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Concepts in *in vivo* siRNA Delivery for Cancer Therapy

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Abstract

In vivo gene silencing using RNAi plays an important role in target validation and is advancing towards the development of RNAi-based therapeutics. RNAs were thought to have just two broad functions in cells as messenger RNAs (mRNAs) and ribosomal RNAs, but recently the relevance of microRNAs is becoming more clearly understood. Messenger RNA molecules transmit information between DNA and protein and, as such, are vital intermediaries for gene expression. Ribosomal and transfer RNAs have structural, catalytic and information-decoding roles in the process of protein synthesis, whereas microRNAs are regulators of gene expression. This review presents the early and intriguing successes of using siRNAs for *in vivo* gene silencing and its use as a possible cancer therapeutics.

Introduction

Observed in the majority of eukaryotes, RNA interference (RNAi) is an evolutionarily conserved process that inhibits gene expression primarily by targeting mRNAs. RNAi targets, including RNA from viruses and transposons, play roles in regulating development and genome maintenance. Small interfering RNA (siRNA) strands are the key to RNAi process as siRNA have nucleotide sequences complementary to the targeted RNA strand. Specific RNAi pathway proteins are guided by the siRNA to the targeted messenger RNA (mRNA) where they “cleave” the target and break it down into smaller portions that can no longer be translated into protein (Fig. 1). The discovery of RNA interference (RNAi) is revolutionary and is transforming biological research in ways not seen before. Short double-stranded RNA (dsRNA) molecules are easily and rapidly available today. These dsRNA molecules can downregulate the expression of a target mRNA molecule in a sequence-specific manner (Dorsett and Tuschl, 2004; Novina and Sharp, 2004).

RNAs were thought to have just two broad functions in cells as messenger (Apirion, 1975) RNAs and ribosomal and transfer RNAs. Messenger RNAs (mRNAs) are vital intermediaries in gene expression, transmitting information between DNA and protein. Ribosomal and transfer RNAs have structural, catalytic and information-decoding roles in the process of protein synthesis. This concept changed in 1998 when Fire *et al.* (Fire *et al.*, 1998) published their discovery of “potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*.” RNAi is now widely accepted as ‘the’ method of choice for rapidly assessing biological mechanisms and pathways through loss-of-function. RNAi libraries, which allow functional phenotypic screening in mammalian cells, have been established in recent years (Berns *et al.*, 2004). Such libraries take advantage of the ability to express short

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hairpin structures from a variety of vector systems, which are processed to generate the functional unit, a small interfering RNA (siRNA). Both short hairpin RNAs (shRNAs) and siRNAs can be used to downregulate mRNA expression in cells. SiRNAs are RNA duplexes commonly composed of two 21-mer oligonucleotides with a 19 nt complementary and a 2 nt single-stranded overhang at each 3' end. Alternative structures of siRNAs also show *in vitro* function (Czauderna *et al*, 2003). Small RNA oligos are easily synthesized and annealed to form functional siRNA molecules, whereas small hairpin RNA can also be chemically synthesized as inverted repeats and allowed to self anneal. The increased length (more than 50 bases) makes the chemical synthesis less efficient. ShRNAs can be efficiently produced in large scale by plasmids expressing inverted repeats. Both siRNAs and shRNAs work similarly and cause efficient knockdown of target mRNAs. The silencing by plasmid based shRNAs is long lasting when compared to siRNAs because of the continuous synthesis of shRNA molecules *in situ* by the plasmids (Huang *et al*, 2008). For *in vitro* use, siRNA can be readily introduced into cell lines by a variety of techniques, including lipid complexes, liposomes, electroporation, etc (Li and Szoka, Jr., 2007). One of the major hurdles for using RNAi *in vivo* is efficient intracellular delivery of a highly charged and structurally rigid siRNA molecule. Progress is being made in delivering siRNA *in vivo*, first through hydrodynamic methods and more recently through local or targeted administration (Herweijer and Wolff, 2007; Li *et al*, 2006). Delivery can be enhanced either by improving siRNA attributes directly through chemical modifications of the duplex or by using different formulations, such as liposomes and ionic polymers (Li and Szoka, Jr., 2007; Yang *et al*, 2006). Significant success has already been seen in using siRNA in many localized settings such as the eye, lungs, and the central nervous system. In addition, encouraging data have been generated in a variety of other *in vivo* settings, including xenograft models and viral infections. All of these studies demonstrate the potential for the systemic delivery of siRNA-based therapeutics.

