

# Function of miRNAs in Tumor Cell Proliferation

Zuoren Yu, Aydin Tozeren and Richard G. Pestell

**Abstract** MicroRNAs (miR) are a class of multifunctional, small, non-coding, singled-stranded molecules that regulate the stability or translational efficiency of targeted messenger RNAs. According to the miRBase Sequence Database (<http://www.mirbase.org/index.shtml>), more than 1,000 miR sequences have been identified from the tissues or cells of human origin. miRNAs are transcribed from the genome mostly by RNA polymerase II into primary miRNAs (called pri-miRNA) which are usually around 1 kb in length. pri-miRNAs are further processed in the nucleus by a ribonucleases complex composed of Drosha and DGCR8 into precursor miRNAs (called pre-miRNAs) which are around 70–90 nucleotides in length with imperfectly complementary stem-loop-stem structures. The pre-miRNA is then transported by exportin-5, a pre-miRNA-specific export carrier, to the cytoplasm where the pre-miRNA is cleaved by another ribonuclease, Dicer, into a double-stranded miRNA which consists of a mature miRNA sequence of about 17–25 nucleotides long and a miRNA\* fragment (derived from the opposite strand to the mature miRNA strand). The mature miRNA is assembled into a ribonucleoprotein complex known as RNA-induced silencing complex (RISC) that includes Argonaute protein [1]. The miR-RISC complex could lead to base-pairing interactions between a miRNA and the binding site of its target mRNAs within the 3' untranslated region (3'UTR). The interaction could lead to endonucleotic cleavage of the target mRNA or interference with its ability to be translated depending on the base-pairing complementarity between the miRNA and the target mRNA [2, 3].

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## 1 Introduction

MicroRNAs (miR) are a class of multifunctional, small, non-coding, singled-stranded molecules that regulate the stability or translational efficiency of targeted messenger RNAs. According to the miRBase Sequence Database (<http://www.mirbase.org/index.shtml>), more than 1,000 miR sequences have been identified from the tissues or cells of human origin. miRNAs are transcribed from the genome mostly by RNA polymerase II into primary miRNAs (called pri-miRNA) which are usually around 1 kb in length. pri-miRNAs are further processed in the nucleus by a ribonucleases complex composed of Drosha and DGCR8 into precursor miRNAs (called pre-miRNAs) which are around 70–90 nucleotides in length with imperfectly complementary stem-loop-stem structures. The pre-miRNA is then transported by exportin-5, a pre-miRNA-specific export carrier, to the cytoplasm where the pre-miRNA is cleaved by another ribonuclease, Dicer, into a double-stranded miRNA which consists of a mature miRNA sequence of about 17–25 nucleotides long and a miRNA\* fragment (derived from the opposite strand to the mature miRNA strand). The mature miRNA is assembled into a ribonucleoprotein complex known as RNA-induced silencing complex (RISC) that includes Argonaute protein [1]. The miR-RISC complex could lead to base-pairing interactions between a miRNA and the binding site of its target mRNAs within the 3' untranslated region (3'UTR). The interaction could lead to endonucleotic cleavage of the target mRNA or interference with its ability to be translated depending on the base-pairing complementarity between the miRNA and the target mRNA [2, 3].

The target identification of miRNAs remains challenging due to the lack of a confident criteria or effective ways to predict targets accurately. Nevertheless, different bioinformatics approaches have been applied to search the putative targets for a particular miRNA. Usually the nucleotides 2–8 (called “seed” sequence) of a miRNA are considered as the most important sequence for binding to target mRNA. A perfect complementarity between target mRNA 3'UTR to the “seed” sequence of a miRNA is required for the target prediction. At this point each vertebrate miRNA is supposed to bind to as many as hundreds gene targets or even more. And each gene may contain multiple binding sites for different miRNAs. miRNAs have the potential to target about one-third of human mRNAs [4]. However, the regulatory interaction between any predicted target gene and a particular miRNA has to be experimentally confirmed, usually through luciferase reporter assays.

