

Antisense Inhibition

Oligonucleotides, Ribozymes, and siRNAs

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1. Introduction

Over a span of more than two decades, antisense strategies for gene therapy have expanded from antisense oligonucleotides (AS-ODNs) solely, to the addition of ribozymes and, more recently, to the inclusion of small interfering RNAs (siRNAs). Antisense therapeutics has also experienced its phases of high expectation, sudden disappointment, and meticulous rediscovery, while maintaining its status as a viable and effective gene therapy approach. With the discovery of RNA interference (RNAi) and development in delivery of these gene drugs, more preclinical and clinical investigations are anticipated to take place in the near future to finally fulfill the promise of antisense therapeutics in humans.

2. Antisense Oligonucleotides

AS-ODNs are typically 18–25 bases in length, consisting of sequences that are complementary to the target RNA. They can be injected directly into tissues or delivered systemically. Once delivered into cells, oligonucleotide binds to its RNA counterpart and suppresses expression of the proteins encoded by target RNA. The specificity of this approach is based on the probability that any sequence longer than a minimal number of nucleotides (nt)—13 for RNA and 17 for DNA—occurs only once within the human genome. The idea of antisense therapy for inhibiting disease-associated proteins has become par-

ticularly appealing since Zamecnik and Stephenson (1) first demonstrated in 1978 the reduction of Rous sarcoma viral RNA translation by a specific oligonucleotide.

2.1. Mechanisms of Antisense Inhibition

Gene expression can be altered by oligonucleotides by means of either posttranscriptional inhibition or splicing shift. Posttranscriptional inhibition is accomplished by several mechanisms including sterical blockade of ribosomal access to the target mRNA, induction of RNase H cleavage of mRNA, and inhibition of ribosomal assembly. The net outcome of this process is the diminished translation of target proteins. Oligonucleotides chemically modified by phosphorothioation are especially effective in activating RNase H, resulting in sequence-specific digestion of the target mRNA molecules. This destruction of RNA while leaving the DNA oligonucleotide intact allows the oligonucleotide to be recycled, which makes AS-ODNs long lasting. A majority of antisense studies so far, including most clinical trials, are aimed at reducing undesired disease-associated proteins by virtue of translational inhibition. Alternatively, oligonucleotides that are RNase H inactive and designed toward a certain exon–intron junction can prevent the pre-mRNA splicing at the targeted site and redirect the splicing to a more favored site. The therapeutic potential of this approach has been exemplified in the correction of the expression of β -globin and the breast cancer gene *BCL-X* in related diseases. Certain forms of β -thalassemia are caused by aberrant splicing of β -globin pre-mRNA that leads to abrogation of the protein production (2). AS-ODNs designed to the untoward splice site have been proven effective at inhibiting aberrant splicing and at restoring β -globin expression in thalassemic patients (3). Likewise, alternative splicing of *BCL-X* pre-mRNA gives rise to two isoforms, *BCL-XL* and *BCL-XS*, with opposing antiapoptotic and proapoptotic activities. Targeting the *BCL-XL* splice site with oligonucleotides favored production of the proapoptotic *BCL-XS* protein that enhances cell death in prostate and breast tumor cells (4).

2.2. Targeting Antisense

Although antisense can be designed against any region of the target RNA in theory, different sequences vary markedly in efficiency of gene inhibition. The accessibility of oligonucleotides to RNA is considered the most important factor in choosing the optimal antisense sequences. Computational analysis of the secondary structure of RNA by programs such as mfold or RNAstructure has been used to facilitate selection of target sites for antisense action (5); however, it does not take into account the three-dimensional structures as well as the instant interaction of RNA molecules with other factors. More commonly

taken routes involve evaluation of accessible sites by use of RNase H mapping (6) or scanning oligonucleotide arrays for the best hybridization signals (7). Nevertheless, in general, targeting the start codon AUG, where mRNA is supposedly open for ribosomal entry, has been a successful strategy, although in many cases other sequences turned out to be more effective. Despite these predictive approaches, the selection of optimal antisense sequences still requires trial-and-error testing initially and, in the end, needs to be confirmed *in vivo*.

2.3. Chemical Modifications

Stability and efficient delivery, prerequisites for oligonucleotides to achieve observable therapeutic effects, have been obstacles due to their macromolecular nature. Numerous chemical modifications and delivery approaches have been developed to overcome this problem (**Fig. 1**). The first generation of antisense agents contains backbone modifications such as replacement of oxygen atom of the phosphate linkage by sulfur (phosphorothioates), methyl group (methylphosphonates), or amines (phosphoramidates). Of these, the phosphorothioates have been the most successful and used for gene silencing because of their sufficient resistance to nucleases and ability to induce RNase H functions. However, their profiles of binding affinity to the target sequences, specificity, and cellular uptake are less satisfactory. The second generation of antisense modifications was aimed at improving these properties, among which substitutions of position 2' of ribose with an alkoxyl group (e.g., methyl or methoxyethyl groups) were most successful. 2'-*O*-methyl and 2'-*O*-methoxyethyl derivatives can be further combined with phosphorothioate linkage (8). The third generation contains structural elements, such as zwitterionic oligonucleotides (possessing both positive and negative charges in the molecule); locked nucleic acids (LNAs)/bridged nucleic acids (BNAs) (9); morpholino (10); peptide nucleic acids (PNAs) (with a pseudopeptide backbone) (11); and, more recently, hexitol nucleic acids (HNA) (12). All of the modifications enhanced AA-ODNs in terms of nuclease resistance; specific binding; and with agents such as PNA and morpholino, cellular uptake. However, the ability of oligonucleotides to induce RNase H cleavage was abolished by these alterations. Therefore, chimeric oligonucleotides with an unmodified RNase H-susceptible core flanked by modified nuclease-resistant nucleotides have recently been proposed to address this issue and applied in a number of investigations (13), including clinical trials.

2.4. Delivery of Antisense

Oligonucleotides are primarily taken up by cells via endocytosis. Only a portion of oligonucleotides are able to escape endosome/lysosome, enter the nucleus, and bind to its RNA complement. Because of the hydrophilic and

