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Mammalian RNAi for the masses

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Just a couple of years ago, only biologists working with plants or *Caenorhabditis elegans* could use RNA-mediated interference (RNAi) technology to gain insight into gene function. However, the recent groundbreaking discovery that *in vitro* synthesized, 21- to 23-nucleotide, double-stranded RNAs can act as small interfering RNAs (siRNAs) to elicit gene-specific inhibition in mammalian cells has made RNAi possible in mammalian systems too. Reported only a year ago, mammalian RNAi is already changing our way of studying gene function in higher eukaryotes. And, a recent exciting advance allows delivery of siRNAs into mammalian cells by a DNA vector. In addition to providing a low-cost alternative to the chemically synthesized siRNAs, this DNA-vector-based strategy is capable of mediating stable target gene inhibition, thus allowing gene function analysis over an extended period of time.

RNA-mediated interference (RNAi), a term coined by Fire and Mello, is the inhibition of expression of specific genes by double-stranded RNAs (dsRNAs) [1]. RNAi is evolutionarily conserved among eukaryotes, and it probably has an essential role in mediating responses to exogenous RNAs (such as viruses) and in stabilizing the genome by sequestering repetitive sequences (such as transposons) [2]. RNAi is a multi-step process involving the generation of small interfering RNAs (siRNAs) *in vivo* through the action of the RNase III endonuclease Dicer. The resulting 21- to 23-nucleotide (nt) siRNAs mediate degradation of their complementary RNA. The biology and mechanisms of RNAi are reviewed in detail in Refs [2–6], so here I limit my discussion to the recent development of DNA vector-based RNAi approaches in mammals and its potential application in gene function analysis and in therapy.

Until recently, the use of RNAi as a reverse genetic tool to study gene function was restricted to plants, *Caenorhabditis elegans* and *Drosophila*, where large dsRNAs can efficiently induce gene-specific silencing [1,7–9]. A major obstacle to achieving RNAi in mammals is that dsRNAs longer than 30 nt activate an antiviral defense mechanism that includes the production of interferon, resulting in non-specific degradation of RNA transcripts and a general shutdown of host cell protein synthesis [10,11]. This obstacle has been overcome recently by using *in vitro* synthesized ~21-nt siRNAs to mediate gene-specific suppression in mammalian cells. These siRNAs are long enough to induce gene-specific suppression, but short enough to evade the host interferon response [12,13]. Following this breakthrough discovery, several reports appeared within a few months describing DNA vector-based strategies for the delivery of siRNA into mammalian cells, further expanding the utility of RNAi in mammals. The new approaches are not only cost effective, but also have unique properties that could be important for the application of the RNAi technology *in vivo*.

Plasmid vectors expressing siRNAs directed by RNA polymerase III

Biochemical analyses show that siRNAs generated by RNase III (Dicer) in *Drosophila* embryonic extracts contain 3′ overhangs of two or three nt [14–16]. This structural feature appears to be important for the *in vitro* synthesized siRNAs to inhibit gene expression effectively in cultured mammalian cells [12]. Furthermore, siRNAs with 3′ overhangs of two uridines have been found to be more efficient than those with 3′ overhangs of AA, CC or GG [15]. Recently, several laboratories have reported the success of using RNA polymerase III (Pol III) promoters to direct *in vivo* synthesis of functional siRNAs that incorporate some of these structural features [17–24]. There are several reasons for using Pol III. First, unlike RNA Pol II, Pol III normally transcribes small, noncoding transcripts that are not capped or polyadenylated at the 5′ and 3′ ends, respectively. Second, Pol III initiates transcription at defined nucleotides, and terminates transcription when it encounters a stretch of four or five thymidines [25]. Consequently, it is possible to design small RNAs synthesized by Pol III that carry 3′ overhangs of one to four uridines, a structural feature resembling that defined for siRNAs to be effective *in vitro* [16].

To date, two approaches, both using Pol III promoters, have yielded robust gene-specific inhibition.

