplant models represents a significant step in the evolution of preclinical models (Singh *et al.*, 2010). Clonal cells with very homogeneous molecular equipment are used in the xenograft transplantation models (e.g. long-term cultivated cell lines), thus the composition of established transplanted tumors is significantly different from endogenously grown tumors. The endogenously grown tumors are genetically heterogeneous and have a lower vascularization and significantly more stroma (Olive *et al.*, 2009). These tumor models are therefore better suited to predict the clinical effectiveness of a specific cancer treatment. For these reasons, the use of an endogenous mouse model of PDAC is believed to be a promising preclinical method to evaluate the systemic effectiveness of polyplex-mediated NIS gene transfer for subsequent radioiodine therapy.

In a recent study, Klutz *et al*. used synthetic polyplexes based on pseudo-dentritic oligoamines with high intrinsic tumor affinity for NIS gene therapy in a subcutaneous human liver carcinoma mouse model (Klutz *et al.*, 2011b). After systemic NIS gene transfer the tumor-selective accumulation of radioiodine was sufficient for a significant therapeutic effect. In addition to the intrinsic tumor affinity due to leaky vasculature, the tumor targeting of polyplexes can be further increased by the attachment of tumor-specific ligands. Therefore, in a subsequent study, Klutz *et al*. used polymers for NIS gene delivery, which were composed of linear polyethylenimine (LPEI), shielded by polyethylene glycol (PEG) and coupled to the synthetic peptide GE11 as an EGFR-specific ligand (LPEI-PEG-GE11) (Klutz *et al.*, 2011a). After systemic application of these polyplexes condensed with NIS cDNA (LPEI-PEG-GE11/NIS), effective and EGFR-specific tumor targeting could be demonstrated by tumor-specific radioiodine accumulation in a high EGFR-expressing xenograft mouse model of hepatocellular carcinoma. After the injection of a therapeutic dose of ^{131}I tumoral iodine uptake was demonstrated to be sufficiently high for a significant delay of tumor growth and prolongation of survival. The endogenous PDAC mouse model examined in this study also expresses EGFR on a very high level and is therefore expected to facilitate active tumor targeting of the NIS gene by the EGFR-specific polyplexes LPEI-PEG-GE11/NIS.

Overall, our preliminary studies in different xenograft tumor mouse models show, that synthetic polymers own an enormous potential for non-viral NIS gene delivery allowing the accumulation of a therapeutically effective ^{131}I or ^{188}Re dose (Grünwald *et al.*, 2012; Grünwald *et al.*, 2013; Klutz *et al.*, 2009; Klutz *et al.*, 2011a; Klutz *et al.*, 2011b; Klutz *et al.*, 2011c; Knoop *et al.*, 2011; Knoop *et al.*, 2013), and thus can offer an innovative and potentially curative treatment option for thyroidal and non-thyroidal tumors. In the current study, we investigated the potential of EGFR-targeted polyplexes for systemic NIS gene therapy in an advanced, more complex endogenous mouse model of PDAC.

5.3 Materials and Methods

Establishment of genetically modified mice

Establishment of the *Ptf1a*$^{+/Cre}$;*Kras*$^{+/LSL-G12D}$;*p53*$^{LoxP/LoxP}$ (*CKP*) and *Ptf1a*$^{+/Cre}$;*Kras*$^{+/LSL-G12D}$;*p53*$^{LoxP/LoxP}$; *Egfr*$^{fl/fl}$ (CKP-EGFR^{KO}) strains has been described previously (Hingorani *et al.*, 2003; Kawaguchi *et al.*, 2002; Lee and Threadgill, 2009; Marino *et al.*, 2000; Natarajan *et al.*, 2007).

Animals were maintained under specific pathogen-free conditions with access to mouse chow and water *ad libitum*. The experimental protocol was approved by the regional governmental commission for animals (Regierung von Oberbayern, Munich, Germany).

Preparation and culture of pancreatic ductal adenocarcinoma (PDAC) explants

PDAC explants were isolated as described previously (Heid *et al.*, 2011) and cultured in DMEM high glucose medium (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (v/v; PAA, Colbe, Germany), 1% penicillin/streptomycin (v/v; Invitrogen) and 1% non-essential amino acids (v/v; Invitrogen). Cells were maintained at 37°C and 5% CO_2 in an incubator with 95% humidity. Cell culture medium was replaced every second day and explants were passaged at about 85% confluency. For each genotype, experiments with cell explants were performed with the following numbers of mice: CKP, n = 3; CKP-EGFR^{KO}, n = 2.

Flow cytometry analysis of cellular EGFR expression was performed as described previously (Klutz *et al.*, 2011a).

Plasmid and polymer synthesis and polyplex formation

The NIS cDNA and LPEI-based conjugates were synthesized as described previously (Klutz *et al.*, 2011a).

Plasmid DNA was condensed with polymers at indicated conjugate/plasmid-ratios (c/p; w/w) in HEPES-buffered glucose [HBG: 20 mmol/l HEPES, 5% glucose (w/v), pH 7.4] as described previously (Russ *et al.*, 2008) and incubated at room temperature for 20 min before use. Final DNA concentration of polyplexes for *in vitro* studies was 2 µg/ml, and for *in vivo* studies 200 µg/ml.

Transient transfection

For *in vitro* transfection experiments, PDAC cell explants were grown to 60–80% confluency. Explants were incubated for 4 hours with polyplexes in the absence of serum and antibiotics followed by incubation with complete growth medium for 24 h. Either LPEI-PEG-GE11/NIS, LPEI-PEG-Cys/NIS, or LPEI-PEG-GE11 without DNA was added in c/p ratios as indicated. Transfection efficiency was evaluated by measurement of iodide uptake activity as described below.

In vitro ^{125}Iodide uptake assay

Following transfections, iodide uptake of PDAC cell explants was determined at steady-state conditions as described previously (Spitzweg *et al.*, 1999; Weiss *et al.*, 1984). Results were normalized to cell survival measured by cell viability assay and expressed as cpm/A490 nm.

Cell viability was measured using the commercially available MTS-assay (Promega, Mannheim, Germany) according to the manufacturer's recommendations as described previously (Willhauck *et al.*, 2007).

Radioiodine uptake studies after systemic NIS gene transfer

Experiments started when mice were about 6-8 weeks of age. For systemic *in vivo* NIS gene transfer, polyplexes (LPEI-PEG-GE11/NIS, c/p 0.8) were applied i.v. via the tail vein at a DNA dose of 2.5 mg/kg (50 µg DNA in 250 µl HBG). For the proof-of-principle of NIS-mediated PDAC-specific radioiodine accumulation *in vivo*, 24 h after i.v. injection of polyplexes mice received 18.5 MBq ^{123}I i.p. (n=9) and radioiodine distribution was monitored by serial imaging on a gamma camera (Forte, ADAC Laboratories, Milpitas, CA, USA) equipped with a VXHR (ultra-high resolution) collimator as described previously (Willhauck *et al.*, 2007). Regions of interest were quantified and expressed as a fraction of the total amount of applied radionuclide per gram tumor tissue (% ID/g). The retention time within the tumor was determined by serial scanning after radioiodine injection. A subset of mice (n=2) was pretreated i.p. with 2 mg of the competitive NIS-inhibitor sodium perchlorate ($NaClO_4$) 30 min before ^{123}I administration. For more detailed high-resolution molecular imaging in order to achieve better discrimination between uptake in the tumor and the adjacent stomach, 24 or 48 h after i.v. injection of polyplexes (LPEI-PEG-GE11/NIS, each time point n=5; LPEI-PEG-GE11/antisense-NIS, each time point n=1) mice received 10 MBq ^{124}I

i.p. and radioiodine biodistribution was monitored by a 15 min static acquisition 3 h post injection using a micro PET system (Inveon, SIEMENS Preclinical Solutions, Erlangen, Germany). Mean tumoral radioiodine uptake was calculated in megabequerel per milliliter (MBq/mL) by manually placing 3D regions of interest in the tumor.

