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Endogenous siRNAs, regulators of internal affairs

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Abstract

Endogenous short interfering RNAs (endo-siRNAs) have recently emerged as versatile regulators of gene expression. They derive from double stranded intrinsic transcripts and are processed by Dicer and associate with Argonaute proteins. In *Caenorhabditis elegans*, endo-siRNAs are known as 22G and 26G RNAs and are involved in genome protection and gene regulation. *Drosophila melanogaster* endo-siRNAs are produced with the help of specific Dicer and Argonaute isoforms and play an essential role in transposon control and the protection from viral infections. Biological functions of endo-siRNAs in vertebrates include repression of transposable elements, chromatin organisation as well as gene regulation at transcriptional and post-transcriptional level.

Keywords

endogenous siRNA; gene regulation; chromatin modification; transposon control

Introduction

The hypothesis that RNA has a regulatory role in gene expression was suggested nearly 50 years ago (ref). However, it was only three decades later when the first regulatory RNA was cloned from *C. elegans* and characterized [1]. Since then, whole genome and transcriptome sequencing projects have identified regulatory small RNAs in all kingdoms of life with various functions in gene regulation and genome defence [2]. In quite general terms, naturally occurring small RNAs can be divided into three distinct families, micro RNAs (miRNAs), Piwi-interacting RNAs (piRNAs) and endogenous small interfering RNAs (endo-siRNAs). The most extensively studied family are miRNAs; RNA molecules of 21-23 nucleotides in length that derive from transcribed hairpin structures. Synthesis involves two RNA selective endonucleases, Drosha and Dicer, an Argonaute protein to form the effector complex (RNA-induced silencing complex, RISC) as well as a number of additional, often pathway or species-specific protein factors [3]. Individual miRNAs share sequence complementarity to the 3′ untranslated region (UTR) of multiple target mRNAs and binding of RISC results in translational arrest and/or deadenylation and degradation of the transcript [4]. piRNAs are typically 25-33 nucleotides in length and derive from distinct piRNA clusters related to transposon sequences. The synthesis of piRNAs is still not fully understood but does neither require Drosha nor Dicer. An amplification step is involved to

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produce the small RNAs that associate with Piwi proteins to form the effector complex. piRNAs are most prominently expressed in germ cells, particularly during spermatogenesis, and are essential in silencing retrotransposons and other repetitive elements [5]. This brief review focusses on the third family of short naturally occurring RNAs, namely endo-siRNAs. These are 20-23 nucleotides long and are synthesized from double-stranded RNAs such as hairpins with a long, perfect stem or sense-antisense transcript hybrids. Endo-siRNAs have a 3′ 2 nucleotide overhang, are produced by Dicer and form an effector complex with an Argonaute protein. The existence of endo-siRNAs has long been debated in flies and vertebrates that lack a siRNA specific amplification mechanism; only powerful parallel sequencing techniques have convincingly demonstrated endo-siRNAs, i.e. Dicer products that reliably mapped to the host genome [6].

The canonical path to endo-siRNAs in animals starts with perfectly complementary RNA hybrids (dsRNA) that are recognized by the RNase III-type enzyme Dicer. Long dsRNA are also substrates for other enzymes such as cytoplasmic protein kinase R and ADAR (adenosine deaminase acting on RNA) [7], therefore dicing is likely to occur in the nucleus [8]. The diced double stranded short RNA fragments are then exported to the cytoplasm and loaded onto an Argonaute protein to form a so-called pre-RISC [9]. This complex can act on complementary RNAs in the cytoplasm or return to the nucleus to regulate transcription or chromatin structure [8, 10]. The entire process involves a considerable number of additional protein factors that show species specific variations. These will be discussed in the context of the specific model systems C. *elegans, Drosophila* and mammalian/human cells.

