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microRNAs: a new emerging class of players for disease diagnostics and gene therapy

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- **Introduction**
  - **Brief history, biogenesis and the regulated mechanism of miRNAs**
- **Roles of miRNAs in human disease and miRNAs as a novel biomarker for cancer and disease diagnostics**
  - **Cancers**
  - **Metabolic disease**
  - **Virus infection**
- **miRNAs as new targets for gene therapy**
  - **miRNAs as a target for gene therapy**
  - **Cancers**
  - **Cardiac diseases**
  - **Strategies for targeting miRNAs**
  - **Designing molecules for targeting miRNAs**
  - **Delivering molecules to specific targeted sites**
- **Concluding remarks and future perspectives**
  - **Early detection of cancers**
  - **Delivery miRNAs and/or anti-miRNAs for miRNA gene therapy**
  - **Potential toxicity effect of miRNA gene therapy**
  - **Potential effect of miRNAs on non-target genes**

Abstract

microRNAs (miRNAs) are a new class of non-protein-coding small RNAs, which regulate the expression of more than 30% protein-coding genes. The unique expression profiles of different miRNAs in different types of cancers and at different stages in one cancer type suggest that miRNAs can function as novel biomarkers for disease diagnostics and may present a new strategy for miRNA gene therapy. Anti-miRNAs and antisense oligonucleotides (ASO) have been employed to inhibit specific miRNA expression *in vitro* and *in vivo* for investigational and clinical purposes. Although miRNA-based diagnostics and gene therapy are still in their infancy, their huge potentials will meet our need for future disease diagnostics and gene therapy. High efficient delivery of miRNAs into targeted sites, designing accurate anti-miRNA/ASOs, and related biosafety issues are three major challenges in this field.

Key words: microRNA • gene therapy • disease • diagnostics • gene regulation • anti-miRNA • biomarker

Introduction

Disease diagnosis and gene therapy are two exciting fields in modern medicine. To better protect people from disease, many scientists have attempted to find new approaches for early detection of diseases and curing these diseases, particularly for serious diseases, such as cancer. Although several biomarkers have been developed for detecting various diseases, it is still difficult to diagnose many in very early stages, especially cancers. Gene therapy is one new technology for curing gene-related diseases, which

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was developed at the later 1980s and the early 1990s. Currently, more than 1000 clinic trials have been reported to cure different diseases, including autosomal dominant disorders, autosomal recessive single gene disorders (such as haemophilia and cystic fibrosis) and some forms of cancers (such as brain tumours, colon cancer) [1]. However, due to the potential toxicity of delivered gene fragments, efficiency of carriers and other potential limitations, there is still a long time before gene therapy becomes a mature technology for curing human diseases.

A recently identified extensive class of small RNAs, called microRNAs (miRNAs), may provide a new insight in this field. Although they were discovered and recognized just a few years ago, miRNAs have become the most important gene regulators at the post transcriptional level, and several studies indicated that miRNAs regulate the expression of more than 30% protein-coding genes [2, 3]. Exponentially growing evidence demonstrates that miRNAs play a versatile function in multiple biological and metabolic progresses although the functions are still unclear for a majority of identified miRNAs [4–7]. Recent investigations demonstrate that miRNAs have a unique expression profiles in different cancer types at different stages and play an important role in many diseases and viral infections. These result suggests that miRNAs can function as a novel biomarker for disease diagnostics and perform a new strategy for miRNA gene therapy.

Brief history, biogenesis and the regulated mechanism of miRNAs

The first miRNA (lin-4) was discovered by Ambros group at Harvard University (now at the Dartmouth Medical School) in 1993 [8]. The story begins with the identification of a mutation in the nematode *Caenorhabditis elegans*, which disrupted the phase change from larval to adult [9]. After more than 10 years of searching for a responsible protein, Lee and colleagues (1993) found that a small non-protein-coding RNA, called *lin-4*, is essential for the phenotype of this mutation. Further investigations demonstrated that *lin-4* negatively controls *lin-14* gene expression by binding to the 3′ untranslated region (3′ UTR) of *lin-14* mRNA *via* an antisense RNA-RNA interaction [8, 10]. However, the important function of miRNAs was not known until another miRNA (*let-7*) was identified in a variety of organisms [11, 12] and an extensive class of similar small RNAs were discovered in *C. elegans, Drosophila melanogaster* and humans [13–15]. Since then, miRNA-related research has become one of the hottest research topics in biology and medicine. It appears that miRNAs, generally speaking, exist and function in every biological and metabolic progress in any eukaryotes; and more than 5000 miRNAs have been identified and deposited in the miRNA database [16,17].