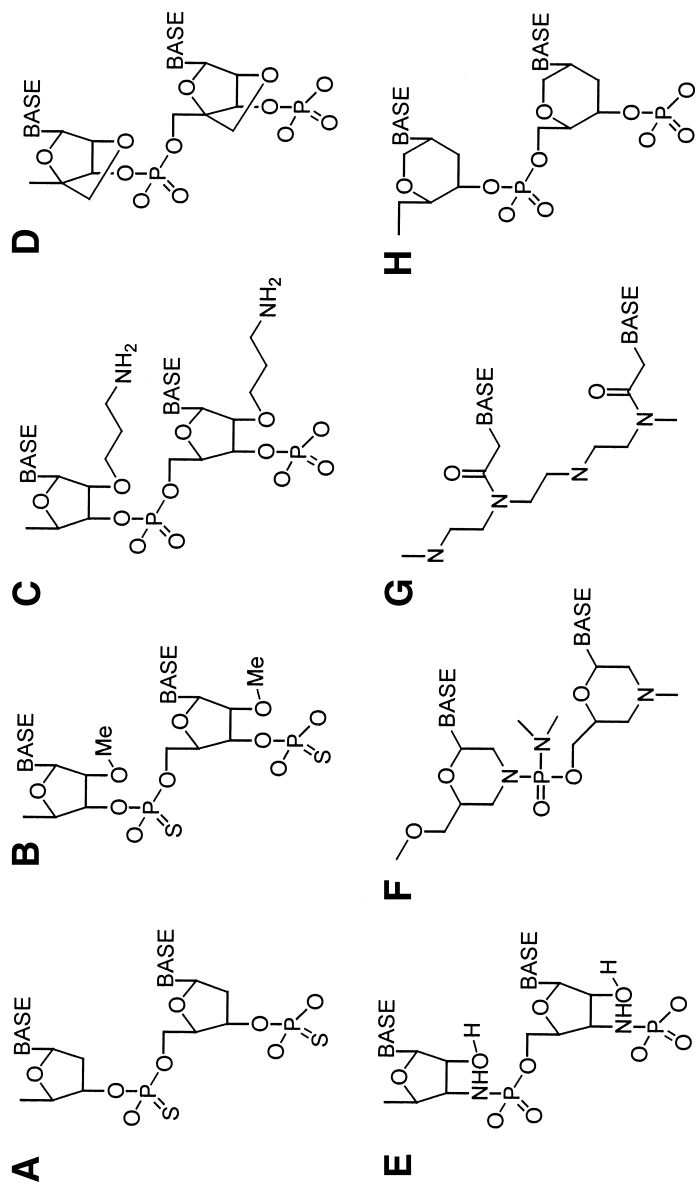


Fig. 1. Structures of synthetic oligonucleotides: (A) phosphorothioate; (B) 2'-O-methyl phosphorothioate; (C) 2'-O-aminopropyl phosphodiester; (D) locked/bridged nucleic acids (LNA/BNA); (E) phosphoramidate; (F) morpholino; (G) peptide nucleic acid (PNA); (H) hexitol nucleic acid (HNA).

macromolecular nature, permeation of oligonucleotides across cell membrane is relatively difficult. Even after two decades of research, safe and efficient delivery of oligonucleotides *in vivo* still remains a major barrier to the clinical success of antisense therapies. Cationic liposomes and electroporation are commonly used carriers. A large variety of liposomal formulas have been developed to facilitate antisense delivery, some of which have entered clinical trials (14). More recently, nanoparticles and oligonucleotide conjugates have shown improved cellular uptake, biodistribution, and targeted delivery, especially in cancer treatment (15,16). A hydrodynamic tail vein injection has proven very effective in delivering oligonucleotides into liver of rodents (17). Inhalable and topical applications of oligonucleotides in patients have shown satisfactory profiles of uptake and distribution (18,19). However, interestingly, most AS-ODNs that are therapeutically valuable in animal models and in patients have been administered in the form of naked compounds, despite the progress in antisense delivery.

2.5. Antisense in Therapies

Antisense therapeutics has seen its ups and downs since the first antisense trial was planned in leukemia in 1992 (20), followed by the excitement over the FDA approval of the first antisense drug, Fomivirsen, for the treatment of cytomegalovirus (CMV) retinitis in 1998 (21). In addition, more recently, a phase III trial reported disappointing results for Affinitak (an antisense inhibitor of protein kinase C- α [PKC- α]) for the treatment of non-small cell lung cancer (NSCLC). Cancer is the major target of ongoing clinical trials using antisense therapies, followed by human immunodeficiency virus (HIV) and other immune-related diseases (Table 1). The targets of antisense for cancer treatment include genes involved in cell growth, apoptosis, angiogenesis, and metastasis. A limitation for antisense as a therapy for cancer may be the single-target approach. Even if the target is successfully inhibited by antisense, other targets may be activated and compensate for the antisense inhibition. Another potential problem is that for successful suppression of cancer growth, the inhibition should be 100%. However, the mechanism of antisense inhibition is always in competition with constitutive copies of mRNA, making a 100% knockdown difficult to achieve. It is noteworthy that after extensive efforts at endogenous expression of antisense RNA by plasmids and viral vectors in a variety of disease models, viral delivery of antisense has recently advanced to human patients; VRX 496 (a lentivirus vector encoding antisense to HIV-1 env protein) started its phase I trial in 2003. Cancer vaccine, a cell therapy using NSCLC cell lines genetically engineered to express transforming growth factor- β (TGF- β) antisense, has also been tested in patients with lung cancer. With the emergence of new generations of modified oligonucleotides and delivery

Table 1
Ongoing Clinical Trials for Antisense Therapy

mRNA target	Drug	Company	Diseases ^a	Phase	Notes ^b
BCL-2	G3139 (Genasense)	Genta/Aventis	Melanoma, MM, CLL, NSCLC	III	18mer/PS
Ha-Ras	ISIS 2503	ISIS	Solid tumors	II	20mer/PS
PKC- α	ISIS 3521 (Affinitak)	ISIS/Eli Lilly	NSCLC, solid tumors	III	20mer/PS
c-RAF	ISIS 5132	ISIS	Solid tumors	Discontinued	20mer/PS
PKC	PKC412	Novartis	Solid tumors, eye infection	II	
PKA-R1 α	GEM 231	Hybridon	Solid tumors	I-II	18mer/AC
Ribonucleotide reductase	GTI 2040, GTI 2501	Lorus	Solid tumors	I-II	21mer/PS
c-RAF	LErafAON	Neopharm	Solid tumors	I-II	
c-MYC	Oncomyc-NG	AVI BioPharma	Cancer	I-II	
c-MYC	AVI 4126	AVI BioPharma	Cancer, kidney disease	I-II	
Clusterin	OGX-011	OncoGenex	Prostate cancer	I	21mer/AC
TGF- β 2	Cancer vaccine	NovaRx	NSCLC	II	NSCLC cells engineered to express TGF- β 2 antisense
Cytochrome P450	AVI 4557 (Neugene)	AVI BioPharma	Drug adverse effects	I-II	
c-MYB	LR/INX-3001	Gewirtz et al.	CML	I-II	24mer/PS
DNA methyltransferase	MG-98	MethylGene	Solid tumors	I-II	20mer/AC
HIV-1	HGTV-43	Enzo Biochem	HIV	I	
HIV gag	GEM-92	Hybridon	HIV	I	
HIV env	VRX496	VIRxSYS	HIV	I	Antisense in lentivirus

c-MYC	Resten-NG	AVI BioPharm	CAD, kidney disease, cancer	I–II
HCV	ISIS 14803	ISIS	HCV	II
ICAM-1	ISIS 2302 (alicaforfen)	ISIS	Crohn disease, psoriasis	II–III
Adenosine A1R	EPI-2010	EpiGenesis	Asthma	II
TNF- α	ISIS 104838	ISIS	Arthritis, Crohn disease, psoriasis	II

^aMM, multiple myeloma; CLL, chronic lymphatic leukemia; CML, chronic myelogenous leukemia; CAD, coronary artery disease.

^bPS, phosphorothioates; AC, advanced chemistry oligonucleotides.

technologies, antisense therapeutics is closer to fulfilling its promise in the clinic for diseases other than cancer, such as cardiovascular disease, psoriasis, and Crohn's disease.

