Published in final edited form as:

Mol Pharm. 2009 June 1; 6(3): 651–658. doi:10.1021/mp800134q.

Non-viral Methods for siRNA Delivery

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Abstract

RNA interference (RNAi) as a mechanism to selectively degrade messenger RNA (mRNA) expression has emerged as a potential novel approach for drug target validation and the study of functional genomics. Small interfering RNAs (siRNA) therapeutics has developed rapidly and already there are clinical trials ongoing or planned. Although other challenges remain, delivery strategies for siRNA become the main hurdle that must be resolved prior to the full-scale clinical development of siRNA therapeutics. This article provides an overview of the current delivery strategies for synthetic siRNA, focusing on the targeted, self-assembled nanoparticles which show potential to become a useful and efficient tool in cancer therapy.

Keywords

RNA interference; small interfering RNA; delivery; nanoparticles

Introduction

Ever since RNA interference (RNAi) was discovered by Fire et al. in 1998¹, this technology has rapidly become a powerful tool in basic research and potentially a new strategy for clinical trials. Small interfering RNA (siRNA), produced from cleavage of longer dsRNA precursors by the RNaseIII endonuclease dicer, can enter the RNA-induced silencing complex (RISC), which is activated upon guide (antisense) strand selection². The selection is based on the relative thermodynamic stabilities of the two duplex ends and it is the least stable 5'end of the duplex that is recognized and asymmetrically unwound by the Piwi-Argonaute-Zwille (PAZ) domain of argonaute 2, a multifunctional protein within the RISC. The incorporated strand acts as a guide for the activated RISC complex to selectively degrade the complementary mRNA³. By targeting an oncogene, siRNA could be applied as a therapeutic agent in cancer therapy⁴. However, due to its relatively large molecular weight and polyanionic nature, naked siRNA does not freely diffuse across the cell membrane, and thus a delivery system is required to facilitate siRNA access to its intracellular sites of action.

The success of gene therapy is highly dependent on the delivery vector, which can be generally categorized into viral and non-viral vector⁵. Viral vectors are highly efficient; they are currently still the most powerful tool for gene transfection. However, some viral vectors show a limited loading capacity, are difficult to produce in large scale and, most importantly, pose severe safety risks due to their oncogenic potential and their inflammatory and immunogenic effects, which prevent them from repeated administration. To overcome these limitations, nonviral

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vectors have emerged as a promising alternative for gene delivery. A number of nonviral siRNA delivery approaches have now been reported in vivo, including in nonhuman primates and humans^{6–14}.

Non-viral vectors for siRNA delivery

Of primary consideration in deciding on a drug delivery system for siRNA is whether the intended disease target lends itself to systemic or local administration. In the case of delivery of DNA encoding for the short hairpin RNA (shRNA) by non-viral delivery systems, nuclear translocation of the DNA is often inadequate. Most attention will be given to nonviral delivery of siRNA. Various strategies for delivering siRNA to specific tissue and organ systems in vivo following systemic administration are summarized here (table 1). The delivery strategy can be classified into the following categories:

A. Hydrodynamic injection

Quick injection of siRNA in a large volume of physiological buffer effectively localizes duplex siRNA in the liver. In rats, administration of a VEGF-specific siRNA resulted in more than 75% inhibition of pathological neovascularization. Due to the invasiveness of the injection technique, hydrodynamics-based transfection is not appropriate for clinical applications at this point^{15,16}. However, recent advances in using a computer-controlled, catheter-guided injection device have greatly improved the precision and reproducibility of this approach¹⁷. To date, the device has only been used for the delivery of DNA, but siRNA should be equally applicable in this approach.

B. Cholesterol conjugation with siRNA

A siRNA with chemically modified backbone conjugated to a lipophilic cholesterol moiety at the 3' end of the sense strand led to suppression of apoB mRNA by approximately 60% in the liver after i.v. injection. Soutschek et al reported that no immune stimulation or off-target effect occurred in mice at a high dose of 50mg/kg^{18,19}. However, safety evaluation of this potential approach needs further study. Recent studies indicated that the cholesterol-conjugated siRNA is delivered into the hepatocytes as a complex of lipoproteins²⁰.

C. Cationic delivery systems

Cationic lipids and liposomes, cationic polymers, cationic dendrimers, and cationic cell-penetrating peptides have been used for the delivery of siRNA. A common characteristic among these vectors is their net positive charge, which contributes to both complex formation with the polyanionic nucleic acid, such as siRNA, and interaction with the negatively charged cell membrane^{6,10–13,21–26}. Nanoparticles and complexes should be <100 nm to reduce renal excretion and be taken up by cells²¹, because they are taken into the cell via endocytosis and/or macropinocytosis.

