RNAi and related mechanisms and their potential use for therapy

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Introduction of double-stranded RNAs into cells can suppress gene expression by mechanisms such as mRNA degradation or inhibition of translation. In mammalian cells, these two responses intersect, a feature that was recently used for the development of novel tools for stable and specific gene inactivation. These new tools were successfully applied to inhibit tumorigenicity and viral replication. Future development of appropriate *in vivo* delivery systems may make this technology useful for disease therapy.

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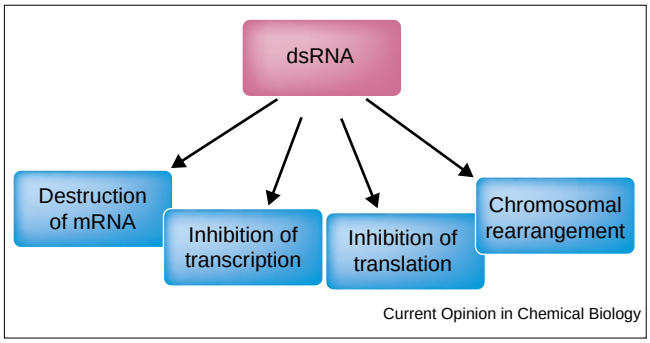
Abbreviations

dsRNA	double-stranded RNA
miRNA	micro RNA
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing protein complex
RNAi	RNA interference
siRNA	short interfering RNA
stRNA	small temporal RNA
UTR	untranslated region

Introduction

The hallmark of RNA interference (RNAi) is that it is triggered by double-stranded RNAs (dsRNAs) that cause selective gene silencing. The term RNAi was first coined after the discovery that the injection of dsRNAs into *Caenorhabditis elegans* interferes with the expression of specific genes that contain a highly homologous region to the delivered dsRNA [1]. dsRNAs can stimulate at least four distinct types of responses that trigger specific gene inactivation (Figure 1). Initial experiments in *C. elegans* have indicated that RNAi occurs at the post-transcriptional level [1]. Supported by later reports this has led to the notion that RNAi works through mRNA destruction [2–5]. This notion has turned out to be not so simple after the discovery that in *Drosophila melanogaster*, *C. elegans* and fungi, RNAi-related mechanisms may also induce effects on chromatin structure and silence transcription of the targeted genes [6–8]. Furthermore, in plants, RNAi directs *de novo* methylation of genomic regions, which can suppress transcriptional activity of target genes [9,10]. Third, a related RNAi mechanism can direct the inhibition of mRNA translation of target genes [11]. Finally, in ciliated protozoa, small RNAs function to mediate chromosomal rearrangements by a mechanism related to RNAi [12••].

Figure 1



Mechanisms of selective gene silencing induced by dsRNA. In various eukaryotes, the introduction of dsRNAs into cells can elicit at least four different types of responses that can selectively suppress gene expression. dsRNA can induce inhibition of protein translation, degradation of mRNAs, transcriptional inhibition and cause chromosomal rearrangements.

