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piRNAs and Their Functions in the Brain

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Abstract

Piwi-interacting RNAs (piRNAs) are the non-coding RNAs with 24–32 nucleotides (nt). They exhibit stark differences in length, expression pattern, abundance, and genomic organization when compared to micro-RNAs (miRNAs). There are hundreds of thousands unique piRNA sequences in each species. Numerous piRNAs have been identified and deposited in public databases. Since the piRNAs were originally discovered and well-studied in the germline, a few other studies have reported the presence of piRNAs in somatic cells including neurons. This paper reviewed the common features, biogenesis, functions, and distributions of piRNAs and summarized their specific functions in the brain. This review may provide new insights and research direction for brain disorders.

Keywords

Biogenesis; Brain; Distribution; Function; piRNA

INTRODUCTION

In recent years, a distinct group of Argonaute family proteins has been found in Drosophila. They are Argonaute 1 (AGO1), AGO2, AGO3, Aubergine (Aub), and Piwi (Gunawardane et al. 2007). These proteins can be grouped into two subfamilies based on their functions. AGO1 and AGO2 belong to the AGO subfamily and AGO3, Aub, and Piwi belong to the Piwi subfamily (Mochizuki et al. 2002). More recently, the Piwi subfamily has also been discovered in other species. In mice, it has 3 members (Mili, Miwi, and Miwi2). However, four proteins (Hili, Hiwi1, Hiwi2, and Hiwi3) in this subfamily have been discovered in humans (Ghildiyal and Zamore 2009). These argonaute proteins have four characteristic domains: the N-terminal, Piwi-Argonaute-Zwille (PAZ), middle, and C-terminal Piwi domains (Tolia and Joshua-Tor 2007). They interact with a group of small RNAs and function as a complex to regulate cellular activities by RNA silencing (Lau et al. 2006). The RNAs interacting with the Piwi subfamily are referred to as piRNA (also called PiwiRNAs).

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piRNAs are a class of small non-coding RNAs originally isolated from mammalian germline cells. They are unevenly distributed across the genome. Over 80 percent of piRNAs map to unique genomic locations, of them 75 percent mapped to loci with multiple transposons and 5 percent mapped to protein-coding genes (Brennecke et al. 2007). The remaining piRNAs are mapped to multiple genomic locations. piRNAs exhibit stark differences in length, expression pattern, abundance, and genomic organization when compared to miRNAs (Mani and Juliano 2013). Usually, miRNAs are conserved across species and exhibit limited diversity in sequences. The human genome is predicted to encode sequences of a total of 1000 to 10000 pre-miRNAs (Bentwich et al. 2005; Miranda et al. 2006). However, there are hundreds of thousands piRNA sequences in each species, and these piRNAs are poorly conserved even between closely related species (Mani and Juliano 2013).

Objective

piRNAs were well-studied in the germline. Beyond germline, some studies have reported the presence of piRNAs in somatic cells including neurons. The objective of this paper is to review all published literature that is related to piRNAs and can be searched from PubMed, and summarize the common features, biogenesis, functions and distributions of piRNAs (majorly in germline) and their specific functions in the brain.

OBSERVATIONS AND DISCUSSION

1. Features of piRNAs

piRNAs may have some common features: (A) length distributing between 24 and 32 nt; (B) 5' uridine signature, adenosine signature at position 10, and 2'-O-methylation at 3' end; and (C) clustering of their coding sequences in the genome. Other sequence features include strand specificity of clusters, and under-representation in repetitive element regions (Yan et al. 2011).

A. A mature piRNA is short and single-stranded RNA molecule with 24–32 nt. In comparison, miRNAs and siRNAs usually have 20–24 nt. This characteristic length profile may be determined by the maximal number of bases protected by Piwi proteins when they bind piRNAs (Boland et al. 2011; Brennecke et al. 2007; Nakanishi et al. 2012; Parker et al. 2004).