Analysis of NIS mRNA expression using qPCR

Total RNA was isolated from PDAC or non-target tissues (liver, lungs) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Single-stranded oligo (dT)-primer cDNA was generated using SuperscriptIII Reverse Transcriptase (Invitrogen). Following primers were used: *h*NIS: (5′-ACACCTTCTGGACCTTCGTG-3′) and (5′-GTCGCAGTCGGTGTAGAACA-3′), GAPDH: (5′-GAGAAGGCTGGGGCTCATTT-3′) and (5′-CAGTGGGGACACGGAAGG-3′). qPCR was performed with the cDNA from 1 µg RNA using the SYBR green PCR master mix (Qiagen, Hilden, Germany) in a Rotor Gene 6000 (Corbett Research; Morthlake, New South Wales, Australia). Relative expression levels were calculated using the comparative $\Delta\Delta C_t$ method and internal GAPDH for normalization.

Immunohistochemical analysis of NIS protein expression

Immunohistochemical staining of paraffin-embedded tissue sections derived from PDAC after NIS gene delivery was performed using a mouse monoclonal antibody directed against aminoacid residues 468–643 of human NIS (kindly provided by John C Morris, Mayo Clinic, Rochester, MN, USA) as described previously (Spitzweg *et al.*, 2007). For histological examination, parallel slides were also routinely stained with hematoxylin and eosin (H/E).

Radioiodine therapy studies

^{124}I-PET imaging revealed highest tumoral radioiodine accumulation 48 h after i.v. NIS gene delivery. Therefore, 48 h after systemic administration of LPEI-PEG-GE11/NIS polyplexes, a therapeutic dose of 55.5 MBq ^{131}I was administered i.p. (LPEI-PEG-GE11/NIS + ^{131}I). Control mice (NaCl-control; n=7) received saline only. The cycle consisting of systemic NIS gene transfer followed by radioiodine was repeated twice on days 4/6 and 8/10 (n=3), or additionally on day 11/13 (n=3). Tumor

sizes were assessed by high resolution magnetic resonance imaging (MRI) on a 3T clinical scanner (Philips Ingenia 3.0T; Royal Philips Electronics, Eindhoven, The Netherlands) before treatment and weekly thereafter (each radioiodine treatment group n=2).

Statistical methods

All *in vitro* experiments were carried out in triplicate. Results are represented as means ± SD of triplicates. Statistical significance was tested using Student's *t*-test (two-tailed).

5.4 Results

Iodide uptake studies in vitro

Transfection conditions using LPEI-PEG-GE11/NIS were optimized in high EGFR-expressing PDAC cell explants, derived from CKP mice, by measurement of perchlorate-sensitive iodide uptake activity 24 hours following application of polyplexes (data not shown). We found an optimal conjugate/plasmid ratio of 0.8 (c/p 0.8) resulting in highest transfection efficiency at lowest cytotoxicity. This ratio was used in all subsequent experiments. Twenty-four hours after transfection with LPEI-PEG-GE11/NIS, cell explants showed a 22-26-fold increase in ^{125}I accumulation as compared to cells incubated with the empty control vector LPEI-PEG-GE11 (Fig. 1A). Transfection with untargeted LPEI-PEG-Cys/NIS polyplexes resulted in significantly lower iodide uptake activity in EGFR-positive PDAC explants (Fig. 1A), thereby demonstrating the advantage in transduction efficiency gained by active EGFR-specific tumor targeting.

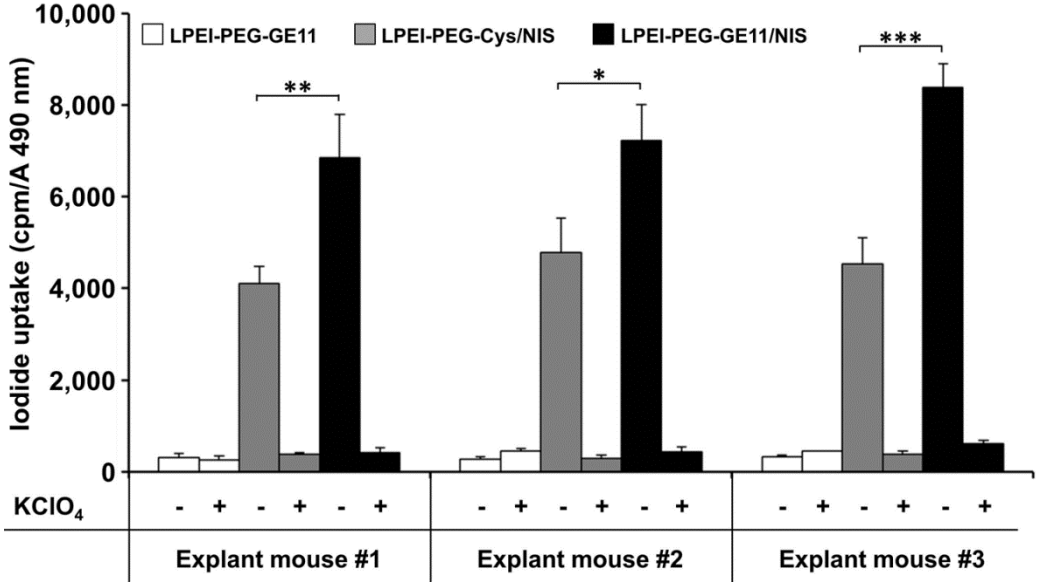

Fig. 1A: Iodide uptake was measured in high EGFR-expressing PDAC cell explants following *in vitro* transfection with LPEI-PEG-GE11/NIS, control polyplexes LPEI-PEG-Cys/NIS, or with LPEI-PEG-GE11 alone. Cells transfected with LPEI-PEG-GE11/NIS showed a significant increase in perchlorate-sensitive ^{125}I accumulation. After transfection with LPEI-PEG-Cys/NIS the iodide uptake was significantly decreased. In contrast, no perchlorate-sensitive iodide uptake above background level was observed in cells transfected with LPEI-PEG-GE11 without DNA.

Additional *in vitro* iodide uptake studies in EGFR-knockout PDAC cell explants with very low EGFR expression levels, as determined by fluorescence-activated cell scanning analysis (data not shown), showed no significant difference between

transfection with targeted LPEI-PEG-GE11/NIS or untargeted LPEI-PEG-Cys/NIS polyplexes, thereby demonstrating EGFR-specificity of the targeting ligand (Fig. 1B). Polyplex-mediated NIS gene transfer did not alter cell viability as measured by MTS assay (data not shown).

