Higher eukaryotes can mount antiviral immune responses induced by dsRNA. This process, called RNA interference, is sequence specific and can therefore be used to target gene expression.

RNA interference: antiviral defense and genetic tool

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Higher eukaryotes have developed a number of innate antimicrobial defense mechanisms based on the recognition of conserved molecular patterns shared by large groups of microorganisms. Double-stranded RNA (dsRNA) represents one such molecular pattern. dsRNA molecules form key intermediates in the genomic replication of many viruses, but are not normally found in eukaryotic cells. All higher eukaryotes, therefore, respond to dsRNA, whether it is produced by a virus or introduced artificially, by activating a range of immune-defense responses. In mammalian cells, the major defensive response used by the immune system upon exposure to dsRNA is the induction of interferon, which can reduce viral spread by inhibiting viral gene expression and causing the apoptosis of infected cells.

In contrast to mammalian cells, many other higher eukaryotes—including plants, nematodes and insects—are unable to mount an interferon response. However, these organisms can mount another antiviral response that is induced by dsRNA and is called RNA interference (RNAi) in animals and post-transcriptional gene silencing (PTGS) in plants[1,2]. The key characteristic of RNAi is its remarkable sequence specificity: these organisms respond to dsRNA by selectively degrading mRNAs that are homologous in sequence to the dsRNA inducer. Therefore, RNAi can be used to specifically block the expression of not only viral, but also host cell, genes upon introduction of a homologous dsRNA. Recent data have shown that the ability to mount sequence-specific RNAi is conserved in mammalian cells[3] and RNAi can therefore provide a potent and simple means with which to perform reverse genetics in cell lines or cultured primary cells.

How RNAi works

Our current mechanistic understanding of RNAi derives largely from work in the *Drosophila* system[4,5]. When dsRNAs are introduced into *Drosophila* cells, or into an *in vitro* Drosophila cell extract, they are cleaved into 21- or 22-nt dsRNA fragments that bear 2- or 3-nt 3′ overhangs (**Fig. 1a**). This cleavage is mediated by the processive RNase III–like enzyme dicer[6]. These ~21-nt dsRNAs, which are referred to as small interfering RNAs (siRNAs), are then selectively incorporated into a large (~500-kD) multiprotein complex—termed the RNA-induced silencing complex (RISC)—that remains poorly defined[7]. It has been suggested that RISC then undergoes an ATP-dependent activation step that involves unwinding of the double-stranded siRNA component to give a single-stranded guide RNA that targets RISC to homologous mRNAs[2] (**Fig. 1a**). After mRNA binding, an unidentified ribonuclease component of RISC cleaves the target mRNA at the center of the region that is complementary to the guide RNA. RISC is then released to seek out additional mRNA molecules, whereas the cleaved mRNA is degraded by cellular exonucleases. If the target RNA is either a viral genome, antigenome or mRNA, RNAi-induced RNA degradation can specifically inhibit, or even entirely block, viral replication. Conversely, if the target mRNA is of cellular origin, then RNAi can post-transcriptionally block the expression of the cognate gene.

An important discovery, made initially in the *Drosophila* system, is that artificial siRNAs can be incorporated into RISC and induce targeted mRNA degradation[5]. Previous efforts to induce RNAi in cultured mammalian cells had largely failed due to the potent nonspecific inhibitory effects induced by long dsRNAs. However, it has been known for some time that dsRNAs of <30 bp are not capable of inducing an interferon response. It has now been demonstrated that transfection of synthetic siRNAs into cultured human cells can effectively inhibit gene expression by inducing the selective degradation of target mRNAs[3]. RNAi induced by synthetic siRNAs has been used to examine the functional importance of a small but rapidly growing list of human gene products.