miRNAs are one class of non-protein-coding small RNAs of 19–24 nucleotides. miRNA can be encoded anywhere in the genome [18]. A majority (61%) of miRNA genes are located at an intronic region of a protein-coding genes; however, miRNA genes can also be located in regions of exons or intergenes [18]. miRNAs are transcribed from genes, called miRNA genes, by RNA polymerase II (pol II); some miRNAs can also be transcribed by pol III [19]. However, miRNA biogenesis is much more complicated than mRNA biogenesis. Producing miRNAs is a multiple steps of processes involving in several different enzymes. Firstly, miRNA genes are transcribed into primary miRNAs (pri-miRNA) with 5' cap and 3' poly A tails by pol II or pol III [20–22]. miRNA genes can be located anywhere in the genome, some in introns, some in exons and some also can be found in intergenic regions [18]. Some miRNA genes are transcribed individually; some are clustered together and co-transcribed as a one polycistronic transcript. The lengths of pri-miRNAs vary from several tenths to more than 1000 nucleotides. Then, a pri-miRNA is recognized by microprocessor complex, which is composed of the nuclear RNase III Drosha together with its double-stranded RNA binding domain (dsRBD) partner DiGeorge syndrome critical region 8 (DGCR8), and cut into a miRNA precursor (pre-miRNA) with an approximately 70 nucleotide stem-loop structure [23–25]. The pre-miRNAs with 2nd hairpin structure are then transported into cytoplasm by the transporter Exportin 5, and this process is RanGTP dependent [26–30]. In the cytoplasm, the pre-miRNAs were further processed into the 19–24 nucleotide double-stranded miRNA:miRNA* complex by another RNase III enzyme, called Dicer, together with its dsRBD partner TRBP [31–33]. Then, the mature miRNA sequences enter the RNA-induced silence complex (RISC) and target specific gene expression awhile the opposite strand miRNA* sequences are degraded by unknown mechanism.

There are many common characteristics between miRNAs and other RNAs, particularly other small RNAs. Usually when we consider a RNA as a miRNA, it should have the following characteristics [34]: (1) all mature miRNAs are processed from long miRNA precursors by multiple processes, although recent evidence suggested that in worm and fruit fly, precursors of some miRNAs derived from excised introns bypass Drosha cleavage step [35]; (2) miRNA precursors can be formed into a secondary stem-loop hairpin structure with high negative minimal folding free energy; (3) miRNAs are located within one arm of the secondary stem-loop hairpin structures; (4) there are no internal loops or bulges in the miRNA:miRNA* complex. Small number of mismatches are allowed in miRNAs, but there is at least 16 base pairs between miRNAs and their miRNA* sequences; (5) some miRNAs are highly evolutionarily conserved from species to species [12, 36]; however, this is not universal characteristic for all miRNAs. There are also many species-specific miRNAs; (6) miRNA precursors have high minimal folding free energy index (MFEI) [37]. Two recent studies demonstrated that miRNA precursors have much lower MFEI than that of other RNAs [37, 38]. The above are biogenesis criteria for identifying miRNAs. In addition to the biogenesis criteria, at least one of the following expression criteria also should be considered when designing a new miRNA: (7) miRNAs should be expressed and detected in at least one tissue or organ by traditional molecular technologies, including northern blotting, microarray and/or real-time PCR; (8) miRNA expression is effect by reduced the expression of miRNA biogenesis-related enzymes, such as Dicer and Drosha.

In the past 7 years, incredible growth has been seen in the field of miRNA biology as evidence by the exponential number of miRNA-related manuscripts published in a wide range of journals [39]. This expansion is shown by the following several aspects: (1) The total number of miRNAs has increased dramatically from 218 in 2002 to 5071 in 2007 according to the public available miRBase miRNA database [16, 17]; although the first miRNA was identified in 1993 in *C. elegans* [8], its function was not recognized until 2000s when an extensive number of miRNAs were identified from three different species (*C. elegans*, fruit fly and human) [11–15]. Currently, more than 400 miRNAs have been identified in human and computational programs have predicted that there are more than 1000 miRNA genes in the human genome [40]. (2) miRNAs are widely distributed in almost all eukaryotic organisms and some types of viruses, including plants and animals. (3) miRNAs regulate the expression of more than 30% of protein-coding genes; currently, miRNAs have become one of the most important gene regulators. (4) miRNAs control a multiple biological and metabolic progresses, ranging from developmental timing, organ and tissue development and signal transduction to diseases including cancers and human immunodeficiency virus (HIV) infection. (5) New technologies, including computational and experimental approaches, have been developed and applied to identify miRNA genes and their targets; for example, in the past several years, more than 10 computational programs have been developed for predicting miRNAs and their targets in plants and animals; in the past several years, quantitative real time PCR (RT qPCR) and miRNA microarray technology also allow us to better understand the expression patterns and functions of miRNAs. (6) miRNAs have huge potentials for clinical applications, including diagnostics and gene therapy.

There are three major currently-known mechanisms for miRNA-mediated gene regulation: translation repression, direct mRNA degradation and miRNA-mediated mRNA decay [41, 42]. Generally speaking, which mechanism controls gene expression is entirely dependent on the degree of miRNA complementarity to their targeted mRNAs. Although both miRNAs and small interfering RNAs (siRNAs) are incorporated into RISC and inhibit gene expression at the post transcriptional levels, their interactions with targeted mRNAs are different [43]. siRNAs perfectly bind to their targeted mRNAs and guide mRNA cleavage endonucleolytically by RISC [44]. In contrast, miRNAs bind, in most cases, with imperfect complementarity to their targeted mRNAs and guide mRNA translation repression [45, 46]. However, there are also several miRNAs which directly degrade their targeted mRNAs. For example, *miR-196* directly cleaves the mRNA of *HOXB8* [47–49], which plays important role in animal development [50–53]. The exact mechanism for miRNA-mediated translation repression is still unknown, and one possible mechanism is miRNA-RISC complex may inhibit the initiation and/or elongation of protein translation by interacting with a various translation factors, such as eIF4F [54–58]. Recently, several investigations