All the major classes of small RNAs mentioned above are intertwined by either shared protein components or similar biological functions. Accordingly, endo-siRNAs share similarities with both miRNAs and piRNAs and a clear separation between families is often difficult to trace and may only relate to some aspects of their biology. Species specific, well documented characteristics in the repertoire of RNA interference (RNAi) effector proteins lead to distinct variations the biology of endo-siRNAs. For example, both synthesis and physiological roles of endo-siRNAs are established in *C. elegans* [11] whereas in flies, the synthesis of endo-siRNAs is well understood but their biological role has predominantly been investigated in the context of transposon control [12]. In vertebrates the understanding of both mechanistic aspects and biological role are rather unclear. Model system related differences also provoked a rather disparate understanding of “endo-siRNA” among different groups of scientists [13]. The scope of this article is to outline the different opinions and give reasons for the discrepancies.

Endo-siRNAs in *C. elegans*

Endo-siRNA processing in *C. elegans* requires multiple proteins including one copy of Dicer, two essential for reproduction and two non-essential forms of RNA-dependent RNA polymerases (RdRP) and 26 different AGO proteins, two of which are linked to miRNA biogenesis [14, 15]. Initial cloning and sequencing efforts identified 3 major categories of endo-siRNAs, 26G-, 22G- and 21U -RNAs [16]. The 21U-RNAs are reminiscent of piRNA and this brief review will therefore focus on 26G and 22G endo-siRNAs. 26G are precursors of 22G endo-siRNAs and generally expressed at a much lower level than the shorter form;

as the nomenclature suggests, both start with a 5′ guanine. 26G endo-siRNAs are synthesized from primary transcripts through the action of an RdRP (RRF-3), Dicer (DCR-1) and the exonuclease ERI-1 [17]. Based on their association with specific Argonaute proteins and their expression pattern 26G endo-siRNAs can be separated in two groups. The first one interacts with the Argonaute ERGO-1 and is expressed in *C.elegans* oocytes, embryos and somatic tissue [11]. A second group is expressed in sperm cells and binds to Argonautes ALG-3/4. A knock-out of both ALG3 and -4 depletes 26G endo-siRNAs, de-represses mRNAs that are expressed during spermatogenesis and affects fertility [18]. Despite a clear distinction made between germline and somatic endo-siRNA their synthesis pathways also share common components.

26G endo-siRNAs are also facultative precursors for the shorter, much more abundant 22G endo-siRNAs [17]. These so-called secondary endo-siRNAs are produced by an RdRP and a significant proportion map to protein coding genes [19]. Their biological function depends crucially on the Ago protein they associate with. For example, a major class of 22G endo-siRNAs that are in complex with the Argonaute CSR-1 target germline expressed coding genes with an essential impact in chromosome segregation [20]. Interestingly, this pathway has recently also been associated with a mechanism to licence intrinsic genes for expression [21-24]. Licencing requires CSR-1 as well as 22G endo-siRNAs and can be demonstrated in *cis* and in *trans*. Dosage effects suggest that a pool of CSR-1 22G RNA needs to be built up before licencing occurs which can then also be transmitted to offsprings. Interplay with the piRNA system guarantees that genomic intruders are recognized and silenced whereas transgenes can build up a pool of licencing complexes to warrant expression [25]. This process may be helped by close “friends” either in the context of genomic location or sequence similarity. A paradigm where endo-siRNAs are being used to scan genetic material to control quality or distinguish between self and nonself is emerging. Examples not only include *C. elegans* but also Paramaecium [26] and Tetrahymena [27] and even in mammalian systems an RNAi basesd quality control mechanism has been proposed in spermiogenesis [28].

22G endo-siRNAs associated with WAGO proteins (Worm-specific AGO) target predominantly pseudogenes and transposons and play a role in genome defence [19, 29]. In addition to the roles in chromosome organisation and transposon control, specific gene regulatory functions are emerging. Somatic cells express an Ago protein, NRDE-3, that in complex with 22G shuttles to the nucleus, recognises the complementary nascent transcript and induces histone H3K9 methylation [30]. Another recent report demonstrated that odour adaptation in *C. elegans* neurons requires the repression of the guanylyl cyclase ODR-1 by endo-siRNAs. Accordingly, odour adaptation leads to increased levels of odr-1 endo-siRNAs that co-immunprecipitate with NRDE-3 [31].

