RNA Interference to Treat Human Diseases, Applications of

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Advanced Article

Article Contents

Discovery of the RNA interference (RNAi) pathway has been revolutionary to biology and biologically based therapeutics. The messenger RNA (mRNA) targets once believed to be ''undruggable'' by conventional methods are now being enthusiastically pursued with RNAi-based therapeutics. Fundamental research into the RNAi pathway and biotechnological research on the application of RNAi may one day make a new class of drugs a reality by targeting the mRNA of disease-causing or disease-promoting genes directly. The whole process begins with the design and validation of short-interference RNA (siRNA) using bioinformatics and cell culture systems. siRNA that has high specificity and potency (knockdown efficiency) then could be applied to animal models of human diseases for more evaluation, with the ultimate hope of advancing to clinical trails. Numerous chemical modifications of siRNA are currently being investigated in an effort to enhance their stability, to extend their stay in the body, and, perhaps, to avoid immune stimulation. Different delivery methods are developed depending on the diseases, tissues, or organs being targeted. In short, this relatively new biotechnology is advancing quickly as we learn from its failures and successes. In the current article, we will explore the background and current state of research as well as discuss some hurdles and future directions in the application of RNAi to treat human diseases.

In 1998, Andrew Fire and Craig Mello (1) published their seminal work on RNA interference (RNAi) with their discovery of double-stranded RNA (dsRNA) as the trigger of posttranscriptional silencing in *Caenorhabditis elegans*. The phenomenon of posttranscriptional gene silencing was first observed in plants (2–5). In the two decades since, many components in the RNAi pathway have been identified and characterized (see also RNA Interference, Mechanisms and Proteins Involved in). Another phenomenon of posttranscriptional silencing comes from microRNA (miRNA). In 1993, Lee et al. (6) cloned a short noncoding RNA (later referred to collectively as microRNA), *lin-4*. In 2000, Reinhart et al. (7) cloned another miRNA called *let-7* that functions similarly to *lin-4*. Both *lin-4* and *let-7* regulate translation via partially complementary sequences in the 3′ untranslated region (UTR) of mRNA they target. Advances in research eventually merged the RNAi pathway with the miRNA pathway by showing that they closely share core components (see also RNA Interference, Mechanisms and Proteins Involved in).

In principle, dsRNA and short-hairpin RNA (shRNA) are cut by Dicer and its associated proteins, such as TRBP, into ∼21-nucleotide (nt) short interference RNA (siRNA) or mature miRNA. One strand (the guide strand) will be integrated into the RNA-induced silencing complex (RISC), which has AGO2 as its core component, whereas the other strand (the passenger strand) will be either degraded (siRNA) or released (miRNA). In the current model, siRNA, by perfect complementarity with the targeted mRNA, triggers the degradation of mRNA in the RISC, whereas miRNA generally suppresses the translation of the target mRNA by incomplete complementarity with the 3′ UTR region (see also RNA Interference, Mechanisms and Proteins Involved in). The discovery that dsRNA, particularly siRNA, can be introduced exogenously into mammalian cells to knock down target mRNAs in a sequence-specific manner generated much enthusiasm for exploring siRNA duplexes as gene-specific therapeutics (8). The subsequent development of siRNAs as drugs has been rapid. Currently, three Phase I studies