miRNAs have been demonstrated to regulate a broad range of biological processes including timing of development, cell cycle progression, embryonic stem cell, cancer stem cell, cancer initiation, cancer cell proliferation, cancer metastasis and apoptosis [5–11]. Cancer is caused by multiple processes including uncontrolled proliferation and the inappropriate survival of damaged cells. Many regulatory factors switch on or off genes that direct cellular proliferation and differentiation. Emerging evidence indicates miRNAs are involved in tumorigenesis and function as tumor suppressors or oncogenes [9]. Altered expression of miRNAs or mutations of miRNA genes have been described in different types of human cancer.

For example, let-7 is downregulated in several cancers including lung cancer [12]; miR-15a and miR16-1 are deleted and/or down-regulated in ~70 % of patients with chronic lymphocytic leukemia [13]; miR-17/20a are decreased in abundance in tumor sample from breast cancer patient compared to the matching sample from same patient [14]. The expression level of a particular miRNA varies by cell type. Thus the same miRNA may perform different functions through distinct pathways dependent on the tissue or cell type. It is important to understand the tumor cell type-specific pathway through which a miRNA regulates cancer cell proliferation and tumorigenesis.

## 2 Aberrant miRNA Expression in Cancer

miRNAs were linked to cancer very soon after their discovery. The first report linking miRNA to cancer patient was in 2002 by Calin et al. showing that miR-15 and miR-16 are located at chromosome 13q14, a region deleted in more than half of B cell chronic lymphocytic leukemias (B-CLL) [13]. Both of the miRNAs are deleted or down-regulated in approximately 68 % of CLL patients. Their further study mapping 186 miRNA locations in the genome indicated that 52.5 % of miRNA genes are located at cancer-associated genomic regions or in fragile sites [8]. In 2005, Lorio et al. identified 29 miRNAs with aberrant expression in human breast cancer by microarray and northern blot analyses on 76 breast tumor samples and 14 human breast cell lines [15]. Jiang et al. detected 222 human miRNA precursors in expression profile of 32 human cell lines from lung, breast, colorectal, hematologic, prostate, pancreatic, and head and neck cancers [16]. Several miRNAs had tissue-specific aberrant expression including miR-205 which showed 36-fold higher abundance in head and neck cancer cell lines than other cell lines. In 2006, Zhang and colleagues performed an analysis of 283 known human miRNA genes by array-based comparative genomic hybridization in 227 human ovarian cancer, breast cancer, and melanoma specimens demonstrating the high-frequency gene copy number abnormality of miRNA-containing regions throughout the genome in human ovarian cancer (37.1 %), breast cancer (72.8 %), and melanoma (85.9 %) [17]. Murakami et al. analyzed the miRNA expression profiles in 25 pairs of hepatocellular carcinoma (HCC), adjacent non-tumorous tissue (NT) and nine additional chronic hepatitis (CH) specimens using a human miRNA microarray [18]. Three miRNAs (miR-224, miR-18 and pre-miR-p18) exhibited higher expression and five miRNAs (miR-199a, miR-199a\*, miR-200a, miR-125a and miR-195) showed lower expression in the HCC samples compared to the NT samples. Yanaihara et al. analyzed the miRNA expression in 104 pairs of primary lung cancers and corresponding non-cancerous lung tissues, and identified 43 miRNAs with differential expression in lung cancer [19]. Many of these miRNAs are located at frequently deleted or amplified regions in several malignancies. For example, miR-21 and miR-205 are located at the region amplified in lung cancer, whereas hsa-mir-126\* and hsa-mir-126 are at a region deleted in lung cancer. Reduced expression of precursor let-7a and let-7f