3. Ribozymes

3.1. What Are Ribozymes?

It was discovered in the early 1980s that some naturally occurring RNA molecules have enzymatic activity (22,23). These enzymatic RNA molecules were termed *ribozymes*. Ribozymes recognize specific RNA sequences and then catalyze a site-specific phosphodiester bond cleavage within the target molecule. Following cleavage, the ribozyme releases itself and binds to another target molecule, repeating the process. The cellular consequence varies depending on the setting. There are many naturally occurring ribozymes, including in plant viroids, ribosomes, self-splicing introns, and the RNA portion of RNase P. In plant and animal cells, as well as in viruses, ribozymes are necessary for some normal cellular processes such as transcription. The goal of most synthetic ribozyme usage, however, is reduction in targeted RNA and, thus, lower levels of the protein encoded by the target RNA.

Ribozyme substrate recognition occurs in the same manner as antisense pairing, through strand complementarity. Therefore, any decrease in target protein following ribozyme treatment could in part be due to antisense inhibition of translation or the recruitment of cellular enzymes to the double-stranded RNA (dsRNA) molecules. However, the ability of each ribozyme molecule to rapidly cleave multiple target molecules gives this technology an advantage over classic antisense that can act only on a single RNA molecule. In fact, the rate constants of ribozyme cleavage reactions can approach and exceed those of protein enzymes, including enzymes with similar functions such as RNase A (24,25).

There are multiple types of ribozymes; the two most commonly used for research and therapeutic purposes are the hammerhead ribozyme and the hairpin ribozyme (Figs. 2 and 3). One of the smallest and most well-understood ribozymes, the hammerhead ribozyme, is composed of 30–40 nt and was originally discovered as a common sequence found in plant viroids that undergo site-specific, self-catalyzed cleavage as part of their replication process (26). All hammerhead ribozymes have a common structure consisting of three base-paired helices connected by two invariant single-stranded regions forming the catalytic core. Helices 1 and 3 contain the antisense arms of the ribozyme. Helix 3 also contains the cleavage triplet, the site that is cut by the catalytic core. The triplet most commonly found in naturally occurring hammerhead ribozymes is GUC; however, mutagenesis studies have shown that any cleav-

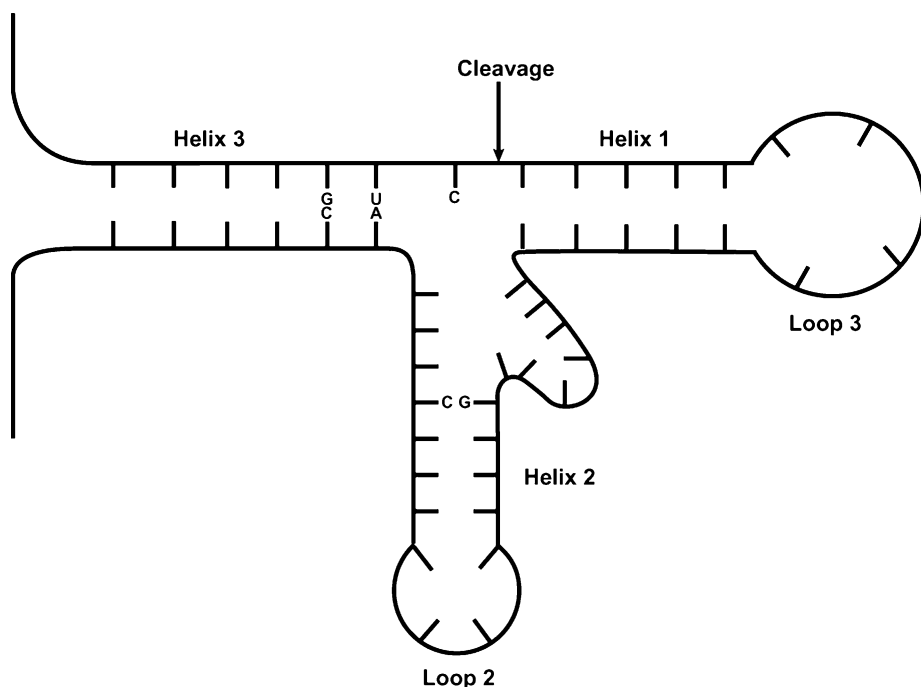


Fig. 2. Schematic of a natural hammerhead ribozyme. Hammerhead ribozymes consist of three helices, formed by complementary base pairing, which are connected by single-stranded regions. Loop 3 is removed to generate a *trans*-cleaving ribozyme; helices 1 and 3 then form the antisense arms. The most commonly found cleavage triplet, GUC, is indicated, as is the cleavage site. The single-stranded domain at the top of helix 2 is the catalytic core. Highly conserved GC residues in helix 2 are necessary for catalytic activity.

age triplet with the sequence NUH is tolerated, in which N is any nucleotide and H is A, U, or C (27). Hammerhead ribozymes catalyze the hydrolysis of the phosphodiester bond at the 3' end of the cleavage triplet. The mechanism requires a divalent metal ion, usually Mg^{2+} , which plays two crucial roles in ribozyme function: it promotes proper folding of the catalytic core and also is a catalytic cofactor (28).

Native hammerhead ribozymes are *cis*-cleaving enzymes, meaning that their targets lie within the same RNA molecule. The ribozyme structure can be engineered to create an intermolecular cleaving ribozyme consisting of two single-stranded antisense arms surrounding the catalytic core and helix 2 so that it will cleave within a different RNA molecule. Because RNA often folds into

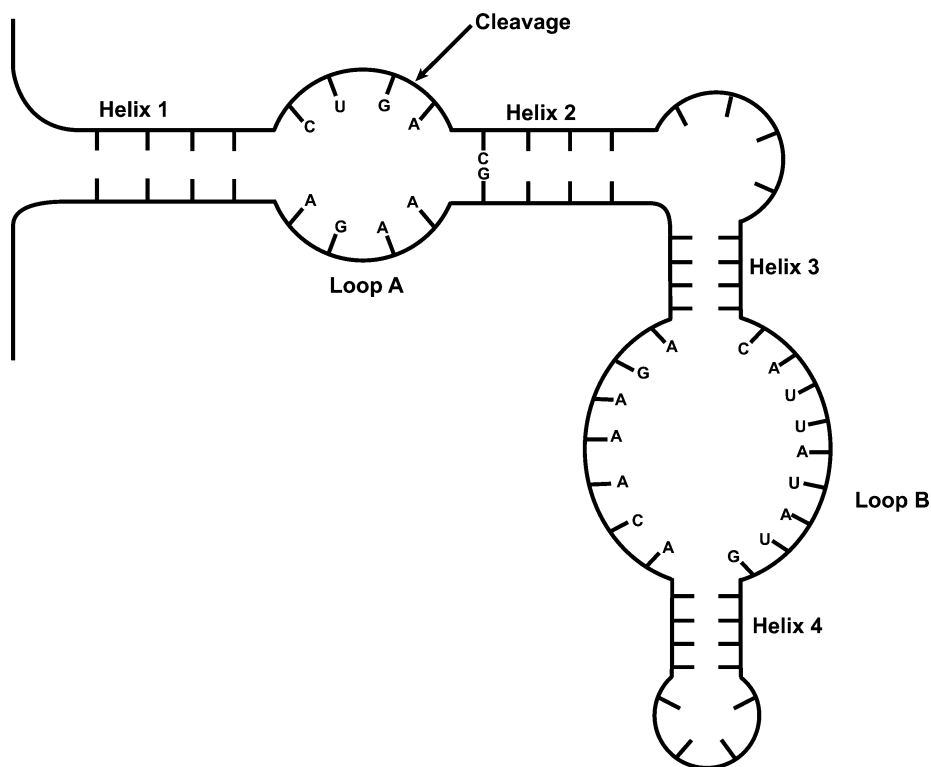


Fig. 3. Schematic of a natural hairpin ribozyme. Hairpin ribozymes consist of 4 helices, formed by complementary base pairing, which are connected by single stranded regions. The small loop at the base of the ribozyme is removed to generate a *trans*-cleaving hairpin ribozyme; helices 1–4 then form portions of the antisense arms. The cleavage site is indicated. Loops A and B comprise the catalytic core domains. The sequences of both loops are highly conserved, as are the GC residues in helix 2.