Table 1 Examples of in vivo application of RNAi-based therapeutics

Gene targeted	Disease model	Delivery strategy and route	References
VEGF	Laser-induced CNV	Lipoplex/Subretinal	9
	Laser-induced CNV	Saline/Intravitreal	10
	Subcutaneuos tumor xenograft	Atelocollagen/Intravenous	11
	Subcutaneuos tumor xenograft	Cholesteryl-R9/Intratumoral	12
VEGF R1	Laser-induced CNV	Saline/Intravitreal	13
VEGF R2	Subcutaneuos tumor xenograft	RDG pepetide-PEI/Intravenous	14
c-myc, MDM2, VEGF	Subcutaneuos tumor xenograft	HIV envelope protamine fusion/Intravenous	15
TGFβ R2	Subconjunctival scarring	Lipoplex/Subconjunctival	16
EWS-FLI1	Ewing's sarcoma model	Transferrin-targeted nanoparticle/Intravenous	17
PLK1, BCL2	Subcutaneuos tumor xenograft	PSMA aptamer/Intratumoral	18
HPV-E6	Subcutaneuos tumor xenograft	Lipoplex/ Intratumoral or peritoneal	19
Pleotrophine	Subcutaneuos and glioblastoma tumor xenograft	PEI/Intraperitoneal or subcutaneuos	20
HER2	Subcutaneuos tumor xenograft	PEI/Intraperitoneal	21
RhoA	Subcutaneuos tumor xenograft	Chitosan nanoparticle/Intravenous	22
RSV-P, PIV-P	RSV, PIV infection	Saline or lipoplex/Intranasal	23
SARS	SARS infection	D5 W or surfactant/Intranasal	24
Influenza A-NP, PA	Influenza virus infection	PEI/Intravenous	25
JEV/WNV-envelope	JEV/WNV-induced encephalitis	Lipoplex/Intracranial	26
HSV2-UL27, UL-29	Herpes simplex 2 virus infection	Lipoplex/Intravaginal	27
ApoB	Dyslipidaemias	Cholesterol conjugated/Intravenous	28
	Dyslipidaemias	Liposome/Intravenous	29
TNF	Collegen-induced arthritis	Lipoplex/Intravenous	30
	Dextran sodium sulfate-induced colitis	Lipoplex/Intrarectal	31
HMOX1	Hyperoxic lung injury	Saline/Intranasal	32
KC, MIP2, Fas	Septic acute lung injury	Saline/Intranasal	33–34
ANGPT2	Hyperoxic lung injury	Saline/Intranasal	35
DDR1	Bleomycin-induced fibrosis	Saline/Intranasal	36
Caveolin-1	Vascular permeability	Liposome/Intravenous	37
AGRP	Metabolic alterations	Saline/Stereotactic injection	38
Dopamine Transporter	Hyperlocomotor response	Saline/Intraventricular	39
Serotonin Transporter	Behavior response	Saline/Intraventricular	40
P2X3	Chronic neuropathic pain	Saline/Intrathecal	41
DOR	DELT-induced nociception	Lipoplex/Intrathecal	42
NMDAR (subunit 2B)	Formalin-induced nociception	PEI/Intrathecal	43
PrP(C)	Scapie infection	Lentivector/Intracranial	44
mir-21	Murine glioma models	LNA-anti-miR-21/Intracranial	45

without unacceptable toxicity are already complete (**Table 1**) (9–45).

RNAi-based therapeutics begin with the design and identification of siRNAs that show high specificity and knockdown potency in the lab. Once several siRNAs have been selected, they are applied to animal models with the diseases of interest. Before delivery, siRNA may also be modified to enhance its stability. siRNA duplexes can be delivered via different routes and even with different strategies. We will now summarize and discuss the entire process, from the initial design of a siRNA duplex to its application in clinical trials.

Design and Evaluation of siRNA

Currently, two methods are available for the endogenous RNAi pathway for therapeutic purposes: either by introducing a viral vector to express shRNA that mimics a miRNA precursor, which then would be processed by Dicer into siRNA, or by delivering siRNA that mimics a Dicer cleavage product into the cytoplasm. Because shRNA has to be processed into siRNA *in vivo* to be functional, the design of a shRNA vector follows the same rules as for siRNA. For either method, the first consideration is to ensure that the siRNA specifically targets the mRNA of interest (specificity), at the same time, the siRNA

should have a minimum desirable (if not the highest possible) knockdown efficiency (potency).

Specificity

The initial siRNA design starts with a bioinformatics-aided search for "targetable" sequences ~21 nt long in the mRNA of interest (46). Because a perfect complement with the targeted mRNA triggers degradation and an imperfect complement triggers translational suppression, the chances of generating a ~21-nt siRNA with an off-target effect is high, without careful attention to design (47, 48). The principle here is to avoid sequences in the siRNA "seed region" complementary to untoward mRNAs (**Fig. 1**). The "seed region" is at positions 2-7 or 2-8 of the guide strand of the miRNA or siRNA duplex. For miRNA function, the seed region usually complements the 3′ UTR of the target mRNA perfectly. Off-targeting of siRNA is found to be associated with perfect complementarity between the seed region and the 3′ UTR of unwanted mRNA (49). It is highly possible that targeting to the coding region of nonrelated mRNA would also contribute to off-target effects. Several software and Internet search programs can be helpful in the selection of siRNA sequences to minimize off-target effects (50, 51). These effects should also be checked with a microarray assay in cell culture systems because currently no ideal strategy completely eliminates off-target effects.

Potency

Although our current understanding of RNAi activity cannot provide us with a precise prediction of individual siRNA potency, algorithms based on common features of empirically tested high-potency siRNAs are available (46, 52). Once the bioinformatic part is complete, a candidate siRNA can be synthesized and tested in cell culture systems for knockdown efficiency. Knockdown efficiency is commonly assessed with real-time polymerase chain reaction (PCR) to check for changes in the mRNA level and with western blot to check for decreases in the protein level. The final goal of this stage is to identify several siRNAs that show high knockdown efficiency and minimal off-target effects at nanomolar or lower concentrations. To be prepared for possible underachievers at any point in the long process from the bench to the bedside, it is better to have more than one siRNA to begin with.