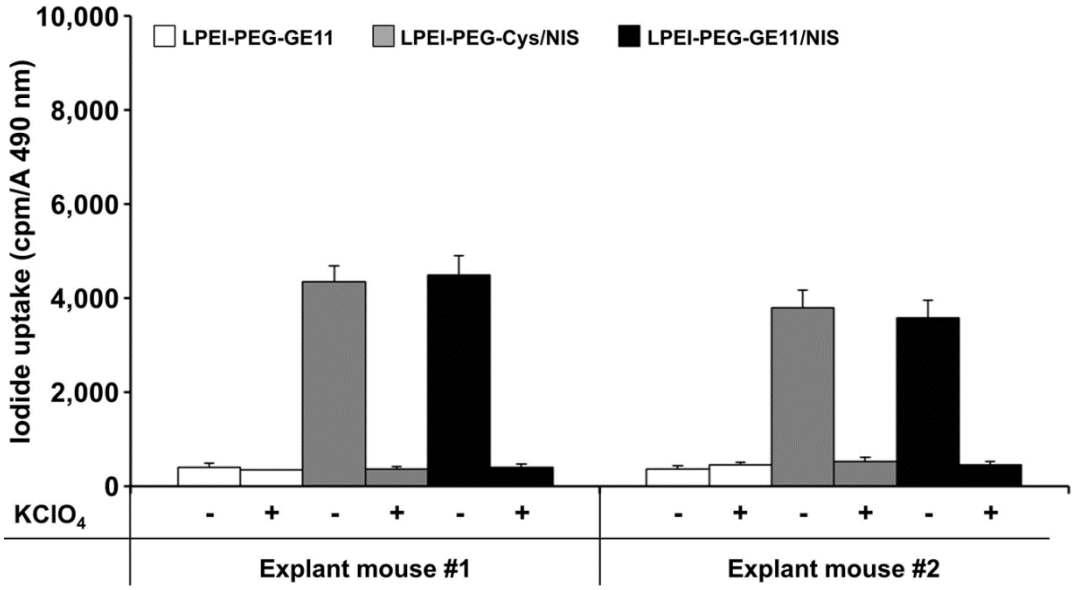

Fig. 1B: Transfection efficiency of LPEI-PEG-GE11/NIS and LPEI-PEG-Cys/NIS was additionally investigated by *in vitro* iodide uptake studies in EGFR-knockout PDAC cell explants and showed no significant difference between transfection with targeted or untargeted polyplexes, thereby demonstrating EGFR-specificity of the targeting ligand.

Preliminary scintigraphy of EGFR-targeted NIS gene delivery

Mice with high EGFR-expressing PDAC (Fig. 2) were imaged after i.v. polyplex administration for functional NIS expression via whole body ^{123}I scintigraphy.

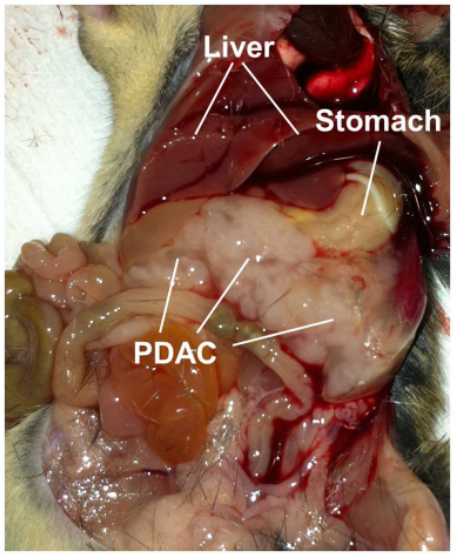

Fig. 2: A genetically engineered mouse at the age of 8 weeks developed a pancreatic ductal adenocarcinoma (PDAC) which occupies a large portion of the abdominal cavity below the stomach.

In vivo ^{123}I gamma camera imaging of radioiodine biodistribution demonstrated high levels of NIS-mediated radionuclide accumulation in the pancreatic tumors of mice after systemic injection of EGFR-targeted LPEI-PEG-GE11/NIS (Fig. 3A).

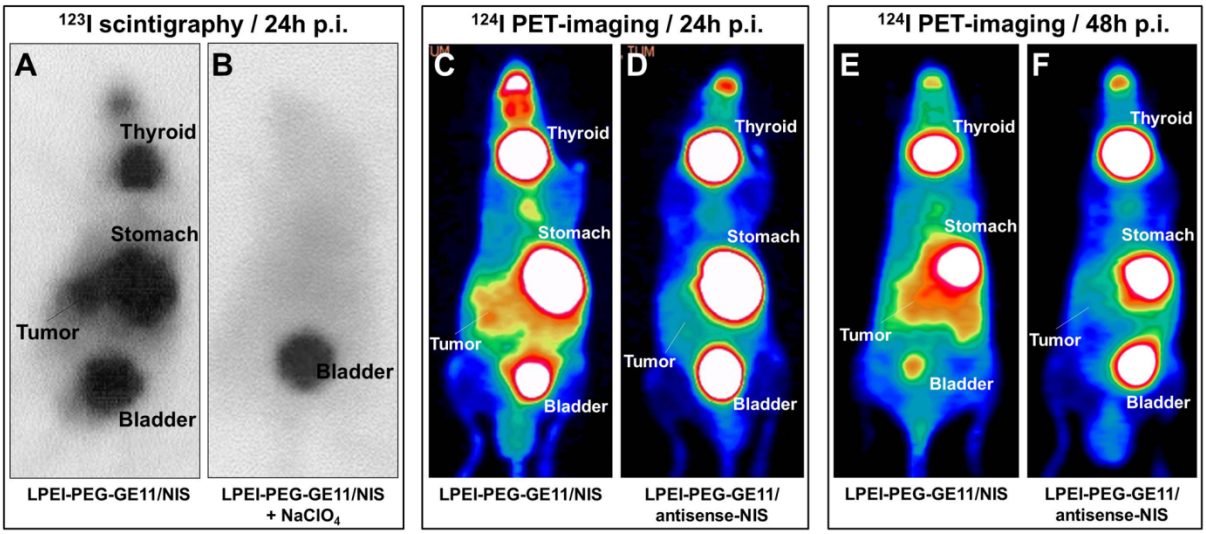

Fig. 3: ^{123}I scintigraphy of mice injected with LPEI-PEG-GE11/NIS demonstrated pancreatic tumoral radioiodine uptake (**A**) to be perchlorate–sensitive (**B**) and therefore indeed NIS-mediated. ^{124}I PET-imaging (**C**-**F**) confirmed findings of scintigraphy and facilitated better differentiation between tumor and stomach. Injection of mice with the control vector LPEI-PEG-GE11/antisense-NIS (**D**, **F**) showed no pancreatic iodide uptake activity above background level.

Serial scanning of mice revealed an accumulated dose of approximately 7-10 % of the injected dose per gram tumor tissue (% ID/g) with an average biological half-life of 4.5 h (Fig. 4).

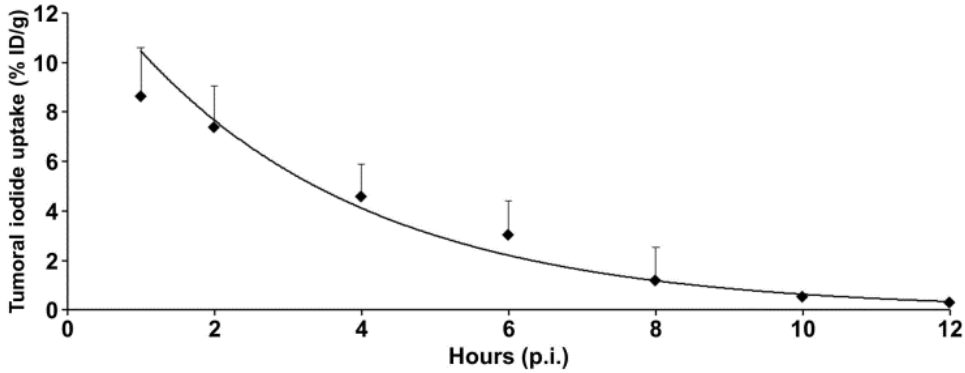

Fig. 4: Serial scanning on a gamma camera 24 h after injection of mice with LPEI-PEG-GE11/NIS demonstrated pancreatic tumors to accumulate 7-10 % ID/g ^{123}I with an average biological half-life of 4.5 h.