complex secondary structures, the accessibility of the target site to the annealing arms of the ribozyme must be considered when designing a ribozyme. Arm lengths of 7 to 8 nt are optimal to convey both specificity and access to most ribozymes (29). These shorter annealing arms also aid in turnover of the ribozyme, enhancing the ability of each ribozyme molecule to cleave multiple target RNA molecules (30).

Hairpin ribozymes, like hammerhead ribozymes, are found in some plant viroids that undergo self-catalyzed cleavage as part of their replication process. Hairpin ribozymes contain four base-paired helices and two unpaired loops. The ribozyme cleavage site resides within loop A. The helices can vary

in length and will tolerate any sequence that maintains complementarity with the exception of a requirement for a guanine residue located at the beginning of helix 2, which is required for cleavage site recognition (31). Nucleotides within the catalytic loop regions, however, must be highly conserved to ensure catalytic activity of the ribozyme (31).

Hairpin ribozymes catalyze site-specific hydrolysis of the phosphodiester bond on the complementary strand of RNA that is one base upstream of the conserved guanine in helix 2. Hairpin ribozymes, like hammerhead ribozymes, require an Mg^{2+} ion to activate proper secondary structure. However, unlike the hammerhead ribozyme, Mg^{2+} does not play a direct role in the catalytic process (32). The exact catalytic mechanism used by hairpin ribozymes is not yet fully understood. A greater understanding of how both hammerhead and hairpin ribozymes work and of methods to optimize their function will enhance their attractiveness as potential therapeutic agents.

3.2. Delivery of Ribozymes

Two major issues in the use of ribozymes for research and therapy are ensuring that the ribozyme is delivered to the target tissues and ensuring that the levels of ribozyme delivered are adequate to produce the desired effect. There are two methods for delivering the ribozyme to cells: exogenous delivery of a presynthesized ribozyme or endogenous expression of the ribozyme. Exogenous delivery is relatively easy and rapid; however, as with antisense, there are two main problems with this technique; cellular uptake of the ribozyme is often difficult to achieve, and once the ribozyme is taken up, it is quickly degraded. Cellular uptake of the ribozyme can be enhanced through the use of cationic liposomes. These cationic lipid micelles have the added benefit of protecting the ribozymes from RNase present in serum. To enhance further the lifespan of ribozymes they are frequently chemically modified. The addition of a 2'-*O*-methyl moiety on some or all of the bases is the most commonly used modification. Work is currently being done to engineer DNazymes, which should be more stable than their ribozyme RNA counterparts (33). One benefit of exogenous ribozyme delivery *in vivo* is that the immune system is fairly tolerant of foreign RNA molecules (34).

The other method for delivering ribozymes, endogenous expression of the ribozyme, is most often accomplished using viral vectors; however, plasmid vectors may also be used. Both retroviral and DNA viral vectors have been used. Expression cassettes can be designed to carry cell type-specific or conditional transcription initiation sites, as well as to include reporter proteins. The big advantage of an endogenous ribozyme is that it can be continuously produced, allowing for the compromise of target protein production over a long period of time.

3.3. Research and Therapeutic Uses of Ribozymes

There are four main uses of ribozymes in the medical field: as a research tool, as a chemotherapeutic agent, as an antiviral agent, and as a method to overcome acquired dominant genetic diseases.

With the recent sequencing of the *Drosophila*, mouse, and human genomes, there was a surge of newly identified proteins whose role in the organism is currently unknown or not fully understood. The use of ribozymes to selectively target these new proteins offers an attractive method to rapidly screen their role in vivo. This method, along with other antisense techniques, offers several advantages over traditional methods of screening proteins. First, only a partial cDNA sequence is required to design a ribozyme. Second, ribozymes can be generated very rapidly, whereas both traditional and conditional knock-out animals as well as transgenic overexpression animals require a significant amount of time to generate. Finally, ribozymes can lead to greater effects for longer periods of time when compared with antibody neutralization of the target protein.

In addition to rapid screening of new proteins, ribozyme technology can also be used to overcome problems with traditional protein function studies. For example, we use ribozymes to target a protein that when knocked out results in embryonic demise in mice and for which conditional knockouts have been unsuccessful (35). Ribozymes can also be used to locally target a protein that is made in many tissues, such as to lower targeted protein levels in brain without altering protein expression in the periphery.

The specificity of ribozymes makes them very attractive as therapeutics in disease states in which a protein is overexpressed or is malfunctioning. Ribozymes have the capability to specifically recognize single nucleotide differences in their targets. This special feature has resulted in the development of ribozymes to target oncogenes that are frequently mutated in tumors. For instance, the oncogene H-ras is mutated at a high frequency in many cancers; therefore, a ribozyme that recognizes only the mutant H-ras transcript has the potential to be a very efficacious treatment. Several ribozymes have been developed that can discriminate between H-ras mutants and the normal H-ras transcript and initial studies have shown that stable expression of H-ras mutant ribozymes leads to reduced tumor formation in athymic mice (36,37).

Alternative uses for ribozymes in cancer therapy are to block the elevation of normal gene products, such as c-fos, that occur in transformed cells or to block angiogenic pathways. One such antiangiogenic ribozyme is targeted to flt-1 mRNA, which encodes for the vascular endothelial growth factor receptor (VEGF-R). The ribozyme has been shown to be well tolerated when administered daily by sc injection, and this dosing schedule leads to prolonged eleva-

tion in the plasma levels of ribozyme (38). This ribozyme is now in phase II clinical trials in which therapeutic efficacy in breast and colorectal cancers is being examined.

A different set of circumstances in which a ribozyme can offer great therapeutic potential is the treatment of acquired dominant genetic diseases. Retinitis pigmentosa is a genetic disease that causes carriers of the dominant P23H rhodopsin allele to slowly lose their vision. Hauswirth and Lewin have developed a ribozyme that recognizes only the dominant version of the gene transcript, which differs by two bases from the wild-type gene. Ribozyme treatment has resulted in a halt of disease progression in various species including rat, dog, and now monkey (39,40). This treatment is currently being prepared to enter the first phase of clinical trials. Promising results from this study could open the door for the development of ribozymes to treat other dominant genetic disorders.