1. Liposomes and lipoplexes—Liposomes consist of an aqueous compartment enclosed in a phospholipid bilayer with hydrophilic drug typically entrapped in the center aqueous layer. The bilayer often contains a lipid component (regularly is a cationic and/or a fusogenic lipid), cholesterol and polyethylene glycol–lipid. The liposomes are particles with stable physicochemical characteristics. In contrast, lipoplexes are spontaneously formed via interaction of positively-charged lipids and negatively-charged nucleic acids. Lipoplexes should be prepared immediately before use because lipoplexes are unstable²⁷. Lipofectin, RNAifect, Oligofectamine, Lipofectamine and TransIT TKO are commercially available as potential enhancers of siRNA delivery in vitro^{24,26,28–42}. DOTAP (N-[1-(2,3-dioleoyloxy)]-N-N-N trimethyl ammonium propane) and Oligofectamine were some of the first lipid formulations to be used for the in vivo delivery of siRNA and effective gene silencing of TNF-

α and β -catenin in mice^{29,30}. Cationic liposomes termed “solid nucleic acid lipid particles” (SNALPs) that have been stabilized by PEGylation for improved pharmacokinetics have also been successfully used to deliver siRNA in mice and nonhuman primates. In monkeys, ApoB were markedly suppressed at a dose of 2.5mg/kg of SNALP-formulated siRNA. Furthermore, it should be noted that PEGylated liposomes are a clinically approved delivery system for doxorubicin and therefore represent a viable option for delivering siRNA in humans²⁸.

2. Polymers and peptide delivery systems—Of the many cationic polymers, polyethyleneimine (PEI) has been widely examined for DNA, oligonucleotide, and siRNA delivery^{13,23,24,26,43–64}. Now, a novel delivery strategy using PEGylated PEI with an RGD (Arg-Gly-Asp) peptide to deliver siRNA targeting VEGF has been demonstrated inhibit tumor growth and reduce angiogenesis after i.v. administration. The use of polyamidoamine polymers in vivo might be hindered owing to their nonspecific toxicity. Atelocollagen (300 kDa) has been used to administer siRNA systemically and locally in tumor models^{48,49,53,65}. In vivo, this polymer was also able to effectively deliver siRNA targeting VEGF to tumor vasculature in an orthotopic model of human testicular cancer, to bone metastases, and to a xenograft model of prostate cancer^{49,53,65}. A cyclodextrin polycation delivery system is well tolerated and even repeat doses failed to elicit a significant delivery system-specific antibody response. More recently, there was a report that repeated administration of RVG (rabies virus glycoprotein)-9R-bound antiviral siRNA did not induce inflammatory cytokines or anti-peptide antibodies. The study also reported for the first time that systemically delivered siRNA-peptide conjugates can cross the blood-brain barrier. (BBB)⁵⁶.

D. Other delivery systems

1. Intracocular delivery—Local delivery to the eye of naked and lipid-complexed siRNA is possible, and such studies have paved the way for human clinical trials^{52,56,66–88}. siRNA targeting VEGF was delivered to prevent laser-induced choroidal neovascularization in an experimental model of age-related macular degeneration (AMD)⁶⁷. There is also an ongoing clinical trial in a similar direction. (ALN-RSV01; Alnylam Pharmaceuticals [Cambridge, MA] press release. AKI-i5; Quark Pharmaceuticals press release.) FDA recently approved a clinical study of a cyclodextrin-containing nanoparticle formulation for siRNA (RONDEL™). Calando's RONDEL™ is two-part siRNA delivery system. The first component is a linear, cyclodextrin-containing polycation that binds to the anionic backbone of the siRNA. The polymer and siRNA self-assemble into nanoparticles with size smaller than 100 nm in diameter that fully protect the siRNA from nuclease degradation in serum. The nanoparticles are additionally modified by adding adamantine and conjugated PEG with or without the targeting ligand transferrin. The siRNA delivery system has been designed to allow for intravenous injection. Upon delivery to the target cell, the targeting ligand binds to membrane receptors on the cell surface and the RNA-containing nanoparticle is taken into the cell by endocytosis.

2. Intratumoral delivery—Direct local injection of siRNA, with or without a delivery system, into the tumor has been an effective anticancer approach in vivo. Delivery of siRNA targeting RasA and RasC in a xenograft breast cancer model in mice has been shown to successfully reduced tumor volume⁷⁴. However, clinically, only a few tumors are suitable for local delivery; systemic approaches described above are necessary for most solid tumors in vivo.

3. Local in vivo electroporation to muscle—Electroporation has been used to deliver siRNA to mouse muscle tissue. Golzio et al showed silencing effects lasted 23 days after administration of siRNA co-transfected with a plasmid encoding GFP in mice⁸¹.

4. Local delivery to the CNS—Local delivery of siRNA to the CNS is a feasible option for targeting CNS-based diseases, though systemic delivery strategies may be clinically more attractive. The delivery of siRNA targeting the NR2B subtype of the NMDA receptor using intrathecal injection led to a 83% decrease in the protein's expression level in the rat brain⁷⁰.

5. Intranasal delivery to the airway—Delivering siRNA to the airway could be a treatment for a number of diseases including asthma, cystic fibrosis, ischemic reperfusion injury, and infection with respiratory viruses^{66,84,87}. Thomas reported marked gene silencing in an acute model of lung injury and Li et al showed treatment the treatment of experimental infection with SARS virus in a rhesus macaque model^{68,81,89}.

