

The MicroRNA (miRNA): Overview of the RNA Genes that Modulate Gene Function

Shao-Yao Ying · Donald C. Chang ·
Shi-Lung Lin

Published online: 13 November 2007
© Humana Press Inc. 2007

Abstract MicroRNAs (miRNAs), widely distributed, small regulatory RNA genes, target both messenger RNA (mRNA) degradation and suppression of protein translation based on sequence complementarity between the miRNA and its targeted mRNA. Different names have been used to describe various types of miRNA. During evolution, RNA retroviruses or transgenes invaded the eukaryotic genome and inserted in the non-coding regions of DNA, conceivably acting as transposon-like jumping genes, providing defense from viral invasion and fine-tuning of gene expression as a secondary level of gene modulation in eukaryotes. When a transposon is inserted in the intron, it becomes an intronic miRNA, taking advantage of the protein synthesis machinery, i.e., mRNA transcription and splicing, as a means for processing and maturation. Recently, miRNAs have been found to play an important, but not life-threatening, role in embryonic development. They might play a pivotal role in diverse biological systems in various organisms, facilitating a quick response and accurate plotting of body physiology and structures. Based on these unique properties, man-made intronic miRNAs have been developed for in vitro evaluation of gene function, in vivo gene therapy and generation of transgenic animal models. The biogenesis and identification of miRNAs, potential applications, and future directions for research are presented, hopefully providing a guideline for further miRNA and gene function studies.

Keywords Small RNA · Non-coding RNAs · siRNA · miRNA · Intronic miRNA · Transposons · Biogenesis · Mechanism · Identification · Targeting · Fine-tuning · Gene function · Gene therapy · Anti-viral vaccine · Drug development · Future directions

Abbreviations

Dicer	RNaseIII-familial endoribonucleases
dsRNA	Double-stranded RNA
Id-miRNA	Intronic miRNA
miRNA	MicroRNA
ncRNA	Non-coding RNA
PTGS	Post-transcriptional gene silencing
RdRp	RNA-directed RNA polymerases
RISC	RNA-induced silencing complex
RNAi	RNA interference
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
smRNA	Small modulatory RNA
snoRNA	Small nucleolar RNA
stRNA	Small temporal RNA
tncRNA	Tiny non-coding RNA

Introduction

The microRNA (miRNA) is a form of small, single-stranded RNA, 18–25 nucleotides long. It is transcribed from DNA, instead of being translated into protein, and regulates the functions of other genes in protein synthesis. Therefore, miRNAs are genes that modulate other protein-coding genes.

Even after considering the thousands of new putative genes identified from sequencing of human genome as well

S.-Y. Ying (✉) · D. C. Chang · S.-L. Lin
Department of Cell and Neurobiology, Keck School of
Medicine, University of Southern California, 1333 San Pablo
Street, BMT-403, Los Angeles, CA 90033, USA
e-mail: sying@usc.edu

as the genes encoding tRNAs, ribosomal RNAs (rRNAs), and snoRNAs, still, nearly 95% of the genome is non-coding DNA, a percentage which varies from species to species. Changes in these sequences are frequently associated with clinical and circumstantial malfunction. Some of these non-coding sequences are responsible for RNA-mediated gene silencing through an RNA interference (RNAi)-like mechanism. One potentially important class of genes corresponding to RNAs that lack significant open reading frames and appear to encode RNA as their final product is the miRNAs. These miRNAs can play critical roles in development, protein secretion, and gene regulation. Some of them are naturally occurring antisense RNAs, whereas others have more complex structures. To understand the diseases caused by dysregulation of these miRNAs, a tissue-specific expression system is needed to recreate the function and mechanism of individual miRNA *in vitro* and *in vivo*.

This article provides a simple and general view of the concept that RNAs can directly regulate gene functions, with particular attention to a step-by-step approach to the study of miRNA. Hopefully, this information will help researchers who are new to this field to overcome problems encountered in the functional analysis of miRNA.

Small RNAs or Non-coding RNAs

A non-coding RNA (ncRNA) is any RNA molecule that functions without being translated into a protein. An ncRNA is also called small RNA (sRNA). Less frequently, it is called non-messenger RNA (nmRNA), small non-messenger RNA (snmRNA), tiny ncRNA (tncRNA), small modulatory RNA (smRNA), or small regulatory RNA. Broadly speaking, the DNA sequence from which an ncRNA is transcribed can be considered to be an RNA gene.

In this article, we will confine our discussion to small RNAs; that is, transcripts of less than 300 nucleotides (nt) that participate directly in RNA processing and degradation, but indirectly in protein synthesis and gene regulation. Because RNA polymerases type II (Pol-II) are inefficient in generating small RNAs of this size, the small RNAs are either directly transcribed by RNA polymerases type III (Pol-III) or indirectly processed from a large transcript of Pol-II.

Transfer RNA

The most prominent example of ncRNA is transfer RNA (tRNA), which is involved in the process of translation and is the first type of small RNA identified and characterized

[1]. Transfer RNA is RNA that transfers a specific amino acid to a growing polypeptide chain at the ribosomal site of protein synthesis during translation. The tRNA is a small RNA, 74–93 nucleotides long, consisting of amino-acid attachment and codon recognition sites, allowing translation of specific amino acids into a polypeptide. The secondary and tertiary structure of tRNAs are cloverleaves with 4–5 domains and an L-shaped 3D structure, respectively.

Nucleolar RNA

Another example of ncRNA is rRNA, which is the primary constituent of ribosomes. rRNA is transcribed from DNA and in eukaryotes it is processed in the nucleolus before being transported through the nuclear membrane. This type of RNA may produce small nucleolar RNA (snoRNAs), the second type of small RNA. Many of the newly discovered snoRNAs are synthesized in an intron-processing pathway. Several snoRNAs and snoRN proteins (RNPs) are known to be needed for processing of ribosomal RNA, but precise functions remain to be defined. In principle, snoRNAs could have several roles in ribosome synthesis including folding of pre-rRNA, formation of rRNP substrates, catalyzing RNA cleavages, base modification, assembly of pre-ribosomal subunits, and export of product rRNP particles from the nucleus to the cytoplasm.

The snoRNA acts as a guide to direct pseudouridylation and 2'-O-ribose methylation of ribosomal RNA (rRNA) in the nucleolus. Consequently, the snoRNA guides the snoRNP complex to the modification site of the target rRNA via sequence hybridization. The proteins then catalyze the modification of bases in the rRNA. Therefore, this type of RNA is also called guided RNA.

The snoRNA is also associated with proteins forming part of the mammalian telomerase as well as with proteins involved in imprinting on the paternal chromosomes. It is encoded in introns of genes transcribed by RNA polymerase II (pol II), even when some of the host genes do not code for proteins. As a result, the intron, but not the exon, of these genes is evolutionarily conserved in vertebrates. In this way, some of the introns of the genes employed in plants or invertebrates are still functioning in vertebrates.

