

Chapter 2

miRNA Interference Technologies: An Overview

Abstract The miRNA pathways are highly responsive to interventions of any kind, being excellent candidates for pharmacological manipulation. Mature miRNAs belong to a category of non-coding small RNAs. They are very different from traditional messenger, ribosomal, or transfer RNA in their biogenesis, structures and functions. The technologies used to study traditional RNAs in basic research and to manipulate traditional RNAs for therapeutic purpose, for the most part, would be ineffective for miRNAs. New technologies for studying miRNA are therefore in need and this demand is being met by the scientific community at a breakneck pace. In the past few years, from the discovery of miRNAs in humans, numerous new technologies have been developed to clone, profile, visualize, quantify and manipulate miRNAs. This book centers on the technologies for manipulating miRNAs.

An overview of the concept of microRNA interference (miRNAi) and another three related concepts and a brief introduction to the miRNAi technologies is given in this chapter, to help readers better understand the technologies. The applications of these technologies to miRNA research and the potential for gene therapy of related diseases are also summarized and speculated. Detailed descriptions of these technologies are provided in the following chapters.

2.1 New Concepts of miRNAi Technologies

2.1.1 “miRNAi”, A New Concept

RNA interference (RNAi) is a well-known strategy for gene silencing; this strategy takes the advantages of the capability of small double-stranded RNA molecules (siRNAs) to bind RNA-induced silencing complex (RISC) on the one hand and to bind target genes (mRNAs) on the other (Xia et al. 2002; Golden et al. 2008; Pushparaj et al. 2008). Through such dual interactions, siRNAs elicit a powerful knockdown of gene expression by degrading their target mRNAs. Two key

characteristics of the RNAi strategy are that the only target of RNAi is mRNAs and that the only outcome of RNAi is silencing of mRNAs. In other words, the RNAi strategy uses siRNAs to interfere directly with mRNAs (mostly protein-coding genes) to silence gene expression.

Taking the same concept, I propose a new concept of microRNA interference (Wang et al. 2008). miRNAi manipulates the function, stability, biogenesis, or expression of miRNAs and as such it indirectly interferes with the expression of protein-coding mRNAs. This concept is based on the thoughts outlined below. The fundamental mechanism of miRNA regulation of gene expression is miRNA:mRNA interaction or binding. A key to interfere with miRNA actions is to disrupt or to facilitate the miRNA:mRNA interaction. In order to achieve this aim, one can either manipulate or to facilitate miRNAs or mRNAs to alter the miRNA:mRNA interaction. For miRNAs, one can either mimic miRNA actions to enhance the miRNA:mRNA interaction or to inhibit miRNAs to break the miRNA:mRNA interaction. Additionally, one can also manipulate mRNA to interrupt the miRNA:mRNA interaction.

I further propose to call the strategies for interfering with miRNAs miRNAi technologies. miRNAi technologies can be categorized based on the following six different perspectives.

1. According to the mechanisms of actions, miRNAi technologies can be divided into two major strategies: miRNA-targeting and targeting-miRNAs strategies.

The “miRNA-targeting strategy of miRNAi” refers to the approaches producing “gain-of-function” of miRNAs to enhance gene targeting so as to alter the gene expression and cellular function (Chaps. 3–6).

The “targeting-miRNA strategy of miRNAi” refers to the approaches leading to “loss-of-function” of miRNAs via inhibiting miRNA expression and/or action to alter the gene expression and cellular function (Chaps. 7–13).

2. In terms of the outcome of actions, miRNAi technologies can be grouped into “miRNA-gain-of-function” (enhancement of miRNA function) and “miRNA-loss-of-function” (inhibition of miRNA expression, biogenesis, or function).

The “miRNA-gain-of-function” approach is in general achieved by overexpression of the endogenous miRNAs and forced expression of exogenous miRNAs, which results in enhanced miRNA targeting (Chaps. 3–6).