E. Self-assembled LPD nanoparticles

We have used surface-modified LPD nanoparticles to deliver siRNA to solid and metastatic tumors. The major components of the delivery systems are cationic lipids and protamine (a cationic polypeptide), which can interact with negatively charged siRNA. Surface steric stabilization is introduced by PEGylation to prevent the aggregation of the resulting complex with serum components. Ligands are attached to the distal end of the PEG chain to increase cellular bioavailability. Cationic lipid is necessary for endosome lysis and intracellular release of siRNA. The mechanism of the endosome membrane destabilization is most likely due to the formation of ion pair complex between the cationic lipid in the nanoparticles and the negatively charged anionic lipids in the endosome membrane, as hypothesized by Xu and Szoka⁹⁰. We have used anisamide as a targeting ligand for tumor cells expressing the sigma receptor^{91,92}. Our ligand targeted, PEGylated LPD formulation showed significant increase in cellular uptake via specific receptor-mediated pathway. It is important to note that the function of anisamide is not the tumor uptake, since non-targeted LPD nanoparticles accumulated in the tumor as efficiently as the targeted particles⁹¹. The surfaced modified LPD nanoparticles showed a strong enhanced permeability and retention (EPR) effect in that approximately 60–80% of the injected dose per g of tissue was found in the tumor⁹³. This targeted formulation also demonstrated its strong gene-silencing effect mediated by RNAi (Figure 1 and see below). Preliminary data showed that the surface-modified LPD delivered siRNA predominantly to the tumor, which was the major uptake organ, after intravenous administration. Our formulation provides an advantage of high tumor targeting and low RES uptake, which implied its potential for RNAi-based tumor therapy⁹³.

Our subsequent study confirmed the cellular entry mechanism for the targeted nanoparticles was via a sigma receptor dependent pathway⁸⁰. The gene silencing activity was significantly improved and nanoparticle formulations were not immunotoxic. In B16F10 melanoma cells targeted nanoparticles showed a four-fold increase in delivery efficiency compared to non-targeted nanoparticles. Addition of free haloperidol, a known agonist for the sigma receptor, significantly reduced the delivery efficiency of targeted nanoparticles but not other formulations. Since the B16F10 cells were stably marked by a luciferase gene by using a retrovirus vector, the in vivo activity of the nanoparticle formulations was assessed by the luciferase gene silencing in the whole B16F10 tumor loaded lungs. Targeted nanoparticles silenced about 75% luciferase activity. Whereas none of the other control formulations showed any significant gene silencing activity. None of the formulations, including the targeted nanoparticles, induced significant proinflammatory cytokines (IL6, IL12, TNF and IFN- α) in the serum.

We combined three different siRNA sequences (MDM2/c-myc/VEGF = 1:1:1, weight ratio) into different formulations to evaluate the therapeutic outcomes⁸⁰. In the B16F10 tumor-loaded lung, siRNA in the targeted nanoparticles significantly reduced the tumor load to 20–30% as compared to the untreated control ($P < 0.01$). The therapeutic outcome was also

analyzed in terms of animal survival. On day 23, the survival rate for the therapeutic siRNA and the control siRNA in the targeted nanoparticles was 90 and 40%, respectively, and the mean survival time was 28, and 22.5 days, respectively ($P < 0.01$). Furthermore, we demonstrated at the therapeutic dose (0.45 mg/kg), the targeted nanoparticles did not induce significant production of all analyzed cytokines, including IL-6, IL-12, TNF- α , and interferon- α . Even after two consecutive injections, the IL-6 and IL-12 levels were not significantly elevated. At the therapeutic dose, none of the formulations caused elevation in cytokine production in the lung. Additionally, the body weight did not significantly decrease during treatment at the therapeutic dose. The targeted nanoparticle formulation did not damage the major organs (heart, liver, spleen, lung, and kidney).

Since the self-assembly of the nanoparticles require the presence of a high molecular weight polyanion for stability, calf thymus DNA was initially used as a carrier for the siRNA which is a low molecular weight nucleic acid. Calf thymus DNA as a foreign DNA to human may cause unwanted toxicity and immune stimulation. Plasmid DNA contains a high amount of unmethylated CpG motifs that is hostile to the cell.⁹⁴ Recently, we have successfully replaced calf thymus DNA with a high molecular weight anionic polysaccharide, i.e., hyaluronic acid^{93,94}. The resulting formulation contains cationic liposome, protamine and hyaluronic acid and is called LPH. LPH showed the same gene silencing activity as the corresponding LPD formulation. The ED₅₀ for the luciferase silencing in the B16F10 melanoma model was 75 μ g/kg in siRNA, same as the LPD formulation. However, the induction of both IL-6 and IL-12 cytokines by LPH was significantly lower than that of the corresponding LPD containing the calf thymus DNA.

Conclusions

Since the discovery of RNA interference, research groups worldwide have sought to invent efficient delivery systems to enhance the ability of siRNA to traverse the cell membrane and elicit a biological response. The most widely used siRNA delivery methodologies consist of cationic lipids and/or cationic polymers that package siRNA into stable nanoparticles capable of translocating across the cellular membrane. Encapsulation of siRNA into the naked nanoparticles dramatically increased the intracellular delivery and the gene silencing activity through the charge-charge interaction of the formulation with the endosome membrane. PEGylation of the naked nanoparticles abolished the non-specific interaction with negatively charged cells or proteins and also enhanced the uptake of siRNA by the tumor due to the EPR effect. Introduction of a targeting ligand at the distal end of the PEG chain restored the intracellular delivery to the receptor positive cells, while the tissue selectivity was maintained. This targeted nanoparticle formulation with improved tissue specificity and delivery efficiency silenced the target gene in the lung metastasis effectively without any significant immunotoxicity. The immunotoxicity of the nanoparticles was further reduced by replacing the high molecular weight DNA with hyaluronic acid. We therefore conclude that siRNA formulated in the targeted nanoparticles has the potential to become a useful tool in clinical cancer therapy.