Theoretically, an alternative method is available for selecting siRNA with high specificity and potency: A library of tiling siRNAs covering the whole mRNA sequence of a disease-causing or relevant gene of interest can be screened with cell culture systems, and individual siRNAs with stringent specificity and high efficacy then can be identified from this library. Drawbacks of this method are that it is more expensive and more labor intensive, although the knowledge gained from this approach can assist in the future design of therapeutic siRNAs.

When considering specificity and potency, it is helpful to bear in mind sequence conservation across species as well. Therapeutic siRNAs usually have to undergo tests that involve nonhuman cell cultures and animal disease models, so it is more reasonable to start with siRNAs that target conserved sequences in disease-related mRNA. One should remember that homologs in different organisms may function differently, and each siRNA could also show different knockdown efficiency in different organisms.

Several practices could enhance the specificity and potency of siRNA once its sequence is decided. For example, because the loading of siRNA into RISC is asymmetric (53, 54), the first nucleotide of the 5′ end of the guide strand is usually made unpaired so that this strand can be loaded into RISC preferentially to increase its specificity and efficiency (**Fig. 1**). siRNA can also be modified chemically to increase specificity and potency, as we will discuss in the next section.

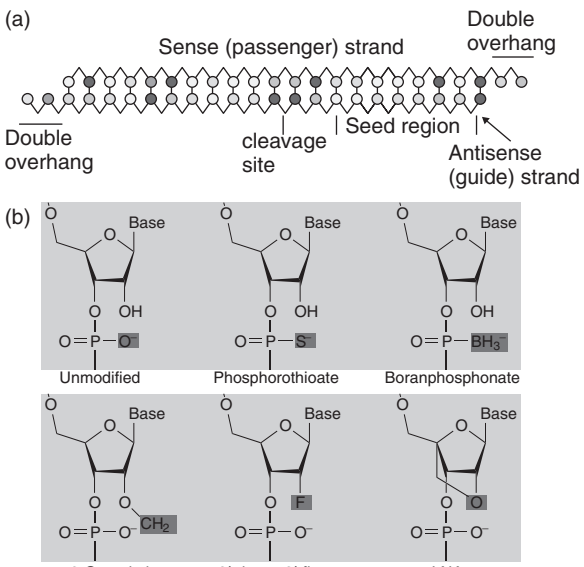

Figure 1 Design and chemical modification of siRNA. a. General features of a siRNA duplex. The first nucleotide at the 5′ end of the guide strand is usually unpaired for asymmetry loading into the RISC. b. Different chemical modifications on the ribose and backbone.

Chemical Modification of siRNA

Some chemical modifications can enhance the specificity of siRNA even more. Chemical modifications of riboses in the guide strand have been found to suppress off-target effects without affecting potency (55, 56). In fact, 2′-*O*-methyl modification at nucleotide 2 of the guide strand is efficient at suppressing off-targeting (57).

Chemical modification to increase siRNA stability

The main purpose of modifying siRNA chemically is to increase its stability so that it remains active in the animal and human body. Unmodified siRNA gets degraded quickly in human plasma, with a half-life of only minutes (58, 59). To convert siRNA into drugs, several ways of modifying siRNA to prolong its half-life have been investigated (**Fig. 1**). Knowledge of

how to avoid nuclease degradation has come to us from previous therapeutics research that use antisense oligonucleotides and aptamers, and this knowledge has been beneficial in the chemical modification of siRNA. Nuclease degradation is prevented mainly via two types of modification.

The first type is phosphodiester modification: Replacing one of the two nonbridging oxygen atoms with a sulfur atom (P = S) or an isophosphonate borane (-BH_3) moiety is found to protect siRNA from exonuclease degradation (58–61; **Fig. 1**). Whereas moderate P = S modifications are well tolerated in terms of knockdown potency and toxicity, extensive P = S modifications should be avoided because of increased binding of the modified siRNA duplex to serum proteins, which may result in cytotoxic cell death in cell culture (62–64). Although -BH_3 modification has not been investigated extensively, current research shows it has some advantages over P = S modification (65); however, one situation to avoid is having a -BH_3 modification at the center of the guide strand, which could reduce the efficacy of the siRNA.

