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Mechanisms and Applications of RNA Interference

Research Article

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Summary

RNA interference (RNAi) and related pathways involving small interfering RNAs (siRNAs), microRNAs (miRNAs), and PIWI-interacting RNAs (piRNAs) regulate processes such as anti-viral defense, genome surveillance, heterochromatin formation, and gene expression in animals, plants, and fungi. Studies on RNAi have revealed a two-step mechanism: (i) Degradation of dsRNA into small interfering RNAs (siRNAs), 21 to 25 nucleotides long, by an RNase III-like activity. (ii) The siRNAs join an RNase complex, RISC (RNA-induced silencing complex), which acts on the mRNA and degrades it. Molecular structures of Dicer, Argonaute proteins, and RNA-bound complexes have offered insights into the underlying mechanisms of RNA-silencing pathways. Sequence-specific gene silencing using small interfering RNA (siRNA) is now being evaluated as a novel therapeutic strategy. Recently, promising data have been obtained from clinical trials for the treatment of respiratory syncytial virus and age-related macular degeneration. The exact mechanism of the RNAi pathways is still unclear. Our review summarizes the RNAi pathways and the known functions of siRNAs, miRNAs, and piRNAs in lower and higher organisms (mostly focusing on mammals) and discusses the potential applications of RNAi.

I. Introduction

Only 2% of the human genome actively encodes proteins. Most transcripts are noncoding RNAs, functional RNA molecules that are not translated into proteins (Morris, B J at al., 2012, Sijen, T at al., 2001). Recently, a large number of diverse small non-coding RNAs have been discovered and characterized in plants and eukaryotic RNA interference (RNAi) pathways. RNAi is involved in the regulation of epigenetic modifications, gene expression, heterochromatin formation, and hostparasite interactions. Over a billion years ago, the common ancestor of all eukaryotes had a functional RNAi pathway that is widely distributed across the eukaryotic phylogeny (Cerutti, H at al., 2006). Most small RNAs can be classified into the following three types: short-interfering RNAs (siRNAs), microRNAs (miRNAs), and PIWI-interacting RNAs (piRNAs) (Carthew, R W at al., 2009, Shi, Z at al., 2013). In animals, these three classes regulate development and defense processes (Yigit, E at al., 2006). Xia *et al*., based on the distinct genomic origins of miRNAs and siRNAs, suggested that a small subset of annotated miRNAs should, in fact, be defined as siRNAs (Xia, J at al., 2013).

siRNA are derived from short double-stranded RNA molecules with both endogenous and exogenous (derived from infection by a RNA viruses or artificial siRNAs) origins. Gene silencing by RNAi is mediated via two main steps. Briefly, the dsRNA is initially recognized by Dicer (a member of the RNase III superfamily) and processed into small double-stranded molecules, termed siRNA. Then, siRNAs are bound by the RNA-induced silencing complex (RISC) with RNase activity which guides the targeted RNA towards degradation (Bantounas, I at al., 2004). Notably, despite the siRNAs that plants produce against viruses, pathogenic viruses have also developed a molecular defense mechanism for their survival against the host anti-viral system and RNAi, known as RNA silencing suppression phenomenon (RSS) (Bivalkar-Mehla, S at al., 2011).

Many studies have shown that miRNAs play an important role in cellular processes, such as growth, differentiation, proliferation, and death. miRNAs are initially transcribed as hairpin-like structures (pre-miRNA) by RNA polymerase II, and then processed to yield mature (single-stranded) miRNAs by two enzymes, Drosha (Class 2 RNase III

enzyme) in the nucleus and Dicer in the cytoplasm. The mature miRNA attaches to the RISC and acts as a guide strand that recognizes target mRNAs (Lee, Y at al., 2004). Based on their sequences so far, miRNAs are the most highly conserved class of small RNAs (Shi, Z at al., 2013). Between *Caenorhabditis elegans* and humans, there are many conserved miRNAs, including let-7 and miR-1, which share 100% sequence identity across species (Lee, R C at al., 2001).

piRNAs in *C. elegans* are 21 bp long and contain a uracil at their 5' termini (Das, P P at al., 2008). Currently, two pathways are known for piRNAs in mice and flies, the primary processing pathway and the ping-pong amplification loop (Mikiko, C S at al., 2011). The primary piRNA pathway produces primary piRNAs, whereas the ping-pong cycle, shapes the piRNA population from the initial pool of piRNAs, which associate with specific PIWI proteins, to target and cleave multiple Transposable Element (TE) transcripts (Bamezai, S at al., 2012). PIWI proteins are a subset of Argonaute proteins. Unlike siRNAs and miRNAs, piRNAs bind to two PIWI-clade Argonautes (Bamezai, S at al., 2012, Cecere, G at al., 2012).

The important difference between mammalian RNAi and RNAi in flies and other lower eukaryotes, is that mammalian cells lack an amplification system for long-term persistence of RNAi (Chiu, Y L at al., 2002). There are three factors that affect the amplification in flies and other lower eukaryotes: (i) The production of smaller 21–23-nt siRNAs from long trigger dsRNAs by Dicer, whereas, in mammalian cells, long trigger dsRNAs invoke interferon response and activates the Protein kinase RNA-activated response (PKR) (Stark, G R at al., 1998). (ii) RNA-dependent RNA polymerase (RdRP) is present in worms, flies, fungi, and plants (Cogoni, C at al., 1999, Dalmay, T at al., 2000, Sijen, T at al., 2001).
It has been suggested that RdRP amplifies target mRNA into dsRNA, through a random and degradative PCR model (Lipardi, C at al., 2001, Sijen, T at al., 2001), that can be targeted by Dicer, whereas no RdRP homologs have been found in mammalian cells (Chiu, Y L;Rana, T M at al., 2002, Schwarz, D S at al., 2003). (iii) The high enzymatic turnover rate of RISC affects amplification during targeting and cleavage of mRNAs, which it is possible to add a degree of amplification to RNAi induction in all eukaryotes (Hutvagner, G at al., 2002). In human cells, persistence of RNAi occurs for only a short time period approximately 66 h before the siRNA is diluted out over the course of several cell Divisions (Chiu and Rana 2002). In this review, the latest studies of human RNAi pathways and their applications are described collectively.

Endo-small interfering RNAs (Endo-siRNAs)

Endogenous siRNAs were first identified in plants, yeasts, and *C. elegans* (Cogoni, C at al., 2000, Sijen, T at al., 2001). Further research in mammals showed that endogenous siRNAs were expressed in murine oocytes, embryonic stem cells, and spermatogenic cells, and play critical roles in normal development of oocytes and embryonic stem cells (Billy, E at al., 2001, Song, R at al., 2011). siRNA or post transcriptional gene silencing (PTGS) pathway is a major arm of the anti-viral immune response in plants, Drosophila, and other insects (Ding, S W at al., 2007, Marques, F Z at al., 2012) **(Figure 1).**

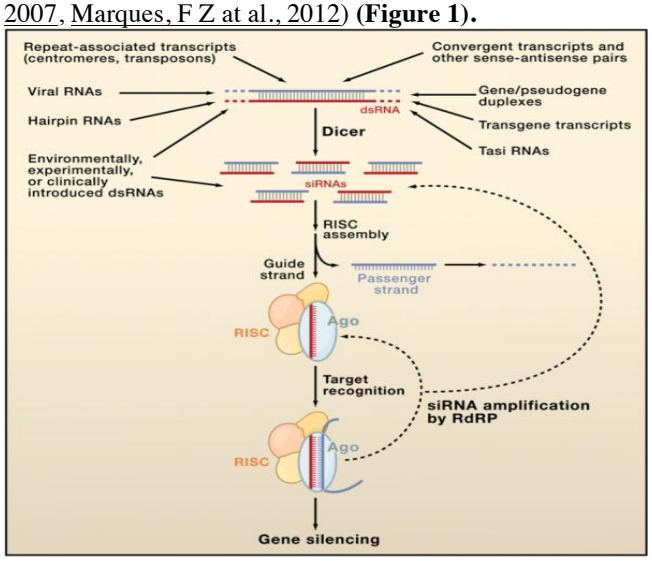

Figure 1: Sources of siRNA. Different sources of processing siRNA by Dicer into siRNAs. An siRNA consists of a guide strand (red), which assembles into functional siRISC, and a passenger strand (blue), which is ejected and degraded (Carthew, R W;Sontheimer, E J at al., 2009).

