DRUG DISCOVERY TODAY TARGETS

Exploring the sounds of silence: RNAi-mediated gene silencing for target identification and validation

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Drug development begins with the identification and early preclinical validation of novel biological targets, a process often called 'target identification and validation'. This process usually uses various approaches, such as observations from literature and findings from animal or clinical studies, together with cutting edge molecular techniques that include analyses of gene and protein expression, interaction and function. The publication of the human genome has increased research in gene and protein expression analysis that, in combination with RNA interference technology, promises the evaluation of novel functions for known genes, as well as hitherto unknown or unstudied genes with functions relevant to disease.

The advent of RNAi

Analysis of gene and protein expression and their association with functionally relevant phenotypes or disease states has been the mainstay of genomics and proteomics approaches to target identification and validation. Use of oligonucleotide and cDNA microarrays has become commonplace in pharmaceutical industry laboratories and is often supported by the use of advanced proteomics tools such as LC–MS, MS–MS, and antibody, peptide and protein arrays. Many laboratories also use tissue microarrays to validate expression data in clinical samples. However, all the technologies used to evaluate gene and protein expression can provide, at best, an association between the expression of a gene or protein with the phenotype under study. Once this association is confirmed, one needs to use techniques to 'validate' the role of the gene in disease causation and pathology by modulating its expression or function, as well as that of its corresponding protein. Until recently, this meant engineering dominant-negative or constitutively active variants of a gene and testing them in functional assays, or using transgenic technologies to knockout, overexpress or knockin the gene in a rodent strain and study its function *in vivo*. Although the analysis of gene modulation *in vivo* using knockout, knockin or transgenic mice remains the gold standard for any target validation project, the process is usually labor intensive and time consuming. As drug discovery timelines and resources shrink across the industry, the search to develop quicker and less labor intensive techniques of validating targets is gaining speed. One option that has been used by some researchers is DNA anti-sense technology, but this suffers from various limitations, including difficulty in identifying effective sequences, low selectivity, limited *in vitro* and *in vivo* delivery and expression options, and little amenability to high-throughput screening [1,2]. Catalytically active nucleic acids, such as ribozymes, are capable of site-specific cleavage of target mRNAs. Unfortunately, the application of ribozymes as target validation tools is limited by their lack of substrate specificity [3,4].

Small RNAs mediating RNA interference (RNAi), named the 2002 'breakthrough of the year' by *Science* [5], have been developed as a viable and more effective alternative to antisense- and ribozyme-based

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techniques for gene perturbation. Several excellent papers and reviews [6–10] document the journey of RNAi from its discovery as a natural phenomenon in lower organisms, such as *Caenorhabditis elegans* [11], to its use in high-throughput target identification screening assays [12] and in the development of 'knockdown' animals [13], and, more recently, its possible use as a therapeutic [14]. The mechanism by which RNA interference works, both in lower organisms and in mammalian systems, has also been extensively studied [15–17]. It begins with the import and recognition of endogenous or exogenously introduced long double-stranded RNA (dsRNA), which is processed into small pieces of 21–25 nucleotide double-stranded small interfering RNA (siRNA) by an RNase III-containing enzyme complex called DICER. The siRNAs contain two perfectly complementary RNA strands which are unwound in an ATP-dependent process and guided to the RNA-induced silencing protein complex (RISC) by interaction between DICER and R2D2 (homologous to RDE-4 in *C. elegans*). The RISC induces cleavage of the complementary mRNA in the middle of the target region, thereby preventing its translation. This siRNA–RISC-mediated cleavage of target mRNA is highly sequence-specific: therein lies the attraction of RNAi as a technique for gene silencing [18].

To induce RNAi, one can transfect cells with chemically synthesized siRNA that is specific for the particular target gene, or clone a siRNA sequence as part of a stem loop or hairpin structure (19–29 sense RNA loop sequence, 19–29 antisense RNA) downstream of, usually, a polymerase III promoter like U6 or H1 and express the construct in cells or *in vivo*. Such RNAi reagents are sometimes referred to as short hairpin RNAs (shRNAs) to distinguish them from chemically synthesized (pre-processed) RNAi reagents or siRNAs. It might also be appropriate at this point to mention microRNA (miRNA), which is a distinct type of short dsRNA that has been observed to regulate developmental processes by modulating the expression of certain crucial genes [10], they are discussed later in this review. Because this review focuses on the possibilities for the use of the RNAi phenomenon to define and design reagents for various aspects of drug discovery, the more generic term 'RNAi reagent' will be used when referring to such reagents throughout this review.