In addition to ^{123}I uptake in the PDAC, radioiodine accumulation was also observed in stomach and thyroid that physiologically express NIS and in the urinary

bladder due to renal radionuclide elimination but in no case in other non-target organs (Fig. 3). To confirm that tumoral iodide uptake was indeed NIS-mediated, LPEI-PEG-GE11/NIS-injected mice were additionally treated with the competitive NIS-inhibitor sodium perchlorate ($NaClO_4$) 30 min before ^{123}I administration, which completely blocked tumoral iodide accumulation in addition to the physiological NIS-mediated iodide uptake in stomach and thyroid gland (Fig. 3B).

High resolution 3-dimensional PET imaging of radioiodine biodistribution

3-dimensional high resolution ^{124}I-PET-imaging was used to better distinguish between tumoral uptake and iodine accumulation in the stomach (Fig. 3C-F). As shown before by ^{123}I-scintigraphy (Fig. 3A), i.v. injection of LPEI-PEG-GE11/NIS resulted in strong transduction of tumor tissue (Fig. 3C, E). In contrast, systemic injection with LPEI-PEG-GE11/antisense-NIS resulted in no significant tumoral radioiodine accumulation (Fig. 3D, F). Quantification of tumoral ^{124}I uptake revealed significantly higher radioiodine accumulation 48 h after i.v. injection of LPEI-PEG-GE11/NIS as compared to 24h after NIS gene transfer (Fig. 5).

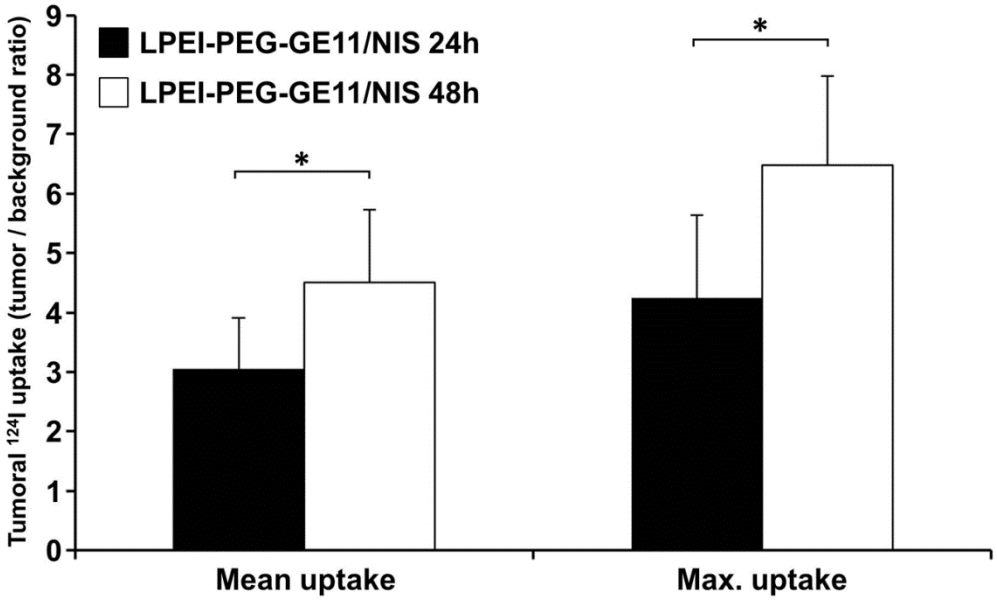

Fig. 5: Scanning of mice at different time points after LPEI-PEG-GE11/NIS-mediated NIS gene transfer demonstrated pancreatic tumors to accumulate significantly higher amounts 48 h after gene transfer as compared to 24h.

Analysis of NIS mRNA expression in PDAC

Ex vivo tumors and non-target organs (liver, lungs) were analyzed for the level of NIS mRNA expression by quantitative real-time PCR (qPCR) (Fig. 6). A 20-fold

increase in NIS mRNA expression of PDAC lesions was detected 48 h after i.v. injection of LPEI-PEG-GE11/NIS as compared to untreated tumors. In contrast, no significant NIS mRNA expression above background level was observed in non-target organs and tumors of mice treated with the control vector LPEI-PEG-GE11/antisense-NIS (Fig. 6). Thereby, NIS mRNA analysis correlated well with the radionuclide biodistribution observed and confirmed the findings of ^{124}I-PET imaging (Fig. 3).

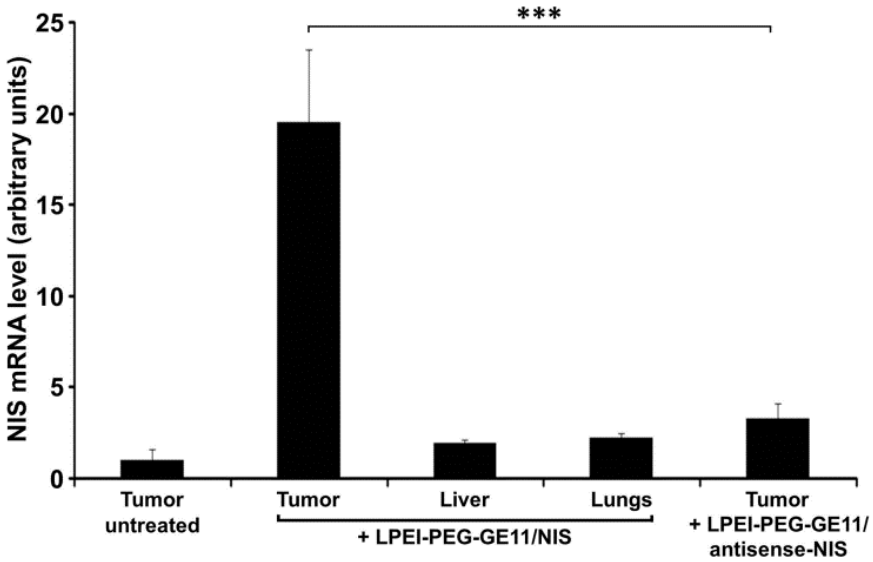

Fig. 6: NIS-specific qPCR analysis revealed a 20-fold increase of NIS mRNA expression level in pancreatic tumors of mice injected with LPEI-PEG-GE11/NIS as compared to tumors of untreated mice. In contrast, NIS mRNA expression was not increased in non-target organs and in tumors of mice injected with the control vector LPEI-PEG-GE11/antisense-NIS.