was also found in adenocarcinoma and squamous cell carcinoma. In 2007, Porkka et al. examined the miRNA expression profiling of six prostate cancer cell lines, nine prostate cancer xenografts samples, four benign prostatic hyperplasia, and nine prostate carcinoma samples using an oligonucleotide microarray [20]. They identified 51 miRNAs aberrantly expressed in prostate cancer including 37 miRNAs down-regulated and 14 miRNAs up-regulated in the prostate carcinoma samples. Gottardo et al. reported four human miRNAs (miR-28, miR-185, miR-27, and let-7f-2) significantly up-regulated in renal cell carcinoma compared to normal kidney, and ten human miRNAs (miR-223, miR-26b, miR-221, miR-103-1, miR-185, miR-23b, miR-203, miR-17-5p, miR-23a, and miR-205) up-regulated in bladder cancers compared to normal bladder mucosa [21]. In 2008, Schepeler et al. profiled the expression of 315 human miRNAs in ten normal mucosa samples and 49 stage II colon cancers using microarray technology [22]. Comparing with normal mucosa, 25 miRNAs were differentially expressed (7 down; 18 up) in microsatellite unstable colon cancers, and 54 miRNAs were differentially expressed (29 down; 25 up) in microsatellite stable colon cancers. miR-145 was identified as the lowest expression in colon cancer relative to normal tissue.

These studies provide evidence for new mechanisms by which aberrant expression of miRNAs and/or the loss or the gain of miRNA-containing genomic regions in a specific type of cancer may contribute to tumorigenesis. miRNAs may therefore serve as new diagnostic biomarkers and/or therapeutic tools for human cancers.

### 3 miRNA Regulation of Tumor Cell Division

Cancer is characterized by loss of cellular growth control, excess of cellular proliferation and altered cellular metabolism, invasion and metastasis. Understanding the mechanisms controlling cell division is important to developing novel anti-cancer therapies. Cyclins and cyclin-dependent kinases (CDKs) determine cell cycle progression. The mechanisms by which miRNAs regulate the cell cycle are increasingly well understood. Controlling cell-cycle represents new approaches to tumor cell inhibition.

#### 3.1 Cell Division Cycle

*Cyclin D1* serves as a cell cycle regulatory switch in actively proliferating cells. The *cyclin D1* gene encodes the regulatory subunit of the holoenzyme that phosphorylates and inactivates the pRb protein to promote G<sub>1</sub>/S transition. Cyclin D1 binds to CDK4/6 forming the active complex, phosphorylates the retinoblastoma (Rb) resulting in the Rb dissociation from E2F complexes resulting in the transcriptional regulation of genes which contain E2F sites in their promoters such as cyclin E, cyclin A, DNA polymerase, thymidine kinase. Cyclin E binds to CDK2 forming the

cyclin E-CDK2 complex, which pushes the cell cycle G<sub>1</sub>/S transition. The cyclin A-CDK2 complex activates DNA synthesis. The cyclin B-cdc2 complex initiates the G<sub>2</sub>/M transition. In contrary to the positive regulation of cell division cycle by cyclins and CDKs, two inhibitory families encode negative regulators of the cell cycle. The *cip/kip* family includes p21<sup>CIP1</sup>, p27<sup>KIP1</sup> and p57<sup>KIP2</sup> which arrest cell cycle at G<sub>1</sub> phase by inactivating cyclin-CDK complexes, and INK4a/ARF family including p16<sup>INK4a</sup> and p14<sup>arf</sup> [23].

*Cyclin D1* is overexpressed in several cancer types including breast, esophageal and thyroid cancer, encoding a rate-limiting factor for proliferation of cancer cells in tissue culture [24–26]. Inhibition of *cyclin D1* expression *in vivo* suppressed breast cancer cell proliferation in nude mice [28]. Cyclin E is overexpressed in 10 % of breast cancers. Emerging evidences have shown that miRNAs interact with *cyclins*, cyclin-dependent kinases (CDKs), *E2F*, *Rb* and CDK inhibitors thereby regulating cellular division and tumor growth [14, 27–30].