The second type is 2′-sugar modifications: Modifications at the 2′ position of the ribose ring protect siRNA from endonuclease degradation. These modifications mainly include 2′-*O*-methyl (2′-OMe), 2′-deoxy-2′-fluoro (2′-F) modifications, and locked nucleic acid (LNA) (57, 60, 66–68; **Fig. 1**). Appropriate 2′-OMe modifications not only increase plasma stability but also ameliorate off-target effects and enhance the *in vivo* potency of siRNA (55, 57). One important point to remember is that 2′-OMe modifications could impair the cleavage of passenger strand and target mRNA, so it is crucial to keep this modification away from the cleavage site in the passenger strand (69, 70). 2′-F modifications dramatically increase the stability of siRNA in human plasma without adverse effects on specificity and potency; these modifications do not enhance the performance of siRNA, unlike 2′-OMe modifications (58). The most commonly used LNA in the siRNA duplex contains a methylene bridge to connect the 2′-Oxygen with the 4′-Carbon of the ribose ring (**Fig. 1**). Besides increasing stability, LNA modifications may also help to reduce off-target effects and enhance potency. Moderate LNA is generally well tolerated in siRNA, with several notable exceptions. LNA modification should be avoided at the 5′ end of the passenger strand because this may affect the asymmetric loading of the guide strand (71). Both extensive LNA and LNA at certain locations (depending on the specific siRNA) may block the RNAi activity of the modified siRNA (72).

Chemical modification to avoid innate immune response

Nuclear acids can trigger innate immune responses (73). dsRNA longer than 30 base pairs (bp) can efficiently trigger serine/threonine protein kinase (PKR). Although siRNA is smaller, at higher concentrations it may trigger this pathway, which leads to global blockade of translation and ultimately cell death (74, 75). Another issue of perhaps greater concern in RNAi therapeutics is that siRNA could activate Toll-like receptors (TLRs), especially the dsRNA receptor TLR7 in plasmacytoid dendritic cells, which triggers the production of type I interferons and proinflammatory cytokines and thus induces nuclear factor-B activation (76). Some siRNAs have a greater tendency to activate TLRs, and, for this reason, they should properly be called immunostimulatory RNA (isRNA) (72, 77). 3′ blunt ends and GU-rich sequences are strong stimulants to PKR; therefore, they should be avoided when designing the siRNA (78). It is important to investigate whether a siRNA duplex is an isRNA using *in vitro* plasmacytoid dendritic cell culture before *in vivo* application to animal models (72). Chemical modifications at the 2′ sugar can be beneficial because they help avoid immunostimulation (**Table 2**).

Different chemical modifications may be used in combination to attain the accumulated benefits of each individual modification, although research in this area has not been exhaustive.

In Vivo Delivery

siRNA duplexes have to be delivered effectively to treat diseases. Efficient and cell type-specific delivery may be the biggest obstacle to the development of RNAi therapeutics. For use in animal models and clinical trails, different delivery strategies have been developed to meet the requirements for different diseases and target tissues (or organs).

Direct application of naked siRNA

This delivery method applies siRNA dissolved in saline or 5% dextrose (D5 W) directly to the targeted tissues, with many successes reported. Most siRNA-saline solutions target specific organs, such as the eye (intravitreal injection), lung (intranasal or orotracheal instillation), or central nervous system (intracerebroventricular, thecal, or parenchymal infusion) (**Table 1**). Because these organs allow direct administration of siRNA, they were a natural focus of initial RNAi therapeutics. Certain cell types can take up naked siRNA efficiently via unknown mechanisms, whereas many other cell types are refractory to naked siRNA. For this reason, siRNA duplexes have to be conjugated or formulated for efficient delivery in most cases.

Delivery via liposomes and lipoplexes

Liposomes are vesicles enclosed by a phospholipid bilayer, which can fuse with a cell membrane and deliver the enclosed contents into the cytoplasm (**Fig. 2**). Liposomes have already delivered many drugs with decreased toxicity and increased pharmacokinetics. In particular, stable nucleic acid lipid particles (SNALPs) that consist of a mixture of cationic and fusogenic lipid bilayers were used for intravenous and intraperitoneal delivery (29, 79). These SNALPs can be coated with a diffusible poly(ethylene glycol)-lipid (PEGylated lipid) conjugate, which stabilizes the liposomes during formation and prevents rapid systemic clearance from the circulating blood. Liposomes have successfully delivered siRNA that target liver diseases like hepatitis B virus (HBV) infection (79, 80; **Table 1**).

siRNAs are commonly transfected into cultured cells with reagents such as Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) or TransIT-TKO (Mirus Bio Corporation, Madison, WI). Complexes formed by siRNA and most commercial

Table 2 Chemical modifications of therapeutic siRNA

	Chemical modifications	Advantages	Negative aspect
2'-sugar modifications	2'-O-methyl	Suppress off-targeting (especially at the position 2 of strand, the guide strand) Enhance *in vivo* potency Increase plasma stability	Avoid from cleavage site in the passenger; may interfere with cleavage, if not.
	2'-deoxy-2'-fluoro	Increase plasma stability	
	Locked naNuclear Acid	Increase stability; Reduce off-targeting; Enhance potency.	Avoid from the 5'end of the passenger strand; Avoid extensive modification.
	All 2'sugar modifications also help to avoid the immunostimulation		
Phosphodiester modifications	P=S modification	Protect from exonuclease degradation.	Extensive modification results in cytotoxic cell death.
	$-BH_3$ modification	Protect from exonuclease degradation.	Avoid from the center of the guide strand, may reduce efficacy, if not.

