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Minireview

Small RNA asymmetry in RNAi: Function in RISC assembly and gene regulation

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Abstract RNAi is a conserved gene-specific regulatory mechanism, which silences target gene expression transcriptionally and post-transcriptionally. The RNAi machinery converts the sequence specific information of a long double stranded RNAs (dsRNAs) into small 21–22 nt long dsRNAs (siRNAs, miRNAs) which assemble into an effector complex, the RNA induced silencing complex (RISC). RISC assembly is asymmetric; one strand of an siRNA or a miRNA preferentially incorporates into the RNA–protein complex. Here, I review the rules of the asymmetric RISC formation and discuss their possible regulatory function in several steps in RNAi.

Keywords: Asymmetry; RISC assembly; siRNA unwinding

1. Introduction

RNA interference (RNAi) is a diverse, evolutionarily conserved mechanism in eukaryotic cells, which inhibits the transcription and translation of target genes in a sequence-specific manner. RNAi is triggered by dsRNA that is converted to small regulatory RNAs. These small RNA species are loaded into the RNAi effector complexes and guide the machinery to the target RNA in a sequence-specific manner. RNAi-related machines control post-transcriptional gene silencing by cleaving, destabilizing the targeted cognate RNAs or preventing their translation. Increasing evidence indicate that a nuclear RNAi mechanism is responsible for transcriptional regulations such as heterochromatin silencing, co-suppression and silencing of transposable elements.

2. Sources and processing of small regulatory RNAs in the RNAi pathways

The sequence specificity of RNAi is assured by a group of small regulatory RNAs such as small interfering RNAs (siRNAs), repeat associated siRNAs (rasiRNAs), *trans*-acting siRNAs (tasiRNAs) [62,83] and small-scan RNAs (scnRNAs) [52] that are processed from long double stranded RNAs or, like microRNAs (miRNAs), from hairpin RNAs with a stem–loop structure [2]. Eukaryotic cells can synthesize long double stranded RNAs (dsRNA) from a wide variety of sources using two distinct mechanisms. Bidirectional transcription from inverted repeats of transgenes and transposons and read-through transcription from convergent promoters directly produces long dsRNA [5]. In addition, organisms whose genomes encode RNA-dependent RNA polymerases (RdRP) such as fission yeast, fungi, worms and plants can convert primary and aberrant transcripts into dsRNA [1,85]. The origin of the dsRNA can also be extracellular. DNA and RNA viruses produce dsRNA intermediates during their replication [84], and some viruses transcribe hairpin like structures that yield functional miRNAs [11,64,65,78]. dsRNA delivery into plants and animal cells using various techniques is a powerful tool in reverse genetics [17,18,28].

Although the origins of dsRNA triggers are diverse, the mechanisms that transform the genetic information of a long dsRNA into the sequence-specific determinant small RNA are very similar. Both siRNAs that originate from long, perfectly-paired dsRNAs and plant miRNAs are processed by Dicer, a class III RNAse III enzyme which processively chops its substrate into 21–28 nucleotide long double-stranded RNA [8,70,79]. However, two separate protein complexes orchestrate the maturation of animal miRNAs. In the nucleus the microprocessor complex, containing Drosha, a class II RNAse III enzyme, processes primary-miRNAs (pri-miRNAs) into pre-miRNAs, defining one end of a miRNA with a cleavage at approximately two helical turns (22 nucleotides) from the loop structure [15,20,21,33,91]. Pre-miRNAs are exported by exportin-5 into the cytoplasm where Dicer cleaves the pre-miRNA near its stem–loop liberating the mature [9,46,59,89] (Hammond review and Chen review, this issue).

According to recent models, Drosha and Dicer cleave in similar ways. The two RNAse III domains of both enzymes form an intramolecular processing center establishing two active sites in near proximity. Each catalytically active site cleaves one phosphodiester bond on the opposite strands of an siRNA or miRNA precursor, which give rise to the characteristic 21–28 nt long double-stranded RNA with 2 nucleotide 3′overhangs [21,92]. Drosha and/or Dicer also determine the end structure of the siRNA and miRNA since RNAse III cleavage characteristically leaves 5′ phosphate and 2′,3′ hydroxy termini. Chemically synthesized small RNAs that contain all the traits of a mature siRNA or miRNA are competent for efficient sequence-specific gene regulation [23].