Designing appropriate RNAi reagents

How does one identify the most efficacious RNAi reagents for use in target identification and validation experiments? Theoretically, all short dsRNAs that correspond to a particular target RNA should have silencing activity; however, algorithms for identification of the most efficacious RNAi reagents are an area of intense study [19–21] because different reagents against the same gene display different levels of gene knockdown. This indicates that RNA secondary structures, binding energy, target accessibility and the thermodynamic characteristics of the target sequence affect the activity of RNAi reagents [22]. Most siRNA identification algorithms take into account the basic constraints set for RNAi design [23] and include a search across the appropriate genome [for example basic local alignment search tool (BLAST-N) or Smith–Waterman-based tools] to ensure sufficient mismatch of the identified sequence with other sequences in that genome. Some algorithms also use parameters such as RNA secondary structure and thermodynamic stability [22]. However, retrospective analysis of several siRNAs, for example those in the human siRNA database (HuSiDa, http://itb.biologie.hu-berlin.de/~nebulus/sirna/v2/) has demonstrated no significant correlation with published criteria for RNAi reagent selection [24]. Thus, despite the publication of several criteria for efficient RNAi activity and the availability of several algorithms for the identification of such sequences, the current consensus among users of RNAi-based (especially shRNA) approaches appears to be that multiple (between 3–10 or in some cases more) oligonucleotides need to be designed to identify RNAi reagents with significant activity (defined ≥75% knockdown of the target gene at low levels of RNAi expression). The term reagents is preferable because the RNAi might be chemically synthesized oligonucleotides or vectors expressing RNAi oligonucleotides.

Although it has been argued that 29mer shRNAs might accord better suppression at lower molar concentrations of the RNAi reagent [25], most RNAi reagents are designed as 21mers with a 19 base complete match to their target RNA sequence and a single base overhang at either end. A recent study has shown that 27mer siRNAs can be up to 100-fold more potent than corresponding 21mer siRNAs, again at low nM and sub-nM concentrations of reagent [26]. The enhanced potency is perhaps linked to the fact that the 27mers are substrates of the DICER endonuclease, which is an end-recognition endonuclease capable of processing long dsRNAs into siRNA duplexes of ~21 nucleotides, thus directly linking the production of siRNAs to incorporation in the RISC [26]. This study also showed that 27mer siRNAs can sometimes be effective in long term silencing (up to 10 days) of genes that are resistant to silencing by 21mer siRNAs. These 27mers have not yet been shown to induce classic anti-viral responses, such as activation of interferon and dsRNA-activated protein kinase signaling (PKR) [26].

Usually, effective RNAi reagents are not specific to cell type, although there are temporal differences in the efficacy and duration (primarily in chemically synthesized reagents) of the knockdown that can be attributed to differences in protein stability and turnover in different cell lines and primary cells. There has been some debate on how easily an effective chemically synthesized RNAi reagent can be converted into an expressed shRNA. It is clear that sequences upstream and downstream of the actual siRNA (including the stem-loop structure) have an important role in determining the efficacy of a shRNA reagent and there have been some reports of miRNA-derived sequences that improve the efficacy of shRNA [27].

Specificity of RNAi

It is generally accepted that an RNAi-mediated change in a given functional phenotype needs to be verified by the use of at least two independent RNAi reagents to ensure that a change is primarily caused by the effect of the knockdown of the gene being studied. This unearths the important and widely debated issue of RNAi specificity and what are called 'off-target' effects of a particular RNAi reagent.

Although the RNAi phenomenon was initially discovered in plants [28] and lower eukaryotic organisms such as *C. elegans* [11], it was later observed to be an important regulatory mechanism in mammalian cells [29]. Double stranded RNA can easily be introduced into *C. elegans* [30,31] but introduction of long (>30 nucleotide) dsRNA into mammalian cells led to activation of the interferon response or a nonspecific shutdown of protein synthesis mediated by dsRNA-mediated kinases such as PKR [32]. This was overcome by using the products of DICER, the short dsRNAs that are the actual mediators of the RNAi response. These dsRNAs, 19–29 nucleotides in length, act as short interfering RNAs [33] and are transfected into mammalian cells (using conventional techniques) either as oligonucleotides or within appropriate expression vectors. Some reports initially indicated that the length of dsRNA used for RNAi-mediated gene silencing might not be directly associated with the development of an interferon or stress response [32], but optimization of transfection conditions and the use of highly efficacious siRNA oligonucleotides (21–29 nucleotides in length) at very low concentrations has been shown to alleviate the non-specific interferon response and suppression of protein synthesis [34].