B. piRNAs have a strong preference [≥60% (Yan et al. 2011)] for the 5' uridine residue. This might be because their first 10 nucleotides could bind to Piwi or Aub (Lin 2007) that typically starts with uridine (Kawaoka et al. 2011) that might authenticate and stabilize piRNA intermediates for further processing (Ross et al. 2014). In addition, their first 10 nucleotides could bind to Ago3 too (Lin 2007) that usually contains an adenosine at position 10, so that some piRNAs have an adenosine signature at position 10 (Yan et al. 2011). Mature piRNAs are then 2'-O-methylated at 3' end by a conserved methyltransferase, Pimet (Saito et al. 2007), perhaps to ensure their stability too (Ross et al. 2014).

C. Approximately 92 percent of piRNAs in the Drosophila ovary are clustered (Brennecke et al. 2007). The number of piRNAs in these clusters

ranges from 40 to 4000 (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006a; Lau et al. 2006). The clusters vary in length from1 to 127 kb (Lee et al. 2011), a majority of them are between 20–100 kb. These clusters are often located in intergenic regions and can make up to 3.5 percent of the genome. The piRNA clusters are enriched for non-functional transposon remnants (Brennecke et al. 2007). Although the individual piRNA sequences are rarely conserved, the genomic locations of the piRNA clusters are usually conserved (Aravin et al. 2006; Girard et al. 2006; Lau et al. 2006). Interestingly, a majority of the piRNA clusters are coded by the same genomic strand and so called the monodirectional clusters), only a few clusters are coded by both strands and so called the bidirectional clusters. These two classes of clusters seem to be evolved differently. The bidirectional clusters distribute across the genome in a divergent, non-overlapping manner, which suggests the possible divergent transcription from the piRNA precursors. The piRNA clusters expressed in germ cells are largely bidirectional, while the somatically expressed piRNA clusters are largely monodirectional (Brennecke et al. 2007; Malone et al. 2009; Mani and Juliano 2013).

2. Detection of piRNAs

Novel piRNAs are mainly discovered by sequencing of the size-selected RNAs from the specific tissues. Small RNAs with 24 to 32 nucleotides can be extracted from the sequencing reads, and then their expression in the tissues can be validated by in situ hybridization and Northern blotting (Lee et al. 2011). Their presence in the piRNA databases (see below) should also be checked. Coimmunoprecipitation with Piwi is the gold standard to verify piRNAs. To test whether a specific piRNA is bound to Piwi, a Piwi antibody can be used (Girard et al. 2006) to pull down Piwi protein, RNAs associated with it can then be extracted and validated by a real-time PCR. The verification of appropriate piRNA features by bioinformatic analyses may be helpful, such as verification of the genomic location of the cluster and transcription orientation. Finally, the functional study of newly discovered piRNAs is essential, which can be accomplished by antisense suppression of the targeted piRNAs (Lee et al. 2011). Since piRNAs are defined as Piwi binding RNAs, sequencing Piwi co-immunoprecipitated RNAs is an alternative method to discover piRNAs.

3. Databases of piRNAs

So far, the large-scale sequencing of the piRNAs from human, rat, and mouse germlines has discovered a large number of piRNA sequences. These sequences have been deposited in many public databases, including the NCBI nucleotide sequence database (Build37) (Girard et al. 2006; Lau et al. 2006; Watanabe et al. 2006), piRNABank (Sai Lakshmi and Agrawal 2008), piRNA cluster database (Rosenkranz 2016), noncoding RNA database (RNAdb 2.0) (Pang et al. 2007), fRNAdb (Kin et al. 2007), NONCODE V3.0 (Bu et al. 2012), RNAcentral (Bateman et al. 2011), NRED (Dinger et al. 2009), UCSC hg19, piRNA cluster – database (Girard et al. 2006; Lau et al. 2006), deepBase (Yang et al. 2010), Piwi-interacting RNA – McManus lab (Grimson et al. 2008), ncRNAimprint (Zhang et al. 2010), BlastR (Bussotti et al. 2011), piRBase (Shao et al. 2012), and Ensembl 37.59. The piRNAs

have also been reported in the Supplementary Data in published literature (Aravin et al. 2006; Grivna et al. 2006a). A large number of these piRNAs in databases and literature have been functionally validated.