The structure of snoRNAs consists of conserved sequences base-paired to their target RNAs. Nearly all vertebrate guide snoRNAs originate from introns of either protein-coding or noncoding RNAs transcribed by pol II, whereas only a few yeast guide snoRNAs derive from introns, suggesting that introns accumulated during evolution reflect the conservation of transgenes incorporated into the introns, as mentioned above [2–4]. These introns are processed through pathways involving endonucleolytic

cleavage by RNase III-related enzymes, exonucleolytic trimming and possibly RNA-mediated cleavage, which occur in large complexes called exosomes [5, 6].

Nuclear RNA

Nuclear RNA (snRNA) is a class of small RNA molecules that are found within the nuclei of eukaryotic cells. They are involved in a variety of important processes such as RNA splicing (removal of introns from hnRNA) and maintaining the telomeres. They are always associated with specific proteins, and the complexes are referred to as small nuclear ribonucleoproteins (snRNP). Some examples of small nuclear RNA are U2 snRNAs, pre-5S rRNAs, and U6 snRNAs. U2 snRNAs in embryonic stem (ES) cells and pre-5S rRNAs in *Xenopus* oocytes facilitate cell survival after UV irradiation by binding to conserved protein R₀. Eukaryotic U6 snRNAs are the five types of spliceosomal RNA involving in mRNA splicing (U1–U6). These small nuclear RNAs have a secondary structure consisting of a stem-loop, internal loop, a stem-closing internal loop, and the conserved protein binding site [7].

Phage and Viral RNA

Another form of small RNAs is 30 ribonucleotides in length and functions as a priming initiator for bacteriophage F1 DNA replication [8, 9]. This function is solely to initiate a given site on the phage DNA, suggesting a primitive defense against foreign pathogen invasion. The phage T4-derived intron is involved in an RNA–RNA interaction in the inhibition of protein synthesis [10].

Small Interfering RNA

The small interfering RNA (siRNA) is a small double-stranded RNA (dsRNA) molecule, 20–25 nt in length, that interferes with the expression of genes via a part of RNAi involving the enzyme Dicer. The story of siRNAs began with the observation of pigment expression in the *Petunia* plant. van der Krol et al. [11] tried to intensify flower pigmentation by introducing additional genes, but unexpectedly observed reduced floral pigmentation in some plants, suggesting that gene silencing may be involved in naturally occurring regulation of gene function. This introduction of multiple transgenic copies of a gene into the *Petunia* plant resulted in gene silencing of not only the transgenic, but also the endogenous gene copy, as has been observed by others [12]. This suggests co-suppression of homologous genes (the transfer gene and the endogenous

gene) and possible methylation are involved [12, 13]. This phenomenon is termed RNAi. Note that the transgene introduced to the *Petunia* plant is a dsRNA, which is perfectly complementary to the target gene.

When dsRNA was injected into *Caenorhabditis elegans*, Fire et al. [14] noticed gene silencing and RNAi. RNAi is a mechanism by which small regulatory RNAs possessing a sequence complementary to that of a portion of a target gene interferes with the expression of that gene. It is thought that the dsRNA, once it enters the cells, is cut up by an RNaseIII-familial endonuclease, known as Dicer. Dicer consists of an amino terminal helicase domain, a PAZ domain, two RNase III motifs, and a dsRNA binding motif. Therefore, Dicer binds to the dsRNA and excises the dsRNA into siRNA. These siRNAs locate other single-stranded RNA molecules that are completely complementary to either strand of the siRNA duplex. Then, the RNA-degrading enzymes (RNases) destroy the RNAs complementary to the siRNAs. This phenomenon is also named post-transcriptional gene silencing (PTGS) or transgene quelling. In other words, gene silencing can be activated by introducing transgenes, RNA viruses, or dsRNA sequences that are completely complementary to the targeted gene transcripts.

In mammals, dsRNAs longer than 30 nt will activate an antiviral response, which will lead to the nonspecific degradation of RNA transcripts, the production of interferon, and the overall shutdown of host cell protein synthesis [15]. As a result, long dsRNA will not produce gene-specific RNAi activity in mammalian cells [16].

Several terms have been used to describe the same or similar phenomenon in different biological systems of different species, including short interfering (si) RNAs [17], small temporal (st) RNAs [18], heterochromatic siRNAs [19], and small modulatory dsRNAs [20].

MicroRNA

miRNAs are small single-stranded RNA genes possessing the reverse complement of another protein-coding gene's mRNA transcript. These miRNAs can inhibit the expression of that target protein-coding gene. MiRNA was first observed in *C. elegans* as RNA molecules of 18–23 nt that are complementary to the 3' untranslated regions of the target transcripts, including *lin-4* [21] and *let-7* [22] genes. As a result, the development of the worm was regulated by these RNA genes. Subsequently, miRNAs were found to occur in diverse organisms ranging from worms, to flies, to humans [23], suggesting these molecules represent a gene family that has evolved from an ancient ancestral small RNA gene.

The miRNA is thought to be transcribed from DNA that is not translated but regulates the expression of other genes.

Primary transcripts of the miRNA genes, pri-miRNAs, are long RNA transcripts consisting of at least a hairpin-like miRNA precursor. Pri-miRNAs are processed in the nucleus to pre-miRNAs by the ribonuclease Droscha with the help of microprocessor [24] and exported from the nucleus by Exportin-5 [25]. The 60–90 nt miRNA precursors form the stem and loop structures, and the cytoplasmic RNaseIII enzyme Dicer excises the miRNA from the pre-miRNA hairpin stem region. MiRNAs and siRNAs seem to be closely related, especially taking the dsRNA and hairpin structures into account. The siRNA can be considered as a duplex form of miRNA in which the RNA molecule contains both miRNA and its reverse complement. Therefore, one can consider siRNAs as a kind of miRNA precursor.

miRNAs suppress gene expression based on their complementarity to a part of one or more messenger RNAs (mRNAs), usually at a site in the 3' UTR. The annealing of the miRNA to the target mRNA inhibits protein translation. In some cases, the formation of dsRNA through the binding of miRNA triggers the degradation of the mRNA transcript through a process similar to RNAi, though in other cases it is believed that the miRNA complex blocks the protein translation machinery or otherwise prevents protein translation without causing the mRNA to be degraded.

Because most of the miRNA suppresses gene function based on partial complementarity, conceivably one miRNA may target more than one mRNA, and many miRNAs may act on one mRNA, coordinately modulating the intensity of gene expression in various tissues and cells. Therefore, miRNAs may have a broad function in fine-tuning the protein-coding genes. Indeed, the discovery of miRNAs has revolutionized our understanding of gene regulation in the post-genome era.