The almost ideal specificity of RNAi is not entirely true in reality. Silencing of off-targets is clearly unwanted. Importantly, off-target effect remains a critical issue for therapeutic applications of RNAi. Hopefully, novel protein array technology will provide a better picture of siRNA effects on cellular protein expression profiles and provide a better way of screening siRNA. Developing and indentifying potent or hyper functional siRNA will help resolve unwanted off-targeting since these siRNAs works at subnanomolar concentration. Likewise intelligent and effective design of siRNA that improves strand selectivity may be possible to significantly avoid off-target effects. While knowledge of immune stimulatory properties calls for research to proceed to animal models, in vitro use of human primary cells with a full

repertoire for immune stimulation are also needed. Long dsRNA would induce interferon responses by binding to double-stranded-RNA-activated protein kinase (PKR), 2',5'-oligoadenylate synthetase RNase L system or several Toll-like receptors (TLRs). A particular sequence motif (5-GUCCUCAA-3'), GU-rich regions and CpG motifs can stimulate innate immune responses. Recently, Dharmacon published that even short 23 nt long siRNA may invoke interferon responses in cell culture assays. Great care and thorough testing are clearly needed before proceeding to clinical use. Importantly, the length threshold seems to vary among cell types which make it hard to predict the outcome of dicer substrate in vivo.

Acknowledgement

The original work in this lab has been supported by NIH grant CA129835.

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806–811. [PubMed: 9486653]
2. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* 2002;110:563–574. [PubMed: 12230974]
3. Akhtar S, Benter IF. Nonviral delivery of synthetic siRNAs in vivo. *J. Clin. Invest* 2007;117:3623–3632. [PubMed: 18060020]
4. Jana S, Chakraborty C, Nandi S, Deb JK. RNA interference: potential therapeutic targets. *Appl. Microbiol. Biotechnol* 2004;65:649–657. [PubMed: 15372214]
5. Li SD, Huang L. Non-viral is superior to viral gene delivery. *J. Control. Release* 2007;123:181–183. [PubMed: 17935817]
6. Xie FY, Woodle MC, Lu PY. Harnessing in vivo siRNA delivery for drug discovery and therapeutic development. *Drug Discov. Today* 2006;11:67–73. [PubMed: 16478693]
7. Martin SE, Caplen NJ. Applications of RNA interference in mammalian systems. *Annu. Rev. Genomics Hum. Genet* 2007;8:81–108. [PubMed: 17477824]
8. Liu G, Wong-Staal F, Li QX. Development of new RNAi therapeutics. *Histol. Histopathol* 2007;22:211–217. [PubMed: 17149694]
9. Leung RK, Whittaker PA. RNA interference: from gene silencing to gene-specific therapeutics. *Pharmacol. Ther* 2005;107:222–239. [PubMed: 15908010]
10. Kim DH, Rossi JJ. Strategies for silencing human disease using RNA interference. *Nat. Rev. Genet* 2007;8:173–184. [PubMed: 17304245]
11. de Fougerolles A, Vornlocher HP, Maraganore J, Lieberman J. Interfering with disease: a progress report on siRNA-based therapeutics. *Nat. Rev. Drug Discov* 2007;6:443–453. [PubMed: 17541417]
12. Bumcrot D, Manoharan M, Kotliansky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat. Chem. Biol* 2006;2:711–719. [PubMed: 17108989]
13. Aigner A. Delivery Systems for the Direct Application of siRNAs to Induce RNA Interference (RNAi) In Vivo. *J. Biomed. Biotechnol* 2006;2006:71659. [PubMed: 17057369]
14. Aagaard L, Rossi JJ. RNAi therapeutics: principles, prospects and challenges. *Adv. Drug Deliv. Rev* 2007;59:75–86. [PubMed: 17449137]
15. Sebestyen MG, Budker VG, Budker T, Subbotin VM, Zhang G, Monahan SD, Lewis DL, Wong SC, Hagstrom JE, Wolff JA. Mechanism of plasmid delivery by hydrodynamic tail vein injection. I. Hepatocyte uptake of various molecules. *J. Gene. Med* 2006;8:852–873. [PubMed: 16724360]
16. Lewis DL, Wolff JA. Systemic siRNA delivery via hydrodynamic intravascular injection. *Adv. Drug Deliv. Rev* 2007;59:115–123. [PubMed: 17442446]
17. Suda T, Suda K, Liu D. Computer-assisted hydrodynamic gene delivery. *Mol. Ther* 2008;16:1098–1104. [PubMed: 18398428]
18. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I,

