Chapter 2
Small RNA Technologies: siRNA, miRNA, antagomiR, Target Mimicry, miRNA Sponge and miRNA Profiling

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Abstract The breakthrough discovery of RNA interference (RNAi) by Fire and Mello in 1998 has ushered in a new wave of RNA-based technological advances in the life sciences. Small RNAs, namely small interfering RNA (siRNA) and microRNA (miRNA), not only play key roles in down regulating gene expression, controlling growth and development, stress response, and various diseases, but also serve as essential tools for the study of gene functions. In this chapter, we provide an overview of the technological aspects of siRNAs and miRNAs and common methods for studying their functions.

Keywords siRNA, miRNA, antagomiR, target mimicry, miRNA sponge, miRNA profiling

2.1 Introduction

RNA interference (RNAi) was the 2006 Nobel Prize winning discovery [135], although related research is still at an early stage and continuing at a rapid pace. RNAi technology has become one of the most important technological tools and is

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now widely applied in almost every research aspect of modern biology. The key elements in RNAi are the small RNAs of ~21 nucleotides (nt), termed small interfering RNAs (siRNAs) [22–24, 39, 138]. SiRNAs regulate their target genes by binding in a sequence-specific manner to target gene transcripts (e.g. mRNAs), and inducing degradation of target RNAs or blocking their translations [20, 138]. The simple structure of siRNAs allows them to be easily generated in large quantities by chemical synthesis [22]. Synthetic siRNAs, as powerful reagents, bypass their biogenesis steps in RNAi and induce potent and specific silencing of any gene of interest in cells [22].

MicroRNA (miRNA), the siRNA cousin, is produced by RNAi-like mechanisms or miRNA pathways [3], and plays a key role in gene regulation, cell developmental control and various disease development [7]. More than 450 miRNAs encoded by the human genome have been identified and are predicated to regulate the expression of about one third of human coding genes [71, 130]. The development of many types of cancers or diseases is related to the abnormal expression or loss of certain miRNAs [11, 12, 17]. The global miRNA expression patterns of specific organs, tissues or cells can serve as an miRNA atlas or as biomarkers for the study of miRNA functions [65]. As a result, one can potentially reverse metastatic cancers by blocking the abnormally-expressed miRNAs or by reintroducing the lost miRNAs during cancer progression [80]. Much evidence indicates that many miRNAs function not only individually but also coordinately in gene regulation or in specific disease development [41]. Thus, this creates a new direction for studying a specific disease at the cellular level by simultaneously manipulating multiple miRNAs. Based on the study of the structures of miRNAs, artificial miRNAs have been developed as powerful tools for potently silencing genes in plants and animals [1, 89, 94, 101, 139, 140]. Compared to previous RNAi technologies, artificial miRNAs more accurately and specifically silence genes of interest and reduce off-target effects.

More than 5,000 miRNAs have been identified from various organisms (*http://microrna.sanger.ac.uk/*) [32, 33], but very few of them have been functionally analyzed. It remains a big challenge to discover the functions of most miRNAs currently stored in the database. The emergence of new technologies, such as high-throughput miRNA array, antagomiR [59], miRNA target mimicry and miRNA sponge [18, 21, 30] provide new tools to understand how, where and when miRNAs are generated and function in specific tissues, cells and organisms. This chapter gives an overview of these different small RNA technologies and their applications.

2.2 The Basic Biology and Chemistry of siRNAs and miRNAs and Their Related Working Mechanisms

SiRNA and miRNA are ~21–23 nt small RNAs produced in cells via a series of enzymatic steps. Long double-stranded RNA (dsRNA) or stem-loop structured RNAs introduced or transcribed in cells are first processed into siRNA or miRNA duplexes by a dsRNA specific RNase-III family of enzymes termed Dicers [137].

The small RNA duplexes are characterized by 2-nt overhangs at the 3′ ends with specific chemical end structures of a monophosphate at the 5′ end and a hydroxyl group at the 3′ end [44, 115]. In addition to differences in biogenesis, the siRNA duplex is a perfect duplex of Watson-Crick base pairs while the miRNA duplex is often an imperfect duplex with mismatches or bulges [114]. Both RNA duplexes need unwinding to form effector complexes: RNA-induced silencing complexes (RISCs) for their functionality. The key protein component of the effector complex is the Argonaute (AGO) protein of an evolutionally conserved protein super family [14]. The end structures of the small RNA duplex play a pivotal role in the assembly of the effectors or RISCs [76, 108]. After RISC assembly and maturation, specific gene transcripts, or messenger RNAs (mRNAs), are recognized and bound by the small RNAs on the RISCs based on sequence complementarities, leading to site-specific cleavage or translational repression of the mRNAs [28].