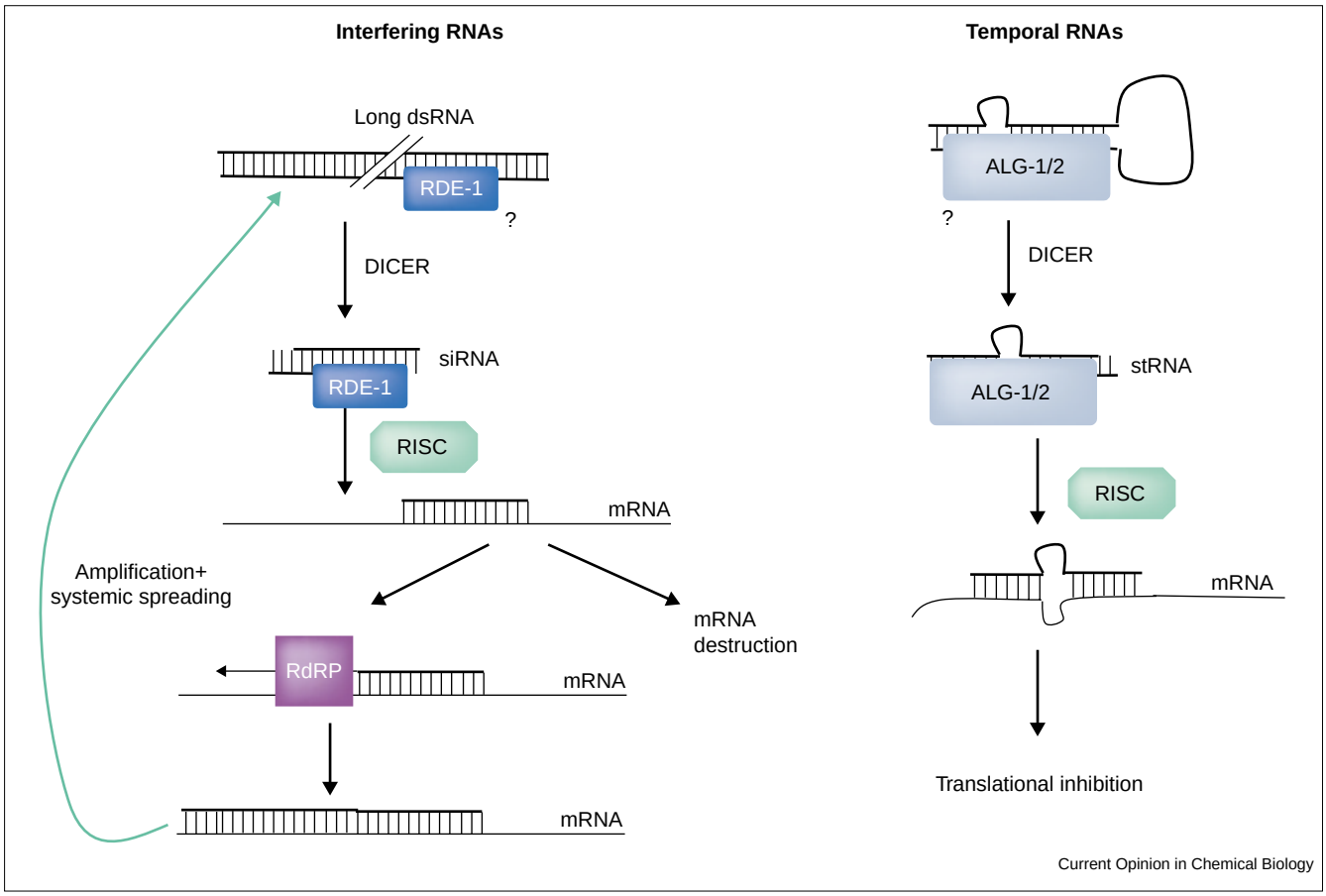
Intersecting mechanisms of destruction and translation inhibition

The ability of the same type of inducer (dsRNA) to provoke diverse responses is intriguing. Why are different responses required and what confers the specificity of each response? These are two important issues that may help us to understand the cellular functions of short RNAs and to improve their future use for gene-function studies and for disease treatments.

In animals and plants, the introduction of long dsRNAs induces selective mRNA destruction (Figure 2) [3–5]. The long dsRNAs are recognized and processed to small pieces of 21–25 nt dsRNA termed short-interfering RNAs (siRNAs) [3,13,14]. Members of the *rde-1* (for RNAi defective)/*ago-1* (argonaute) family of proteins and the Dicer multi-domain RNase-III enzyme mediate these processes. Nematodes mutated for rde-1 are insensitive to RNAi but no other distinguishing phenotypes appear [8]. By contrast, Dicer mutants display developmental abnormalities, a much more severe phenotype [15,16]. The siRNAs generated by the RNAi process invariably contain two perfectly complementary RNA strands [3,14]. They function to guide the RNA-induced silencing protein complex (RISC) to the target mRNAs and induce their destruction through cleaving the mRNA in the middle of the target region by an as-yet unknown nuclease [4]. This guidance of RISC to target mRNA is highly sequence specific, to the extent that even 1–2 nt difference in the targeting recognition sequence hampers RNAi function [17••,18–20].