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In the first case, the design is modeled after the naturally occurring microRNAs (miRNA) that are ~22-nt hairpins and can modulate gene expression *in vivo* [26]. Two Pol III promoters, U6 and H1, are used to direct transcription of small inverted repeats separated by a spacer region of varying lengths. The U6 or the H1 promoter initiates transcription at guanine or adenine, respectively. The resulting RNAs are predicted to form hairpins containing 19- to 29-nt stems that match target sequences precisely, three- to nine-nt loops and 3′ overhangs of four or fewer uridines (Fig. 1a). It is believed that these hairpin RNAs are processed by Dicer to active siRNAs *in vivo* [18]. In the second case, two U6 promoters are placed in tandem [21,23] or on two separate vectors [20] to direct transcription of a sense and an antisense strand of a small RNA with 19 nt matching the target gene sequence precisely and four or fewer Us as 3′ overhangs (Fig. 1b). The sense and antisense strands are believed to form a duplex *in vivo*, similar to the chemically synthesized siRNAs described previously [16]. However, the hairpin siRNA strategy appears to inhibit gene expression more efficiently than the duplex siRNAs expressed from two separate plasmids [20].

RNAi plasmid vectors expressing modified miRNAs that act as siRNAs

Another way of generating small RNAs that can function as siRNAs from DNA templates is through the generation of modified miRNAs. Naturally occurring miRNAs are noncoding RNAs, and they have been identified in a range of organisms from *C. elegans* to humans [26]. The best characterized miRNAs (also known as stRNAs for small temporal RNAs) are *C. elegans lin-4* and *let-7*, both of which are crucial in the control of developmental timing [27,28]. miRNAs are single-stranded ~22-nt RNAs that are processed from ~70-nt hairpin RNA precursors by the same RNase III nuclease Dicer that is responsible for the generation of siRNAs *in vivo* [29–32]. A significant mechanistic difference between siRNAs and miRNAs is that, whereas siRNAs target RNA substrates for degradation, miRNAs appear to inhibit gene expression at the level of translation [26]. This is believed to be due to the fact that siRNAs share complete sequence match with the substrates, whereas miRNAs have multiple mismatches (Fig. 2). Consistent with this, recent studies show that *let-7* can enter the RNAi pathway to induce degradation of a fully complementary artificial target RNA [33].

Based on the above findings, artificial 'designer' miRNAs whose sequences are completely complementary to the target RNAs have been shown to function as siRNAs that inhibit gene expression by reducing RNA transcript levels [24,34]. Unlike the small hairpin RNAs directed by U6 or H1 promoters (Fig. 1), the siRNA-acting miRNAs are generated from ~70-nt miRNA precursors. The artificial miRNA precursor contains a substitution of the stem sequence with a sequence entirely complementary to the intended target gene (Fig. 2b), enabling the resulting miRNA to function as a siRNA to induce target RNA degradation [34].

Both types of RNAi plasmid vectors have been used to achieve impressive inhibition of the expression of a large number of genes with diverse biological functions, including genes encoding the structural proteins lamin A/C [17,22] and neuronal β-tubulin [20]; viral proteins such as HIV rev and tat, as well as its co-receptor CD4 and CD8α [21,24,34]; the tumor suppressor p53 [18,19]; the cell cycle regulators cdk2 [17] and CDH1 [19]; and the Wnt signaling protein β-catenin [23]. The DNA vector-based approaches also appear to function in a wide variety of cells, including primary neurons [35] and established cell lines, such as fibroblasts (IMR90 and NIH3T3) [18], T cells (E-10) [24], pluripotent embryonic carcinoma cells (p19) [20], and cell lines derived from cervical carcinoma (HeLa and C33A) [17–19,22–24], osteosarcoma (U2OS) [17], breast cancer (MCF-7) [19] and kidney (293, 293T and Cos-1) [18,21,34]. These findings, together with the results using synthetic siRNAs, suggest that RNAi could inhibit expression of