## 3.2 miRNAs Inhibiting Tumor Cell Proliferation

### 3.2.1 miR-15a and miR-16-1

miR-15a and miR-16-1 are deleted and/or down-regulated in chronic lymphocytic leukemia patients [13], prostate cancer [31] and pituitary tumors [32]. The anti-apoptotic gene BCL2 is negatively regulated by miR-15a and miR-16-1, leading to the inhibition of tumor growth. miR-15a and miR-16-1 induce cell cycle arrest at the G<sub>1</sub> phase by targeting cell cycle regulators including cyclin D1, cyclin E1, cyclin D3 and CDK6 [33, 34].

### 3.2.2 miR-17/20

miR-17/20 expression is decreased in human breast cancer specimen compared to the matching normal tissue suggesting a tumor suppressor function in breast cancer [14]. miR-17/20 binds to the cyclin D1 3'UTR in the MCF-7 breast cancer cells, inhibits the expression of cyclin D1, resulting in cell cycle arrest and suppression of cell proliferation [14, 35]. Besides cyclin D1, other cell-cycle related genes are regulated by miR-17/20 thereby controlling cell cycle progression [35].

### 3.2.3 miR-221/222

miR-221/222 is a miRNA cluster targeting the CDK inhibitors p27<sup>KIP1</sup> and p57<sup>KIP2</sup> [36]. miR-221/222 cluster ectopic expression decreases p27<sup>KIP1</sup> and p57<sup>KIP2</sup> abundance, activating cyclin E-CDK2 and cyclin A-CDK2 complexes, facilitating the G<sub>1</sub>/S phase transition and DNA synthesis. This has been demonstrated in both human breast cancer and gastric cancer [30, 36].

### 3.2.4 Let-7

Let-7 is a tumor suppressor miRNA family. Let-7 family members are down-regulated in lung [12], colon [37], ovarian [38] and breast cancer [39]. Let-7 regulates tumorigenesis via *Ras*, *HMG2*, *MYC* and/or *caspase-3* [39–43]. Let-7 overexpression inhibits tumor cell proliferation by targeting cyclin D1, cyclin D3, cyclin A, CDK 4 [44] and *CCNA2*, *CDC25 A*, *CDK6* and *CDK8* [45].

### 3.2.5 miR-29

miR-29 family (miR-29a, 29b, and 29c) have been associated with acute myelogenous leukemia (AML), rhabdomyosarcoma, hepatocellular carcinoma and mantle cell lymphoma [46–49] by regulating cell apoptosis, cell cycle, and cell proliferation pathways. miR-29 overexpression induces apoptosis and inhibits tumor cell proliferation *in vitro*. In mantle cell lymphoma miR-29 inhibits CDK6 protein and mRNA levels. The down-regulation of miR-29 may cooperate with cyclin D1 in MCL pathogenesis [49].

### 3.2.6 miR-34, miR-192 and miR-215

miR-34, miR-192 and miR-215 regulate the p53 tumor suppressor network [50]. miR-34 overexpression arrests the cell cycle, induces apoptosis and inhibits cancer cell proliferation and colony formation by downregulating cyclin D1, cyclin E2, E2Fs and CDK4/6 [50–53]. miR-192 and miR-215 expression is reduced in colon cancer samples, and miR-192/ miR-215 suppresses colony formation and carcinogenesis via p21<sup>CIP1</sup> accumulation and cell cycle arrest [54]. p21<sup>CIP1</sup> accumulation is partially dependent on the presence of wild-type p53.

## 3.3 miRNAs Enhancing Tumor Cell Proliferation

### 3.3.1 miR-21

miR-21 overexpression has been observed frequently in a wide variety of cancers including breast cancer [55, 56], lung cancer [19] and liver cancer [57]. miR-21 induces MCF-7 cell-derived breast tumor growth in the xenograft mouse model [55]. In MCF-7 cells, miR-21 targets the antiapoptotic gene Bcl-2 [55], the tumor-suppressor gene *tropomyosin 1 (TPM1)* [58] and the tumor suppressor protein Programmed Cell Death 4 (PDCD4) [56]. In human hepatocellular cancer cells miR-21 targets the tumorsuppressor gene PTEN, thereby enhancing tumor cell proliferation, migration, and invasion [57].