The “miRNA-loss-of-function” approach can be conferred through targeting-miRNAs by knockdown or knockout of miRNA expression and inhibition of miRNA action (Chaps. 7–13).

3. With respect to the target miRNAs, miRNAi technologies can be miRNA-specific or non-miRNA-specific.

The miRNA-specific miRNAi technologies interfere only with miRNA function and through this, they produce derepression of target protein-coding genes. They belong to the “targeting-miRNA” strategy being miRNA-specific but non-gene-specific.

The non-miRNA-specific miRNAi technologies interfere with the biogenesis of miRNAs such as inhibition of Dicer, affecting the levels of the whole population

of miRNAs but not a particular miRNA. These approaches are therefore neither miRNA-specific nor gene-specific.

4. As to the target genes of miRNAs, miRNAi technologies comprise non-gene-specific and gene-specific approaches, which can be either “gain-of-function” of miRNAs or “loss-of-function” of miRNAs.

The non-gene-specific miRNAi technologies direct their actions to miRNAs without interaction with the target genes of the miRNAs. Such actions are miRNA-specific but not gene-specific given that a miRNA can target multiple protein-coding genes. Most of the currently available miRNAi technologies belong to non-gene-specific approaches.

The gene-specific miRNAi acts on the target gene of a given miRNA but not on the miRNA per se. This is achieved by enhancing or removing the actions of miRNAs but leaving miRNAs intact.

5. From the perspective of target protein-coding genes (mRNAs), miRNAi technologies may result in either “mRNA-gain-of-function” (derepression of genes) or “mRNA-loss-of-function” (enhancement or establishment of repression).

The “targeting-miRNA” strategy causes “mRNA-gain-of-function” by relieving the repressive actions induced by the targeted miRNAs. The “mRNA-gain-of-function” miRNAi technologies could be gene-specific or non-gene-specific but they must be miRNA-specific.

The “miRNA-targeting” strategy causes “mRNA-loss-of-function” effects by enhancing the repressive actions of the miRNAs. The “mRNA-loss-of-function” miRNAi technologies could be gene-specific or non-gene-specific but they must be miRNA-specific.

6. Taking the mode of actions into consideration, miRNAi technologies can act by creating AMO:miRNA interaction (AMO: anti-miRNA antisense oligomer) or by creating ASO:mRNA interaction (ASO: anti-mRNA antisense oligomer) to disrupt normal miRNA:mRNA interaction.

The AMO:miRNA-interacting miRNAi technologies suppress miRNA function using AMO approaches. They are all miRNA-specific and non-gene-specific, belonging to the targeting-miRNAs strategies and the miRNA-loss-of-function and mRNA-gain-of-function class.

The ASO:mRNA-interacting miRNAi technologies interrupt miRNA function using ASO approaches. They belong to the targeting-miRNAs strategies and the miRNA-loss-of-function and mRNA-gain-of-function class.

Based on above classifications, some miRNAs are both miRNA- and gene-specific, some are miRNA-specific but non-gene-specific and others are neither miRNA-specific nor gene-specific. Table 2.1 summarizes the six miRNAi strategies. In the following chapters, each miRNAi technology will be specified for its classification.

Table 2.1 General Introduction to miRNAi

Mechanism of action	miRNA-targeting	producing “miRNA-gain-of-function” to enhance “mRNA-loss-of-function”
	Targeting-miRNAs	producing “miRNA-gain-of-function” to enhance “mRNA-loss-of-function”
Mode of action	AMO:miRNA interaction	anti-miRNA oligomer inhibiting miRNA level or activity
	ASO:mRNA interaction	anti-mRNA oligomer inhibiting miRNA function
Outcome of miRNA	miRNA-gain-of-function	enhancement of miRNA function
	miRNA-loss-of-function	inhibition of miRNA expression, biogenesis, or function
Outcome of target gene	mRNA-gain-of-function	causing derepression of protein-coding genes
	mRNA-loss-of-function	enhancing repression of protein-coding genes
miRNA specificity	miRNA-specific	targeting a specific miRNA or a specific group of miRNAs
	Non-miRNA-specific	targeting the whole population of miRNAs
Gene specificity	Non-gene-specific	a miRNA targeting multiple genes with its binding site
	Gene-specific	a miRNA targeting a particular gene