4. Biogenesis of piRNAs

Unlike miRNAs and siRNAs, piRNAs are not derived from the dsRNA precursors. There are two major mechanisms for the biogenesis of piRNAs, primary biogenesis and secondary biogenesis (Lin 2007; Mani and Juliano 2013; Yan et al. 2011). Primary piRNA biogenesis may occur in the germline and somatic cells including neurons (Mani and Juliano 2013). Primary biogenesis processes a transcript covering the entire piRNA cluster into a mature piRNA (Mani and Juliano 2013). A primary piRNA has 5' bias for uridine, but does not have nucleotide bias at position 10 (Mani and Juliano 2013). The secondary biogenesis is also called “ping-pong” model (Mani and Juliano 2013) that is used to produce secondary piRNA using a primary piRNA-guided cleavage (Mani and Juliano 2013). The first 10 nucleotides of primary piRNAs, which typically begin with uridine and are bound to Aub or Piwi (Lin 2007), are often complementary to the first 10 nucleotides of secondary piRNAs, which usually, at position 10, contain an adenosine and are bound to Ago3 (Lin 2007). This complementarity mediates the amplification that generates the new secondary piRNAs (Lin 2007), which occurs as a ‘ping-pong’ (back-and-forth) mechanism between Ago3 and Aub (Brennecke et al. 2007). A secondary piRNA has a bias for adenine at position 10, but does not have a bias at 5' (Mani and Juliano 2013).

5. Function of piRNAs

Individual piRNAs are not conservative, which makes the deductions of their functions challenging. In spite of this challenging, numerous lines of evidence indicate that piRNAs have important functional roles in biology, including suppressing transposon (Mani and Juliano 2013), preserving genomic integrity (Czech and Hannon 2016; Stefani and Slack 2008), remodeling euchromatin and epigenetic programming (Akkouche et al. 2013; Ross et al. 2014), regulating translation (Grivna et al. 2006a), regulating target mRNAs (Lee et al. 2011), modulating mRNA stability (Grivna et al. 2006a), and developmental regulation.

The most widely-recognized and well-characterized function of piRNAs is to suppress the activities of transposable elements at genomic and epigenetic levels, particularly in the germline, where piRNAs are highly abundant, and in somatic cells like neurons, where piRNAs are modestly abundant (Aravin et al. 2007a; Aravin et al. 2007b; Brennecke et al. 2007; Brennecke et al. 2008; Dharap et al. 2011; Kuramochi-Miyagawa et al. 2008; Lee et al. 2011; Malone and Hannon 2009; Mani and Juliano 2013; Peng and Lin 2013; Rajasethupathy et al. 2012; Stefani and Slack 2008). A transposable element (or transposon) is a small piece of DNA sequence. It can be transcribed into RNA, reversely-transcribed into double-stranded DNA, and inserted into a new genomic location; as a result, it changes its position within the genome (that is, transposition) (Cordaux and Batzer 2009; Gogvadze and Buzdin 2009). The transposition often results in the duplication of transposons, which can make up to 40 percent of the genome in eukaryotes. Numerous piRNAs are produced from the disruption of transposons in the genome (Halic and Moazed 2009; Sai Lakshmi and Agrawal 2008); that is, most piRNAs overlap with these transposons or transposon remnants

in sequences (Brennecke et al. 2007). piRNAs selectively target and silence the RNAs transcribed from transposons in the germline or the somatic cells (Brennecke et al. 2007; Gunawardane et al. 2007). This silencing balances the fitness of the genome to maintain the genetic equilibrium in a species. Experimental data supported this notion. Mili- and Miwi-2 null mice have been found to have increased activity of retrotransposons, which suggests that piRNAs could protect the genome from deleterious transposon insertions to preserve the genomic integrity (Stefani and Slack 2008). Any disruption in the piRNAome can cause loss of control of transposons (Dharap et al. 2011). Uncontrolled transposition can cause disaster to the cell. It threats the genomic stability and integrity (Mani and Juliano 2013). Therefore, there is a hypothesis that the Piwi/piRNA complex uses the transposons to regulate a large group of gene expression and cellular function (Mani and Juliano 2013).