also demonstrated that miRNAs mediate gene expression by guiding mRNA decay through de-adenylation and de-capping process of targeted mRNAs [59–61], which is completely different from normal translation repression and/or direct mRNA degradation. It is well known that the 3′ poly(A) tail and 5′ cap are very important for mRNA stability and avoiding mRNA decay. When miRNAs guide the removal of the 3′ poly(A) tail and 5′ cap of the targeted mRNAs, these targeted mRNAs will be quickly degraded by cellular enzymes.

In a majority of cases, miRNAs bind to their targeted mRNAs at the 3′ UTR with multiple sites. However, miRNAs targeted to the 5′ UTR and/or the open reading frame (ORF) can also repress gene expression [62–64]. miRNAs interact with their targeted mRNAs primary through the six to eight nucleotides at the 5′ end of miRNAs, which is perfectly bound to the targeted mRNAs. This region is called 'seed' sequence in miRNAs and is high conserved in a same miRNA family from species to species [2, 65, 66]. This characteristic has been employed to design different computational programs for predicting miRNAs and their targets in animals [65–69].

Roles of miRNAs in human disease and miRNAs as a novel biomarker for cancer and disease diagnostics

More and more evidence indicates that miRNAs play an important role in many human diseases, ranging widely from cancers, HIV to metabolic diseases. This evidence includes, but not limited to, (1) a unique set of miRNAs exists in a specific disease; (2) a unique expression of miRNAs in a certain human disease and (3) aberrant expression of miRNAs in human disease.

Cancers

The first evidence that miRNAs is related to cancers came from Croce group. In their study, they found two miRNA genes (miR-15 and miR-16) are located at the chromosome 13q14 region, which is frequently deleted or down-regulated in the majority (~68%) of B cell chronic lymphocytic leukaemias (B-CLL) cases [70]. Subsequent investigations demonstrated that almost all cancers have alternative miRNA expression profile compared to their adjunct normal tissues. These cancer types include several important cancers, for example lung cancer, leukaemia, brain cancer and breast cancer, which together cause the majority of cancer-related death in the past decades. Significant progress has been made on the relationship between miRNAs and cancers, and the important function of miRNAs in a variety of cancers has been reviewed by several research groups [41, 71–73]. More interestingly, recently studies also demonstrated tumour invasion and metastasis is also initiated by miRNAs [74].

Table 1 summarizes the alternative miRNA expression in the major cancer types. It should be noted that for every cancer type, at least two miRNAs are aberrantly expressed. Table 1 also clearly shows that some miRNAs are overexpressed in cancers, for example *miR-17-92* is highly expressed in lung cancer cell; in this case the miRNA functions as oncogene. In contrast, some miRNAs, for example *let-7* in lung cancer tissue, are down-regulated, and therefore function as tumour suppresses genes. More importantly, some miRNAs have different expression profile pattern in different cancer types [75]. Several studies also demonstrated that miRNA expression aberration exists at the early stage of cancer pathogenesis and the expression pattern varies as the carcinogenesis develops, suggesting the important function of miRNAs in the developmental lineage and differentiation state of the tested tumours [75]. This alternative and unique expression pattern allows miRNA become a novel biomarker for early detection of cancers and may lead to clinical applications.

It is a challenge to identify a poorly differentiated tumour [76]. To test the possible that miRNAs as a biomarker for early detection of cancers, Lu and colleagues (2005) employed a new bead-based flow cytometric miRNA expression profiling method to analyse 17 poorly differentiated tumours. Their results demonstrated that miRNAs can be used to correctly diagnose and classify these non-histological-diagnostic poorly differential tumours with a great accuracy although the global levels of miRNAs were lower than the more-differentiated tumours [75]. miRNA microarray expression profiles have been correlated with survival of human lung adenocarcinomas, including those classified as disease stage I [77]. Blenkiron and colleagues (2007) employed a