Most substrates required producing endo-siRNAs in flies and mice are derived from transposable elements, long 'fold-back' transcripts, or hairpin RNAs and complementary annealed transcripts. The precise mechanism of how the dsRNA substrates of siRNAs are derived from transposable elements is still unknown. siRNAs that are derived from hairpin RNAs are long and inverted repeat transcripts. siRNAs derived from cis-natural antisense transcripts (cis-NATs) involve bidirectional transcription of the same genomic DNA. Cis-NATs can be convergent, divergent, or involve internal exons or annotated introns. siRNA derived Trans-NAT dsRNAs form between transcripts are usually comprise mRNA and an antisense transcribed pseudogene (Okamura, K at al., 2008, Watanabe, T at al., 2008).

siRNAs can elicit RNAi in mammalian cells without producing an interferon response (Elbashir, S M at al., 2001). siRNAs are usually short nucleotides (20–25 bp in length) double-stranded RNA with phosphorylated 5′ ends and hydroxylated 3′ ends, along with 2 overhanging nucleotides. Long dsRNAs are cleaved by Dicer into short dsRNA duplexes or siRNAs in the cytoplasm. Endogenous and exogenous siRNAs obtained from their precursors are sorted by a protein complex composed of Dicer-2 (Dcr-2) in association with a dsRNA-binding protein (dsRBP) called R2D2 and loaded onto Argonaute-2 (Ago2) (Marques, J T at al., 2010). One strand of the siRNA duplex is ejected by Ago2 to generate a mature RISC, containing only the guide strand of the siRNA (Kim, K at al., 2007). The mature Ago2-RISC then cleaves complementary ssRNAs into the guide siRNA (Carthew, R W;Sontheimer, E J at al., 2009).

Billy *et al*. exploited Entero chromaffin (EC) cells to gain some insight into the RNAi machinery in mammals. They

found 23-nt siRNAs, the RNaseIII-like enzyme, and Dicer localized to the cytoplasm of P19, F9, and HeLa cells. The observation confirmed previous findings that these cells are deficient in some of the dsRNA and interferon (IFN) activated enzymes (Billy, E at al., 2001, Harada, H at al., 1990). Recent studies indicate that siRNAs produced in mammalian cells (Song, R at al., 2011) and Drosophila embryos (Zamore, P D at al., 2000) belong to the 21-nt class, while siRNAs in plants and fungi are divided into two distinct classes, including a short (21-nt) and a long (24-nt) size class (Llave, C at al., 2002, Tang, G at al., 2003). Endogenous siRNA sequences are generally not conserved between species and their identification requires experimental approaches (Sunkar, R at al., 2005).

In eukaryotes, various naturally occurring siRNA-like molecules have been described, including (i) animal microRNAs (miRNAs) (Lee, R C at al., 1993, Pasquinelli, A E at al., 2000), (ii) scanRNAs in *Tetrahymena thermophila* (Mochizuki, K at al., 2002), centromeric repeat-originating siRNAs in *Schizosaccharomyces pombe* (Reinhart, B J at al., 2002), (iii) siRNAs produced by viruses in plants (Alvarado, V at al., 2009, Hamilton, A at al., 2002), and transposons in *C. elegans* (Sijen, T at al., 2003).

Viral RNA Silencing Suppressors (RSSs)

Pathogenic viruses have developed a molecular defense mechanism for their survival against the host anti-viral system or RNA interference, known as RNA silencing suppression phenomenon (Bivalkar-Mehla, S at al., 2011), which was first discovered in plants infected with the potato virus and the cucumber mosaic virus (Alvarado, V;Scholthof, H B at al., 2009).

Investigations on RSSs showed that viral proteins and non-coding viral RNAs could inhibit the RNAi (miRNA/siRNA) pathway through different mechanisms. Most of the pathogenic viruses use viral proteins to perform the RSS activity, except adenovirus, which uses its non-coding RNA (VA) (McKenna, S A at al., 2006). Other viruses such as human influenza A virus uses the NS1 protein to interact with various types of RNA (Hatada, E at al., 1997). In mammalian cells, HIV-1 transactivator of transcription (Tat) protein has been reported to block the mi/siRNA processing step performed by Dicer (Bennasser, Y at al., 2006). Similar to HIV and the Influenza virus, the hepatitis C virus (HCV) core and envelope (E2) proteins act as RSSs (Wang, Y at al., 2006, Yasui, K at al., 1998). E2 protein has the ability to interact with the Argonaute 2 (Ago 2) protein and make it non-functional. In the cytoplasm, Ago2 forms the effector complex called RISC, along with Dicer and the mature siRNA or miRNA (Bivalkar-Mehla, S at al., 2011). dsRNA-binding regions and GW/WG motifs of mammalian viruses appear to have a high chance of conferring RSS activity. Data showed that the GW/WG motifs in viral proteins act as Ago hooks and play critical roles in disabling RISC by sequestering Ago proteins in plant viruses. The GW/WG motifs are conserved in the C-terminal domains of plant viruses, but were found in the N-terminal domains as well (Bivalkar-Mehla, S at al., 2011).

The miRNA pathways in plants and animals

MicroRNAs or miRNAs are conserved across species and expressed across cell types. They are short non-coding RNAs that regulate gene expression post-transcriptionally and generally bind to the 3′-UTR of their target mRNAs and decrease protein production by destabilizing the mRNA transcripts resulting in translational silencing (Cannell, I G at al., 2008). More than 60 % of human protein-coding genes are possibly regulated by miRNAs. So far, more than 700 human miRNAs have been cloned (Friedman, R C at al., 2009).

MicroRNAs are transcribed by RNA polymerase II as part larger fold-back transcripts (pri-miRNA). These transcripts are processed in the nucleus of vertebrates by the DGCR8 (also known as Pasha). DGCR8 binds to the RNase III enzyme, DROSHA, to form short stem-loops (pre-miRNAs), which are then exported to the cytoplasm by Exportin five (Exp5). In the cytoplasm, pre-miRNAs are processed by the RNase III enzyme, Dicer-1, complexed with TRBP, and transformed into a duplex consisting of a guide strand (miRNA) and a passenger star strand (miRNA* strand). The mature miRNA is loaded onto an Argonaute-containing effector complex (RISC) and acts as a guide strand that recognizes target mRNAs based on sequence complementarity. The RISC subsequently represses targets by inhibiting translation or promoting destabilization of target mRNAs (Castel, S E at al., 2013, Gurtan, A M at al., 2013, Pillai, R S at al., 2007, Pritchard, C C at al., 2012, Yeom, K H at al., 2006) **(Figure 2).**

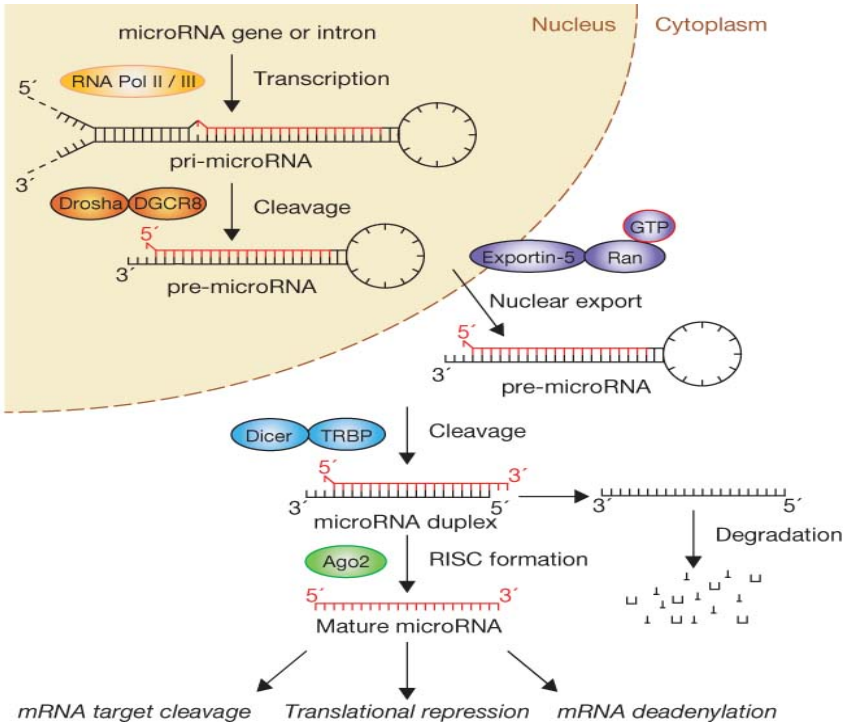

Figure 2: The miRNA processing pathway. In the nucleus, the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8. Result, produced precursor hairpin (the pre-miRNA) that is exported from the nucleus by Exportin-5–Ran-GTP. Then, in the cytoplasm, the RNase Dicer in complex with the dsRNA-binding protein TRBP cleaves

the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RISC, where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression, whereas the passenger strand (black) is degraded(Winter, J at al., 2009).