A genetic link between RNAi and development

Although a role for RNAi in the antiviral immune response of animals is clearly a reasonable hypothesis, evidence to support this proposal currently derives exclusively from studies of immune defense (PTGS) in plants. Specifically, mutant plants that are unable to mount the PTGS response are hypersusceptible to infection by certain viral pathogens[8]. Interestingly, several plant viruses have also evolved gene products that either attenuate or block the ability of plants to mount PTGS, and these gene products play a key role in enhancing viral replication and pathogenesis[9]. Similarly, an animal virus, termed flock house virus (FHV), that infects Drosophila has recently been shown to encode a protein that can suppress the ability of insect cells to mount an RNAi response[10]. Importantly, this viral protein is essential for FHV replication in culture, thus supporting the hypothesis that RNAi also functions as an antiviral defense in animal cells. However, it currently remains unclear whether RNAi in mammals plays a significant role in antiviral defense, and, if so, whether pathogenic human viruses have also evolved mechanisms to inhibit RNAi.

Although the importance of RNAi in mediating an innate antiviral immune response in animals remains to be directly addressed, mutants attenuated in their ability to mount RNAi have been derived in several nonmammalian species. Surprisingly, in several mutants of the nematode *Caenorhabditis elegans* that have lost the ability to mount RNAi, a significant number of endogenous transposons had mobilized[2]. In addition, analysis of endogenous ~21-bp siRNAs from a protozoan and from fruit flies revealed a range of transposon-derived sequences[5]. It is therefore likely that RNAi plays an important

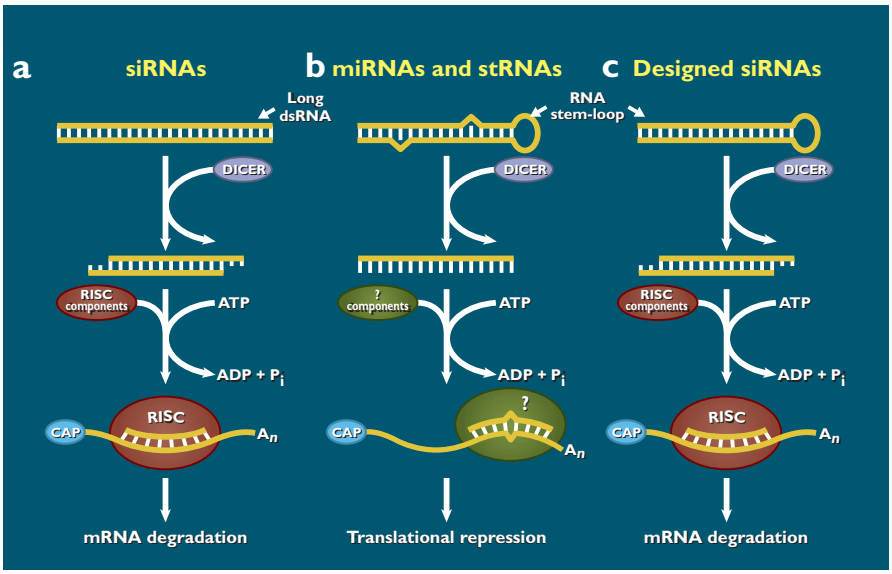

Figure 1. Biosynthesis of small, noncoding RNAs. (**a**) siRNAs are derived from long dsRNA molecules by dicer cleavage. The siRNAs are then incorporated into a RISC and unwound in an ATP-dependent manner. The resultant active RISC is targeted to homologous mRNAs by the incorporated guide RNA and induces their degradation. (**b**) miRNAs and stRNAs are encoded within the host genome as stem-loop RNA precursors of ~70 nt. After transcription, the mature miRNA or stRNA is excised from the precursor by cleavage with dicer and incorporated into a protein complex that may be partly distinct from RISC. Mature miRNAs of stRNAs are recovered in a single-stranded form, but it is unclear whether unwinding occurs before or after complex formation and whether ATP is required. The stRNA complex binds to partly mismatched target sequences found in the 3′ UTR or mRNAs to selectively inhibit their translation. (**c**) Designer siRNAs are also transcribed as stem-loop RNA precursors, which are encoded by an expression vector. However, after cleavage by dicer, these appear to be treated exactly like siRNAs, leading to the specific degradation of homologous mRNAs.