Table 1 Cancer-related miRNAs and their aberrant expression

Cancer	miRNAs involved *	References
Brain cancer (Glioblastoma)	miR-21↑, miR-221↑, miR-12↓, miR-181a,b,c↓	[166] [150]
Breast cancer	miR-21↑, miR-146↑, miR-155↑, miR-10b↓, miR-17-5p↓, miR-125b↓, miR-145↓, miR-125b↓	[167] [168] [169] [170] [74]
Cholangiocarcinoma	miR-21↑, miR-141↑, miR-200b↑	[171]
Chronic lymphocytic leukemia (CLL)	miR-15↓, miR-16↓	[70] [172] [173]
Colorectal neoplasia	miR-10a↑, miR-17-92↑, miR-20a↑, miR-31↑, miR-96↑, miR-183↑, let-7↓, miR-143↓, miR-145↓	[174] [170] [109] [175] [176] [174]
Diffuse large B cell lymphoma (DLBCL)	miR-21↑, miR-155↑, miR-221↑	[80]
Head and neck cancer	miR-21↑, miR-205↑	[177]
Hepatocellular carcinoma (HCC)	miR-18↑, miR-224↑, miR-199↓, miR-195↓, miR-200↓, miR-125↓	[178]
Lung cancer	let-7↓, miR-17-92↑	[114] [109] [179] [77] [180]
Lymphomas	miR-155↑, miR-17-92↑	[181] [182] [183] [184]
Ovarian cancer	miR-200a,b,c↑, miR-141↑, miR-199a↓, miR-140↓, miR-145↓, miR-125b↓	[185]
Pancreatic cancer	miR-221↑, miR-181a↑, miR-21↑, miR148a,b↓	[186]
Papillary thyroid carcinoma	miR-221↑, miR-222↑, miR-146↑, miR-181↑	[183] [187]
Pituitary adenomas	miR-212↑, miR-026a↑, miR-150↑, miR-152↑, miR-191↑, miR-192↑, miR-024-1↓, miR-098↓, miR-15a↓, miR-16-1↓	[188] [189]
Prostate cancer	let-7↑, miR-195↑, miR-203↑, miR-128a↓	[170]
Stomach cancer	miR-21↑, miR-103↑, miR223↑, miR-218↓	[170]
Testicular germ cell tumours	miR-372↑, miR-373↑	[190]

* Listed are miRNAs that are significantly changed in expression between cancer tissue and adjacent normal tissue ↑ overexpression; ↓ down-regulation

bead-based flow cytometric miRNA expression profiling method to analyse the expression profiles of 309 human miRNAs in human breast and breast tumours. Their results show that miRNAs had different expression profiles in the different stages of human breast cancers and individual miRNAs are associated with clinicopathological factors [78]. According to their finding, they classified the breast tumours into four subtypes: Luminal A, Luminal B, Basal-like, $HER2^{+}$ and Normal-like. In an independent data set, miRNAs could be employed to classify basal *versus* luminal tumour subtypes suggesting that miRNA expression profiling might be a suitable platform to classify breast cancer into prognostic molecular subtypes [78]. One recent study indicated that miRNA *let-7* has a different expression profile in two differentiation stages (SC1 and SC2) of human tumour cell line NCI 60, and SC1 cells express low and SC2 cells high levels of miRNA *let-7*, respectively, suggesting that let-7 is a marker for less advanced cancers [79]. miRNA expression profiles can also be used to distinguish two subtypes of diffuse large B cell lymphoma (DLBCL): germinal center B cell-like (GCB) and activated B cell-like (ABC) DLBCL, in which miR-21, miR-155 and miR-221 were more highly expressed in ABC-type than GCB-type DLBCL [80]. Those suggest that miRNA expression profile is a feasible novel biomarker for early detection of cancers, particularly for poorly understand tumours.

Metabolic disease

In the past several years, metabolic disease (such as diabetes) has become the attention of scientists and a major challenge for global health. Any process that disrupts normal metabolism can cause metabolic disease. Several studies demonstrated that miRNAs may have an important function in metabolism. The first evidence for miRNA function in metabolism came from a forward genetic screen in the fruit fly [81]. Xu and colleagues (2003) observed that loss-of-function of *miR-14* significantly increased the amount of total fly body triacylglycerides. Although the overall cellular architecture was normal, the adipocyte lipid droplets were greatly enlarged in *mir-14*Δ^{1} flies and this phenotype was suppressed and triacylglyceride contain was decreased in fly body by over-expressed miR-14 [81]. A subsequent study demonstrated that *miR-278* plays a role in the control of insulin production and loss-of-function of miR-278 elevated insulin production and circulating sugar in the fruit fly [82]. Another study demonstrated that islet-specific *miR-375* regulated glucose-dependent insulin secretion [83]. In this study, Poy and colleague (2004) show that overexpression of *miR-375* inhibited glucose-induced insulin secretion. In conversion, down-regulated *miR-375* promoted insulin secretion. It is obvious that diabetes is related to insulin resistance. This suggests that *miR-278* and *miR-375* is a regulator of insulin secretion and may become a novel pharmacological target for the treatment of diabetes [83].

A recent study demonstrated that another miRNA, *miR-122*, regulated lipid metabolism in liver [84]. Esau and colleagues (2006) observed the decrease in plasma cholesterol level and a significant improvement in liver steatosis in mice by inhibiting *miR-122* expression through an antisense oligonucleotide (ASO) technique. This phenomenon was also observed by Krutzfeldt and colleagues [85]. They employed a new class of miRNA inhibitors (called antagomirs) to knockdown *miR-122* and they found that down-regulation of *miR-122* significantly decreased the plasma cholesterol levels after four days of treatment. All these evidences suggest that *miR-122* is a key regulator of cholesterol and fatty-acid metabolism in the adult liver and an attractive therapeutic target for metabolic disease [84, 86, 87].

miRNAs also participate amino acid metabolism. In a bioinformatics paper, Stark and colleagues predicted that several amino-acid-metabolism-related enzymes are targeted by several miRNAs [88]. A followed study demonstrated that *miR-29b* controls the amount of branched chain (α-ketoacid dehydrogenase complex [89], which play an important role in the first irreversible step in branched-chain amino acid synthesis. As we know, several human metabolic diseases are related to a specific amino acid synthesis, this suggests that a specific miRNA may become a therapeutic target for amino-acid-related metabolic disease.