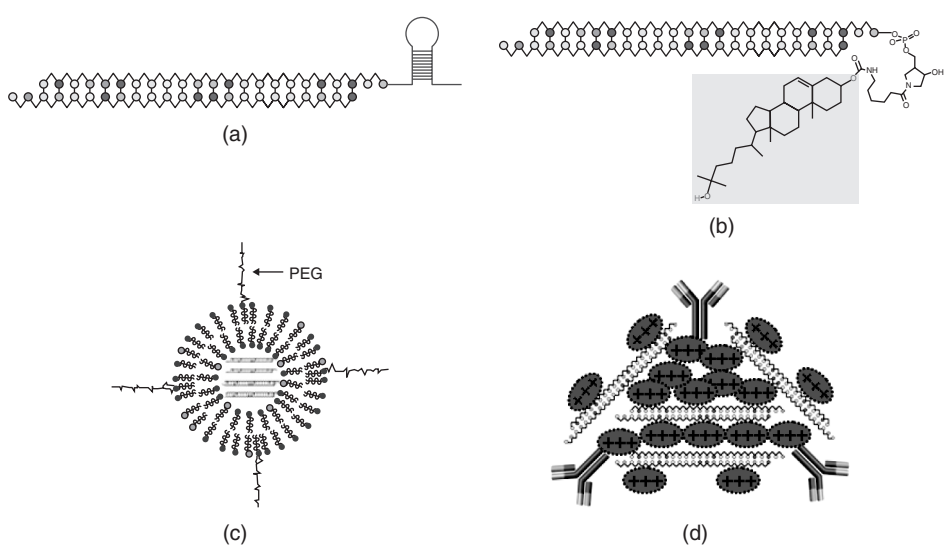

Figure 2 Examples of therapeutic siRNA delivery strategies. a. RNA aptamer conjugation. b. Cholesterol conjugation. c. PEG-liposome. d. Antibody-protamine conjugation. Different chemical modifications and delivery strategies can also be used in combination.

transfection reagents are called lipoplexes. Lipoplexes are relatively easy to form, and they have been widely used for siRNA delivery. Successful applications have been reported via almost all the delivery routes, including subconjunctival, intrathecal, intracranial, intratumoral, intraperitoneal, intravenous, intravaginal, and intrarectal administration (**Table 1**).

Chemical conjugation to small molecules

The idea of conjugating covalently small molecules, such as cholesterol and siRNA, was borrowed from previous research based on antisense oligonucleotide therapeutics (81). Both the 5′ and 3′ ends of the passenger strand tolerate conjugations well. 5′-end cholesterol conjugation of the passenger strand has been shown to efficiently deliver siRNA intravenously for targeting the *ApoB* gene in the liver and jejunum (82).

RNA aptamers can also be conjugated to siRNA for the purpose of cell-specific delivery. Aptamers are artificial DNA or RNA molecules that bind to specific molecular targets (83). Theoretically, RNA aptamer binding to cell-specific receptors or antigens can be linked to siRNA for the purpose of cell type-specific RNAi. One report using a siRNA conjugated with an RNA aptamer that has a high affinity for prostate-specific membrane receptors showed that it silenced survival genes in prostate cancer cells with a high cell specificity and efficacy (18).

Conjugation with peptides and antibodies

When it comes to cell-specific delivery, peptides or antibodies can also be conjugated noncovalently with siRNA. The extreme specificity of an antibody to recognize and bind to a cell-specific antigen makes antibody conjugation a very attractive approach for delivering siRNA. A fusion protein with a specific antibody and a protamine fragment, which is arginine-rich and thus positively charged, can bind to siRNA (negatively charged) for delivery. Some successful *in vitro* and *in vivo* applications include the delivery of siRNA to B16 melanoma expressing HIV envelope protein or HIV-infected primary CD4+T cells using a fusion protein of protamine and Fab fragment of antibody to HIV envelope protein (**Tables 1** and **3**).

The simplest peptide conjugation uses cholesteryl oligo-D-arginine (Chol-R9, chosen because of the positive charge of arginines). Noncovalent formation of a complex of siRNA with Chol-R9 efficiently delivered siRNA targeting VEGF into cells. Moreover, in a mouse model bearing a subcutaneous tumor, this complex led to regression of the tumor (12). Other peptides that have been investigated include MPG, derived from the fusion peptide domain of HIV-1 gp41 protein and the nuclear localization sequence (NLS) of SV40 large T antigen. To be used for siRNA delivery, a mutation that affects the NLS of MPG was generated to prevent the nuclear entry of siRNA. In cell culture, the peptide enables rapid delivery of the siRNA into the cytoplasm, which results in robust downregulation of target mRNA (84). Similar to MPG, Penetratin (Qbiogene Inc., Irvine, CA), which is a peptide derived from the homeodomain of the *Drosophila* protein Antennapedia, is known to deliver cargo into cells. Indeed, Penetratin 1 can be conjugated to siRNA for rapid and efficient delivery into cultured primary mammalian hippocampal and sympathetic neurons (85). For all these peptides, only one report of successful *in vivo* delivery has been made to date, in an animal model with chol-R9.