Immunohistochemical analysis of NIS protein expression in PDAC

48 h after the start of treatment, mice were sacrificed and PDAC lesions were dissected and processed for immunohistochemical analysis using a *h*NIS-specific antibody (red staining; yellow arrows). Analysis revealed a patchy staining pattern with areas of NIS-specific immunoreactivity in tumors after systemic application of LPEI-PEG-GE11/NIS (Fig. 7, left). In contrast, tumors treated with the control vector (LPEI-PEG-GE11/antisense-NIS) showed no NIS-specific immunoreactivity (Fig. 7, right). Parallel control slides with the primary and secondary antibodies replaced in turn by PBS and isotype-matched non immune immunoglobulin were negative (data not shown).

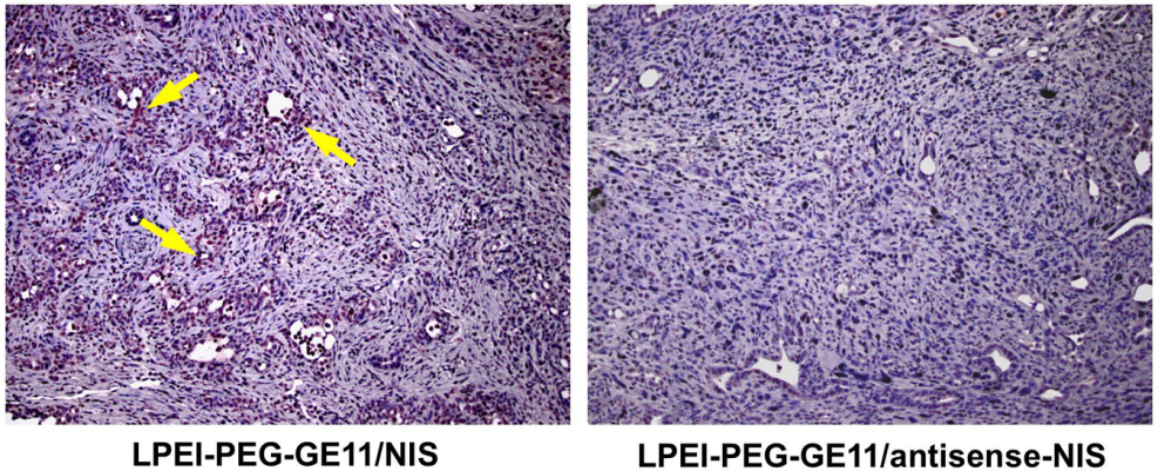

Fig. 7: Immunohistochemical staining of sections of paraffin-embedded pancreatic tumors revealed a patchy staining pattern with areas of NIS-specific immunoreactivity in tumors after systemic application of LPEI-PEG-GE11/NIS. In contrast, tumors treated with the control vector (LPEI-PEG-GE11/antisense-NIS) showed no NIS-specific immunoreactivity.

Preliminary radioiodine therapy studies

Mice treated with three (empty labels) or four cycles (filled labels) of LPEI-PEG-GE11/NIS followed by ^{131}I showed a strong reduction of tumor volume (Fig. 8). No tumor regrowth was detectable until mice died after the end of therapy.

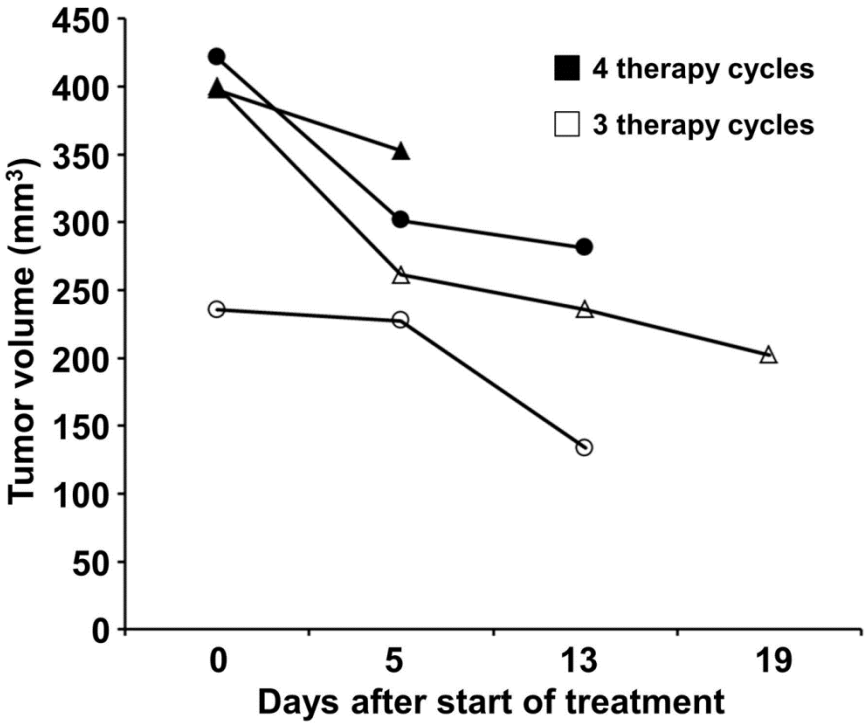

Fig. 8: Mice treated with 3 (empty labels) or 4 (filled labels) cycles of LPEI-PEG-GE11/NIS + ^{131}I showed a strong reduction in tumor volume as determined by magnetic resonance imaging.

5.5 Discussion

Despite numerous chemotherapy trials based on different clinical approaches the prognosis of patients with unresectable pancreatic cancer has not significantly changed over the last 15 years (Mazur and Siveke, 2012). The commonly used Gemcitabine in patients with advanced pancreatic cancer shows a significant, but small survival benefit and primarily improves the health-related quality of life of patients (Schneider *et al.*, 2005). So far newer compounds have shown virtually no improvement in survival. Because the substances that have reached the clinical arena had previously shown promising therapeutic efficacy in *in vitro* cell culture systems as well as in *in vivo* xenograft models, it has to be adopted that such preclinical tests do not adequately simulate the complexity of the disease (Mazur and Siveke, 2012; Olive and Tuveson, 2006). This finding is not surprising, since the PDAC is characterized by a high rate of chromosomal instability with very heterogeneous genetic changes, high chemical resistance, very strong fibrosis as well as low vascularization and immunogenic activity. Since genetically engineered mouse models (GEMMs) are now available that are extremely similar to the carcinogenesis of human carcinoma, both the complex carcinogenesis and the pharmacological method of action of potential therapeutic substances can be evaluated in preclinical studies in a much more suitable manner. As an example of a non-clinical therapy platform, Singh *et al*. evaluated the utility of two state-of-the-art, mutant Kras-driven GEMMs - one of non-small-cell lung carcinoma and another of pancreatic adenocarcinoma - by assessing responses to existing standard-of-care chemotherapeutics, and subsequently in combination with EGFR and VEGF inhibitors (Singh *et al.*, 2010). Comparisons with corresponding clinical trials indicated that these GEMMs model human responses well and these data built the foundation for the use of validated GEMMs in predicting outcome and interrogating mechanisms of therapeutic response and resistance.

Human PDAC develop lesions usually from premalignant PanIN (intraepithelial pancreatic neoplasia) and IPMN (intraductal papillary mucinous neoplasia). In the endogenous PDAC mouse model used in this study (CKP) the combined pancreas-specific recognition of a constitutively active Kras with G12D mutation and a deletion of p53 lead to the accelerated formation of invasive pancreatic carcinoma from PanIN lesions (Bardeesy *et al.*, 2006). Pancreas-specific Cre secures that Kras and p53 are induced only in the pancreas. Normally, in this so-called “CKP” model the formation

of tumors begins 4 - 6 weeks after birth and the life span of mice usually does not exceed 60 days. Therefore, the life-prolonging effect of potential test substances in these mice can be determined very easily. In addition, the rapidly growing tumors show significant fibrosis similar to human tumors, which is regarded as an obstacle to effective availability of agents within the tumor.

