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Review

Piwi-interacting RNAs (piRNAs) in animals: The story so far

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Piwi-interacting RNAs (piRNAs) are small RNA molecules of between 24 to 31 nucleotides (nts) sequence and interact with Piwi subfamily proteins. These piRNAs play important regulatory roles in germline stem cell maintenance, epigenetic regulation, and transposition. Although the biogenesis pathways of piRNAs are not well understood but recent scientific advances have revealed some important aspects of their synthesis. Studies on regulatory mechanism of piRNAs are in their early stages. Yet, the current findings suggest that piRNA based regulation has direct implications on germline development and transposon silencing through heterochromatin formation or RNA destabilization. This review summarizes the knowledge about all aspects of piRNAs since its discovery.

Key words: Transposition, piRNA, RNA biology, germline, small RNAs, PIWI.

INTRODUCTION

P element wimpy testis–induced (PIWI)-interacting RNAs (piRNAs) were first isolated in 2006 from mouse testis (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Watanabe et al., 2006). About 30 nucleotide (nt) long piRNAs were initially recognized by simply looking at the total testis RNAs on ethidium bromide-stained gel (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Watanabe et al., 2006). The piRNAs are abundantly expressed in testis (Kim, 2006; Ro et al., 2007). Subsequently many small RNAs, ranging from 26 to 31 nt, were discovered through cloning techniques (Kim, 2006). Most of these RNAs correspond to the intergenic repetitive elements, including retrotransposons (Klattenhoff and Theurkauf, 2008) and thus were alternatively named as repeat-associated small interfering RNAs (rasiRNAs). These were found to be associated with Piwi subfamily proteins (Aravin et al., 2003). Recent studies show that unlike microRNAs (miRNAs), the piRNAs are produced by a Dicer-independent mechanism and associates with Piwi-class Argonaute proteins. Currently, it is well known that piRNAs can be derived from either repeated or complex DNA sequence elements (Klattenhoff and Theurkauf, 2008). At the present, all those RNAs which associate with Piwi proteins are termed as piRNAs and rasiRNAs are considered as a subspecies of pi RNAs.

The piRNAs have been shown to have an important role in germline as they are abundantly expressed in testis than other small RNAs. Most animal studies have revealed that Piwi proteins are expressed specifically in the germline (Houwing et al., 2007). In flies, Piwi proteins have been shown to be essential for the maintenance and behavior of germline stem cells (Houwing et al., 2007). This suggests that piRNAs might be involved in stem cell development (Houwing et al., 2007). Recently it has been found that MILI, a Piwi-interacting RNA-binding protein, is required for the self-renewal of germline stem cell which also supports the role of piRNAs in stem cell development (Unhavaithaya et al., 2009). Similarly, Piwi associates with chromatin and interacts directly with heterochromatin protein 1a (HP1a) (Brower-Toland et al., 2007). This suggests the possible involvement of Piwi in epigenetic control of genome. Likewise, proteins involved in piRNA production have been implicated in the process of learning and memory (Ashraf et al., 2006). All these findings indicate that piRNAs might be involved in diverse biological process.

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Table 1. Piwi proteins and their homologs in different animals, and the origin and mechanism of actions of piRNAs.

Ago-family Protein	Origin of piRNA	Length of piRNA	Mechanism of action	Species
MILI (PIWIL2 In human	Transposon, and piRNA clusters	24-28 nts	Heterochromatin formation (DNA methylation)	Homo-sapiens
MIWI (PIWIL1 In humans)	piRNA clusters	29-31 nts	Unknown	Homo-sapiens
MIWI2 (PIWL4 In humans)	Transposon, and piRNA clusters	27-29 nts	Heterochromatin formation (DNA methylation)	Homo-sapiens
PIWIL3 In humans	Unknown	Unknown	Unknown	Homo-sapiens
AUB	Transposon, repeats, piRNA clusters, and SU (ste) locus	23-27 nts	RNA cleavage	*Drosophila melanogaster*
AGO3	Transposon, and Repeats (unknown in testis)	24-27 nts	RNA cleavage	*Drosophila melanogaster*
PIWI	Transposon, repeats and piRNA clusters	24-29 nts	Heterochromatin formation	*Drosophila melanogaster*
AGO4 and AGO6	Transposon, and repetitive elements	24 nts	Heterochromatin formation	*Arabidopsis thaliana*

This review summarizes the knowledge about piRNAs till date. The major topics discussed include Piwi proteins and their association with piRNAs, major findings in pi RNA genomics, and the possible biogenesis pathways of piRNAs in flies and mammals.

