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*Expert Review*

# Cell and Tissue Targeting of Nucleic Acids for Cancer Gene Therapy

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***Abstract.*** Tumor targeting—per definition—includes any strategy to improve the specificity of the therapeutic nucleic acid towards the tumor site, while highest biological activity should be maintained. Targeting has been successfully achieved at the transcriptional, transductional or delivery level. For tumor-specific delivery, physical targeting methods like electroporation, hyperthermia, magnetofection, photochemical internalization or ultrasound, and biological targeting systems, including active and passive tumor targeting, have been developed. Therapeutic effects could be demonstrated with various targeted nucleic acid formulations, such as tumor-targeted DNA plasmids expressing p53 or tumor necrosis factor alpha, small interfering RNAs knocking down gene expression from tumor specific chromosomal translocations or gene expression of tumor neoangiogenic processes, as well as double stranded RNA poly inosine-cytosine which triggers apoptosis in targeted tumor cells.

**KEY WORDS:** gene therapy; nucleic acids; siRNA; targeting; tumor.

## INTRODUCTION

Generally, the term of "gene therapy" alludes to transfer of genetic material into cells for a therapeutic purpose. The concept has been investigated for the treatment of inherent genetic diseases such as e.g., cystic fibrosis or severe combined immunodeficiencies. Most present gene therapy studies concern the treatment of cancer (1). Either as monotherapy or in combination with other regimens like radiation and/or chemotherapy, gene therapy offers an alternative to treat unresectable, metastasized or therapy refractory solid tumors.

Nucleic acids with therapeutic potential which have been investigated extensively for cancer gene therapy include plasmid DNA (pDNA) or synthetic nucleic acids such as antisense oligonucleotides, small interfering RNA (siRNA), or other double stranded RNAs like poly inosine-cytosine (pIC). The various types of nucleic acids achieve different effects at the molecular genetic level. Thus, pDNA vectors are mainly used for intra-nuclear delivery to replace or to substitute a specific genetic function in the target cell resulting in a "*gain of gene function*." In contrary, "*loss of gene function*" is often mediated by intra-cytoplasmatic delivery of synthetic antisense oligonucleotides or siRNA reducing the expression of endogenous genes in a sequence-specific manner (2). Therefore the mechanisms of therapeutic effects are diverse including inhibition of neoangiogenesis (3–5), activation of cytokine or immunostimulatory responses (6), induction of apoptosis (7,8), reduction of tumor cell proliferation (9–11) or strategies to replace deleted genes or to over-express beneficial genes (12).

In order to succeed in cancer gene therapy, the efficient delivery of therapeutic genes to a target site is a major challenge. Various gene delivery systems have been developed such as viral or nonviral vectors and their potential advantages and disadvantages have been defined. While viruses are very effective gene delivery systems, they are limited in use due to immunogenicity of viral proteins, risk of oncogenesis and inadvertent creation of infectious viral particles. Nonviral vectors have several advantages regarding safety reasons (lack of immunogenicity) and pharmaceutical issues (easy synthesis and large-scale production), however they tend to show poor transfection efficiencies compared to viral vectors.

Within the last decades numerous nonviral gene delivery systems have been developed. They comprise naked and chemically modified nucleic acids as well as particle-based vectors. These vectors compact nucleic acids to protect them from degradation by serum nucleases and to facilitate cellular uptake by charge-mediated interactions with the cell surface. The investigated particle-based systems can be divided into three main groups: (1) "polyplexes" formed by nucleic acids and polycationic polymers like polylysine (PLL), polyethylenimine (PEI) or polyamidoamine (PAMAM) dendrimers (13–16), (2) "lipoplexes" containing cationic lipids like DOTMA or DOTAP and nucleic acids (13,17,18) and (3) nanoparticles which bind or encapsulate nucleic acids to "nanoplexes" (19–25). Gene transfer efficiency strongly varies between the different formulations. Regarding "polyplex" formulations polyethylenimine (PEI) is the most popularly used polycationic polymer due to its excellent and consistent transfection efficiency levels on several cell lines. The buffering capability of PEI offers the opportunity to escape from the endosome ("proton sponge effect" (26)). Drawbacks of PEI are significant toxicity and lack of degradability. Polylysine (PLL), in

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contrast, is biodegradable, is ineffective in transfection unless endosomal disruptive agents or chloroquine is included. “Lipoplex” formation is dependent on several physical conditions (pH, charge) as well as on the structural characteristics of the lipids so that the specificity and the structural demands for successful transfection are determined by the variation in the liposomal arrangement. At least *in vitro*, PAMAM, PEI and some liposomal formulations are the most effective gene transfecting agents.

