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Review

microRNA, Cell Cycle, and Human Breast Cancer

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The discovery of microRNAs as a novel class of gene expression regulators has led to a new strategy for disease diagnostics and therapeutics. Cell cycle, cell proliferation, and tumorigenesis are all regulated by microRNAs. Several general principles linking microRNAs and cancer have been recently reviewed; therefore, the current review focuses specifically on the perspective of microRNAs in control of cell cycle, stem cells, and heterotypic signaling, as well as the role of these processes in breast cancer. Altered abundance of cell cycle regulation proteins and aberrant expression of microRNAs frequently coexist in human breast cancers. Altered microRNA expression in breast cancer cell lines is associated with altered cell cycle progression and cell proliferation. Indeed, recent studies have demonstrated a causal role for microRNA in governing breast tumor suppression or collaborative oncogenesis. This review summarizes the current understanding of the role for microRNA in regulating the cell cycle and summarizes the evidence for aberrant microRNA expression in breast cancer. The new evidence for microRNA regulation by annotated genes and the involvement of microRNA in breast cancer metastasis are discussed, as is the potential for microRNA to improve breast cancer diagnosis and therapy. ***(Am J Pathol 2010, 176:1058–1064; DOI: 10.2353/ajpath.2010.090664)***

microRNA (miR) are a new class of multifunctional small molecules that regulate the stability or translational efficiency of targeted messenger RNAs. According to the miRBase Sequence Database (Release 13.0 in March 2009), 706 miRs have been identified in humans and 547 in mice to date. Mature miRs are assembled into a ribonucleoprotein complex known as RNA-induced silencing complex (RISC) that includes Argonaute protein Ago2.[1] The miR-RISC complex may lead to base-pairing interactions between miRs and the 3′ untranslated region (3′UTR) of their target mRNAs, often repressing the gene translation or cleaving the target mRNA, depending on the base-pairing features between the miR and the target mRNA.[2,3] Because each vertebrate miR may bind to as many as 200 gene targets, and each gene may contain multiple binding sites for different miRs, miRs potentially could regulate the expression of about one-third of human mRNAs.[4] miRs often target various components of cellular networks or signaling pathways.[5,6] As such, miRs have been predicted to play a prominent role in regulating a broad range of biological processes including development, apoptosis, cell cycle progression, cellular proliferation, cancer initiation, and cancer metastasis.[7–14]

Altered expression of miRs has been demonstrated in different types of human cancer. Thus, the potential of miRs to be robust biomarkers for cancer diagnosis, prognosis, and pathogenesis has been predicted.[14,15] miR-encoding genes are frequently located at fragile sites and in minimal regions of loss of heterozygosity, minimal regions of amplification, and in common breakpoint regions involved in cancers.[11]

Altered miR expression in human breast cancer was first demonstrated in 2005.[16] In breast cancer, abnormalities of the cell cycle are frequently observed, including loss of retinoblastoma (Rb) function, reduced cyclin-dependent kinase (CDK) inhibitor p21 (Waf1/Cip1) and p27 (Kip1) abundances, as well as increased abundance of D and E type cyclins. Cyclin D1 encodes a key regulator of the cell cycle transition from G_1 to the DNA synthetic

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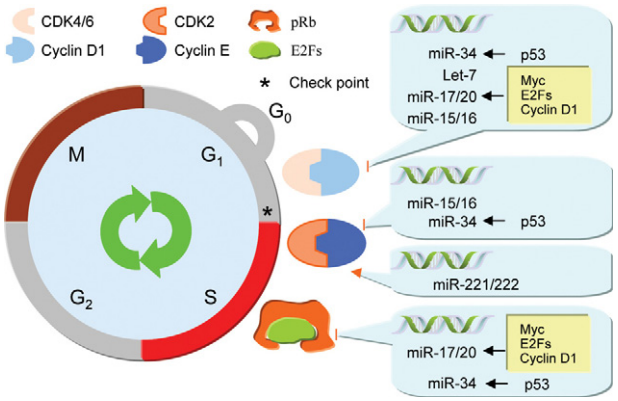

Figure 1. miRNA–cell cycle regulation network. The miR-34 family is involved in cell cycle regulation by repressing E2F, cyclin D1, and cyclin E expression. miR-34 itself is a direct transcriptional target of p53. The miR-17/20 cluster is transcriptionally regulated by myc, E2Fs, and cyclin D1, and it in turn regulates E2F, pRb, and cyclin D1 expression at the translation level. miR-15/16 regulates cell cycle control by inhibiting cyclin D1, cyclin E, and CDK4/6.