In contrast to siRNAs, small temporal RNA molecules (stRNAs), which represent a large group of small transcripts

Figure 2



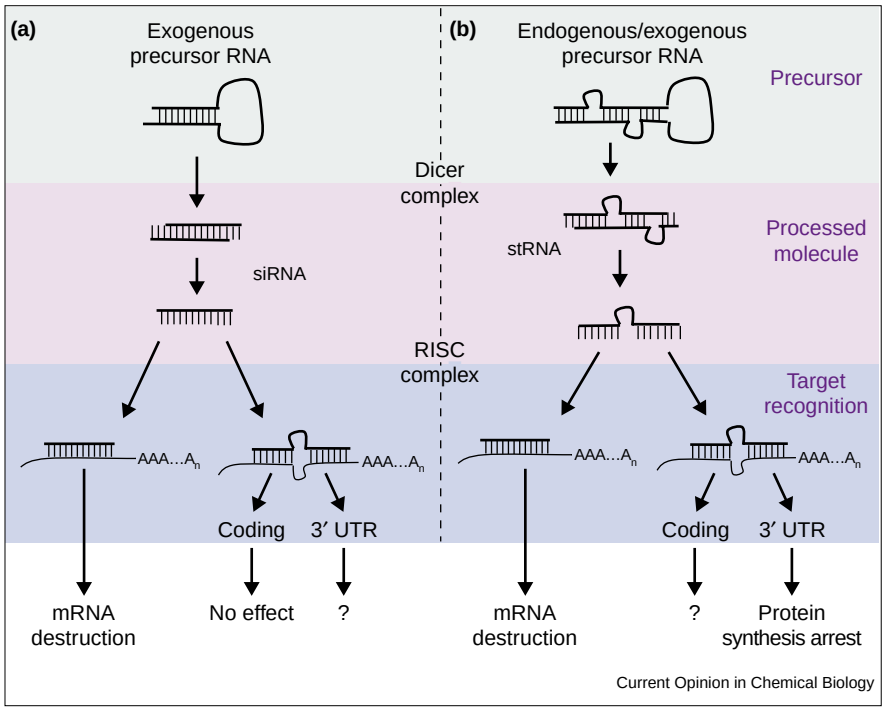
A schematic model for the gene silencing mechanisms induced by long dsRNA and stRNAs in *C. elegans*. Long dsRNAs and precursor-stRNAs are recognized and processed to siRNAs and stRNAs by the Dicer complex. Specific factors for each pathway are the RDE-1 for long dsRNAs and ALG-1/2 for the stRNAs. The processed transcripts guide the RISC complex to induce mRNA destruction or to inhibit translation. siRNAs can be amplified by RdRPs to generate more siRNAs, in a process named transitive RNAi, and also can be systemically spread from cell to cell to silence genes in most of the cells in the organism.

called micro RNAs (miRNAs), mediate gene suppression by inhibiting translation of target mRNA (Figure 2) [11,21,22]. stRNAs are ~70 nt RNA molecules that are predicted to adopt stem-loop folds which are further processed to 20–25 nt transcripts. The prototype stRNA molecules are *lin-4* and *let-7*, which control developmental timing in the nematode, hence the name temporal RNAs [21,23–25]. Typically, stRNAs recognize the target mRNA by a partial-complementary interaction to regions at the 3′ untranslated region (UTR) of the target mRNA. For example, *lin-4* is processed to a 22 nt RNA that bears imperfect complementarity to multiple sequences at the 3′ UTR of *lin-14* and *lin-28* mRNAs [26]. These interactions direct the inhibition of translation of these genes by an as-yet unknown mechanism [11]. In common with siRNAs, the processing of stRNAs from their precursors requires Dicer and most likely also involves the generation of small RNA duplexes [15,16,27]. However, in contrast to RNAi, *rde-1* is not required and instead two other members of its family, *alg-1* and *alg-2*, function to recognize stRNAs [15]. These observations explained the developmental defects of nematodes lacking Dicer.