- Toudjarska I, Wang G, Wuschko S, Bumcrot D, Koteliensky V, Limmer S, Manoharan M, Vornlocher HP. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 2004;432:173–178. [PubMed: 15538359]
19. Moore VA, Dunnion DJ, Brown T, Irwin WJ, Akhtar S. Interaction of oligonucleotide-conjugates with the dipeptide transporter system in Caco-2 cells. *Biochem. Pharmacol* 1997;53:1223–1228. [PubMed: 9214682]
 20. Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandey RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM, Zimmermann T, Koteliensky V, Manoharan M, Stoffel M. Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nat. Biotechnol* 2007;25:1149–1157. [PubMed: 17873866]
 21. Li W, Szoka FC Jr. Lipid-based nanoparticles for nucleic acid delivery. *Pharm. Res* 2007;24:438–449. [PubMed: 17252188]
 22. Kuhn R, Streif S, Wurst W. RNA interference in mice. *Handb. Exp. Pharmacol* 2007:149–176. [PubMed: 17203655]
 23. Kawakami S, Hashida M. Targeted delivery systems of small interfering RNA by systemic administration. *Drug Metab. Pharmacokinet* 2007;22:142–151. [PubMed: 17603214]
 24. Gilmore IR, Fox SP, Hollins AJ, Sohail M, Akhtar S. The design and exogenous delivery of siRNA for post-transcriptional gene silencing. *J. Drug Target* 2004;12:315–340. [PubMed: 15545082]
 25. Ebbesen M, Jensen TG. Nanomedicine: techniques, potentials, and ethical implications. *J. Biomed. Biotechnol* 2006;2006:51516. [PubMed: 17489016]
 26. Akhtar S, Benter I. Toxicogenomics of non-viral drug delivery systems for RNAi: potential impact on siRNA-mediated gene silencing activity and specificity. *Adv. Drug Deliv. Rev* 2007;59:164–182. [PubMed: 17481774]
 27. de Fougères AR. Delivery vehicles for small interfering RNA in vivo. *Hum Gene Ther* 2008;19:125–132. [PubMed: 18257677]
 28. Zimmermann TS, Lee AC, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, Harborth J, Heyes JA, Jeffs LB, John M, Judge AD, Lam K, McClintock K, Nechev LV, Palmer LR, Racie T, Rohl I, Seiffert S, Shanmugam S, Sood V, Soutschek J, Toudjarska I, Wheat AJ, Yaworski E, Zedalis W, Koteliensky V, Manoharan M, Vornlocher HP, MacLachlan I. RNAi-mediated gene silencing in non-human primates. *Nature* 2006;441:111–114. [PubMed: 16565705]
 29. Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB. Small interfering RNAs directed against beta-catenin inhibit the in vitro and in vivo growth of colon cancer cells. *Clin. Cancer Res* 2003;9:1291–1300. [PubMed: 12684397]
 30. Sorensen DR, Leirdal M, Sioud M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J. Mol. Biol* 2003;327:761–766. [PubMed: 12654261]
 31. Semple SC, Harasym TO, Clow KA, Ansell SM, Klimuk SK, Hope MJ. Immunogenicity and rapid blood clearance of liposomes containing polyethylene glycol-lipid conjugates and nucleic Acid. *J. Pharmacol. Exp. Ther* 2005;312:1020–1026. [PubMed: 15525796]
 32. Pirollo KF, Rait A, Zhou Q, Hwang SH, Dagata JA, Zon G, Hogrefe RI, Palchik G, Chang EH. Materializing the potential of small interfering RNA via a tumor-targeting nanodelivery system. *Cancer Res* 2007;67:2938–2943. [PubMed: 17409398]
 33. Pal A, Ahmad A, Khan S, Sakabe I, Zhang C, Kasid UN, Ahmad I. Systemic delivery of RafsiRNA using cationic cardiolipin liposomes silences Raf-1 expression and inhibits tumor growth in xenograft model of human prostate cancer. *Int. J. Oncol* 2005;26:1087–1091. [PubMed: 15754006]
 34. Omid Y, Hollins AJ, Benboubetra M, Drayton R, Benter IF, Akhtar S. Toxicogenomics of non-viral vectors for gene therapy: a microarray study of lipofectin- and oligofectamine-induced gene expression changes in human epithelial cells. *J. Drug Target* 2003;11:311–323. [PubMed: 14668052]
 35. Morrissey DV, Lockridge JA, Shaw L, Blanchard K, Jensen K, Breen W, Hartsough K, Machemer L, Radka S, Jadhav V, Vaish N, Zinnen S, Vargeese C, Bowman K, Shaffer CS, Jeffs LB, Judge A, MacLachlan I, Polisky B. Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat. Biotechnol* 2005;23:1002–1007. [PubMed: 16041363]
 36. Khan A, Benboubetra M, Sayyed PZ, Ng KW, Fox S, Beck G, Benter IF, Akhtar S. Sustained polymeric delivery of gene silencing antisense ODNs, siRNA, DNazymes and ribozymes: in vitro and in vivo studies. *J. Drug Target* 2004;12:393–404. [PubMed: 15545089]

37. Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol* 2005;23:457–462. [PubMed: 15778705]
38. Ishida T, Wang X, Shimizu T, Nawata K, Kiwada H. PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. *J. Control. Release* 2007;122:349–355. [PubMed: 17610982]
39. Halder J, Kamat AA, Landen CN Jr, Han LY, Lutgendorf SK, Lin YG, Merritt WM, Jennings NB, Chavez-Reyes A, Coleman RL, Gershenson DM, Schmandt R, Cole SW, Lopez-Berestein G, Sood AK. Focal adhesion kinase targeting using in vivo short interfering RNA delivery in neutral liposomes for ovarian carcinoma therapy. *Clin. Cancer Res* 2006;12:4916–4924. [PubMed: 16914580]
40. Geisbert TW, Hensley LE, Kagan E, Yu EZ, Geisbert JB, Daddario-DiCaprio K, Fritz EA, Jahrling PB, McClintock K, Phelps JR, Lee AC, Judge A, Jeffs LB, MacLachlan I. Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by RNA interference. *J. Infect. Dis* 2006;193:1650–1657. [PubMed: 16703508]
41. Chien PY, Wang J, Carbonaro D, Lei S, Miller B, Sheikh S, Ali SM, Ahmad MU, Ahmad I. Novel cationic cardiolipin analogue-based liposome for efficient DNA and small interfering RNA delivery in vitro and in vivo. *Cancer Gene. Ther* 2005;12:321–328. [PubMed: 15578064]
42. Arnold AS, Tang YL, Qian K, Shen L, Valencia V, Phillips MI, Zhang YC. Specific beta1-adrenergic receptor silencing with small interfering RNA lowers high blood pressure and improves cardiac function in myocardial ischemia. *J. Hypertens* 2007;25:197–205. [PubMed: 17143192]
43. Yoo H, Sazani P, Juliano RL. PAMAM dendrimers as delivery agents for antisense oligonucleotides. *Pharm. Res* 1999;16:1799–1804. [PubMed: 10644065]
44. Vangasseri DP, Han SJ, Huang L. Lipid-protamine-DNA-mediated antigen delivery. *Curr. Drug Deliv* 2005;2:401–406. [PubMed: 16305443]
45. Urban-Klein B, Werth S, Abuharbeid S, Czabayko F, Aigner A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene Ther* 2005;12:461–466. [PubMed: 15616603]
46. Tsubouchi A, Sakakura J, Yagi R, Mazaki Y, Schaefer E, Yano H, Sabe H. Localized suppression of RhoA activity by Tyr31/118-phosphorylated paxillin in cell adhesion and migration. *J. Cell Biol* 2002;159:673–683. [PubMed: 12446743]
47. Tomalia DA, Reyna LA, Svenson S. Dendrimers as multi-purpose nanodevices for oncology drug delivery and diagnostic imaging. *Biochem. Soc. Trans* 2007;35:61–67. [PubMed: 17233602]
48. Takeshita F, Ochiya T. Therapeutic potential of RNA interference against cancer. *Cancer Sci* 2006;97:689–696. [PubMed: 16863503]
49. Takeshita F, Minakuchi Y, Nagahara S, Honma K, Sasaki H, Hirai K, Teratani T, Namatame N, Yamamoto Y, Hanai K, Kato T, Sano A, Ochiya T. Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo. *Proc. Natl. Acad. Sci. U S A* 2005;102:12177–12182. [PubMed: 16091473]
50. Song E, Zhu P, Lee SK, Chowdhury D, Kussman S, Dykxhoorn DM, Feng Y, Palliser D, Weiner DB, Shankar P, Marasco WA, Lieberman J. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol* 2005;23:709–717. [PubMed: 15908939]
51. Schifferers RM, Ansari A, Xu J, Zhou Q, Tang Q, Storm G, Molema G, Lu PY, Scaria PV, Woodle MC. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res* 2004;32:e149. [PubMed: 15520458]
52. Pardridge WM. shRNA and siRNA delivery to the brain. *Adv. Drug Deliv. Rev* 2007;59:141–152. [PubMed: 17434235]
53. Minakuchi Y, Takeshita F, Kosaka N, Sasaki H, Yamamoto Y, Kouno M, Honma K, Nagahara S, Hanai K, Sano A, Kato T, Terada M, Ochiya T. Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo. *Nucleic Acids Res* 2004;32:e109. [PubMed: 15272050]
54. Meade BR, Dowdy SF. Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. *Adv. Drug Deliv. Rev* 2007;59:134–140. [PubMed: 17451840]
55. Liu S. Radiolabeled multimeric cyclic RGD peptides as integrin alphavbeta3 targeted radiotracers for tumor imaging. *Mol. Pharm* 2006;3:472–187. [PubMed: 17009846]