Intronic miRNA

Some small regulatory RNAs are produced from intronic RNA fragments. For example, snoRNAs are produced from intronic segments from genes encoding ribosomal proteins and nucleolar proteins. In addition, some small RNAs are produced from genes in which exons no longer have the capacity to encode proteins. This type of intron processing involves RNase III-related enzymes, exonucleolytic trimming, and possibly RNA-mediated cleavage. Therefore, intronic miRNA is a new class of miRNAs derived from the processing of introns of a protein-coding gene.

The major difference between the intronic miRNAs and previously described intergenic miRNAs is the requirement of type II RNA polymerases (Pol-II) and spliceosomal components for the biogenesis of intronic miRNAs [26]. Both intronic and intergenic miRNAs may share the same

assembly process, namely the RNA-induced silencing complex (RISC), the effector of RNAi-related gene silencing. Although siRNA-associated RISC assembly has been used to predict miRISC assembly, the link between final miRNA maturation and RISC assembly remains to be determined. The characteristics of Dicer and RISC in siRNA versus miRNA mechanisms are distinctly different [27, 28].

The intronic miRNAs need to fulfill the following requirements. First, they must share the same promoter with their encoding gene transcripts. Second, they are located in the non-protein-coding region of a primary gene transcript (the pre-mRNA). Third, they are co-expressed with the gene transcripts. Lastly, they are removed from the transcript of their coding genes by nuclear RNA splicing and excision processes to form mature miRNAs.

Certain of the currently identified miRNAs are encoded in the genomic intron region of a gene, but they are of an orientation opposite to that of the protein-coding gene transcript. Therefore, these miRNAs are not considered to be intronic miRNAs because they do not share the same promoter with the gene and they are not released from the protein-coding gene transcript by RNA splicing. The promoters of these miRNAs are located in the antisense direction to the gene, probably using the gene transcript as a potential target for the antisense miRNAs. A good example of this type of miRNA is *let-7c*, which is an intergenic miRNA located in the antisense region of the intron of a gene.

Transposon and Intronic miRNA

The intronic and other ncRNAs may have evolved to provide a second level of gene expression in eukaryotes, enabling a fine-tuning of the complex network of gene activity. In bacterial and organellar genomes, group II introns contain both catalytic RNAs and retrotransposable elements. The retrotransposable elements make this type of intron mobile. Therefore, these introns are reversely spliced directly into a DNA target site and subsequently reverse transcribed by the intron-encoded gene. After inserting into the DNA, the introns are spliced out of the gene transcript to minimize the damage to the host.

There is a potential evolutionary relationship between group II introns and both eukaryotic spliceosomal introns and non-LTR-retrotransposons. Taking advantage of this feature, it is feasible to design mobile group II introns to be incorporated into gene-targeting vectors as “targetrons,” to specifically target various genes [29]. There is evidence that introns in *Caenorhabditis* genes are recently gained and some of them are actually derived from “donor” introns present in the same genome. Further, a few of these

new introns apparently derive from other introns in the same gene [30]. Perhaps the splicing machinery determines where introns are added to genes. On the other hand, some newly discovered brain-specific snoRNAs of unknown function are encoded in introns of tandem repeats and the expression of these introns is paternally imprinted.

From an evolutionary vantage, transposons are probably very old and may exist in the common ancestor genome. They may enter the host multiple times at some point for selfish parasitical reasons. This feature of transposons is similar to that of retroviruses. Too much transposon activity can destroy a genome. To counterattack the activity of transposons and viruses, some organisms developed a mechanism to remove and/or silence the activity of transposons and viruses. For example, bacteria frequently delete their genes so that transposons and retroviruses incorporated in the genome are removed. In eukaryotes, miRNA is a way of reducing transposon activity. Conceivably, miRNA may be involved in resistance against viruses, similar to the diversity of antibody production in an immune system, or in a to-be-identified mechanism for fighting disease.

Identical twins derived from the same zygote have the same genetic information in their nuclear DNA. Any differences between monozygotic twins later in life are mostly the result of environmental influences rather than genetic inheritance. But monozygotic twins may not share all of their DNA sequences. Female monozygotic twins can differ because of differences in X-chromosome inactivation. Consequently, one female twin can have an X-linked condition such as muscular dystrophy and the other twin can be free of it. Monozygotic twins frequently demonstrate slightly, but definitely distinguishing, disease susceptibility and physiology more generally. For example, myotonic dystrophy (DM) is a dominantly inherited, multisystemic disease with a consistent constellation of seemingly unrelated and rare clinical features including myotonia, muscular dystrophy, cardiac conduction defects, posterior iridescent cataracts, and endocrine disorders [31]. DM2 (Type 2) is caused by a CCTG expansion (mean ~5,000 repeats) located in intron 1 of the zinc finger protein 9 (*ZNF9*) gene [32]. It is possible that monozygotic twins with this disorder display symptom heterogeneity because of miRNAs or different levels of insertion of intronic genes.

Class II transposons can cut and paste. The enzyme transposase binds to the ends of the transposon, which are repeats, and the target site on the genome, which is cut to leave sticky ends. These two components are joined together by ligases. In this way, transposons increase the size of the genome because they leave multiple copies of themselves in the genome. It is highly possible that transposons are selectively advantageous for the genome to modulate

gene regulation via miRNAs. It is not too far-fetched to suggest that when transposons are inserted in the introns of the protein-coding gene, under appropriate conditions, they, a part of them, or their secondary structures, may become intronic miRNAs.

Piwi-interacting RNAs

PiwiRNAs (Piwi-interacting RNA, piRNA) are a class of small, 29–30 nt, RNA molecules that are expressed uniquely in mammalian testes and forms RNA-protein complexes with Piwi proteins such as MIWI and MILI, members of the argonaute family. These piRNA complexes (piRCs) have been linked to transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells, particularly those in spermatogenesis. PiwiRNAs silence gene expression via the formation of an RISC [33–39].

RasiRNAs

RasiRNAs (the repeat-associated siRNAs) are new members of the piwiRNA family that interact with a class of short regulatory RNAs, Piwi-interacting RNAs (piRNAs). This type of miRNAs uses a different biogenesis of miRNAs in which certain debranched introns mimic the structural features of pre-miRNAs to enter the miRNA-processing with Drosha-mediated cleavage. This type of pre-miRNA-like introns is referred to as mirtrons, which are selectively maintained and conserved during evolution, probably plays an important regulatory role in the animal [40].

Biogenesis and Mechanism of miRNAs

The investigation of the biogenesis and mechanism of miRNAs still is in its infancy. siRNA appears to be a form of miRNA duplex predominantly occurring in plants and lower animals. The biogenesis and mechanism of siRNAs are very similar to those of miRNA. However, there are some differences between these two pathways.

