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Approaches for the sequence-specific knockdown of mRNA

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Over the past 25 years there have been thousands of published reports describing applications of antisense nucleic acid derivatives for targeted inhibition of gene function. The major classes of antisense agents currently used by investigators for sequence-specific mRNA knockdowns are antisense oligonucleotides (ODNs), ribozymes, DNAzymes and RNA interference (RNAi). Whatever the method, the problems for effective application are remarkably similar: efficient delivery, enhanced stability, minimization of off-target effects and identification of sensitive sites in the target RNAs. These challenges have been in existence from the first attempts to use antisense research tools, and need to be met before any antisense molecule can become widely accepted as a therapeutic agent.

Over the past two decades, the use of nucleic acid–based inhibitors of gene expression (antisense agents) has come in and out of fashion. Initial excitement in this area came in the mid-1980s as synthetic DNA chemistry hit full stride, enabling sequence-specific antisense ODNs to be rapidly synthesized and tested for target-specific knockdown of gene expression. It was quickly realized that certain backbone modifications were necessary for full activity of these compounds and that efficient delivery to target cells was a critical requirement. Chemists quickly addressed these challenges by developing a variety of backbone modifications that stabilized antisense ODNs without inhibiting their biological activities. Delivery challenges were also addressed with the introduction of anionic and cationic lipid formulations for packaging and delivering the net negatively charged ODN compounds to a variety of cells in culture. In subsequent years, however, interest declined because the predicted utility of these compounds as therapeutic agents was slow to materialize and, in fact, remains limited to a handful of compounds.

The second wave of interest in nucleic acid–based inhibitors of gene expression followed the discoveries of catalytic RNAs (ribozymes) in the early 1980s. The full potential of ribozymes for target-specific inhibition of gene expression was not completely realized until the late 1980s and early 1990s when simplified catalytic motifs were defined, making these molecules amenable to chemical synthesis. The exploitation of ribozymes as therapeutic agents also depended heavily upon stabilizing backbone modifications that did not inhibit activity and efficient delivery. Fortunately, these issues could be addressed by drawing on the extensive experience of the antisense ODN field. Ribozymes have an advantage over ODNs in that ribozyme genes can be delivered to cells with plasmid or viral vectors, and ribozyme expression can be controlled with promoter-based expression.

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The most recent explosion of interest in the antisense world followed the discoveries of Mello and colleagues[1] in *Caenorhabditis elegans* in 1998, and of others in mammalian cells in 2001 (refs. 2,3), that double-stranded RNAs (dsRNAs) elicit potent targeted degradation of complementary RNA sequences, termed RNA interference (RNAi). Moreover, it was shown that the active component of the RNAi pathway, termed small interfering RNAs (siRNAs), can be chemically synthesized or expressed from vector backbones, similar to ribozymes. The interest in RNAi has been fueled—to an even greater extent than interest in antisense ODNs and ribozymes—by the completion of the human genome sequence initiative because siRNAs can elicit potent, target-specific knockdown of any mRNA, creating a useful and proven surrogate genetic tool. Although RNAi provides a powerful new tool for targeted inhibition of gene expression, there are, nevertheless, concerns and limitations in the use of this technology as well, including efficient delivery and potential side effects.

There have been important developments in the other areas of antisense technologies, giving the investigator several options, depending upon the experimental system and desired outcome. This article explores the basic mechanisms of action of only the popular antisense inhibitory agents. We then compare the advantages and disadvantages for each class of inhibitory agent. We do not intend to present a comprehensive review of the antisense world, but rather to provide a framework for thinking about which agent best matches the goals of an experimental or therapeutic application.

Antisense oligonucleotides

The notion that small ODNs could be used to specifically inhibit gene expression was first put forth in 1978 by Zamecnik and Stephenson[4,5]. Their studies demonstrated that a tridecamer (13-mer) ODN complementary to terminally repeated sequences in Rous sarcoma virus (RSV) long terminal repeat (LTR) inhibited both RSV translation in a cell-free system and viral replication in cultured cells[4,5]. It took several years after these elegant experiments for investigators to begin to fully realize the potential of antisense-mediated gene inhibition. With the

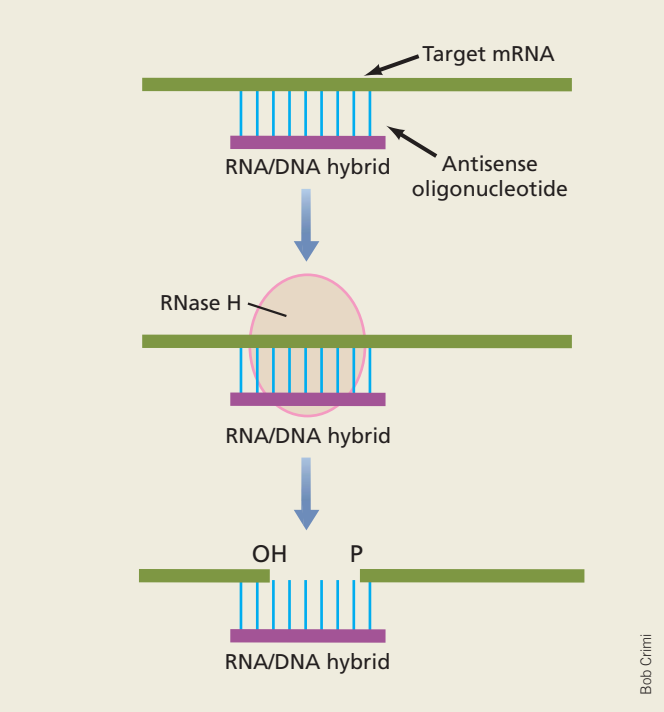

Figure 1 Model for antisense DNA ODN recruitment of RNAse H. Negatively charged ODNs interact with the target mRNA by Watson-Crick base pairing. The DNA-RNA hybrid is a substrate for endogenous RNAse H, which cleaves the RNA within the hybridized region, allowing the ODN to recycle.

automation of ODN synthesis in the early 1980s, it became relatively straightforward to obtain ODN s of any sequence and to test their ability to block gene expression via antisense base pairing.