role in protecting the germ line from the deleterious mutations that would result from transposon accumulation. In addition to effects on transposon mobilization, some mutations that block RNAi were also found to interfere with appropriate development. Most strikingly, mutational inactivation of the dicer ribonuclease, a key mediator of siRNA production, resulted in obvious developmental defects and in sterility in the nematode *C. elegans*[11]. These data were consistent with earlier results[11,12], which indicated that small noncoding RNAs also play an important role in the appropriate development of this experimental organism.

stRNAs and miRNAs

Two developmentally important small noncoding RNAs, called small temporal RNAs (stRNAs) have been identified in *C. elegans*[11]. Inactivation of either of these stRNAs, termed lin-4 and let-7, disturbs the timing of larval development and thereby disrupts the appropriate differentiation of certain adult tissues[11,12]. Although these stRNAs are both 21-nt in length—that is, the same length as each strand of an siRNA—they are found in a single-stranded form *in vivo*. Both let-7 and lin-4 are first transcribed as part of one arm of an imperfect ~70-nt RNA stem-loop structure; they are then precisely excised from the stem-loop by the dicer ribonuclease[11] (**Fig. 1b**). Both let-7 and lin-4 inhibit the expression of specific, developmentally important mRNAs by forming duplexes with conserved sequence elements present in their 3′ untranslated regions[12,13]. However, unlike siRNAs, this interaction specifically blocks the translation of the target mRNAs, rather than inducing their degradation (**Fig. 1b**). Interestingly, known let-7 and lin-4 target sequences all contain a central mismatch, which may be relevant to this functional distinction. In contrast, siRNAs generally no longer functionally recognize target sequences that bear central mismatches.

Although the let-7 and lin-4 regulatory RNAs were initially viewed as a possibly idiosyncratic attribute of *C. elegans*, the more general importance of this regulatory pathway was implied by the demonstration that the let-7 stRNA is highly conserved in other animal species, including humans[12]. More recently, efforts to clone and sequence small noncoding RNAs have revealed a large number of so-called micro RNAs (miRNAs) in species as diverse as nematodes, fruit flies and humans[2]. Many of these miRNAs—which are again ~21 nt in length—are conserved across species boundaries, whereas the expression of others is developmentally regulated. However, at this early stage, no function has been ascribed to any mammalian miRNA. Like the closely similar stRNAs, miRNAs are encoded within the host genome in the form of ~70-nt RNA stem-loop structures from which they are precisely excised by the dicer ribonuclease (**Fig. 1b**). Like let-7 and lin-4, and unlike siRNAs, mammalian miRNAs are usually recovered as single-stranded RNAs. It remains unclear why the other strand—which is presumably excised simultaneously—is selectively lost in miRNAs and in the similar stRNAs, but remains readily detectable with siRNAs.

Given that mutational inactivation of the RNAi machinery affects at least three distinct eukaryotic processes—antiviral immune defense, transposon mobility and the development of multicellular organisms—it is of interest to speculate on how RNAi originally evolved. As RNAi is also seen in some single-celled eukaryotes, it is likely that RNAi evolved very early in eukaryotic evolution, before the appearance of multicellular eukaryotes. Therefore, the involvement of genomically encoded miRNAs in development may reflect the adaptation of a pre-existing mechanism for post-transcriptional inactivation of target mRNAs to a new use. It remains unclear whether RNAi first evolved for antiviral immunity or as a host defense against transposon-mediated mutagenesis.

Derivation and utility of designed siRNAs

Although transfected synthetic siRNAs can induce the effective degradation of target mRNAs, and hence inhibit expression of the encoded human gene product[8], this technique presents a number of problems. One of the most significant of these is the fact that many cells, including primary cells, are difficult to efficiently transfect with synthetic RNA molecules. Also important is the intrinsically transient nature of the observed inhibition. There has, therefore, been considerable interest in developing technology that would allow the stable production of siRNAs from introduced expression plasmids, while avoiding activation of nonspecific cellular responses to dsRNAs.