PIWI PROTEINS AND THEIR ASSOCIATION WITH piRNAs

The Argonaute (Ago) family proteins are composed of three distinctive domains: the PAZ, MID, and PIWI domains. However, the Ago family can be classified into two subclasses on the basis of amino acid sequence similarities: the Ago subfamily and the Piwi subfamily. The Ago proteins are expressed globally and interact with miRNAs or small interfering RNAs (siRNAs). The Piwi proteins are mostly expressed in germ cells and stem cells, and recent findings proposed their interaction with piRNAs (Kim et al., 2009).

Three Piwi members have been identified in mice: MIWI, MILI/PIWIL2 and MIWI2/PIWIL4. The MILI and MIWI expression kinetics are different from each other. MILI is expressed in germline development up to the pachytene spermatocyte phase. MIWI has been identified to exist from mid-pachytene to the early spermatids stage (Kim, 2006; Miyagawa et al., 2001; Deng and Lin, 2002). The two Piwi homologs Ziwi and Zili (also known as Piwil1 and Piwil2, respectively) have been reported in zebra fish (*Drosophila rerio*) (Houwing et al., 2007). The Piwi proteins in flies are known as Aubergine (Aub) and Ago3 and are capable to cleave their target mRNA (Nishida et al., 2007). The Piwi proteins and their homologs in different animals, their associated piRNAs length, and possible mechanisms of action are summarized in Table 1.

GENOMICS OF piRNAs

Many of the cloned piRNAs show an irregular distribution among chromosomes (Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Kim, 2006; Watanabe et al., 2006). The piRNA genes are mostly located on chromosomes 2, 4, 5 and 17 but seem to be absent on sex chromosomes (Kim, 2006). The piRNAs can be divided into three major classes based on genomic localization: non-repeats, simple repeat, and repeat associated piRNAs. The non-repeats piRNAs can be further divided into three subclasses: intergenic, intronic and exonic piRNAs. The majority of piRNAs sequences (34% in the ovary and 21% in the testis library) map to the sequences that are annotated as transposon. Similarly, more than 80% of the non-repetitive sequences map to intergenic regions (Houwing et al., 2007). Most of the piRNAs are clustered in relatively short genome loci ranges from <1kb to >100kb (Kim, 2006). All sequences, both repetitive and nonrepetitive, originate from the same clusters. In turn, piRNAs in a given cluster are derived from the same orientation (Kim, 2006).

SYNTHESIS OF piRNAs

Synthesis of piRNAs in flies

RNase III enzyme, known as Dicer, mainly produces 21 to 22 nt products from double-stranded precursors

(Bernstein et al., 2001). In contrast, the length of piRNAs (24 to 31 nts) demonstrates that they are not processed by Dicer. Recent genetic studies also concluded that piRNA production is a Dicer-independent process (Houwing et al., 2007; Vagin et al., 2006). The piRNAs production is strand bias. Aub and Piwi associated piRNAs are mainly produced from antisense transcript of retrotransposons. On the other hand, Ago3 interacting piRNAs are mostly derived from sense transcripts (Brennecke et al., 2007; Gunawardane et al., 2007). Furthermore, piRNA also show a strong bias towards nucleotides. Aub and Piwi associated piRNAs show tendency towards uracil at their 5' ends. Ago3 associated piRNAs show a strong preference to the adenine residue at position 10 but no preference towards the 5' end (Brennecke et al., 2007; Gunawardane et al., 2007). The Piwi, Aub, and Ago3, in complex with piRNAs, can cleave the target RNAs between positions 10 and 11 with reference to the 5' end of the guide strand. Recently, a 'ping pong' model has been proposed for piRNA production which suggests that Piwi proteins themselves might be involved in piRNA synthesis (Figure 1) (Brennecke et al., 2007; Gunawardane et al., 2007). According to this model, Ago3 is bound to sense strand piRNAs and cleaves antisense strands at an A:U base pair. This phenomenon creates the 5' end of antisense piRNA. The antisense piRNA in turn associate with Aub or Piwi. With subsequent nucleolytic processing of the 3' overhangs, mature 24 ~ 31 nts antisense piRNAs are generated. The newly synthesized Aub or Piwi, associated with antisense piRNA, have been proposed to form a complex. This complex binds and cleaves the sense strand RNAs, silences target gene expression, and generates the 5' end of sense strand piRNAs transcripts that associate with Ago3. In the last step, processing of the 3' overhang produces mature sense strand piRNAs and thus completes the cycle. The major factors that are responsible for the formation of the 3' end are still not clear. The 'ping pong' cycle is thought to be occurring continuously *in vivo,* thereby generating the large population of piRNAs (Brennecke et al., 2007; Gunawardane et al., 2007).