One final area where ribozyme therapy holds much promise is as antiviral agents, particularly in the treatment of retroviral infections. Many RNA viruses such as HIV have very high mutation rates throughout much of their genome that renders the mutated viruses resistant to current treatments. However, some sequences, including promoters and slicing signals, are highly conserved in HIV and among other RNA viruses. These regions provide excellent targets for ribozymes. In fact, some groups have designed ribozymes against conserved areas of HIV and have shown that ribozyme treatment can provide long-term HIV resistance and decrease HIV replication in infected cells (41). Several companies now have ribozymes directed against HIV in various stages of clinical trials. Other viruses for which ribozyme treatments are also being designed include hepatitis B, hepatitis C, and the herpes viruses. **Table 2** summarizes ongoing clinical trials using ribozymes.

The use of ribozymes for the inhibition of gene expression holds great promise in both therapeutics and research; however, we have only begun to understand the potential of these molecules. Efforts to improve the stability and delivery of ribozymes will enhance their usefulness as therapeutic agents and lead to a greater recognition of the role of novel proteins in selected tissues and the body as a whole.

4. RNA Interference with siRNA

4.1. What Is siRNA?

RNAi is a form of antiviral immune response mounted by many higher eukaryotes—including plants, nematodes, and insects—on exposure to dsRNA. dsRNA molecules are key intermediates in the genomic replication of many viruses but are not normally found in eukaryotic cells. In contrast to the inter-

Table 2

Ongoing Clinical Trials for Ribozyme Therapy

mRNA target	Drug	Company	Diseases	Phase	Notes
VEGF-R	Angiozyme	Ribozyme	Solid tumors	II	
HCV genome	LY466700				
	Heptazyme	Ribozyme	Hepatitis C	II	
EGF-R type 2	Herzyme	Ribozyme	Breast, ovarian cancer	I	
Proliferating cell nuclear antigen	ViirenASE	Immusol	Retinal disorders	II	
HIV genome		Ribozyme	non-Hodgkin lymphoma in HIV patients	II	Stem cells transduced with retrovirus expressing anti-HIV ribozymes

feron responses of mammalian cells in the face of viral infection, RNAi is used by many other eukaryotes to defend against viruses through dsRNA-induced degradation of viral RNAs.

The first evidence that dsRNA could suppress gene functions came from the work in *Caenorhabditis elegans* (42). In 1998, Fire et al. (43) found that sense RNA was as effective as antisense RNA for inhibiting genes. Subsequently, Zamore et al. (44) demonstrated that dsRNA was at least 10-fold more potent as a silencing trigger than was sense or antisense RNA alone. Since then, gene silencing by dsRNA has been termed RNAi, and its mechanisms have been elucidated vigorously. Our current mechanistic understanding of RNAi derives largely from work in the *Drosophila* system (44,45). The first step of RNAi is to process longer dsRNA into 21- to 23-nt fragments that bear 3' overhangs by an RNase III-like enzyme called Dicer (46). These approx 21 nt dsRNAs, which are termed as siRNA, are essential to form a large (approx 500-kDa) RNA-induced silencing complex (RISC) (47). Through a yet-undefined mechanism, RISC cleaves the target mRNA that is complementary to the guide siRNA, whether the target RNA is a viral mRNA or a cognate gene.

4.2. Application in Mammalian Cells

The key characteristics of RNAi are its remarkable sequence specificity, and it can therefore be used to target gene expression. It was found in *Drosophila* that artificial siRNAs can be incorporated into RISC and induce degradation of target mRNA. However, previous efforts to induce RNAi in cultured mammalian cells had largely failed because long dsRNAs (>30 bp) could induce a potent, nonspecific interferon response and activation of the protein kinase PKR and 2',5'-oligoadenylate synthetase (48,49). dsRNAs shorter than 30 bp do not activate the PKR and interferon pathways. In 2001, a pioneering work by Elbashir et al. (50) demonstrated that transfection of 21 nt synthetic siRNAs into cultured human cells can effectively inhibit gene expression in a sequence-specific manner with extremely high inhibitory efficiency. The advantages and great potential of RNAi technology, including high efficiency (typically >85%), have since raised tremendous interest in this field and generated rapidly emerging progress (reviewed in refs. 51 and 52). These advances have expanded RNAi technology from the use of synthetic siRNA for the endogenous production of small hairpin RNA (shRNA) by plasmid and viral vectors, and from transient inhibition in vitro to longer-lasting effects in vivo and in transgenic animals. This makes possible the utilization of RNAi in suppressing undesirable genes for human gene therapy.

siRNA-mediated gene silencing is sequence specific and dose dependent. As with antisense oligonucleotides and ribozymes, the efficient delivery of siRNA into cells of choice is currently the limiting factor to successful gene

inhibition. The delivery of siRNA can be in the form of naked compounds (53), or markedly improved by cationic liposomes (54) or electroporation (55), depending on cell types. Structural variations and sequence mutations have been made to investigate the structural and sequence requirements for siRNA-induced gene silencing. It was found that the status of the 5' hydroxyl terminus of the antisense strand of an siRNA determines RNAi activity, while a 3' terminus block is tolerated (56–58). Sequence mutations, on the other hand, are generally tolerated at the 5' end, but not the 3' end (59). Chemical modifications such as phosphorothioation and 2'-*O*-methylation and 2'-*O*-allylation were developed to improve the nuclease resistance of synthetic siRNAs (56,59,60). Certain chemical modifications at selected sites prolonged the siRNA activities, whereas others compromised the efficiency. Simultaneous knockdown of more than two genes is possible, as illustrated by the double suppression of the nuclear mitotic apparatus protein (NUMA1) and lamin in HeLa cells (61). However, different siRNA species can possibly undergo reversible competition in a sequence-independent manner (58,62), suggesting that the RNAi machinery might be titratable or limited in mammalian cells.

4.3. siRNA as an Antiviral Agent

The ability of RNAi to protect plants and insects from viral infection can be applied to mammals, although mammalian cells themselves do not possess inherent RNAi mechanisms. siRNAs designed against a variety of viruses, HIV (63–68), hepatitis C virus (HCV) (69–74), hepatitis B virus (75–77), papillomavirus (78), herpesvirus (79), rotavirus (80), and influenza virus (81), have been tested in cell cultures and displayed high efficiency in inhibiting viral infection and replication. In experiments aimed at HIV suppression, siRNAs have been targeted to various regions of the HIV genome including the long-terminal repeats and all five encoded genes, and typically a 30- to 50-fold decrease was observed in the viral levels in cell lines as well as in primary T-lymphocytes (63–68). Similar cases were reported in the siRNA-mediated HCV suppression (69–74). Silencing of HCV RNAs was dose dependent and specific, resulting in a dramatic decrease in HCV RNAs and clearance of HCV in hepatoma cell lines bearing an HCV subgenome. The effects lasted for 3 to 4 d with synthetic siRNA and for more than 3 wk with expression vectors (70,72). It is noteworthy that siRNA-resistant virus strains might already be present in the original viral population and thereby selectively survive the treatment with siRNA, as reported in an attempt to inhibit poliovirus with siRNAs (82). These results suggest that a pool of different siRNA sequences should be used to avoid the selection pressure for favoring siRNA-immune virus.