56. Kumar P, Wu H, McBride JL, Jung KE, Kim MH, Davidson BL, Lee SK, Shankar P, Manjunath N. Transvascular delivery of small interfering RNA to the central nervous system. *Nature* 2007;448:39–43. [PubMed: 17572664]
57. Kichler A. Gene transfer with modified polyethylenimines. *J. Gene Med* 2004;6:S3–S10. [PubMed: 14978746]
58. Kang H, DeLong R, Fisher MH, Juliano RL. Tat-conjugated PAMAM dendrimers as delivery agents for antisense and siRNA oligonucleotides. *Pharm. Res* 2005;22:2099–2106. [PubMed: 16184444]
59. Juliano RL. Intracellular delivery of oligonucleotide conjugates and dendrimer complexes. *Ann. N Y Acad Sci* 2006;1082:18–26. [PubMed: 17145920]
60. Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res* 2005;65:8984–8992. [PubMed: 16204072]
61. Huang YZ, Zang M, Xiong WC, Luo Z, Mei L. Erbin suppresses the MAP kinase pathway. *J. Biol. Chem* 2003;278:1108–1114. [PubMed: 12379659]
62. Hollins AJ, Omidi Y, Benter IF, Akhtar S. Toxicogenomics of drug delivery systems: Exploiting delivery system-induced changes in target gene expression to enhance siRNA activity. *J. Drug Target* 2007;15:83–88. [PubMed: 17365277]
63. Heidel JD, Yu Z, Liu JY, Rele SM, Liang Y, Zeidan RK, Kornbrust DJ, Davis ME. Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proc. Natl. Acad. Sci. U S A* 2007;104:5715–5721. [PubMed: 17379663]
64. Ge Q, Filip L, Bai A, Nguyen T, Eisen HN, Chen J. Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl. Acad. Sci. U S A* 2004;101:8676–8681. [PubMed: 15173599]
65. Takei Y, Kadomatsu K, Yuzawa Y, Matsuo S, Muramatsu T. A small interfering RNA targeting vascular endothelial growth factor as cancer therapeutics. *Cancer Res* 2004;64:3365–3370. [PubMed: 15150085]
66. Zhang X, Shan P, Jiang D, Noble PW, Abraham NG, Kappas A, Lee PJ. Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. *J. Biol. Chem* 2004;279:10677–10684. [PubMed: 14688267]
67. Tolentino MJ, Brucker AJ, Fosnot J, Ying GS, Wu IH, Malik G, Wan S, Reich SJ. Intravitreal injection of vascular endothelial growth factor small interfering RNA inhibits growth and leakage in a nonhuman primate, laser-induced model of choroidal neovascularization. *Retina* 2004;24:660. [PubMed: 15473063]
68. Thomas M, Lu JJ, Chen J, Klivanov AM. Non-viral siRNA delivery to the lung. *Adv. Drug Deliv. Rev* 2007;59:124–133. [PubMed: 17459519]
69. Thakker DR, Natt F, Husken D, Maier R, Muller M, van der Putten H, Hoyer D, Cryan JF. Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. *Proc. Natl. Acad. Sci. U S A* 2004;101:17270–17275. [PubMed: 15569935]
70. Tan PH, Yang LC, Shih HC, Lan KC, Cheng JT. Gene knockdown with intrathecal siRNA of NMDA receptor NR2B subunit reduces formalin-induced nociception in the rat. *Gene Ther* 2005;12:59–66. [PubMed: 15470478]
71. Song E, Lee SK, Wang J, Ince N, Ouyang N, Min J, Chen J, Shankar P, Lieberman J. RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med* 2003;9:347–351. [PubMed: 12579197]
72. Shishkina GT, Kalinina TS, Dygalo NN. Attenuation of alpha2A-adrenergic receptor expression in neonatal rat brain by RNA interference or antisense oligonucleotide reduced anxiety in adulthood. *Neuroscience* 2004;129:521–528. [PubMed: 15541874]
73. Reich SJ, Fosnot J, Kuroki A, Tang W, Yang X, Maguire AM, Bennett J, Tolentino MJ. Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis* 2003;9:210–216. [PubMed: 12789138]
74. Pille JY, Denoyelle C, Varet J, Bertrand JR, Soria J, Opolon P, Lu H, Pritchard LL, Vannier JP, Malvy C, Soria C, Li H. Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of