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3. Strand selection in RISC formation

Processing of long dsRNAs or pre-miRNAs results in the production of regulatory small RNAs with double-stranded attributes. However, early biochemical data and transfection experiments in human cell culture suggested that the active regulatory RNA species in the RNAi pathways indeed are single-stranded [49,55,72]. *Drosophila* and human cell extracts can be programmed with single-stranded small RNAs to produce efficient RISC activity in vitro, and single-stranded siRNAs initiate gene specific regulation upon transfection into human cells. Recently, the minimal cleavage competent human RISC, using recombinant human AGO2, could be reconstituted only with single stranded siRNA [68]. In addition, the majority of miRNAs, the most abundant sequence specific determinant in RNAi, are represented as single-stranded RNAs at steady state RNA levels supporting the idea that miRNAs, just like siRNAs, are incorporated into the RNA-protein complex as single-stranded RNA.

If RISC chooses one of the strands of an siRNA or miRNA, is there a bias in the strand selection or are both strands of a small regulatory RNA equally potent to incorporate into the effector complex? The quest to answer this question lead two research groups to the discovery that the relative thermodynamic characteristics of the two 5′ termini of an siRNA and miRNA determine which strand has inherent regulatory function. One group analyzed the thermodynamic properties of the cloned animal miRNAs, hypothesizing that the mature, cloned miRNAs represent the strand from the stem of a pre-miRNA that preferentially incorporate into RISC. Their comparative sequence analysis demonstrated that the 5′ end of mature miRNA strands have significantly lower thermodynamic stability compared to the 5′ termini of the opposite strands in the stem of the pre-miRNA which are under-represented in miRNA cloning experiments. Next they showed with transfection experiments in human cells that chemically synthesized siRNAs that are efficient in gene silencing have similar thermodynamic profiles. The 5′ end of the guiding (anti-sense) strands of the competent siRNAs are less stable than the 5′ end of the passenger (sense) strands [29]. In vitro experiments with chemically synthesized siRNAs and radiolabeled RNA targets in *Drosophila* cell-free embryo extract came to the same conclusion and provided additional insight into the mechanism of asymmetric RISC assembly [71]. The advantage of the in vitro system is that the sequence of both strands of an siRNA can be changed and modified together with the corresponding target sequences and in addition, both the guiding and the passenger-strand RNAi activity can be monitored by providing cognate, in vitro transcribed target RNAs to each of the strands of the siRNA separately. Furthermore, in vitro it is possible to measure the concentration of the active RISC by capturing the incorporated single-stranded RNA. This study showed that asymmetric RISC formation is defined by the relative thermodynamic strength of the first four nucleotide-pairs of the 5′ termini of an siRNA calculated by the nearest-neighbor method (Fig. 1). The mechanism which monitors the thermodynamic properties of the two 5′ ends of an siRNA can sense variation as small as a single hydrogen bond since altering a G:C base-pair to a I:C base-pair of a closely symmetric siRNA made the modified strand more preferable for RISC assembly. An siRNA which consists of only unmodified base-pairs still undergoes asymmetric RISC formation, even if the energy difference is only ~0.3 kcal/mol. The 5′ end strength of an siRNA not only defines which strand is selected to incorporate into the regulatory complex, but to some extent has an effect on general RISC formation since loosening both ends of a symmetric siRNA at the same time increase either the sense or anti-sense strand derived cleavage activity.

4. Role of siRNA asymmetry in RISC assembly

Between processing of the long dsRNA into small RNAs and the formation of the active regulatory complex there is an intermediate step in which small RNAs are activated and handed over to the catalytic core of the RNAi machinery. Combined genetic and biochemical approaches in flies provided the first insight into how the RISC loading complex (RLC) places one strand of an siRNA into RISC. In flies, two similar but specialized mechanisms exist to process and activate either siRNAs or miRNAs. Two distinct Dicers, Dcr-2 and Dcr-1, process long dsRNAs and pre-miRNAs respectively, into siRNAs and miRNAs that are incorporated into separate, but similar effector complexes [35,57,66]. In RLC, Dcr-2 and R2D2, an RNA binding protein with tandem dsRNA binding motifs, form a heterodimer and bind to double-stranded siRNAs [42,81] (Birchler review, this issue). This complex recognizes the