In animals, miRNAs usually pair imperfectly with one or more targets in the 3′-UTR, and do not induce mRNA endonucleolytic cleavage. In contrast, in plants, miRNAs usually match exactly with their messenger RNA targets and cause the cleavage and degradation of the target transcript. (Pillai, R S at al., 2007).

The piRNA pathways

In addition to siRNAs and miRNAs, a third RNA interference system has been uncovered that prevents the invasion of selfish genetic elements such as transposons and exogenous retroviruses, which are threats for the host genome especially in germline cells (Kawaoka, S at al., 2013, Malone, C D at al., 2009). piRNAs are part of a complex class of small non-coding RNAs with 24-31 bp in length, which are associated with PIWI proteins (Beyret, E at al., 2012).

piRNAs arise from intergenic repetitive elements in the genome known as piRNA clusters (Brennecke, J at al., 2007). The biogenesis of piRNA was initially described in the *D. melanogaster* germ line and is known as 'the ping-pong cycle (Brennecke, J at al., 2007, Gunawardane, L S at al., 2007).

In the *Drosophila melanogaster*, many aspects of piRNA biogenesis remain unclear. The primary antisense transcripts that are transcribed from transposons or piRNA clusters are converted into piRNAs by unknown mechanisms, and are loaded onto Aubergine (AUB) or PIWI (Mikiko, C S at al., 2011). Saito *et al*. determined that piRNAs in Drosophila are 2′-O-methylated at their 3′ ends, as in mouse and rat (Saito, K at al., 2007). piRNAs derived from the flamingo locus are exclusively loaded onto PIWI. piRNA-RISCs produced through this mechanism act as a 'trigger' for the amplification loop (Siomi, M C at al., 2011). Studies showed that fish and mammals may have similar mechanisms (Aravin, A A at al., 2007, Houwing, S at al., 2007) **(Figure 3).**

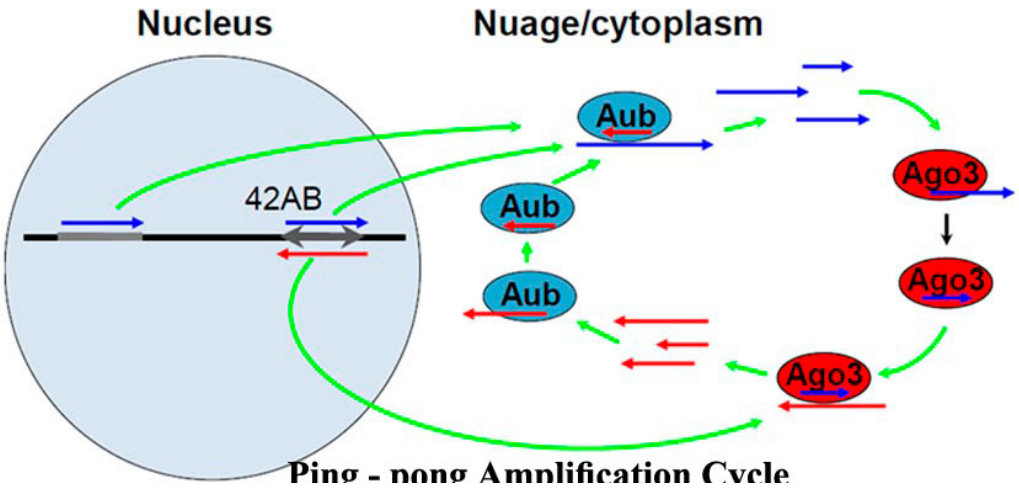

Figure 3: Ping-pong amplification in the germline. Transcripts from piRNA clusters (blue and red) and functional transposons (blue) are exported from the nucleus. Aubergine (Aub), preprogrammed with piRNAs generated through the primary biogenesis pathway, cleaves complementary transposon and cluster transcripts (blue), yielding randomly sized RNA fragments that bind to Ago3. 3'-end trimming produces mature Ago3-sense strand piRNA complexes, which cleave anti-sense cluster transcripts (red). The resulting fragments bind to Aubergine (Aub), and 3'-end processing generates anti-sense piRNAs, completing the amplification cycle(Khurana, J S at al., 2010).

MILI (PIWI-like protein 2) and MIWI2 (PIWI-like protein 4) are two PIWI proteins that are engaged in ping-pong cycle in the mouse primordial germ cells of 16.5 dpc testes (Aravin et al 2008) , where MIWI2 piRNAs are produced in the presence of MILI (located in pi-bodies). However, MIWI2 (located in cellular bodies) expression can be detected at 4 dpp (Kuramochi-Miyagawa, S at al., 2008). Furthermore, high production of piRNA occurs at 14 dpp (Beyret, E at al., 2012, Lau, N C at al., 2006). Primary piRNAs in mouse prospermatogonia are processed from TEs and cluster transcripts, associated with MILI. MILI, loaded with sense piRNA, cleaves antisense transcripts, generating 5′ ends of secondary piRNAs, which are then transferred into MIWI2. MIWI2 subsequently cleaves sense transcripts (producing new sense piRNAs) that are loaded into MILI (Beyret, E at al., 2012).

Dicer protein in Humans

Dicer protein is an endoribonuclease from the RNase III family. This protein is responsible for generating short, non-coding interfering RNAs and microRNAs duplexes from dsRNA fragments about 20–25 bp long, with a two-base overhang at the 3′ end, which leads to gene silencing (Koscianska, E at al., 2011, Macrae, I J at al., 2006). In human cells, Dicer acts with protein partners such as members of the AGO family (MacRae, I J at al., 2008), a protein activator of PKR (PACT) (Kok, K H at al., 2007), HIV-1 TAR RNA-binding protein (TRBP) (Sanghvi, V R at al., 2011), and possibly other accessory proteins. Human Dicer contains an N-terminal DExH-box RNA helicase-like domain, originally termed the domain of unknown function (DUF283), a PAZ domain, two RNase III domains (type A and B), and a double-stranded RNA-binding domain (dsRBD) (MacRae, I J at al., 2008).

Dicer protein contains two different RNA-binding sites including: (i) a position for cleavage of long dsRNA, and (ii) a position for the rejoining of cleaved siRNA after cleavage via TRBP (Meister et al 2013). The RNase III type A domain cleaves the 3′-arm of pre-miRNA and the RNase III

type B domain cleaves its 5′-arm, and both domains must exhibit their activity to generate a miRNA-miRNA* duplex. (Zhang, H at al., 2004). In humans, Dicers interact with TRBP proteins. These partner proteins could be involved in determining the substrate specificity of Dicer proteins (Cenik, E S at al., 2011).

RNA-induced silencing complex (RISC)

RNA-induced silencing complex (RISC) is a multiprotein complex that incorporates one strand of siRNA or miRNA. RISC uses the siRNA or miRNA strand as a template to recognize complementary mRNA. When it finds a complementary strand, it activates RNase and cleaves the mRNA. The cytoplasmic RISC contains dsRNA binding proteins, including: (i) protein kinase RNA activator (PACT), or eukaryotic translation initiation factor 2-alpha kinase 2 (EIF2AK2), which is an enzyme that is encoded by the *EIF2AK2* gene in humans (Feng, G S at al., 1992), (ii) transactivation response RNA binding protein (TRBP) that has a physiological role in spermatogenesis and growth control during development, and Dicer, which processes pre-microRNAs into mature microRNAs (miRNAs) targeting specific mRNA species for regulation (Koscianska, E at al., 2011, Redfern, A D at al., 2013). Staphylococcal nuclease domain containing 1 (SND1) or p100 co-activator, is a multifunctional protein. In the cytoplasm, SND1 functions as a nuclease in the RNA-induced silencing complex (RISC) and facilitates RNAi-mediated gene silencing (Caudy, A A at al., 2003). Argonaute proteins are the catalytic components of the RISC, which bind to different classes of small non-coding RNAs, including siRNAs, miRNAs, and piRNAs (Ghildiyal, M at al., 2009).

Argonaute protein subfamilies

Argonaute proteins are key players in gene-silencing pathways guided by small RNAs. They were first identified in plants and their family members were subsequently found in all eukaryotes. They are highly conserved between species (Drinnenberg, I A at al., 2009). Argonaute proteins typically have a molecular weight of ~100 kDa and are highly specialized. These proteins contain amino-terminal (N) PAZ (PIWI–ARGONAUTE–ZWILLE) (also common in Dicer enzymes), MID (middle), and carboxy-terminal PIWI domains. The PAZ domain forms a specific binding pocket for the 3′-protruding end of the small RNA with which it associates. The PAZ domain anchors the 3′ end of the small RNA by bending it into a specific binding pocket (Jinek, M at al., 2009, Meister, G at al., 2013).