2.1.2 “miRNA as a Regulator of a Cellular Function”, Second New Concept

As already mentioned in the previous chapter, with respect to its pathophysiological role, a miRNA can be viewed as a regulator of a particular cellular function or a particular cellular program, not of a single gene. I propose this concept based upon the following facts (Wang et al. 2008).

1. Each miRNA normally has multiple target genes on the one hand and each gene may be a target for multiple miRNAs on the other hand. Focusing on any particular one of the target genes may lead to incomplete understanding of function of a miRNA.
2. The target genes of a given miRNA may encode proteins that have different or even opposing functions (e.g., cell growth vs. cell death). Focusing on any particular one of the target genes could be misleading to our understanding of exact function of a miRNA. For example, the oncoprotein Bcl-2 and the tumor suppressor p53 are both the target genes of miR-150 and miR-214. If one looks at only Bcl-2, then one may conclude that both miR-214 and miR-150 are miRNA tumor suppressors; conversely, if one focuses on p53, then one will believe both miR-214 and miR-150 to be oncomiRs: two contradictory views of the same miRNAs.

3. The net outcome of integral actions of a miRNA on its multiple target genes defines the function of that miRNA. Following this idea, whether miR-214 and miR-150 are tumor suppressors or oncomiRs will depend upon which of the target genes p53 and Bcl-2 predominates under a particular cellular context. If the effects on p53 predominate, then miR-214 and miR-150 are oncomiRs but if it is the other way around, then miR-214 and miR-150 are tumor suppressors.

Therefore, a more reasonable, correct view of function of a miRNA is that a miRNA is a regulator of a cellular function or a cellular program.

This concept is important to keep in mind when designing or applying miRNAi technologies in one's studies.

2.1.3 “One-Drug, Multiple-Target”, Third New Concept

Human diseases are mostly multifactorial and multistep processes. Targeting a single factor (molecule) may not be adequate and certainly not optimal in disease therapy, because single agents are limited by incomplete efficacy and dose-limiting adverse effects. If related factors are concomitantly attacked, better outcomes are expected and the combination pharmacotherapy has been developed based on this reasoning: a combination of two or more drugs or therapeutic agents given as a single treatment that produces improved therapeutic results. The “drug cocktail” therapy of AIDS is one example of such a strategy (Henkel 1999), and similar approaches have been used for a variety of other diseases, including cancers (Konlee 1998; Charpentier 2002; Ogihara 2003; Kumar, 2005; Lin et al. 2005; Nabholz and Gligorov 2005). However, the current drug-cocktail therapy is costly and may involve a complicated treatment regimen, undesired drug-drug interactions and increased side effects (Konlee 1998). There is a need to develop a strategy to avoid these problems.

In 2006, I proposed the “One-Drug, Multiple-Target” concept: a single agent capable of acting on multiple selected key targets to treat a disease (Gao et al. 2006). One example of such a drug is amiodarone, a class III antiarrhythmic agent. While most of the specific ion channel blockers can increase mortality by producing *de nova* lethal arrhythmias, amiodarone, by targeting multiple ion channels without channel specificity, can produce beneficial antiarrhythmic effects devoid of an increase in the risk of mortality. However, with respect to its highly desirable actions, amiodarone is an unexpected, accidental pharmaceutical product, not from a purposed design; it is nearly impossible to confer to single compounds the ability to act on multiple target molecules with the traditional pharmaceutical approaches or the currently known antigene strategies. My laboratory developed a complex decoy oligonucleotides strategy based on the “One-Drug, Multiple-Target” concept, which has demonstrated its superiority to inhibit tumor growth. In theory, the “One-Drug, Multiple-Target” strategy mimics the well-known drug cocktail therapy of AIDS. Nevertheless, this “One-Drug, Multiple-Target” strategy may be devoid of the weaknesses of the drug cocktail therapy, without involving complicated

treatment regimens, undesirable drug-drug interactions and increased side effects. It opens the door to one-drug, multiple-target intervention, providing promising prototypes of gene therapeutic agents for a wide range of human disease.