Efficient delivery and expression of nucleic acids can only succeed in therapy if properly directed towards the tumor site. Thus, targeting is one of the major bottlenecks in cancer gene therapy. The current review reports on a variety of achievements of specific nucleic acid formulations, with an emphasis on the options for physical and biological targeting of cancer tissue.

## TARGETING NUCLEIC ACID DELIVERY

Targeting—per definition—includes any strategy to improve the specificity of gene expression and/or delivery of nucleic acids towards the tumor site, while highest transfection levels should be achieved. Different targeting policies have been investigated in the past; including the targeted delivery of nucleic acids as well as the transductional and transcriptional targeting at the intracellular site of tumor cells (see Fig. 1). Transductional targeting comprises of all methods which improve the intracellular release and transport of transgenes towards the nucleus of the target cell. Triggered endosomal release, enhanced cytosolic trafficking and specific nuclear import of transgenes (27) are the major objectives of this targeting strategy. As soon as the genes are delivered into the nucleus they can only be expressed if adequate promoter and/or enhancer elements are included in the gene expression cassette. Transcriptional targeting makes use of tumor-specific promoter/enhancer systems through which specifically high levels of transcription controlled transgene expression can be obtained within the target tumor cell (28–31).

The cellular uptake of particle-based gene delivery systems usually happens via charge-mediated interactions with the cell surface followed by endocytosis. Upon systemic administration, however, such non-specific interactions also take place with blood components and non-target tissues and hamper gene delivery. Specific delivery of lipo-, nano- or polyplexes towards the tumor site can be mediated by physical or biological targeting technologies.

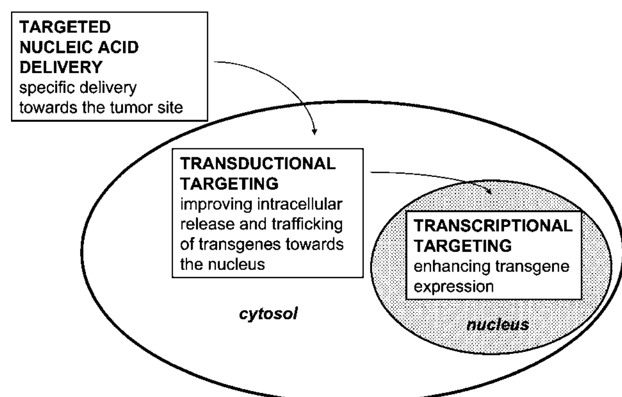


**Fig. 1.** Tumor targeting principles for therapeutic nucleic acids.

## PHYSICAL TARGETING

Physical targeting consists of a series of physical techniques to enhance nucleic acid delivery at a specific site. Specificity is often achieved by the localized action of physical forces. These can be e.g., mechanical forces, an electric or a magnetic field, light or thermic effects. Mechanical forces are exploited for example with the gene gun (32,33) or hydrodynamic delivery, especially using hydrodynamic limb vein delivery (34–36). Methods commonly used are described in the following paragraphs.

### Electroporation

In electroporation, electrical pulses are used to enhance the cellular uptake of nucleic acids. Although the detailed mechanism remains unclear, it is believed that the electrical impulses destabilize the cell membranes and allow direct migration of nucleic acids into the cytosol, avoiding the endocytosis pathway (37). Brunner *et al.* found that elutriated cells show a cell cycle independent gene expression, indicating that nuclear import and transcription of transgenes is less limiting with electorporation than with particle-based methods (38). Electroporation is deemed to be safe, inexpensive and easy to handle.

This method has been efficiently used since a long time to transport DNA into living cells *in vitro* (39). Recently, using *in vitro* tissue electroporation with naked pDNA on multicellular tumor spheroids (MCTS) a more than 10-fold enhanced gene expression compared to the polycation polyethylenimine PEI 22 kDa was observed comprising quiescent cells (40). *In vivo*, local injection of pDNA followed by electroporation of muscle, tumor or skin tissue has been shown to enhance gene transfer 100- to 1000-fold over plasmid injection alone (41–45). Intramuscular pDNA electroporation gave excellent gene expression levels in mice and expression can be maintained for several months (46). Goto *et al.* found by using electroporation in CT26 bearing mice that after intratumoral application of herpes simplex virus thymidine kinase gene or diphtheria toxin “A” gene a siginificant decrease in tumor growth occurs finding up to 90% retarded tumor growth compard to control mice (43).

