

Technology review

Towards *in vivo* application of RNA interference – new toys, old problems

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Abstract

RNA interference (RNAi) is the sequence-specific degradation of mRNA by short double-stranded RNA molecules. The technology, introduced only 5 years ago, has stimulated many fantasies regarding the future of functional gene analysis and gene therapy. Given its ease of application, its high efficiency and remarkable specificity, RNAi holds great promise for broad *in vitro* and *in vivo* application in all areas of biomedicine. Despite its potential, the major obstacle to the use of RNAi (as for all previous gene silencing approaches) is the need for efficient and sustained delivery of small interfering RNA into primary mammalian cells, and specific targeting of particular cell types *in vivo*.

Keywords: functional genomics, gene silencing, primary mammalian cell, small interfering RNA, transfection

Introduction

In the postgenomic era it has become a major challenge to develop efficient reverse genetic approaches (i.e. from genotype to phenotype) to evaluate the function of a vast number of newly identified genes. Furthermore, specific silencing of disease-relevant genes (e.g. from tumours, pathogens, or inflammatory mediators) is an interesting therapeutic strategy. In this respect RNA interference (RNAi) technology, which allows targeted 'knockdown' of individual genes by so-called small interfering RNAs (siRNAs) [1], has already opened up new avenues for functional analyses *in vitro*, and holds great promise for analytical as well as therapeutic applications *in vivo*.

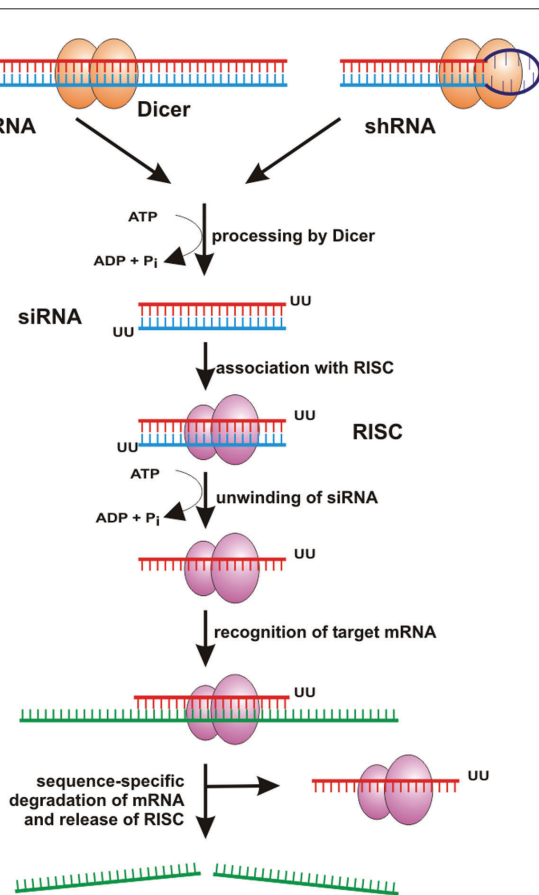
Although other gene silencing approaches, using anti-sense oligonucleotides, ribozymes, or DNAzymes, have been introduced over the past 25 years, their application has been restricted to certain areas. Only one antisense-based pharmacological agent has thus far been approved. In contrast to those technologies, RNAi represents a physiological process that occurs naturally in many eukaryotes, where it has evolved probably as a mechanism

to defend against invading nucleic acids such as viruses and transposons [2,3], and therefore it is easily applicable to a large variety of organisms, cell types and genes. The technology has remarkable target specificity and requires only low amounts of siRNA effector molecules per cell, which can even be expressed directly *in situ*, allowing long-term silencing of target genes. This makes RNAi an interesting tool for the analysis of loss-of-function phenotypes *in vivo* and it may also lead to the development of new gene therapeutic approaches.

As for all gene silencing approaches, the critical step toward application of RNAi in mammals is the delivery of effector molecules into the target cell. What has been accomplished rather easily in cell lines represents a much greater challenge in hard-to-transfect primary mammalian cells, which are of course the ultimate targets.