Soon after the demonstration that phosphodiester backbone ODNs were effective as target-specific agents for blocking gene expression, several new backbone modifications were developed to improve the stability of the ODNs and to enhance their effectiveness. The most widely used modification is one in which the nonbridging oxygen is replaced by a sulfur atom, creating phosphorothioate ODNs[6]. This type of backbone formed the basis for the only Food and Drug Administration (FDA, Rockville, MD, USA)-approved antisense drug, Vitravene (Isis Pharmaceuticals, Carlsbad, CA, USA), which targets the cytomegalovirus *IE2* mRNA and is used to treat cytomegalovirus-associated retinitis. A second ODN, Genasense, which targets *Bcl2* (Genta, Berkely Heights, NJ, USA), has recently completed a phase III clinical trial for metastatic melanoma where it is being used in conjunction with standard chemotherapy, which the antisense potentiates. Several other phosphorothioate antisense ODNs are in earlier stages of clinical trials for a variety of cancers and inflammatory diseases.

The mechanisms of action of ODNs with respect to blocking gene function vary depending upon the backbone of the ODN[7–10]. Net negatively charged ODNs, such as phosphodiesters and phorphorothioates, elicit RNAse H–mediated cleavage of the target mRNA (**Fig. 1**). Other backbone modifications that do not recruit RNAse H, because of their lack of charge or the type of helix formed with the target RNA, can be classified as steric hindrance ODNs. Popularly used members of this latter group include morpholinos, 2′-*O*-methyls, 2′-*O*-allyls, locked nucleic acids and peptide nucleic acids (PNAs). These ODNs can block splicing, translation, nuclear-cytoplasmic transport and translation, among other inhibition targets. It is well beyond the scope of this article to delve further into the mechanisms of action of this diverse array of ODN modifications and for more detailed information, the reader is referred to specific reviews on this subject, which describe each of these modifications in detail[6,8].

Ribozymes

Ribozymes are RNA molecules that act as enzymes, even in the complete absence of proteins. They have the catalytic activity of breaking and/or forming covalent bonds with extraordinary specificity, thereby accelerating the spontaneous rates of targeted reactions by many orders of magnitude. The ability of RNA to serve as a catalyst was first shown for the self-splicing group I intron of *Tetrahymena thermophila* and the RNA moiety of RNAse P[11–13]. After the discovery of these two RNA enzymes, RNA-mediated catalysis has been found associated with the self-splicing group II introns of yeast, fungal and plant mitochondria (as well as chloroplasts)[14], single-stranded plant viroid and virusoid RNAs[15–17], hepatitis delta virus[18] and a satellite RNA from *Neurospora crassa* mitochondria[19]. Ribozymes occur naturally, but can also be artificially engineered for expression and targeting of specific sequences in *cis* (on the same nucleic acid strand) or *trans* (a noncovalently linked nucleic acid). New biochemical activities are being developed using *in vitro* selection protocols as well as generating new ribozyme motifs that act on substrates other than RNA[20].

The group I intron of *T. thermophila* was the first *cis*-cleaving ribozyme to be converted into a *trans*-reacting form, which we refer to as an intron/ribozyme[12,21], making it useful both in genomic research and as a possible therapeutic. In the *trans*-splicing reaction, a defective exon of a targeted mRNA can be exchanged for a correct exon that is covalently attached to the intron/ribozyme[22–24]. This occurs via a splicing reaction in which the exon attached to the intron is positioned by base pairing to the target mRNA so that it can be covalently joined to the 5′ end of the target transcript in a transesterification reaction. This reaction has been used to *trans*-splice wild-type sequences into sickle cell β-globin transcripts[25] and mutant *p53* transcripts[26] and replace the expanded triplets in the 3′-UTR of protein kinase transcripts in a myotonic dystrophy allele[27].

The endoribonuclease RNAse P is found in organisms throughout nature. This enzyme has RNA and one or more protein components depending upon the organism from which it is isolated. The RNA component from the *Escherichia coli* and *Bacillus subtilis* enzymes can act as a site-specific cleavage agent in the absence of the protein under certain salt and ionic conditions[28]. Studies of the substrate requirements for human and bacterial enzymes have shown that the minimal substrates for either enzyme resemble a segment of a transfer RNA molecule[29,30]. This structure can be mimicked by uniquely designed antisense RNAs, which pair to the target RNA, and serve as substrates for RNAse P–mediated, site-specific cleavage both in the test tube and in cells. It has also been shown that the antisense component can be covalently joined to the RNAse P RNA, thereby directing the enzyme only to the target RNA of interest[31]. Investigators have taken advantage of this property in the design of antisense RNAs, which pair with target mRNAs of interest to stimulate site-specific cleavage of the target[32] and for targeted inhibition of both herpes simplex virus and cytomegalovirus in cell culture[33–36].