- MDA-MB-231 breast cancer cells in vitro and in vivo. *Mol. Ther* 2005;11:267–274. [PubMed: 15668138]
75. Nakamura H, Siddiqui SS, Shen X, Malik AB, Pulido JS, Kumar NM, Yue BY. RNA interference targeting transforming growth factor-beta type II receptor suppresses ocular inflammation and fibrosis. *Mol. Vis* 2004;10:703–711. [PubMed: 15475878]
 76. Medarova Z, Pham W, Farrar C, Petkova V, Moore A. In vivo imaging of siRNA delivery and silencing in tumors. *Nat. Med* 2007;13:372–377. [PubMed: 17322898]
 77. McNamara JO 2nd, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat. Biotechnol* 2006;24:1005–1015. [PubMed: 16823371]
 78. Massaro D, Massaro GD, Clerch LB. Noninvasive delivery of small inhibitory RNA and other reagents to pulmonary alveoli in mice. *Am. J. Physiol. Lung Cell Mol. Physiol* 2004;287:L1066–L1070. [PubMed: 15234906]
 79. Makimura H, Mizuno TM, Mastaitis JW, Agami R, Mobbs CV. Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci* 2002;3:18. [PubMed: 12423556]
 80. Li SD, Chono S, Huang L. Efficient oncogene silencing and metastasis inhibition via systemic delivery of siRNA. *Mol. Ther* 2008;16:942–946. [PubMed: 18388916]
 81. Golzio M, Mazzolini L, Moller P, Rols MP, Teissie J. Inhibition of gene expression in mice muscle by in vivo electrically mediated siRNA delivery. *Gene Ther* 2005;12:246–251. [PubMed: 15592423]
 82. Kishida T, Asada H, Gojo S, Ohashi S, Shin-Ya M, Yasutomi K, Terauchi R, Takahashi KA, Kubo T, Imanishi J, Mazda, O. Sequence-specific gene silencing in murine muscle induced by electroporation-mediated transfer of short interfering RNA. *J. Gene. Med* 2004;6:105–110. [PubMed: 14716682]
 83. Kim WJ, Christensen LV, Jo S, Yockman JW, Jeong JH, Kim YH, Kim SW. Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. *Mol. Ther* 2006;14:343–350. [PubMed: 16765648]
 84. Howard KA, Rahbek UL, Liu X, Damgaard CK, Glud SZ, Andersen MO, Hovgaard MB, Schmitz A, Nyengaard JR, Besenbacher F, Kjems J. RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system. *Mol. Ther* 2006;14:476–484. [PubMed: 16829204]
 85. Herard AS, Besret L, Dubois A, Dauge J, Delzescaux T, Hantraye P, Bonvento G, Moya KL. siRNA targeted against amyloid precursor protein impairs synaptic activity in vivo. *Neurobiol. Aging* 2006;27:1740–1750. [PubMed: 16337035]
 86. Dorn G, Patel S, Wotherspoon G, Hemmings-Mieszcak M, Barclay J, Natt FJ, Martin P, Bevan S, Fox A, Ganju P, Wishart W, Hall J. siRNA relieves chronic neuropathic pain. *Nucleic Acids Res* 2004;32:e49. [PubMed: 15026538]
 87. Bitko V, Musiyenko A, Shulyayeva O, Barik S. Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med* 2005;11:50–55. [PubMed: 15619632]
 88. Akaneya Y, Jiang B, Tsumoto T. RNAi-induced gene silencing by local electroporation in targeting brain region. *J. Neurophysiol* 2005;93:594–602. [PubMed: 15604463]
 89. Li BJ, Tang Q, Cheng D, Qin C, Xie FY, Wei Q, Xu J, Liu Y, Zheng BJ, Woodle MC, Zhong N, Lu PY. Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat. Med* 2005;11:944–951. [PubMed: 16116432]
 90. Xu Y, Szoka FC Jr. Mechanism of DNA release from cationic liposome/DNA complexes used in cell transfection. *Biochemistry* 1996;35:5616–5623. [PubMed: 8639519]
 91. Li SD, Huang L. Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. *Mol. Pharm* 2006;3:579–588. [PubMed: 17009857]
 92. Banerjee R, Tyagi P, Li S, Huang L. Anisamide-targeted stealth liposomes: a potent carrier for targeting doxorubicin to human prostate cancer cells. *Int. J. Cancer* 2004;112:693–700. [PubMed: 15382053]
 93. Li SD, Huang L. Surface-modified LPD nanoparticles for tumor targeting. *Ann. N Y Acad Sci* 2006;1082:1–8. [PubMed: 17145918]

94. Chono S, Li SD, Conwell CC, Huang L. An efficient and low immunostimulatory nanoparticle formulation for systemic siRNA delivery to the tumor. *J. Control. Release* 2008;131:64–69. [PubMed: 18674578]
95. Li SD, Chono S, Huang L. Efficient gene silencing in metastatic tumor by siRNA formulated in surface-modified nanoparticles. *J. Control. Release* 2008;126:77–84. [PubMed: 18083264]

Figure 1.

A schematic depiction of the structural components required of a targeted cationic complex or nanoparticle for siRNA delivery in vivo. Optimally designed and formulated siRNA can cross the endosome membrane and provide a sequence-specific gene-silencing effect.

Table 1

Delivery systems for siRNA in vivo

Delivery system	Mechanism	Target tissue or model	Characteristics
Hydrodynamic i.v. or direct injection	High pressure contributes to penetration across the cell membrane	Rat brain ⁸⁶ , mouse liver ¹⁶ , mouse lung ⁶⁶ .	Relative simplicity of local administration
Cholesterol conjugation with siRNA	Promote distribution and cellular uptake via lipoprotein as a carrier	Dyslipidemia in mice ¹⁸ and non-human primate ²⁰ .	Significantly decrease the complexity by conjugated with the sense strand.
Liposomes and lipoplexes	Improve pharmacokinetic properties and/or reduce toxicity profiles.	Dyslipidemia in monkeys ⁴⁰ , pancreatic tumor xenografts in mice ³² , breast cancer xenografts in mice ⁶⁴ , Prostate cancer xenograft in mice ⁷⁷ .	Similar to commercial transfection agents.
Polymers and peptide delivery systems for siRNA	Endosomal escape takes place because of "proton sponge" effect. Improve selectively and specifically deliver siRNA in vivo.	Ewing Sarcoma in mice ⁶⁰ , mouse brain ⁵⁶ , melanoma xenografts in mice ⁵⁰ .	Condensed nanoparticles with siRNA. Can be modified with a targeting element for receptor mediated uptake.
Surface modified LPD nanoparticles	siRNA condensed with protamine to form a core, which is wrapped with cationic lipid membrane. Final PEGylation provides surface protection and targeting specificity.	Oncogenes in solid and metastatic tumors can be effectively silenced in mouse models ^{21,80,93,95} .	Very high tumor uptake and low immunotoxicity.