



Small non-coding RNAs as magic bullets

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RNA interference (RNAi) – inhibition of gene expression by small, non-coding RNAs [small interfering RNAs (siRNAs) or microRNAs (miRNAs)] – has changed our view of regulation of expression dramatically. The application of siRNAs for both functional analysis of genes and medication raises several questions. These include the design of the double-stranded oligonucleotides, their preparation and introduction into cells or animals either as chemically synthesized entities or as transcripts from a suitable vector. Delivery of the oligonucleotides, choice of vector, chemical modification to stabilize against nucleases and avoidance of side effects (e.g. stimulation of interferons) are major challenges. Work to identify the multiple targets of miRNAs is still in its infancy, and a clear distinction between siRNAs and miRNAs is difficult in some instances. Moreover, transcriptional silencing by RNAi is poorly understood; it is evident that the siRNA machinery is involved but the details await clarification. Given the multitude of interactions of the small non-coding RNAs revealed so far, we should be prepared to encounter, as yet, undiscovered interactions and mechanisms.

Introduction

In an unbelievably short amount of time, the discovery of RNA interference (RNAi) directed researchers to the short pieces of RNA that had previously escaped attention. Not surprisingly this flourishing field has been the subject of numerous recent reviews (see, for example, Refs [1–6]). Two types of such short oligoribonucleotide duplexes of 21–23 nucleotides with two- or three-nucleotide 3' overhangs exist: short interfering RNAs (siRNAs) and microRNAs (miRNAs). siRNAs are generated by random processing of long double-stranded RNAs (dsRNAs), whereas miRNA duplexes are excised in a sequence-defined manner from short hairpin dsRNA precursors. siRNAs can be introduced into cells as chemically synthesized entities by transfection (Figure 1). Alternatively, both siRNAs and miRNAs can also be processed from endogenously transcribed short hairpin RNAs that are expressed from transfected plasmids or engineered viruses. So far, only very few endogenously expressed siRNAs have been detected in mammals. Only one of the strands of a siRNA duplex is incorporated into the ribonucleoprotein particle RNA-induced silencing

complex (RISC), which is responsible for the cleavage of the target mRNA as long as there is significant complementarity with the mRNA [7]. In human and *Drosophila melanogaster*, Argonaute 2 (Ago2) is responsible for this cleavage [8–10], the result of which is a 5' phosphate and a 3' hydroxyl group at the termini of the products [11,12]. X-ray structures suggest that the PIWI domain of Ago2, which adopts an RNase H fold, is responsible for the cleavage [13–15] and that the PAZ domain recognizes the siRNA by its two-nucleotide 3' overhang [16]. The X-ray structure also supports the notion that Ago2 measures the position of cleavage from the 5' end of the siRNA [17].

However, in contrast to artificially introduced siRNAs, the structurally related miRNAs are encoded by endogenous genes and have been isolated from a wide range of organisms and cell types [18,19]. They originate from stem-loop structures of ~80 nucleotides that are components of longer RNA transcripts. These primary transcripts are processed in the nucleus by Drosha into hairpins of ~60–70 nucleotides (pre-miRNAs) [20,21]. These pre-miRNAs are exported to the cytoplasm, where Dicer further processes them into the final duplex miRNAs of 21–23 base pairs [18,22] (Figure 1). Finally, one of the miRNA strands is incorporated into a ribonucleoprotein particle (RNP) in the same process as siRNA duplexes [5]. In contrast to siRNAs, which have been selected to be fully complementary to their target mRNAs, the miRNAs bind in most cases to the 3' untranslated regions (UTRs) with only partial complementarity, resulting in translational arrest without cleavage of the target [23,24] (Figure 2).

siRNA

Application and design

The introduction of siRNAs into cells can be achieved in various ways [25]. Probably the most commonly used method is the transfection of chemically synthesized siRNAs. For cleavage of a given target RNA, the antisense (or guide) strand should preferably be incorporated into RISC (Figure 2). This complex only contains single-stranded RNA and details of the rules that promote asymmetric assembly of the antisense strand of a siRNA duplex have been elucidated. Thus, for example, the base pair at the 5' end of the antisense strand should be thermodynamically weak compared with the 3' end to achieve asymmetry in incorporation into RISC [26,27]. However, further parameters for optimal efficiency of siRNAs have been identified, such as low guanine or