Because piRNAs are highly abundant in the germline, piRNAs play a key role in the germline development via restricting transposable elements. Numerous lines of evidence indicate that piRNAs have critical roles in spermatogenesis (Grivna et al. 2006a; Grivna et al. 2006b; Schubert 2016). Genomic instability caused by transposon upregulation can lead to sterility; that is, piRNAs are essential for the maintenance of fertility (Houwing et al. 2007; Mani and Juliano 2013).

Experimental data suggest that piRNAs can inhibit transposons at either genomic or epigenetic levels. The restriction of transposons by piRNAs has been demonstrated by the upregulation of transposons resulting from the mutations of the Piwi/piRNA complex; for example, when an experiment depleted the Piwi proteins in Drosophila, upregulation of transposon was observed (Reiss et al. 2004; Sarot et al. 2004; Savitsky et al. 2006). This upregulation may be the consequence of DNA damage and genomic instability caused by Piwi mutations (Klattenhoff et al. 2007; Mani and Juliano 2013).

piRNAs regulate the transposable elements possibly via *de novo* DNA methylation (Aravin et al. 2007b; Brennecke et al. 2008; Kuramochi-Miyagawa et al. 2008). Mice lacking the Piwi homologs showed substantial demethylation and repression of transposable elements targeted by piRNAs (Rajasethupathy et al. 2012). Mili and Miwi2 regulate DNA methylation at the transposon loci too (Aravin et al. 2008; Aravin et al. 2007b; Kuramochi-Miyagawa et al. 2008; Manakov et al. 2015). Additionally, there are reports that piRNAs direct the DNA methylation on the non-transposon genomic loci (Watanabe et al. 2011).

There is also evidence that piRNAs can directly regulate chromatin architecture to control genomic stability (Mani and Juliano 2013). The Drosophila Piwi protein may influence the histone modification targeted by piRNAs (Yin and Lin 2007) and associate to HP1a and histone H3 lysine9 methylation (H3K9me) (Brower-Toland et al. 2007; Mendez et al. 2011). HP1a and H3K9me are two key marks for the transcriptionally silenced chromatin.

In addition to transposon repression, piRNAs have other functions. For example, piRNAs were found to modulate the stability and translation of those mRNAs encoding proteins required for spermatogenesis (Grivna et al. 2006a). piRNAs from the intergenic regions were also found to regulate the mRNAs from early embryos and gonads (Lee et al. 2011). The Piwi/piRNA complex also regulates some protein-coding genes (Mani and Juliano 2013).

For example, it targets some genes that control spine shape, playing roles in spine morphogenesis (Lee et al. 2011). It can mediate canalization to enhance developmental robustness (Peng and Lin 2013).

6. Distribution of piRNAs

So far, in mouse testes, people have identified over 52,000 piRNAs that are associated with Miwi and over 1000 piRNAs that are associated with MILI (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006a; Grivna et al. 2006b; Lau et al. 2006; Watanabe et al. 2006). Around 24,000 piRNAs were identified in human testes. Although Piwi proteins were originally identified in the germline, they are also expressed in the somatic tissues. Sharma et al. (2001) reported that the Piwi and Hiwi mRNAs were most abundant in the testis, followed by the kidney and brain (Sharma et al. 2001). Lee et al. (2011) reported that Miwi showed a similar rank order of expression in mice (testis > kidney > brain) (Lee et al. 2011). Miwi protein was observed to be expressed in the hippocampal neurons, and also in the heart, liver, and lung (Lee et al. 2011; Sharma et al. 2001). By deep sequencing analysis, Yan et al. (2011) identified piRNA-like species in mammalian tissues that included the cortex, prostate, epididymis, and hematopoietic system (Yan et al. 2011). Piwi proteins are also expressed in other somatic tissues, including various adult stem cells (Juliano et al. 2011), prostate, ovary, small intestine, heart, liver, skeletal muscle, kidney, and pancreas (human) (Sharma et al. 2001), human Natural Killer (NK) cells (Cichocki et al. 2010), and a variety of mouse tissues (Ro et al. 2007).