To date, nearly 200 miRNAs have been identified collectively from *C. elegans*, fruit fly, plants and humans. These exhibit diverse sequence, structure, abundance and expression profile, but invariably fold into a stRNA-like imperfect complementary stem-loop structure whose stem is disrupted by one or more 1–3 nt long unpaired sequences [28–30,31••,32,33]. Except for plants, all the other organisms do not contain in their mRNA collection a perfect targeting sequence to the miRNAs. The mechanism of action of these miRNAs is not known but is likely to be translation control as in *Drosophila*, where a large subset of miRNAs contain complementary sequences to several classes of 3′ UTR motifs that mediate post-transcriptional regulation [34]. Plants, however, contain many miRNAs that have nearly or identical complementary sequences in their mRNA collection [35•]. Interestingly, the majority of the target mRNAs are transcription factors that regulate

Figure 3

Gene silencing by endogenous and exogenous hairpin-like transcripts. A schematic model showing how (a) foreign stRNAs-like transcripts and (b) endogenous stRNAs affect gene expression. Depending on the type of stRNA molecule and its target sequence, different outcomes are possible.



developmental events, and the region complementary to the miRNA is almost invariably placed within the coding region. These observations lead to the speculation that plants miRNAs function also as siRNAs to mediate specific destruction of target mRNAs [35•]. It remains to be experimentally shown whether this is indeed the case.

To some extent, mammalian stRNAs may resemble plant miRNAs. The human *let-7* paralog, miR-98, is incorporated into the miRNP multi-protein complex that contain Gemin3, Gemin4 and eIF2C2, members of the PIWI and PAZ domains (PPD) protein family that are required for RNAi [31••]. *let-7* is also co-purified from human cells with an RNA-cleavage activity that is functionally identical to siRNAs, as it requires perfect target sequence recognition [31••]. Therefore, in human cells, and perhaps in plants as well, both siRNAs and stRNAs are being incorporated into the RISC complexes that are able to mediate mRNA destruction.

What makes them different?

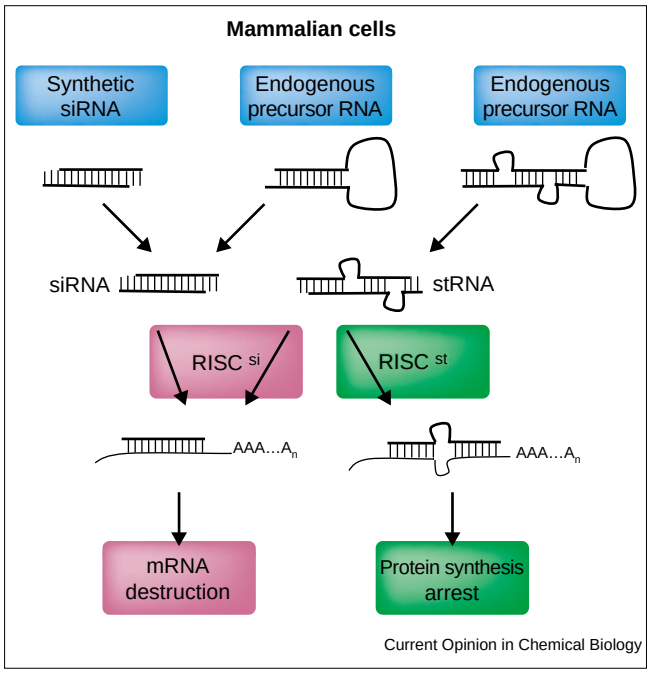
At least three molecular differences distinguish between mRNA destruction and inhibition of translation by RNAi: the primary structure of the precursor dsRNA, its internal complementarity, and its association with the target mRNA (Figure 3). siRNAs contain a fully complementary dsRNA-targeting sequence and require a perfectly matched target mRNA sequence for functionality. By contrast, miRNAs are stem-loops that are processed into an imperfect complementary dsRNA that inhibit protein translation of an imperfectly matched target sequence which is almost invariably located at the 3′UTR of the target mRNA. Which of these differences is critical for determining the specific response?