Virus infection

It is well known that plants and some animals employ the RNA interference (RNAi) pathway to against viral infection [90]. Several recent investigations demonstrated that miRNAs, with a similar gene regulation mechanism to RNAi, also play an important role in both animal and plant response to viral infection

Table 2 Disease-related miRNAs and their aberrant expression

Disease	miRNAs involved *	Reference
Cardiac hypertrophy	miR-1↓, miR-133↓	[132] [191]
Alzheimer's disease	miR124a↓, miR-9↑, miR-128↑	[107]
Psoriasis	miR-203↑, miR-146a↑, miR-21↑, miR-125b↑	[192]
HCV infection	miR-122↑	[101]
PFV-1 infection	miR-32↓	[91]
Tourette's syndrome (TS)	miR-189↑	[106]
Parkinson's disease	miR-133b↓	[193]
Schizophrenia	miR-130b	[194]

* ↑ overexpression; ↓ down-regulation.

[91–94]. Many miRNAs have also identified in several viruses, including HIV, Epstein Barr virus (EBV) and human cytomegalovirus (HCMV), by computational and experimental methods [38, 95–99], although the mechanisms for virus-encoded miRNA biogenesis are still unclear.

One important function of miRNAs is to control viral replication when the virus infects a cell and to further control virus infection. For example, hepatitis C virus (HCV) is a small (50 nm in size) single-stranded positive-sense RNA virus belonging to the family *Flaviviridae*, which is one of the major causes of chronic liver disease, including cirrhosis and live cancer, with an estimated 170 million people infected [100]. A recent study shows that a liver-specific miRNA *miR-122* modulates HCV RNA abundance and HCV replication. In that study, the authors observed that knockdown *miR-122* using an antagomir antisense 2'-O-methylated RNA oligonucleotide (2'-OMe-anti-miR-122) resulted in a significant loss (about 80%) of autonomously replicating hepatitis C viral RNAs [101]. However, studies with replication-defective RNAs demonstrated that *miR-122* did not significantly influence mRNA translation or RNA stability, suggesting that *miR-122* is likely to facilitate replication of the viral RNA [101]. This conclusion is also confirmed by the same study on two types of human hepatocytes: Huh7 and HepG2, in which Jopling and colleagues (2005) only observed HCV RNA was only replicated in Huh7 cells (miR-122 positive), but not in HepG2 cells (miR-122 negative). A further study demonstrated that a partial sequence in the 5' non-coding region (NCR) is responsible for *miR-122* targeting although there is also a potential binding sequence in 3'NCR of HCV RNA [101]. This system is remarkably different from previous observations that a majority of miRNAs target the 3' UTR to repress protein translation or to degrade the targeted mRNAs in animals [42]. Currently, the molecular mechanism that *miR-122* regulate HCV replication is still unclear. However, due to this phenomena and the fact those current therapies against HCV are frequently ineffective, *miR-122* may present a novel alternative target for antiviral intervention and also provide a possible antiviral tool against a rapidly evolving viral genome.

Host miRNAs also can target a specific virus RNA to restrict viral infection and protect cell itself. A recent study demonstrated that a cellular miRNA (*miR-32*) effectively restricts the accumulation of the retrovirus primate foamy virus type 1 (PFV-1) in human cells by targeting a PFV-1-encoding protein, Tas [91].

In addition to the diseases reviewed above, miRNAs also regulate several other diseases (Table 2). For example, miRNAs regulate brain and neuron development [102–104]; aberrant expression of these miRNAs are associated with several neuronal diseases [105], including Tourette's syndrome [106], Alzheimer's disease [107], schizophrenia and schizoaffective disorder [108].

miRNAs as new targets for gene therapy

The unique aberrant (up- or down) expression of specific miRNAs in a specific disease state suggests new targets and strategies for gene therapy. As the expression profiles of miRNAs in certain diseases, by overexpression or down-regulation of the specific miRNAs it may become possible to treat and perhaps even cure the specific genetic disease.

miRNAs as a target for gene therapy

Cancers

Cancer is one of the most obstinate group of diseases currently and in the past several decades. To date, the most popular therapeutic tool is surgical removal of the tumour with subsequent chemotherapy and/or radiation therapy. However, the cure rate is low, particularly for a majority of malignant tumours. The surprising aberrant expression of specific miRNAs in a specific cancer type suggests that those miRNAs may serve as a novel target for cancer treatment and a possible new approach for gene therapy.

