Review

Delivery of RNA Interference

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ABSTRACT

Over the last few years, RNA Interference (RNAi), a naturally occurring mechanism of gene regulation conserved in plant and mammalian cells, has opened numerous novel opportunities for basic research across the field of biology. While RNAi has helped accelerate discovery and understanding of gene functions, it also has great potential as a therapeutic and potentially prophylactic modality. Challenging diseases failing conventional therapeutics could become treatable by specific silencing of key pathogenic genes. More specifically, therapeutic targets previously deemed “undruggable” by small molecules, are now coming within reach of RNAi based therapy. For RNAi to be effective and elicit gene silencing response, the double-stranded RNA molecules must be delivered to the target cell. Unfortunately, delivery of these RNA duplexes has been challenging, halting rapid development of RNAi-based therapies. In this review we present current advancements in the field of siRNA delivery methods, including the pros and cons of each method.

INTRODUCTION

RNA Interference (RNAi) is a natural mechanism of gene silencing conserved in plant and mammalian cells. Since the discovery of RNAi activity in mammalian cells,1,2 RNAi has quickly been adopted by the research community as a versatile tool with a wide range of applications from reverse genetics to high throughput screening of drug targets. RNAi’s potential therapeutic role may benefit a wide variety of diseases, including malignant, infectious and autoimmune diseases. The advantages of RNAi over other therapeutic modalities include its high specificity (in some cases, a single point mutation can abolish silencing effect), versatility (interfering RNA can be designed against virtually any gene), and efficiency (in many cases, genes can be silenced by over 90%).

RNAi is triggered by small interfering RNA (siRNA), short RNA duplexes typically consisting of two 19- to 21-nucleotide single stranded RNAs with 2- to 4-nucleotide overhangs at the 3’ ends. Within the cytoplasm of a given cell, these duplexes become separated by a multi-protein complex called RISC (RNA-induced silencing complex). One of the RNA strands (the antisense strand) remains bound to the RISC complex. This single strand RNA-RISC complex locates mRNA sequences within the cytoplasm with homologous nucleotide sequences and induces cleavage of mRNA, thereby preventing its translation into protein.

Development of RNAi-based therapies has been slow, and current approaches are limited to few indications. However, novel therapeutic methods for systemic use of RNAi are being actively developed. More specifically, phase I clinical trials were initiated by three companies, Alnylam for Respiratory Syncytial Virus (RSV) infection, Sirna and Acuity for treatment of age-related macular degeneration (AMD), a vascular-proliferative disorder of the retina through direct injection of the siRNA into the eyes. The blocking of angiogenesis in the retina via interruption of VEGF signaling is the therapeutic goal. These trials are exciting as they are the first human trials testing the application of this novel approach. Unfortunately, these trials are also limited in scope, as they only address the issue of target organ delivery via direct injection into a confined organ.

For systemic use, the biggest challenge in the use of siRNA-based therapies is the difficulty of delivery. In order to become effective and induce silencing, it is imperative that the siRNAs reach the cytoplasm of the target cell. Naked RNA cannot penetrate cellular lipid membranes and therefore, systemic application of unmodified siRNA is unlikely to be successful. Clearly, the question of delivery remains the largest hurdle and has to be solved to avoid setbacks such as those experienced with gene therapy and viral vectors.

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The purpose of this paper is to review published literature regarding the use of RNAi in animal models and to provide an overview of methods currently available for the application of siRNA in vivo, with a special focus on systemic in vivo delivery.

We review the available literature along the lines of viral delivery versus non-viral delivery, and briefly touch upon the most successful applications in animal models by organ site.