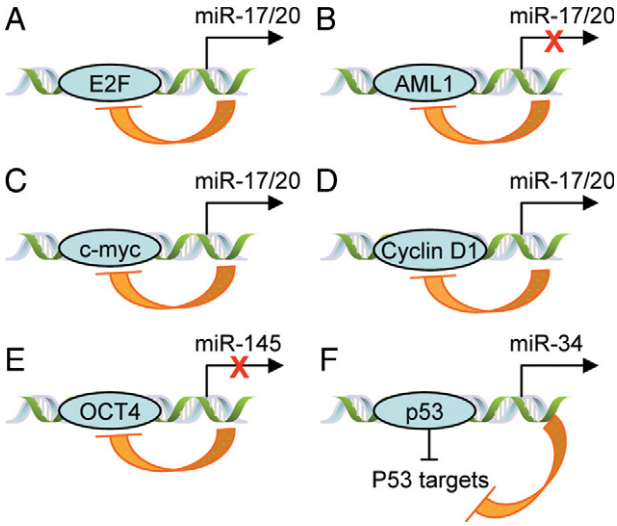

Figure 2. miR-target regulatory loop. **A:** The miR-17/20 cluster, which is transcriptionally induced by E2F, in turn translationally represses E2F expression. **B:** AML1 transcriptionally inhibits miR-17/20 expression, and miR-17/20 inhibits AML1 translation. **C:** Myc induces the miR-17/20 cluster, and miR-17/20 inhibits myc expression. **D:** Cyclin D1 induces miR-17/20 expression, and miR-17/20 translationally inhibits cyclin D1 expression. **E:** OCT4 inhibits miR-145 expression, and miR-145 in turn inhibits OCT4 by 3′UTR binding. **F:** p53 transcriptionally induce the expression of the miR-34 family, and miR-34 negatively targets the downstream genes of p53.

phase and is overexpressed in more than 50% of breast cancers, functioning as a rate-limiting factor for human breast cancer cell proliferation *in vivo* and *in vitro*.[17,18] Cyclin E, another important cell cycle regulator, is overexpressed in more than 10% of breast cancers and is a powerful prognostic predictor in early stage breast cancer as well as a significant determinant of tumor aggressiveness.[19] miRs interact with *E2F*, *Rb*, *cyclins*, CDKs, and CDK inhibitors, providing the potential to regulate cellular division and cell cycle progression.[20–23]

miR Regulation of Cell Cycle Progression Genes

The mechanisms governing cell cycle control by miRs are increasingly well understood. Five groups of miRs, including the miR-15a/16 cluster, the miR-17/20 cluster, the miR-221/222 cluster, and the let-7 and miR-34 families, can regulate cell cycle progression by directly targeting cell cycle regulators (Figure 1).

miR-15a/16 Cluster

The miR-15a/16 cluster is deleted and/or down-regulated in ≈70% of chronic lymphocytic leukemia patients,[24] as well as in pituitary adenomas[25] and in a gastric cancer cell line,[26] suggesting an important role in cell proliferation and tumor growth. The miR-15a/16 cluster induces cell cycle arrest at the G_1 phase by targeting cyclin D1, cyclin E1, cyclin D3, and CDK6.[27] In addition, through a high-throughput profiling analysis Calin et al identified a gene signature regulated by miR-15a/16 in a leukemic cell line that included cell cycle and apoptosis signaling genes.[28]