However, not all off-target effects of RNAi reagents are concentration-specific, it has been observed that some of these effects appear to be cell-type- and assay-specific. Others have noted sequence-specific off-target effects [35] that can probably be explained by the mode of action of miRNAs. Although miRNAs, like siRNAs, require recognition of a short stretch of mRNA by distinct dsRNAs, there are three important differences between the processes by which miRNAs and siRNAs regulate gene expression. First, miRNAs can affect the expression of genes distinct from the ones their sequence comes from [36]. Second, miRNA synthesis results from the activity of Drosha (a RNase III endonuclease) on long primary nuclear miRNA sequences. This process releases pre-miRNA sequences that are then transported into the cytoplasm, processed further by DICER into mature miRNAs and, in animal systems, enter the RISC-mediated RNAi pathway. However, unlike siRNAs, the perfect match recognition sequence for miRNAs can be as short as 7–8 nucleotides and the complete sequence can tolerate mismatches and bulges [37]. Finally, miRNAs do not merely direct cleavage of mRNAs; they also affect the progression of the translational complex by binding to a site of partial complementarity in the 3′ untranslated region of the gene [38]. The similarities between siRNA and miRNA processing and recognition sites imply that a subset of siRNAs could have miRNA-like function [39] and could explain some of the off-target effects of siRNAs, especially those observed at low concentrations [40]. Because only partial mRNA complementarity is required for miRNA action, a siRNA that is rendered inactive against its target gene by a single mismatch in the sequence can still demonstrate nonspecific gene-silencing due to miRNA-like effects. Some off-target effects – including interferon induction as well as other cytotoxic effects – have also been attributed to the vectors used for RNAi expression [41]. It has been argued that pooling several siRNAs that target the same gene could actually improve specificity without affecting gene-silencing efficacy [42]. This might result from the fact that pooling allows lower concentrations of any one reagent to be used, thereby potentially decreasing non-specific effects while maintaining activity of the most effective siRNA. Despite discussions regarding the effect of siRNA pooling on knockdown efficacy and specificity, the use of pooled siRNAs as reagents in high-throughput RNAi library screens has advantages in terms of resource consumption.

To overcome any issues with specificity of RNAi reagents in target identification and validation experiments, it is important to use appropriate controls in RNAi-based experiments. In most studies where RNAi expression is vector-driven, either the empty vector or a vector containing scrambled versions of the shRNA sequence is used as the control. This is a viable option when dealing with a small number of target genes; however, for high throughput approaches using RNAi reagents, it becomes important to understand what the most appropriate controls should be. Several vendors provide scrambled or 'non-targeting' siRNAs (19mers chosen for the absence of any sequence matches across the genome) to be used as the control in experiments with chemically synthesized RNAi reagents. Because it has been observed that each RNAi reagent (including any non-targeting RNAi reagent) has a individual molecular signature in expression profiling experiments (B. Li, A. Hill and M. Chatterjee-Kishore, unpublished), it is advisable to evaluate and use a panel of non-targeting siRNAs that can best signify the range of signals that should be called negative in a screen. However, the definition of the right controls for RNAi-based experiments is still a debatable issue and a particular cause for concern in high-throughput target identification studies using RNAi libraries. For virally expressed RNAi libraries, the signal intensities have been normalized to background signal from the vector alone or a separate normalization vector. Several laboratories have developed vectors that have genetic 'barcode' sequences (either as part of the shRNA sequence itself or as a separate sequence on the vector) that can be scored using microarray analysis to identify the shRNAs that had the observed effect in the screen. This is particularly useful when screening is carried out using pooled reagents.