Based on sequence homology, the Argonaute protein family can be divided into the Ago subfamily (resembles *Arabidopsis* AGO1) and the PIWI subfamily (related to the *Drosophila* PIWI protein) (Meister, G at al., 2013, Peters, L at al., 2007). AGO1, AGO2, AGO3, and AGO4 are members of the human Ago subfamily that associate with miRNAs and siRNAs (Ender, C at al., 2010). In humans, only AGO2 has been shown to cleave the phosphodiester bond of a target RNA (Meister, G at al., 2013). The structure of the PIWI domain is similar to bacterial RNase H, which has been shown to cleave the RNA strand of an RNA-DNA hybrid (Jinek, M;Doudna, J A at al., 2009).

Argonaute proteins bind different classes of small non-coding RNAs, including siRNAs, miRNAs, and piRNAs (Jinek, M;Doudna, J A at al., 2009).http://en.wikipedia.org/wiki/Argonaute - cite_note-Zamore-2 The degree of complementarity between the small RNA and the target mRNA is the key for the regulatory mechanism of RNA silencing. Perfect complementarity promotes AGO2-mediated endonucleolytic cleavage, whereas mismatches in the middle region of the small RNA lead to repression of gene expression at the translational level (Filipowicz, W at al., 2008, Pillai, R S at al., 2007).

GW182 protein was identified in human cells as the antigen that is recognized by the serum from a patient suffering from motor and sensory neuropathy (Eystathioy, T at al., 2002). In animal cells, GW182 family proteins are essential for microRNA-mediated gene silencing through direct interactions with Argonaute proteins. Studies show that GW182 proteins interact with the multiprotein complex, including the cytoplasmic poly (A)-binding protein (PABP), CCR4-NOT deadenylase complexes, and PAN2-PAN3, which silences miRNA targets (Eulalio, A at al., 2009, Huntzinger, E at al., 2013). There are two domains in the GW182 protein that play essential roles in silencing. One is the N-terminal domain, which provides multiple binding sites for Argonaute proteins and contributes to the assembly of silencing complexes in the *D. melanogaster* protein. The other is a bipartite C-terminal silencing domain that promotes translational repression and decay of mRNA targets (Eulalio, A at al., 2009). Some GW motifs, known as Ago ‘hooks’, form Ago-interaction platforms connecting with the MID domain of Ago proteins. GW-repeats outside of the N-terminal region do not contribute to Argonaute binding (Eulalio, A at al., 2009, Takimoto, K at al., 2009, Zipprich, J T at al., 2009). A phylogenic analysis of Argonautes from *Homo sapiens* (Hs), *C. elegans* (Ce), C. *briggsae* (Cb), *Drosophila melanogaster* (Dm), *Arabidopsis thaliana* (At), and *Schizosaccharomyces pombe* (Sp) showed that there are three distinct clades within the Argonaute family, including: Ago-like, Piwi-like, and Wago. The AGO and the PIWI subfamilies apply different mechanisms for small RNA loading (Czech, B at al., 2011). Humans have eight Argonaute-like proteins, four within the eIF2C/AGO subfamily (EIF2C1/hAGO1, EIF2C2/hAGO2, EIF2C3/hAGO3, and EIF2C4/hAGO4) and four within the PIWI subfamily (PIWIL1/HIWI, PIWIL2/HILI, PIWIL3, and PIWIL4/HIWI2) (Li, L at al., 2010, Sasaki, T at al., 2003).

The Ago-like subfamily proteins were defined based on their similarity to Ago1, which are present in animals, plants, and fission yeast, with genes expressed ubiquitously (Joshua-Tor, L at al., 2006, Kawaoka, S at al., 2008). In mammals, miRNAs usually bind to partially complementary sites. AGO clade proteins preferentially use a portion of the guide to identify targets from this region, known as the “seed sequence” of the miRNA that encompasses two to eight nucleotides of the small RNA (Braun, J E at al., 2013).

The Piwi subfamily is an animal-specific clade. In contrast to the AGO subfamily, the PIWI subfamily has been identified only in animals and the genes are expressed mainly in germ cells, where they form the core of animal transposon silencing pathways (Aravin, A A at al., 2007, Kawaoka, S at al., 2008, Reddien, P W at al., 2005). The human genome encodes four Piwi proteins, including HIWI, HILI, HIWI3, and HIWI2. In mice, there are three Piwi proteins, known as MIWI, MILI, and MIWI2 (Ender, C;Meister, G at al., 2010, Peters, L;Meister, G at al., 2007).

The Wago clade is entirely constituted of worm Argonautes. In the nematode *C. elegans*, WAGO proteins are important for endogenous and exogenous RNAi pathways (Yigit, E at al., 2006). The Wago proteins are generally occupied by "secondary siRNAs", which bear 5′ triphosphate and 3′OH termini, and they are paramount for silencing responses to exogenously delivered dsRNAs, small RNA-based regulation of endogenous genes, and chromosome structure and segregation (Conine, C C at al., 2010, Tabara, H at al., 1999, Yigit, E at al., 2006).

Loading AGO clade members:

Recent biochemical studies described the loading events of AGO clade in humans (Noland, C L at al., 2011). First, it was confirmed that human TRBP functions similar to R2D2 in *D. melanogaster* and helps Dicer rebind the cleaved siRNA after the first cleavage. Double-stranded siRNA or miRNA transfer to the AGO protein through mediation by multi-proteins, including: chaperone complex Hsp70–Hsp90 (heat shock cognate protein 70 kDa–heat shock protein 90) in *D. melanogaster,* or Hsp90 in plants and humans; these complexes hydrolyze ATP to keep AGO proteins in an open state, allowing them to accommodate the RNA duplex (Iki, T at al., 2010, Johnston, M at al., 2010). In miRNA and the siRNA pathways, the strand with the less stable paired 5′ end is preferentially loaded into AGO proteins. The principle governing these thermodynamic differences between small RNA ends is known as the asymmetry rule (Khvorova, A at al., 2003, Schwarz, D S at al., 2003).

In the next step, with the support of the N domain of AGO proteins, the duplex is unwound, and the passenger strand of the siRNA or the miRNA* strand of the miRNA is removed (Kwak, P B at al., 2012). A study showed that human putative helicases, such as RNA helicase A (RHA), MOV10, or Armitage, are involved in duplex unwinding (Robb, G B at al., 2007). Next, perfectly paired siRNA or miRNA duplexes are cleaved by catalytically active AGO2 proteins (Leuschner, P J at al., 2006). In humans, the passenger strand nicked by AGO2 is removed from the RISC-loading complex by the endonuclease C3PO. The interaction between C3PO and AGO2 activated RISC then stops, enabling the latter to fully cleave complementary target RNAs (Liu, Y at al., 2009, Ye, X at al., 2011).

Loading PIWI clade members:

The piRNA-loading mechanisms are still largely unknown. piRNAs are associated with members of the PIWI clade (Das, P P at al., 2008), whereas miRNAs are associated with Argonautes from the AGO clade (Czech, B;Hannon, G J at al., 2011). In eukaryotes, Argonaute proteins have been found in high concentrations in processing bodies (P-bodies) of the cell's cytoplasm and participate in mRNA decay pathways (Parker, R at al., 2007, Sen, G L at al., 2005). In humans, the Argonaute-like and PIWI-like proteins are required for the miRNA and PIWI-interacting RNA (piRNA) pathways (Hutvagner, G at al., 2008). The Armitage ortholog in mice (MOV10L1) is a germ cell–specific putative RNA helicase associated with PIWI proteins. The detailed mechanism is still unclear (Zheng, K at al., 2010).

Therapeutic applications of RNAi

Synthetic siRNAs are new tools towards nucleic acid therapeutics for treatments of various infectious and genetic diseases, including cancer. RNAi, like other forms of gene therapy in systemic diseases, is challenged by the method of delivery and stability *in vivo* (Hammond, S M at al., 2000). Numerous anticancer gene therapy strategies had proven their efficacy in experimental animal models and some of them have already been tested in clinical trials (Azzam, T at al., 2004).

siRNA technology enables selective gene knockdown which is used to identify critical genes or pathways. This outcome provides a new insight into innovation in drug development for the treatment of various diseases and cancers (Gewirtz, A M at al., 2007). In oncology, the applications of RNAi have been focused on amplifying and translocating dominant mutant oncogenes and targeting viral oncogenes (Chen, J at al., 2004). siRNA technology has been applied to DNA repair through knockdown of the Ataxia-Telangiectasia and Rad3-related interacting protein (ATRIP). Three different siRNAs, targeting different regions of the ATRIP mRNA, were used for gene silencing. The results showed that ATRIP were phosphorylated by Ataxia-Telangiectasia and Rad3-related protein kinase (ATR) and was an essential component of the DNA damage checkpoint pathway (Cortez, D at al., 2001).