Endo-siRNAs in Drosophila

It was long argued that the synthesis of endo-siRNAs was essentially dependent on RdRP and, since Drosophila and vertebrates lack such enzyme, that these specific kinds of RNA were absent in those organisms [6]. However, this view was challenged in 2008 when a series of papers were published reporting siRNAs mapping to endogenous genic and non-

genic loci in both *Drosophila* and mice [32-34]. *Drosophila* differs from other eukaryotes in that they have two clearly distinct pathways for miRNA and endo-siRNA synthesis involving specific Dicer and Argonaute isoforms. After trimming of the hairpin structure by the Drosha/Pasha complex, miRNA synthesis involves Dicer-1 with the co factor Loqs (Loquacious). The imperfect duplexes are then transferred to Ago-1, an isoform that shows reduced slicing activity [35]. Perfect RNA duplexes are processed by the Dicer-2/R2D2 complex and loaded onto Ago-2. Segregation of the two pathways is also achieved by localizing Dicer-2/R2D2-RNA complexes and Ago-2 to specific cytoplasmic foci, so-called D2 bodies, where Ago-1 is excluded and Ago-2 loading occurs [36]. *Drosophila* cells handle exogenously introduced siRNAs differently as compared to endo-siRNAs, the latter involving a specific isoform of Loqs (Loqs-PD) that associates with Dicer-2 [37]. It was shown that Loqs-PD aids dsRNA processing whereas D2R2 contributes essentially to Ago-2 loading; accordingly, flies with single deletions show reduced endo-siRNA levels whereas the effect is more pronounced in double knock-outs [38]. Interestingly, the processing of endo-siRNAs slightly differs in germ line and soma as in somatic cells the contribution of R2D2 is minimal. In the germline, however, it appears that R2D2 competes with Loqs for Dcr-2 binding and, in the context of high transposon mobilization, slows down the process at the cost of specificity in Ago-2 loading [36, 39].

Large scale sequencing projects and gene knock-out studies have clearly linked endo-siRNAs to transposon silencing in *Drosophila* both at post-transcriptional and transcriptional level. Fagegaltier et al found that mutants lacking essential mediators of endo-siRNA biogenesis (Dicer-2, R2D2, Ago-2) showed a disturbed pattern of chromatin modifying enzymes (histone methyl transferase Su(var)3-9 and Heterochromatin Protein 1). Interestingly, that phenotype could be reproduced by scavenging dsRNAs (longer precursors or endo-siRNAs) with the viral RNAi suppressor proteins P19 or B2. However, these dsRNA binding proteins were only active in transcriptional silencing if expressed and localized in the nucleus [40].

The question whether endo-siRNAs are involved in the regulation of protein coding genes is still unsolved. Whereas in the germline a large proportion of endo-siRNAs (80-90%) map to repetitive sequences, the percentage is lower in somatic cells and around 40% of reads map to protein coding genes. Precursors for these endo-siRNAs include transcripts with stem loop structures or sense/antisense transcript hybrids that form Dicer substrates [32-34]. A number of studies focusing on the phenotypic consequences of a Dcr-2 or Ago-2 deletion in Drosophila suggest an impact of endo-siRNAs in the regulation of somatic genes. For example, mutant embryos became significantly less resistant to temperature perturbations showing abnormalities in segmentation [41]. Dcr-2 mutants also proved more sensitive to other forms of stress (oxidative, ER, starvation) and in general had a shorter life span [42]. Expression profiling in Dcr-2 mutant and wild type pupae identified more than 300 significantly up-and down regulated genes, respectively, suggesting a direct interference of endo-siRNAs rather than an indirect consequence of relaxed transposon suppression [43].