### 3.3.2 miR-27a

miR-27a expression is upregulated in kidney cancers [21] and breast cancer cell lines [59]. The zincfinger *ZBTB10* gene is a direct target of miR-27a [59]. In MDA-MB-231 human breast cancer cells, miR-27a inactivation induced *ZBTB10* expression and reduced expression of oncogene *specificity proteins* (*Sp1*, *Sp3*, and *Sp4*) at the mRNA and protein levels [60]. The cyclin B-cdc2 complex inhibitor *Myt-1* is another target of miR-27a [60]. In MDA-MB-231 cells miR-27a inhibits *Myt-1* expression increasing Cyclin B-cdc2 activity thereby promoting breast cancer cell proliferation.

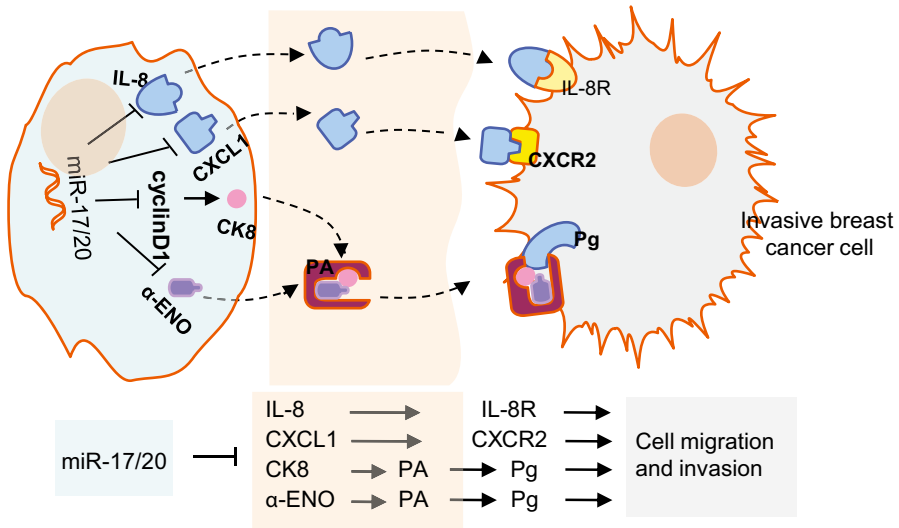
### 3.3.3 miR-155

miR-155 is frequently up-regulated in breast cancer [61], lung cancer [19], pancreatic [62] and lymphomas [63]. In breast cancer, miR-155 expression induces cell survival, growth and chemoresistance by targeting the FOXO3a gene [61]. In pancreatic ductal adenocarcinoma (PDAC) cells, miR-155 is oncogenic by targeting the tumor suppressor gene *tumor protein 53-induced nuclear protein 1* (TP53INP1) [62].

## 4 miRNA Regulation of Cancer Stem Cells

Cancer stem cells (CSCs) are a subpopulation of stem-like cells within tumors. CSCs are characterized by their self-renewal capacity, an ability to differentiate into non-tumorigenic cell progeny, and their ability to seed tumors when transplanted into animal hosts [65]. CSCs have been demonstrated in several solid tumors including human breast cancer and brain cancer [64, 66], and melanoma, glioblastoma colon, pancreas, lung and prostate cancers. CSCs are isolated and enriched on the basis of cell surface markers (CD44, CD24 and/or CD133) dependent on tumor type. The leukaemic stem-like cells are fractioned by CD34<sup>++</sup> CD38<sup>-</sup> [67]; the mammary tumorigenic CSCs are isolated by CD44<sup>+</sup> CD24<sup>-/low</sup> lineage<sup>-</sup> [65]; the colon CSCs are isolated by CD133<sup>+</sup> [68]. Recently epithelial-specific antigen (ESA) and aldehyde dehydrogenase-1 (ALDH-1) were added into the candidate list of CSC-specific markers [69, 70, 71].