Researchers have demonstrated that many oncogenes are potential molecular targets for siRNAs. For example, one of the normal cellular genes, with the potential to enhance cell growth and tumor formation, is cellular oncogene (c-onc). Many proteins, such as signal transducers and transcription factors associated with tumor initiation and progression, and growth factors and their receptors, are encoded by c-onc. C-onc siRNAs/cationic lipid complexes have been transfected into different types of cells such as lung adenocarcinoma, HeLa, ovarian carcinoma, melanoma, and hepatoma cells. The results demonstrated that siRNAs suppressed the proliferation of these cells (Yin, J Q at al., 2003).

Over-expression of CXCR4 was associated with lymph node metastases in breast cancer, oral squamous cell carcinoma, and other cancers (Kato, M at al., 2003). It was shown that silencing of CXCR4 in MDA-MB-231 human breast cancer cells resulted in significant inhibition of *in vitro* breast cancer cell migration (Chen, Y at al., 2003, Schimanski, C C at al., 2005). Shimanski *et al*. also reported that expression of CXCR4 and CCR7 were elevated in 96 patients with histologically confirmed colorectal cancers and in four colorectal cancer cell lines by immunohistochemical staining. Their results showed that strong expression of CXCR4 in colorectal cancer cells was

significantly associated with lymphatic and distant dissemination in patients with colorectal cancer and a migratory phenotype *in vitro* (Schimanski, C C at al., 2005).

Researchers demonstrated that inhibiting the signaling by *CXCR4* and its ligand, *CXCL12,* either by antibodies, peptide analogs, or siRNA knockdown, reduced metastasis in various metastatic models of breast and colorectal cancer (Abedini, F at al., 2011, Abedini, F at al., 2012, Abedini, F at al., 2011, Liang, Z at al., 2005).

Tabernero et al. initiated the first Phase I trial utilizing ALN-VSP, a lipid nanoparticle (LNP) formulation of siRNAs targeting vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP), on 41 patients with endometrial cancer metastases to the liver. Their results demonstrated clinical benefits with the cancer either stabilized after six months or regresses metastasis in some patients. Pharmacodynamic analysis of biopsy samples from the patients revealed the presence of drug in tumor biopsies, siRNA-mediated mRNA cleavage in the liver and antitumor activity including complete regression of liver metastases in endometrial cancer. For the first time, their research showed that RNAi is effective in the treatment of cancer patients (Tabernero, J at al., 2013).

Another phase I clinical trial involving systemic administration of siRNA was conducted by Davis *et al*. on patients with solid cancers (Melanoma). They generated nanoparticles comprising a synthetic delivery system containing a linear cyclodextrin-based polymer (CDP), a human transferrin protein (TF), polyethylene glycol (PEG), and an siRNA targeting *RRM2*. They observed a reduction in both the specific mRNA (M2 subunit of ribonucleotide reductase (RRM2)) and the RRM2 protein levels as compared with those in the pre-dosed tissue (Davis, M E at al., 2010).

The role of piRNAs in cancer is just beginning to be investigated. Piwi family members are commonly expressed in germline stem cells and lose their expression during development. Researchers have found that human Piwi orthologs known as Hiwi and Hili can be overexpressed in a variety of human cancers (Sun, G at al., 2011, Taubert, H at al., 2007). Hiwi plays an important role in tumorigenesis by increasing DNA methylation levels and consequently, silencing the cyclin-dependent kinase inhibitor (CDKI). Furthermore, treatment with a DNA methyltransferase inhibitor could reduce DNA methylation levels and tumor formation (Siddiqi, S at al., 2012).

The RNAi pathway is present in all mammalian cell types. The primary challenge for effective systemic gene silencing is delivery of the siRNA or miRNA to the appropriate organ(s) with productive cellular uptake leading to engagement of RISC in the cytoplasm (Gollob, J A at al., 2011). Polymers such as dextran-spermine and lipid nanoparticles have proven effective in delivery of siRNAs to the colon, liver, and lung, in animals (Abedini, F at al., 2011, Abedini, F at al., 2012, Tabernero, J at al., 2013).

Figures and Legends

Figure 1: Sources of siRNA. Different sources of processing siRNA by Dicer into siRNAs. An siRNA consists of a guide strand (red), which assembles into functional siRISC, and a passenger strand (blue), which is ejected and degraded (Carthew, R W;Sontheimer, E J at al., 2009).

Figure 2: The miRNA processing pathway. In the nucleus, the production of the primary miRNA transcript (pri-miRNA) by RNA polymerase II or III and cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8. Result, produced precursor hairpin (the pre-miRNA) that is exported from the nucleus by Exportin-5–Ran-GTP. Then, in the cytoplasm, the RNase Dicer in complex with the dsRNA-binding protein TRBP cleaves the pre-miRNA hairpin to its mature length. The functional strand of the mature miRNA is loaded together with Argonaute (Ago2) proteins into the RISC, where it guides RISC to silence target mRNAs through mRNA cleavage, translational repression, whereas the passenger strand (black) is degraded(Winter, J at al., 2009).

Figure 3: Ping-pong amplification in the germline. Transcripts from piRNA clusters (blue and red) and functional transposons (blue) are exported from the nucleus. Aubergine (Aub), preprogrammed with piRNAs generated through the primary biogenesis pathway, cleaves complementary transposon and cluster transcripts (blue), yielding randomly sized RNA fragments that bind to Ago3. 3'-end trimming produces mature Ago3-sense strand piRNA complexes, which cleave anti-sense cluster transcripts (red). The resulting fragments bind to Aubergine (Aub), and 3'-end processing generates anti-sense piRNAs, completing the amplification cycle(Khurana, J S;Theurkauf, W at al., 2010).

VIII. References

Abedini F, Hosseinkhani H, Ismail M, Chen Y R, Omar A, PeiPei C ,Domb A J **(2011)** In vitro intracellular trafficking of biodegradable nanoparticles dextran-spermine in cancer cell lines. **Int. Journal of Nanotechnology**, 712-723.

Abedini F, Hosseinkhani H, Ismail M, Domb A J, Omar A R, Chong P P, Hong P D, Yu D S ,Farber I Y **(2012)** Cationized dextran nanoparticle-encapsulated cxcr4-sirna enhanced correlation between cxcr4 expression and serum alkaline phosphatase in a mouse model of colorectal cancer. **Int J Nanomedicine,** 4159-68.

Abedini F, Ismail M, Hosseinkhani H, Ibrahim T A, Omar A R, Chong P P, Bejo M H ,Domb A J **(2011)** Effects of cxcr4 sirna/dextran-spermine nanoparticles on cxcr4 expression and serum ldh levels in a mouse model of colorectal cancer metastasis to the liver. **Cancer Manag Res**, 301-9.

Alvarado V ,Scholthof H B **(2009)** Plant responses against invasive nucleic acids: Rna silencing and its suppression by plant viral pathogens. Semin Cell Dev Biol, 1032-40.

Aravin A A, Hannon G J ,Brennecke J (2007) The piwi-pirna pathway provides an adaptive defense in the transposon arms race. **Science**, 761-4.

Azzam T, Eliyahu H, Makovitzki A, Linial M ,Domb A J **(2004)** Hydrophobized dextran-spermine conjugate as potential vector for in vitro gene transfection. J Control Release, 309-23.

Bamezai S, Rawat V P ,Buske C **(2012)** Concise review: The piwi-pirna axis: Pivotal beyond transposon silencing. **Stem Cells,** 2603-11.

Bantounas I, Phylactou L A ,Uney J B **(2004)** Rna interference and the use of small interfering rna to study gene function in mammalian systems. **J Mol Endocrinol**, 545-57.

Bennasser Y ,Jeang K T **(2006)** Hiv-1 tat interaction with dicer: Requirement for rna. **Retrovirology,** 95.

Beyret E, Liu N ,Lin H **(2012)** Pirna biogenesis during adult spermatogenesis in mice is independent of the ping-pong mechanism. **Cell Res**, 1429-39.

Billy E, Brondani V, Zhang H, Muller U ,Filipowicz W **(2001)** Specific interference with gene expression induced by long, double-stranded rna in mouse embryonal teratocarcinoma cell lines. **Proc Natl Acad Sci U S A,** 14428-33.