The proteins that mediate the proposed steps in 'ping pong' model are yet to be identified. However, mutation in several genes including Squash and Zucchini (both encode putative nucleases), Spindle-E, Krimper, and Maelstrom, cause depletion of piRNA population in fly ovaries. This suggests that they are involved in the piRNAs synthesis (Lim and Kai, 2007; Kim et al., 2009; Pane et al., 2007; Vagin et al., 2006; Findley et al., 2003).

The piRNAs transcribed from the flamenco (flam-piRNAs) are not dependent on the 'ping pong' cycle because they are found only in Piwi. A separate pathway, known as primary processing pathway, has been recently proposed for flam-piRNAs (Aravin et al, 2007). According to this pathway, as in *Drosophila melanogaster,* at least Aub and probably Piwi are passed on to the next generation by germline transmission (Brennecke et al., 2007; Kim et al., 2009; Nishida et al., 2007). The maternal transmission has also been reported in fish (Houwing et al., 2007). Recently it has been shown that these maternally inherited piRNAs have an important epigenetic regulatory role in transposon silencing (Brennecke et al., 2008). These inherited piRNAs are probably in a Piwi proteins associated form and apparently act as a primary source that starts an amplification cycle of piRNAs synthesis in embryos. The cycle may, therefore, continue from generation to generation (Kim et al., 2009).

Synthesis of piRNAs in mammals (mice)

Recent findings suggest that the 'ping pong' model, originally proposed in *D. melanogaster*, may also apply to the mouse pre-pachytene piRNAs (Aravin et al., 2008). However, the differences among embryonic, neonatal, and adult piRNAs propose that the cycle does not continue throughout male germ-cell development (Miyagawa-Kuramochi et al., 2008). On the other hand, pachytene piRNAs have also exhibited characteristic differences with piRNAs produced by the 'ping pong' pathway. These observations suggest that the primary processing pathway, as in the case of *D. melanogaster,* may also be involved in the production of piRNAs in mice (Yu et al., 2005; Kim et al., 2009). As in plant miRNAs, piRNAs have a methyl group at their 3` ends (Saito et al., 2007; Horwich et al., 2007; Kirino and Mourelatos 2007; Ohara et al., 2007). Plant homolog HEN1 methyltransferase carries out this modification at the 3' end (Kim et al., 2009). The interaction of HEN1 with Piwi proteins have also been discovered in ovaries. The biological importance of the 2`-O-methyl modification is unknown because *D. melanogaster* mutants of HEN1 show no obvious phenotypic alterations (Kim et al., 2009).

FUTURE PROSPECTIVE

The piRNAs synthesis pathway in animals is well studied during past few years but the precise piRNA pathway is still mysterious. Because the 'ping pong' pathway alone is not sufficient to explain all the characteristics of piRNAs, it is expected that an additional pathway may exist for certain piRNAs. This may be true mainly for those piRNAs which are associated with Piwi in *D. melanogaster* and pachytene piRNAs in mice. The exact mechanism of piRNA based gene silencing is still elusive and it would be interesting to find out how piRNAs relate to heterochromatin formation.