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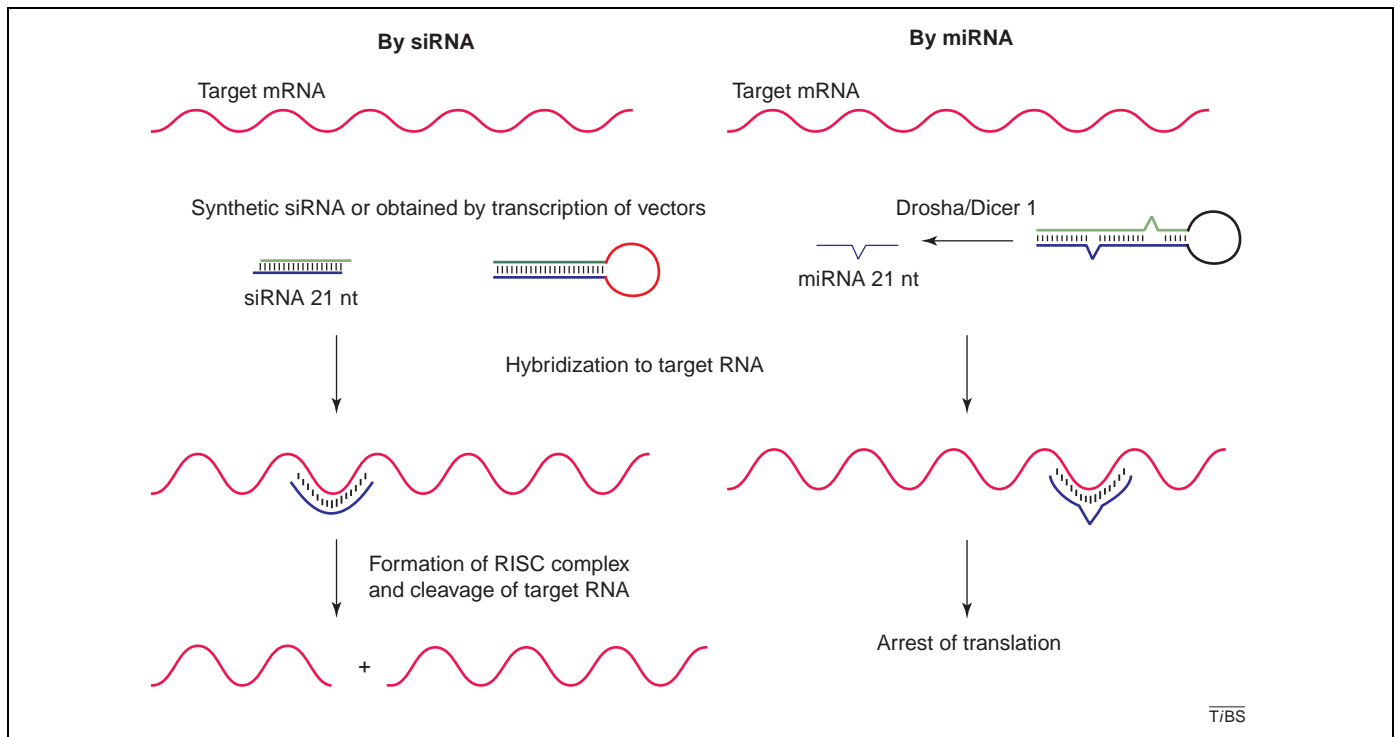


Figure 1. The general scheme for RNAi by siRNAs and miRNAs. Complete complementarity of siRNAs (left), either chemically synthesized or cleaved from transcripts, with target RNA in RISC results in cleavage of the target in the RISC complex. Incomplete complementarity between miRNA and the 3' UTR of the target results in inhibition of translation. Colour code: red, target mRNA; blue, antisense strand of siRNA or miRNA; green, sense strand of siRNA or miRNA.

cytosine content, lack of inverted repeats and sense-strand base preferences at positions 3, 10, 13 and 19 [28]. However, it should be noted that siRNAs are sometimes found to be highly active even when they do not obey these, or other, rules, thus indicating the need for experimental verification of activity [29]. As

our understanding of the enzymology of siRNA-guided cleavage progresses we will probably be in a position to further improve these selection rules. Interestingly, it seems that the 5' end of the siRNA is responsible for target affinity, whereas the 3' end contributes mainly to catalysis [30].