**VIRAL DELIVERY**

Employed in the field of gene therapy for over a decade, viral vectors have recently been applied to RNA interference. Due to their intrinsic mechanism for gene transduction, viruses readily function as vectors in a cost-effective and delivery-efficient way. The five types of viral vectors that are currently in use for RNAi include the Retrovirus, Lentivirus, Adenovirus, Adeno-Associated-Virus (AAV), and Baculovirus.3

**RETROVIRUS**

Retroviruses were among the first vectors used to transfect cells with hairpin-RNA expressing plasmids. A study by Brummelkamp et al used retroviruses and highlighted the extreme specificity of the RNAi concept and greatly fanned the interest in using RNAi for therapeutic applications, including cancer therapy.4 But while retroviruses work well on cell lines in vitro, for use in vivo, and especially for possible use in human applications, retroviruses have serious drawbacks, namely safety concerns and limitations in efficiency. Retroviruses work by integrating viral genome into the host genome, using the latter for viral replicative efficiency. Unfortunately, genomic integration carries the grave risk of insertional mutagenesis and potential carcinogenesis. Unfortunate development of leukemia in two pediatric patients undergoing experimental gene therapy treatment of x-linked severe immune deficiency syndrome (x-SCID), is the main reason gene therapy trials using retroviral vectors have been halted and why other vectors are currently favored for in vivo RNAi experiments.5,7 Another limitation for in vivo use of retroviruses as vectors for RNAi therapy is that they are limited to actively dividing cells. Because most mammalian cells are in resting state and not actively going through the cell cycle, retroviruses simply may not be able to reach the cells of interest.

**LENTIVIRUS**

Lentiviruses, a subclass of retroviruses, have emerged as appealing vectors for in vivo application and are widely used for proof of concept experiments. While safety concerns still exist, lentiviruses are free of some of the major disadvantages hindering use of retroviruses. Namely, there is no risk of insertional mutagenesis and lentiviruses are able to efficiently transduce primary and non-dividing cells, thereby abolishing two major shortcomings of retroviruses for in vivo use. Additionally, lentiviruses can accommodate large amounts of data in their genomes, and are less immunogenic than adenoviral vectors. Lentiviral vectors are mostly used for local application, and their ability to transduce nondividing cells such as neurons makes them preferred vectors for nervous system applications. In literature, lentiviruses have been employed successfully to regulate target genes in the brain after local injection.8-10 Singer et al used lentiviral vectors and RNAs to silence BACE1, a gene involved in the cleavage of amyloid precursor protein in a mouse model of Alzheimer’s disease, and showed a reduced formation of Alzheimer-typical plaques in the injected region. The effect of lentiviral delivery remained localized as no change in BACE1 expression or formation of amyloid plaques was found in untreated regions of the brains of treated animals, such as the neocortex.11

Additionally, following intramuscular injection, the lentivirus Equine Infectious Anemia Virus (EIAV) was able to infect the relevant motoneurons in the spinal cord and brain stem via retrograde transport. In a mouse model of amyotrophic lateral sclerosis (ALS), transgenic expression of mutant human Superoxide Dismutase-1 (SOD-1) results in development of a degenerative motoneuron disease with similar clinical features as ALS. Using intramuscular injection of lentiviral vectors, and hairpin induced silencing of SOD-1 expression, the onset of disease symptoms in the treated animals was significantly delayed while survival was increased.

One promising avenue of research, which may increase the usefulness of lentiviral vectors for RNAi delivery, concerns the development of lentiviruses with certain tissue tropism. Inclusion of specific envelope signals in such lentiviruses, or “pseudotyping”, employs integration of envelope material from other viruses to increase tropism towards certain cell types. Examples include Mokola or Ebola virus glycoprotein or vesicular stomatitis virus glycoprotein (VSV-G) which confer certain tissue tropism toward heart and skeletal muscle cells (MVG and EVG) or hepatocytes (VSV-G), respectively, when injected into mice embryos.12

**ADENOVIRUS**

Adenoviral vectors are popular for gene therapy trials, and approximately 25% of clinical gene therapy trials currently underway are conducted using adenovirus (reviewed in ref. 13). These are vectors of choice for tumor-targeting gene therapy, where a relatively short duration of action is sufficient, unlike the need for long-term gene replacement therapy in treatment of genetic disorders (e.g., cystic fibrosis). In adenoviruses the genetic information propagates outside of the target cell’s nucleus and there is little risk of viral DNA integration into the host cell genome. While this means that the genetic information is less stably conserved and may be lost through repetitive cell divisions, adenoviral use has evolved to be the preferred route for many applications in which a transient effect is sufficient, such as cancer gene therapy with suicide genes. One major disadvantage of adenoviral vectors, however, is the lack of clear tissue tropism and dependence on defined surface receptors of the target cell, which are often absent in the tissues of interest (especially tumor cells). Furthermore, adenovirus toxicity to the liver is significant and can be dose-limiting, thereby reducing possible therapeutic effects in the target tissue.