Five steps are involved in miRNA biogenesis in vertebrates. First, miRNA is generated as a long primary precursor miRNA (pri-miRNA), most likely mediated by RNA polymerases type II (Pol-II) [41, 42]. The pri-miRNA is transcribed from the genome. Second, the long pri-miRNA is excised by Drosha-like RNase III endonucleases and/or spliceosomal components to form the ~60–70 nt precursor miRNA (pre-miRNA). The pre-miRNA exhibits considerable secondary structure including regions of

imperfectly paired dsRNA, which are sequentially cleaved to one or more miRNAs. This step depends on the origin of the pri-miRNA, whether located in an exon or an intron respectively [41, 42]. Third, the pre-miRNA is exported out of the nucleus by Ran-GTP and a receptor, Exportin-5 [25, 43]. In the cytoplasm, Dicer-like endonucleases cleave the pre-miRNA to form mature 18–25 nt miRNA. Lastly, the mature miRNA is incorporated into a ribonuclear particle (RNP) to form the RNA-induced gene silencing complex (RISC) which executes RNAi-related gene silencing [44, 45]. Only one of the two strands is the miRNA; the other counterpart is named miRNA*. The mature miRNA can block mRNA translation based on partial complementarity between the miRNA and the targeted mRNA, particularly via base pairing with the 3'-untranslated region of the mRNA. If there is a perfect complementarity between the miRNA and the targeted mRNA, mRNA degradation occurs similarly to that mediated by siRNA. Auto-regulatory negative feedback via miRNAs regulates some genes, including those involved in the RNA silencing mechanism itself.

The assembly of RISC for siRNA has been reported in an in vitro system, and a similar assembly probably also occurs for miRNA. But the link between final miRNA maturation and RISC assembly remains unknown. However, there is evidence that the actions of Dicer and RISC in siRNA and miRNA processing are distinct [27, 28]. In recent studies using zebrafish, it was demonstrated that the stem-loop structure of the pre-miRNAs is involved in strand selection for mature miRNA during RISC assembly. These findings further suggest that the duplex structure of siRNA may not be strictly required for the assembly of miRNA-associated RISC in vivo. Proposed pathways for the biogenesis of miRNA are based on the in vitro model developed for siRNA. For these reasons, future work needs to focus on distinguishing the individual properties and differences in action of Dicer and RISC in siRNA and miRNA processing. Conceivably, siRNA is a defence mechanism against immediate insertion of viral genes or transposons in plants and lower animals. In contrast, miRNA over evolutionary time selects segments of transposons for incorporation into the genome for fine-tuning of gene regulation in vertebrates, including human beings. This hypothesis for the differences between siRNA and miRNA gene silencing may provide a clue toward explaining the prevalence of native siRNAs in invertebrates but relative scarcity in mammals.

In plants, siRNAs and their dsRNA precursors trigger DNA methylation as well as RNAi [46–48]. Another function of siRNAs is a specialized ncRNA molecule, which is X chromosome-encoded. This non-coding RNA is named Xist. Xist is preferentially expressed from only one

of the two female X chromosomes and builds up in cis along the chromosome from which it was transcribed. That X chromosome is tightly packaged in transcriptionally inactive heterochromatin; therefore, only one female X-chromosome is active. This phenomenon is associated with DNA methylation. By the same token, the viruses, transgenes and transposons, which have been incorporated into the introns of the mammalian genome during evolution, may take advantage of these characteristics by splicing the pri-miRNAs and incorporating them into Dicer-like proteins for gene silencing and mRNA degradation.

AGO I is a key protein that is required for both the siRNA and miRNA pathways and is likely the endonuclease that cleaves the mRNA targeted by the RISC [49, 50]. Elucidating the roles of the full complement of the AGO protein family will reveal further modulation of the RISC, and small RNA regulation more generally. Additional proteins involved in the RISC at the convergence of the PTGS and miRNA pathways have been reported [51, 52].

Introns account for the largest proportion of non-coding sequences in the protein-coding DNA of the genome. The transcription of the genomic protein-coding DNA generates precursor messenger RNA (pre-mRNA), which contains four major parts including the 5'-untranslated region (UTR), the protein-coding exon, the non-coding intron and the 3'-UTR. In broad terms, both 5'- and 3'-UTR can be seen as a kind of intron extension; however, their processing during mRNA translation is different from that of the intron located between two protein-coding exons, termed the in-frame intron. The in-frame intron was originally thought to be a huge genetic wasteland in gene transcripts, but this stereotypic misconception was abandoned because of the finding of intronic miRNAs. To this day, the biogenesis of intronic miRNAs remains to be determined (Fig 1).

Identification

To this day, there are four major ways to identify miRNAs. They are (a) direct cloning, (b) computer search of the genome, (c) miRNA microarray search in different species, and (d) artificial preparation of intronic miRNA for targeting known gene sequences.

The conventional direct cloning of short RNA molecules, as in the cloning of *let-7* and *lin-4*, is still the method of choice to identify new miRNAs. Conceivably, one can isolate the small RNAs and sequence them individually. So far, results have been dominated by a few highly expressed miRNAs. But, once the miRNA is identified, its role in other organisms, including human beings, can be explored.