miR-17/20 Cluster

The miR-17/20 cluster, which encodes six mature miRs within a 1-kb genomic region, inhibits tumor growth in a human B cell line[20] and breast cancer cell lines.[22] miR-17/20 coordinates the timing of cell cycle by targeting multiple cell cycle regulators (E2F, c-*myc*, Rb, and cyclin D1) as shown in Figure 1. In the G_1 phase of a cell cycle, *c-myc* and *cyclin D1* are induced while *E2F1* is inactivated by binding to Rb. The miR-17/20 cluster is involved in the G_1 to S transition of the cell cycle. Previous publications have indicated that miR-17/20 dampens the reciprocal activation of *E2F* by *c-myc* through inhibiting *E2F* translation.[20] A Rb family member, Rbl2, is also a target of miR-17-5p.[21] The first studies demonstrating that miRs directly inhibit cyclin D1 showed that miR-17/20 targeted the cyclin D1 3′UTR in the MCF-7 breast cancer cell line, resulting in cell cycle arrest and suppression of cell proliferation.[22] miR-17-5p also inhibits the estrogen receptor α (*ER-*α) coactivator AIB1 in breast cancer cells.[29] Collectively, these studies suggested the miR-17/20 cluster may function as a breast tumor suppressor through the regulation of cell cycle progression genes.

It has been hypothesized that the miR-17/20 cluster regulates cell cycle progression via E2F, c-Myc, and cyclin D1, as these proteins can both regulate and be regulated by miR-17/20 (Figure 2, A–D). These regulatory loops provide mechanisms to tightly control cell cycle progression and cell proliferative signals. In contrast with the mammary gland,[22] the expression of this miR cluster was increased and cell growth was enhanced in both lung cancer and lymphomas,[30,31] indicating that miR-17/20 function is cell type–dependent.

miR-221/222

miR-221/222 regulates the cell cycle by targeting CDK inhibitors. Ectopic expression of the miR-221/222 cluster

activates CDK2, facilitates G_1/S phase transition, and enhances tumor growth by negatively regulating $p27^{kip1}$ and $p57^{kip2}$.[32] This has been demonstrated in both the MCF-7 cell line and HER2/neu-positive primary human breast cancer tissues.[23] Furthermore, increased miR-221/222 cluster expression is associated with tamoxifen resistance in breast cancer.[23,33]

let-7

let-7 controls the timing of cell cycle exit and terminal differentiation in *C. elegans*.[34] The abundance of let-7 family members is reduced in several types of cancer including lung and breast cancer.[35,36] let-7 overexpression in lung cancer cell lines suppressed cell cycle progression and reduced cell division.[37] The mechanism by which let-7 regulates tumor growth is via targeting the *Ras*, *HMGA2*, and *caspase-3* genes.[36,38–40] Multiple important cell cycle control genes are repressed by let-7 including cyclin D1, cyclin D3, cyclin A, CDK4[41] and *CCNA2*, *CDC25A*, *CDK6*, and *CDK8*.[37]

miR-34

The miR-34 family (miR-34a, miR-34b, and miR-34c) is an important component of the p53 tumor suppressor network.[42] DNA damage and oncogenic stress activate p53. p53 binds to the promoter of miR-34a and miR-34b/c, inducing their expression at the transcriptional level (Figure 2F). Ectopic expression of the miR-34 family induces cell cycle arrest and apoptosis by down-regulating cyclin D1, cyclin E2, E2Fs, and CDK4/6.[42,43,44] miR-34b/c overexpression inhibits cell proliferation and colony formation in soft agar.[45] Two additional miRs, miR-192 and miR-215, are also involved in the p53 network, and upregulation of these two miRs suppresses carcinogenesis via $p21^{CIP1}$ accumulation.[46]

miRs in Breast Cancer

Liu et al[16] first reported the aberrant expression of miRs in human breast cancers compared with normal tissue. They identified 29 miRs with aberrant expression in human breast cancer by microarray and Northern blot analyses on 76 breast tumor samples and 14 human breast cell lines. Of the altered miRs in breast cancers, a substantial proportion has been aligned to genomic fragile sites or regions associated with cancers.[11,47] Zhang and colleagues performed an analysis on 283 known human miR genes by array-based comparative genomic hybridization in 73 breast cancer specimens (55 primary tumors and 18 cell lines), demonstrating the high-frequency gene copy number abnormality of miR-containing regions throughout the genome in human breast cancers.[47] Approximately 73% (206 of 283) of miR genes were located in regions that exhibited DNA copy number abnormalities.[47] The location of miRs in a genomic region amenable to alterations is not a random event, suggesting that the loss or the gain of genomic regions including miRs in a specific type of cancer participates in the cause of the malignancy.[48]