I introduce here this concept because several of the miRNAi technologies have been developed based on this concept. Indeed, considering the fact that a single miRNA has the potential to regulate multiple target genes and a single gene may be regulated by multiple miRNAs, it is often necessary to interfere with multiple miRNAs or multiple genes to acquire effective manipulation of gene expression and cellular function. The “One-Drug, Multiple-Target” strategy opens up new opportunities for creative and rational designs of a variety of combinations integrating varying miRNA-related elements for a therapeutic purpose and provides an exquisite tool for functional analysis of miRNAs in a gene controlling program. It can also be used as a simple and straightforward approach for studying any other biological process involving multiple factors, multiple genes and multiple signaling pathways.

2.1.4 “miRNA Seed Family”, Another New Concept

We have introduced the “Seed Site” concept proposed by Lewis et al. (2003, 2005) in Sect. 1.2 to define the mechanism of target recognition and action of miRNAs (i.e., miRNA:mRNA interactions). Based on this concept, miRNAs possessing a same seed motif (5'-end 2–8 nts) should have the same repertoire of target genes thereby the same cellular function. This concept has indeed been verified by numerous experimental investigations.

For the sake of easiness and clarity in understanding the function of miRNAs, I propose to categorize miRNAs into families based on their function or seed motifs. According to this classification system, miRNAs with a same seed motif 5'-end 2–8 nts are grouped into the same miRNA seed family. Further, miRNAs carrying exactly the same seed motif are grouped into the same miRNA seed subfamily. For example, miR-17-5p and miR-20b have identical 5'-end 1–8 nts seed sequence CAAAGUGC; miR-520g and miR-520h have ACAAGUG; miR-20a and miR-106b contains UAAAGUGC; miR-106a, AAAAGUGC; miR-93, miR-372 and miR-520a-e all have AAAGUGCU; miR-519b and miR-519c have AAAGUGCA. Intriguingly, if 7 of 8 nts in the seed motif base-pairing with target genes is sufficient to produce post-transcriptional repression (as already shown by an enormous volume of studies) (Lewis et al. 2003, 2005; Pillai et al. 2007), then these six seed motifs should all give the same cellular effects. Based on this view, I consider all these miRNAs as the members of one miRNA seed family while belonging to six different subfamilies.

This classification provides a guideline of pivotal importance for interfering with a cellular process involving gene expression regulation by a multi-member miRNA seed family. Enhancing or inhibiting any one of the members of a miRNA seed family may not be able to elicit efficient, thorough changes of gene expression and cellular function. In this case, manipulation of all members of a miRNA seed family