Some peptides can be recognized by cell-specific receptors and thus can be conjugated with siRNA for cell-specific delivery. Recently, a 29-amino-acid peptide derived from rabies virus glycoprotein (RVG) was shown to be capable of delivering conjugated siRNA (via a nonamer arginine fusion, RVG-R9) transvascularly to the brain, which resulted in efficient gene silencing. This peptide was found to bind specifically to the acetylcholine receptor expressed by neuronal cells (82).

Delivery with nanopolymers

Nanotechnology involves the manipulation of atoms and molecules to construct structures on the nanometer scale (often 100 nm or smaller). Liposomal vesicles, lipoplexes, as well as antibody- and some peptide-conjugated siRNA complexes discussed above are actually nanoparticles. Nanoscale technologies are changing the foundations of therapeutics; in fact, this branch of medicine is now referred to as "nanomedicine" by the National Institutes of Health. Nanoparticles have special advantages in drug delivery. First, therapeutic agents can be encapsulated and hence protected from degradation, clearance, and nonspecific binding. Second, the release speed and location (tissue or even subcellular localization) of drugs can be well controlled by manipulating the composition of nanoparticles. And finally, the pharmacokinetics of the drugs can be optimized (86).

Currently, the most widely used nanoparticles for siRNA delivery are nanopolymers formed with polyethylenimine (PEI). PEI polymers are synthetic, are highly cationic charged, and can be used directly to form complexes with siRNA duplexes. The resulting PEI–siRNA polyplexes are thought to enter cells via endosomes, in which PEI disrupts the low endosomal pH, leading to the eventual release of PEI–siRNA complexes into the cytoplasm. Several reports using PEI polymers demonstrated efficient siRNA delivery in animal models of influenza, Ebola virus infection, and tumors (**Table 1**). Components such as peptides and small compounds (e.g., folate) for cell-specific delivery can also be added into the PEI–siRNA polyplexes. For example, arginine-glycine-aspartic acid-(RGD)-peptide, which is known to bind to both tumors and tumor-endothelial cells *in vivo* (87), has been successfully used to deliver PEI–siRNA into tumor tissues (14). One drawback of PEI has been its extreme toxicity at high concentrations. Its methylene backbone (-CH(2)CH(2)N(x)-) and high charge density make for poor biodegradability and high toxicity to cells. Much effort has been expended to optimize the PEI structure to expand its safety margin (88–91).

Another material of current interest is chitosan. Chitosan is produced commercially by the deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans. Chitosan is positively charged and is bioadhesive to negatively charged molecules, such as nuclear acids, and surfaces, such as mucosal membranes. Chitosan enhances the transport of polar drugs across epithelial surfaces and is biocompatible and biodegradable. Previously, it was used for DNA delivery in

Table 3 Delivery strategies of therapeutic siRNA

Delivery strategy	Method	Advantages	Negative aspect
Naked siRNA	siRNA duplex dissolved in saline or 5% dextrose	Cheap and straightforward	Applicable only to some tissues with easy direct accessibility
Lipoplexes	siRNA duplex in complexes with transfection reagents (cationic lipids and neutral lipids).	Convenient to make in the lab	Not tissue specific delivery
Liposomes	siRNA duplex enclosed in a bilayer vesicle of cationic lipids and fusogenic lipids. To stabilize them, or for other purposes, molecules such as cholesterol and PEG are often incorporated in liposomes.	Decrease toxicity and increase pharmacokinetics	Not easy to prepare. Not tissue specific delivery at this stage.
RNA aptamer conjugation	Chemically conjugate RNA aptamer to the passenger strand of siRNA duplex during RNA synthesis.	Tissue specific delivery; can design all kinds of aptamer for different specificity.	Relatively expensive; large RNA molecule may be immuno- stimulative.
Cholesterol conjugation	Chemically conjugate cholesterol to the passenger strand of siRNA duplex.	Specific delivery to cells (e.g., liver cells) uptake cholesterol.	Sometimes it may be hard to find a small molecule for a specific cell type.
Peptide conjugation	Positive charged pepetide (e.g., chlolesteryl-R9 and RVG-R9) conjugate non-covalently to siRNA	Tissue specific delivery	Need more *in vivo* experiments
Antibody conjugation	Antibody-protamine fusion protein conjugates non-covalently to siRNA duplex.	Cell specific delivery	Need more *in vivo* experiments
Nanopolymers	Encapsulate siRNA duplex into nanopolymers formed with PEI or other materials.	All the benefits of using nanoparticles for drug delivery. Maybe next generation of siRNA delivery strategy	PEI polymers still need optimized to reduce toxicity. Need more *in vivo* experiments