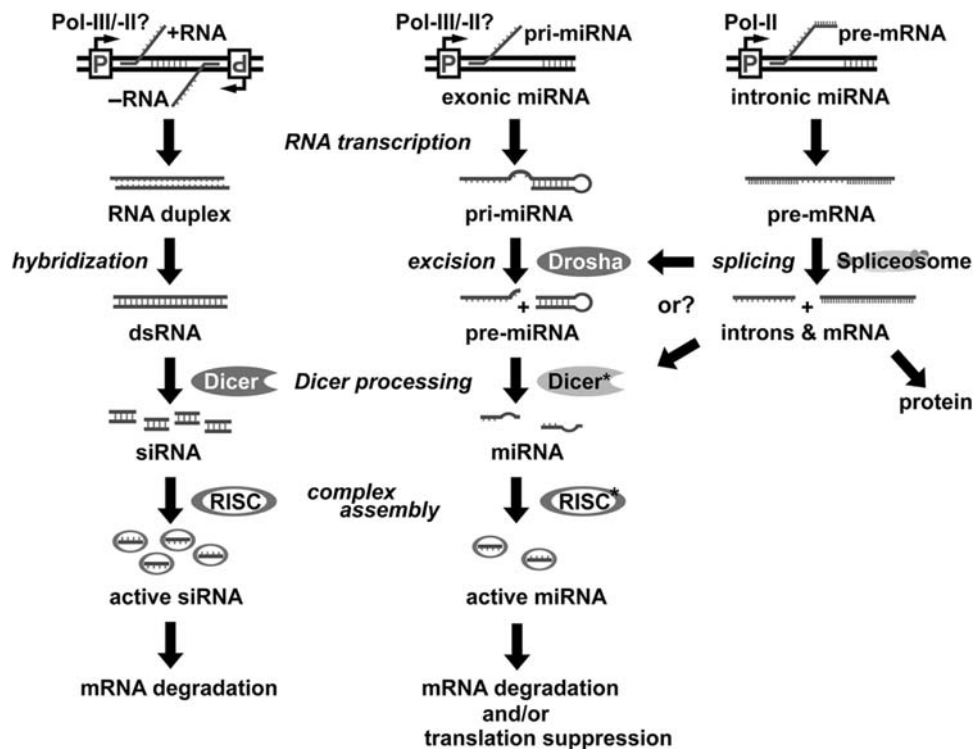


Fig. 1 Comparison of biogenesis and RNAi mechanisms among siRNA, intergenic (exonic) miRNA, and intronic miRNA. siRNA is likely formed by two perfectly complementary RNAs transcribed from two different promoters (remains to be determined) and further processing into 19–22 bp duplexes by the RNase III-familial endonuclease, Dicer. The biogenesis of intergenic miRNAs, e.g., *lin-4* and *let-7*, involves a long transcript precursor (pri-miRNA), which is probably generated by Pol-II or Pol-III RNA promoters, while intronic miRNAs are transcribed by the Pol-II promoters of its encoded genes and co-expressed in the intron regions of the gene transcripts (pre-mRNA). After RNA splicing and further processing, the spliced intron may function as a pri-miRNA for intronic miRNA generation. In the nucleus, the pri-miRNA is excised by Drosha

to form a hairpin-like pre-miRNA template and then exported to the cytoplasm for further processing by Dicer* to form mature miRNAs. The Dicers for siRNA and miRNA pathways are different. All three small regulatory RNAs are finally incorporated into an RNA-induced silencing complex (RISC), which contains either strand of siRNA or the single-strand of miRNA. The effect of miRNA is considered to be more specific and less adverse than that of siRNA because only one strand is involved. On the other hand, siRNAs primarily trigger mRNA degradation, whereas miRNAs can induce either mRNA degradation or suppression of protein synthesis depending on the sequence complementarity to the target gene transcripts

For example, *let-7* was originally identified in *C. elegans*. Subsequently, reduced expression of the *let-7* miRNA [53] and Dicer [54] in human lung cancers suggested that the alteration of *let-7* expression is associated with clinical and biological effects.

There are numerous new computational methods that provide ways to estimate the total number of miRNA genes in different animals [55–58]. Fundamentally, each program identifies highly conserved genomic non-coding regions that possess stem-loop structures with specific “seed” sequences, and complementarity of the first 8–10 nt. Then, the secondary structure is examined in terms of both the forward and reverse complements of the sequence. In addition, the following criteria help to identify miRNAs: the longest helical arm, free energy of the arm, short internal loops, and asymmetric and bulged loops. The identified miRNAs usually are more heterogeneous than those discovered experimentally, suggesting

traditional cloning has a high false negative or miss rate. However, computational techniques may suffer from a high false alarm rate. Therefore, validation of the identified miRNAs by Northern blot analysis and functional study is critical. These methods are still evolving and there is a possibility of one-to-many and many-to-one relationships between the miRNAs and their targets. Potentially thousands of mammalian targets may be identified with this approach.

To facilitate such investigations, an oligonucleotide microchip for genome-wide miRNA profiling in diverse tissues of various species was developed [59–62]. Some of these chips use locked nucleic acid (LNA)-modified oligonucleotides so as to allow both miRNA in situ hybridization and miRNA expression profiling [63]. Again, this approach can identify regulation via a large class of miRNAs. A good example is the studies of miRNAs regulating brain morphogenesis in zebrafish [64].

Applications

Application of miRNA is common in plants. The first experimentally observed RNAi was demonstrated by the discoloration of flowers after introduction of dsRNAs. MiRNAs do not just mediate gene regulation in flowering plants; the regulation of a similar type of plant gene can be utilized to silence a fruit-ripening gene. The latter approach has been applied to create hardier tomatoes by engineered repression of the tomato-ripening gene through homology-based silencing.

The following areas are potential applications of miRNAs in vertebrates: (a) analysis of gene function, (b) evaluation of function and effectiveness of miRNA, (c) design and development of novel gene therapy, (d) design and development of anti-viral vaccines, and (e) development of loss-of-function transgenic animals.

Analysis of Gene Function

The human genome contains more than 22 billion bases. Analyzing and understanding these sequences is a challenge to all of natural science. Numerous high-throughput screening programs have been developed in the post-genome-era. Good examples of the utility of such programs are the discoveries that *lin-4* and *let-7* miRNAs, originally identified in *C. elegans*, control the timing of fate specification of neuronal and hypodermal cells during larval development. Subsequently, these miRNAs were found to be conserved in mammals with potential functions in development and disease [65, 66]. Although most programs are focused on global computational screens, others aim at screening or isolating all miRNAs with a specific function in the genome. For instance, retroviral insertional mutagenesis in mouse hematopoietic tumors provides a potent cancer gene discovery tool in the post-genome-sequence era. The multiple high-throughput insertional mutagenesis screening projects were thus designed for identifying new cancer genes. Using an shRNAi expression library against the whole human transcriptome, attempts were made to screen for small RNAs in the genome [57].

With the completion of genome-sequencing projects, a major challenge will be to understand gene function and regulation. Achieving this goal will require determining how miRNAs modulate gene expression. The various gene-silencing mechanisms based on complete or partial complementarity and their intertwined actions are beginning to reveal the sensitive control mechanisms that modify gene expression at the post-transcriptional and RNA turnover levels.

Intronic miRNA also represents a new frontier in genetics research. The evidence of intronic miRNA-

induced silencing of gene expression in cell lines, zebrafish, chicken embryos, and mouse skin demonstrates that this ancient intron-mediated gene regulation system is highly conserved in eukaryotes. Intronic regulation of gene expression is mediated through the activation of miRNA-mediated RNAi effects. From an evolutionary vantage point, the genome exhibits a remarkable increase in the complexity and variety of introns in higher plants and animals; therefore, the influence of intronic gene regulation to facilitate genome stability and gene coordination progressively increases phylogenetically. Conceivably, dysregulation of intronic miRNAs is highly likely to reveal the intertwined actions between intronic miRNA and gene expression, leading to a better understanding of the genetic etiology of human diseases. The success of intronic miRNA generation by artificial means in vivo may provide a powerful tool to study the mechanism by which miRNAs induce diseases and will hopefully provide novel gene therapies.

Evaluation of miRNA Function and Effectiveness

Prediction of miRNA candidates using analytical software has identified thousands of genomic hairpin sequences. For instance, the human DGCR8, the DiGeorge syndrome critical region gene 8, and its *Drosophila melanogaster* homolog were identified in this fashion [67]. The biochemical and whole cell-based data demonstrating the requirement of DGCR8 for the maturation of miRNA primary transcripts has been acquired. Further, RNAi knockdown experiments with fly and human DGCR8 showed both accumulation and reduction of pri-miRNAs as well as mature miRNAs. In this manner, the function of, effectiveness of, and interaction between miRNAs and enzyme processing complexes can be demonstrated [67].