This review briefly summarizes our current knowledge of the mechanism of RNAi, the technical basis for its application to functional gene analysis in mammalian cells *in vitro* and *in vivo*, and potential therapeutic applications.

dsRNA = double-stranded RNA; ES = embryonic stem (cell); nt = nucleotide; RISC = RNA-induced silencing protein complex; RNAi = RNA interference; shRNA = small hairpin RNA; siRNA = small interfering RNA.

Figure 1

The RNA interference pathway. Long double-stranded RNA (dsRNA) or small hairpin RNA (shRNA) is processed by Dicer to form a small interfering RNA (siRNA), which associates with RNA-induced silencing protein complex (RISC) and mediates target sequence specificity for subsequent mRNA cleavage. (See text for further details.)

Mechanism of RNA interference

The phenomenon of RNAi, originally described in the nematode worm *C. elegans* by Fire and colleagues [4] in 1998, has been recognized as a general mechanism in many organisms (Fig. 1) [1,5]. Basically, RNAi is induced within the cytoplasm when long, double-stranded RNA (dsRNA) is recognized by Dicer, a multidomain RNase III enzyme. Dicer processes dsRNA into short (21–25 nucleotide [nt]) duplexes that are termed siRNAs [6–10]. Like products of other RNase III enzymes, siRNA duplexes contain 5′ phosphate and 3′ hydroxyl termini, and two single-stranded nucleotide overhangs on their 3′ ends [10]. These structural features are important for the entry of siRNAs into the RNAi pathway because blunt-ended siRNAs or those that lack a 5′ phosphate group are ineffective in triggering gene silencing [11,12]. The generated siRNA associates with a multiprotein complex, the RNA-induced silencing protein complex (RISC), which

becomes activated on ATP-dependent unwinding of the siRNA duplex [6,12]. One of the two siRNA strands is retained within the complex and confers sequence specificity in targeting of the mRNA by Watson–Crick base-pairing [6,11,13,14]. A perfectly homologous mRNA is cleaved at a single site in the centre of the duplex region formed with the guide siRNA, 10 nt from the 5′ end of the latter [10,12,13,15]. Finally, RISC is released and the cleaved mRNA is further degraded by cellular exonucleases [16]. The specific degradation of mRNA in turn leads to decreased synthesis of the respective protein and eventually to a loss of protein function.

Concentrations of only a few siRNA molecules per cell can lead to a pronounced silencing effect, demonstrating the catalytic action of RISC [1,4]. Generally, although greatly diminished, residual mRNA levels can be detected. Hence, the RNAi-mediated silencing of a particular gene is generally referred to as a ‘knockdown’ rather than a ‘knockout’.

RNA interference in mammalian cells

Originally, the RNAi pathway was thought to be nonfunctional in mammalian cells, where dsRNA longer than 30 base pairs induces a nonspecific antiviral response. This so-called interferon response is characterized by the activation of the RNA-dependent protein kinase [17], leading to phosphorylation of the translation initiation factor eIF-2 α and thereby to a nonspecific arrest in translation and induction of apoptosis [18]. Moreover, the synthesis of 2′–5′ polyadenylic acid results in the activation of the sequence nonspecific RNaseL [19].

The breakthrough for the use of RNAi in mammalian cells came when Elbashir and coworkers [20] and Caplen and colleagues [21] showed that siRNA, when directly introduced into mammalian cells, does not trigger the RNA-dependent protein kinase response but effectively elicits RNAi, presumably by directly associating with RISC. Targeted gene silencing in mammalian cells by the application of siRNA is well established. The high degree of sequence specificity inherent to the technology is emphasized by several reports showing that even a 1–2 nt mismatch in the siRNA sequence hampers targeted gene silencing [11,16,20,22,23].

Recently, evaluation of target gene specificity on a genome-wide level by applying gene expression profiling led to conflicting results. In two studies [24,25] no effects on nontarget genes were observed, although high concentrations (100 nmol/l) of siRNA were shown to induce stress-response and apoptosis-related genes. In contrast, Jackson and coworkers [26] challenged the idea of perfect sequence specificity of siRNA; they detected silencing of nontargeted genes with limited sequence similarity. As few as 11 contiguous nucleotides of identity