A number of small plant pathogenic RNAs (viroids, satellite RNAs and virusoids), a transcript from a *N. crassa* mitochondrial DNA plasmid and the animal hepatitis delta virus undergo a self-cleavage reaction *in vitro* in the absence of protein. The reactions require neutral pH and Mg^{2+}. The self-cleavage reaction is an integral part of the *in vivo* rolling circle mechanism of replication. These self-cleaving RNAs

can be subdivided into groups depending on the sequence and secondary structure formed about the cleavage site. Small ribozymes have been derived from a motif found in single-stranded plant viroid and virusoid RNAs. On the basis of a shared secondary structure and a conserved set of nucleotides, the term 'hammerhead' has been given to one group of this self-cleavage domain[37,38] (**Fig. 2a**). The hammerhead ribozyme is composed of ~30 nucleotides. The simplicity of the hammerhead catalytic domain has made it a popular choice in the design of *trans*-acting ribozymes. Using Watson-Crick base pairing, the hammerhead ribozyme can be designed to cleave any target RNA. The requirements at the cleavage site are relatively simple, and virtually any UH sequence motif (where H is U, C or A) can be targeted.

A second plant-derived, self-cleavage motif, initially identified in the negative strand of the tobacco ringspot satellite RNA, has been termed the 'hairpin' or 'paperclip' (**Fig. 2b**)[17]. The hairpin ribozymes cleave RNA substrates in a reversible reaction that generates 2′,3′-cyclic phosphate and 5′-hydroxyl termini. Engineered versions of this catalytic motif also cleave and turn over multiple copies of a variety of targets in *trans*[39]. Substrate requirements for the hairpin include a GUC, with cleavage occurring immediately upstream of the G. The hairpin ribozyme also catalyzes a ligation reaction, although it is more frequently used for cleavage reactions.

There have been numerous applications of both hammerhead and hairpin ribozymes in cells for downregulating specific cellular and viral targets. Haseloff and Gerlach[40] designed a hammerhead motif in 1988 that can be engineered to cleave any target by modifying the arms that base pair with the target. Our laboratory first demonstrated that this hammerhead ribozyme motif had potential therapeutic applications was a study of cells engineered to express an anti-human immunodeficiency virus (HIV) *gag* ribozyme in which there was virtually complete inhibition of viral gene expression and replication[41]. Since this study, there have been literally thousands of applications of ribozymes targeting cellular and viral targets. A number of comprehensive reviews have been written that survey these applications, and the reader is referred to these for further treatment of this subject[42–46].

DNAzymes

A category of site-specific cleaving nucleic agents that has received considerable attention in the past several years is that of catalytic DNAs. Small DNAs capable of site specifically cleaving RNA targets have been developed via *in vitro* evolution (as no known DNA enzymes occur in nature)[47,48]. Two different catalytic motifs, with different cleavage site specificities, were found via this search. The most commonly used 10–20 enzymes (**Fig. 2c**) bind to their RNA substrates via Watson-Crick base pairing and site specifically cleave the target RNA, as do the hammerhead and hairpin ribozymes, resulting in 2′,3′-cyclic phosphate and 5′-OH termini. Cleavage of the target mRNAs results in their destruction and the DNAzymes recycle and cleave multiple substrates. Catalytic DNAs are relatively inexpensive to synthesize and have good catalytic properties[49–51], making them useful substitutes for either antisense DNA or ribozymes.

Several applications of DNAzymes in cell culture have been published including the inhibition of *veg F* mRNA and consequent prevention of angiogenesis[52], and inhibition of expression of the *bcr/abl* fusion transcript characteristic of chronic myelogenous leukemia[53]. A drawback of catalytic DNAs compared to ribozymes is that they can only be delivered exogenously, but they can be backbone-modified, perhaps allowing them to be delivered systemically in the absence of a carrier.

RNAi and siRNAs

RNAi refers to a group of related gene-silencing mechanisms sharing many common biochemical components in which the terminal effector molecule is a small 21–23-nucleotide antisense RNA. One mechanism uses a relatively long, dsRNA 'trigger,' which is processed by the cellular enzyme Dicer into short, 21–23-nucleotide dsRNAs, referred to as siRNAs (**Fig. 3**). The strand of the siRNA complementary to the target RNA becomes incorporated into a multi-protein complex termed the RNA-induced silencing complex (RISC), where it serves as a guide for endonucleolytic cleavage of the mRNA strand within the target site. This leads to degradation of the entire mRNA; the antisense siRNA can then be recycled[54]. In lower organisms, RNA-dependent RNA polymerase also uses the annealed guide siRNA as a primer, generating more dsRNA from the target, which serves in turn as a Dicer substrate, generating more siRNAs and amplifying the siRNA signal. This pathway is commonly used as a viral defense mechanism in plants.