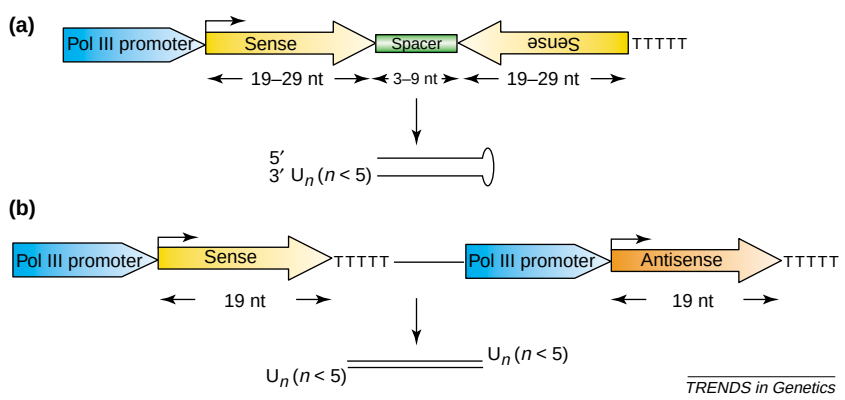

Fig. 1. The DNA vector-based RNA interference (RNAi) technology. (a) Generation of a hairpin siRNA directed by a Pol III promoter. An inverted repeat is inserted at the +1 position of the U6 promoter (−351 to +1). The individual motif is 19–29 nt, corresponding to the coding region of the gene of interest. The two motifs that form the inverted repeat are separated by a spacer of three to nine nt. The transcriptional termination signal of five Ts are added at the 3′ end of the inverted repeat. The resulting RNA is predicted to fold back to form a hairpin dsRNA as shown. The resulting siRNA starts with either a G or an A at the 5′ end, dependent on the promoter used (U6 or H1) and ends with one to four uridines, forming a 3′ overhang that is not complementary to the target sequences. (b) Generation of two complementary siRNA strands synthesized by two U6 promoters. Two U6 promoters either placed in tandem or on two separate plasmids (not shown) direct transcription of a sense and an antisense strand of 19-nt RNAs. The two RNA strands are predicted to form a duplex siRNA in the transfected cells, with 3′ overhangs of one to four uridines.

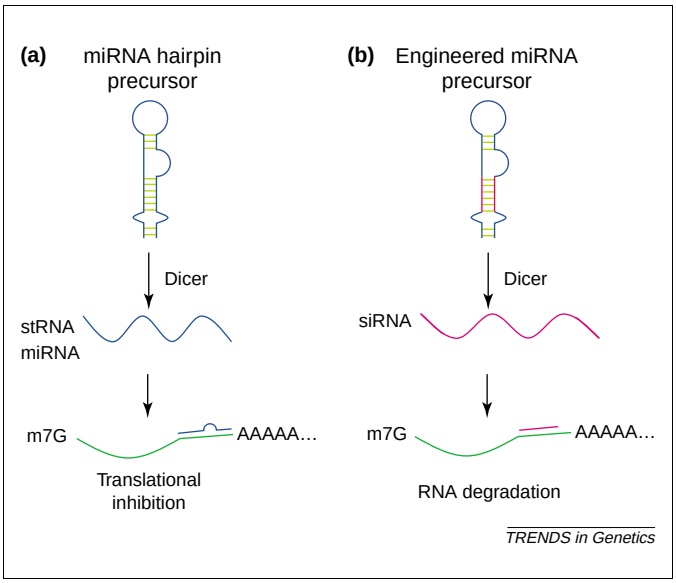

Fig. 2. Naturally occurring and artificial miRNAs that can function as siRNAs (adapted, with permission, from Ref. [2], see http://www.nature.com). Naturally occurring miRNA processors (A) are ~70-nt hairpins that are processed by the RNase III enzyme Dicer to generate single-stranded miRNAs (e.g. *lin-4, let-7* and *mir-30*). The resulting miRNAs show imperfect sequence complementarity to the target genes and regulate their expression by inhibiting translation. The engineered artificial miRNAs (b) are similarly processed to generate single-stranded miRNAs that display full complementarity to the targets, resulting in target RNA degradation. StRNA, small temporal RNA; miRNA: microRNA.

genes of interest in almost every type of cell. However, in mammalian cells, siRNA-mediated gene inhibition, although impressive (80 to >90%), never completely eliminates the gene product, and must be considered as a 'knock-down' rather than a 'knockout' approach. Nevertheless, it is possible that RNAi could in some instances result in phenotypes that mimic genetic knockouts.