TABLE 1

A list of vendors of libraries of RNAi reagents

Application	RNAi platform	Advantages	Disadvantages
High throughput assays for target identification			
Libraries of chemically synthesized RNAi reagents		No library maintenance requirements (i.e. DNA and/or virus preparation)	Finite resource
		Quick screens possible Easy to use	Cells need to be easily transfectable with siRNAs
			Assays need to be of relatively short duration (2–144 h on average)
			Kinetics of gene/protein knockdown needs to be relatively short
	Libraries of pooled siRNAs	Lower number of plates in primary screen, lower automation needs	Need to re-confirm data with individual siRNAs against each 'hit'
	Libraries of multiple individual siRNAs per gene	Ability to go directly from primary screen data to hit validation if more than one siRNA per gene scores in screen	Significantly higher automation needed
Libraries of vector-expressed RNAi reagents		Unlimited resource Longer term assays possible	Significant library maintenance requirements (i.e. DNA and/or virus preparation)
	Plasmid-expressed		Cells need to be easily transfectable with plasmids
	Virus-expressed	Significantly larger repertoire of cells can be used	Special containment might be needed for some viruses
Target validation			
Chemically synthesized RNAi reagents		Reagents are easily available from commercial vendors	Cells need to be transfectable with siRNA
		Easy to use	Assays need to be of relatively short duration (24–144 h on average)
			Kinetics of gene/protein knockdown needs to be relatively short
			Delivery of siRNAs and stability of the siRNAs *in vivo* can be issues.
Vector-expressed RNAi reagents		Can be used in a larger number of cells, especially if expressed from viral vectors	Vector construction and virus preparation can be issues
		Long-term assays possible, particularly when using lentiviral vectors which integrate into host genome	Special containment might be needed for some viruses
		In vivo delivery might be less challenging for certain organ systems	

RNAi as a tool for gene perturbation and target validation

Gene expression profiling has become an important approach for target identification. However, as discussed, this approach only offers a chance to evaluate the association between the expression of a particular gene with the phenotype under study. The next step requires demonstration of a direct causal link between the identified genes and the phenotype. RNAi (in conjunction with conventional approaches such as the use of dominant negative and/or constitutive active versions of the genes and the use of knockout, knockin and transgenic animals) offers a spectrum of tools for target validation. There are many advantages to using RNAi for gene perturbation experiments: Table 1 provides an initial checklist that can help decide which form of RNAi reagents should be used for a particular experiment. RNAi invokes post-transcriptional gene silencing and therefore does not lead to compensatory transcription, which can be observed in cases of gene deletion (including knockouts) [10], because appropriately designed RNAi reagents are specific for their target genes [18]. RNAi (particularly the chemically synthesized oligonucleotide approach) is a fast and inexpensive route for initial target validation in cultured cells. The disadvantage of experiments using chemically synthesized RNAi is that over a relatively short period of time (typically a week) these dsRNAs are removed from the cells by nuclease action [43]. One of the ways one can use RNAi for longer-term experiments is to express RNAi using plasmid or viral vectors. When expressed from viral vectors, expression of shRNAs has been shown to be long-term (over a month or more). Virally expressed shRNA is

currently being used for long-term expression studies and for the modulation of gene expression in cell lines that are difficult to transfect, primary cells and *in vivo* [44–47].

It is important to keep in mind that although gene knockdown can offer a quick and easy experimental approach to evaluate whether the target needs further follow-up, the phenotypic effect of gene knockdown might not necessarily reflect a drug-mimetic phenotype. For example, a drug against a kinase would target its kinase activity but an RNAi reagent would stop expression of the kinase, thereby shutting down activities associated with domains that are not associated with its kinase activity. Thus, both for cultured cells and *in vivo* target-validation experiments, chemically synthesized and expressed RNAi reagents can be used. However, it is always advisable to use RNAi-based approaches in conjunction with more conventional techniques, such as expression of functional dominant negative and constitutionally active versions of the genes, that might alter gene function and not gene expression. RNAi could offer significant advantage over conventional knockout technologies in terms of the speed with which one can obtain a cohort of animals to initiate a study. It is still early days for the use of RNAi *in vivo* but it appears that this approach could allow for the study of *in vivo* phenotypes mediated by the dose-dependent reduction of gene expression, which is not the case for gene knockouts. This area of RNAi use is still in its infancy, at least with regard to use in the pharmaceutical industry, and needs further evidence to confirm reproducible efficacy.