Let-7 is a remarkable miRNA in lung cancer. Several investigations demonstrated that *let-7* expression level is associated with the pathogenesis of lung cancer and the expression of *let-7* is significantly reduced in lung cancer tissues [109–114]. Takamizawa *et al.* (2004) found that *let-7* was poorly expressed in lung cancers and reduced *let-7* expression was significantly associated with shortened after operative survival independent of disease stage [114]. More interesting and importantly, an *in vitro* study show that transient overexpression of miRNA *let-7* in the A549 lung adenocarcinoma cell lines inhibited lung cancer cell proliferation [114]. Recent studies indicate that *let-7* inhibits tumour growth by targeting several oncogenes including RAS and high-mobility group A2 (HMGA2) [109, 111, 112, 114, 115]. *Hmgo2* was found to be expressed in a wide variety of benign and malignant tumours [116–122] and overexpression of HMGA2 gene caused the onset of specific tumours [123]. miRNA *let-7* regulates the HMGA2 gene expression by binding to seven conserved HMGA2 3' UTR complementary sites [2, 111]. Disrupting the bind pairing between *let-7* and HMGA2 caused HMGA2 overexpression, which enhanced anchorage-independent growth in several cell lines, and further promoted oncogenic transformation [112]. These results suggest that overexpressing miRNA *let-7* may inhibit lung cancer growth and even cure lung cancer in the future. However, this possibility still needs to be tested *in vivo* in a live animal model, and finally in clinical trials.

Cardiac diseases

Cardiac disease is one primary cause of morbidity and mortality in industrialized countries as well as the most frequent reason for non-infectious mortality in infants [124–126]. Recently, several investigations demonstrated that miRNAs play an important role in cardiac development and contractility, and several heart diseases are associated with the aberrant expression of certain miRNAs [124, 127–133]. Cardiac-specific overexpression of miR-1 inhibits cardiomyocyte in the embryonic heart [133] and exacerbated arrhythmogenesis [131]. In contrast, inhibiting miR-1 expression in infarcted rat hearts relieved arrhythmogenesis [131]. These suggest that miR-1 may have an important pathophysiological functions in heart failure and may serve as a potential antiarrhythmic target for gene therapy.

Recent studies show that another miRNA, miR-133, was associated with cardiac hypertrophy [129, 132, 134, 135]. Overexpression of miR-133 inhibits hypertrophic symptom in both neonatal and adult mouse myocytes [132]. In contrast, down regulation of miR-133 enhances hypertrophic growth in mouse heart [132]. This suggests that miR-133 controls the pathogenesis of heart hypertrophy and overexpressing miR-133 in heart hypertrophic patients may serve as a novel gene therapy for preventing pathological cardiac hypertrophy.

Strategies for targeting miRNAs

There are two critical processes for targeting a specific miRNA: designing molecules for targeting miRNAs and delivering these molecules to the specific targeted sites.

Designing molecules for targeting miRNAs

Antisense oligonucleotide (ASO) technology is a powerful technique to selectively modify gene

expression *in vitro* and *in vivo*. In the past several years, ASO technology has been employed to clinic application on gene therapy for human disease [136]. Currently, ASOs are becoming the most accepted approaches technology for controlling miRNA expression experimentally and/or therapeutically [43]. According to the specific characteristics of miRNAs, ASOs have been chemically modified to single-stranded RNA analogues complementary to specific miRNAs, and a new name has been given to these modified ASOs: anti-miRNA ASOs (AMOs). Some people also termed these oligonucleotides as antagomirs [85]. In the past several years, many groups have employed the AMOs/antagomirs technology to inhibit miRNA activity in both *in vitro* and *in vivo* studies for investigating the function of miRNAs and their potential application on the clinic.

To the best of our knowledge, the first report on inhibiting miRNAs using AMOs came from a group of scientists working in Greece. In their study, antisense DNA oligonucleotides complementary to 11 miRNAs were synthesized and injected to *Drosophila* embryos; after injection, a variety of developmental defects were observed, suggesting that AMOs inhibited miRNA activity [137]. However, this study and a later study show that unmodified DNA oligonucleotides were not enough to inhibit miRNA activity [137, 138] possibly due to the low-binding affinity between DNA oligonucleotides and miRNAs. In the following years, several investigations have been performed to modify AMOs for increasing the AMO-binding affinity for miRNAs and further inhibiting miRNA activity *in vitro* and *in vivo*. The most effective modifications are ASOs with the addition of a chemical group to the 2'-hydroxyl group, locked nucleic acid (LNA) ASOs and ASO with phosphorothioate backbone modification [139]. However, in a majority of cases, adding a chemical group and modifying the backbone with phosphoramidate are used together to further increase the efficiency of AMO inhibition [84, 85].

Three successful modifications with addition of a chemical group to the 2'-hydroxyl group are 2'-O-methyl (2'-OMe), 2'-O-methoxyethyl (2'-MOE) and 2'-flouro (2'-F). 2'-OMe is one of the oldest, simplest and most frequently used chemicals to modify oligonucleotides; in this modification, the methyl group enhance RNA resistance to nuclease cleavage and improve AMO-binding affinity to miRNAs [139]. Krutzfeldt and colleagues (2005) employed 2'-OMe and phosphoramidate to modify AMO and synthesized antogomir-122, an AMO specifically inhibiting a live-specific miR-122. After injection of antagomir-122 to normal mice, expression level of miR-122 was significantly decreased; in contrast, the mRNA levels of miR-122 targeted genes are increased in liver by up to 6.8 fold [85]. In a same study, Krutzfeldt and colleagues (2005) also observed that the same antagomir technology significantly reduced the expression of three other miRNAs (miR-16, miR-192 and miR-194) in 11 different tissues or organs (liver, lung, kidney, heart, intestine, fat, skin, bone marrow, muscle, ovaries and adrenal). Almost at a same time, another independent laboratory also significantly inhibited miR-122 expression in mice with a 2'-MOE phosphorothioat-modified ASO [84]. Both studies found that silencing miR-122 with antisense AMO reduced plasma cholesterol levels [84, 85].

