DDT Vol. 7, No. 20 October 2002

RNAi: gene-silencing in therapeutic intervention

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Several rapidly developing RNA interference (RNAi) methodologies hold the promise to selectively inhibit gene expression in mammals. RNAi is an innate cellular process activated when a double-stranded RNA (dsRNA) molecule of greater than 19 duplex nucleotides enters the cell, causing the degradation of not only the invading dsRNA molecule, but also single-stranded (ssRNAs) RNAs of identical sequences, including endogenous mRNAs. As such, RNAi technology is currently being evaluated not only as an extremely powerful instrument for functional genomic analyses, but also as a potentially useful method to develop highly specific dsRNA based gene-silencing therapeutics.

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▼ There are essentially three potential sites for therapeutic intervention: transcriptional, post-transcriptional, and post-translational. The vast majority of traditional drug targets has been proteins, largely enzymes and receptors, and by definition fall into the post-translational class. The therapeutic agents most commonly used to regulate these proteins are generally small molecules with a molecular weight of 500 daltons or less. These usually consist of synthetic or naturally occurring organic compounds and peptides.

The mid-1980s presented a potential new avenue for therapeutic intervention with the discovery of antisense technologies where target mRNA transcripts hybridize in a sequence-specific manner to homologous RNA, DNA or chemically altered nucleic acids, thereby inhibiting their expression [1] post-transcriptionally. In theory, this type of approach could selectively silence any gene product before it was translated, and was therefore regarded with great enthusiasm. Although antisense has been useful in the validation of many targets, its success as a therapeutic has been limited to one approved product – Vitravene™ (ISIS; http://www.isip.com). Unlike classical small molecules, antisense nucleic acids have molecular weights >1000 Da resulting in significant delivery problems. Other strategies for therapeutic intervention at the post-transcriptional level involving small molecules are showing great promise [2] but it is still too early to determine whether these approaches will ultimately result in drugs.

In the early 1990s, nucleic acid molecules were used to directly target the transcriptional regulation of gene expression. 'Triplex' generating reagents opened the window for researchers to inhibit the transcription process itself by introducing a nucleic acid molecule that hybridizes to a specific sequence of DNA within a cell to block cellular machinery from acting to initiate or elongate gene transcription [3]. Like antisense, delivery issues and transitory inhibitory effects have limited the success of this strategy.

RNA interference (RNAi) methods are also based on nucleic acid technology, however unlike antisense and triplex approaches, the dsRNA activates a normal cellular process leading to a highly specific RNA degradation, and perhaps more importantly, a cell-to-cell spreading of this gene silencing effect in several RNAi models. As yet this 'systemic' spreading has not been observed in mammals; however, several recent RNAi studies coupled with extensive mouse and human cDNA homology to a newly discovered molecular component of cell-to-cell RNAi spreading in worms are truly encouraging [4]. This systemic property would provide great promise for the use of RNAi as a therapeutic because the delivery problems that have plagued the other nucleic acid based technologies could be at least partially alleviated in RNAi-based gene silencing applications. Other features of this gene silencing platform and its use as a therapeutic are discussed below.

PII: S1359-6446(02)02474-1

RNAi: a brief mechanistic description

In both plant and animal cells, intracellular exposure of a double-stranded RNA (dsRNA) sequence can result in the specific post-transcriptional gene silencing (PTGS) of the homologous cellular RNA; this phenomenon is known as RNAi [5]. The precise biochemical mechanisms involved are currently being elucidated in several systems [6] and it appears that many of the steps in this process are well conserved. To briefly summarize, the RNAi pathway consists of the presentation of a 'triggering' dsRNA that is subsequently processed into 21–25 bp small interfering RNAs (siRNA) by an RNaseIII-like enzyme called Dicer [7,8]. Interestingly, this enzyme also includes a helicase domain, suggesting that unwinding of the triggering dsRNA might be required for the subsequent target recognition event, presumably guided by traditional Watson–Crick base-pairing in the enzyme–substrate complex. This 21–25 bp siRNA species is then incorporated into an, as yet, crudely defined multi-subunit RNA-induced silencing complex (RISC), which targets the unique cellular RNA transcript for enzymatic degradation. RNA hydrolysis occurs within the region of homology directed by the original siRNA [9], thereby selectively inhibiting target gene expression (Fig. 1). A cellular mechanism involving the RNA-dependent RNA polymerase (RdRp)-mediated generation and amplification of single-stranded RNA into dsRNA precursors is also depicted [10].