The term siRNA is now generally used whenever the antisense

Table 1 Relative strengths and weaknesses of antisense technologies

Approach	Advantages	Disadvantages
Antisense ODNs	Can be modified to improve selectivity and efficacy	Can induce interferon (if long and has CpG)
	Can be targeted to introns	Can bind proteins (aptamer activity)
	Easy to make	Only exogenous delivery possible (synthetic)
		Off-target effects
Ribozymes	Can discriminate single base polymorphisms	Requires GUC triplet—limits choice of target
	Can be used to correct defects	Binds proteins (aptamer activity)
	Sequences can be appended to change target specificity	
	Simple catalytic domain	
	Can target introns/subcellular compartments	
DNAzymes	Inexpensive to make	Only exogenous activity
	Good catalytic properties	Off-target effects?
	Can be modified for systemic delivery	
RNAi	Effective at low concentrations	Cannot target nuclear RNAs or introns
	Bypasses interferon pathway	No option for improving if target refractory
	Can be delivered by multiple pathways	Some reports of off-target effects
	Tissue-specific expression possible	
	Nontoxic?	
	Lasts longer?	

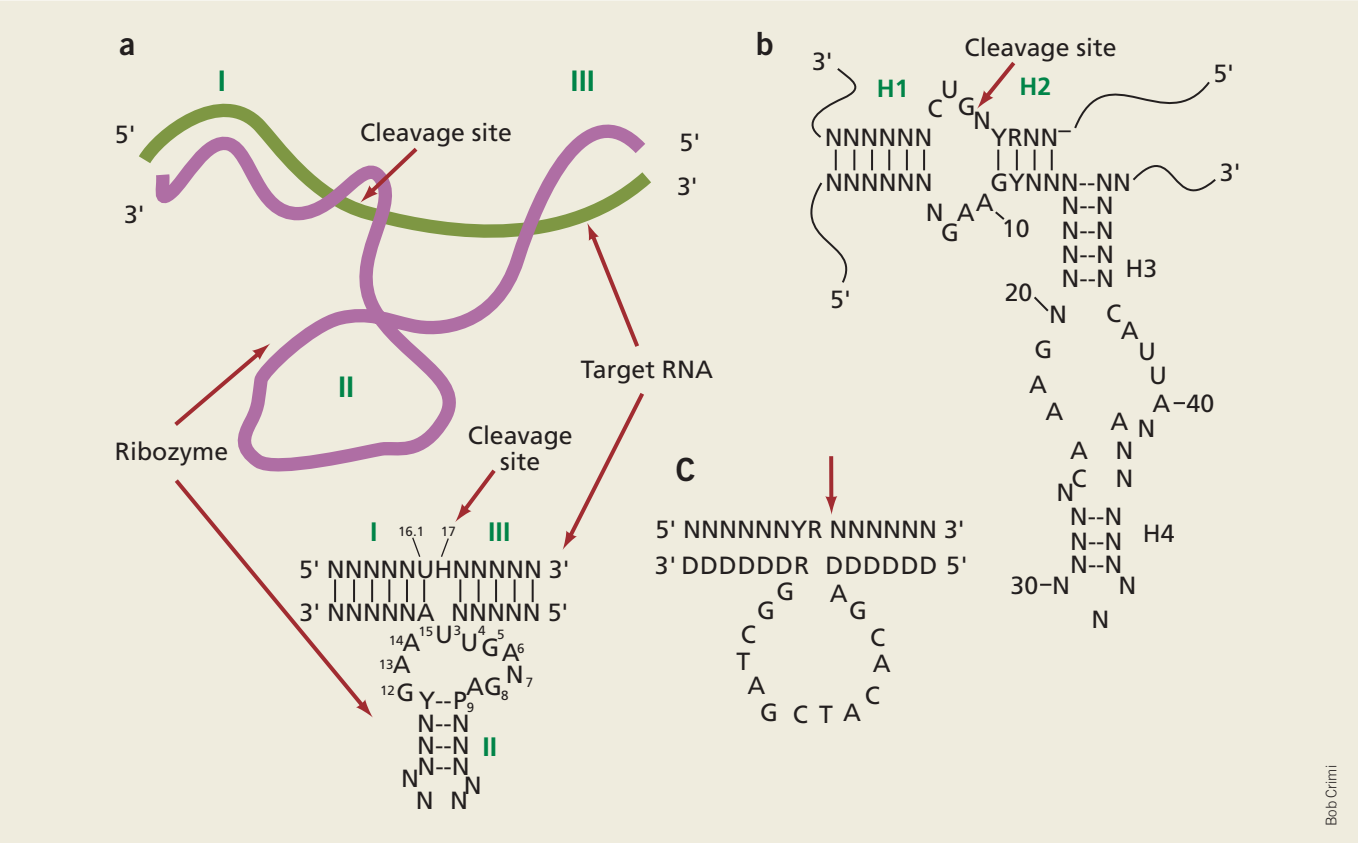

Figure 2 Generalized hammerhead ribozyme, hairpin ribozyme and DNAzyme motifs. (**a**) The hammerhead ribozyme in a *trans* configuration with a target RNA. The upper portion depicts an outline of the three-dimensional structure of the ribozyme bound to its substrate[98,99]. N represents A, C, G or U; H represents A, C or U. The numbering of the hammerhead ribozyme conforms to the standard convention adopted for this ribozyme[100]. The sites of cleavage are depicted by the arrows. (**b**) The hairpin ribozyme base-paired to a target RNA, using the same conventions as in **a**. (**c**) The 10–20 DNA enzyme[48]. The DNAzyme is depicted by D (for DNA) or A, G, C, T; Y, pyrimidine; and R, purine. The arrow depicts the site of cleavage. For further details, see text.

strand is completely complementary to the mRNA target site. The siRNA may consist of two separate, annealed single strands of 21 nucleotides, where the terminal two 3′-nucleotides are unpaired (3′ overhang). Alternatively, the siRNA may be in the form of a single stem-loop, often referred to as a short hairpin RNA (shRNA). Typically, but not always, the antisense strand of siRNAs is also completely complementary to the sense partner strand of the si/shRNA.