Target site selection

The effectiveness of an siRNA is likely to be determined by the accessibility of its target sequence in the intended substrate. Many of the siRNAs reported to date are designed to target coding sequences. In our laboratory, we typically select sequences located 100–200 bases away from the translation initiation sequence AUG [17]. However, successful gene inhibition has been reported for siRNAs targeting various sequences, including the 3′ untranslated region [24]. At the moment, there are no reliable ways to predict or identify the 'ideal' sequence for an siRNA. However, Rossi and colleagues reported a successful experimental approach to determine the accessibility of the chosen sequences [21]. This approach involves the use of oligonucleotides complementary to the target sequences as probes to determine substrate accessibility in the cell extracts. After forming a duplex with the oligonucleotide probe, the substrate becomes susceptible to RNase H. Therefore, the degree of the RNase H sensitivity of a given probe reflects the accessibility of the chosen site, and could predict how well the siRNA will perform. However, it might be just as easy to construct and test a few siRNA vectors. In addition to the sequences that form the stems of the hairpin siRNAs, the loop size and the sequences at the base of the loop might also have a role in determining siRNA efficacy [19]. A future challenge is to determine these requirements for effective siRNAs.

siRNA delivery

The inhibitory effects of the transfected, *in vitro* synthesized siRNAs are usually transient because mammalian cells lack the mechanism to support the amplification of siRNA-mediated silencing observed in *C. elegans* and *Drosophila* [2]. Thus, a distinct advantage of the DNA vector-based RNA approach is that it can be used to express siRNAs stably in cells and thus confer long-term gene inhibition. This principle was demonstrated recently by Brummelkamp *et al.* who reported sustained inhibition of p53 by stably integrated siRNA-expressing DNA templates [19]. In addition, the delivery of siRNA from DNA templates can be adapted to viral vectors, and thus can facilitate entry of siRNAs into cells and tissues that are otherwise difficult to transfect. Retroviruses and adeno-associated virus (AAV) have already been shown to be effective vehicles for delivering siRNA into cells ([19,36,37]; Y. Shi *et al.*, unpublished). Other viral vectors such as adenovirus and lentivirus should also be amenable as vehicles carrying siRNA-expressing DNA templates.

Potential medical application

One of the unique features of RNAi is its exquisite sequence specificity. Introduction of a single base mismatch in the body of the hairpin siRNA abrogates its RNAi effect [19]. This high degree of sequence specificity enables the use of RNAi to selectively knock-down expression of alleles carrying point mutations, insertions or deletions, and it therefore has important medical implications. Conceptually, RNAi technology can be used alone, or in combination with other existing therapeutic tools, to curb diseases that are caused by dominantly acting mutant alleles, such as cancer and neurodegenerative diseases, as well as to combat viral infection. For instance, RNAi can be used to inhibit oncogenes resulting from chromosomal translocations, or carrying point mutations. Many hematopoietic cancers are caused by dominantly acting oncoproteins encoded by fusion RNA transcripts resulting from chromosomal translocations. The chimeric RNA transcripts are therefore ideal targets for selective inhibition by siRNAs targeting the fusion sites, as has been demonstrated recently [38]. Selective siRNAs also suppress tumorigenesis by inhibiting expression of the oncogenic K-ras^{v12} allele, but not that of the wild-type alleles [36]. In addition to cancers, siRNAs also successfully inhibit viral replication in cell culture [39,40] and suppress the cytotoxicity caused by overexpression of a androgen receptor with an expanded polyglutamine tract, which is associated with the neurodegenerative disorder, spinobulbar muscular atrophy (SBMA) [41]. More recently, both *in vitro* synthesized and vector-directed siRNAs were demonstrated to selectively silence the amyotrophic lateral sclerosis (ALS)-causing, but not the wild-type SOD1 alleles in the same cells (H. Ding *et al.*, unpublished). These findings have therefore laid the

foundation for future experiments in ALS mouse models using the RNAi technology.

Perspectives

Currently, selection of siRNA sequence is largely empirical. It will be important to determine whether there are rules that can guide the design of an effective siRNA. In addition, inducible siRNA-expressing systems and various viral vectors that can efficiently deliver siRNAs to proliferating and resting cells will greatly enhance the application of the RNAi technology in gene function analysis, and in potential therapeutic use. To translate the RNAi technology to medical use, an immediate challenge is to determine the effectiveness of siRNAs in living animals. As a step towards this goal, siRNAs synthesized *in vitro* or from a DNA template were recently used effectively to inhibit transgene in mice [42]. Given the fast pace of the RNAi field, it will not be surprising if the outstanding issues are resolved in the near future.

Note added in proof

Xia *et al.* have recently shown that adenoviral vectors can be used to carry siRNA-expression DNA templates to mediate gene-specific silencing in mice [43].

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