The elucidation of siRNA or miRNA chemical structures provides chemical approaches to generate them in large quantities for RNAi applications. Although single-stranded siRNA can be assembled into RISCs [81], synthetic siRNAs or miRNAs usually need to be double-stranded to be recognized by Dicer for initiating effective RISC assembly [77]. Thus, two short complementary RNA strands need to be synthesized and annealed to form siRNA or miRNA duplex characteristics with *bona fide* siRNA or miRNA end structures. In *Drosophila*, structurally distinct siRNA and miRNA duplexes are recognized by different Dicer proteins (DCR-1 and DCR-2) and sorted into distinct AGO proteins (AGO-1 and AGO-2) [29, 69, 91, 117]. DCR-2 sorts the siRNA duplex to be assembled into AGO-2 while DCR-1 sorts the miRNA duplex into the AGO-1 protein complex. Sometimes the two sorting systems are interchangeable based on siRNA or miRNA duplex structures or nucleotide compositions [29, 117].

SiRNA or miRNA duplexes can be divided into two types according to their end structures and thermodynamic stability: symmetric and asymmetric siRNA or miRNA [114]. A symmetric siRNA or miRNA assembles into two kinds of RISCs with either a sense strand or an antisense strand and can potentially interact with two different complementary target mRNAs for regulation [114]. However, siRNAs or artificial miRNAs are normally designed to target one specific gene transcript rather than two. Thus, symmetric siRNAs or miRNAs have a higher chance of targeting unwanted mRNAs due to their ability to target two different gene transcripts, leading to off-target effects. In contrast, asymmetric siRNAs or miRNAs preferentially favor only one specific strand of the small RNA duplex assembled into a RISC while the other strand is excluded from RISCs and is subsequently degraded [102]. This allows a maximum assembly of one specific strand of small RNA duplex into RISC components, considerably reducing off-target effects coming from the RISC assembled by the unwanted strand.

Intriguingly, a major fraction of endogenous miRNAs are structurally asymmetric and display high specificity in the regulation of their target genes [50, 102], although a substantial number of miRNAs seem to have dual functions coming from each strand of the miRNA duplex (personal communication with Eric Lai). The asymmetric character seems a result of natural selection to reduce unwanted

off-target effects for fine control of development during evolution. The asymmetric structure of endogenous miRNAs has been successfully adopted to design highly efficient artificial miRNAs to target genes of interest in plants and animals [1, 89, 94, 101, 139, 140]. Specific programs have also been developed to help design highly specific artificial miRNAs [101].

How can asymmetric siRNAs or miRNAs ensure that one specific strand is going into the RISC and excluding the other strand to prevent off-target effects? Recent research results indicate that RISC assembly and activation involve strand selection and exclusion by the RISCs. In the case of siRNAs, guide strand-associated RISCs exclude the passenger strand by guide-associated RISC directed cleavage of the passenger [70, 82, 95]. This cleavage has the same characteristics of RISC-directed target mRNA cleavage. That is, the cleavage site on the passenger strand is between the bases 10 and 11 from the 5′ end of the guider siRNA strand [24]. However, this cleavage depends on several prerequisites [82]. First, the AGO protein on the RISC must have "slicer" (endonuclease) activity. Second, there must be no mismatches or bulges between the guide strand and the passenger strand around the cleavage site. Thus, miRNA duplexes, which naturally have bulges or mismatches around the potential cleavage sites, likely employ other mechanisms to exclude and eliminate the passenger strand. This mechanism should be also true for RISCs on which the slicer activity is missing. In such cases, it remains unknown how the passenger strands are degraded after their exclusion from the RISCs, rather than cleaved by the slicer.

2.3 MiRNA Functional Analysis and miRNA Inhibitors

Like transcription factors, miRNA-directed regulation of post-transcriptional gene expression is wide-ranging [41]. First, the expression of many transcription factors themselves are regulated by miRNAs in plants and animals. Second, miRNA-directed gene regulation seems more extensive in animals than in plants due to distinct working mechanisms. One third of the human protein-coding genes are predicted to be regulated by different miRNAs [71, 130], but relatively very few miRNA-target interactions have been experimentally validated. Most identified or predicted miRNAs are functionally unknown. Thus, the study of miRNA functions constitutes a unique aspect of miRNA genomics. Due to the small size of miRNAs and their functions associated with specific target genes, functional analysis of miRNAs is substantially different from coding genes and as a result, various methods have been developed.