Several papers have reported use of adenoviral vectors for transduction of RNAi-based therapy in vivo. The first study employing an adenoviral vector for in vivo RNAi was published in 2002 for application in the central nervous system. Using a hairpin-encoding adenoviral system targeting green fluorescent protein (GFP) in GFP-transgenic mice, Xia et al. were able to demonstrate efficient knockdown of GFP in the brain after local stereotactic injection. This early trial, although it used a transgenic marker gene and did not include a therapeutic result, carries promise for therapeutic application of siRNA-based treatments for a whole class of degenerative brain disorders as well as detailed RNAi-based studies of brain function.8

A study by Chen et al. used intra-ventricular injection of an adenoviral shRNA vector to examine the effect of angiotensin receptor 1a (AT1a) silencing and was able to demonstrate changes on water intake associated with the silencing of AT1a.15
In an effort to use RNAi based therapy against cancer, a number of groups used local injection of adenoviral vectors expressing hairpin RNA into subcutaneous or orthotopic tumors with the aim to suppress tumor growth. A variety of oncopgenes in different tumor subtypes have been targeted, and these studies often resulted in significant tumor growth reduction. Although each of these reports is a first in their field, and each is valuable for understanding of the respective therapeutic targets, it is unlikely that local injection of tumor tissue will become a widely used therapeutic tool and a way to administer RNAi-based therapy, as not all tumors are amenable to local injection. Using a different kind of topical application, and one closer to a possible clinical application, Gou et al. used an adenoviral formulation with intratracheal application to reach lung epithelial cells in rats and silence annexin A2 expression. Using the cell-type specific promotor SP-C (surfactant protein C, exclusively expressed by type II alveolar epithelial cells), the authors were able to limit silencing to this specific group of target cells. Slightly disappointing, however, was that gene silencing was only achieved in an estimated 15% of the target cells, which may not be enough to create appreciable clinical benefits. Nevertheless, this result of cell-type specific silencing opens an important avenue for future applications, as it will contribute another facet to the target specificity inherent in the RNAi technology and will thereby open new indications for RNAi based therapy, in which tissue or cell-specific silencing is required.

Table 1: Vectors used for delivery of siRNA and hairpin-encoding DNA for in vivo experiments