Table 1**Guidelines for siRNA design**

General guidelines for siRNA design	<p>Select 23-nt long sequences from the mRNA conforming to the consensus 5'-AA[N19]UU-3' or 5'-NA[N19]NN-3' (where N is any nucleotide)</p> <p>Avoid targeting of regions that are likely to bind regulatory proteins, such as 5'-UTR, 3'-UTR and regions close to the start site</p> <p>Choose sequences with GC content between 30% and 70%</p> <p>Avoid highly G-rich sequences</p> <p>Design sense and antisense N19 sequences, add two 2-deoxythymidine residues to the 3' ends</p> <p>Perform BLAST search to exclude potential homology to other genes</p>
Additional considerations for vector-based siRNA expression	<p>Avoid more than three consecutive As or Ts in the targeting sequence</p> <p>U6 promotor requires a guanine at position +1</p> <p>H1 promotor prefers adenosine at position +1</p> <p>Design oligonucleotides containing N19 targeting sequence, a loop forming spacer sequence (often 5'-TTCAAGAGA-3'), followed by the reverse complementary targeting sequence and five to six consecutive thymidine residues for termination of transcription</p> <p>Add respective restriction sites for cloning</p>
siRNA design tools on the internet	<p>http://www.ambion.com</p> <p>http://www.qiagen.com/siRNA</p> <p>http://jura.wi.mit.edu/bio/</p> <p>http://www.dharmacon.com</p> <p>http://sinc.sunysb.edu/Stu/shilin/rnai.html</p>

Rules for the design of synthetic siRNAs according to Tuschl and coworkers [27] and some further considerations for vector-based small hairpin RNA (shRNA) expression are given. A collection of links to small interfering RNA (siRNA) design tools on the internet is provided. nt, nucleotide; UTR, untranslated region.

to the siRNA were sufficient. Apparently, this off-target silencing was mediated not only by the antisense but also the sense strand of the siRNA. These findings highlight the need for careful selection of the siRNA sequences and appropriate specificity controls to verify functional effects.

Small interfering RNA selection

A synthetic siRNA consists of a 19 base-pair double-stranded region that is complementary to the gene of interest, contains 5' phosphate and 3' hydroxyl termini, and possesses two single-stranded nucleotides on the 3' ends [20].

Tuschl and coworkers [27] reported a number of guidelines for the design of siRNA molecules (Table 1). Several design tools are also available from the internet (Table 1). Although one can follow these guidelines it is still necessary to test several siRNAs, targeting distinct regions within the gene of interest, because there is great

variability in the capacity of an individual siRNA to induce silencing [16,28]. One may have to test three or four siRNAs in order to find one that results in more than 90% reduction in target gene expression (unpublished data). The reason for this is not entirely understood but it may be related to one or more of the following factors: incorporation of siRNA into RISC and stability of RISC; base pairing with mRNA; cleavage of mRNA and turnover after mRNA cleavage; secondary and tertiary structures of mRNA; and binding of mRNA-associated proteins. Accordingly, Vickers and coworkers [28] found a significant correlation between mRNA sites that are RNase H sensitive (i.e. accessible) and sites that promote efficient siRNA-mediated mRNA degradation. Moreover, placing the recognition site of an efficient siRNA into a highly structured RNA region abrogated silencing.

Two recent reports [29,30] found that the decision regarding which of the two strands of a siRNA molecule is

incorporated into RISC was crucial in determining the efficiency of gene silencing. In order to target specifically a given mRNA for degradation, the antisense strand of the siRNA duplex, which is complementary to the mRNA, must be incorporated into the activated RISC. Schwarz and coworkers [29] and Khvorova and colleagues [30] found that the absolute and relative stabilities of the base pairs at the 5' ends of the two siRNA strands determine the degree to which each strand participates in the RNAi pathway. The strand with lower 5' end stability is preferred. As a consequence, a highly functional siRNA is characterized by lower internal stability at the 5' end of the antisense strand as compared with less effective duplexes. A further improved algorithm for the prediction of siRNA efficiency is highly desirable and will enable us to improve quality and efficiency, and reduce the cost of the technology.