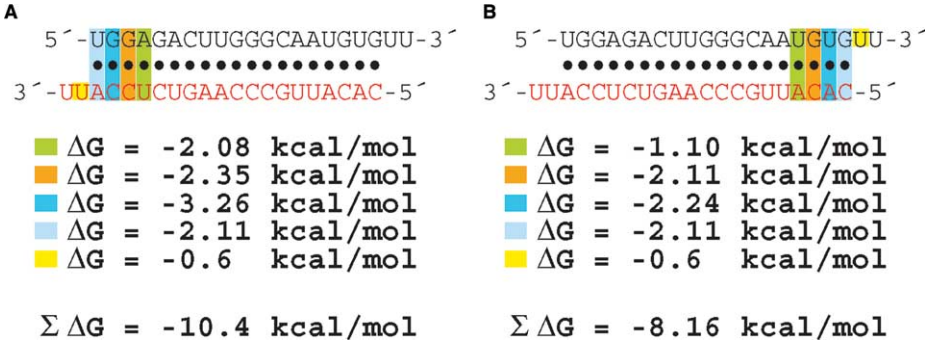

Fig. 1. 5′ end strength calculation of an asymmetric siRNA [71] using the nearest-neighbor method. Thermodynamic properties of the passenger (A) and the guiding (B) strands. The color code represents the base pair and corresponding nearest-neighbor ΔG^{0}_{37} values. The thermodynamically favored strand in RISC formation is highlighted in red.

authenticity of the siRNA and senses the free energy differences between the two 5′ ends. R2D2 binds to the more thermodynamically stable end of an siRNA, and its binding is facilitated by the characteristic 5′ phosphate. By blocking the non-preferred strand of the siRNA, R2D2 positions Dcr-2 at the opposite end of the duplex (Fig. 2). Dcr-2 involvement in the recognition of the asymmetry suggests that after the siRNA is processed by Dicer and is released, the RLC binds to it again placing Dicer at the thermodynamically favorable end of the siRNA. In spite of the fact that both Dcr-2 and R2D2 are required for unwinding in vivo, neither of these proteins alone, nor the Dcr-2/R2D2 heterodimer, can unwind dsRNA in vitro [82]. However, Ago2 mutant flies are impaired in siRNA unwinding despite the fact that RLC forms in Ago2 fly lysate [57]. This data suggests that the catalytic engine of the siRNA-programmed RISC is required for siRNA unwinding.

Argonautes are the core proteins in every described RNAi complex. They have two conserved protein domains, the PAZ domain, which was proposed to bind single-stranded RNA [39,75,86], and the PIWI motif, which shows structural homology to the active center of RNAse H [47,60,61,76] (Cheng review, this issue). RNAi-mediated RNA cleavage shares several similarities with RNAse H activity. RISC leaves 5′ phosphates on the 3′ cleavage product, 3′ hydroxy group on the termini of the 5′ cleavage product and its activity depends on the presence of divalent cations [50,73] (Hammond review, this issue). Several lines of evidence support that the Ago protein physically cooperates with the small RNA loading machinery. The dsRNA binding domain of human Dicer directly interacts with the PIWI domain of the human Ago2 protein [67] and Dicers generally co-immunoprecipitate with Argonautes [57,79]. Recently it was demonstrated that the PIWI