<table>
<thead>
<tr>
<th>Vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retrovirus</td>
<td>High efficiency, stable transfer of gene, gene passed on to daughter cells</td>
<td>Integration into genome, effects limited to dividing cells, no primary cell lines (in culture)</td>
<td>Through pseudotyping, tissue tropism may have implications for targeting</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>Non-dividing cells reached, can carry large amount of data</td>
<td>So far, only topical (local) use reported, no systemic application possible</td>
<td>Adenoviral targeting, cell type specific silencing for lung application</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>No risk of insertional mutagenesis, transient duration of treatment, useful for liver applications</td>
<td>No tissue tropism (except liver), dose limiting hepatotoxicity, transient effect</td>
<td>Required carrier/vector, limited experience, high cost</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>No known pathogenicity</td>
<td>Spectrum includes non-dividing cells</td>
<td>Limited experience</td>
</tr>
<tr>
<td>Baculovirus</td>
<td>Probably safe</td>
<td>Requires large amount of siRNA (expensive)</td>
<td>Spectrum includes non-dividing cells, limited experience</td>
</tr>
<tr>
<td>Naked siRNA</td>
<td>Least experimental steps required, no rational mechanism of siRNA uptake into cells</td>
<td>Only useful in mice</td>
<td>Other organs except liver not accessible, no potential clinical use</td>
</tr>
<tr>
<td>High pressure injection (hydrodynamic injection)</td>
<td>Reliable, cheap method for delivery into mouse liver</td>
<td>Other organs except liver not accessible, no potential clinical use</td>
<td>No potential clinical use, limited experience</td>
</tr>
<tr>
<td>Chemically modified siRNA</td>
<td>Increased serum stability</td>
<td>Still requires carrier/vector, higher cost</td>
<td>Limited experience</td>
</tr>
<tr>
<td>Liposomes</td>
<td>Readily available for most laboratory scientists, ip injection shown to be efficient, distribution in numerous organs after ip injection</td>
<td>No target cell/tissue specificity, distribution in most organs, electric charge of liposome might influence delivery efficiency and distribution pattern</td>
<td>New system, limited experience and safety data</td>
</tr>
<tr>
<td>PEI nanoparticles</td>
<td>Good vehicle for lung delivery, useful for both siRNA/DNA delivery, systemic availability</td>
<td>Difficulty of preparation, not yet generally available, expensive</td>
<td>New system, limited experience and safety data</td>
</tr>
<tr>
<td>Selective particles</td>
<td>Tissue selective delivery, potential as clinical solution for RNAi therapy</td>
<td>Expensive complicated</td>
<td>New system, limited experience and safety data</td>
</tr>
<tr>
<td>Bacteria</td>
<td>Cheap, versatile, controllable through antibiotics/auxotrophy</td>
<td>New system, limited experience and safety data</td>
<td>New system, limited experience and safety data</td>
</tr>
</tbody>
</table>

In an effort to use RNAi based therapy against cancer, a number of groups used local injection of adenoviral vectors expressing hairpin RNA into subcutaneous or orthotopic tumors with the aim to suppress tumor growth. A variety of oncopgenes in different tumor subtypes have been targeted, and these studies often resulted in significant tumor growth reduction. Although each of these reports is a first in their field, and each is valuable for understanding of the respective therapeutic targets, it is unlikely that local injection of tumor tissue will become a widely used therapeutic tool and a way to administer RNAi-based therapy, as not all tumors are amenable to local injection. Using a different kind of topical application, and one closer to a possible clinical application, Gou et al. used an adenoviral formulation with intratracheal application to reach lung epithelial cells in rats and silence annexin A2 expression. Using the cell-type specific promotor SP-C (surfactant protein C, exclusively expressed by type II alveolar epithelial cells), the authors were able to limit silencing to this specific group of target cells. Slightly disappointing, however, was that gene silencing was only achieved in an estimated 15% of the target cells, which may not be enough to create appreciable clinical benefits. Nevertheless, this result of cell-type specific silencing opens an important avenue for future applications, as it will contribute another facet to the target specificity inherent in the RNAi technology and will thereby open new indications for RNAi based therapy, in which tissue or cell-specific silencing is required.

Thus far, there are only a limited number of reports discussing systemic (other than local injection) use of adenoviral vectors for RNAi. One such report includes study by Xia et al., in which an adenoviral vector was injected intravenously with an RNAi construct targeting mouse β-glucuronidase. A modest (12%) decrease of β-glucuronidase RNA was subsequently found in the liver, showing that it is possible to reach the liver with this treatment modality, but also clearly indicating the need to optimize this strategy.

Additionally, Koo et al. used intravenous injection of an adenoviral vector targeting nuclear hormone receptor coactivator PGC-1 (peroxisome proliferator-activated-γ-coactivator-1) to study its role in diabetes. The authors were able to show important metabolic changes in glucose homeostasis induced by silencing of this nuclear receptor coactivator, such as reduced fasting glucose levels, increased triglyceride levels, and changes in expression of enzymes involved in gluconeogenesis.

Finally, in an experiment directly targeting the liver, Ragozin et al. used adenovirus-mediated RNAi against ABCA1, a lipid regulating protein in hepatocytes, to clarify its functional role in mice. Following systemic application, silencing of hepatic ABCA1 lead to changes in lipid metabolism, including reduced systemic levels of high density lipoprotein (HDL) cholesterol, lowered systemic apolipoprotein E levels, and increased postprandial triglyceride levels.