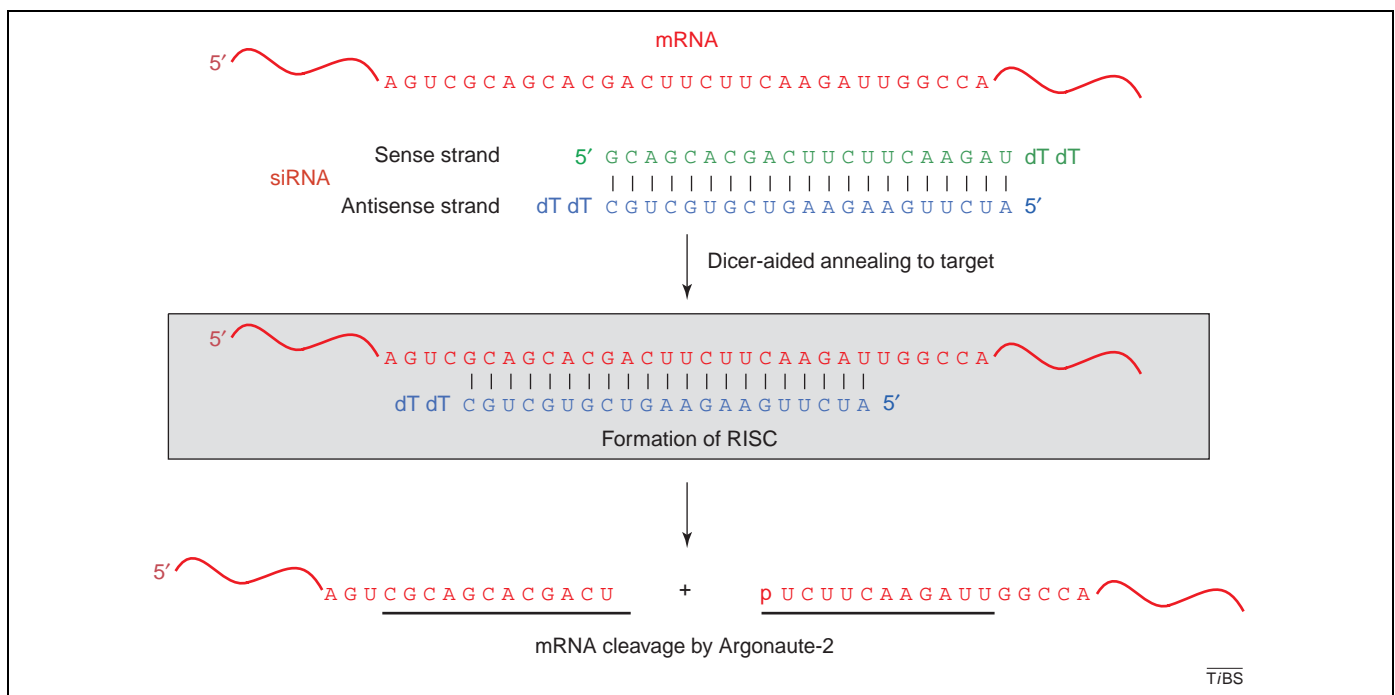


Figure 2. Interaction of siRNA and its target. Sequence-specific interaction of the antisense strand of siRNA with the target mRNA in RISC results in cleavage. The two-nucleotide 3' overhangs of the siRNA can vary (see, for example, Ref. [38]). The dsRNA cleavage products of Dicer also have such overhangs. Cleavage of the target RNA occurs in the middle of the complementary region, ten nucleotides from the 5' end of the antisense siRNA. Colour code: red, target mRNA; blue, antisense strand of siRNA; green, sense strand of siRNA.

Although 21-mer siRNAs are considered to be the most active, two groups recently observed that 27- to 29-mer dsRNAs can be more active than 21-mer siRNAs [31,32]. The reasoning is that these RNAs are processed by Dicer and, thus, the siRNAs are directly and efficiently incorporated into RISC [31,32]. In addition, the siRNA duplexes, which resemble the Dicer processing products, should also bind to Dicer effectively owing to the principle of microscopic reversibility. Of course, as is the case for all oligonucleotide-based silencing methods, there is also the problem of target-RNA accessibility for the siRNAs. This accessibility can be analysed by experimental or computational approaches, and the latter have been used to explore the local folding of three sites of the intracellular adhesion molecule (ICAM)-1 mRNA and inhibition by siRNA. ICAM-1 mRNA is one of the best-studied targets of antisense oligodeoxynucleotides; interestingly, similarly to antisense oligonucleotides, regions of ICAM-1 mRNA that are not involved in intramolecular folding are the optimal targets [33].