***In vivo* delivery strategies**

Delivery of siRNA *in vivo* can basically be divided into two classes, systemic and local. Systemic delivery involves the delivery of siRNA into the whole organism and usually requires large quantity of siRNA to achieve the desired downregulation. In contrast, localized delivery of siRNA to a specific site (e.g., brain, eye or a tumor) is more achievable since large quantities of siRNA are not required and subsequently do not affect the whole organism (Pardridge, 2007; Pushparaj *et al*, 2008; Shen, 2008). Numerous *in vivo* delivery approaches have been published, ranging from simple direct injection of siRNA to complex procedures involving structurally modified molecules (Alshamsan *et al*, 2009; Watts *et al*, 2008; Zhang *et al*, 2009). Animal studies using siRNA have either employed saline-based formulations or used conjugates, including liposome/lipoplexes and complexes with peptides, polymers, or antibodies (Aigner, 2008; Law *et al*, 2008; Wang *et al*, 2008; Whitehead *et al*, 2009; Xia *et al*, 2008). Local delivery has particular advantage in that, as with any pharmacologic approach, doses of siRNA required for efficacy are substantially lower when siRNAs are injected into or administered at or near the target tissue (Aigner, 2006; Li and Huang, 2008). Direct delivery also allows for more focused delivery of siRNA, which might circumvent any theoretical, undesired side effects resulting from systemic delivery.

Systemic delivery of siRNA, especially with cholesterol conjugates, liposomes, and polymer-based nanoparticle approaches, has also been widely explored with moderate success. Other approaches using antibodies, peptides, and aptamers have also been reported (Neidhardt *et al*, 2005; Turner *et al*, 2007). The various delivery methods developed to date are represented in Table 1 (Takeshita and Ochiya, 2006). Delivery of siRNA can vary from direct to indirect. The direct method can be classified as hydrodynamic delivery, which involves naked siRNA molecules and employs brute force to deliver siRNA to an organism. This method is only partially effective when large volumes are used (Herweijer and Wolff, 2003). As delivery

methods are developed, the use of carriers of genetic material (adenoviruses, lentiviral vectors, adeno associated viruses) has gained popularity (Whitehead et al, 2009). The use of viruses as a delivery method was questioned when undesirable side effects related to antiviral response, hyper immune reactions and viral genome integration into host genome were reported (Braun, 2006). These drawbacks paved the way for the development of lipids, lipid complexes and chemically modified siRNA molecules as delivery agents (Fig. 2).