Recent experiments indicate that in fission yeast, dsRNA encoded by the centromeric DNA also mediates silencing of centromeric heterochromatin, and is dependent on components of the RNAi pathway[55,56]. Similar RNAi-like mechanisms are involved in silencing of the *Schizosaccharomyces pombe* mating type locus[57]. Chromatin silencing of an endogenous *ura4*$^+$ gene in *trans* is initiated by a *ura4*$^+$ long-stemmed (280 base pairs) hairpin encoded on an extra-chromosomal plasmid requiring both RNAi components and Clr4 (a histone methylase); spreading of heterochromatin through euchromatin requires the *S. pombe* ortholog of Swi6. Moreover, the same mechanism, using naturally occurring siRNAs derived from endogenous transposons, has been implicated in regulating normal host gene expression in *S. pombe* during meiosis[58].

In mammalian cells, long dsRNAs (usually greater than 30 nucleotides in length) trigger the interferon pathway, activating protein kinase R and 2′,5′-oligoadenylate synthetase[2]. Activation of the interferon pathway can lead to global downregulation of translation as well as global RNA degradation. However, shorter siRNAs exogenously introduced into mammalian cells have been reported to bypass the interferon pathway, although recent evidence suggests this may not always be the case[59].

The siRNA antisense product can also be derived from endogenous microRNAs. Data drawn from experiments in several paradigm systems, such as the *C. elegans* lin4/lin14 pathway, suggest the following pathway for microRNA biogenesis and gene regulation in animal cells. The ends of a transcript are removed in the nucleus by an exo III RNAse (Drosha, in human cells), forming a ~70 nucleotide pre-micro RNA fold-back intermediate[60]. Pre-microRNAs may be multicistronic, containing multiple hairpins directed against different target RNAs. The pre-microRNA is actively exported to the cytoplasm where Dicer processing trims the hairpin stem and removes the loop and sense strand to create the final 21–23-nucleotide antisense RNAi effector. In contrast to the prototypical si/shRNAs, the sense and antisense stem partner strands are not completely complementary, containing bubbles or bulges; both the structure and thermodynamic properties of the base pairing are critical for proper processing[61,62]. Moreover, the antisense strand contains mismatches to one or more sites in the 3′ untranslated region of the target mRNA, where binding mediates translational repression rather than mRNA degradation. MicroRNAs are widespread phylogenetically and conserved in some instances; they also exhibit temporal and spatial regulation[63]. A recent estimate for the

number of human microRNAs is 200–250 (ref. 64).

In human cells, experiments with siRNAs and microRNAs indicate that, regardless of the initial form or processing pathway, a final mature 21–23-nucleotide antisense RNA that is completely homologous to the mRNA will direct mRNA cleavage. In general, the effect of mismatches between siRNAs and target sites can vary from almost none to complete abrogation of activity, for reasons that are only partially understood; however, in at least one case, partial homology resulted in mRNA translation inhibition. In this report, an siRNAwith target mismatches designed to mimic a prototypical microRNA-target interaction mediated varying degrees of translational repression, depending on both the specific interaction and the number of target sites in the mRNA[65–67]. Consequently, it is likely that the structural features typical of siRNAs or microRNAs are important for processing and selection of the antisense strand in RISC and have important implications for the design of RNAi-inducing agents (see 'Target sequence' below).

RNAi can be activated by either exogenous delivery of preformed siRNAs or via promoter-based expression of siRNAs or shRNAs[68] (**Fig. 4**). Thus, RNAi has emerged as a potent mechanism to specifically knockdown mRNA transcripts to a few percent of their original levels by most methods of detection. RNAi appears to be more potent than antisense RNAs, ribozyme or RNAzymes for targeted message destruction, presumably because it exploits cellular machinery that efficiently directs the antisense component to the target mRNA for site-directed cleavage.

Comparative analyses

In the light of the above discussion, the question of what roles still exist for older technologies, such as antisense ODNs, ribozymes or DNAzymes versus RNAi is an important one. The various unique potential uses for each of the technologies are summarized below and in **Table 1**.

Targeting precursor RNA molecules. The majority of published evidence indicates that RNAi targets RNA molecules primarily in the cytoplasm in animal cells[69]. It is unclear at this time whether RNAi in mammals also affects chromatin organization and gene expression as it does in some lower eukaryotes and plants. RNAi-mediated chromatin silencing as found in yeast may also require the action of a group of clustered siRNAs in a localized area, as opposed to the one or a few siRNAs typically necessary for mRNA target downregulation. Short inhibitory RNAs have not been effective against intron target sites and may not be effective against RNAs that are exclusively nuclear, such as spliceosomal RNAs.

Ribozymes and antisense ODNs, on the other hand, can be designed to target introns and nuclear-localized RNAs. These agents may be more useful when it is necessary to selectively downregulate a sequence derived from a gene family of highly homologous sequences in which only the introns have grossly different sequences. By the same token, ribozymes can be used under circumstances where it is highly advantageous to degrade mRNA before it reaches the cytoplasm. For example, work in our laboratory has shown that an α-HIV ribozyme directed to the nucleolar compartment can successfully inhibit HIV replication[70]. Such selectivity in intracellular compartmentalization is not possible with antisense ODNs, DNAzymes or siRNAs.