Mammalian endo-siRNAs

Significant expression of endo-siRNAs in vertebrate systems and especially mammals was reported in the same issues of “Nature” as above cited research from Drosophila [44, 45]. Concurrently, endo-siRNAs from a specific zebrafish gene (encoding a Na-phosphate transport protein) were demonstrated and published [46]. Progress towards understanding mechanistic details of endo-siRNA biosynthesis and their biological role, however, has proven rather slow; probably because their mere existence conflicted with some aspects of the common understanding of RNAi in mammalian cells. First, the lack of an amplifying mechanism involving RdRP was though to prevent biologically significant (and detectable) accumulation. Second, the occurrence of dsRNA in mammalian cells represents the necessary precursor for endo-siRNAs but also signals virus infection and triggers a strong immune response via protein kinase R/ interferon. Third, Dicer was perceived as a cytoplasmic enzyme thus conflicting with the toxicity dsRNA and only recently nuclear actions of Dicer were demonstrated (ref).

In contrast to Drosophila, vertebrates only express one isoform of Dicer and three Argonaute proteins that can accommodate endo-siRNAs (Ago-1,2,4; Ago-3 is related to piRNAs) [9]. Since Dicer is essential to both miRNA- and endo-siRNA synthesis, the two branches of RNAi are potentially intertwined. Despite these conceptual difficulties, emerging evidence suggests that endo-siRNAs are involved in genome defence and gene regulation in vertebrates.

The initial reports focused on endo-siRNA expression in mouse oocytes which do not raise an interferon response against dsRNA. Significant numbers of reads mapped to hairpin structures, pseudogenes, protein coding genes with natural antisense transcripts but most prominently to repetitive elements [44, 45]. Samples from conditional Dicer knock-out ovaries showed decreased counts whereas immunopreciptation of Ago-2 enriched endo-siRNAs, indicating that both RNAi effector proteins are involved in the synthesis of these short RNAs. Endo-siRNAs mapping to complementary regions between either sense/antisense pairs from the same locus or a pseudogene derived antisense transcript suggest RNAi could have a regulatory impact on the sense mRNA-encoded protein expression, as recently demonstrated in detail for PTEN/PTENpseudogene [47]. The observation that the majority of reads map to repetitive sequences, yet lack hallmarks of piRNAs, suggest that endo-siRNAs contribute to the repression of transposons.

The role of endo-siRNAs in transposon control has been investigated in some detail with particular focus on LINE-1 retrotransposons (Long interspersed nuclear element 1). LINE-1 transcripts are significantly enriched in breast cancer cells while LINE-1 related endo-siRNAs are depleted. Over-expression of a specific set of differentially expressed LINE-1 endo-siRNAs silences transcription of the retrotransposons by promoter targeted DNA methylation [48]. Interestingly, differential expression of LINE-1 proteins within invasive cancer cells were linked to disease progression and patient survival [49].

The impact of endo-siRNAs on the regulation of specific genes is not well understood and only few examples have been described. Ameyar-Zazoua et al. reported that endo-siRNAs

associated with either Ago-1 or Ago-2 influence alternative splicing of CD44 in HeLa cells. The fact that the demonstrated splicing effect coincided with increased H3K9 methylation and slowed RNA polymerase II (RNAPolII) elongation indicates that the impact of endo-siRNAs on splicing may well have broader significance [50].

Endo-siRNAs and natural antisense transcripts

There are various possibilities to generate the dsRNA to be diced into endo-siRNAs, though hybrids between sense and natural antisense transcripts (NATs) are an intriguing option. Many genes express NATs in a tissue and development specific way, and the two transcripts tend to be co-expressed in the same tissue [51]. Somewhat puzzling, however, is that NATs are usually an order of magnitude less abundant than the cognate sense transcripts. Nevertheless, there is evidence that NATs contribute to low levels of endo-siRNAs in somatic cells while associating with Ago-1 and RNAPolII [52]. Intriguingly, only about 350 genes give rise to endo-siRNAs and no correlation between read counts and expression levels could be detected.