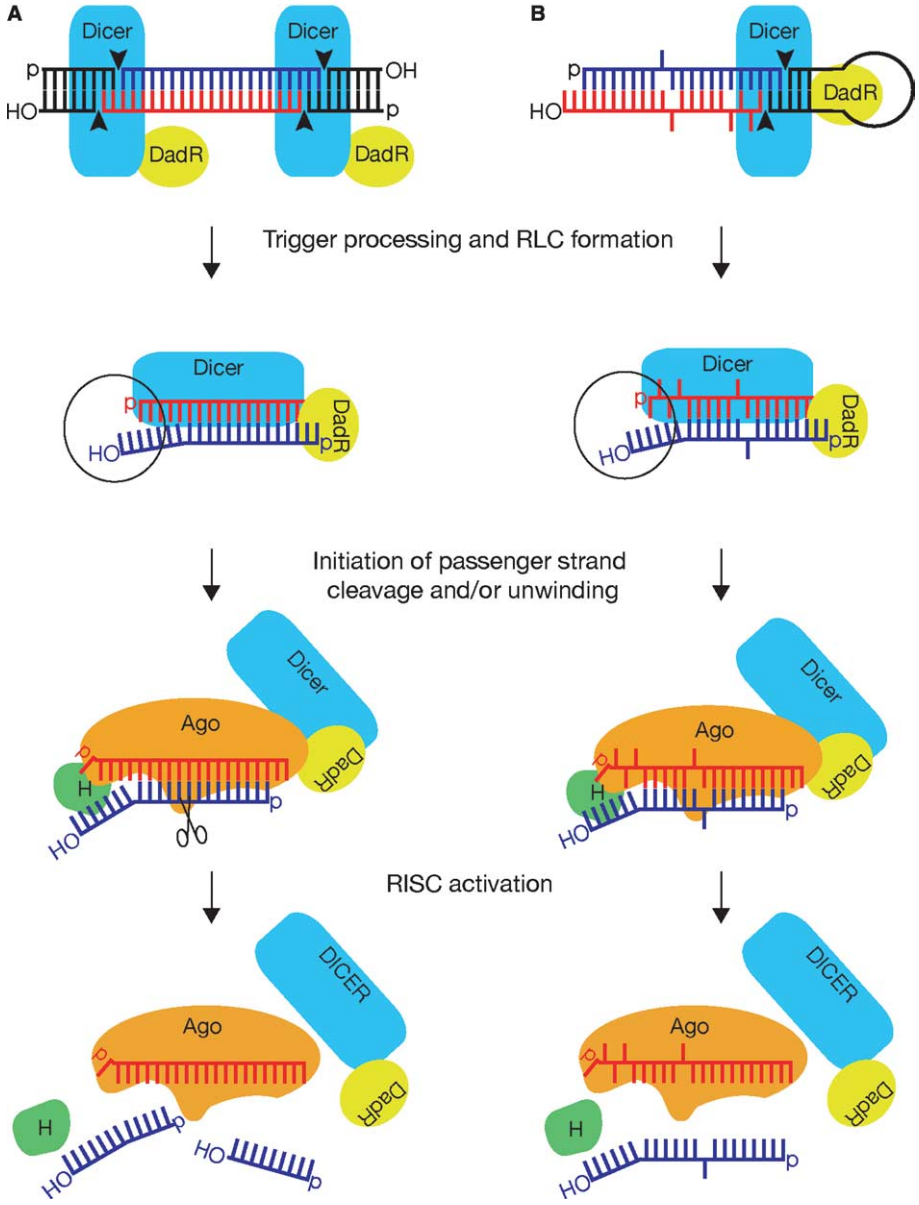

Fig. 2. The thermodynamically favored siRNA (A) and miRNA (B) strand (labeled in red) are loaded into RISC. DadR: Dicer associated double-stranded RNA binding protein. RLC: RISC loading complex. H: putative helicase. Ago: Argonaute protein. p: 5′phosphate, OH: 2′,3′hydroxy. ✂: Argonaute catalytic activity. Open circle shows the thermodynamically less stable end of the siRNA and miRNA.

domain of *Archeoglobus fulgidus* Piwi protein can be co-crystallized with siRNA-like duplex, suggesting that Argonaute proteins can bind double-stranded siRNA to some extent [47,61].

Do similar loading complexes that recognize the thermodynamic properties of the miRNA exist in flies or in other animals where only a single Dicer processes dsRNAs and miRNAs? The answer is very likely yes. miRNA maturation follows the asymmetry "rule", which means that the thermodynamically more favorable strand of the stem of the pre-miRNAs incorporate into the miRNA programmed regulatory complex. RDE-4, an R2D2-like protein in *C. elegans*, is requi- red for RNAi and interacts with the worm Dicer [79] (Grishok review, this issue). In plants the dsRNA binding protein HYL1 interacts with DCL1 [22], and the HYL1 paralog DRB4 binds to DCL4 [24]. Very recently several groups identified *Loquacious*, a fly R2D2 paralog, that interacts with fly Dcr-1 and is essential for miRNA maturation [19,69]. TRBP, a human ortholog of R2D2 and *Loquacious*, is required both siRNA mediated RISC activity and miRNA processing [12]. However, it is not determined yet if these dsRNA binding proteins have simi- lar functions to R2D2 or if they help Dicer to position and cleave the mature miRNA from its precursor similar to how Drosha/DGCR8 orients Drosha in the pre-miRNA producing microprocessor complex (Fig. 2B).