For a long time, it has been thought that the machinery for siRNA-mediated RNA cleavage is located in the cytoplasm. However, efficient cleavage of small nuclear RNA (snRNA; 331 nucleotides), as part of the 7SK RNP, after delivery of the corresponding siRNA has recently been described to take place in the nucleus of HeLa cells [34]. If 7SK snRNA, as suggested, never leaves the nucleus, this indicates that the necessary proteins for RISC are also located in the nucleus.

Vectors

Besides applying the siRNAs exogenously, they can also be transcribed from appropriate expression plasmids or viral vectors inside the cell (Figure 3). In most cases, they are expressed as 20-base-pair hairpin transcripts, most often from a polymerase III U6-promoter. These transcripts are likely to be too short to be processed by Drosha, but are substrates for Dicer.

The use of RNA polymerase II (pol II) promoters is essential for tissue-specific transcription. Such transcripts obtained from the pol II promoter are, in most cases, long dsRNA that, when transferred to the cytosol, induce interferon response. However, deviation from the strict double-stranded nature of the transcript, such as introduction of a short hairpin or bulge, might prevent interferon stimulation, as seems to be the case for primary miRNA transcripts (pri-miRNAs). An alternative mechanism, which could avoid interferon stimulation, is the transcription of RNAs that lack the necessary signals for transport to the cytoplasm. Thus, a vector has been developed to express transcripts from pol II promoters that lack the 5'-cap structure and the 3'-poly(A) tail, which are responsible for the export from the nucleus; thus, the interferon response is not induced [35]. Attempting to obtain increased tissue specificity, U6-based lentiviral vectors for conditional Cre-lox-regulated RNAi that are applicable to Cre-expressing transgenic mice have been described [36]. Ubiquitous gene knock-down in mice can be achieved by a short hairpin RNA (shRNA) transgene under the control of either the U6 or the H1 promoter when integrated at the *rosa26* locus [37].

Animal model studies

It is of great interest to interfere with gene expression in an animal model to explore whether siRNAs have the potential for therapy in humans. Several mouse models, in which chemically synthesized siRNA has been either injected intravenously [38] or instilled intranasally, have been described [29,39]. In one study [38], siRNA directed against the endogenous apolipoprotein B (ApoB) was modestly chemically modified to carry a cholesterol group and a phosphorothioate at the 3' end of the sense strand, and two 2'-O-methyl groups and two phosphorothioates at the 3' end of the antisense strand. This siRNA was injected without a transfection reagent to avoid the risk of eliciting unwanted side effects. The siRNA reduced the mRNA level and decreased the plasma concentration of the ApoB protein in liver and jejunum, and, as a consequence, led to a reduction in total cholesterol in the blood. In a transgenic mouse model, the siRNA silenced the human ApoB. This is an encouraging result because it demonstrates that siRNAs have the potential to silence endogenous genes by conventional low-pressure intravenous injection.

To fight influenza infection, siRNAs directed against several parts of the viral genome were applied to mice either as chemically synthesized oligonucleotides in a complex with polyethylene-imine as transfection reagent or as shRNAs via lentiviral transduction [40]. Both methods seemed to be successful.

It has been found that inhibition of respiratory syncytial virus and parainfluenza virus by intranasally instilled siRNA either with or without a transfection reagent can be achieved [29,41], although delivery of the siRNA without transfection reagent is slightly more effective. Although the data were obtained in a mouse model, they point towards an easily administrable antiviral compound against respiratory viral disease in humans for which there is no reliable vaccine or antiviral drug at present.

To interfere with expression of hepatitis B virus (HBV) in transgenic mice, an adenovirus vector encoding the sequences of siRNAs as shRNAs was intravenously injected in the tail vein [42]. The HBV transcript was degraded by one of two shRNAs in the liver, and HBV expression could be suppressed for at least 26 days. Interestingly, a stable pool of viral 3.5-kb RNA transcripts persisted even though the shRNAs contained the proper sequences to target them. It was deemed possible that this population of viral RNA might have been protected within the RNA-protein complex.

Resistance

There are several examples whereby mechanisms against silencing by siRNA have been developed. Thus, HIV-1 has found ways to escape inhibition of replication by siRNA [43]. The *Nef* gene has developed two modes for this purpose: (i) to undergo mutations to perturb the pairing between the siRNA and target duplex, which results in RNAi resistance, thus retaining HIV-1 replication for these mutants; and (ii) a mutation leading to alternative folding of the RNA, which renders the original target inaccessible, leading to resistance of cleavage.