Therapeutic effects were observed with a plasmid encoding granulocyte-macrophage colony-stimulating factor (GM-CSF) and the B7-1 costimulatory immune molecule in the murine fibrosarcoma model resulting in complete and permanent tumor regression of more than 60% of treated mice (47,48). Also upon intratumoral electroporation of IL-12 plasmid in the B16F10 melanoma model in mice (49) tumor regression was observed in the absence of critical side effects.

The simplicity and high efficiency of DNA electroporation led to first clinical trials in late 2004 (50). Clinical phase I melanoma studies for intratumoral IL-12 DNA electroporation have been performed finding encouraging results including up-regulated IL-12 levels, tumor necrosis and infiltrating lymphocytes. Further clinical trials concerning melanoma have already started using a intralesion-

ally electroporated plasmid encoding IL-2. Phase I studies for electroporation supported vaccines for prostate cancer and tumors expressing HER-2 and/or carcinoembryonic antigen have been initiated (50).

### Magnetofection

Magnetofection is a technique which shows promising results both for *in vitro* and *in vivo* targeted gene delivery (51). In this method the nucleic acid is reversibly attached to superparamagnetic nanoparticles which are then focused to the target site via a high-energy magnetic field. *In vitro* magnetofection promotes rapid transfection and excellent gene expression levels as well. Same was demonstrated *in vivo* when applied to the gastrointestinal tract and in blood vessels (51). Recently promising *in vivo* results of gene transfection to airway epithelium have been reported by Xenariou *et al.* (52).

Regarding the mechanism it was demonstrated that the magnetic field itself does not alter the uptake of PEI-DNA polyplexes which were physically associated with the paramagnetic nanoparticles. It is proposed that the magnetic forces lead to an accelerated accumulation of complexes on the cell surface but not to traction into the cell (53).

### Ultrasound

Ultrasound (US) can provoke non-thermal effects via acoustic cavitations. Cavitation is a result of the interaction between ultrasonic waves and a gaseous inclusion in an aqueous medium leading to cavitation bubbles (54). This effect causes a cell membrane damage which leads to a higher permeability for macromolecules, followed by membrane sealing and cell survival (55). This US-mediated increase of cell permeability is also termed “sonoporation” and is used for gene delivery (54).

On the base of promising *in vitro* results, *in vivo* US treatment has also shown to increase gene transfer. After pDNA injection into the tumor, transfection efficiency was enhanced in prostate (56) and colon carcinoma (57). Systemic application of naked pDNA in combination with US was inefficient, probably due to degradation of naked DNA by serum nucleases and low DNA concentrations in the surrounding of sonoporated cells (56). Complexes of pDNA with cationic lipids (“lipoplexes”) showed after i.v. injection combined with sonoporation an enhanced gene transfer in a SCCVII tumor model highlightening the use of particle-based systems in combination with physical targeting techniques (58).

Another approach regarding US is to use so called microbubbles which are clinically established as contrast agents. The DNA was either directly attached to the surfaces of the microbubbles or just mixed with them prior to administration. In some studies these vehicles showed enhanced pDNA transfection efficiency both *in vitro* and *in vivo* (59–63). Furthermore, gas-filled microparticles (GFMP) were developed as gene delivery systems. The incorporation of nucleic acids into the GFMP should provide a better protection against degradation by serum nucleases. Gene expression in rodent colon carcinoma tumor models after GFMP/US treatment was recently demonstrated (64,65).

### Photochemical Internalization (PCI)

Photochemical internalization (PCI) is a technology which improves and focuses gene delivery to a specific light-exposed target site. Acidic amphiphilic photosensitizers (PS) localize in endosomal membranes where they meet the gene carrier after endocytosis. Illumination leads to photochemical damage and rupture of the endosomal membranes, releasing the gene carrying complexes into the cytosol.