5. Passenger strand elimination

What is the mechanism that separates the two strands of an siRNA or miRNA and destroys the strand with the relative higher 5′ termini free energy profile. In vitro experiments in fly embryo extract showed that the thermodynamically non-preferred strand is eliminated after unwinding [71]. It has been suggested that fly Ago2 can eliminate the passenger strand through the same activity that can cleave the cognate RNA target [32] (Fig. 2A). There are several experimental results which can support this idea. The PIWI motif of Argonautes could bind duplex siRNAs and directly interact with Dicer suggesting that RLC can forward the siRNA in double-stranded form to an Argonaute protein. Affinity purified human Ago2 containing single-stranded RNA can cleave the exogenously added passenger strand through RISC activity suggesting that the small size of the target is not a limiting factor in the sequence specific cleavage [50]. However, there are some facts which discourage this hypothesis as an exclusive mechanism to eliminate the passenger strand or the miRNA* sequence. It was shown that siRNA or miRNA programmed RISC does not require ATP for the initial cleavage of the target, still it is well-established that siRNA unwinding is an energy dependent step in RISC formation [55]. Furthermore, not every Argonaute protein is cleavage competent. Perhaps plants are the best candidates for using the passenger strand cleavage for RISC activation because both siRNAs and miRNAs are near-perfectly complementary and plant miRNAs regulate the target mRNA through sequence-specific cleavage [43]. In fly both the miRNA-programmed Ago1 and the siRNA associated Ago2 can mediate target cleavage if the cognate RNA is provided [57]. In mammals only Ago2, one of the four Argonautes, has catalytic activity in spite of the fact that all ectopically expressed Argonautes equally bind transfected siRNAs [40,51]. Since in worm the classical RNAi activity has yet to be recapitulated we do not know how many, if any, among the 27 members of the Argonaute gene family have catalytic activity. In addition, recombinant human Ago2 can only show cleavage competent RISC activity when it is programmed with single-stranded siRNA suggesting that Argonautes alone are not capable of cleaving double-stranded siRNA [68]. It would be difficult to explain the elimination of the miRNA* sequence in animal systems with selective strand cleavage. Extensive complementary between the guiding strand or the miRNA and the target RNA is required for efficient target cleavage. Since the stems of pre-miRNAs are not perfectly paired, they contain mismatches and bulges, it is unlikely that miRNA maturation can rely on cleavage of the miRNA* sequences even if they are incorporated in cleavage competent Argonaute complexes.

What other possible mechanism can select one of the strands of siRNAs or miRNAs? DEAD-box RNA helicases rearrange RNA duplexes in an ATP-dependent manner in distinct steps in RNA metabolism. siRNA "unwinding" is an energy dependent step which implies the participation of such RNA helicases. RNA helicases bind to single-stranded RNA to destabilize the nearby short RNA duplexes. The RLC positions siRNAs by blocking the thermodynamically less favorable strand and offering the 5′ end of the duplex that has propensity to fray for RISC formation [82]. It can be envisioned that a non-processive helicase binds to the end of the siRNA that offers more single-stranded feature, by producing more terminal "breathing" further destabilizing the duplex (Fig. 2). Indeed, genetic data and immunoprecipitation experiments identified several RNA helicases that are necessary for or associated with RNAi in a wide range of eukaryotic organisms. Which among the described RNAi related helicases are the best candidates to melt the double-stranded siRNA or miRNAs to facilitate RISC formation?

In *C. elegans* two closely related DEAD-box helicases, (DRH-1/DRH-2) were co-immunoprecipitated with the worm Dicer (DCR-1) and it was shown that they are required for efficient RNAi activity [79]. Intriguingly, these helicases are in a protein complex that is very similar in context to the fly RLC. DRH-1/DRH-2 interact with DCR-1, RDE-4, a paralog of the fly R2D2, and the Argonaute protein RDE-1 that is required for long dsRNA initiated RNAi. DRH-1/DRH-2 homologues are present in flies and vertebrates also. The human DRH-1/DRH-2 homologue RIG-1 is implicated in the interferon response by sensing viral dsRNA [90].