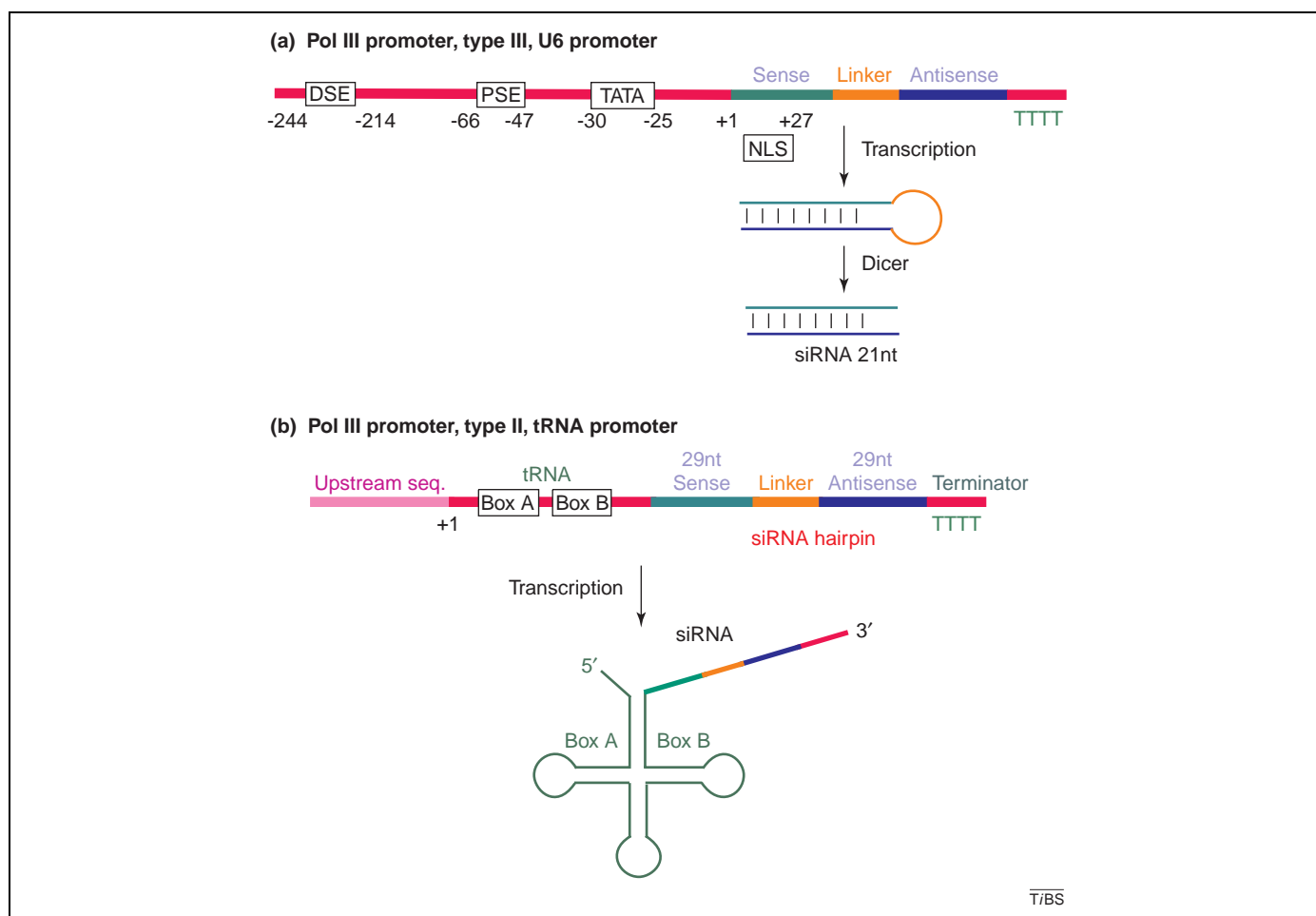


Figure 3. Two polymerase III promoter designs for transcription of siRNAs. Generation of transcripts for siRNAs using a U6 promoter (a) and tRNA promoter (b). Transcripts of the hairpin-loop structure are further processed by Dicer to produce active siRNAs. Blue, antisense strand of siRNA; green, sense strand. Abbreviations: DSE, distal sequence element; NLS, nuclear localization signal; PSE, proximal sequence element.