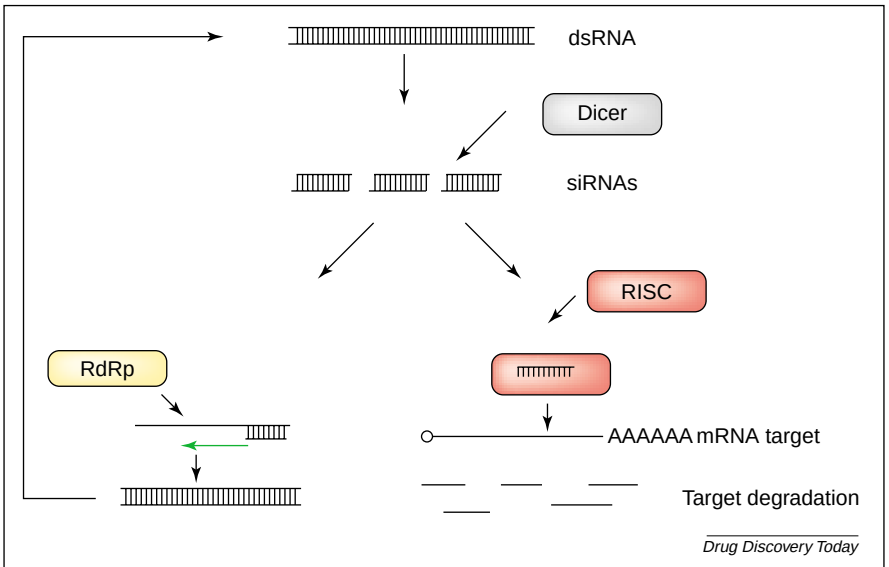

Figure 1. Overview of RNAi pathway. Intracellularly synthesized or exogenously administered dsRNA is cleaved by the enzyme Dicer into 21–25 nucleotide siRNAs. siRNAs become associated with the RNA-induced silencing complex (RISC), which uses the antisense strand of the siRNA to bind to and cleave the target mRNA. The siRNAs can also be used as primers for the generation of new dsRNA by RNA-dependent RNA polymerase (RdRp). This newly formed dsRNA can then also serve as a target for the Dicer enzyme.

The discovery and conservation of RNAi

RNAi was originally discovered as an endogenous property of plants [11,12], and perhaps exists as a defense mechanism against viral pathogens or uncontrolled transposon mobilization [10]. Subsequently this phenomenon was observed and experimentally demonstrated in worms [5,13], flies [14,15], and vertebrates [16–18]. Indeed a variety of endogenous small, regulatory RNAs have been identified in several organisms, and are assumed to elicit their roles via an RNAi degradative pathway [19–21]. A collection of excellent reviews that provide comprehensive coverage of the botanical, mechanistic, evolutionary and historical perspectives of RNAi are available [8,22–25]. This review will instead focus on recent technical advances of RNAi model systems in mammalian cells and whole animals and attempt to provide an early assessment of the potential for using RNAi technologies to directly treat human disease.

dsRNA and the mammalian PKR response

One potential obstacle to the therapeutic use of RNAi in mammalian cells is the activation of a dsRNA-dependent protein kinase (PKR) by dsRNA [26]. PKR activation leads to a generalized inhibition of translation as well as to the induction of apoptosis. Exposure of cells to dsRNA can lead to activation of the type 1 interferon response and the STAT-mediated expression of PKR. In addition to activating the expression of this kinase, the binding of dsRNA to PKR directly activates it, leading to the phosphorylation of the small subunit of the eukaryotic initiation factor 2 (eIF2α) resulting in a global shutdown of translation. dsRNA also promotes the synthesis of 2′–5′ polyadenylic acid, which, in turn, activates the non-specific Rnase L. These collective phenomena can dramatically alter cellular metabolism and often activate apoptotic pathways [27]. It seems likely that the direct transfection of large dsRNA will be severely limited as a gene-silencing tool in higher organisms. This pathway can be circumvented, albeit transiently, with the use of smaller dsRNAs and recent evidence suggests that vector-mediated delivery of larger dsRNAs can also bypass this apparent limitation (see later).