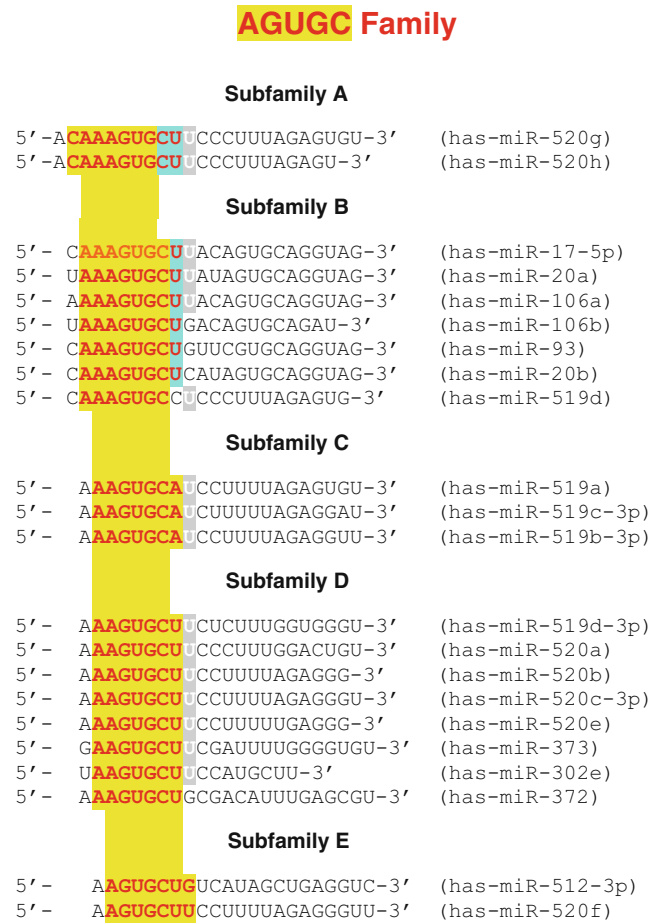


Fig. 2.1 An example of miRNA seed family concept. The seed site of each listed miRNA is shown in red-bold-face and highlighted in yellow. The miRNAs bearing exactly the same seed sites (5'-end 2–7 nts) are grouped into a subfamily. Assuming that 6-nt base-pairing out of 7-nt seed site is sufficient to produce gene targeting actions, these five subfamilies labeled A–E are supposed to have same target genes and cellular functions and they are therefore placed into one same family

is definitely required to achieve a level with sufficient miRNA-promoting effects or anti-miRNA effects.

We have effected out all miRNAs registered in miRBase by their seed motifs and are able to categorize these miRNAs into 498 seed families. For convenience, I designate these families according to their 4–7 nts (Fig. 2.1). Some families contain subfamilies with varying number of miRNAs and some currently contain only one member.

2.2 General Introduction to miRNAi Technologies

2.2.1 *miRNA-Targeting Technologies*

An array of technologies has been developed for achieving gain-of-function of miRNAs by forced expression or overexpression. These include Synthetic Canonical miRNA technology, miRNA Mimic technology, Multi-miRNA Hairpins technology, Multi-miRNA Mimics technology and miRNA Transgene technology.

The Synthetic Canonical MiRNA technology involves an application of synthetic miRNAs that are identical in sequence to their counterpart endogenous miRNAs (Luo et al. 2007, 2008; Yang et al. 2007; Xiao et al. 2007a).

The key for the miRNA Mimic technology is to generate non-naturally existing double-stranded RNA fragments that are able to produce miRNA-like actions: the post-transcriptional repression of gene expression or inhibition of protein translation (Xiao et al. 2007b). It is a gene-specific miRNA-targeting strategy.

The Multi-miRNA Hairpins technology uses a single artificial construct in the form of double-stranded RNA to produce multiple mature miRNAs once introduced into cells (Sun et al. 2006; Xia et al. 2006).

The Multi-miRNA Mimics technology incorporates multiple miRNA Mimic units for targeting different mRNAs into a single construct that is able to silence multiple targeted genes (Chen et al. 2008a).

MiRNA Transgene technology requires production of mice by incorporating a non-native segment of DNA containing a pre-miRNA-coding sequence of interest into mice's germline, which retains the ability to overexpress that miRNA in the transgenic mice (Zhang et al. 2009).

2.2.2 *Targeting-miRNA Technologies*

Targeting-miRNA technologies include Anti-miRNA Antisense Inhibitor Oligoribonucleotides (AMO) technology, Multiple-Target AMO technology (MT-AMO technology), miRNA Sponge technology, miRNA-Masking Antisense Oligonucleotides technology, Sponge miR-Mask technology and MiRNA Knockout technology.

The Anti-miRNA Antisense Inhibitor Oligoribonucleotides (AMOs) are single-stranded 2'-O-methyl-modified oligoribonucleotide fragments exactly antisense to their target miRNAs and can bind to their target miRNAs to inhibit their actions.