For most sequences, though, cytoplasmic targeting is sufficient to achieve the desired downregulation, and here siRNAs can be the most effective reagents because of their reactivity at concentrations lower than those required for the same level of gene silencing mediated by the other agents[71,72]. (It should be pointed out, however, that the effective concentrations of RNAse H–dependent ODNs and siRNAs for a given target have been found to be equivalent in one study[72].) Thus far, there have been no direct comparisons between siRNAs and ribozymes or DNAzymes. Furthermore, as siRNAs can be produced by intracellular expression of siRNAs or shRNAs, it would be useful to compare expressed si/shRNAs with these other types of inhibitors.

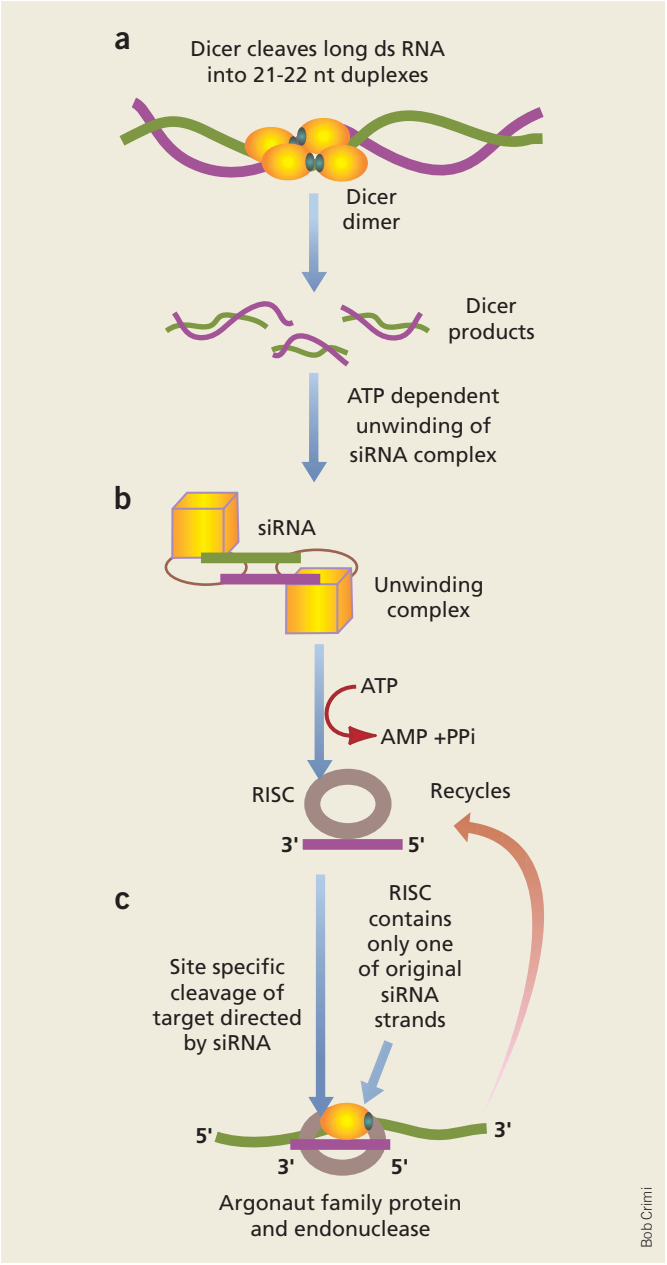

Figure 3 The RNAi pathway. (**a**) DsRNAs are cleaved by the enzyme Dicer into siRNAs. (**b**) The siRNAs are unwound before entry into RISC and the strand complementary to the target mRNA is incorporated into RISC. (**c**) RISC contains an endonuclease that cleaves only the target mRNA within the hybridized region.

Target sequence selection. Finding an effective target site within an mRNA can be problematic for antisense ODNs, ribozymes, DNAzymes and siRNAs. It is clear that there are sequences that are refractory to siRNAs[73,74] as well as to antisense ODNs and ribozymes[6,75]. The major limitation for each of these approaches is the