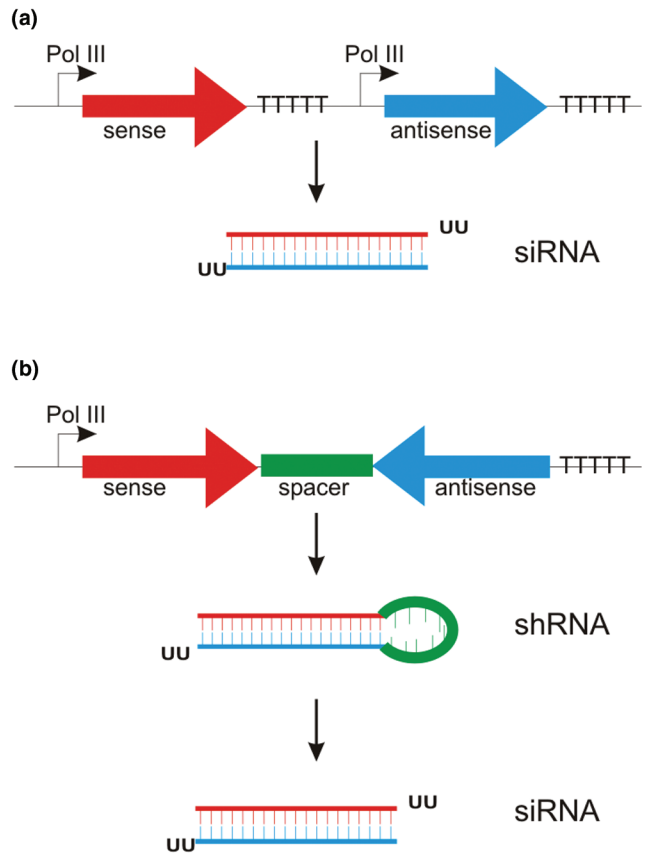
Modes of application and routes into the cell

To induce RNAi in mammalian cells, siRNA can either be directly transfected or produced endogenously within the target cell from expression plasmids [22,31–34]. Synthetic siRNA can be generated by chemical synthesis, by *in vitro* transcription using a T7 polymerase [34,35], or by Dicer digestion of long dsRNA [36]. Synthesized siRNA induces potent silencing at concentrations of 1–10 nmol/l [13].

siRNA expression vectors utilize mostly U6-snrRNA or H1 (RNase P) promoters, both of which are members of the RNA polymerase III promoter family, which lack downstream transcriptional elements and produce a transcript without a cap or poly-A tail [37]. Transcription is terminated at a stretch of five to six thymidine residues, leading to the incorporation of two to three uracil residues at the 3' end, which is compatible with the two or three nt overhangs that are found to be indispensable for silencing activity in natural siRNAs.

Sense and antisense strands are either produced from two independent promoters and anneal within the cell [31], or more commonly the two strands are linked by a 9 base pair spacer leading to the expression of a stem-loop structure termed short hairpin RNA (shRNA). The hairpin is subsequently cleaved by Dicer to generate effective siRNA molecules [22,33,34,38] (Fig. 2). By incorporating a drug resistance gene or via episomally replicating plasmids, a long-lived knockdown effect can be achieved in cultured cells [31,39]. To facilitate the analysis of genes that are essential for cell survival and cell cycle regulation, two groups have generated inducible shRNA expression systems [40,41]. However, the specificity of gene knockdown must be tightly controlled, because Bridge and coworkers [42] recently reported the induction of an interferon response by a substantial number of shRNA expression vectors tested, perhaps caused by the accumulation of nonprocessed Pol III transcripts within the cell.

Figure 2



Approaches to endogenous expression of small interfering RNAs (siRNAs) in mammalian cells. **(a)** Sense and antisense strand of the siRNA duplex are expressed from separate promoters. **(b)** siRNA duplex is expressed as a stem-loop structure (small hairpin RNA [shRNA]) from a single promoter. Sense and antisense strands are separated by a loop-forming spacer. The construct is further processed by Dicer within the cell to form a functional siRNA. In both cases transcription is terminated by six consecutive thymidine residues.

Gene silencing occurs very rapidly after the transfection of an efficient siRNA. Although the kinetics may vary depending on the gene of interest, usually target mRNA levels will be diminished after 48 hours, reaching a minimum at 72 hours after transfection. A knockdown efficiency of 90–95% reduction in the amount of target mRNA can be achieved. However, the major drawback of the method is its transient gene silencing effect. The duration of the knockdown using synthetic siRNA is generally in the range of 3–5 days. Protein levels will return to normal 5–7 days after transfection [16,27]. The longevity of silencing depends on factors such as the abundance of target mRNA and protein, the stability of target protein, transcriptional feedback loops, and the number of cell divisions diluting the siRNA, rather than on the degradation of the siRNA itself.