Presumably, these methods for resistance can only be adopted by a virus with a high mutation rate and should be kept in mind when designing therapeutic strategies that require longer lasting exposure to siRNA or shRNA. To be on the safe side, multiple targets should be chosen. For example, a vector for the simultaneous expression of two distinct shRNAs against two different regions in the RNA polymerase of coxsackievirus B3 has been developed [44]. Many plant viruses encode proteins that specifically inhibit their silencing by siRNAs; the proteins are produced by the plant after infection as a defence mechanism of the virus to preserve its survival. The best understood is the p19 protein from the Tombusvirus. The X-ray structural analysis shows it to bind the siRNA duplex predominantly by water-mediated intermolecular contacts via phosphates and 2'OH groups [45]; this guarantees sequence-independent recognition. Another strategy to modulate RNAi, unrelated to viruses, was observed in the nervous system of *Caenorhabditis elegans* [46]. An RNase specific to neurons, ERI-1, specifically degrades siRNAs, thus rendering the nervous system refractory to RNAi. In the nematode worm, ERI-1 is predominantly cytoplasmic and mainly expressed in the gonad and a subset of neurons. Thus, it is a negative regulator that limits the half-life of siRNA, cell-type specificity or endogenous functions of siRNAs. The

function of the human orthologue remains to be investigated. Another example of RNAi resistance is the presence of a protein in the retrovirus primate foamy virus type 1 (PFV-1), which suppresses the miRNA-directed inhibition of accumulation of this virus in human cells [47].

Chemical modification

For more detailed reviews of chemical modifications of the siRNAs see Refs [48] and [4]. One of the few examples of chemically modified siRNA tested *in vivo* is that directed against ApoB (as discussed earlier); it carries two phosphorothioates and 2'-O-methyl groups near the 3' end of the antisense strand (23 nucleotides) for stabilization against degradation by 3' nucleases [38]. A cholesterol and phosphorothioate was placed at the 3' end of the sense strand (21 nucleotides). The importance of these modifications is illustrated by the fact that the unmodified and unconjugated siRNA showed no down-regulation of ApoB. Another study tested the 2'-fluoropyrimidine-nucleotide modification in both strands of a siRNA directed against a luciferase gene [49]. The siRNA and the luciferase reporter plasmid were introduced to the mice by tail-vein hydrodynamic injection. Although the siRNA was greatly stabilized against degradation, this did not translate into enhanced or prolonged inhibition of the target. This is in contrast to the ApoB experiments

discussed earlier [38]. There might be several reasons behind this difference in effect of chemical modification. For example, it is possible that the uptake of the cholesterol-conjugated siRNA was superior to that of the non-conjugated, whereas the 2'-fluoro modification had no effect in this respect. Another reason could be differences in the life times of the targeted proteins in the two studies. Thus, at present, it seems that each modification and conjugation will have to be tested until we have a better understanding of which step(s) they benefit.

A systematic study for evaluating the effect of siRNA modification on targeting was executed in cell culture by Chiu and Rana [50]. They showed that a great deal of modification might be tolerated in the sense and the antisense strands but, because this study was performed only with one siRNA sequence, generalization of rules applicable to other sequences is premature. Harborth *et al.* [51] conducted a more thorough study in which many siRNAs directed against lamin A/C with a variety of modifications were tested. Most, or all, chemical modifications were applied to one specific sequence. They found that, for alternating positions containing a phosphorothioate linkage, no serious activity loss is observed but there is some cytotoxicity. However, toxicity was absent when only a few phosphorothioates were placed at the termini. They also observed that 2'-fluoro derivatives did not severely interfere with activity.

A more indirect type of modification is the attachment of a peptide – for example, of nuclear localization signal (NLS) – to the DNA sequence serving as the template for transcription for the desired shRNA. The NLS should facilitate the uptake in the nucleus and improve the generation of the siRNA [52]. The cellular uptake and localization of oligonucleotide-peptide conjugates, specifically for siRNAs, requires further research [53,54].