With regard to further development of the NIS gene therapy concept towards clinical application with the main challenge of sufficiently high tumor-specific NIS expression after systemic vector application, the genetically engineered PDAC mouse model is a highly interesting preclinical model to test the potential of synthetic polyplexes that have recently shown promising results in subcutaneous xenograft mouse models (Klutz *et al.*, 2009; Klutz *et al.*, 2011a; Klutz *et al.*, 2011b). Based on the high expression levels of EGFR in the PDAC model, we chose the EGFR-targeted LPEI-PEG-GE11 polymers (Klutz *et al.*, 2011a; Li *et al.*, 2005). We first investigated transduction efficiency and EGFR-specificity of LPEI-PEG-GE11 polymers *in vitro* in tumor cells derived from PDAC explant cultures. After transduction with LPEI-PEG-GE11/NIS, the high EGFR-expressing PDAC cells showed significant perchlorate-sensitive accumulation of radioiodine, which was significantly reduced after transduction with non-targeted LPEI-PEG-Cys/NIS, thereby representing improved transduction efficiency by the use of the targeting ligand GE11. EGFR-specificity of targeting was further demonstrated in EGFR-negative PDAC explant cultures derived from CKP-EGFR^{KO} mice that showed no significant difference between transduction with EGFR-targeted or non-targeted vectors. As additional control, *in vitro* transduction with the empty vector LPEI-PEG-GE11 led to no significant iodide accumulation above background level, thereby corroborating that accumulation of radioiodine is indeed NIS-mediated.

After successful demonstration of feasibility of transduction *in vitro*, the next question was whether tumor-specific transduction can also be achieved *in vivo* after systemic vector application. Intravenous administration of LPEI-PEG-GE11/NIS resulted in a significant tumor-specific iodide uptake in mice bearing endogenous PDAC tumors, as demonstrated by ^{123}I gamma camera imaging. The uptake was shown to be perchlorate-sensitive and of a magnitude that is comparable to what we have observed in previous xenograft studies using the same vector construct (Klutz *et al.*, 2011a). Pancreatic tumors accumulated approx. 7-10 % ID/g with an average biological half-life of 4.5 h. Quantification of the tumoral radioiodine uptake was more

challenging in this endogenous tumor model due to partial overlap of the pancreatic tumor with the radioiodine accumulating stomach on 2-dimensional gamma camera images. Therefore, after the proof-of-principle of polyplex-mediated tumor-selective NIS gene transfer by gamma camera imaging, ^{124}I PET imaging was employed as a more detailed high resolution 3-dimensional imaging technique with increased sensitivity and resolution. Mice injected systemically with LPEI-PEG-GE11/NIS were confirmed to accumulate high levels of radioiodine in endogenous tumors. Analysis of mice at different time points after NIS gene transfer demonstrated maximal tracer uptake 48 h after NIS gene transfer. Control experiments with LPEI-PEG-GE11/antisense-NIS showed no significant tumoral radioiodine accumulation above background level and thereby confirmed LPEI-PEG-GE11/NIS to cause NIS-specific tumoral tracer uptake. These molecular imaging data were further corroborated by NIS-specific immunohistochemistry as well as qPCR analysis.

High resolution and a certain degree of sensitivity is a crucial prerequisite of functional molecular imaging of NIS expression using 3-dimensional imaging techniques (Baril *et al.*, 2010; Richard-Fiardo *et al.*, 2011). To further improve resolution and sensitivity of PET-imaging of NIS expression, [^{18}F]-TFB, a well-known substrate of NIS, had recently been characterized as PET-imaging agent (Jauregui-Osoro *et al.*, 2010; Weeks *et al.*, 2011) with significantly improved imaging characteristics and resolution as compared to ^{124}I. This is particularly important for systemic NIS gene delivery approaches in orthotopic and metastatic tumor models with low volume disease and/or overlap with organs that physiologically accumulate iodide, in particular stomach.

In a next step we have evaluated the therapeutic effectiveness of ^{131}I in this genetically engineered PDAC mouse model after LPEI-PEG-GE11-mediated systemic NIS gene delivery and in preliminary results we were able to demonstrate a strong reduction in tumor growth after application of 3 to 4 cycles of LPEI-PEG-GE11/NIS followed by 55.5 MBq ^{131}I 48 h later.

In conclusion, our data clearly show the high potential of EGFR-targeted nanoparticle vectors for targeting the NIS gene to PDAC in a genetically engineered mouse model. Based on the role of NIS as potent and well characterized reporter gene allowing non-invasive imaging of functional NIS expression, this study allowed detailed characterization of *in vivo* biodistribution of functional NIS expression by ^{123}I gamma camera imaging as well as ^{124}I PET imaging, which is 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. In a first therapy study, tumor-specific iodide accumulation was further demonstrated to be sufficiently high for a remarkable reduction of tumor growth in genetically engineered mice after three to four cycles of polyplex application followed by ^{131}I therapy. Although the results will certainly have to be confirmed in a larger series, the data gained so far clearly demonstrate feasibility of the use of a genetically engineered mouse model of PDAC to predict the outcome and therapeutic response of systemic nanoparticle-mediated NIS gene therapy. This study therefore opens the exciting prospect of NIS-targeted radionuclide therapy of pancreatic ductal adenocarcinoma using EGFR-targeted polyplexes for systemic NIS gene delivery.

5.6 Acknowledgments

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6. Summary

The sodium iodide symporter (NIS) represents one of the oldest and most successful targets for molecular imaging and targeted radionuclide therapy. The capacity of the NIS gene to induce radioiodine accumulation in non-thyroidal tumors has been investigated in a variety of tumor models and the enormous potential of NIS as novel reporter and therapy gene has been convincingly demonstrated. However, one of the major hurdles on the way to efficient and safe application of the NIS gene therapy concept in the clinical setting, in particular in metastatic disease, is optimal tumor-specific targeting in the presence of low toxicity and high transduction efficiency of gene delivery vectors, with the ultimate goal of systemic vector application.

In the course of this thesis, the establishment and characterization of novel tumor-targeted adenovirus-based gene delivery vectors for the purpose of systemic NIS gene therapy in a liver cancer xenograft mouse model as well as systemic non-viral NIS gene therapy in a genetically engineered mouse model of pancreatic ductal adenocarcinoma are reported.

In a first step, tumor selectivity and transduction efficacy of a genetically engineered replication-selective oncolytic adenovirus have been evaluated, in which E1A is driven by the AFP promoter and NIS is inserted in the E3 region driven by the replication-dependent E3 promoter (Ad5-E1/AFP-E3/NIS). The use of NIS as reporter gene provided us with the possibility of non-invasive imaging of vector biodistribution as well as monitoring of biodistribution, level and duration of transgene expression after local application of Ad5-E1/AFP-E3/NIS in a xenograft mouse model of hepatocellular carcinoma (HCC). The application of NIS as reporter gene confirmed high tumor-selectivity of virus replication as well as high transduction efficacy of Ad5-E1/AFP-E3/NIS after local intratumoral injection, which also resulted in a significant reduction of tumor growth due to strong oncolytic activity in HCC xenografts (virotherapy). Moreover, combination of oncolytic virotherapy with radioiodine treatment (radiovirotherapy) led to an additional reduction of tumor growth that resulted in markedly improved survival as compared to virotherapy alone. These data clearly demonstrated that tumor-specific NIS gene transfer using a replication-selective adenoviral gene delivery vector allows for targeted NIS-mediated, imaging-guided radionuclide therapy of extrathyroidal tumors, which enhances the therapeutic effect of oncolytic virotherapy and proves its potential for clinical application.