Direct delivery—Hydrodynamic delivery involves rapid large-volume intravenous injection of siRNA equivalent to the total blood volume. The first successful *in vivo* delivery of siRNA into cells used a hydrodynamic methodology that was initially developed to deliver gene therapy vectors into hepatocytes (Song *et al*, 2003). In preliminary applications of siRNA *in vivo*, combinations of enhanced green fluorescent protein (EGFP) (Lewis *et al*, 2002) or luciferase (McCaffrey *et al*, 2002) encoding plasmids together with the siRNA yielded significant downregulation of target gene expression in the liver, suggesting that once delivered to hepatocytes, siRNAs were functional *in vivo*. Using this approach, several therapeutically relevant hepatic targets were downregulated, including Fas (Song *et al*, 2003) and caspase 8 (Zender *et al*, 2003). In both of these cases, the target genes were downregulated by 60-90%. Downregulation of Fas was sustained for 10 days and resulted in protection from hepatitis and liver failure. Similarly, hydrodynamically delivered siRNA directed toward hepatitis B inhibited viral replication in mice when co-injected with HBV-expressing plasmids (Giladi *et al*, 2003). More recently, 2'-F modified siRNAs with enhanced serum stability showed efficacy in silencing luciferase expressed from a co-injected plasmid, thereby demonstrating that chemically modified siRNAs are active *in vivo* (Layzer *et al*, 2004). Also, plasmids using H1 or U6 promoters have been used with promising results. Hao *et al*. have demonstrated that p53 downregulation efficiencies of H1- and U6-driven shRNAs were almost identical (Hao *et al*, 2005). Recently, Takahashi *et al*. have studied the gene silencing kinetics of short hairpin RNA (shRNA)-expressing plasmid DNA (pDNA) driven by U6, H1 or tRNA promoter (pU6-shLuc, pH1-shLuc, and ptRNA-shLuc) in melanoma cells expressing firefly luciferase (Takahashi *et al*, 2008). These studies have demonstrated that U6 promoter-driven shRNA expressing pDNA is the most effective in inducing gene silencing effect as far as the intensity and duration of RNAi effect is concerned, they. The main drawback of using Pol III promoters is their inability to process multi-cistronic constructs efficiently unlike CMV, which is a Pol II promoter. Recently, researchers have also described tRNA(Lys3)-shRNA chimeric expression cassettes, which produce siRNAs with comparable efficacy and strand selectivity to U6-expressed shRNAs and show that their activity is consistent with processing by endogenous 3' tRNase (Scherer *et al*, 2007).

Although hydrodynamic injection provides an important proof-of-concept demonstration that RNAi can function *in vivo*, it does not represent a therapeutically viable method due to the large volumes required. Interestingly, when plasmid vectors expressing siRNAs are used, large quantities of injection are not required due to plasmid transcription activity. We used plasmid-mediated RNAi targeting uPAR and uPA, which did not induce 2',5'-oligoadenylate synthetase 1 (OAS1) expression, indicating no non-specific activation of the antiviral interferon response pathway in mice with pre-established intracranial tumors. Furthermore, the small interfering RNA plasmid targeting uPAR and uPA caused regression of pre-established intracranial tumors when compared with controls (Gondi *et al*, 2007). We have also demonstrated that intraperitoneal injections of RNAi expressing plasmids targeting uPAR, MMP-9, cathepsin B and uPA are capable of regressing intracranial tumors. In these studies, we used novel bi- or multi-cistronic plasmid construction method where a single plasmid was developed to generate multiple RNAi molecules mediated via hpRNAs driven by a single CMV promoter and which were processed to siRNAs via internal RNAi machinery (Gondi *et al*, 2004a;Gondi *et al*, 2004b;Gondi *et al*, 2007;Lakka *et al*, 2004). Given that the RNAi effect is limited only by the half life of the plasmids *in vivo*, the advantage of using multi-cistronic constructs over single

constructs is that a single plasmid molecule can produce multiple RNAi molecules specific to more than one target, thereby enhancing the RNAi effect and resulting in higher efficiency. In contrast, methods utilizing the direct injection of siRNA molecules are limited by the volume that can be injected.

Viral delivery—Currently, researchers are exploring the use of both viral and nonviral vectors for siRNA delivery. The viability of inserting genetic material into a vector encoding shRNA, which efficiently blocks production of a specific protein, has been demonstrated (Zentilin and Giacca, 2004). Retroviral vectors have been designed to produce siRNA driven by either U6 or H1-RNA promoters for efficient, uniform delivery and immediate selection of stable knockdown in cells. Adenoviral vectors have been demonstrated to mediate gene silencing in *in vitro* lung and breast models (Shen *et al*, 2003) and to induce RNAi in a range of animal tissues. Our studies have shown effective downregulation of MMP-2 using RNAi mediated by adenovirus in lung and meduloblastoma models (Lakka *et al*, 2005b; Lakka *et al*, 2005a). Recently, Choy *et al*. have demonstrated a new set of plasmid vectors driven by promoters of the Epstein-Barr virus (EBV)-encoded small RNAs (EBERs). The EBERs are the most abundant transcript in infected nasopharyngeal carcinoma cells and are transcribed by Pol III. The authors also showed that the EBER promoters were able to drive the expression of shRNA fusion transcripts (Choy *et al*, 2008). Even though no *in vivo* studies using these constructs were conducted, the potential for *in vivo* use is very promising. Researchers using a lentiviral transfection system have shown that the U6 promoter is more efficient than H1 in GFP silencing *in vitro*, leading to 80% GFP knockdown at an average of one integrated vector genome per target cell genome. In addition, the U6 promoter was shown to be superior to H1 *in vivo* and led to stable GFP knockdown in mouse brain for at least 9 months, thereby indicating that LV-mediated RNAi is a powerful gene-silencing method for the long-term inhibition of gene expression *in vitro* and *in vivo* (Makinen *et al*, 2006). Other viral methods are also being developed using retroviral vectors but their *in vivo* use has not been fully explored (Hao *et al*, 2005; Wadhwa *et al*, 2004). The potential to use viral vectors as targeting agents carrying siRNA-expressing constructs is very promising if issues relating to immunogenicity and toxicity are addressed. However, no conclusive reports employing targeted viruses carrying siRNA-expressing constructs have been published to date.