gene therapy. Several labs have now reported efficient delivery of siRNA using chitosan nanopolymers intranasally or intratumorally (22, 92; **Tables 1** and **3**). Cyclodextrins and atelocollagen are the other two polymers for possible application. Cyclodextrins, which are sometimes called cycloamyloses, are produced from starch by means of enzymatic conversion. Atelocollagen is produced by treating collagen with protease. Both of these polymers can form nanopolymers. Hu-Lieskovan et al. (17) used transferrin-targeted cyclodextrin nanoparticles to silence EWS-FLI1 oncogene in a mouse model of sarcoma tumor. Atelocollagen has been used to deliver siRNA into tumor tissues (11, 93; **Table 1**).

Different delivery strategies can be used in combination. For example, siRNA can be conjugated covalently with small compounds and noncovalently with other molecules, while being packed simultaneously into liposomes or other nanoparticles with or without components (e.g., antibody or peptides) for tissue-specific delivery.

Disease Models and Clinical Trials

Current RNAi therapeutics are focused on infectious diseases caused by viruses, neurological diseases, and cancers or other overgrowth-related diseases (summary in **Table 1**).

Infectious diseases caused by viruses

Current viral diseases under consideration for RNAi therapeutics include those that infect the respiratory system, such as respiratory syncytial virus (RSV), parainfluenza virus (PIV), severe acute respiratory syndrome-associated coronavirus (SARS-

CoV), and influenza viruses; those that infect the central nervous system, such as Japanese encephalitis virus (JEV); viruses that infect the liver, such as HBV and Ebola virus; and those that infect other organs or are tumorigenic, such as herpes simplex viruses type 2 (HSV2) and human papillomaviruses (HPV). From these efforts, one Phase I clinical trial has been completed without untoward toxicity for siRNA-based therapeutics for RSV through intranasal administration of naked or lipoplex complexes. Notably, RSV infections are the leading cause of pediatric hospitalization in the United States today.

Tumors and other overgrowth-related diseases

The most investigated target for RNAi therapeutics to treat tumors and overgrowth-related diseases is vascular endothelial growth factor (VEGF) or its receptor VEGFR. siRNAs that target to VEGFR for age-related macular degeneration (AMD) have passed Phase I clinical trials. VEGF is overproduced in AMD, which resulted in the overgrowth of choroidal blood vessels into the subretinal space. AMD is a leading cause of blindness. Choroidal neovascularization (CNV) is the advanced stage of AMD and accounts for >80% of the vision loss in AMD. Mouse or rat models of laser-induced CNV have been used to test the efficacy, potency, and delivery of siVEGFR1 (**Table 1**).

Most RNAi therapeutics use subcutaneous tumor xenograft mouse models. siRNA targeting overgrowth-related genes, such as VEGF, VEGFR, HER2, c-Myc, pleiotrophin, and RhoA, have been tested with different delivery methods and demonstrated varying degrees of success (**Table 1**).

Dominant diseases

Hopes of applying RNAi-based therapeutics to treat dominant inheritable diseases continue. siRNA can distinguish a single-nucleotide difference between wild-type and mutant alleles when well designed (53, 94). Another strategy for applying RNAi therapy to dominant diseases was initiated by Kiang et al. (95) and validated by O'Reilly et al. (96). Their strategy comprises two elements: gene suppression in conjunction with gene replacement. This strategy was tested in a model of retinitis pigmentosa, which is caused by single-site, dominant mutations in the rhodopsin gene. Using recombinant adeno-associated virus (AAV), researchers delivered a siRNA targeting a site independent of the mutation for both mutant and wild-type alleles while they applied in conjunction a codon-modified replacement gene refractory to that siRNA. The strategy proved successful both *in vitro* and *in vivo*.

Other diseases

The first report of efficient *in vivo* RNAi therapeutics involved a mice model of fulminant hepatitis (acute liver failure) (97). Here, intravenous injection of Fas (also known as Tnfrsf6) siRNA protected hepatocytes from apoptosis. Some chronic diseases, such as dyslipidaemias, arthritis, and colitis, are also under investigation for RNAi therapeutics. siTNF (for arthritis and colitis) and siApoB (for dyslipidemias) have already been developed in animal models. Diseases of the nervous system are another category of special interest. siRNAs that target neurotransmitters, such as dopamine transporter (hyperlocomotor response), serotonin transporter (behavior response), P2X3 (chronic neuropathic pain), DOR (DELT-induced nociception), AGRP (metabolic alterations), and NR2B (formalin-induced nociception), are among those tested with animal models (**Table 1**).