With respect to the major hurdles for clinical application of systemic adenovirus-mediated gene therapy, we have investigated the potential of adenovirus surface coating with synthetic dendrimers to detarget adenoviral vectors away from the liver towards tumor-specific targets after systemic application. For this purpose, we first coated a replication-deficient adenovirus carrying the NIS gene under the control of the CMV promoter (Ad5-CMV/NIS) with poly(amidoamine) dendrimers (PAMAM-G5) and studied its biodistribution using NIS as reporter gene. ^{123}I γ-camera imaging showed high levels of radioiodine accumulation in the liver of mice after i.v. injection of Ad5-CMV/NIS, which was significantly reduced by 70% after i.v. injection of dendrimer-coated virus particles. Inhibition of adenovirus liver pooling resulted in increased tumoral transduction efficiency as demonstrated by increased levels of tumoral iodide uptake after i.v. virus application. In a next step, we coated the replication-selective Ad5-E1/AFPE3/NIS and performed biodistribution analysis after i.v. injection in the HCC xenograft mouse model using ^{123}I-scintigraphy. Serial ^{123}I γ-camera imaging showed significantly higher levels of tumor-specific iodide accumulation, which resulted in a significant delay in tumor growth in contrast to uncoated virus demonstrating, that dendrimer coating is very effective in increasing the load of viable virus reaching peripheral tumors by decreasing liver pooling. These results indicate that non-covalent coating of adenoviral vectors with synthetic dendrimers shows high promise for effective adenovirus liver detargeting and tumor retargeting taking advantage of the merge of non-viral and viral vector technology, and therefore has the potential to improve current systemic gene delivery and tumor targeting strategies. It represents an innovative strategy to optimize efficacy and safety of systemic NIS gene delivery that allows imaging and radiovirotherapy of non-thyroidal cancers exploiting synergies between oncolytic virotherapy and NIS-mediated radionuclide therapy.

To further improve shielding and targeting of adenovirus-based vectors, we physically coated replication-selective adenoviruses carrying the *h*NIS gene with a conjugate consisting of cationic poly(amidoamine) (PAMAM) dendrimer linked to the peptidic, epidermal growth factor receptor (EGFR)-specific ligand GE11. *In vitro* experiments demonstrated CAR-independent but EGFR-specific transduction efficiency. Systemic injection of the uncoated adenovirus in a HCC xenograft mouse model led to high levels of NIS expression in the liver due to hepatic sequestration, which were significantly reduced after coating as demonstrated by ^{123}I-scintigraphy.

Evasion from liver pooling resulted in decreased hepatotoxicity and increased transduction efficiency in peripheral xenograft tumors. ^{124}I-PET-imaging confirmed EGFR specificity by significantly lower tumoral radioiodine accumulation after pretreatment with the EGFR-specific antibody cetuximab. A significantly enhanced oncolytic effect was observed following systemic application of dendrimer-coated adenovirus that was further increased by additional treatment with a therapeutic dose of ^{131}I. These results demonstrate that we successfully developed new adenovirus-based vectors by EGFR-targeted dendrimeric surface modification that retained the superior characteristics of dendrimer coating and additionally improved its biodistribution and selective transduction efficiency of peripheral tumor tissues upon systemic vector administration by EGFR-specific targeting. The ability of the coated vector to improve NIS gene delivery to EGFR-expressing tumor cells, combined with its reduced hepatic tropism and toxicity profile highlights its potential as a prototype virus for future clinical investigation and warrants further investigation in more advanced tumor models.

Genetically engineered mouse models with endogenous tumors may be more suitable to predict the clinical effectiveness of a specific cancer treatment. As a logical consequence of the proof-of-principle studies in xenograft-bearing mice and as a next step towards clinical application of the NIS gene therapy concept, we investigated tumor specificity and transduction efficiency of tumor-targeted polyplexes as systemic NIS gene delivery vehicles in an advanced genetically engineered mouse model (GEMM) of pancreatic ductal adenocarcinoma (PDAC). For this purpose, we used tumor-targeted polyplexes based on linear polyethylenimine (LPEI), shielded with polyethylene glycol (PEG), and coupled with the EGFR-specific peptidic ligand (LPEI-PEG-GE11) to target a NIS-expressing plasmid to the high EGFR-expressing PDAC. *In vitro* iodide uptake studies with cell explants derived from murine EGFR-positive and EGFR-knockout PDAC lesions demonstrated high transduction efficiency of LPEI-PEG-GE11/NIS as well as EGFR-specificity of targeting. *In vivo* 2-dimensional ^{123}I γ-camera and 3-dimensional high-resolution ^{124}I-PET imaging experiments were performed at different time points after systemic EGFR-targeted NIS gene transfer and showed significant tumor-specific accumulation of radioiodine in the pancreatic tumors of mice, that is expected to result in a therapeutic effect of ^{131}I. In first therapy studies a strong reduction of tumor

growth was demonstrated after NIS-mediated radioiodine treatment that will have to be confirmed in ongoing therapy studies in a larger series.

In conclusion, the data reported in this thesis clearly demonstrate the high potential of local and particularly systemic NIS gene therapy using targeted adenoviral, synthetic and combined gene transfer vehicles, opening the exciting prospect of clinical application of targeted NIS-mediated radionuclide therapy of non-thyroidal cancers, even in the metastatic stage.

7. Publications

7.1 Original papers

Grünwald GK, Vetter A, Klutz K, Willhauck MJ, Schwenk N, Senekowitsch-Schmidtke R, Schwaiger M, Zach C, Wagner E, Göke B, Holm PS, Ogris M, Spitzweg C.
EGFR-Targeted Adenovirus for Improved Systemic Delivery of the Theranostic NIS Gene and Image-Guided Radiovirotherapy. Mol Ther Nucleic Acids. 2013 [in revision]

Grünwald GK, Vetter A, Klutz K, Willhauck MJ, Schwenk N, Senekowitsch-Schmidtke R, Schwaiger M, Zach C, Wagner E, Göke B, Holm PS, Ogris M, Spitzweg C.
Systemic Image-Guided Liver Cancer Radiovirotherapy Using Dendrimer-Coated Adenovirus Encoding the Sodium Iodide Symporter (NIS) as Theranostic Gene. J Nucl Med. 2013 [in press]

Grünwald GK, Klutz K, Willhauck MJ, Schwenk N, Senekowitsch-Schmidtke R, Schwaiger M, Zach C, Göke B, Holm PS, Spitzweg C.
Sodium iodide symporter (NIS)-mediated radiovirotherapy of hepatocellular cancer using a conditionally replicating adenovirus. Gene Ther. 2012 Oct 4. doi: 10.1038/gt.2012.79. [Epub ahead of print]

Klutz K, Schaffert D, Willhauck MJ, **Grünwald GK**, Haase R, Wunderlich N, Zach C, Gildehaus FJ, Senekowitsch-Schmidtke R, Göke B, Wagner E, Ogris M, Spitzweg C.
Epidermal growth factor receptor-targeted (131)I-therapy of liver cancer following systemic delivery of the sodium iodide symporter gene. Mol Ther. 2011 Apr;19(4):676-85. doi: 10.1038/mt.2010.296. Epub 2011 Jan 18.