Lipid-based complex delivery—The use of lipids as carriers of genetic material is not new (Ledley, 1994). With the advent of RNAi, research in the use of liposomes to deliver drugs into cells has surged. Liposomes are vesicles that consist of an aqueous compartment enclosed in a phospholipid bilayer with the drug typically entrapped in the center aqueous layer. The bilayer is composed of multicomponents, typically comprising a lipid component, cholesterol, and polyethylene glycol (PEG)-lipid. The liposomes that are formed yield particles with stable physicochemical characteristics, thus making them suitable drug delivery vehicles. In contrast, lipoplexes are spontaneously formed with the interaction of cationic lipids and negatively charged nucleic acids and are typical of most commercial transfection agents, such as Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and TransIT-TKO. Lipoplexes tend to be structurally more heterogeneous and unstable, aggregating over time in solution, and as a result, lipoplexes must be prepared immediately before use. These represent major disadvantages from the standpoint of reproducibility, manufacturing, and drug administration.

Significant and repeated success with liposome-mediated delivery has been reported *in vivo*. One of the most important advances in the development of RNAi as a therapeutic came with the demonstration that systemically delivered siRNA in a stable nucleic-acid-lipid form targeting ApoB was able to silence ApoB in mice and non-human primates (Zimmermann *et al*, 2006). While most lipid-based nucleic acid delivery systems make use of a cationic lipid to associate with the negatively charged siRNA backbone, neutral liposomal delivery systems have also proven effective (Spagnou *et al*, 2004; Welz *et al*, 2000). Using the same dioleoyl

phosphatidylcholine (DOPC)-based delivery system, studies using siRNAs directed against EphA2 (Landen, Jr. *et al*, 2005) and focal adhesion kinase (Halder *et al*, 2006) were able to demonstrate specific target protein knockdown and inhibition of tumor growth in an orthotopic mouse model of ovarian cancer. In both studies, animals were treated with formulated siRNA at a dose of 150 mg/kg twice a week for 3 weeks. More recently, the authors have reported the ability of neuropilin-2 siRNA formulated in DOPC liposomes to inhibit the growth of colorectal carcinoma cells implanted in the murine liver (Gray *et al*, 2008). As neutral lipid-based formulations are generally well tolerated, these results are encouraging. No conclusive studies using siRNA plasmids or expression cassettes employing the use of lipids or lipid complexes have been reported yet in an *in vivo* setting. These types of studies could aid in the faster development of therapy models for effective *in vivo* siRNA delivery.