Heterochromatin formation

Apart from gene silencing via mRNA cleavage or translational repression, miRNAs are involved in regulatory processes at the transcriptional level in the nucleus. This is owing to two mechanisms: (i) RNA-directed methylation of DNA and (ii) RNAi-mediated heterochromatin formation with methylation of lysine in histone H3 [55,56]. DNA methylation by a site-specific methyltransferase is nearly absent in fission yeast, flies and nematodes, but is well established in plants. Transcriptional silencing via DNA methylation of promoter sequences has also been documented for human cells and requires transport or expression of siRNA in the nucleus [57,58]. It is thought that the siRNA directs the methyltransferase to the DNA of complementary sequences. The enzyme might recognize the heteroduplex. The DNA-methylation is not always preceded by histone H3 Lys9 methylation; this can differ between organisms. In plants, at least, histone methylation helps to maintain DNA methylation.

The RNAi machinery has also been shown to be responsible for heterochromatin formation in fission yeast, *D. melanogaster* and vertebrate cells [59,60]. Processing of the dsRNAs by Dicer is considered to provide small RNAs to guide the histone

methyltransferase to the target sites on the chromosome to methylate H3 Lys9 [56,61].

In addition to regulation of transcription, these reactions at the genomic level represent yet another RNAi mechanism of defence against invasive sequences [62,63]. Interestingly, these modifications can imprint chromosomal sequences so that they are maintained through cell division, which could account for epigenetic silencing.

miRNA

In contrast to siRNAs, which cleave the targeted mRNA, the association of miRNAs with the 3' UTR of the targeted message results in inhibition of translation. The main interaction is apparently the complete complementarity between nucleotides at position 2–7 of the miRNA and the target RNA [23,24]. However, there are exceptions to this translational inhibition. In plants, the majority of miRNAs lead to cleavage, and some mammalian miRNAs might also guide cleavage of their targets [64,65]. The protein in RISC that is responsible for the mRNA cleavage in mammals and flies is Ago2 [8,9]. The complementarity of the target encountered by the different small RNA-loaded Ago2 complexes might be the biochemical basis that siRNAs can also function as miRNAs [24,66,67].

Several hundred miRNAs have been found in plants and mammals but their role, particularly in mammals, has not yet been linked to particular mechanisms. Poy *et al.* [68] have investigated a particular miRNA (miR-375) that is naturally expressed in primary pancreatic endocrine MIN6 cells. When miR-375 was over-expressed in these cells via an adenovirus vector, glucose-induced insulin secretion was suppressed by reducing the levels of the protein myotrophin (encoded by one of 64 putative miR-375-target genes). The inhibition of endogenous miR-375 enhanced this secretion. This demonstrates the power of miRNAs to identify as yet unknown pathways that, in this case, might lead to novel therapeutic approaches.

Mansfield *et al.* [69] have described tissue-specific expression of several miRNAs during mouse embryogenesis. The emphasis of this study was to follow the spatial and temporal expression of Hox clusters using two miRNAs for which there are target sequences at the 3' UTRs of the Hox genes [64]. The expression of Hox genes identified the distribution of miRNAs and suggests that these miRNAs participate in the fine-tuning of at least some of the Hox genes during development. Another example of the role of miRNAs is that of regulation of brain morphogenesis in Zebra fish [70]. Yet another function of miRNAs is the observation that a human cellular miRNA effectively restricts the accumulation of the retrovirus PFV-1 in human cells [47].

Frequency of miRNA occurrence

Genes encoding miRNAs are surprisingly frequent, representing ~1–2% of known genes in eukaryotes. The identification of targets for these miRNAs in animals is complicated because of the incomplete complementarity that is sufficient for conferring miRNA action. Nevertheless, using the apparently desirable complementarity of the first 2–7 nucleotides as a starting point, the

currently known 218 mammalian miRNAs have been computed to target ~2000–5300 human genes, thus regulating protein production of 10–30% of all human genes [71–74]. This evaluation indicates that one miRNA has several targets, which has been shown experimentally in human cells [65]. It is often observed that more than one miRNA site at the 3' UTR is necessary for efficient inhibition of translation, but also that – at least in *Drosophila* – one is often sufficient [75]. Prediction of miRNA targets is complicated because the binding of a miRNA to a target at a weak seed region can be supplemented by sequences with complementarity at the 3' end. This has been documented for miRNAs in *Drosophila* where it is estimated that each of the 96–124 miRNAs has ~100 sites in the genome [75]. Unfortunately, experimental verification lags far behind the prediction from the computational assessment. Once a more complete correlation has been established we will learn more about the structure–function relationship of this system. The problems associated with such an assignment are obvious from the two examples in mammalian systems, particularly *in vivo*, mentioned here.