Gemin3, a DEAD-box helicase originally identified in the Survival of Motor Neuron (SMN) protein complex, co-immunoprecipitates with human Ago2 and many miRNAs as a component of the human miRNP [53]. Interestingly, Gemin3 is the only RNA helicase among those that are implicated in RNAi pathways which ATP-dependent RNA unwinding activity was confirmed in vitro [87].

Recently in flies a genetic screen for embryonic axis specification combined with biochemical analysis revealed that a non-canonical RNA helicase, *armitage*, is required for RNAi in vivo and for RISC formation in vitro [13,81]. In the absence of *armi*, RISC formation is impaired. However, addition of single-stranded siRNA to *armi* mutant fly lysate could not restore RISC activity suggesting that it has role in RISC assembly upstream of siRNA unwinding. SDE3, the closest plant homologue of *armitage* is also involved in post-trancriptional

gene silencing (PTGS) and participates in the cell-to-cell propagation of the silencing trigger [14].

RNAi based screens in *C. elegans* revealed three additional DEAD-box helicases that are required for gene silencing. All three genes have homologues in *Drosophila* and vertebrates [30].

6. Small RNA asymmetry in gene regulation

Inarguably, the biggest impact of the asymmetric RISC formation on gene regulation is to determine which strand of a miRNA is incorporated into RISC. miRNAs are abundant regulatory RNAs that consist of ~1–2% of the worm, fly, and human gene pools, and recent bio-informatic predictions suggest they may regulate the expression of up to one third of the expressed genes which makes miRNAs the biggest player in eukaryotic post-transcriptional gene regulation [31,36,77] (Bentwich review, this issue). Since miRNAs are predicted to regulate a large number of transcription factors, their influence on gene expression could extend far beyond the post-transcriptional gene expression phenomenon. miRNA mediated gene regulation is fundamentally different in plants and animals. The majority of plant miRNAs eliminate the expression of their target RNAs by cleaving them within a near-perfectly complementary site [48]. miRNA-driven sequence specific target cleavage is rare in animal systems [88] as the complementarity between the majority of miRNAs and their putative or validated target sites, are only restricted to the seed sequences, 2–7 nucleotides of the 5′ end of the miRNA. This level of complementarity is insufficient to initiate cleavage of the target sites even if the miRNA is bound to a catalytically active Argonaute protein; therefore, animal miRNAs co-operatively (targeting multiple target sites on the mRNA) silence gene expression [16]. The general view is that non-cleaving miRNAs repress target gene expression by inhibiting protein translation after the initiation step without degrading the mRNA [58]. However, increasing evidence suggest that animal miRNAs also can destabilize their target mRNAs. Putative target genes were identified for a brain and muscle specific miRNA by using mRNA arrays [38] and human Ago2 and human miR-16 were demonstrated to participate in the AU-rich element-mediated RNA decay [26]. Furthermore, ectopically expressed human Ago1-3 co-localize with miRNAs in the P-bodies, which are cytoplasmatic foci of mRNA storage and cap-dependent RNA degradation [41,74]. Recently it was proposed that animal miRNAs micromanage the transcriptome by fine-tuning the expression of their target mRNAs [7]. To fulfill the requirements of this complex regulatory role, miRNAs have to be spatially and temporarily regulated together with their target transcripts. The known examples of animal pri-miRNAs are Polymerase II (Pol II) transcripts with 5′cap structures and poly (A) tails [34]. *C. elegans* pri-*let-7* undergoes trans-splicing [10] and also in worms a *cis*-regulatory element in the genome was identified that regulates *let-7* expression [27]. C-Myc transcription factor regulates the expression of the miR-17 cluster in human cells [56].Together the evidence suggest that the regulation of miRNAs and their targets is very similar at the transcriptional level.