*In vitro*, PCI already showed encouraging results in improving gene delivery of polyplexes (66,67). For lipoplexes the PCI effect was variable and depends on the liposomes used (68). Moreover, receptor-mediated gene delivery plus PCI worked very well, thus combining biological and physical targeting principles. Kloeckner *et al.* showed a 2- to 600-fold enhanced transfection efficiency of EGF receptor-targeted polyplexes *in vitro* compared to transfections without PCI treatment (69). Ndoye *et al.* recently showed promising therapeutic effects in an HNSCC model *in vivo* with PEI-mediated p53 gene transfer combined with PCI (70).

### Hyperthermia

Hyperthermia with controlled increase of temperature in the target area is applied in different concepts for tumor treatment. Hyperthermia is already in clinical use. Besides the direct cytotoxic effect of hyperthermia (>42.5°C) in the heated tissue, immunomodulatory effects as well as radiation and chemotherapy sensitizing properties were found (71). Additionally, it has been demonstrated that the application of heat at the target site increases the permeability of tumor vessels to a variety of macromolecules (72–74). A leading hypothesis assumes that due to the heat stress the endothelial cells of tumor neovasculature lose their cytosceletal structure and contract, which causes widening of the intercellular gap junctions. According to cytostatic drug delivery, liposomes plus hyperthermia are well investigated (72,73). Local hyperthermia has also been successfully used for systemically targeting recombinant vaccinia virus to tumors (75). The combination of nonviral gene therapy with hyperthermia was recently established with encouraging results *in vitro* (76). DNA polyplexes with PNIPAM/PEI- based block copolymers were formed and after transient hyperthermic treatment (30 min, 42°C followed by incubation at physiological temperature) these particles undergo phase transition and aggregate. It was found that gene expression after the hyperthermic regimen was increased by two orders of magnitude presumably due to the fact that after internalization the aggregated particles show a more effective release from the endosome due to the enhanced proton sponge effect induced by accumulated PEI (76).

## BIOLOGICAL TARGETING

Biological targeting strategies towards the tumor site can be achieved by taking advantage of the special tumor architecture and unique tumor properties. Basically, these strategies can be divided into two main groups: *passive* and *active* tumor targeting. To exploit these strategies, lipoplexes,

nanoplexes or polyplexes can be further modified with surface shielding and tumor targeting molecules.

## Passive Tumor Targeting

The imperfect and leaky tumor vasculature, due to abnormal neovascularisation required to serve fast-growing tumors, combined with an inadequate lymphatic drainage results in an effect termed "enhanced permeability and retention" (EPR effect) (77). To take advantage of this for tumor targeting, particles should own certain properties. First, they should show an elongated plasma circulation time in order to increase the ability of the particles to extravasate at the tumor site. Second, the particles should be hydrophilic to avoid the RES (78,79) and third, the molecular weight of the vectors should exceed approx. 50 kDa to circumvent renal excretion.

Hydrophilic surface modifications of lipo- and polyplexes e.g., with polyethylene glycol (PEG) are required to benefit from the EPR effect. PEG-lipids have been incorporated into lipoplexes (80,81) and also polyplexes have been modified using different PEGylation strategies. PEGylation of polyplex particles can either be done after complex formation with nucleic acids ("post PEGylation") (82,83) or prior to complex formation ("pre PEGylation") (84–86).

While PEG shielding is beneficial for passive targeting to the tumor site, it is unfavourable for intracellular release of nucleic acids because the interaction of polycations with membranes like the endosomal membrane is reduced and in consequence endosomal release of complexes is decreased. At best, PEG might shield the complexes during systemic circulation and dismantle after reaching the targeted site. For this, different methods for triggered deshielding have been established in the past taking advantage of given physiological parameters in the cell including changes in pH, enzyme concentration or redox potential (81,87–91). For example, Szoka *et al.* developed a pH-sensitive PEG lipid consisting of a hydrophilic PEG headgroup which is connected via an orthoester with a hydrophobic tail. Orthoesters can easily break down under acidic conditions such as in the endosome. It was demonstrated that the pH-sensitive PEG-lipid was able to rapidly release liposome-encapsulated payload when the pH was reduced to endosomal pH of 5–6 (92). Further optimization led to lipoplexes with high transfection efficiency at low cytotoxicity (93). Walker *et al.* (86) investigated hydrazone bonds as pH-sensitive linkers in PEGylated polyplexes. With such polyplexes a 10- to 100-fold enhanced transfection efficiency compared to stable PEG shielded polyplexes was found *in vitro* as well as *in vivo* in a hepatocellular carcinoma tumor model. Other bioresponsive linkers were investigated by Shin *et al.* using vinyl ethers as pH-sensitive bonds in liposomes (88). Murthy *et al.* studied acid-labile acetals in polyplex formulations (94,95).