Potential side effects

Side effects generated by siRNAs aimed at silencing a specific gene by mRNA cleavage can arise for several reasons. There are two types to be considered: (i) off-target effects, which are concentration-independent, and (ii) unspecific effects, which are unrelated to the siRNA sequence. There are several reports of such side effects, but no unified picture as yet [76].

For example, in a study by Jackson *et al.* [77], two families of siRNAs consisting of 16 and eight members, respectively, directed against two mRNAs were analysed in HeLa cells [77]. The majority of the altered transcript expressions were siRNA specific rather than target specific. Usually, such effects come to light only when microarray-expression profiles are performed. Even a luciferase-siRNA regulated the expression of several genes. In contrast to other studies (for example, Ref. [78]), these unspecific effects were not simply concentration-dependent. The explanation provided by Jackson *et al.* is that there was enough partial sequence identity between transcripts and siRNAs to elicit gene silencing. These effects were observed both for sense and antisense siRNAs. However, a different group finds the concentration-dependent off-effect not to be caused by partial complementarity between target and siRNA but, rather, by the activation of some protein kinases other than dsRNA-dependent protein kinase PKR [78]. The authors concluded that the siRNA pathway overlaps but is not identical with that involving long dsRNA or interferon. These two reports, with such different observations even though they both use HeLa cells and the same transfecting reagent, illustrate that it is difficult to find a common ground.

Interferon activation by siRNAs has been of great concern. Long dsRNA and the polynucleotide poly(I:C) are known to trigger interferon response by interaction either with PKR or with Toll-like receptor (TLR)-3 [79]. But,

because this is considered length-dependent for sequences >30 nucleotides, the siRNAs, because of their considerably shorter length, were thought to be inactive in this respect. However, off-target gene suppression in cell culture has been associated with up-regulation of interferon by siRNAs as ligands to TLRs [80–82]. It is, thus, encouraging to find that 'naked' (i.e. not complexed to a cationic lipid), synthetic siRNAs down-regulated their target in immunocompromised BAB/c mice without an interferon intervention upon intraperitoneal injection, or low-pressure or high-pressure tail-vein injection [83]. The importance of liposome complexation is also seen by injection of siRNA into mice, which induced systemic immune responses by interaction with the TLR7 in plasmacytoid dendritic cells only when complexed with a cationic liposome [79]. A sequence motif for the sense strand is suggested to be active in the induction of interferon. The details of the role of liposomes are not known.

Another study reports on the unexpected results obtained with siRNAs [84]. Ten different siRNAs directed against *MEN1*, the gene encoding menin 1, were examined in HeLa cells, and the expression of two additional genes was followed, those encoding p53 and p21, which are markers of cell state but functionally unrelated to menin. The siRNAs had different effects on the expression of the genes encoding p53 and p21: some down-regulated both, some showed no effect and some down-regulated p21 only. The down-regulation is thought to be due to the action of miRNAs for which there are partial complementary sequences in these two genes.

At this time, we are still far from understanding siRNA-sequence-related off-target and sequence-unrelated unspecific effects.

Concluding remarks

RNAi is a fascinating area with enormous potential. This is manifold because the small non-coding RNAs siRNA and miRNA can interfere with gene expression in several ways: by cleaving the mRNA in a sequence-specific manner, by preventing translation of mRNA or by transcriptional silencing. Although the enzymology of these processes is just beginning to be understood, these RNAs are ready for application in plants and animals. At present, siRNAs are most often used for gene functional analysis as the method of choice because of efficiency and ease of use. Therapeutic applications are a logical consequence, and the first animal model studies look encouraging. Delivery of the RNAs, preferentially to particular cells or organs, is one of the challenging tasks. A most puzzling question concerns the miRNAs as individual members of the class that seem to have a multitude of targets, only few of which have been identified. Studies indicate that the role of these miRNAs is the spatial and temporal expression pattern of genes. The possibility that 20% or more of the human genes might be subject to miRNA targeting opens a vast field for inquiry. How a certain miRNA selects for an individual target from the many it can affect is still unclear. Maybe these targets are components of a regulatory system or cascade. The least understood function of these RNAs is the transcriptional silencing and heterochromatin

formation by methylation of DNA and histone. This particular subject is still too poorly understood to render itself to an application.

There are many opportunities offered by the RNAi methodology; it is a challenging field with many unanswered questions, but with considerable potential in many areas. It is evident that these small non-coding RNAs regulate gene expression by aiming, like bullets, at an unpredicted number of targets.

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