The observation that dicer, the ribonuclease responsible for siRNA production from long dsRNAs, could also selectively cleave short RNA stem-loop structures—as seen during the biosynthesis of miRNAs and stRNAs (**Fig. 1b**)—indicated that it should be possible to generate designed siRNAs from RNA stem-loop structures encoded

by introduced expression plasmids. In fact, several groups have demonstrated the production of biologically active siRNAs derived from expression plasmids that contain promoters dependent either on RNA polymerase II (pol II) or RNA polymerase III (pol III)[14–16]. Pol III has the advantage that its transcripts are not necessarily post-transcriptionally modified and that pol III–dependent promoters are highly active when introduced into human cells. The reported siRNA expression plasmids rely on a class of pol III–dependent promoter element, for example U6 or H1 that is located entirely 5′ to the transcription start site[14,15]. The pol III expression plasmids are designed to transcribe a predicted RNA stem-loop structure that contains a perfect 19–29-bp stem that is homologous to the target mRNA. At present, the sequence of the terminal loop, which is from six to nine nucleotides long, does not appear to be significant. Flanking the siRNA precursor sequence is a pol III transcription termination signal, which consists of five "T" residues and adds two "U" residues to the 3′ end of the stem-loop. The resultant siRNA precursor is cleaved by dicer to produce designed siRNAs that can specifically inhibit target gene expression either when produced transiently or expressed in a stable form[14,15] (**Fig. 1c**).

An alternative approach relies not on pol III, but on pol II: it uses, as a starting point, a natural miRNA precursor RNA stem-loop structure rather than a totally artificial RNA stem-loop[16]. When the human mir-30 miRNA precursor was transcribed as part of a longer mRNA transcript, it was nevertheless effectively processed, presumably by dicer, to give two miRNAs encoded by both the 5′ and the 3′ arm of the precursor RNA stem-loop. These two miRNAs, if annealed, would give rise to a 21-nt dsRNA bearing 2-nt 3′ overhangs, that is, the structure typical of an siRNA. Substitution of the stem sequences of the mir-30 miRNA precursor with designed, base-paired sequences generated new miRNAs that were able to inhibit the expression of either cotransfected or endogenous target genes by inducing the selective degradation of the relevant mRNA species[16]. At present, it remains unclear whether the pol II– or the pol III–based vectors will provide the most effective means of expressing designed siRNAs and, hence, of inhibiting endogenous gene expression. However, the former do have the advantage that they can be more readily incorporated into viral expression vectors, such as retroviral or adeno-associated virus–derived vectors, which are useful for the stable transduction of cells, including primary cells. Also, several inducible or tissue-specific pol II–dependent promoters have been reported, whereas functionally equivalent pol III–dependent promoters remain to be developed.

The development of systems that allow the stable production of designed siRNAs—and, hence, presumably stable inhibition of target gene expression—suggests that it should now be possible to perform reverse genetic experiments, including epistasis experiments, in mammalian cells in culture. For example, in the case of cultured lymphoid cells, it should be possible to selectively block the expression of specific cell surface receptors, signaling molecules or transcription factors and thereby determine their importance in mediating a particular immunologically important cellular response. This technology may also prove useful in the design of transgenic animals that constitutively or inducibly produce siRNAs that can block the expression of selected target genes, including genes encoded by pathogenic viruses. Finally, this technology might be used in the future to inhibit the expression of deleterious gene products in selected human cells *in vivo*, with a gene therapy approach. Although the usefulness of RNAi in the treatment of diseases characterized by the inappropriate expression of wild-type or mutant forms of specific human gene products remains to be determined, the advent of RNAi in human cells clearly represents a major technical advance that has the potential to dramatically affect how biomedical research is conducted.

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