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The dose limiting hepatotoxicity of adenoviral vectors and its lack of target cell specificity has spurred the development of novel strategies to improve tissue and cell tropism of these vectors (reviewed in ref. 23), and although these new generation vectors are just beginning to arrive for in vivo usage in gene therapy, and there are currently no reports of their use for RNAi-based therapy in vivo, there is great promise for targeted adenoviral delivery of RNAi as well.

OTHER VIRAL SYSTEMS

Adeno-associated virus (AAV) has been proposed as a promising vector for siRNA delivery based on several advantages over existing viral vectors. Firstly, AAV is not known to be pathogenic and has a broad range of possible target cells, including nondividing cells. Several papers have described successful use of AAV for in vitro transport of RNAi.24,25 but only two papers have reported use of AAV for in vivo transfection of siRNA, both of them using the method of local stereotactic injection into the brain.26,14 The place of AAV for RNAi based treatment in vivo remains to be seen.

The insect Baculovirus is in its very early testing stages as a possible vector for in vivo use and has several advantages. One advantage it its capacity to transport large amounts of genetic data, thereby allowing for the combined use as gene therapy vector and a silencing vector. The second advantage addresses possible concerns of viral safety. Namely, because the baculovirus is unable to replicate and express viral proteins in mammalian cells, it can be more controllable and safer than other viral vectors which take advantage of compatible cell machinery for replication. In a study reporting successful use of baculovirus in an animal model, Theng Ong et al.3 were able to reduce luciferase gene expression in rat brain after co-injection of a shRNA producing baculovirus with a luciferase expression vector.

The use of viral vectors for RNA interference has proven to be fairly successful. Because viruses are naturally adept at infecting target cells and transferring genetic material, they were a logical choice for the introduction of hairpin-RNA encoding DNA material. Nevertheless, viral vectors are still cause for certain concerns. The main drawback of these vectors is the potential to cause disease, and the lack of effective agents such as antibiotics to block a wayward viral infection.

NON-VIRAL DELIVERY

Delivery of RNAi without viral vectors has been tried with a wide variety of agents, such as: injection of pure, unmodified siRNA, chemically stabilized or modified RNA, encapsulating the siRNA in microparticles or liposomes, and binding siRNA to cationic or other particulate carriers.

UNMODIFIED “NAKED” siRNA OR DNA PLASMID AND HYDRODYNAMIC METHODS

Theoretically, direct injection of siRNA should be inefficient due to presence of RNA degrading enzymes (RNAses) in serum. Still, a few papers report successful use of unmodified siRNA through direct injection in animals. Ocker et al.27 studied the distribution of naked siRNA in the mouse following intravenous injection over time and showed some therapeutic benefit on pancreatic cancer xenografts after silencing blc-2. However, such reports are isolated. Other groups reported successful treatment in vivo following systemic application of hairpin-RNA encoding plasmid DNA.28 Such studies face numerous difficulties, including distribution to unwanted sites27 and degradation by nucleases in serum. Activity of RNAses in serum resulted in the decision to use stabilized or packaged RNA molecules.

The problem of distribution of siRNA treatment can be addressed successfully in mice when the liver is the only target organ. In this case, RNAi can be delivered using the so-called hydrodynamic method.29 With this approach, successful silencing of marker genes in hepatocytes was shown following injection of naked siRNA,30 as well as following injection of hairpin-RNA encoding DNA plasmids.31,32 With this approach, thus far described only in mice, relatively high volumes ranging from 500–2000 μL were injected into the tail vein at high pressures and over a short time (within seconds). This method has been used in the past for DNA delivery, and leads to relatively efficient uptake of nucleotides into the liver and expression in up to 40% of hepatocytes within eight hours. The mechanism of uptake is not entirely clear, but is likely based on endothelial leakage through liver sinusoids combined with transient formation of membrane pores in hepatocytes.33 Hydrodynamic delivery may be an option for delivery into lung tissues as well, as demonstrated by one study in which hydrodynamic injection was used to deliver naked siRNA into lung. In this study, protective effects were shown for siRNA directed against influenza proteins PA and NP in mice challenged with lethal doses of human influenza virus.34

Hydrodynamic delivery is an elegant method for mouse applications with appreciable delivery efficiency into hepatocytes and relatively few side effects. However, application of a comparable volume in a human adult would amount to sudden injection of several liters of fluid, resulting in volume overload and most certainly leading to lethal right heart failure.