Ambegia *et al.* introduced a novel strategy to PEGylate liposomes reversibly (81). They assembled PEG into stabilized plasmid-lipid particles (SPLP) using diffusible hydrophobic anchors which are able to be extracted from a liposomal bilayer at physiological conditions. They hypothesized that the length of the PEG anchor determines the dissociation rate of PEG and hence the pharmacokinetics of SPLPs. *In vitro*, they observed highest gene expression levels with the shorter anchor due to fast diffusion of the PEG shield resulting in enhanced complex-cell interaction. In contrast, *in vivo* data showed that a higher chain length increased the levels of gene expression in a murine neuroblastoma model due to accumulation on the tumor site because of persistent shielding by the larger anchors and decreased renal clearance. Moreover, for these SPLP conjugates a tumor selective targeting was observed. MacLachlan *et al.* recently found very encouraging results, using similar stable nucleic acid lipid particles (SNALP) for apolipoprotein B (APOB)-specific siRNA delivery. A single siRNA injection resulted in maximal APOB messenger RNA silencing of >90% and significant reductions in APOB, serum cholesterol and low-density lipoprotein levels (96).

A completely different approach to PEGylate particle based systems was taken by Oishi *et al.*, who linked PEG directly to siRNA or antisense oligonucleotides (97,98). For this they used an acid-labile thioproprionate linker and mixed the grafted oligonucleotide afterwards with polylysine to form polyion complex micelles (PIC). Targeted acid-labile PICs showed significant higher inhibition of gene expression compared to the stable PICs in a human hepatoma carcinoma cell line *in vitro*.

## Active Tumor Targeting

Typical for fast dividing cells, tumor cells and tumor endothelial cells over-express growth factors and tumor specific receptors on their surfaces. To benefit from this for active targeting to the tumor site, corresponding ligands can be coupled to nucleic acid delivery systems.

One commonly used ligand is the serum glycoprotein transferrin (Tf) using the transferrin receptor (TfR) for targeted delivery. TfR required for iron uptake into cells is over-expressed in tumor cells due to the higher demand of iron for their growth (99). Tf as protein ligand combines both a shielding function of the vectors and a targeting moiety towards the tumor site (100,101). It was found that the incorporation of Tf or anti-Tf receptor single-chain antibody Fv fragments into polyplexes and lipoplexes resulted in enhanced gene transfer efficiencies *in vitro* as well as *in vivo* (102–110). PEGylation of PEI/DNA polyplexes or lipoplexes containing Tf or anti-Tf scFv fragments as targeting ligand further improved the *in vivo* application. After systemic administration of Tf-PEG-coated vectors gene expression was mainly found at the tumor site (84,108,109). For example, tail vein injection of transferrin-shielded PEI/DNA complexes into mice bearing subcutaneous neuroblastoma tumors resulted in 100- to 500-fold higher luciferase reporter gene expression in the distant tumors as compared with the major organs (104,108) and maintains over three days (upon single injection) or one week (after two injections). Within the tumor, expression was associated mainly with tumor cells near structures resembling primitive blood vessels. As evaluated in different tumor models, gene expression is affected by tissue-dependent factors; uptake of DNA depends on vascularisation, while necrosis and macrophage infiltration facilitates degradation of DNA and gene expression product (111).

Therapeutic effects were observed with such Tf-tumor-targeted formulations in murine models (see Table I). Tf lipoplexes were successfully applied for systemic p53 gene therapy which in combination with radiation resulted in tumor regression (100). Systemic application of Tf polyplexes of pDNA encoding TNF-alpha into tumor-bearing mice induced tumor necrosis and inhibition of tumor growth in several mouse tumor models (84,105). As TNF-alpha expression was largely localized within the tumor, no significant TNF-related toxicities were observed. A cationic cyclodextrin carrier containing transferrin as targeting ligand and PEG for shielding (see Fig. 2a) was developed for systemic siRNA delivery (112). Repeated systemic delivery of siRNA against the Ewing's sarcoma specific chromosomal translocation t (11,22) strongly inhibited growth of metastatic Ewing's sarcoma in a murine model. Thereby the presence of transferrin and PEG in the formulation was required for this therapeutic effect.