The Multiple-Target AMO technology is a modified AMO strategy, which allows designing single-stranded 2'-O-methyl-modified oligoribonucleotides carrying multiple antisense units that are engineered into a single fragment that is able to simultaneously silence multiple targeted miRNAs or multiple miRNA seed families (Lu et al. 2009).

The miRNA Sponge technology involves synthesis of RNAs containing multiple, tandem binding sites for miRNAs with the same seed sequence (a miRNA seed family) of interest and is able to target all members of that miRNA seed family (Ebert et al. 2007; Hammond 2007).

The MiRNA-Masking Antisense Oligonucleotides technology involves synthesis of single-stranded 2'-*O*-methyl-modified oligoribonucleotides that does not directly interact with its target miRNA but binds the binding site of that miRNA in the 3' UTR of the target mRNA by a fully complementary mechanism (Xiao et al. 2007b). In this way, the miR-Mask covers up the access of its target miRNA to the binding site to derepress its target gene via blocking the action of its target miRNA. It is a gene-specific targeting-miRNA strategy.

The Sponge miR-Mask technology combines the principle of actions of the MiRNA Sponge and the miR-Mask technologies for targeting miRNAs (Chen et al. 2008b). It is a gene-specific targeting-miRNA strategy.

The miRNA or Dicer Knockout technology enables to generate targeted deletion of a specific miRNA or Dicer; the former is miRNA-specific but non-mRNA-specific and the latter is non-miRNA-, non-mRNA-specific.

These miRNAi technologies are summarized in Table 2.2 and the strategies of miRNAi are illustrated in Fig. 2.2.

2.3 miRNAi Technologies in Basic Research and Drug Design

miRNAs are universally expressed in mammalian cells, being involved in nearly every aspect of the life of organisms (Lagos-Quintana et al. 2002; Landgraf et al. 2007). The level of miRNAs in cells is dynamic depending on developmental stage, cell cycle, metabolic alteration and pathophysiological conditions. Both upregulation and downregulation of miRNAs have been frequently implicated in a variety of pathological conditions. Both gain-of-function and loss-of-function technologies are necessary tools for understanding miRNAs. The miRNA-Targeting strategy resulting in gain-of-function of miRNAs is an essential way for gain-of-knowledge about miRNA targets and functions. On the other hand, the Targeting-miRNA technologies leading to loss-of-function of miRNAs is also an indispensable strategy for miRNA research. These two approaches are mutually complementary for elucidating miRNA biology and pathophysiology. The miRNAi technologies have opened up new opportunities for creative and rational designs of a variety of combinations integrating varying nucleotide fragments for various purposes and providing exquisite tools for functional analysis related to identification and characterization of targets of miRNAs and their functions in a gene controlling program.

In addition, the miRNAi technologies offer the strategies and tools for designing new agents for gene therapy of human disease (Ambros et al. 2003; Lee et al. 2005; Bartel, 2004; Wang et al. 2008; Calin & Croce, 2006). These agents will possess a backbone structure in the form of oligoribonucleotides or oligodeoxyribonucleotides. Like other types of nucleic acids for gene therapy, such as antisense