An earlier approach to the study of miRNA targets was focused on computational predications based on the base-pairing conditions between miRNAs and their target genes [16, 51, 56, 96, 100, 106, 123, 124, 131, 134, 141]. The completion of the whole genome sequencing of various organisms expedited the discovery of new miRNA genes and their targets by a computational approach. The regulatory roles of miRNAs are reflected in the cellular functions of miRNA target genes.

The prediction that one-third of human protein-coding genes are miRNA targets was mainly based on the assumption that a short stretch of "seed" region (~7 base pairs of the position 2 to 8 from the 5′ end of the miRNAs) between miRNAs and the 3′ untranslated region (UTR) of their targets is sufficient for regulation [71, 72, 130]. In contrast, regulation of target gene transcripts in plants by miRNAs seems predominantly reliant on extensive sequence complementarities between the miRNAs and their target genes. Thus, the number of predicted miRNA targets in plants is much more limited [98]. The outcomes of the interactions between plant miRNAs and their targets, in most cases, are the cleavage and degradation of the target mRNAs. Thus, validation of plant miRNA targets is relatively simple and can be done by assaying for target cleavage in vitro and in vivo.

The first validations of miRNA-target interactions were reported in plants [78, 115]. Two experimental approaches were established for these validations: direct visualization of the target cleavage *in vitro* and cloning of the target cleavage products *in vivo* by 3′ or 5′ RACE-PCR. Traditional wheat germ extracts or newly established maize germ extracts (G. Tang, 2007) are convenient cell-free systems for miRNA target validation. Both systems contain abundant endogenous miRNAs that have already been loaded on the RISCs, and exogenous target mRNAs are readily cleaved by the existing miRNA-associated RISCs. However, these cell-free systems have limitations when validating miRNA-target interactions whose corresponding miRNAs do not exist in wheat or maize germ extracts. *In vitro* programming of active RISCs by synthetic miRNA duplexes in plant systems needs further exploration [83, 93, 115]. In contrast, *Drosophila* embryo extracts are capable of RISC assembly programmed by various kinds of small RNAs and can thus serve as a platform for animal miRNA target validations, as well as a useful heterologous system for validation of plant miRNA targets [38].

The validation of animal miRNA-target interactions is not trivial. First, most miRNA targets in animals are not directly cleaved but rather translationally repressed by miRNA-associated RISCs. Therefore, the validation needs to be conducted at the protein level. Detection of the change in protein expression of the target genes using specific antibodies is preferred for such validation [143]. Alternatively, reporter genes are often fused with the 3′ UTR of the target mRNAs for the purpose of validating translational repression by miRNAs [58]. A mammalian cell-free system was recently developed to recapitulate *let-7* miRNA-directed translational repression *in vitro*, which will be useful in the validation of animal miRNA-target interactions [121]. This in vitro system was established with extracts from HEK293F cells transfected with expression vectors that contain genes encoding various miRNA pathway components, such as Dicer, TRBP2, Argonaute2 and GW182. This system is capable of processing chemically synthesized *let-7* miRNA precursors into mature let-7 that is likely to be further assembled into RISC to direct translational repression. Based on this system, Wakiyama et al. found that *let-7* miRNP complexes induced the deadenylation of the let-7 target mRNAs and the abolishment of cap-poly(A) synergy, leading to target mRNA translation blockage [121]. These in vivo and in vitro approaches are often used together to validate animal miRNA targets.

Approaches to inducing a loss of function of miRNAs represent a powerful functional genomics tool. Traditional screening for mutation of miRNA genes has not proved very successful. This is because miRNAs are often encoded by multigene family members and the loss of function of one miRNA member is often obscured by redundant functions of other miRNA members that have almost identical sequences and the ability to bind and regulate the same target gene transcripts. Fortunately, different methods and strategies have been developed to block the regulatory functions of all members of any miRNA family. In vitro chemically modified miRNA inhibitors, such as 'antagomirs', or in vivo target mimicry in planta and miRNA sponge in mammalian cells have recently proved to be effective in blocking functions of specific miRNA families [21, 30, 59].