miRNAs regulate self-renewal and differentiation of ES cells, adult tissue stem cells, and CSCs. A subset of miRNAs (miR-142-3p, miR-451, miR-106a, miR-142-5p, miR-15b, miR-20a, miR-106b, miR-25 and miR-486) has altered expression in lung cancer progenitor cells [72]. Thirty seven miRNAs were deregulated in human breast cancer stem cells (CD44<sup>+</sup> CD24<sup>-/low</sup> lineage<sup>-</sup>) compared to the lineage<sup>-</sup> nontumorigenic breast cancer cells [73]. Notably, members of miR-200 family including miR-200a, miR-200b and miR-200c, were down-regulated in the breast cancer stem cell population. The miR-200 family regulates epithelial to mesenchymal transition (EMT) in breast cancer [74]. Let-7 has a low expression in breast CSCs and



**Fig. 1** miR-17/20 regulates cancer cell migration and invasion via heterotypic secreted signals Pg plasminogen, PA plasminogen activator, IL-8R IL-8 receptor, CXCR2 CXCL1 receptor

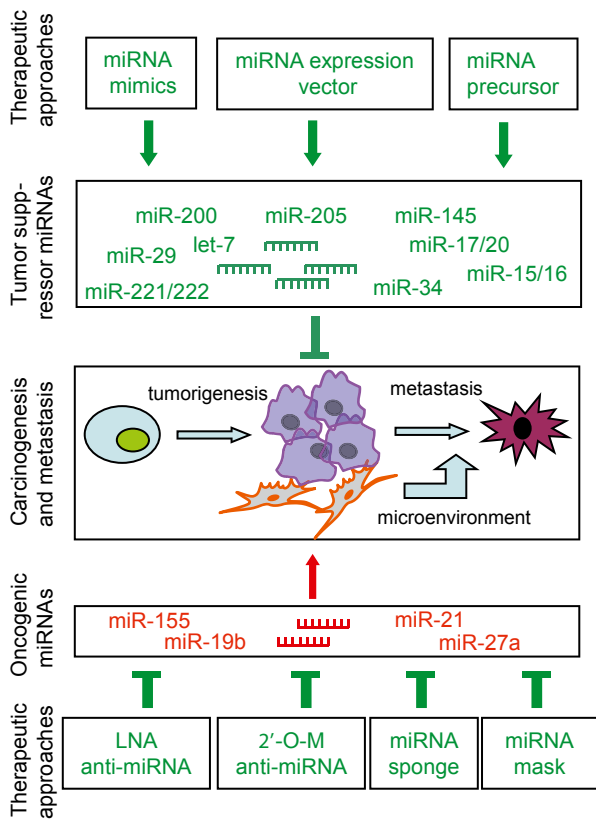
increases with differentiation. Let-7 regulates self renewal and tumorigenicity of breast cancer stem cells [39]. Expression of let-7 in breast CSCs reduced proliferation, mammosphere formation, and the proportion of undifferentiated cells *in vitro* and tumor formation and metastasis in NOD/SCID mice [39].

### 5 miRNA Regulation of Tumor Microenvironment and Cancer Metastasis

The regulation of the tumor microenvironment to promote tumorigenesis was proposed by Paget in his ‘soil and seed’ hypothesis of cancer metastasis. Carcinogenesis and metastasis are controlled by both internal and external “heterotypic” signals from the surrounding cells and environment. Cancer metastases represent a complex process by which primary solid tumor cells invade adjacent tissue and grow into secondary tumors. miR-373 and miR-520c stimulate breast cancer cell migration and invasion by suppressing the gene *CD44* [75]; miRNA-200 and miR 205 inhibit EMT in breast cancer [74]. Our recent studies demonstrated a novel mechanism by which miRNA regulates cancer cell migration and invasion via heterotypic secreted signals (Fig. 1) [76]. miR-17/20 conditioned medium from cultures of MCF7 cells (a non-metastatic line) inhibited the migration and invasion of MDA-MB-231 cells (a metastatic line). miR-17/20 decreased the abundance of secreted factors such as cytokines (IL-8, CXCL1) and plasminogen activators (cytokeratin 8/18 and α-enolase) in MCF-7 cells. These secreted factors were essential for migration of the cancer cells. As miRNAs and the cancer microenvironment have a