To date, the function of the vast majority of miRNAs so identified remains to be determined. Because direct transfection of hairpin-like miRNA precursors (pre-miRNAs) in mammalian cells is not always sufficient to trigger effective RISC assembly, a key step for RNAi-related gene silencing, our intronic miRNA-expressing system was developed to overcome this problem and indeed successfully increased the efficiency and effectiveness of miRNA-associated RNAi induction in vitro and in vivo. Nevertheless, there are still problems in the efficient use of miRNA. Indeed, evaluating the function and effectiveness of the miRNAs so far identified may contribute greatly to our understanding of gene regulation and the control of the differentiation and development of cells.

Based on the strand complementarity between the designed miRNA and its target gene sequence, we have

also developed a miRNA isolation protocol to purify and identify the mature miRNAs generated by the intronic miRNA-expressing system. Several intronic miRNAs have been confirmed active *in vitro* and *in vivo*. As shown by this proof-of-principle method, we now have the necessary knowledge to design more efficient and effective pre-miRNA inserts for the intronic miRNA-expressing system.

Design and Development of Novel Gene Therapy

We are undergoing an epoch-marking transition into the post-genome era, which opens up data sources of unprecedented scale. This information can be used for designing and developing potential drugs as novel gene therapies. Furthermore, the elucidation of genomic control of gene activities mediated via miRNA may play a crucial role in the characterization and treatment of disease at the molecular level. At the same time, our still very limited knowledge of the biological functions of genes and proteins at different levels of cellular organization is preventing full exploitation of the available data. We believe that the recent discovery of miRNAs will fill the gap and lead to unlimited functional prediction based on the DNA–miRNA and RNA–miRNA paradigm. In theory, oncogene and transgene expression could be inhibited by synthetic miRNAs, a simple, effective gene therapy. Thus, miRNAs or their machineries are now known to be involved in several human diseases, including cancer and neurological disorders. Specific removal of the target genes by miRNAs or their associate mediators can be developed as a simple gene therapy [68].

Design and Development of Anti-viral Vaccines

By the same token, anti-viral vaccines can be developed. The problems in preventing viral infections such as HIV are as follows: first, the global prevalence of epidemic proportions is mainly caused by the high mutation rate of the HIV genome that gradually generates more resistant strains to highly active antiretroviral therapies. Second, the HIV provirus is capable of integrating into a host cellular genome to escape from the inhibitory effects of the treatment, thus inactivating the viral replication cycle rather than destroying the latent viral genome, resulting in an increased number of HIV carriers. Such an increase of drug-resistant HIV strains and their carriers has posed great challenges and financial burdens for the AIDS prevention programs. To alleviate such problems, miRNAs can trigger either translation repression or RNA degradation

depending on their degree of complementarity with the target genes. Our approach, using vector-based miRNA, to overcome the complications of HIV mutation is a breakthrough in the field. We believe the knowledge obtained from this research will facilitate the development of anti-viral drugs and vaccines against HIV infections [69, 70]. Similar approaches for the treatment of viral diseases will target avian flu, SARS, HBV, human papillomavirus, and polio.

Development of Loss-of-function Transgenic Animals

The ability to utilize miRNA and its machinery for silencing target-gene expression has created a lot of excitement as a novel and simple means to develop loss-of-function transgenic animals. To define the function of a critical molecule in miRNA processing, zebrafish models have been developed that carry loss-of-function mutations.

This type of animal model has provided an unprecedented resource for miRNA research because this approach can be used to create miRNAs for use in loss-of-function studies. It is clear that miRNAs also hold great promise as therapeutic tools because of their sequence-specific targeting, particularly against infectious diseases with frequent mutations. Another potential use of transgenic animal models using miRNAs is the testing of gene functions and drug mechanisms *in vivo*.

Using the intronic miRNA described above, one can establish loss-of-function in zebrafish, chicken and mice. The loss-of-function transgenic zebrafish could not have been achieved with siRNAs due to promoter incompatibility, but have been developed with intronic miRNAs. The zebrafish, possessing numerous features similar to human biological systems, is most suitable for etiological and pathological studies of human diseases, particularly mechanisms by which the loss of a specific signal molecule causes a disease or disorder. All pharmaceutically developed drugs can be screened with this approach in loss-of-function transgenic zebrafish. In addition, this approach may shed light on the effects of miRNAs on embryonic development, environmental impacts, and micro-modulation of gene functions, particularly brain and heart functions. Indeed, insight regarding structure-function features of a candidate gene involved in pathobiology and the mechanisms in which the candidate gene operates can be illuminated with the help of miRNA transgenic animal models carrying a loss-of-function mutation within the candidate gene.

Acknowledgment The work was supported by a grant from the National Institutes of Health, CA 85722.