7. Distribution and Functions of piRNAs in Brains

In addition to the above three reports by Lee et al. (2011), Sharma et al. (2001), and Yan et al. (2011), more studies have reported the distribution of piRNAs in brains. For example, in several brain regions, including the hippocampus, the expression of Miwi mRNA in the mouse brain was observed by in situ hybridization. These observations were confirmed by real-time PCR. Lee et al. (2011) further showed that, in mouse dendritic spines, there were abundant piRNA complexes, and that the knockdown of piRNAs resulted in reduced spine density in the axons (Lee et al. 2011). Rajasethupathy et al. (2012) found that piRNAs were amply present in neurons in the *Aplysia* brain (Rajasethupathy et al. 2012). Ghildiyal et al. (2008) identified thousands of piRNA-like small non-coding RNAs (pilRNAs) in Drosophila heads (Ghildiyal et al. 2008). These pilRNAs displayed known piRNA sequence features, including the presence of 2'-O-methyl group at the 3'-end (Ghildiyal et al. 2008). Additionally, the expression of two Drosophila PIWIs, that is, Aub and AGO3, was found in specific regions of the brain (Perrat et al. 2013). Their mutations had effects on brain development and neurons. More studies also reported a potential role of piRNA in the brain of multiple organisms (Iyengar et al. 2014; Ross et al. 2014; Weick and Miska 2014).

Lee et al. (2011) sequenced small RNA libraries from mouse hippocampus for small non-coding RNAs (≤35bp) using RNA-Seq technology (30×). This study produced 14.83×10^6 35-bp reads in the male mouse and 9.18×10^6 reads in the female mouse, respectively. Of these reads, 66.7 percent mapped to the mouse genome, accounting for 9.89×10^6 (male) and 6.12×10^6 (female) unique RNA transcripts. Among these transcripts, after stringently filtering out tRNAs, rRNAs, adaptors, miRNAs and small RNAs <25 nt, 11.3 percent of

these transcripts ranged from 25 to 32 nt. Among these piRNA-like small RNAs with 25 nucleotides or more, 2,297 (0.76%) were the known piRNAs deposited in the piRNA bank (pirnabank.ibab.ac.in) or the noncoding RNA database (RNAdb). Most of these piRNAs coimmunoprecipitated with Miwi protein. More than half (59.3%) of these piRNAs had a "U" at the first position. Many of these piRNAs were mapped to chromosomes in clusters. Most of the highly expressed piRNAs mapped to unique intergenic genomic regions. The expression of five most highly expressed piRNAs was validated by Northern blotting. The piRNAs in the brain usually have unique biogenesis patterns and a predominant nuclear localization (Rajasethupathy et al. 2012). The amount of piRNAs in the brain is about one-tenth of those found in the germline, consistent with other reports that showed 1 percent - 10 percent or 9.7 percent (Dharap et al. 2011; Lee et al. 2011; Peng and Lin 2013; Yan et al. 2011).