The most prominent expression of NATs occurs in testis [52-54] where also a significant level of endo-siRNAs has been reported [55]. The latter dataset, however, identified only 73 different endo-siRNA species mapping to multiple sites on all chromosomes, which is somewhat counterintuitive to the pervasive expression of sense transcripts and NATs in testis. It remains to be tested whether most of the sense/antisense pairs do not give rise to endo-siRNAs or, alternatively, have escaped detection because of cell specific expression or low abundance. Despite this discrepancy, the pervasive high level expression of both sense transcripts and NATs in spermatids is strongly suggestive of a biological rational that may well involve endo-siRNAs.

Conclusions

Endo-siRNAs are probably the least understood member of the miRNA-piRNA-endo-siRNA world of small RNAs. Per definition, endo-siRNAs derive from perfectly complementary, endogenous RNA precursors that are processed by Dicer. The resulting siRNAs combine with a particular Ago protein that eventually defines the biological impact of the complex. The mechanism of synthesis as well as the various biological roles of endo-siRNAs show essential species specific differences. Endo-siRNAs can repress gene expression post transcriptionally but have also been shown to affect transcription of specific loci and chromosome structure. Thus endo-siRNAs represent potent mediators of intrinsic affairs ranging from gene regulation, transposon defence to self-nonself decisions and quality control.

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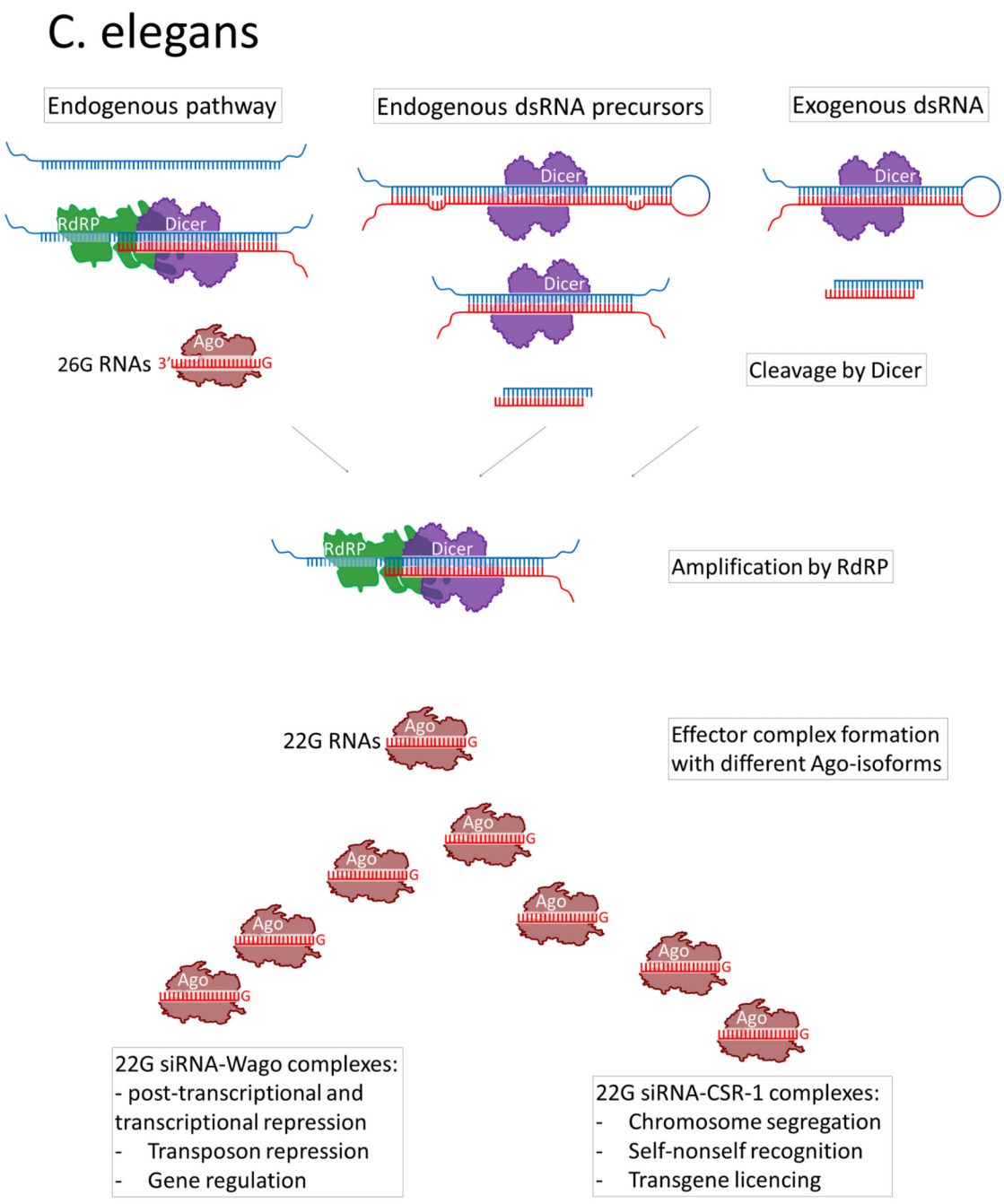