CHEMICALLY MODIFIED siRNA MOLECULES

Duration of siRNA activity in vivo and any possible therapeutic effect will depend on its stability in the serum. Double stranded RNA is more stable than single strand RNA in serum, but will still be degraded within a few hours due to RNAase activity. The search is on for chemical modifications which may increase stability of siRNA molecules in serum without affecting RNA-Interference effect. Ideally, chemical modifications could also contribute to increase thermal stability, cellular tropism, silencing activity and pharmacokinetic properties of the siRNA.

One of attempted chemical modifications is the use of locked nucleic acid (LNA) residues. LNAs increase stability of oligonucleotides and have been used successfully to enhance the effect of antisense RNA and ribozyme RNA =. A methylene bridge connects the 2’O with the 4’C without disturbing the steric conformation of the RNA. Recently, this technique has been applied to RNAi by integrating LNA nucleotides at both ends or other positions of the siRNA strand. This was shown to not have any detrimental effect on the activity of the siRNA in vitro35 and greatly increased stability of the siRNA in serum.36 Furthermore, the integration of LNA might have beneficial effects on the integration of otherwise only moderately active siRNA constructs into the RISC complex, thereby increasing its activity.36 Overall, LNA modification of siRNA seems to be promising, however, thus far successful in vivo studies have yet to be performed.

Another way to chemically stabilize RNA molecules is via use of phosphothioates (PS). Phospho-sulfur bindings replace phosphodiester linkages in the sugar-backbone of the RNA. PS linkages increase the half-life of oligonucleotides in vivo and were used with
antisense nucleotide approaches in several clinical trials to increase drug stability.\textsuperscript{37}

Other reportedly useful chemical alterations include modifications of residues added at the 2' position of the ribose, 2'-O-Me, 2'-O-allyl, and 2'-deoxyfluorouridine modifications. These modifications were based on early reports showing that the 2'OH group is not required for silencing in siRNA.\textsuperscript{38} Systematic in vitro testing is underway in many laboratories to develop the most useful modifications for siRNA molecules. Out of the possible modifications under review, only the 2'deoxyfluoridine (2'-F) modification has been used in a head-to-head comparison with its natural counterpart in vivo. In this report the 2'F modified siRNAs were found to have much better stability when exposed to serum in vitro, and retained their activity in vivo. On the other hand, no beneficial effect was observed in vivo either on the magnitude or the duration of target gene silencing when compared to the natural siRNA.\textsuperscript{39} A report by SiRNA Therapeutics showed successful use in vivo of a synthetic siRNA derivative with multiple chemical modifications (2'-deoxy-, 2'-F, 2'-O-Me). In this study, the heavily modified siRNA was effective against a mouse model of HBV while the unmodified siRNA molecule showed no effect.\textsuperscript{40}

While the primary aim of these modifications is to stabilize the molecules, more research is required, as some of these modifications seem to inhibit siRNA silencing activity, especially when introduced into the 5' end of the antisense strand. It has been speculated that the bulkier modifications (such as 2'-O-Me) may interfere with the unwinding of RNA or integration of the antisense strand into the RISC complex.\textsuperscript{41}

\section*{LIPOSOMES AND NANOPARTICLES}

Unmodified siRNA has a half-life of less than an hour in human plasma,\textsuperscript{39} and circulating siRNAs are rapidly excreted by kidneys thanks to their small size.

To address the concerns of low stability in serum and rapid renal excretion, several different approaches have been developed to deliver envelope packeted siRNA. The most popular envelopes included liposomes and other nanoparticles, both as simple packages, but also in combination with specific homing signals designed to direct the preferential uptake by a specific target tissue or a group of target cells.