'Systemic' gene silencing

The systemic effect in plants

Local exposure to dsRNA, or to a viral or plasmid vector expressing dsRNA, is often followed by a widespread gene silencing effect throughout most, if not all, tissues of the

organism. This effect is termed 'systemic' suppression and is thought to involve at least two components; a previously described local and cellular PTGS effect, and a separate, but related global gene-silencing mechanism often referred to as transcriptional gene silencing (TGS).

TGS appears to have strong epigenetic (i.e. genomic) components to gene silencing in plants and is also heritable [28–30]. As with most RNAi-mediated phenomena, TGS was first identified in plants [11]. Subsequently, this apparently bimodal systemic effect has been demonstrated in animals (see later). Many current investigations attempting to define the molecular components of RNAi systemic response in plants are focusing on the TGS component especially the chromatin reformatting produced by alkylation of both DNA and histone substrates that appears to be initiated by dsRNA [31].

The systemic response in animals

Several cases of systemic RNAi-mediated gene silencing have been reported in nematodes [5,32,33] but until recently, these remained the only examples of the systemic response within the animal kingdom.

However, a recent presentation by the Mirus Corporation (http://www.genetransfer.com) at the *2002 Taos Conference on RNA Interference, Cosuppression, and Related Phenomena,* describes an RNAi experiment using chemically synthesized siRNA probes that generate 30–90% suppression of a stably integrated green fluorescent protein (GFP) transgene in mouse [34]. Initiated with a single high-pressure hydrodynamic tail vein injection, gene silencing in an adult mouse encompassed most tissues analyzed, but was unfortunately short lived, with a half-life of only 2–3 days. This study, although encouraging, is clearly not relevant to human therapies as it involves high pressure and massive volume delivery schemes.

Pachuk and colleagues from Nucleonics (http://www.nucleonicsinc.com) went further and presented the nearly complete *in vivo* silencing of co-injected interleukin-12 (IL-12) gene expression in an adult mouse model using single intra-muscular injection protocols of plasmid-based vectors to express intracellular dsRNAs [35]. Significantly, this response elicited by a pharmacologically relevant delivery system was long-term, exhibiting duration of at least 120 days.

A crucial question for pharmaceutical intervention remains; can researchers devise experimental, and subsequently therapeutic applications of RNAi that will be selective, long term, and systemic to modulate gene targets distal from the dsRNA inoculation area? That is, can biologists reproduce the well-documented systemic effect already observed in plants and worms within the human body? Answers to this pivotal question will determine to a large extent the potential application of this gene silencing technology to human therapeutics.

Towards a molecular description of the systemic silencing

A novel class of genetic loci named systemic RNA interference deficient (*sid*) mutants [4] has been isolated from the worm *Caenorhabditis elegans*. Employing a novel RNAi screening regimen, 106 independent mutant alleles were characterized and could be assigned to three complementation groups, *sid-1*, *-2*, and *-3*. These gene products are not required for the onset of gene silencing, but are required for its spread throughout the worm. The *sid-1* mutant was analyzed and appears to be a membrane protein with 11 transmembrane spanning domains, not unlike the ABC family of transporters involved in macromolecular membrane transit. Interestingly, this protein has homologues in both mouse and human sharing significant amino acid identity and a strikingly similar predicted transmembrane profile. *Drosophila*, thought not to exhibit the systemic RNAi effect, apparently lacks such a gene homologue.

It is postulated that this *sid-1* membrane protein may pass the RNAi signal from cell to cell. Such a transfer would necessarily involve a sequence-specific component, most probably nucleic acid. If demonstrated in humans, the modulation of the systemic response could prove be a fertile area of therapeutic intervention where biologists may wish to limit or amplify this phenomenon. For now the scientific community eagerly awaits the molecular description of the sibling *sid-2* and *sid-3* systemic complementation groups.

dsRNA delivery strategies

Chemically synthesized siRNA

Tuschl and co-workers demonstrated that long transfected dsRNAs were processed into shorter 19–21 bp siRNAs [7]. This observation directed them to exploit these molecules as a means to selectively target and suppress messages in a diverse range of cell types. Significantly, it was found that these smaller siRNAs did not induce the PKR suppressive effects of their longer dsRNA relatives [36].

Thus, the types of siRNAs used to generate the RNAi mediated silencing have been synthesized to closely mimic those found *in vivo* following the digestion of dsRNA by Dicer. Usually these are chemically synthesized, coding and non-coding 21-mers, which are annealed before delivery to the test cells or organisms.

This type of technology has been used to elicit transient RNAi responses in *Drosophila* embryos [9], *C. elegans* [37,38], *Xenopus* embryos [17], and several mammalian cell lines [36,39,40].