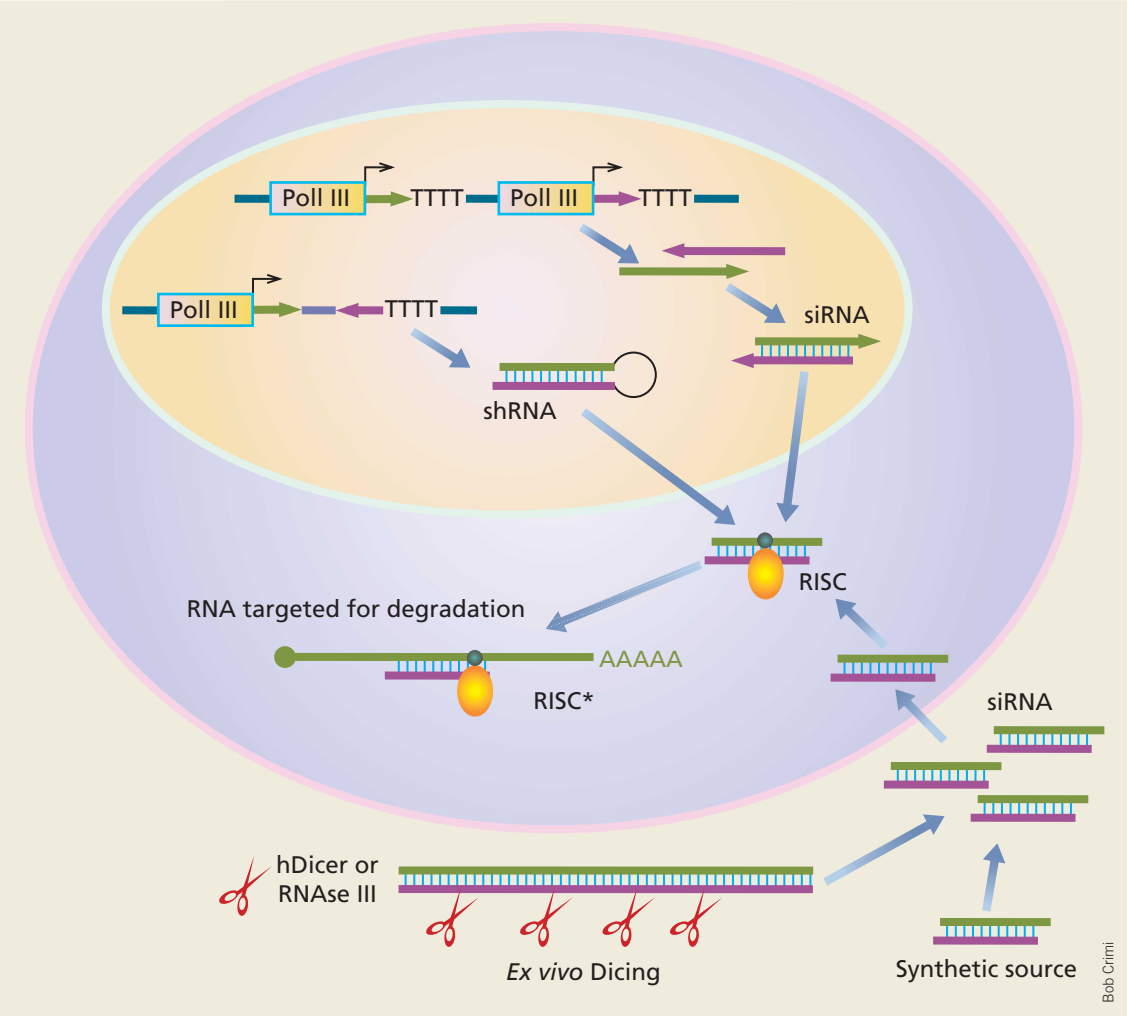

Figure 4 Various methods for introduction of siRNAs or shRNAs into mammalian cells. Small inhibitory RNAs can be generated from long dsRNAs *in vitro* via recombinant Dicer, they can be transcribed *in vitro* from plasmids or they can be chemically synthesized. These RNAs are transfected into cells using cationic lipid formulations. The expression cassettes depicted within the cell can create shRNAs or siRNAs as depicted. Both Pol II and Pol III systems have been used for intracellular promoter-based expression.

identification of a sequence/antisense combination that provides the most potent knockdown at the lowest possible concentration of antisense agent. The mere predicted folding pattern of a target mRNA is usually not sufficient to find such a combination. To date, target site selection for all of these approaches is best done by systematically testing a variety of potential targets[72] although there are several commercial and academic websites that have algorithms for identifying accessible target sequences. However, recent publications suggest that the thermodynamic properties of the sense/antisense base pairing in the siRNA are very important in the choice of target sequence, and may explain the weak suppression by some previously tested siRNAs[61,76].

If the choice of accessible target sites is limited, use of a ribozyme may not be possible if the site does not contain an appropriate triplet cleavage site—a limitation not shared by antisense ODNs or siRNA design. In addition, if a specific target site is refractive to siRNA, there are currently no options for improving cleavage of that site. A number of colocalization options exist to improve ribozyme accessibility by direction to specific cell compartments and (sequence-directed) colocalization with the target[71,77–80]. The size requirement of siRNAs and the need to get cytoplasmic accumulation restricts the use of appended sequences, although there are exceptions[81], and more may emerge as the biochemical processing pathways of siRNAs and the related microRNAs are better understood. Hybrid RNAs sharing both microRNA and siRNA characteristics could be used for processing multiple siRNAs and/or microRNAs from a single transcript, possibly providing multiple targeting for treating genetically variable viruses, such as HIV and hepatitis C virus.

Off-target effects. In choosing a method for targeted knockdown of gene expression, an important consideration has to be the potential for off-target, non-sequence specific effects. Each of the respective methods (antisense ODNs, ribozyme and siRNA) has the potential for such effects. For instance, antisense ODNs have been shown to direct RNAse H cleavage of nontargeted RNAs by virtue of the fact that only six or seven contiguous base pairs with the target RNA are required to direct cleavage[82]. In contrast, ribozymes are much more sensitive to polymorphisms at the cleavage site (though relatively less so in the hybridizing arms, depending upon position) and have therefore been used for discriminating between single nucleotide polymorphisms[83–85]. There is at least one report in the literature of a multitude of off-target effects by synthetic siRNAs[86], although a separate study has suggested that siRNAs were highly specific[87]. These two studies differed in the choice of targets for siRNA inhibition. In the former, the targets were endogenous transcripts, whereas in the latter a reporter construct was the target. It is too soon to draw strong conclusions about the generality of off-targeting by siRNAs, but clearly this potential problem needs to be further addressed by investigators in the field.