Chemically modified oligonucleotides have been widely used in the study of the loss-of-function of miRNAs. Based on antisense strategy, oligonucleotides complementary to the miRNAs act as competitive inhibitors of endogenous target mRNA binding to the miRNAs, leading to a suppression of miRNA functions. These have been developed and demonstrated to be very specific and potent inhibitors of targeted miRNAs [45, 59]. The major modification in antagomirs is 2′-O-methylation of the ribose, sometimes combined with other kinds of modifications such as phosphorothioate linkage near 5′ and 3′ ends and a cholesterol-moiety conjugated at 3′end. Antagomir, usually 21–33 nt in length, sequence-specifically binds to specific target miRNAs through base-pairing [45, 59]. A traditional modified antisense oligo, such as morpholino that was previously used to knock down the expression of protein coding gene transcripts, also successfully knocked down specific miRNAs by binding to the miRNAs or their precursors [54]. These chemically modified miRNA antisense RNAs can effectively compete with miRNA target mRNAs by a stronger bind to their specific target miRNAs on the miRISCs, resulting in inhibition of the miRNA activities. Antagomirs also induced the degradation of the targeted miRNAs with as of yet unknown mechanisms [57, 59]. Antagomirs were delivered to mouse tissues via intravenous injections, absorbed by tissues, and were highly resistant to various RNAses in cells. It was shown that antagomirs specifically inactivated multiple target miRNAs in various mouse tissues for over 20 days following a single intravenous injection, resulting in changes in the abundance of distinct target mRNAs [57, 59]. The introduction of antagomirs against specific miRNAs in cells will release the repression of the *bona fide* miRNA target mRNAs from translation into proteins, thus indirectly validating the miRNA targets.

The strategy of target mimicry to block miRNA functions was enlightened by a study of the interactive relationships between the phosphate (Pi) starvation-induced miR-399 and its naturally occurring RNA transcripts from *IPS1* gene in *Arabidopsis thaliana* [30]. *IPS1* contains a 23-nucleotide motif that is almost complementary to miR-399 but with a mismatched loop at the expected miRNA-directed cleavage site. Over-expressed *IPS1* RNAs can bind to mature miR-399 associated RISCs, and prevent miR-399 mediated cleavage of the target mRNAs, including *PHO* mRNA. Mutation of the *IPS1* motif to be perfectly complementary to miR-399 abolished *IPS1* inhibitory activity on miR-399, indicating that miR-399 associated RISCs are highly efficient and multiple-turnover enzymes to cleave their perfectly

complementary targets, but may be stuck in the interaction between RISCs and their non-cleavable targets. This research not only revealed the phenomenon that miRNA activity in planta can be regulated by its mimic non-coding RNA, but also provided a genetic tool for the miRNA functional analysis. That is, introduction of an artificial non-cleavable miRNA target mimic is capable of knocking down complementary miRNA activity *in vivo*. Using this strategy and the *IPS* gene backbone [30], successfully inhibited the activities of the miR-156 and miR-319 separately by over-expressing target mimicries with non-cleavable sites for these miRNAs, respectively, in *Arabidopsis thaliana*.

The principle of 'miRNA sponges' developed by [21] is similar to target mimicry of miRNAs described in plants. Artificial target RNAs are designed to contain several tandem complementary binding sites to the miRNA of interest, but with a bulge or mismatch in the RISC cleavage site, and are genetically engineered to be stably expressed in mammalian cells. These artificial RNAs, like sponges, absorb a high level of their complementary miRNAs and release the translational repression of the bona fide targets by the miRNA-associated RISCs (miRISCs). The mismatch in the cleavage site prevents the decoy RNA from being degraded by miRISCs, but binds more firmly to the target miRNA loaded in RISC, sequestering it away from its *bona fide* mRNA targets in the cell. These miRNA sponges are experimentally proven to function as highly competitive miRNA inhibitors and depress miRNA targets effectively in mammalian cells. In addition, the sponges can be designed to bind effectively to multiple miRNAs that contain the same "seed' region (position 2–8 from the 5′ end of the miRNA) [21].