Although chemical synthesis of siRNAs has been the most commonly used method to generate siRNAs, T7-transcribed

siRNAs [39], as well as siRNAs isolated from *Drosophila* embryo protein extracts [41], have also been effective.

Factors which could ultimately limit the usefulness of siRNAs include a relatively short and transient period of activity, particularly in human cells, and often a strong preference for certain sequences of the mRNA target for optimal activity [40].

siRNA expressing plasmids

In an effort to circumvent the temporally limited activity of exogenously added siRNAs, several groups have used RNA polymerase III (pol III) promoters, such as U6 or H1, to allow for the intracellular expression of siRNAs [42–44]. These promoters have the advantage of being 'self-contained' pol III promoters, in that all essential transcription elements are located upstream. Transcripts derived from these promoters end in a run of 4–5 thymidines, which allows for the specific determination of a transcript length [45]. Pol III transcripts terminate following the second thymidine resulting in a two nucleotide overhang reminiscent of the most effective siRNAs incorporated into the RISC complex.

Intracellular expression of siRNAs from pol III promoters has recently been shown to be effective in establishing RNAi in mammalian cells [42–44]. These siRNAs have been expressed in two ways: coding and non-coding strands of a potential siRNA are driven from separate promoters and the expressed transcripts anneal in the cell nucleus, or, alternatively, hairpin constructs are expressed from a single pol III promoter so that the transcript folds back upon itself to form a double-stranded structure. In both cases, the endogenous RISC-mediated RNAi pathway is initiated resulting in efficient gene silencing. Levels of RNAi following intracellular expression in this manner appear to compare favorably with, or outperform, the earlier exogenous administration of synthetic siRNAs. The use of a plasmid-based, pol III promoter system to intracellularly produce siRNAs could allow for a longer period of expression as compared with exogenously added siRNAs, particularly in cells where the expression unit becomes integrated into the host genome [42,44,46].

Although the pol III system appears to be an effective means to elicit RNAi, some questions and concerns remain. The relatively small size limit of transcripts expressed by a pol III promoter [45] might be adequate for the current siRNA technology, but could ultimately put an upper limit on the number of different siRNAs that can be generated from a single transcript.

Vector-based 'large' dsRNA delivery

Different siRNAs and siRNA-expressing plasmids have shown varying abilities to induce RNAi for an identical target mRNA [40,43]. Therefore, previous knowledge of the suppressive activities of individual siRNAs, usually involving numerous synthetic siRNA pilot experiments, is required for their use. Longer dsRNA molecules (>50 bp) have the advantage of presenting multiple Dicer-derived siRNAs to the cell thus allowing the cell to employ the endogenous dsRNA silencing pathway to choose the most effective silencing siRNA(s).

The general and sequence-independent inhibitory effects of exogenously delivered long dsRNAs mentioned above has caused concern over their effectiveness in mammals and their eventual use as therapeutics. Long dsRNAs have been successfully used to generate RNAi in *Drosophila* [7], *C. elegans* [5], and *Trypanosoma brucei* [47]; however, these organisms do not possess the same stress response pathways as vertebrates. In mammalian cells, the direct transfection of long dsRNAs leads to both sequence-specific gene silencing as well as a generalized, sequence-independent suppression [36] of cellular processes, presumably mediated by the activation of the stress response pathway. Importantly, the RNAi pathway does function as expected in these cells as the transfected dsRNA serves as a substrate leading to the intracellular appearance of 21 nt siRNAs. A dramatic example of vertebrate gene silencing by long dsRNA is shown in Fig. 2, where direct RNA transfections were shown to elicit an anti-GFP RNAi response in zebrafish embryos [16]. It appears, however, that embryos and some embryonic cell lines could be refractory to the cellular stress induced by the direct transfection of longer dsRNA, as they might be incapable of generating the generalized suppressive PKR response seen in other cell lines and adult animals [16,48].

The ability to express intracellular, small, double-stranded siRNA has been demonstrated by several groups mainly through the use of plasmids containing RNA pol III promoters. Significantly, Paddison *et al.* have shown that RNA pol II-generated intracellular expression of a relatively large 500 nt hairpin-structured dsRNA in a mouse embryonic cell line induces RNAi and stable gene silencing in those cells [48].