One possibility is that the characteristic hairpin structure of stRNAs determines the mechanism of RNAi. From the following observations, this seems not to be the case. It has been shown that artificially expressed stRNAs inhibit translation of mRNAs containing imperfect complementary sequences at their 3′ UTR. By contrast, artificially expressed stRNA-like molecules that contain a perfect stretch of duplexed RNA mediate mRNA destruction of a perfect match but are inactive towards an imperfect target recognition site (do not inhibit its translation) [19,36,37]. Finally, mammalian endogenous stRNAs are able to induce destruction of target mRNAs that contain a perfect complementary sequence [31••].

A second possibility is that the perfect/imperfect nature of the precursor's duplex RNA determines the different response. An internally imperfect duplexed RNA may attract factors to the mRNA that act to inhibit translation, whereas a perfect duplex may bind factors that cause transcript destruction. Such a model predicts that two types of RISC complexes exist, one contains specific factor(s) that cleave mRNAs and one contains factors that inhibit mRNA translation (Figure 4). At this point, assuming the existence of two different RISC complexes, one can only speculate why one RISC complex prefers perfect duplexes whereas the other imperfect ones.

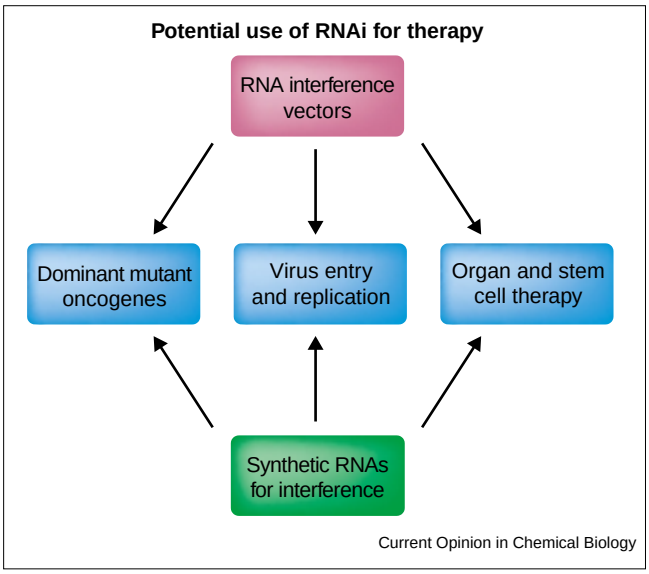
A third option is that the bulge sequences of stRNAs determine the binding of specific factors that induce translation inhibition. One observation that may support such a model is that a bulged cytosine residue of the *lin-4* stRNA in *C. elegans* is essential for its activity [23]. Finally,

Figure 4



Intersection of siRNA and stRNA pathways in mammalian cells. A schematic model showing that in mammalian cells stRNAs and stRNA-like molecules are processed to induce intersecting responses.

Figure 5



Potential use of RNAi tools. Synthetic siRNAs and vectors for induction of gene silencing by RNAi can now be applied for selective inactivation of dominant oncogenes, inhibition of viral infection and organ and stem cell therapies.

the position of the target sequence on the mRNA may play a role in determining the mechanism chosen. However, as mRNA destruction mediated by siRNAs can be induced by target sequences placed at the 3′ UTRs as well as in the coding regions, this seems unlikely [37]. It remains to be explored whether coding regions can mediate translation inhibition by stRNAs (Figure 3).