Table 2.2 Summary of miRNAi technologies

Name of miRNAi Technology	Characteristics	Applications
miRNA-targeting miRNA-gain-of-function mRNA-loss-of-function	Enhancing miRNA action to enhance gene silencing	
Synthetic Canonical miRNA (SC-miRNA)	miRNA-specific Non-gene-specific	(1) transient miRNA-gain-of-function in miRNA research; (2) miRNA replacement therapy
miRNA Mimic (miR-Mimic)	Non-miRNA-specific Gene-specific	(1) transient miRNA-gain-of-function in a gene-specific manner for miRNA research; (2) miRNA replacement therapy
Multi-miRNA Hairpins	miRNA-specific Non-gene-specific	(1) transient miRNA-gain-of-function in miRNA research; (2) miRNA replacement therapy
Multi-miRNA Mimics (Multi-miR-Mimic)	Non-miRNA-specific Gene-specific	(1) transient miRNA-gain-of-function in a gene-specific manner for miRNA research; (2) miRNA replacement therapy
miRNA Transgene	miRNA-specific Non-gene-specific	(1) long-lasting miRNA-gain-of-function in miRNA research; (2) controllable or conditional overexpression of miRNA; (3) in vivo animal model; (4) miRNA replacement therapy
Targeting-miRNA miRNA-loss-of-function mRNA-gain-of-function	Knockdown or knockout miRNA to relieve gene silencing	
Anti-miRNA Antisense Oligonucleotides (AMO)	miRNA-specific Non-gene-specific	(1) To knockdown target miRNA for validating the miRNA targets & function; (2) To achieve upregulation of the cognate target protein; (3) To reverse the pathological process
Multiple-Target Anti-miRNA Antisense Oligonucleotides (MT-AMO)	miRNA-specific Non-gene-specific	(1) To knockdown multiple miRNAs from same or different seed families; (2) To achieve upregulation of multiple target proteins; (3) To reverse the pathological process
miRNA Sponge	miRNA-specific Non- gene-specific	(1) To knockdown multiple miRNAs from a same seed family; (2) To achieve upregulation of the cognate target proteins; (3) To reverse the pathological process

(continued)

Table 2.2 (Continued)

Name of miRNAi Technology	Characteristics	Applications
miRNA-Masking Antisense Oligonucleotides (miR-Mask)	miRNA-specific Gene-specific	(1) To block action of a miRNA without knocking down the miRNA; (2) To achieve upregulation of the cognate target protein; (3) To reverse the pathological process
Sponge miR-Mask	miRNA-specific Non-gene-specific	(1) To knockdown multiple miRNAs from a same seed family; (2) To achieve upregulation of the cognate target proteins; (3) To reverse the pathological process
miRNA Knockout (miR-KO)	miRNA-specific Non-gene-specific	(1) To acquire permanent removal of a target miRNA; (2) To allow for controllable or conditional miRNA silencing; (3) To allow for studying miRNA-loss-of-function in vivo whole-animal context
Dicer Inactivation	Non-miRNA-specific Non-gene-specific	(1) To achieve a global loss-of-function of literally all cellular miRNAs; (2) To allow for controllable or conditional miRNA silencing; (3) To allow for studying miRNA-loss-of-function in vivo whole-animal context

oligodeoxynucleotides (Alvarez-Salas 2008; Koizumi 2007), decoy oligodeoxynucleotides (Gao et al. 2006), siRNA (Alvarez-Salas 2008; Pushparaj et al. 2008), triplex-forming oligodeoxynucleotides (Mahato et al. 2005), aptamer (Kaur and Roy 2008) and DNAzyme (Benson et al. 2008), the oligomer fragments generated using miRNAi technologies can be chemically modified to improve stability and are constructed into plasmids for easier delivery into organisms to treat diseases.

The Synthetic Canonical miRNA technology is an indispensable and most essential approach for miRNA gain-of-function in the fundamental research of miRNAs and of miRNA-related biological processes. It has been usually used to force expression of miRNAs of interest to investigate the pathophysiological outcome of upregulation of the miRNAs. The approach can also be used to supplement the loss of the miRNAs under certain situations and to maintain the normal levels to alleviate the pathological conditions as a result of downregulation of those miRNAs. The Multi-miRNA Hairpins technology can be employed if more than one miRNAs need to be applied under certain conditions.

miRNA Mimics can be used when a particular protein-coding gene needs to be knocked down. miRNA Mimics and siRNAs are quite similar in terms of