Table 1. miRs in Breast Cancer

	Target(s)	Reference(s)
Tumor suppressor miRs		
Let-7	Ras, Hmga2	36,38,39
miR-125a/b	ErBb2, ErBb3	51
miR-206	ERα	55,56
miR-17/20	AIB1, Cyclin D1, E2F	20,22,29
miR-145	RTNK	57
miR-205	HER3 receptor	58
Onco-miRs		
miR-21	Tpm1, PDCD4	59,60
miR-27	ZBTB10, Myt1	61,62

The miR distribution in breast tumor tissues from more than 100 patients using *in situ* hybridization[49] shows a different distribution and expression of many miRs in the breast cancer tissues compared with normal tissues. miR-145 and miR-205 were restricted to the myoepithelial/basal cell compartment of normal mammary ducts and lobules. However, their accumulation was reduced in matched tumor specimens. Compared with luminal epithelial cells in normal tissue, expression of miR-21 was frequently increased, whereas let-7a was decreased in malignant cells. Careful comparison of normal tissue and tumorous tissue in the same patient demonstrated altered expression of miR-17/20 in breast cancer. In normal tissue, the relative abundance of miR-17/20 is higher compared with matched tumor tissue from the same patient.[22]

Suppressor miRs in Breast Cancer

Tumor suppressor miRs can inhibit tumorigenesis by repressing oncogenes (Table 1). The *ErbB* family plays an important role in organismal development, cellular proliferation, and survival in human epithelial malignancies.[50] *ErbB2* is amplified and/or overexpressed in about 20% to 30% of human breast cancers. miR-125 targets the *ErbB2* gene in breast cancer cells.[51] miR-125a and mir-125b overexpression in SKBR3 cells decreased *ErbB2* protein level ≈40% to 65% and decreased *ErbB3* level ≈60% to 80%. SKBR3 cells overexpressing miR-125a or miR-125b were impaired in their anchorage-dependent growth, migration, and invasion capacities.[51]

The human miR-17/20 cluster's genomic location, chromosome 13q31, correlates with loss of heterozygosity in a number of different cancers including breast cancer.[52,53] miR-17/20 decreased *cyclin D1* abundance, suppressed MCF-7 cell proliferation, and inhibited G_1/S phase transition of cell cycle.[22] In human breast cancer cell lines, reduced miR-17/20 expression was inversely correlated with high *cyclin D1* abundance. In human breast cancer specimens, decreased miR-17/20 expression correlated with high cyclin D1 abundance compared with matched normal breast tissues. Targeted gene deletion revealed that mice deficient for the miR-17/20 cluster die shortly after birth,[54] therefore conditional gene

deletion or tissue-specific transgenic techniques will be required to determine the function of miR-17/20 in mammary tumorigenesis.

Estrogen, which binds *ER-α*, is a risk factor for breast cancer development. *ER-α* is overexpressed in approximately 75% of primary breast cancers. It was reported that miR-206 expression is strongly inhibited by *ER-α* agonists, and miR-206 levels are higher in ER-α–negative MB-MDA-231 cells than ER-α–positive MCF-7 cells.[55] miR-206 overexpression reduces ER-α level in MCF-7 cells, indicating an autoregulatory feedback loop between ER-α and miR-206. Decreased expression of miR-206 occurs in ER-α–positive human breast cancer tissue. miR-206 expression and ER-α protein level are inversely correlated in human breast cancer.[56] In addition, miR-145 targets RTNK and inhibits breast tumor growth.[57] miR-205 targets HER3 receptor, thereby inhibiting HER-mediated proliferative signaling.[58]

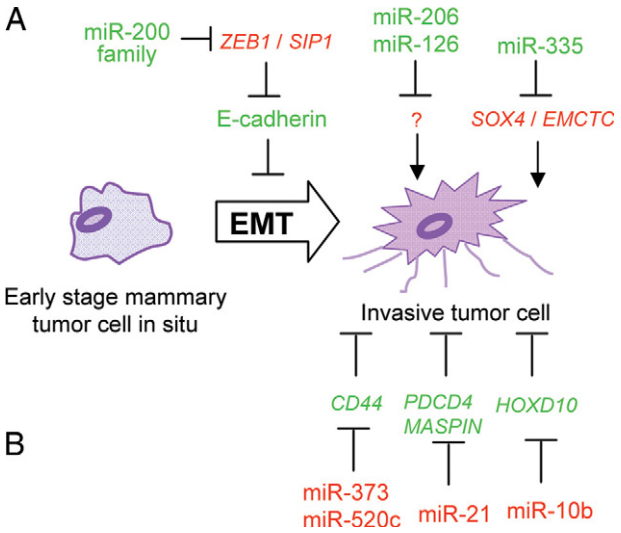

Figure 3. miRNA regulation of breast cancer metastasis. **A:** Metastasis inhibitor miRs in breast cancer. **B:** Metastasis inducer miRs in breast cancer.