Both chemically synthesized siRNA and shRNA expression plasmids can be delivered to cells using standard transfection methods. Thereby, the efficiency mainly depends on the type of cell that is targeted. Because of their small size, transfection of synthetic siRNAs is usually very efficient, even in primary mammalian cells. A number of cationic lipid-based or liposome-based transfection reagents optimized for the transfection of oligonucleotides are commercially available. In cells that are more resistant to chemical transfection methods (e.g. suspension cells), electroporation may achieve an efficient induction of RNAi. Transduction rates with siRNA of up to 80–90% have been reported for some haematopoietic cell lines and primary cells [43,44]. Optimized for the transfection of primary human cells with siRNA, Nucleofection™ technology (Amaxa biosystems, Cologne, Germany) appears to be a very efficient and convenient approach [45,46].

When using these conventional transfection strategies, the silencing effect is only transient. Exceptions are established cell lines that allow selection for integrated vectors. Viral gene delivery systems are perfectly suited to overcome these limitations; they are well established tools for efficient transduction of primary cells and some of them have the inherent ability to integrate into the host cell genome, thereby leading to stable transgene expression. Several adenoviral [47,48], onco-retroviral [49–51] and lentiviral [52–54] vectors have been utilized for the efficient delivery of shRNA expression cassettes. Adenoviral infection is transient whereas onco-retroviral vectors, based on the Moloney murine leukaemia virus or the murine stem cell virus, integrate into the host cell genome, leading to a prolonged silencing effect. Lentiviral vectors based on HIV-1 bear the additional advantage of efficiently transducing both dividing as well as nondividing cells, such as stem cells and terminally differentiated cells. Moreover, they are resistant to developmental silencing after integration of the provirus, and therefore they can be used to generate transgenic animals. Several groups have reported the use of lentiviral systems for the silencing of genes in a variety of cultured as well as primary cells, such as human and murine T cells [52,53], haematopoietic stem cells [53] and mouse dendritic cells [53,54]. Although onco-retroviruses and lentiviruses hold great promise as vehicles for gene therapy, two patients who were undergoing retroviral based therapy for X-linked severe combined immunodeficiency developed leukaemia [55,56]. This indicates that improved safety standards and ways to control the integration of the provirus are needed before retroviruses can be used to deliver siRNA for therapeutic purposes.

Towards *in vivo* application of small interfering RNA

RNAi has already been proven to be a powerful tool for dissecting and elucidating gene function, even on a

genome-wide basis. The first example comes from *C. elegans*, in which Kamath and coworkers [57] reported the construction of a library of bacterial clones that express dsRNA, which corresponds to approximately 86% of the total gene products made by *C. elegans*. Also, the library has been used to screen for genes that are involved in body fat regulation, longevity and genome stability [58–60].

Thus far, *in vivo* gene silencing approaches are very limited in the mammalian system. Nonetheless, a number of potential candidate genes, especially in viral infections, cancers and inherited genetic disorders but also in chronic inflammatory diseases such as autoimmune arthritis, has been defined and successfully targeted *in vitro*. Consistent with its natural function as an antiviral defence mechanism, siRNA was found to inhibit *in vitro* replication of several viruses effectively, including HIV, hepatitis C virus and influenza virus, by interfering with various stages of the virus life cycles [38,52,61–67].

Similarly, several cancer-related genes have been targeted in proof-of-principle experiments, including cellular oncogenes and drug resistance genes. In these studies, RNAi was efficient and highly selective in targeting oncogenes resulting from chromosomal translocations [43,68] or carrying single point mutations, without affecting the wild-type allele [50,69].

Protocols must be established for efficient delivery of siRNA and selective targeting of specific cell types in order to allow future therapeutic applications and *in vivo* verification of results obtained from *in vitro* silencing experiments. Moreover, it must be determined whether transient gene silencing, as obtained by introduction of synthetic siRNA or expression plasmids, is sufficient for treatment, or whether the target gene must be silenced for an extended period of time by the use of viral expression systems.