7.2 Manuscripts in preparation

Grünwald GK, Trajkovic-Arsic M, Klutz K, Gupta A, Schwenk N, Braren R, Settles M, Senekowitsch-Schmidtke R, Schwaiger M, Wagner E, Göke B, Ogris M, Siveke J, Spitzweg C.

Systemic epidermal growth factor receptor-targeted sodium iodide symporter (NIS) gene therapy in a genetically engineered mouse model of pancreatic ductal adenocarcinoma, *in preparation*

Klutz K, **Grünwald GK**, Willhauck MJ, Vetter A, Schwenk N, Hacker M, Zach C, Senekowitsch-Schmidtke R, Gildehaus FJ, Göke B, Wagner E, Ogris M, Spitzweg C.
Systemic sodium iodide symporter (NIS) gene transfer in hepatocellular carcinoma using Transferrin-receptor (Tfr) targeted non-viral gene delivery vectors, *in preparation*

7.3 Oral presentations

95th Annual Meeting of the Endocrine Society, San Francisco, CA, USA, June 2013
Grünwald GK, Vetter A, Klutz K, Willhauck MJ, Schwenk N, Senekowitsch-Schmidtke R, Schwaiger M, Zach C, Wagner E, Göke B, Holm PS, Ogris M, Spitzweg C.
Epidermal Growth Factor Receptor (EGFR)-Specific Retargeting of a Dendrimer-Coated Adenovirus Encoding the Theranostic Sodium Iodide Symporter (NIS) Gene for Systemic Image-Guided Radiovirotherapy.

19th Annual Meeting of the German Society for Gene Therapy, Hamburg, February 2013
GK Grünwald, A Vetter, K Klutz, MJ Willhauck, N Schwenk, R Senekowitsch-Schmidtke, M Schwaiger, C Zach, E Wagner, B Göke, PS Holm, M Ogris, C Spitzweg.
EGFR-Specific Retargeting of a Dendrimer-Coated Adenovirus Encoding the Theranostic NIS Gene for Systemic Radiovirotherapy.

2nd Molecular Imaging in Biology and Oncology (MIBO) Meeting, Nice, France, January 2012
GK Grünwald, K Klutz, A Vetter, N Schwenk, R Senekowitsch-Schmidtke, M Schwaiger, PS Holm, E Wagner, B Göke, M Ogris, C Spitzweg.
Polymer Coating of Adenoviral Vectors for Effective Systemic Gene Delivery Using the Sodium Iodide Symporter (NIS) as Reporter and Therapy Gene.

17th Annual Meeting of the German Society for Gene Therapy, Munich, October 2010
GK Grünwald, K Klutz, MJ Willhauck, N Wunderlich, R Senekowitsch-Schmidtke, M Ogris, PS Holm, B Göke, C Spitzweg.
An α-fetoprotein promoter driven, conditionally replicating adenovirus that expresses the sodium iodide symporter (NIS) for radiovirotherapy of HCC.

7.4 Poster presentations

Collaborative Congress of the European Society of Gene and Cell Therapy and the French Society of Gene and Cell Therapy, Versailles, France, October 2012
Grünwald GK, Vetter A, Klutz K, Willhauck MJ, Schwenk N, Senekowitsch-Schmidtke R, Schwaiger M, Göke B, Wagner E, Holm PS, Ogris M, Spitzweg C.
EGFR-Specific Retargeting of a Dendrimer-Coated Adenovirus Carrying the Sodium Iodide Symporter (NIS) for Systemic Radiovirotherapy of Liver Cancer.

Joint Conference of the Germany Society for Gene Therapy and the LOEWE Center for Cell and Gene Therapy Frankfurt, Frankfurt, March 2012
Grünwald GK, Klutz K, Vetter A, Schwenk N, Senekowitsch-Schmidtke R, Schwaiger M, Holm PS, Wagner E, Göke B, Ogris M, Spitzweg C.
Polymer Coating of Adenoviral Vectors for Effective Systemic Gene Delivery Using the Sodium Iodide Symporter (NIS) as Reporter and Therapy Gene.

2nd Molecular Imaging in Biology and Oncology (MIBO) Meeting, Nice, France, January 2012
Grünwald GK, Klutz K, Vetter A, Schwenk N, Senekowitsch-Schmidtke R, Schwaiger M, Holm PS, Wagner E, Göke B, Ogris M, Spitzweg C.
Polymer Coating of Adenoviral Vectors for Effective Systemic Gene Delivery Using the Sodium Iodide Symporter (NIS) as Reporter and Therapy Gene.

Collaborative Congress of the European Society of Gene and Cell Therapy and the British Society of Gene Therapy, Brighton, UK, October 2011
Grünwald GK, Klutz K, Willhauck MJ, Schwenk N, Senekowitsch-Schmidtke R, Holm PS, Göke B, Spitzweg C.

Radioiodine imaging-guided analysis of transduction efficiency, tumor-selectivity and therapeutic efficacy of an α-fetoprotein promoter-driven, conditionally replicating adenovirus that expresses the sodium iodide symporter (NIS).

Collaborative Congress of the European Society of Gene and Cell Therapy and the British Society of Gene Therapy, Brighton, UK, October 2011
Grünwald GK, Vetter A, Klutz K, Schwenk N, Senekowitsch-Schmidtke R, Holm PS, Wagner E, Göke B, Ogris M, Spitzweg C.
Liver detargeting of adenoviral vectors by polymer coating after systemic delivery using the sodium iodide symporter (NIS) as reporter gene.

Collaborative Congress of the European Society of Gene and Cell Therapy and the British Society of Gene Therapy, Brighton, UK, October 2011
Grünwald GK, Vetter A, Klutz K, Senekowitsch-Schmidtke R, Holm PS, Wagner E, Göke B, Ogris M, Spitzweg C.
Enhanced oncolytic efficacy after polymer coating of an α-fetoprotein promoter driven, conditionally replicating adenovirus that expresses the sodium iodide symporter (NIS).

21st Annual Meeting of the German Society of Virology, Freiburg, March 2011
Grünwald GK, Vetter A, Klutz K, Schwenk N, Senekowitsch-Schmidtke R, Holm PS, Wagner E, Göke B, Ogris M, Spitzweg C.
Liver detargeting of adenoviral vectors by polymer coating after systemic delivery using the sodium iodide symporter (NIS) as reporter gene.

93rd Annual Meeting of the Endocrine Society, Boston, MA, USA, June 2011
Grünwald GK, Vetter A, Klutz K, Schwenk N, Senekowitsch-Schmidtke R, Holm P, Wagner E, Göke B, Ogris M, Spitzweg C.
Liver detargeting of adenoviral vectors by polymer coating after systemic delivery using the sodium iodide symporter (NIS) as reporter gene.

54th Annual Meeting of the German Society of Endocrinology, Hamburg, March 2011
Grünwald GK, Klutz K, Willhauck MJ, Wunderlich N, Senekowitsch-Schmidtke R, Holm PS, Göke B, Spitzweg C.

An α-fetoprotein promoter driven, conditionally replicating adenovirus that expresses the sodium iodide symporter (NIS) for radiovirotherapy of hepatocellular cancer.

7.5 Awards

Travel grant, German Society of Endocrinology
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10. Curriculum Vitae

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