RNAi delivery

One of the major challenges for using the RNAi platform in cultured cells or *in vivo* is delivery. Chemically synthesized RNAi reagents can easily be transfected into most cell lines; however, for efficient performance of the RNAi reagents, optimization of transfection conditions is essential. The availability of a large number of siRNA transfection reagents probably reflects the need to evaluate transfection reagents for every new cell system. The use of siRNA reagents under optimal transfection conditions (maximal efficacy and minimal toxicity) leads to a significant decrease in the stress-related transcriptional signatures often associated with the use of siRNAs. Commercially available cationic and anionic lipid-based transfection reagents are most commonly used, for example, Lipofectamine 2000™ (www.invitrogen.com), TransitTKO® (www.mirusbio.com) and HiPerFect (www.qiagen.com). However, for some cell types that are not amenable to lipid-based transfection, electroporation [recently introduced by Ambion (www.ambion.com)] and nucleofection [introduced by amaxa (www.amaxa.com)] have been used. However, electroporation and nucleofection require the use of large amounts of siRNAs (up to 1 μM in the final solution in some cell lines), which can be associated with greater incidence of off-target effects. Expression of shRNAs in such cell lines and primary cells is sometimes possible using viral vectors, as described in a previous section. One recent approach to delivery of RNAi reagents into cultured cells is the use of reverse transfection and cell-array-based approaches [48]. These techniques have the potential to significantly increase throughput for RNAi-based screens but not all cell types have been shown to be amenable to these approaches.

Efficient delivery is vital for evaluation of any reagent (chemical compounds, biotherapeutics or DNA based reagents) in vivo. Following an initial surge in the use of RNAi in cultured cells and *ex vivo* cellular systems, a lot of effort has recently been applied to developing novel delivery strategies that can make use of RNAi technology *in vivo*. Much of this thrust has come from the interest in using RNAi for therapeutics but increasingly the same level of effort is being applied to *in vivo* target validation efforts [49]. Delivery of RNAi reagents *in vivo* almost completely depends on the target organ or cells and on the desired range of expression (i.e. whether it needs to be focal, systemic, targeted or inducible). For example, tail vein injections of either naked siRNA or vector expressed RNAi reagents induce accumulation of the injected material mostly in the liver, which makes this approach most suitable when the liver is the target organ [50]. Strategies have recently been evaluated for delivery of RNAi reagents into the lung using the intra-nasal delivery route [51] and for delivery into tumors [52]. Other delivery options that have been used with compounds or antisense reagents, and can therefore be considered for RNAi, are intrathecal injections into the spinal column, intraosseous delivery or calvarial injections into the bone. For systemic or organ-specific delivery of RNAi reagents, depending on the environment into which the RNAi reagent is delivered, care needs to be taken to prevent, or at least slow down, nuclease-mediated degradation. Modifications of the dsRNA used for RNAi, either in the nucleic acid backbone or with the use of alternate nucleic acids is one way of trying to improve *in vivo* stability with sustained efficacy. A recent review describes the need for such modifications, especially for *in vivo* studies, and evaluates the various modifications that have been used [53]. Formulation is also crucial to help ensure the delivery of a sufficient amount of the RNAi reagent at the site where its activity is required. Delivery approaches include the use of viruses expressing the RNAi reagent [54], modified liposomes [55], cholesterol [56] and nanoparticles [52].

RNAi-based approaches for drug target identification

One of the greatest benefits of RNAi, which is now being used for drug target discovery, is the ability to carry out high-throughput RNAi library screens to assay the direct correlation between the modulation of gene expression and functional phenotypes [12]. This potential use of RNAi was identified early, and several vendors offer chemically synthesized RNAi libraries that span entire gene families [Dharmacon (www.dharmacon.com) and Ambion), the