Bivalkar-Mehla S, Vakharia J, Mehla R, Abreha M, Kanwar J R, Tikoo A ,Chauhan A **(2011)** Viral rna silencing suppressors (rss): Novel strategy of viruses to ablate the host rna interference (rnai) defense system. **Virus Res,** 1-9.

Braun J E, Huntzinger E ,Izaurralde E **(2013)** The role of gw182 proteins in mirna-mediated gene silencing. **Adv Exp Med Biol,** 147-63.

Brennecke J, Aravin A A, Stark A, Dus M, Kellis M, Sachidanandam R ,Hannon G J **(2007)** Discrete small rna-generating loci as master regulators of transposon activity in drosophila. **Cell,** 1089-103.

Cannell I G, Kong Y W ,Bushell M (2008) How do micrornas regulate gene expression? Biochem Soc Trans, 1224-31.

Carthew R W ,Sontheimer E J **(2009)** Origins and mechanisms of mirnas and sirnas. Cell, 642-55.

Castel S E ,Martienssen R A (2013) Rna interference in the nucleus: Roles for small rnas in transcription, epigenetics and beyond. **Nat Rev Genet,** 100-12.

Caudy A A, Ketting R F, Hammond S M, Denli A M, Bathoorn A M, Tops B B, Silva J M, Myers M M, Hannon G J ,Plasterk R H **(2003)** A micrococcal nuclease homologue in rnai effector complexes. **Nature,** 411-4.

Cecere G, Zheng G X, Mansisidor A R, Klymko K E ,Grishok A **(2012)** Promoters recognized by forkhead proteins exist for individual 21u-rnas. **Mol Cell,** 734-45.

Cenik E S ,Zamore P D **(2011)** Argonaute proteins. **Curr Biol,** R446-9.

Cerutti H ,Casas-Mollano J A **(2006)** On the origin and functions of rna-mediated silencing: From protists to man. **Curr Genet,** 81-99.

Chen J, Wall N R, Kocher K, Duclos N, Fabbro D, Neuberg D, Griffin J D, Shi Y ,Gilliland D G **(2004)** Stable expression of small interfering rna sensitizes tel-pdgfbetar to inhibition with imatinib or rapamycin. **J Clin Invest,** 1784-91.

Chen Y, Stamatoyannopoulos G ,Song C Z **(2003)** Down-regulation of cxcr4 by inducible small interfering rna inhibits breast cancer cell invasion in vitro. **Cancer Res**, 4801-4.

Chiu Y L ,Rana T M **(2002)** Rnai in human cells: Basic structural and functional features of small interfering rna. **Mol Cell,** 549-61.

Cogoni C ,Macino G **(1999)** Gene silencing in neurospora crassa requires a protein homologous to rna-dependent rna polymerase. **Nature**, 166-9.

Cogoni C ,Macino G **(2000)** Post-transcriptional gene silencing across kingdoms. **Curr Opin Genet Dev**, 638-43.

Conine C C, Batista P J, Gu W, Claycomb J M, Chaves D A, Shirayama M ,Mello C C **(2010)** Argonautes alg-3 and alg-4 are required for spermatogenesis-specific 26g-rnas and thermotolerant sperm in caenorhabditis elegans. **Proc Natl Acad Sci U S A,** 3588-93.

Cortez D, Guntuku S, Qin J ,Elledge S J **(2001)** Atr and atrip: Partners in checkpoint signaling. **Science,** 1713-6.

Czech B ,Hannon G J **(2011)** Small rna sorting: Matchmaking for argonautes. Nat Rev Genet, 19-31.

Dalmay T, Hamilton A, Rudd S, Angell S ,Baulcombe D C **(2000)** An rna-dependent rna polymerase gene in arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. **Cell,** 543-53.

Das P P, Bagijn M P, Goldstein L D, Woolford J R, Lehrbach N J, Sapetschnig A, Buhecha H R, Gilchrist M J, Howe K L, Stark R, Matthews N, Berezikov E, Ketting R F, Tavare S ,Miska E A **(2008)** Piwi and pirnas act upstream of an endogenous sirna pathway to suppress tc3 transposon mobility in the caenorhabditis elegans germline. **Mol Cell,** 79-90.

Davis M E, Zuckerman J E, Choi C H, Seligson D, Tolcher A, Alabi C A, Yen Y, Heidel J D ,Ribas A **(2010)** Evidence of rnai in humans from systemically administered sirna via targeted nanoparticles. **Nature,** 1067-70.

Ding S W ,Voinnet O (2**007)** Antiviral immunity directed by small rnas. **Cell,** 413-26.

Drinnenberg I A, Weinberg D E, Xie K T, Mower J P, Wolfe K H, Fink G R ,Bartel D P **(2009)** Rnai in budding yeast. **Science**, 544-50.

Elbashir S M, Lendeckel W ,Tuschl T (2001) Rna interference is mediated by 21- and 22-nucleotide rnas. **Genes Dev**, 188-200.

Ender C ,Meister G **(2010)** Argonaute proteins at a glance. **J Cell Sci,** 1819-23.

Eulalio A, Tritschler F ,Izaurralde E **(2009)** The gw182 protein family in animal cells: New insights into domains required for mirna-mediated gene silencing. **Rna**, 1433-42.

Eystathioy T, Chan E K, Tenenbaum S A, Keene J D, Griffith K ,Fritzler M J **(2002)** A phosphorylated cytoplasmic autoantigen, gw182, associates with a unique population of human mrnas within novel cytoplasmic speckles. **Mol Biol Cell**, 1338-51.

Feng G S, Chong K, Kumar A ,Williams B R **(1992)** Identification of double-stranded rna-binding domains in the interferon-induced double-stranded rna-activated p68 kinase. **Proc Natl Acad Sci U S A,** 5447-51.

Filipowicz W, Bhattacharyya S N ,Sonenberg N **(2008)** Mechanisms of post-transcriptional regulation by micrornas: Are the answers in sight? **Nat Rev Genet,** 102-14.

Friedman R C, Farh K K, Burge C B ,Bartel D P **(2009)** Most mammalian mrnas are conserved targets of micrornas. **Genome Res**, 92-105.

Gewirtz A M **(2007)** On future's doorstep: Rna interference and the pharmacopeia of tomorrow. **J Clin Invest**, 3612-4.

Ghildiyal M ,Zamore P D **(2009)** Small silencing rnas: An expanding universe. Nat Rev Genet, 94-108.

Gollob J A, Bumcrot D, Toudjarska I, Bastian B C ,Sah D W (2011) Progress in the delivery of sirna therapeutics: Potential in uveal melanoma. **Drugs of the Future**, 229.

Gunawardane L S, Saito K, Nishida K M, Miyoshi K, Kawamura Y, Nagami T, Siomi H ,Siomi M C **(2007)** A slicer-mediated mechanism for repeat-associated sirna 5' end formation in drosophila. **Science,** 1587-90.

Gurtan A M ,Sharp P A **(2013)** The role of mirnas in regulating gene expression networks. **J Mol Biol**, 3582-600.

Hamilton A, Voinnet O, Chappell L ,Baulcombe D **(2002)** Two classes of short interfering rna in rna silencing. **Embo j**, 4671-9.

Hammond S M, Bernstein E, Beach D ,Hannon G J **(2000)** An rna-directed nuclease mediates post-transcriptional gene silencing in drosophila cells. **Nature**, 293-6.

Harada H, Willison K, Sakakibara J, Miyamoto M, Fujita T ,Taniguchi T **(1990)** Absence of the type i ifn system in ec cells: Transcriptional activator (irf-1) and repressor (irf-2) genes are developmentally regulated. **Cell**, 303-12.

Hatada E, Saito S, Okishio N ,Fukuda R **(1997)** Binding of the influenza virus ns1 protein to model genome rnas. **J Gen Virol,** 1059-63.

Houwing S, Kamminga L M, Berezikov E, Cronembold D, Girard A, van den Elst H, Filippov D V, Blaser H, Raz E, Moens C B, Plasterk R H, Hannon G J, Draper B W ,Ketting R F **(2007)** A role for piwi and pirnas in germ cell maintenance and transposon silencing in zebrafish. **Cell,** 69-82.

Huntzinger E, Kuzuoglu-Ozturk D, Braun J E, Eulalio A, Wohlbold L ,Izaurralde E **(2013)** The interactions of gw182 proteins with pabp and deadenylases are required for both translational repression and degradation of mirna targets. **Nucleic Acids Res,** 978-94.

Hutvagner G ,Simard M J **(2008)** Argonaute proteins: Key players in rna silencing. **Nat Rev Mol Cell Biol,** 22-32.