Oncogenic miRs in Breast Cancer

Several groups have noticed the frequent overexpression of miR-21 in breast tumors compared with the matched normal breast tissues (Table 1).[49,59,60] miR-21 knock down inhibits MCF-7 cell growth *in vitro* and suppresses MCF-7 cell–derived breast tumor growth in a murine xenograft model.[59] The tumor suppressor *tropomyosin 1* (*TPM1*) was a target of miR-21 in MCF-7 cells. A genome-wide screen for miR-21 targets identified several p53-regulated mRNAs, including *FAM3C*, *ACTA2*, *APAF1*, *BTG2*, *FAS*, *CDKN1A (p21)*, *PDCD4*, and *SESN1*[60] in MCF-7 cells, suggesting a functional link between miR-21 and the p53 tumor suppressor pathway.[60]

miR-27a was reported as a breast cancer oncomiR that regulates the zinc finger *ZBTB10* gene,[61] a putative repressor of oncogene *specificity proteins* (*Sp*). Inactivation of miR-27a in MDA-MB-231 cells induced *ZBTB10* expression and reduced abundance of the *Sp* genes.[62] miR-27a suppressed the expression of cdc2/cyclin B inhibitor *Myt-1* in MDA-MB-231 cells, thereby increasing the cdc2/cyclin B activity and inducing breast cancer cell proliferation. Thus miRs regulate distinct signaling cascades in breast cancer cells.

miRs in Breast Cancer Metastases

Metastasis represents a complex process by which primary solid tumor cells invade adjacent tissue and grow into secondary tumor (Figure 3, A and B).[63] Breast cancer cells have the potential to spread to almost any region of the body where they continue to grow and multiply. Expression of a metastasis suppressor gene, *CD44*, is reduced during progression of breast cancer to the metastatic phenotype.[64] miR-373 and miR-520c stimulated human breast cancer cell migration and invasion *in vitro* and *in vivo* by suppressing the expression of *CD44*.[65] miR-21 promotes breast cancer cell invasion and metastasis by targeting multiple tumor/metastasis suppressor genes.[66] miR-10b is markedly upregulated in breast cancer cells compared with either primary human mammary epithelial cells or MCF-10A cells.[67] miR-10b promotes cell migration *in vitro* and initiates breast tumor invasion *in vivo* by targeting gene *HOXD10*.[67] Twist promotes epithelial to mesenchymal transition (EMT) and mammary tumor metastasis,[68] and miR-10b is directly regulated by Twist through a transcriptional manner.[67]

In contrast, miR-335, miR-206, and miR-126 have been identified as human breast cancer metastasis suppressor miRs.[63] miR-335 or miR-206 overexpression reduced cell migration of lung metastatic LM2 cells and bone metastatic BoM1 cells. The expression of miR-335 and miR-126 in human mammary tumors is inversely associated with metastatic relapse. Breast cancer metastasis and migration are inhibited by miR-335 through targeting the transcription factor *SOX4*, which is known to regulate progenitor cell development and migration.[63]

miR-200 family and miR-205 regulate EMT, which is thought to be an essential early step in tumor metastasis.[69] Expression of these miRs is reduced in invasive breast cancer cell lines with a mesenchymal phenotype. Enforced expression of the miRs prevents TGF-β–induced EMT. Conversely, overexpression of these microRNAs in mesenchymal cells initiated mesenchymal to epithelial transition. The miR-200 family repressed ZEB1 and SIP1, which have been implicated in EMT induction and tumor metastasis.[70] A ZEB1-miR200 family-feed-forward loop may stabilize EMT and hereby promote cancer cell invasion.[71] Because most of the evidence for miRs in regulating metastasis has been performed in cultured cells, *in vivo* studies of miR using transgenic and knockout mice will be important.[72]