LNA-modified ASOs is structurally different from 2'-O-modifed ASOs. In LNA-modified ASOs, the 2'-oxygen is bridged to the 4'-postion of ribose *via* a methylene linker to form a rigid bicycle [139–141]. Due to its unprecedented affinity, good or even improved mismatch discrimination, low toxicity and increased metabolic stability, LNA has attracted the attention of scientists for *in vivo* applications of inhibition of miRNA activity [141]. In the past several years, LNA technology has been employed to detect miRNA function, to identify miRNA targets, and to inhibit miRNA activity by LNA-modified ASOs/AMOs [142–149]. A recent study demonstrated that LNA-modified oligonucleotides (ASOs) stoichiometrically and reliably inhibited miRNAs with high specificity in a heterologous human embryonic kidney cell line HEK293 and in fruit fly cells [148]. A mixture of 2'-O-methyl- and LNA-modified ASOs was also employed to inhibit miR-21 expression for understanding the function of miRNAs in apoptosis progress in human glioblastoma cells [150].

To further investigate the potential application of ASOs and AMOs/antagomirs, the subcellular localization and effect factors on ASOs/AMOs have been studied. Davis and colleagues (2006) evaluated the effect of 2'-sugar and backbone ASO modification on anti-miRNA activity of ASO/AMO using a luciferase reporter mRNA assay in Hela cells. Their results demonstrated that all 2'-sugar substitution significantly increased AMO-binding affinity to miRNAs and increased the ASO/AMO efficiency on silencing miRNA activities [151]. High activity of AMOs/antagomirs required an optimal number

of phosphorothioate modifications and minimum length (>19 nt) [152].

The above evidence suggests that ASOs/AMOs/antagomirs may become a powerful tool to inhibit specific miRNA activity and a therapeutic strategy for miRNA therapy in a specific human disease, including cancers.

Delivering molecules to specific targeted sites

The success of miRNA therapy depends on effective systems to deliver ASOs/AMOs/antagomirs to the targeted sites. RNAi has been employed for gene therapy in the past 5 years [153]. Because both RNAi-mediated gene therapy and miRNA therapy are based on the development of antisense therapeutics that knockdown gene expression after transcription, and miRNAs are also chemically identical to small interfering RNAs (siRNAs), all methods used in RNAi gene therapy can be theoretically employed to deliver therapeutic molecules in miRNA therapy.

It is well established that cholesterol-conjugated miRNA and siRNAs can be delivered into cell and cause gene silence *in vivo*. Recently, Wolfrum and colleagues (2007) demonstrated that the efficiency of siRNA delivery into cells depend on the interactions with lipoprotein particles, lipoprotein receptors and transmembrane proteins. siRNA conjugation to lipophilic molecules increased the efficiency of siRNA delivery into cells. High-density lipoprotein (HDL) targets siRNA delivery into liver, gut, kidney and steroidogenic organs; however, low-density lipoprotein (LDL) delivers siRNA primarily to the liver [154]. Therefore, these molecules will provide a potential mechanism for miRNA delivery into certain tissues.

Viruses have been widely used as vectors to deliver siRNA and/or short haipin RNA (shRNA) into cells for gene function studies *in vitro* and *in vivo*. For example, modified adenovirus, adeno-associated virus (AAV) and lentivirus have been employed to successfully deliver siRNA/shRNA into cells and stably integrate siRNA/shRNA into targeted genome [155–158]. This method also has been employed successfully to deliver miRNAs into cells *in vitro* or animals *in vivo*. Krutzfeldt and colleagues (2005) inserted miR-122 and antagomirs into adenovirus genome and then transferred them into mice by direct intravenous injection to study the function of miRNAs and the efficiency of antagomir-induced miRNA silencing.

Currently, several companies have developed several RNAi expression vectors, which are also capable to deliver miRNAs into cells and to integrate into the genome. To enhance the delivery of miRNA-vectors, specific chemicals are also being developed for this transfection. Currently, more and more scientists are employing the commercially-developed vectors and chemical reagents for delivering miRNAs into cells.

Concluding remarks and future perspectives

Rapid progression of miRNA-related research has been revealing the huge potential of miRNAs as novel diagnostic and gene therapy tool as well as a novel class of drug targets for cancers, anti-virals and potentially many other diseases. However, many miRNA-related therapeutic fields are still in their infancy. Before miRNA therapy can become a widespread therapeutic tool for detecting and treating diseases, including cancers, new technologies and new strategies need to be developed. It is likely that much significant progress will be achieved in the therapeutic usage of miRNAs in the next few years.