The function

stRNAs and siRNAs are messenger molecules of different entities. siRNAs are processed from foreign genomes such as viruses, transposones and transgenes. In lower eukaryotes and plants, they act to protect the organism from these intruders by a robust, yet very specific, inhibition of gene expression. Through mechanisms that require RNA-dependent RNA polymerases (RdRPs) and specific spreading factors (such as SID-1), the signal is 'amplified' to generate more siRNAs also from other parts of the gene (a process named transitive RNAi) and is delivered to other cells, resulting in a highly specific and long-lasting target gene suppression [38,39]. By contrast, stRNAs are derived from endogenously expressed precursor RNAs that function to synchronously regulate multiple genes in a temporally and spatially restricted manner; for example, during development or tissue differentiation [32]. The space–time issues are the basis for a strict functional distinction between the responses. One could only imagine what would have happened if a given stRNA in *C. elegans* would be recognized as siRNA that by the transitive RNAi and systemically spread mechanisms will lead to whole body gene inactivation.

With the development of the cellular immune system, which served as the 'new' defense mechanism against foreign infections, possibly some ancient RNAi mechanisms became irrelevant. It is clear that mammalian cells have lost the transitive RNAi (no RdRP) and, possibly, also the mechanisms of systemic spreading, two characteristics of RNAi in plants and worms. It looks as if these losses have given enough flexibility to allow stRNAs to be incorporated into complexes that function like siRNAs to degrade mRNAs.

RNAi tools for disease and gene therapy: challenges for the future

Just over a year ago, an ingenious method was developed that utilizes synthetic short (21–25) nt interfering (si) dsRNAs to induce selective mRNA destruction, avoiding the toxic effects associated with long dsRNAs in somatic mammalian cells [17••]. Using this method it was possible to suppress gene expression to the extent that the gene function is lost and to inhibit the replication of HIV and RNA viruses in human cells [40••–42••]. As it stands, the application of siRNAs for disease and gene therapies can follow the existing tools that are already applicable for clinical trials of anti-sense strategies to inhibit gene expression. However, a major drawback of this technology is its transient effect. Genes could only be inactivated for a week. To overcome this limitation, several systems were designed where the expression of siRNAs was derived from vectors and viral vectors that produce stRNAs-like molecules [19,43,44,45••]. These stem-loop RNAs were

transcribed using either polymerase III or II promoters and then were processed, in the cells, by the Dicer enzyme to siRNAs that function to direct selective mRNA destruction [19,36,46–50]. Much like stRNAs, the cellular processing of either in vitro synthesized hairpin precursors or exogenously expressed molecules to siRNAs was highly efficient [19,37,44,46,47]. As discussed above, it is very likely that the ability of mammalian cells to process stRNAs as siRNA molecules and provoke mRNA destruction stands behind the successful development of the vector-based gene silencing technology [31••]. In the future, it will be interesting to examine whether and under which conditions such artificial molecules can also be manipulated to provoke other RNAi-related responses such as inhibition of protein translation.

Conclusions

In any case, the ability to express siRNAs from plasmids and viral vectors allows us now to generate stable mammalian populations of cells carrying specific sets of inactive genes [19]. This permits long-term gene function studies in mammalian cells, stable inhibition of viral infections and suppression of human oncogenesis [41••,42••,51]. For example, we recently developed such a viral vector to stably suppress the expression of the oncogenic mutant allele *K-RAS^{V12}* through RNAi [45••]. *K-RAS* mutations are frequently found in many human cancers where they are required to maintain tumorigenic growth. Indeed, when *K-RAS^{V12}* expression was selectively suppressed, the cancerous cells lost their tumorigenic phenotype. In the future, these viral vectors can be designed and applied to suppress other oncogenes in other types of cancers. However, the employment of this technology for cancer therapy in humans awaits the development of an efficient *in vivo* delivery system. A recent report employed a high-pressure tail-vein injection in postnatal mice to deliver siRNA-mediated gene inactivation [52•]. As expected, the effect was transient but possibly can be made stable by the use of vectors similar to those described above. RNAi-mediated disease therapy will necessitate the development of similar or novel delivery systems of short dsRNAs into humans.

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- •• of outstanding interest

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