Therapeutic effects in targeted delivery of siRNA polyplexes were also observed by Song *et al.* (113). Cell-type specific delivery was obtained with single-chain antibody-protamine fusion proteins as siRNA binding carrier targeting either the HIV envelope protein (as an artificial model receptor) or ErbB2. Intravenous injection of a cocktail of siRNAs for c-myc, MDM2 and VEGF complexed with the carrier reduced the growth of envelope-expressing but not unmodified melanomas.

The epidermal growth factor (EGF) is another widely investigated ligand for tumor targeting (8,69,114–116). The EGF receptor (EGFR) is up-regulated in many tumors including epithelial tumors, glioblastoma and hepatocellular carcinoma. Incorporation of EGF into PEGylated PEI polyplexes enhanced the gene expression *in vitro* up to 100-fold (115). Also upon systemic application in hepatocellular carcinoma bearing mice, a 10-fold enhanced *in vivo* gene expression at the tumor site was found (117). Therapeutic effects of EGFR-targeted polyplexes containing the double stranded RNA poly Inosine-Cytosine (polyIC) were recently reported by Shir *et al.* (see Fig. 2b); the complete regression of established EGFR overexpressing glioblastomas in nude mice was observed (8).

As another targeting ligand/receptor system, folate receptors show a narrow expression on healthy tissue whereas in a huge number of cancer types it is upregulated (118). Efforts in the development of folate receptor targeted vectors have focused on the use of folate itself as a targeting ligand finding promising tumor specific gene transfer in several in *in vitro* and *in vivo* models (119–122).

Like tumor cells, also tumor vascular endothelial cells over-express certain surface markers which are only up-regulated in neoangiogenic tumor blood vessels or exist either at very low levels or not on normal blood vessels (123). Such surface receptors include e.g., the integrins like $\alpha v\beta 3$ and $\alpha v\beta 5$ (124). Several approaches targeting gene carrying systems towards the integrins use synthetic peptides with the RGD sequence (125–127). RGD-targeted PEI polyplexes have shown enhanced gene transfer levels compared to PEI alone. Thereby, the receptor-mediated gene delivery depended on the degree of RGD substitution of targeted PEI conjugates (128). Coupling RGD via a PEG spacer to PEI, targeting was partially reduced, possibly because of hiding of the RGD sequence inside the PEG cloud (127,128). Suh *et al.* demonstrated the importance of optimal composition of RGD-PEG-PEI conjugates showing that transfection efficiency decreased as the degree of PEG-RGD grafting onto PEI increased (127). Recently RGD-PEG-PEI conjugates have been successfully tested for systemic antiangiogenic siRNA therapy (129). Repeated intravenous administration into neuroblastoma bearing mice resulted in sequence-specific inhibition of tumor growth (see Table I).

As a further peptide for targeting angiogenic blood vessels, the NGR peptide has been investigated (123). NGR shows highest affinity for aminopeptidase N (APN; also

**Table I.** Therapeutic Effects Obtained with Tumor-targeted Nucleic Acids

| Nucleic acid | Gene/target | Formulation/Application | Reference |
|---|---|---|---|
| pDNA | p53 | Tf lipoplex, systemic, head and neck cancer (s.c.) | Xu *et al.* (100) |
| | TNF alpha | Transferrin-PEI, systemic, various cancer types (s.c.) | Kircheis *et al.* (105) |
| | p53 | APN/CD13 targeting peptide, PEI polyplex, systemic, human non-small-cell lung cancer (s.c.) | Moffat *et al.* (131, 132) |
| siRNA | EWS-FLI1 | Tf-PEG-cationized cyclodextrin systemic, Ewing's sarcoma (i.v.) | Hu-Lieskovan *et al.* (112) |
| | c-myc/MDM2/VEGF | Protamine antibody fusion, systemic or local, melanoma (s.c.) | Song *et al.* (113) |
| | VEGF receptor | RGD-PEG-PEI, systemic, neuroblastoma (s.c.) | Schiffelers *et al.* (129) |
| others | Pleotropin ribozyme | PEI, systemic (intraperitoneal), melanoma (s.c.) | Aigner *et al.* (10) |
| | HER2 antisense oligonucleotide | folate-lipoplex, systemic, with docetaxel, breast cancer (s.c.) | Rait *et al.* (9) |
| | Poly IC (Poly Inosine-Cytosin) | EGF-PEG-PEI/melittin, local, glioblastoma (s.c.) | Shir *et al.* (8) |