Regulation of miRs by Annotated Genes

miRs are also regulated by products of annotated genes, which opens the possibility of regulating miRs expression by external agents. For instance, a number of miRs are induced by all-*trans*-retinoic acid, and the promoters of

several miRs have putative RARα receptor binding sites.[73] p53 is known to induce miR-34,[42] which in turn targets several genes whose expression is decreased by p53. The induction of miR-34 family by p53 allows p53 to regulate a large number of genes that are downstream targets of miR-34. The targeting of p53-regulated mRNAs by miR-34 may contribute to the fine tuning of the p53 response.[74] This finding suggests a dual model for gene down-regulation of gene expression by p53, one by direct action on the target genes transcription or stability, and a second action via a specific miR (Figure 2F). Increased miR-223 expression in differentiating myeloid cells occurs via the binding of C/EBPα (an inducer of differentiation) to the miR-223 promoter.[75] Another example of feedback regulation by annotated genes is the finding by Yu et al,[22] in which cyclin D1 is targeted to the miR-17/20 cluster, which in turn regulates the expression of the same cluster. Thus cyclin D1, like C/EBPα and RARα, binds to the promoter region of miR to regulate their expression. The finding that cyclin D1 regulates miR promoter is consistent with previous finding that cyclin D1 targets gene promoter elements in the context of local chromatin.[76,77] Feedback regulation of miR and annotated genes may be quite frequent, and a feedback between OCT4 and miR-145 will be discussed below.

miRs in Human Stem Cell Renewal and Differentiation

miRs were discovered as modulators of differentiation in embryos of lower animals. miR-145, which was reported to act as a tumor suppressor,[16,78] also plays an important role in human stem cell growth and differentiation by targeting the 3′UTR of OCT4, SOX2, and KLF4.[79] OCT4 in turn represses miR-145 expression (Figure 2E). Shi et al[6] showed that miR-145 inhibits human cancer cell growth *in vitro* via the 3′UTR of the type 1 insulin-like growth factor receptor and its docking protein, the insulin receptor substrate-1 (IRS-1). Rubin et al[80] found IRS-1 was highly expressed in undifferentiated murine embryonic stem cells (mESC), with decreased expression when the cells differentiated. Forced expression of IRS-1 inhibited mESC differentiation suggesting miR-145 and IRS-1 function in stem cell differentiation.

It is believed that only a small proportion of cancer cells display the stem/progenitor cell characteristics and retain the ability to form new tumors. These cells are termed tumor-initiating cells (T-ICs) or cancer stem cells.[81] Breast tumor-initiating cells (BT-ICs) have been identified and isolated as CD44(+)CD24(−/low)Lineage(−) cells by Al-Hajj and colleagues.[82] The miR let-7, which is reduced in BT-ICs and increased with differentiation, can regulate self renewal and tumorigenicity of breast cancer cells.[36] Let-7 overexpression in BT-ICs inhibited cell proliferation and mammosphere formation, blocking tumor formation and metastasis in NOD/SCID mice.[36] Two targets of let-7, *RAS*[39] and *HMGA2*,[38] showed high expression in BT-ICs and were silenced during differentiation.

Concluding Remarks

The regulation of miR expression by annotated genes indicates the importance of miR promoter regions and new possibilities for therapeutic intervention by using compounds to regulate miR expression. miRs have been identified that arrest cell cycle progression and suppress cell proliferation by negatively regulating cell cycle modulators in breast cancer cells. miRs that are overexpressed in breast cancers can limit the proliferation of tumor cells by targeting oncogenic genes. Because miRs are involved in breast cancer from the onset of the malignant state through the progression to metastasis, miR may be ideal targets for the development of new drug therapies for treating breast cancer patients.

Although the up- or down-regulation of a miR expression in cancers depends on the individual miR itself and tumor type, the global decrease of miRs in cancers reflects the tendency in the direction of the differential expression of miRs in human cancers.[83] This finding suggests that miRs may serve a general tumor suppressor function. The regulation of miRs may therefore form the basis for a therapeutic strategy for cancers. Synthetic miR mimics, DNA expression vectors containing artificial miR precursor sequences and/or miR inhibitors (modified miR antisense oligonucleotides), have strong potential as new therapeutic tools.[83]

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