Other delivery methods—In addition to the *in vivo* delivery methods already described, other methods are also being developed and show significant potential as efficient strategies for the delivery of therapeutic siRNAs. Rozema *et al*. (Rozema *et al*, 2007) have demonstrated an ingenious targeted delivery method using dynamic polyconjugates and also shown effective delivery of siRNA *in vivo* in mice by silencing endogenous ApoB and peroxisome proliferator-activated receptor [PPAR]- α mRNA in hepatocytes. Although this study did not deal with cancer, it is a very significant demonstration of a novel method that could be used to deliver siRNAs of interest to cancer therapeutics. Another polymer approach involves using transferrin-targeted, cyclodextrin-containing polycation nanoparticles. These nanoparticles have demonstrated targeted silencing of the EWS-FLI1 gene product in transferrin receptor-expressing Ewing's sarcoma tumor cells (Hu-Lieskovan *et al*, 2005). The siRNA formulated in these nanoparticles was well tolerated in non-human primates (Heidel *et al*, 2007). Both of these delivery strategies are appealing in that they incorporate rational approaches using targeted delivery and endosomal escape mechanisms.

In addition to these methods, there are several other methods that are being developed for the delivery of siRNA *in vivo*, such as the direct siRNA conjugation method that utilizes the conjugation of the siRNA molecules to larger molecules like cholesterol (Wolfrum *et al*, 2007). However, the utility of this method for cancer therapy has not been explored fully. Conjugation of siRNA molecules with proteins or peptides is being explored, especially conjugation with signal peptides targeting specific cells (Kumar *et al*, 2007). The concept of conjugating antibodies to siRNA molecules has also been proposed (Peer *et al*, 2007) and holds great promise as a targeted therapeutic approach for various cancers.

One of the earlier methods showing promise as a delivery strategy involves the use of atelocollagen. Atelocollagen was the first biomaterial with the potential for use as a carrier for gene delivery (Ochiya *et al*, 1999). Atelocollagen has been obtained from type I collagen of calf dermis by pepsin treatment (Ochiya *et al*, 2001; Sano *et al*, 2003). Atelocollagen obtained by pepsin treatment is low in immunogenicity because it is free from telopeptides, and is used clinically for a wide range of purposes. The surface of atelocollagen molecules is positively charged, and the molecules can bond electrostatically with negatively charged nucleic acid molecules. The size of the complex particles can be changed by altering the ratio of nucleic acid to atelocollagen. When the concentration of atelocollagen is high, the complex persists locally for a long time, which functions as a sustained release carrier. One problem for systemic treatment *in vivo* is that, although most endogenous RNases are inactive against dsRNA, some serum RNases can degrade siRNA. However, siRNA complexed with atelocollagen is resistant to nucleases and is transduced efficiently into cells, thereby allowing long-term gene silencing at the site of delivery (Minakuchi *et al*, 2004).

Clinical Trials

RNAi has rapidly advanced from research discovery to clinical trials with several new RNAi-based drugs expected to enter trials in 2008. Many researchers have developed strategies to target specific organs or tissues (Pirollo and Chang, 2008). Initial RNAi involving trials have focused on direct local delivery of siRNA and on well-validated therapeutic targets, such as the VEGF pathway for the treatment of the wet form of age-related macular degeneration (AMD) (Grunweller and Hartmann, 2005) and the respiratory syncytial virus (RSV) genome for the treatment of RSV (Whitehead et al, 2009) infection. The RNAi therapeutics being developed for ophthalmic indications all employ direct injection into the vitreal cavity to efficiently target the siRNA to the back of the eye whereas the RSV RNAi therapeutic uses direct delivery to the lung.

The development of RNAi-based therapies targeted against cancer is quickly progressing. Calando Pharmaceuticals has initiated the first anti-cancer, RNAi-based clinical trial (Clinical Trial #NCT00689065, 2008) using CALAA-01, which is a targeted molecule designed to inhibit tumor growth and/or reduce tumor size. The active ingredient in CALAA-01 is a small interfering RNA (siRNA) and inhibits tumor growth by targeting the expression of the M2 subunit of ribonucleotide reductase (RRM2). It was originally developed for pancreatic adenocarcinoma resistant to gemcitabine and to assess the efficacy of siRNA directed against RRM2 in suppressing resistance to gemcitabine (Duxbury *et al*, 2004b). The CALAA-01 siRNA is protected from nuclease degradation within a stabilized nanoparticle targeted to tumor cells. The outcome of this study will initiate a new era in cancer therapies.