known as CD13) which is known to play an important role in tumor invasion (130). Moffatt *et al.* recently reported promising *in vivo* results using a CD13 targeted PEG-PEI polyplex yielding in enhanced transgene expression on the tumor site (131). In these polyplexes (see Fig. 2c) CNGRC, the cyclic form of a pentapeptide containing the NGR motif, in PEG-conjugated form is attached through non-covalent phenyldiboronic acid/salicylhydroxamic acid bridges to PEI. When applied for p53 gene transfer polyplexes showed encouraging therapeutic effects, such as significant H1299 (human non-small-cell lung carcinoma) tumor regression and 95% of animal survival after 60 days. Moreover, this vector targeted only tumor tissue and tumor-associated endothelial cells but not any normal cells (132).

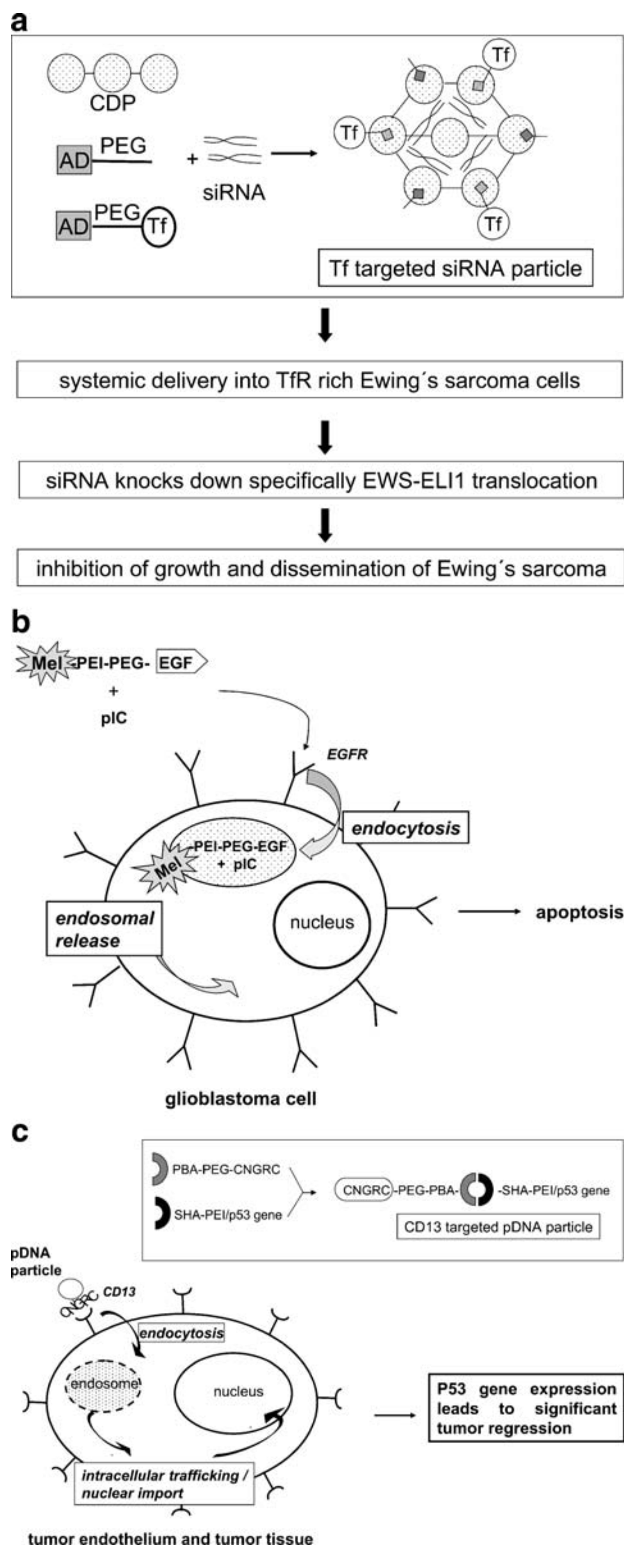


**Fig. 2.** (**a**) TfR targeting for siRNA delivery. Cyclodextrin polycation (CDP) condenses siRNA, shielding and targeting molecules are included by adamantane-cyclodextrin inclusion complex formation. Adamantane-PEG (AD-PEG) stabilizes the complex and adamantane-PEG-Tf (AD-PEG-Tf) provides the targeting ligand. Systemic delivery leads to inhibited EWS-ELI1 translocation and reduced growth and dissemination of Ewing's sarcoma. (**b**) EGFR targeting of pIC. pIC as a strong intracellular activator of apoptosis leads due to EGF targeting to selective cell death of EGFR-overexpressing glioblastoma multiforme. pIC is electrostatically complexed to the EGF-PEG-PEI-Mel conjugate. Mellitin (Mel) ruptures endosomal membrane and enhances the endosomal release of the polyplex formulation. (**c**) CD13 targeting of pDNA. PBA (phenyldiboronic acid) -PEG-CNGRC and SHA (salicylhydoxamic acid)-PEI/p53gene self-assemble through non-covalent bridges. CD13 targeting of p53 gene leads to specific tumor endothelium and tumor tissue targeting resulting in significant H1299 tumor regression.

## CONCLUSIONS AND CHALLENGES FOR NUCLEIC ACID DELIVERY

Extended clinical trials using lipoplexes have been performed (133–137). Galanis *et al.* found in clinical phase I/II studies promising results using a lipoplex formulation encoding for interleukin (IL)-2 in patients with metastatic renal cell carcinoma. After intratumoral application, 10% of 31 patients experienced partial response and 23% stable disease after one cycle of treatment without any critical side effects indicating the safety of this lipoplex administration (136). Also polyethylenimine (PEI 22 kDa) based DNA particle systems were recently tested in humans for bladder cancer showing extensive tumor regression after intravesical vector installation in treated patients. A significant reduction in tumor size by more than 75% was observed (138). This indicates the upcoming importance of nonviral vectors while the specific delivery of nucleic acids towards the tumor site after systemic delivery remains a major challenge in optimization of these gene vector formulations.