Glossary

Argonaute (AGO):	A large protein family that constitutes key components of RISCs. AGO proteins are characterized by two unique domains, PAZ and PIWI, whose functions are not fully understood. Current evidence suggests that the PAZ domain binds the 3'-end two-nucleotide overhangs of the siRNA duplex, whereas the PIWI domain of some AGO proteins confers slicer activity. PAZ and PIWI domains are both essential to guide the interaction between the siRNA and the target mRNA for cleavage or translational repression. Distinct AGO members have distinct functions. For example, human AGO2 programs miRNA-associated RISCs to cleave the mRNA target, whereas AGO1 do not.	MicroRNA (miRNA):	A type of non-coding small RNA (~21–23 nucleotides) produced by DCR from a stem-loop structured RNA precursor. miRNAs are widely expressed in animal and plant cells as RNA-protein complexes (RNPs) termed miRISCs, and have been implicated in the control of development because they target specific gene transcripts for destruction or translational suppression. An intracellular gene regulation mechanism in which a transgene or double-stranded RNA (dsRNA) triggers the degradation or the translational suppression of a gene transcript containing high complementarity to the transgene or dsRNA.
Cosuppression:	A phenomenon similar to PTGS but silencing multiple homologous genes via a transgene or dsRNA.	Post-transcriptional gene silencing (PTGS):	An intracellular gene regulation mechanism in which a transgene or double-stranded RNA (dsRNA) triggers the degradation or the translational suppression of a gene transcript containing high complementarity to the transgene or dsRNA.
Dicer:	An enzyme of the RNase III endonuclease family consisting of multiple domains and cleaves dsRNA or stem-loop structured RNA precursors into small RNAs and form siRNAs and miRNAs, respectively. It possesses a potential helicase activity and an additional domain of undetermined function and is essential for RNAi in <i>Drosophila</i> and <i>C. elegans</i> .	Pseudogene:	A defective segment of DNA that resembles a gene but cannot be transcribed into RNA.
Drosha:	A nuclear RNase III endonuclease that is implicated in the cleavage of pri-miRNA into pre-miRNA prior to the nuclear export of miRNA precursors.	RNA-induced silencing complex (RISC):	An RNA-protein complex that targets its perfectly or partially complementary mRNA for cleavage or translational repression. siRNA programs a siRISC and miRNA programs a miRISC. RISCs (both siRISC and miRISC) can be divided into two types: cleaving and non-cleaving. Current evidence suggests that the type of AGO protein, an essential RISC component, determines whether a RISC is cleaving or non-cleaving. An RNP complex that is the effector of RNAi mechanism consisting of AGO familial proteins, Dicer endonuclease, RNA helicase, and other accessory factors.
Epigenetics:	Changes in gene expression not caused by the DNA code and that occur across at least one generation.	RNA interference (RNAi):	A post-transcriptional gene silencing phenomenon induced by either single-stranded (ssRNA) miRNA or double-stranded (dsRNA) siRNA.
Exportin-5:	A nuclear transmembrane protein that transports precursor miRNA (pri-miRNA) from the nucleus to the cytosol.	Small hairpin RNA:	A single-stranded oligonucleotide containing two complementary regions which form a duplex structure with a short hairpin loop.
Microprocessor:	A nuclear complex composed of Drosha and Pasha that functions in miRNA biogenesis from the primary miRNA (pri-miRNA) to the precursor miRNA (pre-miRNA).	Small interfering RNA (siRNA):	A type of small RNA (~21–25 nucleotides) produced by DCR, a double-stranded RNA-specific enzyme of the RNase III family. The siRNA is the key component of siRISCs and triggers the silencing of its complementary mRNA.

Transgene: Double-stranded DNA or DNA-RNA hybrid duplex capable of being transcribed by RNA polymerases and affecting the expression of an intracellular gene.

References

- Holley, R. W. (1965). Structure of an alanine transfer ribonucleic acid. *Journal of the American Medical Association*, *194*, 868–871.
- Maxwell, E. S., & Fournier, M. J. (1995). The small nucleolar RNAs. *Annual Review of Biochemistry*, *64*, 897–934.
- Tycowski, K. T., Shu, M. D., & Steitz, J. A. (1996). A mammalian gene with introns instead of exons generating stable RNA products. *Nature*, *379*, 464–466.
- Filipowicz, W. (2000). Imprinted expression of small nucleolar RNAs in brain: time for RNomics. *Proceedings of the National Academy of Science USA*, *97*, 14035–14037.
- Allmang, C., Kufel, J., Chanfreau, G., Mitchell, P., Petfalski, E., & Tollervey, D. (1999). Functions of the exosome in rRNA, snoRNA and snRNA synthesis. *EMBO Journal*, *18*, 5399–5410.
- van Hoof, A., & Parker, R. (1999). The exosome: a proteasome for RNA? *Cell*, *99*, 347–350.
- Frank, D. N., Roiha, H., & Guthrie, C. (1994). Architecture of the U5 small nuclear RNA. *Molecular and Cellular Biology*, *14*, 2180–2190.
- Stavianoopoulos, J. G., Karkus, J. D., & Charguff, E. (1971). Nucleic acid polymerase of the developing chicken embryos: a DNA polymerase preferring a hybrid template. *Proceedings of the National Academy of Science USA*, *68*, 2207–2211.
- Stavianoopoulos, J. G., Karkus, J. D., & Charguff, E. (1972). Mechanism of DNA replication by highly purified DNA polymerase of chicken embryos. *Proceedings of the National Academy of Science USA*, *69*, 2609–2613.
- Wank, H., & Schroeder, R. (1996). Antibiotic-induced oligomerisation of group I intron RNA. *Journal of Molecular Biology*, *258*, 53–61.
- van der Krol, A. R., Mur, L. A., Beld, M., Mol, J. N., & Stuitje, A. R. (1990). Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell*, *2*, 291–299.
- Napoli, C., Lemieux, C., & Jorgensen, R. A. (1990). Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes *in trans*. *Plant Cell*, *2*, 279–289.
- Matzke, M. A., Primig, M. J., Trnovsky, J., & Matzke, A. J. M. (1989). Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. *EMBO Journal*, *8*, 643–649.
- Fire, A., Xu, S., Montgomery, M. K., et al. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, *391*, 806–811.
- Shi, Y. (2003). Mammalian RNAi for the masses. *Trends in Genetics*, *19*, 9–12.
- Sui, G., Soohoo, C., Affar, el B., Gay, F., Shi, Y., Forrester, W.C., & Shi, Y. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proceedings of the National Academy of Science USA*, *99*, 5515–5520.
- Elbashir, S. M., Lendeckel, W., & Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes & Development*, *15*, 188–200.
- 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*, *408*, 86–89.
- Reinhart, B. J., & Bartel, D. P. (2002). Small RNAs correspond to centromere heterochromatic repeats. *Science*, *297*, 1831.
- Kuwabara, T., Hsieh, J., Nakashima, K., Taira, K., & Gage, F. H. (2004). A small modulatory dsRNA specifies the fate of adult neural stem cells. *Cell*, *116*, 779–793.
- 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*, *75*, 843–854.
- Lau, N. C., Lim, L. P., Weinstein, E. G., & Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. *Science*, *294*, 858–862.
- Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., & Tuschl, T. (2003). New microRNAs from mouse and human. *RNA*, *9*, 175–179.
- Lee, Y., Ahn, C., Han, J., Choe, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., & Kim, V. N. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature*, *425*, 415–419.
- Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E., & Kutay, U. (2003). Nuclear export of microRNA precursors. *Science*, *303*, 95–98.
- Ying, S. Y., & Lin, S. L. (2005). Intronic microRNAs (miRNAs). *Biochemical and Biophysical Research Communications*, *326*, 515–520.
- Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., & Carthew, R. W. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, *117*, 69–81.
- Tang, G. (2005). siRNA and miRNA: an insight into RISCs. *Trends in Biochemical Sciences*, *30*, 106–114.
- Lambowitz, A. M., & Zimmerly, S. (2004). Mobile group II introns. *Annual Review of Genetics*, *38*, 1–35.
- Coghlan, A., & Wolfe, K. H. (2004). Origins of recently gained introns in *Caenorhabditis*. *Proceedings of the National Academy of Science USA*, *101*, 11362–11367.
- Harper, P. S. (1989). *Myotonic dystrophy*, 2nd edn. London: Saunders.
- Liquori, C. L., Ricker, K., Moseley, M. L., Jacobsen, J. F., Kress, W., Naylor, S. L., Day, J. W., & Ranum, L. P. (2001). Myotonic dystrophy type 2 caused by a CCTG expansion in intron 1 of ZNF9. *Science*, *293*, 864–867.
- 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*, *313*, 363–367.
- Kim, V. N. (2006). Small RNAs just got bigger: Piwi-interacting RNAs (piRNAs) in mammalian testes. *Genes & Development*, *20*, 1993–1997.
- Girard, A., Sachidanandam, R., Hannon, G. J., & Carmell, M. A. (2006). A germline-specific class of small RNAs binds mammalian Piwi proteins. *Nature*, *442*, 199–202.
- Aravin, A. A., Sachidanandam, R., Girard, A., Fejes-Toth, K., & Hannon, G. J. (2007). Developmentally regulated piRNA clusters implicate MILI in transposon control. *Science*, *316*, 744–747.
- Carmell, M. A., Girard, A., van de Kant, H. J., Bourc'his, D., Bestor, T. H., de Rooij, D. G., & Hannon, G. J. (2007). MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Developmental Cell*, *12*, 503–514.
- Grivna, S. T., Pyhtila, B., & Lin, H. (2006). MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in