Direct injection of siRNA into the blood would be ineffective because of rapid degradation of the RNA by serum ribonucleases. However, it was recently demonstrated that chemical modification can protect the siRNA molecule from degradation [70] and might even prolong the silencing effect due to slower depletion within the cell [71]. Thus far, synthetic siRNAs have been applied in animals via hydrodynamic transfection [72] (i.e. the intravenous injection of a substantial dose of siRNA within a large volume of liquid), resulting in a knockdown efficiency up to 70–80%, at least in some organs, including liver, kidney, spleen, lung and pancreas [73]. Using this method, the silencing of either Fas receptor [74] or caspase-8 [75] resulted in a clearly measurable protection from severe Fas-induced liver damage. *In vivo* application of siRNA against genes of the hepatitis B virus also led to an effective inhibition of virus replication [76].

This method is of course not applicable to humans. It is also limited by the fact that siRNA can only be delivered to a certain set of organs and it is not possible to target specific organs or cells. Development of cell-specific or organ-specific delivery systems for siRNA, as is required for broad *in vivo* application of this technique, is indeed a demanding task.

Prolonged gene silencing by stable integration of a siRNA expression vector is currently only possible *in vitro*. The subsequent *in vivo* adoptive transfer of these *in vitro* manipulated cells is an option in situations where a small number of cells can develop a dominant phenotype *in vivo*. This is the case for stem cells (e.g. embryonic stem [ES] cells) or haematopoietic stem cells, which either give rise to a complete new animal or at least generate defined organs.

An approach using siRNA-modified stem cells would be particularly useful for the analysis of gene function *in vivo*. So far this has mainly been done in knockout mice, which carry a nonfunctional mutation of the target gene, generated by homologous recombination in ES cells. The technique suffers from a number of limitations that could be overcome by RNAi technology, such as the need for cloning of the target gene, the time and effort required for generating a knockout mouse, and the potential embryonic lethality. In contrast to the all-or-nothing phenotype obtained from knockout animals, analysis of gene dosage effects may be possible by using siRNAs with variable silencing efficiency. Finally, the combination of multiple loss-of-function phenotypes in one generation would be possible. Lentiviral siRNA vectors have been used to generate stable transgenic 'knockdown' animals by infection of fertilized eggs [77]. In another study, Rubinson and coworkers [53] used lentiviral vectors expressing green fluorescent protein as a selection marker and an siRNA targeting CD8 for embryo infection. Between 25% and 50% of the resulting mice were transgenic and expressed both green fluorescent protein and siRNA in all tissues tested. Transgenic mice exhibited a reduction in CD8 expression of about 90%; however, the percentage of cells affected by gene silencing varied among individual mice and correlated with the number of integrated viruses per genome. Therefore, different siRNA expression levels may account for this variance. In an alternative approach, not involving the use of lentiviral vectors, transgenic 'knockdown' mice were generated by transfecting ES cells with a siRNA expression plasmid containing a drug resistance gene [78].

The adoptive transfer of *in vitro* modified cells may also be applicable to the modulation of an antigen-specific immune response (e.g. for the treatment of autoimmune diseases, allergies, or organ rejection). In these situations, a relatively small population of antigen-specific lymphocytes or antigen-presenting cells, previously modified by

siRNA *in vitro*, may later dominate an antigen-specific immune response *in vivo*. This has recently been demonstrated by transfer of dendritic cells transfected with an siRNA against the immunomodulatory cytokine interleukin-12 [79]. However, for therapeutic use in humans, both the safety of stably transfected cells and the target specificity of the siRNA must be controlled more closely.

Conclusion

RNAi has rapidly evolved as a potent technology for the analysis of gene function in many organisms *in vitro* and *in vivo*. In mammals, at present RNAi is mainly restricted to the analysis of easily transfectable cell lines *in vitro*, but here it has already proven its efficiency in targeting a number of therapeutically relevant genes with high specificity. Recent work has set the scene for addressing gene function in primary cells both *in vitro* and *in vivo*, which is more pertinent to the definition of disease-related pathways and potential therapeutic targets. However, for therapeutic applications of siRNA in humans, new strategies must be developed that will allow the efficient and specific targeting of distinct organs or cell types.

Competing interests

None declared.

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