For each of the antisense reagents discussed in this review, the off-

target effects may be a consequence of the levels of the agent delivered to cells. As an example, a standard tissue culture experiment might use ~5×10^5 cells in 2.0 ml of medium. If one applies a 10 nM concentration of a single strand of an antisense agent to these wells, roughly10^7 molecules of the antisense agent per cell is present. If the uptake is only 1%, there will still be 10^5 molecules per cell.

For siRNA, it is critical to keep the concentration as low as possible, because the double-stranded molecules can yield two strands capable of eliciting off-target effects. An additional concern for siRNAs is that the capacity of RISC for interaction with transfected or expressed siRNAs is unknown. It is highly unlikely that all the RNA entering the cytoplasm will be incorporated into RISC or be immediately degraded, and therefore it will be available for other cellular processes.

High concentrations of the other antisense agents is risky because ODNs and ribozymes act by diffusing to their targets, a process in which they could encounter many partially homologous sequences in the process. The lesson here is that all of these agents must be used at the lowest effective concentration to minimize unwanted side activities.

Side effects. Other potential issues of importance in comparing approaches for sequence-specific knockdown of mRNAs are 'unanticipated side effects.' For instance, CpG motifs in antisense DNA ODNs elicit strong innate and acquired immune responses *in vivo*, most likely via interactions with Toll-like receptor[88,89]. Both antisense ODNs and ribozymes could function as aptamers, binding proteins that are unrelated to the targets of these ODNs[90]. Finally, at least two recent reports suggest that siRNAs and shRNAs can activate arms of the interferon response pathways, which could lead to nonspecific inhibition of protein synthesis and global RNA degradation[59,91]. As powerful as all three of these target knockdown technologies can be, there is always the concern that one can be led astray by such side effects. Again, there is a strong requirement for further experimentation to sort out which of these agents is most likely to generate unwanted side effects.

Duration of effect. Another consideration in choosing the 'most efficacious' antisense agent is whether the application requires long-term or short-term knockdown of the target molecule. Applications involving chronic infections, such as HIV and hepatitis C virus, recalcitrant cancers, some dominant or codominant genetic abnormalities and generation of knockdown animals, to name a few, will require continuous application or expression of the antisense agent. Moreover, if endogenous expression is the best route for application, then ribozyme, RNAi and antisense RNA are the best approaches as these RNAs can be expressed from vector backbones, whereas synthetic ODNs and DNAzymes can only be delivered exogenously. Ribozymes, RNAi and antisense RNAs can also be expressed using inducible or tissue-specific promoter systems, making controlled expression possible[75,92,93]. For transient applications, each of these approaches can be applied, provided that the desired cellular delivery can be achieved. The use of backbone modifications that enhance the serum/cellular half-lives of antisense ODNs, ribozymes, DNAzymes and siRNAs makes each of these reagents useful for short-term inhibition of gene expression[6,94]. The efficiency of delivery will continue to be the limiting factor for stabilized antisense compounds. Delivery is a key concern if the antisense agents are going to be used in a therapeutic setting. To date, there is no single reagent or backbone modification that can be effectively used for all the different antisense agents.

Conclusions

As the biochemical mechanisms of RNAi become better understood, the use of siRNA will continue to expand; however, RNAi is unlikely to supplant the use of antisense, ribozymes, DNAzymes and related approaches for many applications. In practice, the choice of antisense methodology will depend on the specific circumstances of the application. Moreover, the addition of new technologies to the antisense toolbox is expected to increase the range of applications and allow fine-tuning of the general approach. One recent example is inhibition by RNAu, where a mutated U1 small nuclear RNA is attached to a 10-nucleotide antisense sequence targeted against a site in an mRNA terminal exon. In this case, the inhibitory RNA prevented polyA addition and triggered mRNA degradation[95].

Regardless of which antisense technology is applied, the challenge of ensuring specificity remains paramount because of the potential for nontargeted alteration of gene expression. One perceived, but not yet demonstrated, advantage of RNAi is that it takes advantage of cellular machinery, RISC, specifically designed for selective inhibition of targeted transcript expression. However, one potential side effect is that the mechanism may be prone to saturation, thereby leaving unprocessed small duplexes of RNAs free to enter into other cellular pathways. Thus, it is imperative to identify target sequences that are effectively downregulated at low nanomolar or even sub-nanomolar concentrations of siRNA. The same qualification holds for the other antisense-based mechanisms. For each of these approaches it would also be in the best interest of investigators to verify the phenotype with a second or third agent directed at a different sequence in the same transcript. For siRNAs, there is always the potential that even low concentrations can result in off-target effects via participation in the microRNA pathway or perhaps even at the level of chromatin remodeling[66,96,97].

Alternatively, siRNAs provide an additional antisense-based tool that may be even more powerful combined with the other nucleic acid–based therapies. The discovery of RNAi has certainly accelerated the pace at which targeted post-transcriptional gene silencing is being applied as a tool for identifying gene function and as a therapeutic agent. It remains to be determined whether the recent concerns about off-target effects and interferon pathway induction become a roadblock or merely a detour.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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