A recent presentation at the previously mentioned *Taos Conference* demonstrates that the plasmid-mediated expression of relatively long intracellular dsRNA in non-embryonic mammalian cells is able to induce efficient RNAi-mediated gene silencing for several weeks without eliciting the generalized PKR-mediated suppressive response [35]. As previously noted, injection of plasmid DNA capable of expressing long cytoplasmic dsRNA elicited a strikingly similar effect in a systemic manner in an adult mouse system.

Ultimately, this intracellular expression of long dsRNA might prove to be the most advantageous method of

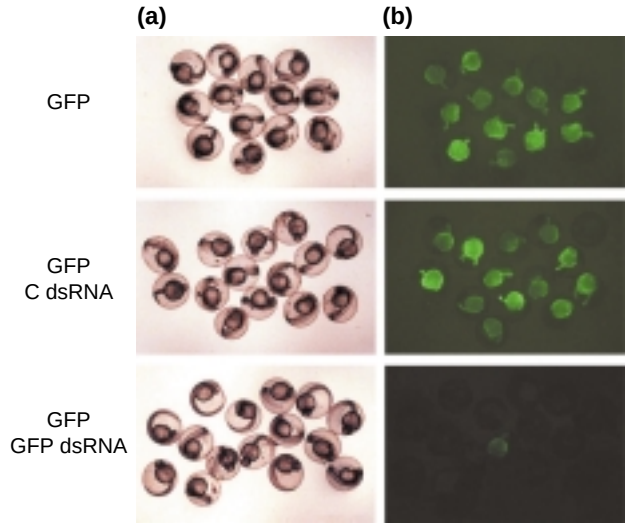

Fig. 2. RNAi in zebrafish embryos. The left panels **(a)** show injected embryos and the right panels **(b)** are the same embryos visualized to reveal green fluorescent protein (GFP) expression. Row 1 has only the control GFP expression vector injected. Row two has the same GFP plasmid but also has a control dsRNA-expressing plasmid (C dsRNA) not targeted to GFP. Row 3 has a GFP-specific dsRNA plasmid co-injected demonstrating the effectiveness and specificity of RNAi in vertebrate embryos. Reproduced, with permission, from [16].

inducing RNAi in mammals. These technologies will essentially allow for the simultaneous expression of a large number of siRNAs that are derived from a single precursor dsRNA, some of which should elicit a strong and sequence-specific RNAi response without inducing a generalized suppressive or apoptotic response. A longer dsRNA would permit targeting of more than one message with a single construct and could also potentially alleviate the development of resistance to potential RNAi therapies resulting from point mutations; both issues should prove to be particularly important for viral or oncological applications. Table 1 provides a summary of existing dsRNA delivery systems.

Table 1. Mammalian dsRNA delivery platforms

Synthetic RNAs	dsRNA size	Duration	PKR stress response	Refs
siRNA	19–21 bp	Transient	–	[9,36–40]
dsRNA	>50 bp	Transient	+++	[36]
Plasmid-generated RNAs (plasmid sizes ~3–6 kb)				
siRNA (pol III)	<200 bp	Transient*	–	[42–44,46]
dsRNA (pol II and T7)	50–2000 bp	Sustained	–	[35,48]

*unless incorporated as a stable transgene (refs [42,44,46])

RNAi and functional genomics

One of the earliest and, to date, most productive uses of RNAi technology in drug development has been its application to functional genomic analyses. In these studies one can isolate and identify components of complex pathways and assess their relevance to various drug discovery applications. For example one could determine with relative ease which specific receptor, kinase, or other effector is involved in an agonist-elicited response. An early experiment employing large dsRNAs demonstrated the use of RNAi in dissecting the insulin signal transduction pathway of *Drosophila* [49]. A particularly elegant use of RNAi has recently been described to elucidate the pathways involved in cellular lipid homeostasis [50].

As one might expect, several commercial vendors already offer products to perform just these types of experiments [e.g. http://www.ambion.com, http://www.dharmacon.com, http://www.neb.com, http://www.promega.com]. Most of these cell-based functional genomic applications have employed synthetic siRNA, which is sufficient for most transient transfection protocols used in pathway-profiling experiments. One drawback of these methods will certainly be the duration of the siRNA-mediated silencing effect, as proteins with longer half-lives might be refractory to this analysis.