Serotonin, that is, 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter. It is largely synthesized in the serotonergic neurons in the central nervous system, where it regulates mood, appetite and sleep. Modulation of serotonin at synapses also contributes to some cognitive functions such as memory and learning and pharmacological effects of antidepressants. It was found that the amount of Piwi/piRNA complex in *Aplysia* brain was sensitive to serotonin modulation (Rajasethupathy et al. 2012). Additionally, cAMP-responsive element-binding protein 2 (CREB2) is a transcriptional repressor binding to the cAMP-responsive element (CRE). It plays a role in the development of the nervous system. CREB2 in neurons is the major inhibitory constraint of memory. There is evidence that the Piwi/piRNA complex in the *Aplysia* brain regulates CREB2 in an activity-dependent manner (Rajasethupathy et al. 2012). The Piwi/piRNA complex may facilitate the serotonin-dependent methylation of a conserved CREB2 promoter CpG island in the neurons, leading to the enhancement of long-term synaptic facilitation, learning-related synaptic plasticity and memory storage in *Aplysia* (Rajasethupathy et al. 2012).

Furthermore, Piwi/piRNA complex may play a role in the local translation in the mouse neuronal dendrites (Lee et al. 2011). Piwi/piRNA complex can potentially modulate the dendritic spine development (Lee et al. 2011). Some Piwi/piRNA complex can target at Astrotactin that may be implicated in the neuronal migration (Adams et al. 2002). Some Piwi/piRNA complex can also potentially regulate genes to control nervous system function (Lee et al. 2011). For example, in mice, (i) Piwi/piRNA complex can regulate Cdk5rap1 that inhibits brain-enriched kinase Cdk5 (Ching et al. 2002). Kinase activity of Cdk5 has been implicated in regulating the dendritic spine growth and shrinkage (Cheung and Ip 2007; Fu and Ip 2007). (ii) Piwi/piRNA complex can regulate microtubule affinity-regulating kinase 1/2 (Mark1/2) that interacts with a key signaling protein, 14-3-3. This protein can regulate dendritic spine development too (Angrand et al. 2006). (iii) Piwi/piRNA complex can regulate AKAP79/150 that regulates the dendritic spine area (Robertson et al. 2009). (iv) Piwi/piRNA complex can regulate Myosin Va. This myosin is enriched in postsynaptic density. It is critical in the organization of postsynaptic density (Naisbitt et al. 2000). Finally, a pioneering work has shown that Piwi/piRNA functions in both transcriptional and posttranscriptional silencing of the alcohol dehydrogenase gene (*Adh*) in Drosophila (Pal-Bhadra et al. 2002; Ross et al. 2014). *Adh* gene is majorly expressed in the liver, and partly in the brain (majorly *Adh5*) (Galter et al. 2003). The homolog in human, that is, the *ADH*

gene, is the most robust risk gene for alcohol dependence demonstrated by numerous GWASs and candidate gene studies (Zuo et al. 2014). Although the functions of piRNAs have been reported in the diverse mammalian brains that might be far different from human due to the poor conservation across species, they provide a potential clue for us to explore the possible roles of Piwi/piRNAs in human brain disorders.

As described above, the main function of piRNAs is to regulate transposons. This raises an interesting question whether piRNAs play a role in the brain tumor since transposition events are frequently observed in human brain tumors. The report that the inactivation of Piwi or Aub in Drosophila suppresses the malignant tumor growth in the sensitized lethal malignant brain tumors (Janic et al. 2010) seems to support this notion. Another possible clue regarding the functions of piRNA in the brain is related to the discovery of the L1 retrotransposons in the human, mouse and rat brains. In the brains, the L1 retrotransposons are involved in the neuronal differentiation, heterogeneity, and somatic mosaicism (Coufal et al. 2009; Muotri et al. 2005). Some piRNAs and retrotransposons co-exist in the brains. These piRNAs regulate the L1 retrotransposons and their mutants elevate the retrotransposon expression in the brains. The co-existence of piRNA and retrotransposons might play important roles in the brain development and the adult brains.

CONCLUSION

piRNAs in the brain might have common features, biogenesis, and functions as those in germline reviewed above. Additionally, piRNAs in the brain may have some specific roles in CNS beyond germline. However, literature is very limited, especially those related to the human brain, and the findings remain preliminary.

RECOMMENDATIONS

More specific functions of piRNAs in the brain are expected to be discovered in near future. Their roles in brain disorders warrant further study.

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