Hutvagner G ,Zamore P D **(2002)** A microrna in a multiple-turnover rnai enzyme complex. **Science**, 2056-60.

Iki T, Yoshikawa M, Nishikiori M, Jaudal M C, Matsumoto-Yokoyama E, Mitsuhara I, Meshi T ,Ishikawa M **(2010)** In vitro assembly of plant rna-induced silencing complexes facilitated by molecular chaperone hsp90. **Mol Cell,** 282-91.

Jinek M ,Doudna J A **(2009)** A three-dimensional view of the molecular machinery of rna interference. **Nature**, 405-12.

Johnston M, Geoffroy M C, Sobala A, Hay R ,Hutvagner G **(2010)** Hsp90 protein stabilizes unloaded argonaute complexes and microscopic p-bodies in human cells. **Mol Biol Cell,** 1462-9.

Joshua-Tor L **(2006)** The argonautes. **Cold Spring Harb Symp Quant Biol,** 67-72.

Kato M, Kitayama J, Kazama S ,Nagawa H **(2003)** Expression pattern of cxc chemokine receptor-4 is correlated with lymph node metastasis in human invasive ductal carcinoma. **Breast Cancer Res**, R144-50.

Kawaoka S, Hara K, Shoji K, Kobayashi M, Shimada T, Sugano S, Tomari Y, Suzuki Y ,Katsuma S **(2013)** The comprehensive epigenome map of pirna clusters. **Nucleic Acids Res**, 1581-90.

Kawaoka S, Minami K, Katsuma S, Mita K ,Shimada T **(2008)** Developmentally synchronized expression of two bombyx mori piwi subfamily genes, siwi and bmago3 in germ-line cells. **Biochem Biophys Res Commun**, 755-60.

Khurana J S ,Theurkauf W **(2010)** Pirnas, transposon silencing, and drosophila germline development. **J Cell Biol,** 905-13.

Khvorova A, Reynolds A ,Jayasena S D **(2003)** Functional sirnas and mirnas exhibit strand bias. Cell, 209-16.

Kim K, Lee Y S ,Carthew R W **(2007)** Conversion of pre-risc to holo-risc by ago2 during assembly of rnai complexes. **Rna**, 22-9.

Kok K H, Ng M H, Ching Y P ,Jin D Y **(2007)** Human trbp and pact directly interact with each other and associate with dicer to facilitate the production of small interfering rna. **J Biol Chem**, 17649-57.

Koscianska E, Starega-Roslan J ,Krzyzosiak W J **(2011)** The role of dicer protein partners in the processing of microrna precursors. **PLoS One,** e28548.

Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, Asada N, Kojima K, Yamaguchi Y, Ijiri T W, Hata K, Li E, Matsuda Y, Kimura T, Okabe M, Sakaki Y, Sasaki H ,Nakano T **(2008)** DNA methylation of retrotransposon genes is regulated by piwi family members mili and miwi2 in murine fetal testes. **Genes Dev**, 908-17.

Kwak P B ,Tomari Y **(2012)** The n domain of argonaute drives duplex unwinding during risc assembly. **Nat Struct Mol Biol**, 145-51.

Lau N C, Seto A G, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel D P ,Kingston R E **(2006)** Characterization of the pirna complex from rat testes. **Science**, 363-7.

Lee R C ,Ambros V **(2001)** An extensive class of small rnas in caenorhabditis elegans. **Science**, 862-4.

Lee R C, Feinbaum R L ,Ambros V **(1993)** The c. Elegans heterochronic gene lin-4 encodes small rnas with antisense complementarity to lin-14. **Cell,** 843-54.

Lee Y, Kim M, Han J, Yeom K H, Lee S, Baek S H ,Kim V N **(2004)** Microrna genes are transcribed by rna polymerase ii. **Embo j**, 4051-60.

Leuschner P J, Ameres S L, Kueng S ,Martinez J **(2006)** Cleavage of the sirna passenger strand during risc assembly in human cells. **EMBO Rep**, 314-20.

Li L, Yu C, Gao H ,Li Y (2010) Argonaute proteins: Potential biomarkers for human colon cancer. **BMC Cancer,** 38.

Liang Z, Yoon Y, Votaw J, Goodman M M, Williams L ,Shim H **(2005)** Silencing of cxcr4 blocks breast cancer metastasis. **Cancer Res**, 967-71.

Lipardi C, Wei Q ,Paterson B M **(2001)** Rnai as random degradative pcr: Sirna primers convert mrna into dsrnas that are degraded to generate new sirnas. **Cell**, 297-307.

Liu Y, Ye X, Jiang F, Liang C, Chen D, Peng J, Kinch L N, Grishin N V ,Liu Q **(2009)** C3po, an endoribonuclease that promotes rnai by facilitating risc activation. **Science**, 750-3.

Llave C, Kasschau K D, Rector M A ,Carrington J C **(2002)** Endogenous and silencing-associated small rnas in plants. **Plant Cell**, 1605-19.

MacRae I J, Ma E, Zhou M, Robinson C V ,Doudna J A **(2008)** In vitro reconstitution of the human risc-loading complex. **Proc Natl Acad Sci U S A**, 512-7.

Macrae I J, Zhou K, Li F, Repic A, Brooks A N, Cande W Z, Adams P D ,Doudna J A **(2006)** Structural basis for double-stranded rna processing by dicer. **Science**, 195-8.

Malone C D ,Hannon G J **(2009)** Molecular evolution of pirna and transposon control pathways in drosophila. **Cold Spring Harb Symp Quant Biol,** 225-34.

Marques F Z ,Morris B J **(2012)** Neurogenic hypertension: Revelations from genome-wide gene expression profiling. **Curr Hypertens Rep,** 485-91.

Marques J T, Kim K, Wu P H, Alleyne T M, Jafari N ,Carthew R W **(2010)** Loqs and r2d2 act sequentially in the sirna pathway in drosophila. **Nat Struct Mol Biol,** 24-30.

McKenna S A, Kim I, Liu C W ,Puglisi J D (2006) Uncoupling of rna binding and pkr kinase activation by viral inhibitor rnas. **J Mol Biol,** 1270-85.

Meister G **(2013)** Argonaute proteins: Functional insights and emerging roles. **Nat Rev Genet,** 447-59.

Mikiko C S, Kaoru S, Dubravka P ,A. A A **(2011)** Piwi-interacting small rnas: The vanguard of genome defence. **Nature Reviews Molecular Cell Biology** 246-258.

Mochizuki K, Fine N A, Fujisawa T ,Gorovsky M A **(2002)** Analysis of a piwi-related gene implicates small rnas in genome rearrangement in tetrahymena. **Cell,** 689-99.

Morris B J **(2012)** Review of non-coding rnas and the epigenetic regulation of gene expression. **Epigenetics.** , 664-666.

Noland C L, Ma E ,Doudna J A **(2011)** Sirna repositioning for guide strand selection by human dicer complexes. **Mol Cell,** 110-21.

Okamura K, Balla S, Martin R, Liu N ,Lai E C **(2008)** Two distinct mechanisms generate endogenous sirnas from bidirectional transcription in drosophila melanogaster. **Nat Struct Mol Biol,** 581-90.

Parker R ,Sheth U **(2007)** P bodies and the control of mrna translation and degradation. **Mol Cell,** 635-46.

Pasquinelli A E, Reinhart B J, Slack F, Martindale M Q, Kuroda M I, Maller B, Hayward D C, Ball E E, Degnan B, Muller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E ,Ruvkun G **(2000)** Conservation of the sequence and temporal expression of let-7 heterochronic regulatory rna. **Nature**, 86-9.

Peters L ,Meister G (2007) Argonaute proteins: Mediators of rna silencing. Mol Cell, 611-23.

Pillai R S, Bhattacharyya S N ,Filipowicz W **(2007)** Repression of protein synthesis by mirnas: How many mechanisms? **Trends Cell Biol**, 118-26.

Pritchard C C, Cheng H H ,Tewari M **(2012)** Microrna profiling: Approaches and considerations. **Nat Rev Genet,** 358-69.

Reddien P W, Oviedo N J, Jennings J R, Jenkin J C ,Sanchez Alvarado A **(2005)** Smedwi-2 is a piwi-like protein that regulates planarian stem cells. **Science**, 1327-30.

Redfern A D, Colley S M, Beveridge D J, Ikeda N, Epis M R, Li X, Foulds C E, Stuart L M, Barker A, Russell V J, Ramsay K, Kobelke S J, Li X, Hatchell E C, Payne C, Giles K M, Messineo A, Gatignol A, Lanz R B, O'Malley B W ,Leedman P J **(2013)** Rna-induced silencing complex (risc) proteins pact, trbp, and dicer are sra binding nuclear receptor coregulators. **Proc Natl Acad Sci U S A**, 6536-41.