The reports described above using RNA pol III plasmid systems will surely open the door to commercially available systems for the routine generation of both transiently and stable RNAi-expressing cell lines. Coupling this plasmid transfection technology with inducible vectors (tet, hormone receptors, and so on) could facilitate gene-silencing analyses by allowing the temporary suppression of normally lethal knockouts (e.g. 'essential genes') and also aid in dissecting the sequential or temporal constraints of certain cellular phenomena.

RNAi methods are also being used in animals to quickly generate phenotypic knockouts circumventing the more time consuming and laborious gene replacement-based genotypic knockout regimens. Inclusion of tissue-specific and rheostatic promoters could further empower RNAi technology in transgenic animals as well.

Other considerations

Designing dsRNA for selective gene silencing

Basic numerical calculations suggest that any 21 nt region of homology (i.e. siRNA) would be sufficient to provide the sequence specificity required to uniquely degrade any mammalian mRNA; $(4\text{ bp})^{21}$ = ~1000 human genomes. This calculation, however, ignores at least one potential source of error: highly homologous multi-gene families resulting from relatively recent gene duplication events. To illustrate this,

one can perform a basic homology alignment within the phosphodiesterase PDE4 gene sub-family, or several other gene families, and detect numerous regions of identity that exceed this minimal 21 nt limit. Thus, it may be useful to avoid highly conserved domains (i.e. catalytic, ligand binding, and so on) when designing dsRNA for RNAi experiments and even consider targeting the more rapidly evolving and divergent 5′ and 3′ untranslated regions (UTRs).

In conclusion, it should be emphasized that when selecting regions of a transcript to target, the specific dsRNA design should minimally account for any potentially cross-reactive mRNA species. Alternatively, this property could be exploited to design dsRNAs that could silence an entire class of targets. For either application, several design programs exist to assist in this effort [see http://www.ambion.com and http://www.dharmacon.com].

RNA and DNA delivery vehicles

Nucleic acid delivery will surely play a significant, if not rate-limiting, role in any mammalian gene therapy application. RNAi-based therapies are surely not insulated from these technical challenges, however, as previously mentioned this delivery platform could provide a unique advantage to RNAi researchers and enable them to harness the process of systemic cell-to-cell gene silencing observed in lower animals [5,32,33]. Clearly, a successful RNAi delivery strategy would also include the necessary assurance that the generalized dsRNA-mediated PKR stress pathway is not widely activated.

Notably, several physical plasmid-mediated and viral vectored technologies, some novel and others using improved reagents, are currently emerging that could improve both the efficacy and tissue specificity of RNA or DNA delivery (reviewed in [51,52]). To this end, researchers have found that the anesthetic bupivicane, when complexed with DNA and cationic lipids, can efficiently deliver plasmid DNA to most tissues of the mammalian body [53]. Unfortunately, as with most nucleic acid delivery schemes, the blood–brain barrier appears to remain refractory.

It is hoped that a simple inoculation of dsRNA, or a dsRNA-expressing vector, might provide a long term and widespread systemic gene-silencing effect and experiments to address the putative systemic transfer and the TGS-mediated components of the RNAi phenomenon in mammals are under way in many laboratories.

As investigations into the molecular mechanisms of these processes advance, it might be possible to design small molecules or even RNAi regulators to control the amplitude, duration and possibly the tissue distribution of any potential RNAi therapy.

Even in the absence of a long term and wholly systemic tissue RNAi response, human serum-based or superficial molecular targets could still be accessible via simple intravenous injection or direct topical application of RNAi reagents. Significantly, this class of pharmacological target is quite large and would include pathogenic or recurrent infectious viral, and perhaps even bacterial, targets.

Future applications – RNAi as a drug?

RNAi is an extremely powerful research tool and clearly holds significant potential for a wide variety of gene silencing applications. Many researchers in the field suspect that this technology will greatly exceed the promise of the much-heralded antisense and triplex methodologies of the past decades. RNAi proponents have generated a huge scientific following in a relatively short time-period, considering that it was only four years ago that this phenomenon was first described in animal cells. One should expect this rapidly acquired enthusiasm to generate significant advances for years to come. The recent publications describing the use of siRNAs expressed by eukaryotic RNA pol III promoters are only the most recent demonstrations of this technical momentum, and it is hoped that crucially needed *in vivo* RNAi experiments in mammals will follow within the year.

It is entirely possible, and maybe even likely, that a better description of the systemic nature of this response in whole animals combined with the ongoing and coincident improvements of *in vivo* nucleic acid delivery technologies could enable RNAi to be used therapeutically far sooner than many scientists now dare to predict.

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