2.4 SiRNA and miRNA Vectors and Their Application in Gene Silencing

Various siRNA vectors have been developed for knocking down genes in plants and animals since the discovery of RNAi [2, 26, 49, 68, 85, 97, 109, 110, 126, 128]. SiRNA vectors are able to generate siRNAs transiently or consistently from double-stranded RNAs or hairpin/stem-loop structure via Dicer enzymes targeting specific gene transcripts in various tissues or cells. However, not all siRNA vectors work well for both plants and animals. For example, the most popular siRNA vectors for gene silencing in plants are inverted repeat sequences coupled with a linker or a spliceable intron between the two repeats to form long (>100 bp) RNA hairpins [27, 62, 107, 125]. Yet, these vectors are not applicable in animal cells because the long double-stranded RNAs (dsRNA) generated from these vectors trigger cellular interferon pathways and lead to non-specific programmed cell death. Consequently, vectors that produce short hairpin RNAs (shRNAs) but which rarely trigger the interferon pathway were developed and have become widely used in animal studies [110, 129]. Similar shRNA vectors were also successfully applied toward directing gene silencing in plants [79]. Most shRNA vectors currently use an RNA polymerase III (Pol III) promoter, usually U6 or H1, and a pol III terminator (a stretch of thymidines)

to transcribe a short hairpin structure with a stem of 19–29 bp and a short loop of 4–10 nt. The shRNA structures are further recognized and cleaved by Dicers to produce a large amount of siRNAs in cells to target specific gene transcripts.

Later on, with the discovery of miRNA genes that produce highly specific short miRNAs, earlier vectors that produce short hairpin structured siRNAs were further modified by using miRNA backbones [9]. The new vectors adapted the flanking and loop regions of endogenous miRNAs to express miRNA-like primary transcripts (pri-miRNA-like RNAs) using pol-II promoters and terminators. These modified miRNA-like small RNA vectors have been demonstrated to be more effective in gene knock-down by many folds, indicating the miRNA backbones are structurally predisposed to produce more effective small RNAs for gene silencing [9].

The most significant discovery that helped to revolutionize small RNA vector technology was the discovery of the asymmetric structures of siRNAs [50, 102]. The realization that most endogenous miRNAs are structurally asymmetric immediately prompted the birth of second-generation RNAi vectors, artificial miRNA vectors [1, 89, 94, 101, 139, 140]. The major difference between miRNA backboned siRNA vectors and artificial miRNA vectors is found on the stem region of the stem-loop structured RNAs produced by both kinds of vectors. While the stem regions of the stem-looped structured RNAs produced by the miRNA backboned siRNA vectors are perfect Watson-Crick base pairs, the ones from the miRNA vectors are often designed to be imperfectly matched with mismatches, GU wobbles, and bulges. The biogenesis of artificial miRNAs produced by miRNA vectors strictly follows the miRNA pathways distinct from the RNAi pathways in cells. Thus, artificial miRNA vectors can be used not only to silence most protein coding genes, but also to knock down genes encoding the enzymes/proteins of RNAi pathways.

Today, artificial miRNAs are widely used for gene silencing in both plants and animals. Compared to siRNA vectors, artificial miRNA vectors have significant advantages, including high specificity, fewer off-target effects, tissue-specific expression and almost no side effects. In contrast to the vectors that are used to silence individual genes, various modified vectors that direct simultaneous silencing of multiple genes have also been developed. For example, modified multi-hairpin structures of miR-30 have successfully knocked down multi-genes in a single construct [111]. We expect future gene silencing vectors to be more powerful in fine-tuning silencing of genes with subtle differences for various therapeutic purposes.

2.5 MiRNA Profiling and miRNA Biomarkers

Over 5,000 miRNAs from 44 organisms have been identified/predicted and stored in the miRBase registry (*http://microrna.sanger.ac.uk/*) [32, 33]. Most of them are not characterized by function. Determination of miRNA functions and miRNA-target interactions is therefore a long-term objective. Roles of miRNAs in post-transcriptional gene regulation are presumed to be extensive. In humans, an individual miRNA is predicted to regulate hundreds of coding gene transcripts [10, 71, 130]. Furthermore,

transcripts from a single gene can be regulated by multiple miRNAs in a coordinated manner [25, 42, 48]. Analysis of miRNA expression profile is thus important in elucidating roles of specific miRNA or miRNA cohorts in the regulation of their target gene transcripts.