Reinhart B J ,Bartel D P **(2002)** Small rnas correspond to centromere heterochromatic repeats. **Science**, 1831.

Robb G B ,Rana T M **(2007)** Rna helicase a interacts with risc in human cells and functions in risc loading. **Mol Cell,** 523-37.

Saito K, Sakaguchi Y, Suzuki T, Suzuki T, Siomi H ,Siomi M C **(2007)** Pimet, the drosophila homolog of hen1, mediates 2'-o-methylation of piwi- interacting rnas at their 3' ends. **Genes Dev,** 1603-8.

Sanghvi V R ,Stee L F **(2011)** The cellular tar rna binding protein, trbp, promotes hiv-1 replication primarily by inhibiting the activation of double-stranded rna-dependent kinase pkr▽. **J Virol.**, 12614-12621.

Sasaki T, Shiohama A, Minoshima S ,Shimizu N **(2003)** Identification of eight members of the argonaute family in the human genome. **Genomics**, 323-30.

Schimanski C C, Schwald S, Simiantonaki N, Jayasinghe C, Gonner U, Wilsberg V, Junginger T, Berger M R, Galle P R ,Moehler M **(2005)** Effect of chemokine receptors cxcr4 and ccr7 on the metastatic behavior of human colorectal cancer. **Clin Cancer Res**, 1743-50.

Schwarz D S, Hutvagner G, Du T, Xu Z, Aronin N ,Zamore P D **(2003)** Asymmetry in the assembly of the rnai enzyme complex. **Cell**, 199-208.

Sen G L ,Blau H M **(2005)** Argonaute 2/risc resides in sites of mammalian mrna decay known as cytoplasmic bodies. **Nat Cell Biol**, 633-6.

Shi Z, Montgomery T A, Qi Y ,Ruvkun G **(2013)** High-throughput sequencing reveals extraordinary fluidity of mirna, pirna, and sirna pathways in nematodes. **Genome Res**, 497-508.

Siddiqi S ,Matushansky I **(2012)** Piwis and piwi-interacting rnas in the epigenetics of cancer. **J Cell Biochem**, 373-80.

Sijen T, Fleenor J, Simmer F, Thijssen K L, Parrish S, Timmons L, Plasterk R H ,Fire A **(2001)** On the role of rna amplification in dsrna-triggered gene silencing. **Cell**, 465-76.

Sijen T ,Plasterk R H **(2003)** Transposon silencing in the caenorhabditis elegans germ line by natural rnai. **Nature**, 310-4.

Siomi M C, Sato K, Pezic D ,Aravin A A **(2011)** Piwi-interacting small rnas: The vanguard of genome defence. **Nat Rev Mol Cell Biol,** 246-58.

Song R, Hennig G W, Wu Q, Jose C, Zheng H ,Yan W **(2011)** Male germ cells express abundant endogenous sirnas. **Proc Natl Acad Sci U S A**, 13159-64.

Stark G R, Kerr I M, Williams B R, Silverman R H ,Schreiber R D **(1998)** How cells respond to interferons. **Annu Rev Biochem**, 227-64.

Sun G, Wang Y, Sun L, Luo H, Liu N, Fu Z ,You Y **(2011)** Clinical significance of hiwi gene expression in gliomas. **Brain Res,** 183-8.

Sunkar R, Girke T ,Zhu J K **(2005)** Identification and characterization of endogenous small interfering rnas from rice. **Nucleic Acids Res**, 4443-54.

Tabara H, Sarkissian M, Kelly W G, Fleenor J, Grishok A, Timmons L, Fire A ,Mello C C **(1999)** The rde-1 gene, rna interference, and transposon silencing in c. Elegans. **Cell,** 123-32.

Tabernero J, Shapiro G I, LoRusso P M, Cervantes A, Schwartz G K, Weiss G J, Paz-Ares L, Cho D C, Infante J R, Alsina M, Gounder M M, Falzone R, Harrop J, White A C, Toudjarska I, Bumcrot D, Meyers R E, Hinkle G, Svrzikapa N, Hutabarat R M, Clausen V A, Cehelsky J, Nochur S V, Gamba-Vitalo C, Vaishnaw A K, Sah D W, Gollob J A ,Burris H A, 3rd **(2013)** First-in-humans trial of an rna interference therapeutic targeting vegf and ksp in cancer patients with liver involvement. **Cancer Discov,** 406-17.

Takimoto K, Wakiyama M ,Yokoyama S **(2009)** Mammalian gw182 contains multiple argonaute-binding

sites and functions in microrna-mediated translational repression. Rna, 1078-89.

Tang G, Reinhart B J, Bartel D P ,Zamore P D **(2003)** A biochemical framework for rna silencing in plants. **Genes Dev**, 49-63.

Taubert H, Greither T, Kaushal D, Wurl P, Bache M, Bartel F, Kehlen A, Lautenschlager C, Harris L, Kraemer K, Meye A, Kappler M, Schmidt H, Holzhausen H J ,Hauptmann S **(2007)** Expression of the stem cell self-renewal gene hiwi and risk of tumour-related death in patients with soft-tissue sarcoma. **Oncogene**, 1098-100.

Wang Y, Kato N, Jazag A, Dharel N, Otsuka M, Taniguchi H, Kawabe T ,Omata M **(2006)** Hepatitis c virus core protein is a potent inhibitor of rna silencing-based antiviral response. **Gastroenterology,** 883-92.

Watanabe T, Totoki Y, Toyoda A, Kaneda M, Kuramochi-Miyagawa S, Obata Y, Chiba H, Kohara Y, Kono T, Nakano T, Surani M A, Sakaki Y ,Sasaki H **(2008)** Endogenous sirnas from naturally formed dsrnas regulate transcripts in mouse oocytes. **Nature**, 539-43.

Winter J, Jung S, Keller S, Gregory R I ,Diederichs S **(2009)** Many roads to maturity: Microrna biogenesis pathways and their regulation. **Nat Cell Biol**, 228-34.

Xia J, Joyce C E, Bowcock A M ,Zhang W **(2013)** Noncanonical micrornas and endogenous sirnas in normal and psoriatic human skin. **Hum Mol Genet**, 737-48.

Yasui K, Wakita T, Tsukiyama-Kohara K, Funahashi S I, Ichikawa M, Kajita T, Moradpour D, Wands J R ,Kohara M **(1998)** The native form and maturation process of hepatitis c virus core protein. **J Virol,** 6048-55.

Ye X, Huang N, Liu Y, Paroo Z, Huerta C, Li P, Chen S, Liu Q ,Zhang H **(2011)** Structure of c3po and mechanism of human risc activation. **Nat Struct Mol Biol,** 650-7.

Yeom K H, Lee Y, Han J, Suh M R ,Kim V N **(2006)** Characterization of dgcr8/pasha, the essential cofactor for drosha in primary mirna processing. **Nucleic Acids Res**, 4622-9.

Yigit E, Batista P J, Bei Y, Pang K M, Chen C C, Tolia N H, Joshua-Tor L, Mitani S, Simard M J ,Mello C C **(2006)** Analysis of the c. Elegans argonaute family reveals that distinct argonautes act sequentially during rnai. **Cell,** 747-57.

Yin J Q, Gao J, Shao R, Tian W N, Wang J ,Wan Y **(2003)** Sirna agents inhibit oncogene expression and attenuate human tumor cell growth. **J Exp Ther Oncol,** 194-204.

Zamore P D, Tuschl T, Sharp P A ,Bartel D P **(2000)** Rnai: Double-stranded rna directs the atp-dependent cleavage of mrna at 21 to 23 nucleotide intervals. **Cell,** 25-33.

Zhang H, Kolb F A, Jaskiewicz L, Westhof E ,Filipowicz W **(2004)** Single processing center models for human dicer and bacterial rnase iii. **Cell,** 57-68.

Zheng K, Xiol J, Reuter M, Eckardt S, Leu N A, McLaughlin K J, Stark A, Sachidanandam R, Pillai R S ,Wang P J **(2010)** Mouse mov10l1 associates with piwi proteins and is an essential component of the piwi-interacting rna (pirna) pathway. **Proc Natl Acad Sci U S A,** 11841-6.

Zipprich J T, Bhattacharyya S, Mathys H ,Filipowicz W **(2009)** Importance of the c-terminal domain of the human gw182 protein tnrc6c for translational repression. **Rna**, 781-93.