4.4. Comparison of siRNA AS-ODNs

Direct comparisons of AS-ODNs and siRNAs to the same targets were made in cell culture and in vivo (83–87). Dose-response experiments revealed that the IC_{50} value for the siRNA was about 100-fold lower than that of the AS-ODNs (83). The effect of siRNA is also longer lasting than of AS-ODNs because siRNAs are more stable in fetal calf serum, human plasma, and cell cultures (84). By contrast, Vickers et al. (87) reported that optimized RNase-H-dependent oligonucleotides and siRNAs are comparable in terms of potency, maximal effectiveness, sequence specificity, and duration of action. An independent combinatorial effect of AS-ODNs and siRNAs has been observed when siRNA was coadministered with nonhomologous antisense oligonucleotides, targeting distant regions of the same mRNA (88).

4.5. Small Hairpin RNA

The silencing activity of synthetic siRNAs is transient, lasting 3–5 d in cell culture and 10 d in vivo (53,70,84). This phenomenon can be attributed to siRNA degradation and dilution of siRNA concentrations over cell divisions. To achieve persistent inhibitory effects of RNAi, various plasmid- and virus-based vectors have been developed to express siRNA or shRNA endogenously. RNA polymerase III-dependent promoters such as U6, H1, and tRNA promoters are among the most commonly used, followed by RNA polymerase II-dependent promoters such as CMV promoter (68,89–97). Pol III is ideal for transcribing small RNAs, and its transcripts are not modified posttranscriptionally. Vectors based on adenovirus, retrovirus, and lentivirus have been used to produce functional shRNA species, resulting in persistent and robust gene silencing in vitro and in vivo (97–105). The structure, length, and composition of hairpins appeared to be crucial in determining siRNA activities; however, they varied greatly in these studies. So far it is unclear what makes the best shRNA. Furthermore, RNAi has provided a rapid and effective means to functionally silence genes in stem cells and transgenic animals (99,100,106,107).

4.6. In Vivo Delivery

Efficient in vivo delivery of siRNAs has been reported in a number of mouse models (53,54,74,97,108–110). After a rapid systemic injection into tail vein, the target gene expression was effectively inhibited in liver, kidney, spleen, lung, and pancreas (111). Intravenous injection of siRNA targeting Fas specifically reduced Fas protein expression in mouse hepatocytes and protected mice from fulminant hepatitis induced by concanavalin A or an agonistic Fas antibody (53). Moreover, siRNA is capable of gene silencing when administered into brain or retina (108,110).

Although RNAi technology is still in the fledgling stage, exhaustive efforts in the past few years have advanced our knowledge of RNAi from an antiviral mechanism in higher eukaryotes, to a powerful tool in functional genetics in mammalian cells, and then to a promising therapeutic approach for gene therapy. While many mechanistic and functional questions await answers, the advantages of RNAi in terms of its extraordinary efficiency and specificity, coupled with extensive research for improving its stability, delivery, and duration of action, warrant further preclinical and clinical explorations in a wide variety of diseases.

References

1. Zamecnik, P. C. and Stephenson, M. L. (1978) Inhibition of Pous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl. Acad. Sci. USA* **75**, 280–284.
2. Schwartz, E. and Benz, E. (1995) *Thalassemia Syndromes*. Churchill Livingston, New York.
3. Lacerra, G., Sierakowska, H., Carestia, C., et al. (2000) Restoration of hemoglobin A synthesis in erythroid cells from peripheral blood of thalassemic patients. *Proc. Natl. Acad. Sci. USA* **97**, 9591–9596.
4. Mercatante, D. R., Bortner, C. D., Cidlowski, J. A., and Kole, R. (2001) Modification of alternative splicing of Bcl-x pre-mRNA in prostate and breast cancer cells. Analysis of apoptosis and cell death. *J. Biol. Chem.* **276**, 16,411–16,417.
5. Mathews, D. H., Sabina, J., Zuker, M., and Turner, D. H. (1999) Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *J. Mol. Biol.* **288**, 911–940.
6. Ho, S. P., Bao, Y., Leshner, T., et al. (1998) Mapping of RNA accessible sites for antisense experiments with oligonucleotide libraries. *Nat. Biotechnol.* **16**, 59–63.
7. Milner, N., Mir, K. U., and Southern, E. M. (1997) Selecting effective antisense reagents on combinatorial oligonucleotide arrays. *Nat. Biotechnol.* **15**, 537–541.
8. Manoharan, M. (1999) 2'-carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. *Biochim. Biophys. Acta.* **1489**, 117–130.
9. Morita, K., Takagi M, Hasegawa C, et al. (2002) 2'-O,4'-C-ethylene-bridged nucleic acids (ENA): highly nuclease-resistant and thermodynamically stable oligonucleotides for antisense drug. *Bioorg. Med. Chem. Lett.* **12**, 73–76.
10. Summerton, J. (1999) Morpholino antisense oligomers: the case for an RNase-independent structure type. *Biochim. Biophys. Acta* **1489**, 141–158.
11. Elayadi, A. N. and Corey, D. R. (2001) Application of PNA and LNA oligomers to chemotherapy. *Curr. Opin. Investig. Drugs* **2**, 558–561.
12. Declercq, R., Van Aerschot, A., Read, R. J., Herdewijn, P., and Van Meervelt, L. (2002) Crystal structure of double helical hexitol nucleic acids. *J. Am. Chem. Soc.* **124**, 928–933.

13. Wang, H., Wang, S., Nan, L., Yu, D., Agrawal, S., and Zhang, R. (2002) Antisense anti-MDM2 mixed-backbone oligonucleotides enhance therapeutic efficacy of topoisomerase I inhibitor irinotecan in nude mice bearing human cancer xenografts: in vivo activity and mechanisms. *Int. J. Oncol.* **20**, 745–752.
14. Maurer, N., Fenske, D. B., and Cullis, P. R. (2001) Developments in liposomal drug delivery systems. *Expert Opin. Biol. Ther.* **1**, 923–947.
15. Brigger, I., Dubernet, C., and Couvreur, P. (2002) Nanoparticles in cancer therapy and diagnosis. *Adv. Drug Deliv. Rev.* **54**, 631–651.
16. Manoharan, M. (2002) Oligonucleotide conjugates as potential antisense drugs with improved uptake, biodistribution, targeted delivery, and mechanism of action. *Antisense Nucleic Acid Drug Dev.* **2**, 103–128.
17. Lecocq, M., Andrianaivo, F., Warnier, M. T., Wattiaux-De Coninck, S., Wattiaux, R., and Jadot, M. (2003) Uptake by mouse liver and intracellular fate of plasmid DNA after a rapid tail vein injection of a small or a large volume. *J. Gene Med.* **5**, 142–156.
18. Sandrasagra, A., Leonard, S. A., et al. (2002) Discovery and development of respirable antisense therapeutics for asthma. *Antisense Nucleic Acid Drug Dev.* **12**, 177–181.
19. Brand, R. M. (2001) Topical and transdermal delivery of antisense oligonucleotides. *Curr. Opin. Mol. Ther.* **3**, 244–248.
20. Reynolds, T. (1992) First antisense drug trials planned in leukemia. *J. Natl. Cancer Inst.* **84**, 288–290.
21. Roehr, B. (1998) Fomivirsen approved for CMV retinitis. *J. Int. Assoc. Physicians AIDS Care* **4**, 14–16.
22. Altman, S. (1990) Ribonuclease P. Postscript. *J. Biol. Chem.* **265**, 20,053–20,056.
23. Cech, T. R. (1987) The chemistry of self-splicing RNA and RNA enzymes. *Science* **236**, 1532–1539.
24. Esteban, J. A., Banerjee, A. R., and Burke, J. M. (1997) Kinetic mechanism of the hairpin ribozyme. *J. Biol. Chem.* **272**, 13,629–13,639.
25. Fedor, M. J. and Uhlenbeck, O. C. (1992) Kinetics of intermolecular cleavage by hammerhead ribozymes. *Biochemistry* **31**, 1242–1254.
26. Symons, R. H. (1992) Small catalytic RNAs. *Annu. Rev. Biochem.* **61**, 641–671.
27. Birikh, K. E., Heaton, P. A., and Eckstein, F. (1997) The structure, function and application of the hammerhead ribozyme. *Eur. J. Biochem.* **245**, 1–16.
28. Dahm, S. C. and Uhlenbeck, O. C. (1991) Role of divalent metal ions in the hammerhead RNA cleavage reaction. *Biochemistry* **30**, 9464–9469.
29. Lieber, A. and Strauss, M. (1995) Selection of efficient cleavage sites in target RNAs by using a ribozyme expression library. *Mol. Cell. Biol.* **15**, 540–551.
30. Hendry, P. and McCall, M. (1996) Unexpected anisotropy in substrate cleavage rates by asymmetric hammerhead ribozymes. *Nucleic Acids Res.* **24**, 2679–2684.
31. Fedor, M. J. (2000) Structure and function of the hairpin ribozyme. *J. Mol. Biol.* **297**, 269–291.
32. Young, K. J., Gill, F., and Grasby, J. A. (1997) Metal ions play a passive role in the hairpin ribozyme catalyzed reaction. *Nucleic Acids Res.* **25**, 3760–3766.