Like the expression of mRNAs, the expression of miRNAs varies considerably from different cells, tissues, organs and species [63, 64]. The big challenge of studying miRNAs is how to effectively analyze thousands of miRNAs simultaneously for a limited amount of given samples. Traditional microarray technology has succeeded in analyzing entire protein-coding gene transcriptomes in various organisms. Similarly, various laboratories and companies have adopted the traditional mRNA array platform for the analysis of miRNAs over the last few years, allowing thousands of miRNAs to be analyzed from samples including plants and animals [6, 13, 15, 36, 47, 55, 67, 73–75, 84, 86, 87, 103–105, 113, 116, 122, 142].

The technologies to be used for miRNA array are not trivial. Compared to array analysis for mRNA expression, miRNA arrays usually involve much more complicated procedures due to the small size of the miRNAs and the lack of a conserved 3′ end for easy sample labeling. These complicated steps include the ligation of RNA adapters to the miRNAs, RT-PCR amplification and T7 RNA polymerase transcription [19, 87]. Current non-isotope miRNA array platforms involve these complicated techniques and require special skills; moreover, process-related systematic biases are unavoidable [19]. To simplify these steps, we have recently optimized the conditions for an earlier version of an isotope labeled miRNA array platform [55, 86], and further developed this system demonstrating its use in mouse miRNA analysis [116]. This optimized miRNA array platform is characterized by several unique features: (1) a careful selection of miRNAs for probe design to reduce potential cross-hybridization between different probes; (2) isolation of small RNAs of 15–28 nt using a 15% sequencing gel to avoid interference of signals between pre-miRNAs and pri-miRNAs; (3) direct labeling of the isolated small RNAs at their 5′end by introducing isotope-labeled phosphates to avoid using adaptors and biased PCR amplification, and directly hybridizing the labeled small RNAs to the membrane containing arrayed miRNA probes. Results can be output as a visual miRNA atlas reflecting the bonafide level of miRNAs in cells; and (4) introduction of a new way of data normalization by using northern blot analysis of a constitutively expressed miRNA for initial data adjustment, and by a set of external controls for the evaluation of process-related loss of signal and quantification of endogenous miRNAs.

Application of various miRNA array platforms reveals numerous miRNA biomarkers for a variety of human diseases including various kinds of cancers. The expression changes of these miRNA biomarkers indicate changes from normal to abnormal genetic and physiological conditions. For example, a specific spectrum of miRNAs including miR-23, -24, -26, -27, -103, -107, -181, -210, and -213 were induced in neoplastic cells under a hypoxic environment compared to normal conditions [61]. Similarly, comparisons of miRNA profiles of tumor and normal tissues have revealed distinct miRNA biomarkers for various tumor cells over the last a few years [11, 12, 46, 60, 112, 120, 132]. Exploration of these miRNA biomarkers will be particularly useful in early diagnostics of human diseases such as cancers,

diabetes, and Alzheimer's disease. Eventually, detailed miRNA atlases of humans, animals and plants will be available with the help of small RNA deep sequencing and miRNA array platforms for a wide variety of applications.

2.6 Conclusions and Perspectives

In conclusion, the study of RNAi and miRNAs has led to a number of small RNA technologies that are and will continue to be extremely useful in the study of small RNAs, individual gene functions, functional genomics, and various biological questions in both plants and animals. The dissection of RNAi, miRNA, and other small RNA pathways is only the beginning. For most organisms, the detailed maps or atlases of small RNAs have not been completed or even started. The small RNA networks in gene regulation are still obscure, and the entire picture of gene regulation by small RNAs and their detailed regulatory steps is a long-term goal. With a deeper understanding of the roles and mechanisms of small RNA-directed gene regulation, new technologies of using small RNAs will continue to be developed and established. We expect that such small RNA technologies will be expanded from the current siRNA or artificial miRNA directed post-transcriptional gene regulation to transcriptional gene regulation, for example, small RNA directed chromatin modifications or DNA methylation, to modulate the expression of any gene of interest. Small RNA technologies will not only used as tools to silence genes of various pathways but also genes of small RNA pathways themselves. For example, since RNAi and miRNA generally belong to different pathways that composed of different sets of enzymes, we expect that RNAi technology will be used to study functions of genes of the miRNA pathway, and vice verse. In addition to siRNAs and miRNAs, other kinds of small RNAs, such as trans-acting siRNAs (ta-siRNAs) [31, 40, 43, 90, 119, 127], repeat-associated small interfering RNAs (rasiRNAs) [37, 52, 53, 92, 118], and piwi-associated siRNAs (piRNAs) [4, 5, 8, 34, 35, 66, 88, 99, 133, 136], may also be able to be used for silencing of genes of interests.

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