**Fig. 2** miRNA-based cancer diagnostic and therapeutic strategies



crucial role in tumorigenesis and metastasis, these studies identify a potential new site of invention via inhibition of miRNA-regulated secreted factors.

## 6 Therapeutic Application of miRNA in Cancer

MiRNA-based cancer diagnostic and therapeutic approaches are being established and tested in animal models. There are two strategies for miRNA-based therapeutic application in cancers: expression-based restoration of tumor suppressor miRNA and functional inhibition of oncogenic miRNA (Fig. 2).

### 6.1 Restoring the Expression of Tumor Suppressor miRNAs

Two lines of evidence suggest miRNAs function as tumor suppressor rather than oncogenes. The global decrease of miRNA expression in cancer tissues compared

to normal control [77], and the enhanced cellular transformation and tumorigenesis by impaired miRNA processing [78]. Synthetic miRNA mimics or miRNA expression vectors carrying either a pre-miRNA sequence or an artificial miRNA hairpin sequence have been widely used to perform the miRNA expression restoring *in vitro*. Recent publications demonstrated that miRNA reintroduction suppresses tumor growth *in vivo* [79, 80, 81]. Intranasal delivery of exogenous let-7 mimics to the lung tumors of mice (non-small-cell lung cancer) reduced the tumor burden [80]. Intravenous delivery of miR-34a mimics using a lipid-based delivery vehicle accumulated miR-34a in the tumor tissue in mouse models of non-small-cell lung cancer, and blocked tumor growth. Furthermore, this approach did not induce an immune response [81]. An adeno-associated virus (AAV)-mediated delivery of *miR-26a* to mouse liver resulted in reduced liver cancer cell proliferation, induction of tumor-specific apoptosis, and protection from disease progression without toxicity [82].

## 6.2 Blocking the Function of Oncogenic miRNAs

Since a small number of miRNAs show oncogenic function, such as miR-21, decreasing the expression level or blocking the function of those oncogenic miRNAs is another strategy for cancer therapy. Chemically modified antisense oligonucleotides (called anti-miRNA) are used most frequently to knock down miRNA *in vitro*. The modification includes addition of 2'-O-methyl, addition of 2'-O-methoxyethyl and locked nucleic acid (LNA) with 2'-O connecting to 4'-C. The modified nucleic acid structure has high affinity and high specificity to bind with target miRNA. Moreover, the anti-miRNA-miRNA structure is highly stable. As such the delivery of a specific anti-miRNA into cells prevents the miRNA from binding to their cognate target genes thereby silencing miRNA function.

Recently two more approaches were reported for blocking miRNAs. One is called the miRNA-sponge [83] which serves as a competitive inhibitor of miRNAs. An expression vector carrying multiple binding sites to a targeted miRNA is introduced into cells. Following the vector gene transcription, the over-expressed synthetic binding sequences occupy the endogenous miRNA in the cells with high affinity blocking miRNA regulation of its target genes. Another approach called the miRNA-mask [84] which uses oligonucleotides perfectly complementary to miRNA binding sites of target mRNAs. The miRNA-mask blocks the access of the miRNA to the binding sequence of the target mRNAs thereby blocking the miRNA-mRNA interaction.

Tumor-targeted delivery and local administration are still major challenges to apply miRNA therapy to the clinic. The possibility of immune response, off-target effects and toxicity of exogenous miRNA mimics or antagonism to normal tissues will have to be taken into account and minimized to a safe level.

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