33. Li, Y. and Breaker, R. R. (1999) Deoxyribozymes: new players in the ancient game of biocatalysis. *Curr. Opin. Struct. Biol.* **9**, 315–323.
34. Riddell, S. R., Elliott, M., Lewinsohn, D. A., et al. (1996) T-cell mediated rejection of gene-modified HIV-specific cytotoxic T lymphocytes in HIV infected patients. *Nat. Med.* **2**, 216–223.
35. Taylor, M. M. and Samson, W. K. (2002) Ribozyme compromise of adrenomedullin mRNA reveals a physiological role in the regulation of water intake. *Am. J. Physiol.* **282**, R1739–R1745.
36. Koizumi, M., Hayase, Y., Iwai, S., et al. (1989) Design of a RNA enzyme distinguishing a single base mutation in RNA. *Nucleic Acids Res.* **17**, 7059–7071.
37. Koizumi, M., Kmiya, H., and Ohtsuka, D. (1992) Ribozymes designed to inhibit transformation of NIH/3T3 cells by the activated c-Ha-ras gene. *Gene* **117**, 179–184.
38. Usman, N. and Blatt, L. M. (2000) Nuclease-resistant synthetic ribozymes: developing a new class of therapeutics. *J. Clin. Invest.* **106**, 1197–1202.
39. Lewin, A. S., Drenser, K. A., Hauswirth, W. W., et al. (1998) Ribozyme rescue of photoreceptor cells in a transgenic rat model of autosomal dominant retinitis pigmentosa. *Nat. Med.* **4**, 967–971.
40. Lavail, M. M., Yasumura, D., Matthes, M. T., et al. (2000) Ribozyme rescue of photoreceptor cells in P23H transgenic rats: long-term survival and late-stage therapy. *Proc. Nat. Acad. Sci. USA* **97**, 11,488–11,493.
41. Muotri, A. R., Pereira, L. V., Vasques, L. R., et al. (1999) Ribozymes and the anti-gene therapy: how a catalytic RNA can be used to inhibit gene function. *Gene* **237**, 303–310.
42. Guo, S. and Kempfues, K. J. (1995) Par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611–620.
43. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806–811.
44. Zamore, P. D., Tuschl, T., Sharp, P. A., and Bartel, D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25–33.
45. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* **15**, 188–200.
46. Bernstein, D., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363–366.
47. Hammond, S. M., Bernstein, F., Beach, D., and Hannon, G. J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* **404**, 293–296.
48. Stark, G. R., Kerr, I. M., Williams, B. R., et al. (1998) How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264.

49. Minks, M. A., West, D. K., Benveniste, S., and Baglioni, C. (1979) Structural requirements of double-stranded RNA for the activation of 2',5'-oligo(A) polymerase and protein kinase of interferon-treated Hela cells. *J. Biol. Chem.* **254**, 10,180–10,183.
50. Elbashir, S. M., Harborth, J., Lendeckel, W., et al. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–498.
51. McManus, M. T. and Sharp, P. A. (2002) Gene silencing in mammals by small interfering RNAs. *Nat. Rev.* **3**, 737–747.
52. Shi, Y. (2003) Mammalian RNAi for the masses. *Trends Genet.* **19**, 9–12.
53. Song, E., Lee, S. K., Wang, J., et al. (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* **9**, 347–351.
54. Sorensen, D. R., Leirdal, M., and Sioud, M. (2003) Gene silencing by systemic delivery of synthetic siRNAs in adult mice. *J. Mol. Biol.* **327**, 761–766.
55. Herweijer, H. and Wolff, J. A. (2003) Progress and prospects: naked DNA gene transfer and therapy. *Gene Ther.* **10**, 453–458.
56. Czauderna, F., Fechtner, M., Dames, S., et al. (2003) Structural variations and stabilizing modifications of synthetic siRNAs in mammalian cells. *Nucleic Acid Res.* **31**, 2705–2716.
57. Chiu, Y. L. and Rana, T. M. (2002) RNAi in human cells: basic structural and functional features of small interfering RNA. *Mol. Cell* **10**, 549–561.
58. Holen, T., Amarzguioui, M., Wiiger, M. T., Babaie, E., and Prydz, H. (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Res.* **30**, 1757–1766.
59. Amarzguioui, M., Holen, T., Babaie, E., and Prydz, H. (2003) Tolerance for mutations and chemical modifications in a siRNA. *Nucleic Acids Res.* **31**, 589–595.
60. Harborth, J., Elbashir, S. M., Vandeburgh, K., et al. (2003) Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucleic Acid Drug Dev.* **13**, 83–105.
61. Elbashir SM, Harborth J, Weber K, Tuschl T. Analysis of gene function in somatic mammalian cells using small interfering RNAs. *Methods* 2002; **26**: 199–213.
62. MaManus, M. T., Haines, B. B., Chen, J., and Sharp, P. A. (2002) SiRNA-mediated gene silencing in T-lymphocytes. *J. Immunol.* **169**, 5754–5760.
63. Jacque, J. M., Triques, K., and Stevenson, M. (2002) Modulation of HIV-1 replication by RNA interference. *Nature* **418**, 435–438.
64. Novina, C. D., Murray, M. F., Dykxhoorn, D. M., et al. (2002) siRNA-directed inhibition of HIV-1 infection. *Nat. Med.* **8**, 681–686.
65. Surabhi, R. M. and Gaynor, R. B. (2002) RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. *J. Virol.* **76**, 12,963–12,973.
66. Capodici, J., Kariko, K., and Weissman, D. (2002) Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J. Immunol.* **169**, 5196–5201.