Sensing the thermodynamic properties of miRNAs could provide an additional mechanism for controlling miRNA levels at the step of RISC formation. In vitro experiments showed that asymmetric RISC formation is not absolute. One strand is preferentially incorporated into RISC, but the strand not preferred thermodynamically could still form active complex to some extent and drive target cleavage if the target RNA is present. Furthermore, absolute RISC activity could be increased by decreasing the 5′ end free energy of a certain siRNA [71]. Theoretically, the relative thermodynamic strength at the 5′ termini of the miRNA determines the miRNA/ miRNA* ratio and the absolute free energy profile of the 5′ends can influence the quantity of the active regulatory complex formation between different miRNAs. In addition, it has recently been demonstrated that a human miRNA, miR-22, undergoes tissue specific editing [45]. The editing changes two terminal A nucleotides to I on the non-preferred 5′end of the pre-miR-22 sequence which results in a dramatic change in the asymmetry, suggesting that a fraction of this miRNA can produce elevated level of the miRNA* sequence. If this is a general phenomenon, what is the impact of the miRNA* in gene regulation? As discussed above, it can mean that it merely regulates the steady state level of the thermodynamically preferred strand in an indirect way. However, it is very likely that some miRNA* sequences bear direct regulatory functions. The best candidates for such miRNA* are processed from thermodynamically symmetric pre-miRNAs in which both miRNA strands are equally competent for regulatory complex formation. Elaborate miRNA cloning experiments revealed the existence of several miRNA* sequences at the steady-state RNA level [3,4,37] (Tuschl review, this issue). Their relative abundance was originally calculated from their cloning frequencies. In some cases, the tissue and developmental-specific occurrence of the miRNA/miRNA* sequences showed different patterns supporting the idea that the two strands might have different roles in gene regulation [4]. Since miRNA cloning involves amplification steps and the bias in cloning preference and efficiency cannot be excluded, a more quantitative experimental approach is necessary to examine the possible role of miRNA* sequences in regulating gene expression. Recently several miRNA micro-array methods have been employed to define tissue-specific miRNA expression patterns and some of these arrays contain a few identified miRNA* sequences [44,54,80]. Most of the miRNA/miRNA* expression patterns were similar, suggesting that the two strands are co-regulated. An interesting exemption is miR-30a/miR-30a*. miR-30a showed more dominant expression in adult mouse tissues, however, only miR-30a* expression was detected in three and 28 day old embryonic bodies [80]. This significant difference in a miRNA/miRNA* expression pattern can be explained with the tissue-specific editing which can reverse the thermodynamic characteristics of the 5′ ends of miR-30. To generalize this potential level of miRNA regulation, more extensive quantitative studies are required to compare the expression of both strands of the miRNAs. In addition, bio-computational expe- riments could shed light on miRNA* associated regulatory functions by extending the target prediction with the miRNA* sequences.

An elegant example of siRNA asymmetry defined regulatory mechanism was recently described in plants. Plants produce a unique class of siRNAs called *trans*-acting siRNAs. tasiRNAs correspond mostly to the sense strand of non-coding RNAs which is converted to dsRNA by RdRp and processed by Dicer into 21-nt increments. Plant miRNAs recognize the nascent, non-coding RNA and initiate its cleavage setting a

21-phase for progressive Dicer cleavage after the tagged primary RNAs are converted to dsRNAs. The asymmetry rule was implicated in preventing RISC formation from the strand of the tasiRNAs that is derived from the miRNA non-targeted RNA, which would initiate the degradation of the tasiRNA precursor [1] (Herr review, this issue).

7. siRNA asymmetry in siRNA design

The discovery of asymmetric RISC formation was delineated from human transfection experiments that characterized the thermodynamic properties of the efficient chemically synthesized siRNAs [6,29]. Designing asymmetric siRNAs can improve their efficiency in several ways. Human cells have limited capacity to form RISC with exogenous siRNA [25], therefore applying the asymmetry rule in siRNA design would result in most of the available RISC being programmed with the guiding strand. In addition, this would decrease the amount of siRNA required for efficient silencing, since asymmetric siRNA is effective in the sub-nM concentration range. Applying asymmetric siRNA rules can also avoid unwanted off-target effects. First, it would decrease the chances to knock down gene expression with the passenger strand since its potential to form active RISC is limited. Second, reduced siRNA concentration precludes the induction of the interferon response which was shown to be activated upon introducing siRNAs at high concentration [63]. Fraying the first nucleotide of an siRNA could facilitate the design of effective siRNAs when the target site is limited, such as targeting individual members of a highly homologous gene families or knocking down dominant mutant RNAs where the polymorphism between the wild type and the mutant alleles are restricted to a single or few nucleotides. In these cases there are only limited or no possibilities to shift the siRNAs on the target to find a thermodynamically favored guiding strand.

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