TABLE 2

Selected sources of RNAi reagents

Commercial Vendor/Academic Institution	Platform	Library details	Status	Contact
MIT/Broad Institute RNAi consortium	Viral expression of shRNA	15,000 genes of each species	Library generation in progress	www.broad.mit.edu
Netherlands Cancer Institute	Viral expression of shRNA	~10,000 human genes	Complete, proprietary	www.nki.nl
Genomic Institute of the Novartis Foundation	Plasmid expression of shRNA	Human druggable genome; ~5000 genes	Complete, proprietary	http://web.gnf.org/scientific/cellmol.html
Exelixis	dsRNA	Whole *C. elegans* genome	Complete, proprietary	www.exelixis.com
Cenix Biosciences	dsRNA	19,075 *C.elegans* genes	Complete, proprietary	www.cenix-bioscience.com
Harvard Medical School	Long dsRNA	All predicted *Drosophila* open reading frames	Complete, version 1 available through Open Biosystems	http://flyrnai.org/RNAi_index.html, www.openbiosystems.com
Cold Spring Harbor Laboratories; Harvard Medical School–Open Biosystems	Viral expression of shRNA	~16,300 human and ~9800 mouse genes	Additions to library ongoing, current collection available through Open Biosystems	www.cshl.edu; www.openbiosystems.com
System Biosciences	Viral expression of shRNA	Up to 50,000 human and 40,000 mouse genes	Available	www.systembio.com
Dharmacon	Chemically synthesized siRNA pools	Human, mouse and rat gene family based sets; siGENOME™ targeting 66,000 mouse, rat and human genes	Available	www.dharmacon.com
Ambion	Chemically synthesized siRNA	Human genome, gene family and custom sets; ~13,000 drosophila reagents	Available	www.ambion.com
Qiagen	Chemically synthesized siRNA	Whole human, mouse and rat genome; gene-family-based sets	Available	www.qiagen.com
Galapagos Genomics / Galdeno	Viral expression of shRNA	4700 human druggable transcripts	Available	www.galadeno.com

whole druggable genome or the entire human transcriptome (Qiagen). Formats range from the use of pools of siRNAs against each gene (e.g. Dharmacon SMART Pools™) to using 2–3 individual siRNAs per gene (Qiagen and Ambion). Several different formats exist for expressed RNAi libraries, chemically synthesized, plasmid or viral vector-expressed, that target either mammalian or invertebrate genomes (Table 2). An effort is currently in progress at the Broad Institute, Massachusetts Institute of Technology to develop a viral expression library that will eventually be able to target the entire human and murine transcriptomes (www.broad.mit.edu/rnai_platform). Other RNAi libraries have been in use for the past couple of years and several publications indicate the power of this technology: a group at the Netherlands Cancer Institute (www.nki.nl) has identified novel genes involved in p53 signaling [57]; a group at the Cold Spring Harbor Laboratories has worked on deciphering pathways deregulated in cancer [58]; another group at the Harvard Medical School is involved in understanding signaling cross-talk using the drosophila RNAi libraries and correlating the data back to the more complex vertebrate genomes (http://flyrnai.org) [59]; and a group at the Genomics Institute of the Novartis Research Foundation (www.gnf.org) has identified novel signaling components in the tumor necrosis factor (TNF)-related apoptosis inducing ligand 1-induced apoptosis [60].

Therapeutic potential of RNAi

Recent studies have created significant excitement about the use of the RNAi reagents as therapeutics [14,61]. One of the most obvious challenges in this arena is delivery. Several avenues of research are currently being pursued, including delivery of 'naked' or unmodified and unformulated chemically synthesized RNAi reagents, viral vector-based delivery and, more recently, delivery using large moieties such as cholesterol, nanoparticles or pegylated immunoliposomes [52,54,55]. Several companies have launched campaigns to evaluate the clinical efficacy of siRNAs in diseases such as age-related macular degeneration (Alnylam and Sirna Therapeutics), Huntington's disease (Sirna Therapeutics) and for more mundane purposes such as hair removal (Sirna Therapeutics). Although the most promising first use of siRNAs as therapeutics is likely to be for diseases where local delivery would be sufficient, ongoing studies, if successful, will provide important data for the development of RNAi as therapeutic option for viral infections, cancer, autoimmune diseases and possibly for genetic disorders.

Summary

RNAi is still a technology in its early stages of development. The pathway leading to post-transcriptional gene silencing mediated by siRNAs is relatively well understood but several

questions, especially regarding the nature and cause of the off-target effects of siRNAs, are just beginning to be answered. However, efforts to harness the immense potential of siRNAs for drug target discovery and to develop these siRNAs as drugs are in full swing. We are entering the next and perhaps the most crucial phase of the development of the RNAi platform. The next 5–7 years will either see the beginning of the use of siRNAs as therapeutic entities or the vanishing glory of what at the moment appears to be a technology with significant promise.

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