Some groups have used liposomes for in vivo delivery with good results. Flynn et al. targeted the expression of IL12-p40 in a model of LPS-induced inflammation using intraperitoneal injection of IL12-p40siRNA in Lipofectamine. They showed significant reduction of immune reaction in treated animals, presumably via reduced IL12 of LPS-induced inflammation using intraperitoneal injection of DOPC-siRNA, but simultaneously raises concerns regarding unwanted side effects of siRNA treatment in organs or tissues other than the intended target.

Furthermore, Landen et al. went on to compare the efficiency of intraperitoneal DOPC-siRNA approach with two other frequently used delivery methods. The group examined uptake of siRNA into the same tumors using tail vein injection of naked siRNA and tail vein injection of siRNA complexed with cationic DOTAP liposomes. Following intravenous injection, both naked siRNA as well as DOTAP delivered siRNA were recovered in tumors, but at much lower rates (10-fold lower for DOTAP-siRNA and 30-fold lower for naked siRNA injection). Furthermore, siRNA were found clustered around tumor neovasculature following intravenous injection. Because of this perivascular distribution pattern the authors doubted that siRNA administered by these delivery methods could reach tumor tissue as efficiently as with the intraperitoneal DOPC approach.\textsuperscript{45} Additionally, the electrical charge of the liposomes may play an important role in determining uptake by tissues or macrophages. Clearly, understanding of liposome-delivery of siRNA is still evolving, and more research is needed. This is the first study to compare different delivery options for a given indication, and should be followed up by more studies. The investigation of liposome-delivery of siRNA in vivo is just in its infancy, but with increased understanding, it may be developed into a promising tool for therapeutic application of siRNA.

siRNA delivery in vivo has also been reported using nanoparticles, especially the cationic polymer, polyethyleneimine (PEI). Ge et al. examined the use of siRNA delivery into lung tissue to treat and prevent potentially deadly influenza infections in the mouse.\textsuperscript{46} It was found that PEI-delivered nucleotides (luciferase expressing DNA) are distributed preferentially to the lungs following intravenous injection. When siRNA or hairpin-encoding DNA targeting influenza viral proteins NP (nucleocapsid protein) or PA (component of influenza virus RNA transcriptase) were given with PEI-intravenous delivery, they conferred significant protection against influenza virus infection and resulted in significantly lower viral titers. When used after the infection occurred, intravenous PEI-siRNA effective as a treatment.\textsuperscript{46}

Urban-Klein et al demonstrated that PEI-siRNA can be applied intraperitoneally to achieve a systemic therapeutic effect.\textsuperscript{37} In their study, the authors used HER-2/neu targeting siRNA to treat SKOV-3 ovarian cancer xenografts and achieved significant suppression of Her-2 expression as well as growth reduction of tumors.\textsuperscript{37} This finding of systemic availability of siRNA following intraperitoneal injection, if confirmed, could facilitate application of siRNA-based treatment in mice, as intra peritoneal injection is technically less challenging compared to tail vein intraavenous injection in mice. These two studies demonstrate easy systemic availability of PEI delivered siRNA and pDNA.
SELECTIVE TISSUE DELIVERY

SiRNA Therapeutics combines the approach of chemically stabilized siRNA with liposome delivery in a concept called SNALP (stable nucleic acid-lipid particle). SNALP confers superior stability and half-life or siRNA over un-stabilised siRNA within vivo applications. With this approach, the liposome is made of a mixture of cationic and neutral lipids, and covered with a hydrophilic PEG-lipid which facilitates formulation and administration in the aqueous phase.