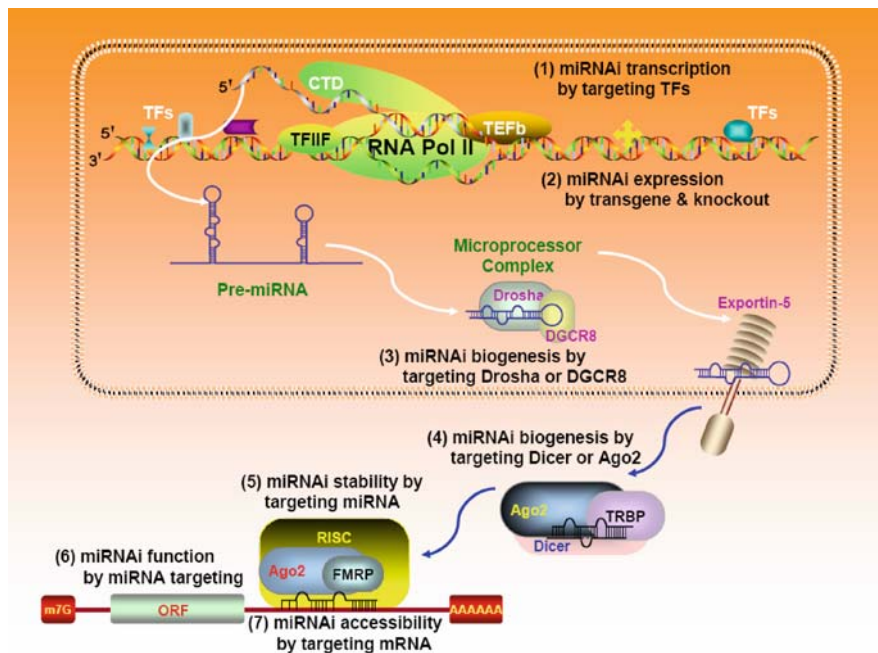


Fig. 2.2 Diagram illustrating the miRNAi strategies: (1) miRNAi can be directed to interfere with miRNA transcription by targeting transcriptional factors to either enhance or repress transcription. (2) miRNAi can change miRNA expression by creating Transgene or knockout for in vivo model studies. (3) and (4) miRNAi can disrupt miRNA biogenesis by targeting Drosha, DGCR8, Dicer and Ago2. (5) miRNAi can be directed to target the stability of miRNAs to knockdown miRNA level. (6) miRNAi can be used to change the function of miRNA by miRNA targeting (miRNA replacement). (7) miRNAi can block miRNA accessibility to its binding site in the target genes

their applications to basic research and their potential for disease treatment. The Multi-miRNA Mimics technology can be used as a simple and straightforward approach for studying the biological processes involving multiple protein-coding genes.

miRNA Transgene technology is an efficient approach to create overexpression of miRNAs of interest to study the role of miRNAs in in vivo conditions.

The AMO technology has been the most commonly and frequently used targeting-miRNA/loss-of-function approach to knock down miRNAs of interest. This method offers a miRNA-specific way to wipe out the function of the targeted miRNAs and can be used to bring back to the normal levels of miRNAs that are abnormally upregulated in the diseases states. The Multiple-Target AMO technology is designed as a simple and straightforward approach for studying the biological processes involving multiple miRNAs.

The MiRNA-Masking Antisense Oligonucleotides technology is a supplement to the AMO technique; while AMO is indispensable for studying the overall function

of a miRNA, the miR-Mask might be more appropriate for studying the specific outcome of regulation of the target gene by the miRNA. The Sponge miR-Mask technology is an alternative for the applications requiring manipulation of multiple target protein-coding genes of miRNAs.

The miRNA Knockout technology aims to generate mouse lines with genetic ablation of specific miRNAs or targeted disruption of miRNA genes. This approach allows for investigations of miRNA function related to the development of particular biological processes and/or pathological conditions in an *in vivo* context and in a permanent manner.

Dicer Inactivation technology aims to disrupt the maturation of miRNAs by inhibiting Dicer function through knocking down or knocking out the Dicer gene. This approach has been widely used to study the global requirement of miRNAs for certain fundamental biological and pathological processes.

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