Early detection of cancers

Although several studies demonstrated that miRNA expression profile can be used to identify and classify poor-differentiated tumours, there remains much work before it can be directly applied to clinical diagnostics. Currently, a majority of studies focus on the comparison of miRNA expression profiles between tumour and normal tissues, but it is more useful to correlate miRNA expression level with tumour subtypes. This issue has beginning to attract the attention of scientists, and several studies started to show the promise in this direction [78, 79, 159]. Recently, a bead-based flow cytometric miRNA expression profiling method was employed to identify the four subtypes of breast cancers: Luminal A, Luminal B, Basal-like, $HER2^{+}$ and Normal-like [78]; a real time qPCR was used to identify the differentiation stages of ovarian cancer [79] and breast cancer [159] based on the miRNA expression profiles. Tissue sampling is another big issue for diagnostic applications. It is probably

not a good strategy to collect samples from all possible tissues or organs due to their heterogeneity and specific location in body. A more useful strategy is collecting blood samples to use for miRNA expression profile analysis, and correlating those results with disease state. Such a data set will make a huge contribution for approaching direct diagnostics. Thus, more data need to be gathered and perhaps more new miRNAs need to be identified in the next few years. In addition, we need to focus on identifying new approaches for developing biomarker kits for early monitoring of potential carcinogenesis.

Delivery miRNAs and/or anti-miRNAs for miRNA gene therapy

As mentioned, RNAi-based gene therapy has been in use for several years, and many strategies and technologies for delivering small interfering RNA (siRNA) into cells also can be used in miRNA delivery. However, novel strategies may need to be developed for miRNA therapy based on the unique characteristics and small size of miRNAs. A major challenge for miRNA therapy is to deliver miRNAs/anti-miRNAs into a specific tissue and express them at certain times for targeting a specific gene and minimize their introduction into other non-targeted sites.

Although ASOs/AMOs are a powerful tool to silence miRNA activity in miRNA functional studies, the ASO-mediated miRNA silencing mechanism is still unclear The efficiency of ASOs/AMOs on therapeutic usage is dependent on miRNA silence mechanism and miRNA activity *in vivo* [152]. Therefore, the better our understanding of the miRNA silencing mechanism, the more efficient strategies we may develop in the future.

Another challenge is that certain tissues/organs may be refractory to miRNA therapy. Possibly due to the blood–brain barrier, systemic infusion of antagomir did not change the miRNA levels in the brain [152]. Thus, more efficient delivery methods and/or strategies need to be developed for brain disease therapy, such as brain tumours.

Potential toxicity effect of miRNA gene therapy

Before miRNA gene therapy can be used clinically, the potential toxicity effect and immune responses of delivered miRNAs or anti-miRNAs should be fully investigated. At the early stage of gene therapy, because of the dose-dependent induction of both innate and adaptive immune responses, delivered vectors and/or genes have resulted in inflammatory toxicity in certain patients [160]; one of the most serious example is to cause patient death after an adenoviral gene transfer because of fatal systemic inflammatory response [161]. miRNA therapy follows the similar strategy as traditional gene therapy, and whether or not miRNA therapy causes innate and/or adaptive immune response is still unclear because of current limited data. In most gene therapy applications, a very large amount of agent, for example miRNAs, is introduced into cells. How large amounts of miRNA affects the homologous miRNA pathway in cells is unknown. A recent study demonstrated that overexpression of shRNA in mice saturated the miRNA pathway and caused severe toxicity in liver [162]. It is possible that introducing a large amount of miRNA caused feedback inhibition that interrupted the normal miRNA synthesis pathway. This possibility raises the safety concern about gene therapy including miRNA therapy.

Another issue is that a majority of current studies are performed for a short-term period, and we need to determine what will happen if a specific miRNA is down-/up-regulated for a long-term period. A recent study demonstrated that miR-122 promotes the HCV RNA replication and may contribute to chronic liver disease; blocking miR-122 expression using antagomir inhibited HCV replication in human hepatocytes [101]. Based on this finding, several research groups propose that blocking miR-122 may become a novel strategy for anti-HCV therapy. Recently, two impendent groups found the antagomirs can efficiently inhibit the expression of miR-122 and no apparent toxicity was observed in both studies based on a limited study period [84, 85]. However, Kutay and colleagues (2006) observed that reduced expression of miR-122 is associated with neoplastic transformation in rodent and human cells after a long-term treatment [163]. This suggests that more experiments should be performed before miRNA therapy can be tested in a clinical trial.

Potential effect of miRNAs on non-target genes

Many miRNAs have hundreds of conserved targets as well as additional non-conserved targets [12, 164,

165]; miRNAs regulate specific targets by expressing at different times in different tissue. However, it is a challenge for miRNA therapy to control the delivery of miRNAs or other molecules expressing at a specific cellular compartment, such as ER and cytosol. For a majority of cases, miRNAs regulate human disease by targeting a specific gene, so it is also a challenge to avoid delivering miRNAs to other non-targeted genes instead of the targeted genes. Because many miRNA targets are unknown and/or poorly understood, it is difficult to predict the potential effect of miRNAs on non-targeted genes.

Currently, more than 500 miRNAs are already found in human genome [17] and computational approach predicts that human may have more than 1000 miRNAs [40]. Computational analysis also demonstrated that more than 30% of protein-coding genes are regulated by at least one miRNAs at the post transcriptional level [2, 3]. In a majority of cases, several miRNAs can regulate the same gene. How these miRNAs relate with each other and what kind of mechanisms control these miRNAs for targeting a same gene are still unclear. When performing miRNA-mediated gene therapy, the interaction of these miRNAs also needs to be considered.

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