In an attempt to confer more specific tissue tropism to liposome-carried RNAi, some groups established methods to increase affinity for defined cell types using either ligands for specific receptors or antibodies against cell surface markers. In one example, PEG (polyethylene glycol) strands are conjugated to outer surface of a liposome, and specific antibodies are attached to these PEG strands, creating a “pegylated immunoliposome” (PIL). Zhang et al. were able to successfully deliver hairpin encoding DNA plasmid into an implanted brain tumor using a construct with a monoclonal antibody against a transferrin receptor which allowed passage of the liposomes through the blood-brain. Selective tissue directed delivery into tumor was demonstrated by Intradigm corporation for a siRNA targeting vascular endothelial growth factor receptor-2 (VEGF-R2) encapsulated in ligand-targeted nanoparticles. These nanoparticles were made of polyethyleneimine (PEI), a cationic polymer conjugated with PEG-peptide constructs. These strands bear a homing peptide motif composed of three amino acids Arginine-Glycine-Asparagine (RGD), which has been shown to lead to preferential binding to integrins highly expressed in tumor cells. Using this approach, preferential delivery into the tumor tissue was achieved. Tumors showed reduced vessel density and inhibition of tumor growth was achieved.

Following a different tactic to achieve tissue selectivity, targeted delivery in vivo was recently reported by Song et al. Using an antibody Fab-fragment directed at HIV surface protein, pg160, the authors were able to selectively target pg160-positive cells following systemic as well as topical injection of a xenograft tumor mouse model. In this method, a protamine residue bound to the antibody fragment allows for temporal binding of siRNA molecules. siRNA in this study was targeted against growth promoting genes c-myc, MDM-2 and VEGF, and treatment resulted in significant tumor growth retardation in treated animals, as well as accumulation of FITC-labelled siRNA in the tumors. Which proved that this novel delivery method works in vivo. This method not only proved that this mode of delivery works in vivo, but was able to target tumor tissue by concentrating systemically administered siRNA in a target organ marked by specific expression of surface antigen. This development carries great promise, especially for targeted RNAi treatment of malignant tumors, following the footsteps of currently used clinical cancer treatments based on antibody-targeting towards Her/2 or EGFR expressing tissues.

BACTERIAL DELIVERY: TRANSKINGDOM RNA INTERFERENCE

We have recently made progress using nonpathogenic bacteria to induce gene silencing in target cells, both in vitro and in vivo. The concept of using invasive bacteria to transfect genetic material is not new and has been explored mainly with the focus of DNA vaccination. Bacteria have great advantage over other delivery vectors and are being discussed as vectors for gene therapy, although clinical trials with bacterial vectors for gene delivery in humans have yet to be developed. Advantages include safety, the ability to control the vector using antibiotics, as resistance profile of bacterial vectors is well known. Additionally, bacteria have potential for easy genetic engineering, allowing tailor-made specific vector strains for specific applications.

Our group has developed a technology called transkingdom RNA Interference (tkRNAi), effective for administration of RNAi both in vitro and in vivo. The silencing hairpin RNA is produced by genetically modified bacteria which invade and release silencing RNA into eukaryotic (mammalian) cells, hence crossing transkingdom borders. Zhao et al. have recently published a report on using bacterial delivery of hairpin RNA to achieve silencing in cell culture. In a modified transkingdom RNA Interference approach, bacteria can be used to transfect hairpin-encoding DNA plasmids. Following cellular entrance and bacterial lysis (we call this technique bacteria-mediated RNAi, bmRNAi), the genes encoded on the plasmids can be expressed intracellularly. Both concepts work well with low toxicity and a very efficient silencing profile. Thus far, we have demonstrated silencing of endogenous genes, oncogenes and transgenes in the gastrointestinal tract, liver and tumor tissue in mice. A wide variety of tissues can be reached when bacterial strains and route of application are selected accordingly. We have seen promising therapeutic effects on xenograft tumors as well as a significant activity for cancer prevention when targeting oncogenes. Bacteria mediated RNAi and transkingdom RNAi are promising avenues for future research for functional genomcis and the RNAi-mediated information transfer between kingdoms.

OUTLOOK

Overall, the excitement observed in the field of RNAi related research seems justified taking into consideration the great potential of this method with its high specificity, limited side effects and potential for targeting a multitude of therapeutic targets. Looking at the past four years of research into RNAi in mammalian cells, rapid development and progress made in understanding the mechanisms and solving some of the newly encountered obstacles support the optimism that this method may soon be developed into a useful tool for therapeutic and preventive applications. Solving the problem of in vivo delivery will be another big step and may soon lead to a number of clinical trials for RNAi based therapies.

References