Future of RNAi in cancer therapy

The growth of RNAi technology and the potential of this phenomenon to control gene expression very precisely have opened avenues for the management of diseases and metabolic conditions previously thought unmanageable. Cancer is the alteration of gene expression causing uncontrolled gene expressions. The identification of these altered expression or mutations is the key for the precise targeting of these molecules using RNAi. Chemotherapy drugs are broad based and usually not targeted to one particular molecule. Even if target-specific these drugs are plagued with side effects causing a deterioration in the quality of life in many cases is worse than the disease itself (Jain, 2006). So far RNAi had shown no such unmanageable side effects and the development of efficient delivery methods will allow for the development of individualized anti-cancer RNAi molecules suited to a particular patient. This growth and development of RNAi delivery systems will assist in controlling cancer to a more manageable disease (Li and Huang, 2008; Moreira et al, 2008; Racz and Hamar, 2008).

Conclusion

The effective targeted delivery of specific siRNA molecules to cancer cells *in vivo* is the key to controlling and, to a certain extent, curing most malignancies. From the direct delivery method to the development of complex targeted liposomes harboring multi-cistronic siRNA expressing plasmids, cancer researchers have focused on RNAi. The discovery of new cancer-specific molecules and pathways would provide a greater impetus for the further development of targeted therapies. The use of multi-cistronic constructs in plasmids expressing multiple siRNA molecules that can be specifically targeted to cancer cells would allow for the treatment cancer as a manageable disease if not a complete cure.

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Figure 1. Mechanism of RNAi using siRNA molecules or siRNA expressing molecules
RNAi involves the use of specific siRNA molecules that form a complex with proteins to form the RNA-induced silencing complex (RISC). In RNAi, one siRNA molecule is capable of targeting multiple mRNA molecules.

Figure 2. *In vivo* siRNA delivery strategies

Delivery of siRNA *in vivo* can be accomplished through direct injections systemically or locally; however, direct delivery of unmodified siRNA cannot be targeted. Other methods of delivery that incorporate the use of viruses, lipids or lipid complexes have potential for use as targeted strategies.

Table 1

Small interfering RNA for cancer therapy in vivo*

Carriers	Routes	Type of cancer (cell line)	Implanted site (target organ)	Target gene	Reference
Naked siRNA	i.p., i.v., s.c., i.t.	Fibrosarcoma (JT8)	s.c.	VEGF	(Filleur <i>et al.</i> , 2003)
Naked siRNA + gemcitabine	i.v.	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3)	Orthotopic pancreas	FAK	(Duxbury <i>et al.</i> , 2003)
Naked siRNA	i.v.	Pancreatic adenocarcinoma (BxPC3)	s.c., Orthotopic pancreas	CEACAM6	(Duxbury <i>et al.</i> , 2004c)
Naked siRNA	i.v.	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3, Capan2)	s.c., Orthotopic pancreas, liver metastasis	EphA2	(Duxbury <i>et al.</i> , 2004a)
Naked siRNA + gemcitabine	i.v.	Pancreatic adenocarcinoma (PANC1, MIAPaCa2, BxPC3, Capan2)	s.c., Liver metastasis	RRM2	(Duxbury <i>et al.</i> , 2004b)
Naked siRNA	i.v.	Breast cancer(MDA-MB-231)	Lung metastasis	CXCR4	(Liang <i>et al.</i> , 2005)
Liposome	i.p.	Colon cancer(HTC116)	s.c., i.p.	β -Catenin	(Verma <i>et al.</i> , 2003)
Liposome	i.v.	Liver metastatic spleen cancer (A549)	Liver	bcl-2	(Yano <i>et al.</i> , 2004)
Liposome	i.t.	Bladder cancer (UM-UC-3-LUC)	Bladder	PLK-1	(Nogawa <i>et al.</i> , 2005)
Liposome	i.t.	Pancreatic carcinoma (Capan-1)	s.c.	Somatostatin	(Carrere <i>et al.</i> , 2005).
CCLA (NeoPhectin-AT)	i.v.	Prostate cancer (PC-3)	s.c.	Raf-1	(Pal <i>et al.</i> , 2005)
CCLA	i.v.	Breast cancer (MDA-MB-231)	s.c.	c-raf	(Chien <i>et al.</i> , 2005)
shRNA plasmid + pegylated immunoliposome	i.v.	Glioma (U87)	Brain	EGFR	(Zhang <i>et al.</i> , 2004b)
PEI	i.p.	Ovarian carcinoma cells (SKOV-3)	s.c.	HER-2	(Urban-Klein <i>et al.</i> , 2005)
shRNA plasmid + PEI	i.t.	Ewing's sarcoma (TC71)	s.c.	VEGF	(Guan <i>et al.</i> , 2005)
Adenovirus vector	i.t.	Cervical adenocarcinoma, colon cancer (HeLa, HTC116)	s.c.	HIF-1 α	(Zhang <i>et al.</i> , 2004a)
Adenovirus vector	i.t.	Lung cancer (ACC-LC-172)	s.c.	Skp-2	(Sumimoto <i>et al.</i> , 2005)
shRNA plasmid	it	Meningioma IOMM-LEE	Brain osmotic pumps	uPAR+MMP-9	(Tummala <i>et al.</i> , 2007)
shRNA plasmid	it	Breast (MDA MB 231)	Orthotopic breast	uPAR+MMP9	(Kunigal <i>et al.</i> , 2007)
shRNA plasmid	ip	Glioblastoma (SNB19)	Brain	uPAR+uPA	(Gondi <i>et al.</i> , 2007)
shRNA plasmid	ip	Glioblastoma (SNB19)	Brain	uPAR+MMP-9	(Lakka <i>et al.</i> , 2005c)
shRNA plasmid	ip	Glioblastoma (SNB19)	Brain	uPAR+uPA+MMP-9	(Gondi <i>et al.</i> , 2004a)
shRNA plasmid	i.t.	Glioblastoma (SNB19)	Brain	MMP-9 + cathepsin B	(Lakka <i>et al.</i> , 2004)