67. Coburn, G. A. and Cullen, B. R. (2002) Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J. Virol.* **76**, 9225–9231.
68. Lee, N. S., Dohjima, T., Bauer, G., Li, H., et al. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat. Biotechnol.* **20**, 500–505.
69. Yokota, T., Sakamoto, N., Enomoto, N., et al. (2003) Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep.* **4**, 602–608.
70. Wilson, J. A., Jayasena, S., Khvorova, A., et al. (2003) RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. *Proc. Natl. Acad. Sci. USA* **100**, 2783–2788.
71. Kapadia, S. B., Brideau-Andersen, A., and Chisari, F. V. (2003) Interference of hepatitis C virus RNA replication by short interfering RNAs. *Proc. Natl. Acad. Sci. USA* **100**, 2014–2018.
72. Randall, G., Grakoui, A., and Rice, C. M. (2003) Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. *Proc. Natl. Acad. Sci. USA* **100**, 235–240.
73. Seo, M. Y., Abrignani, S., Houghton, M., and Han, J. H. (2003) Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7. *J. Virol.* **77**, 810–812.
74. McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A. (2002) RNA interference in adult mice. *Nature* **418**, 38–39.
75. Hamasaki, K., Nakao, K., Matsumoto, K., Ichikawa, T., Ishikawa, H., and Eguchi, K. (2003) Short interfering RNA-directed inhibition of hepatitis B virus replication. *FEBS Lett.* **543**, 51–54.
76. McCaffery, A. P., Nakai, H., Pandey, K., et al. (2003) Inhibition of hepatitis B virus in mice by RNA interference. *Nat. Biotechnol.* **21**, 639–644.
77. Shlomai, A. and Shaul, Y. (2003) Inhibition of hepatitis B virus expression and replication by RNA interference. *Hepatology* **37**, 764–770.
78. Hall, A. H. and Alexander, K. A. (2003) RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in Hela cells. *J. Virol.* **77**, 6066–6069.
79. Jia, Q. and Sun, R. (2003) Inhibition of gammaherpesvirus replication by RNA interference. *J. Virol.* **77**, 3301–3306.
80. Dector, M. A., Romero, P., Lopez, S., and Arias, F. (2002) Rotavirus gene silencing by small interfering RNAs. *EMBO Rep.* **3**, 1175–1180.
81. Ge, Q., McManus, M. T., Nguyen, T., et al. (2003) RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. *Proc. Natl. Acad. Sci. USA* **100**, 2718–2723.
82. Gitlin, L., Karelsky, S., and Andino, R. (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* **418**, 430–434.
83. Miyagishi, M., Hayashi, T., and Taira, K. (2003) Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. *Antisense Nucleic Acid Drug Dev.* **13**, 1–7.

84. Bertrand, J. R., Pottier, M., Vekris, A., et al. (2002) Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. *Biochem. Biophys. Res. Commun.* **29**, 1000–1004.
85. Aoki, Y., Cioca, D. P., Oidaira, H., Kamiya, J., and Kiyosawa, K. (2003) RNA interference may be more potent than antisense RNA in human cancer cell lines. *Clin. Exp. Pharmacol. Physiol.* **30**, 96–102.
86. Garber, K. (2003) Better blocker: RNA interference dazzles research community. *J. Natl. Cancer Inst.* **95**, 500–502.
87. Vickers, T. A., Koo, S., Bennett, C. F., Crooke, S. T., Dean, N. M., and Baker, B. F. (2003) Efficient reduction of target RNAs by small interfering RNA and RNase H-dependent antisense agents. *J. Biol. Chem.* **278**, 7108–7118.
88. Hemmings-Mieszczak, M., Dorn, G., Natt, F. J., et al. (2003) Independent combinatorial effect of antisense oligonucleotides and RNAi-mediated specific inhibition of the recombinant rat P2X3 receptor. *Nucleic Acids Res.* **31**, 2117–2126.
89. Yu, J. Y., DeRuijter, S. L., and Turner, D. L. (2002) RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**, 6047–6052.
90. Paddison, P. J., Caudy, A. A., Bernstein, E., et al. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* **16**, 948–958.
91. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550–553.
92. Paul, C. P., Good, P. D., Winer, I., and Engelke, D. R. (2002) Effective expression of small interfering RNA in human cells. *Nat. Biotechnol.* **20**, 505–508.
93. Miyagishi, M. and Taira, K. (2002) U6 promoter driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat. Biotechnol.* **20**, 497–500.
94. Sui, G., Soohoo, C., Affar, B., et al. (2002) A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc. Natl. Acad. Sci. USA* **99**, 5515–5520.
95. Kawasaki, H. and Taira, K. (2003) Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. *Nucleic Acids Res.* **31**, 700–707.
96. Zeng, Y., Wagner, E. J., and Cullen, B. R. (2002) Both natural and designed microRNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol. Cell.* **9**, 1–20.
97. Xia, H., Mao, Q., Paulson, H. L., and Davidson, B. (2002) siRNA-mediated gene silencing in vitro and in vivo. *Nat. Biotechnol.* **20**, 1006–1010.
98. Barton, G. M. and Medzhitov, R. (2002) Retroviral delivery of small interfering RNA into primary cells. *Proc. Natl. Acad. Sci. USA* **99**, 14,943–14,945.
99. Robinson, D. A., Dillon, C. P., Dwiatkowski, A. U., et al. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* **33**, 401–406.

100. Tiscornia, G., Singer, O., Ikawa, M., and Verma, I. M. (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc. Natl. Acad. Sci. USA* **100**, 1844–1848.
101. Shen, C., Buck, A. K., Liu, X., Winkler, M., and Reske, S. W. (2003) Gene silencing by adenovirus-delivered siRNA. *FEBS Lett.* **539**, 111–114.
102. Stewart, S. A., Dykxhoorn, D. M., Palliser, D., et al. (2003) Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* **9**, 493–501.
103. Dirac, A. M. and Bernards, R. (2003) Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *J. Biol. Chem.* **278**, 11,731–11,734.
104. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* **2**, 243–247.
105. Hasuwa, H., Kaseda, K., Einarsdottir, T., and Okabe, M. (2002) Small interfering RNA and gene silencing in transgenic mice and rats. *FEBS Lett.* **532**, 227–230.
106. Kunath, T., Gish, G., Lickert, H., Jones, N., Pawson, T., and Rossant, J. (2003) Transgenic RNA interference in ES cell-derived embryos recapitulates a genetic null phenotype. *Nat. Biotechnol.* **21**, 559–561.
107. Reich, S. J., Fosnot, J., Kuroki, A., et al. (2003) Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis.* **9**, 210–216.
108. Verma UN, Surabhi, R. M., Schmalties, A., Becerra, C., and Gaynor, R. B. (2003) Small interfering RNAs directed against beta-catenin inhibits the in vitro and in vivo growth of colon cancer cells. *Clin. Cancer Res.* **9**, 1291–300.
109. Makimura, H., Mizunno, T. M., Mastaitis, J. W., Agami, R., and Mobbs, C. V. (2002) Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci.* **3**, 18.
110. Lewis, D. L., Hagstrom, J. E., Loomis, A. G., Wolff, J. A., and Herweijer, H. (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. *Nat. Genet.* **32**, 107–108.



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