RNAi-based therapeutics also holds out new hope for the treatment of neurodegenerative diseases, such as spinocerebellar ataxia, amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, and prion diseases (98–100; **Table 1**). Some neurodegenerative and other inheritable diseases are mediated by dominant alleles with trinucleotide repeats; allele-specific RNAi was developed for this kind of disease, including Huntington's disease and myotonic dystrophy (101–103). Among preclinical research that is not related to trinucleotide repeats, Pfeifer et al. (44) used lentivectors to deliver PrP(C)-specific shRNA to both cell cultures and a mouse model of scrapie infection. They achieved efficient and stable suppression of PrP(Sc) accumulation in cultured neurons and a significant extension of survival in the mouse model. Hong et al. (104) generated replication-defective herpes simplex virus vectors for APP-specific shRNA and neprilysin-specific shRNA. These viral vectors inhibited Abeta accumulation, both *in vitro* and *in vivo*.

Current Hurdles and Future Promises

With three Phase I clinical trials having passed to date, the area of RNAi therapeutics has moved promptly from the first demonstration of efficient gene knockdown using siRNA duplex in mammalian cell culture in 2001 to many reports of the successful application of siRNA in animal disease models by 2003 (**Table 1**). More recently, John et al. (105) demonstrated that long-term systemic administration of siRNA effectively silenced hepatocyte gene expression in rodents and primates, without significant changes in the levels of three hepatocyte-expressed miRNAs (miR-122, miR-16, and let-7a) or any effect on miRNA activity.

Many negative concerns that once surrounded RNAi therapeutics have been eliminated or at least addressed. Side effects that could be caused by off-target effects and immunostimulation from siRNA can now be reduced to acceptable levels through improvements in design, modification, and delivery strategies, and all these have greatly improved *in vivo* knockdown efficiency.

Despite all the progress, every aspect of RNAi-based therapeutics bears improvement. Algorithms for siRNA design are still far from ideal. In many cases, the required dosage is still high, in the range of 100 micrograms per administration in mice. Chemical modifications and delivery strategies remain to be optimized, with the goal of tissue-specific delivery and high knockdown efficiency.

Another strategy for RNAi therapeutics, in parallel with siRNA, is shRNA delivered with recombinant viral vectors,

such as adenovirus and retrovirus. Viral shRNA-based RNAi therapy has its own advantages. It can be delivered efficiently into the genome or nucleus of transfected cells and expressed and processed the same as endogenous miRNA. Thus, the effect from a single infection lasts for a long time and normally does not require repeated administration, unlike siRNA. Also, for this reason, it is more financially feasible than the expensive chemical synthesis, modification, and delivery of siRNA. Nevertheless, drawbacks to virus-based RNAi therapy also exist. Retrovirus must insert into the genome to function and therefore may cause hazardous mutations, which have already hindered the use of retrovirus in gene therapy (106). Grimm et al. (107) investigated adeno-associated virus type 8 (AAV8) as a viral vector for shRNA expression in liver. Lethality was found to be widespread at higher viral titers because of oversaturation of the endogenous miRNA pathway. Thus, the application of adenovirus for RNAi therapy requires caution in optimizing the viral dosage and sequence of the encoded shRNA.

Dramatic changes in the expression of specific miRNAs are found in a variety of diseases, especially cancers (108). Some of these changes contribute to the etiology of certain diseases. It has been proposed that miRNA-based therapeutics could be developed by delivery of either miRNA duplex to compensate for decreased levels or antisense oligos to block the effect of elevated levels of specific miRNA (109). For example, miR-21 levels were found to be elevated in gliomas, and their knockdown is associated with increased apoptotic activity (45, 110). Recently, Corsten et al. (45) showed that application of (LNA)-anti-miR-21 oligonucleotides has a synergistic effect with another anticancer treatment in increasing the caspase activity and decreasing cell viability in human glioma cells both *in vitro* and *in vivo*. miRNA-based strategies can also be considered as RNAi-based therapeutics in a general way. Future development of this type of therapeutics would be a boon to patients.

Given the potential to control the level of any mRNA through the RNAi pathway, RNAi therapeutics hold great promise for the treatment of virtually any disease with an etiology or pathology associated with an elevated or bad mRNA. As a phenomenon that biologists just began to uncover at the end of the last century, RNAi has generated enormous interest among scientists in both basic and medical research. As the secrets of the RNAi pathway itself emerge, and as techniques and strategy development advance, we are optimistic about the miraculous potential of RNAi-based therapeutics in the early part of this century.

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