Carriers	Routes	Type of cancer (cell line)	Implanted site (target organ)	Target gene	Reference
shRNA plasmid	i.t.	Glioma (SNB19)	Brain	Cathepsin B, uPAR	(Gondi <i>et al.</i> , 2004b)
shRNA plasmid + ATA	i.v.	Cervical adenocarcinoma, lung cancer (HeLa S3, A549)	s.c.	PLK1	(Spankuch <i>et al.</i> , 2004)
PEI-PEG-RGD	i.v.	Neuroblastoma (N2A)	s.c.	VEGF-R2	(Schiffielders <i>et al.</i> , 2004)
CDP-AD-PEG-transferrin	i.v.	Ewing's sarcoma (TC71)	Multiple organ metastasis	EWS-FLI1	(Hu-Lieskovan <i>et al.</i> , 2005)
HVJ envelope vector + cisplatin	i.t.	Cervical adenocarcinoma (HeLa)	Intradermally	Rad51	(Ito <i>et al.</i> , 2005)
ErbB2-protamine fusion protein	i.t., i.v.	Melanoma (B16)	s.c.	c-myc MDM2 VEGF (mix)	(Song <i>et al.</i> , 2005)
Atelocollagen	i.t.	Prostate cancer (PC-3)	s.c.	VEGF	(Takei <i>et al.</i> , 2004)
Atelocollagen	i.t.	Germ-cell tumor (NEC8)	Testis	FGF-4	(Minakuchi <i>et al.</i> , 2004)
Atelocollagen	i.v.	Prostate cancer (PC-3M-Luc)	Bone metastasis	EZH2, p110a	(Takeshita <i>et al.</i> , 2005)

AD-PEG, adamantane-PEG5000; ATA, aurointracarboxylic acid, nuclease inhibitor; CCLA, cationic cardiolipin analog-based liposome; CDP, cyclodextrin-containing polycations; i.p., intraperitoneal; i.t., intratumoral; i.v., intravenous; PEG, polyethylene glycol; PEI, polyethylenimine; RGD, Arg-Gly-Asp; s.c., subcutaneous; HVJ, Hemagglutinating virus of Japan.

* Modified from (Takeshita and Ochiya, 2006).