Figure 1.

Schematic representation of endo-siRNA formation in *C. elegans*. Only the generic type of the enzymes Dicer, RdRP and Ago are given. For more details refer to the text.

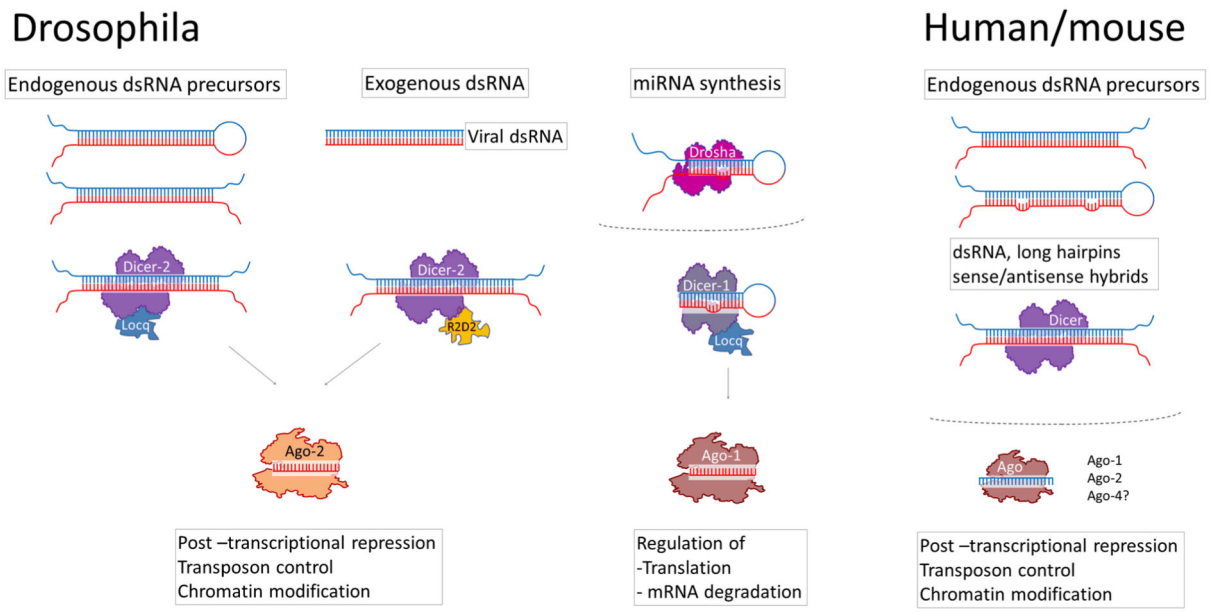

Figure 2.

Summary of endo-siRNA formation in *Drosophila* (left) and Human/mouse (right). The specific isoforms Dicer-1 and -2 as well as Ago-1 and -2 are indicated in *Drosophila*. MicroRNA synthesis in *Drosophila* with the relevant enzyme isoforms is shown for comparison. More details are given in the text.

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