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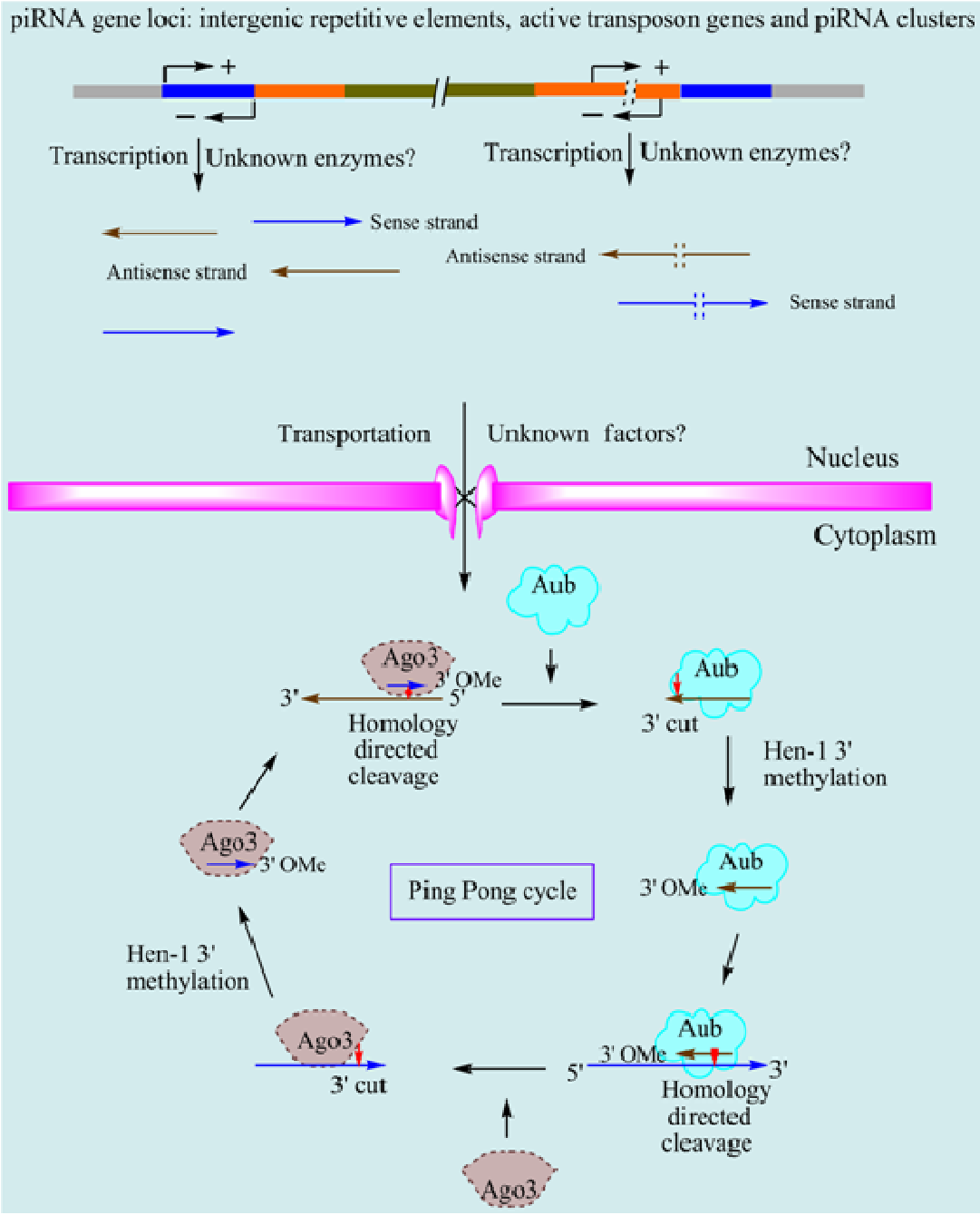

Figure 1. The piRNA 'ping pong' synthesis pathway in flies. Piwi-interacting RNAs (piRNAs) are processed from single-stranded RNA transcripts that are transcribed from intergenic repetitive elements, transposons or large piRNA clusters. The piRNAs associate with Piwi-subfamily proteins, and they may use the nuclease activity of the Piwi proteins themselves for their processing. Primary processing and loading might occur in the cytoplasm because Piwi proteins (Argonaute 3, Aubergine and Piwi) are localized in the cytoplasm. Factors that are needed for primary processing which occurs in the nucleus are unknown. In the secondary processing step (ping-pong cycle), the Piwi-class Argonaute protein Argonaute 3 (Ago3) binds to sense-strand piRNAs and cleaves the target antisense-strand precursor, producing the 5' end of antisense-strand piRNAs. The Aubergine (Aub) or Piwi (not shown) bind to the processed piRNA transcript, and processed to its final length. This might be catalyzed by the putative nucleases Squash and Zucchini. Drosophila Hen1 may carry out methylation step and methylate the 3' ends of piRNAs (3' OMe). The Aub–antisense-strand piRNA complexes are responsible for the cleavage of sense transcripts, producing the 5' end of sense piRNAs. The Ago3 binds the resulting sense piRNA precursors, which are processed and methylated, as illustrated for antisense-strand piRNAs. This pathway is based on the models proposed by Brennecke et al. (2007) and Kim et al. (2009).

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