Physical targeting techniques result in rather high specific and localized transgene delivery and they are already in clinical use (50). However, they are limited to the application side and are not capable for systemic delivery. Biological targeting strategies are well-suitable for systemic delivery. Nevertheless, they still face several critical limitations. Significant delivery barriers both on the intracellular and extracellular side have to be overcome for efficient *and* safe nucleic acid delivery. One limiting factor for successful receptor-mediated gene transfer is the intracellular release of internalized contents out of the endosome. Membrane active peptides and proteins such as e.g., influenza HA2, listeriolysin, or mellitin which cause a rupture of endosomal membranes, have been coupled to DNA binding polycations finding strongly enhanced transgene expression levels due

to improved endosomal release of polyplexes into the cytosol (139–142). The transport of transfected nucleic acids into the nucleus places the next huge barrier for pDNA delivery (143), whereas for siRNA the release into the cytosol is sufficient and nuclear uptake is not necessary. Once delivered into the nucleus, pDNA can only be expressed if efficient and specific promoter/enhancers are involved; for this, different transcriptional targeting strategies can be assessed. Overcoming all these intracellular barriers, additional extracellular challenges remain for safe gene delivery. Cytotoxic effects of nonviral vectors should be minimized or completely eliminated. Unspecific interactions with blood components (101) or non-targeted sites (82), and undesired activation of the immune response or the complement system (144) can be reduced by modifying the vector surfaces with hydrophilic compounds like PEG (see "Passive Tumor Targeting"). In addition, purification of nucleic acid complexes may lead to further reduced acute cytotoxicity in the host, as recently demonstrated by Boeckle *et al.* (145). To further increase biocompatibility, biodegradable gene carriers should be applied to reduce long-term cytotoxicity. Such carriers are degradable by reductive cleavages of disulfide bonds (146–150) hydrolysis of pH-sensitive hydrazones (151) or ester or amide bonds (116,146,152–161).

In summary, the optimization of target-specific, safe and efficient nonviral delivery systems for nucleic acids remains an ongoing challenge. Nevertheless, unique tumor characteristics and various emerging delivery technologies offer the opportunity to form tailor-made powerful therapeutic nucleic acid carriers for cancer gene therapy.

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