- regulating spermatogenesis. *Proceedings of the National Academy of Science USA*, 103, 13415–13420.
39. Seto, A. G., Kingston, R. E., & Lau, N. C. (2007). The coming of age for Piwi proteins. *Molecular Cell*, 26, 603–609.
 40. Ruby, J. G., Jan, C. H., & Bartel, D. P. (2007). Intronic microRNA precursors that bypass Drosha processing. *Nature*, 448, 83–86.
 41. Lin, S. L., Chang, D., Wu, D. Y., & Ying, S. Y. (2003). A novel RNA splicing-mediated gene silencing mechanism potential for genome evolution. *Biochemical and Biophysical Research Communications*, 310, 754–760.
 42. 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 Journal*, 23, 4051–4060.
 43. Yi, R., Qin, Y., Macara, I. G., & Cullen, B. R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes & Development*, 17, 3011–3016.
 44. 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*, 115, 199–208.
 45. Khvorova, A., Reynolds, A., & Jayasena, S. D. (2003). Functional siRNAs and miRNAs exhibit strand bias. *Cell*, 115, 209–216.
 46. Jones, L., Hamilton, A. J., Voinnet, O., Thomas, C. L., Maule, A. J., & Baulcombe, D. C. (1999). RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. *Plant Cell*, 11, 2291–2301.
 47. Vaistij, F. E., Jones, L., & Baulcombe, D. C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. *Plant Cell*, 14, 857–867.
 48. Béclin, C., Boutet, S., Waterhouse, P., & Vaucheret, H. (2002). A branched pathway for transgene-induced RNA silencing in plants. *Current Biology*, 12, 684–688.
 49. Okamura, K., Ishizuka, A., Siomi, H., & Siomi, M. C. (2004). Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. *Genes & Development*, 18, 1655–1666.
 50. Kidner, C. A., & Martienssen, R. A. (2005). The role of ARGONAUTE1 (AGO1) in meristem formation and identity. *Developmental Biology*, 280, 504–517.
 51. Liu, J., Valencia-Sanchez, M. A., Hannon, G. J., & Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nature Cell Biology*, 7, 719–723.
 52. Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., & Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Molecular Cell*, 15, 185–197.
 53. Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., Harano, T., Yatabe, Y., Nagino, M., Nimura, Y., Mitsudomi, T., & Takahashi, T. (2004). Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Research*, 64, 3753–3756.
 54. Karube, Y., Tanaka, H., Osada, H., Tomida, S., Tatematsu, Y., Yanagisawa, K., Yatabe, Y., Takamizawa, J., Miyoshi, S., Mitsudomi, T., & Takahashi, T. (2005). Reduced expression of Dicer associated with poor prognosis in lung cancer patients. *Cancer Science*, 96, 111–115.
 55. Lai, E. C., Tomancak, P., Williams, R. W., & Rubin, G. M. (2003). Computational identification of Drosophila microRNA genes. *Genome Biology*, 4, R42.
 56. Brown, J. R., & Sanseau, P. (2005). A computational view of microRNAs and their targets. *Drug Discovery Today*, 10, 595–601.
 57. Miyagishi, M., Matsumoto, S., & Taira, K. (2004). Generation of and shRNAi expression library against the whole human transcripts. *Virus Research*, 102, 117–124.
 58. Nagl, S. B. (2002). Computational function assignment for potential drug targets: From single genes to cellular systems. *Current Drug Targets*, 3, 387–399.
 59. Gesellchen, V., & Boutros, M. (2004). Managing the genome: microRNAs in Drosophila. *Differentiation*, 72, 74–80.
 60. McManus, M. T. (2003). MicroRNAs and cancer. *Seminars in Cancer Biology*, 13, 253–258.
 61. Liu, C. G., Calin, G. A., Meloon, B., Gamliel, N., Sevignani, C., Ferracin, M., Dumitru, C. D., Shimizu, M., Zupo, S., Dono, M., Alder, H., Bullrich, F., Negrini, M., & Croce, C. M. (2004). An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proceedings of the National Academy of Science USA*, 101, 9740–9744.
 62. Miska, E. A., Alvarez-Saavedra, E., Townsend, M., Yoshii, A., Sestan, N., Rakic, P., Constantine-Paton, M., & Horvitz, H. R. (2004). Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biology*, 5, R68.
 63. Valoczi, A., Hornyik, C., Varga, N., Burgyan, J., Kauppinen, S., & Havelda, Z. (2004). Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Research*, 32, e175.
 64. Giraldez, A. J., Cinalli, R. M., Glasner, M. E., Enright, A. J., Thomson, J. M., Baskerville, S., Hammond, S. M., Bartel, D. P., & Schier, A. F. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. *Science*, 308, 833–838.
 65. Xu, P., Guo, M., & Hay, B. A. (2004). MicroRNAs and the regulation of cell death. *Trends in Genetics*, 20, 617–624.
 66. Jin, P., Alisch, R. S., & Warren, S. T. (2004). RNA and microRNAs in fragile X mental retardation. *Nature Cell Biology*, 6, 1048–1053.
 67. Landthaler, M., Yalcin, A., & Tuschl, T. (2004). The human DiGeorge syndrome critical region gene 8 and its *D. melanogaster* homolog are required for miRNA biogenesis. *Curr Biol*, 14, 2162–2167.
 68. Pardridge, W. M. (2004). Intravenous, non-viral RNAi gene therapy of brain cancer. *Expert Opinion on Biological Therapy*, 4, 1103–1113.
 69. Zamore, P. D. (2004). Plant RNAi: How a viral silencing suppressor inactivates siRNA. *Current Biology*, 9, R198–R200.
 70. Lecellier, C. H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saub, A., & Voinnet, O. (2005). A cellular microRNA mediates antiviral defense in human cells. *Science*, 308, 557–561.