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Non-Coding RNAs as Theranostics in Human Cancers

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ABSTRACT

Theranostics was coined originally as a term used to describe a system that combines diagnosis and therapy, aiming to provide the tools for personalized medicine. This review reasserts the grounds for regarding non-coding RNAs (ncRNA) as theranostics in human cancers. The microRNAs (miRNAs) are the most well studied ncRNAs in recent years; their pivotal role in orchestrating tumor initiation and progression has been confirmed in all types of cancers. Hence, these small ncRNAs have emerged as attractive therapeutic targets and diagnostic tool. Various approaches to use their therapeutic potential have been taken, here we summarize the most important ones. In the near future, the focus of theranostics will be shifted towards longer and mechanistically more versatile ncRNAs, and we included some recent advances supporting this view. J. Cell. Biochem. 113: 1451–1459, 2012.

KEY WORDS: miRNA; THERAPY; CANCER

The discovery that around 98% of all transcriptional output in humans is actually non-coding RNA (ncRNA), questioned the traditional opinion that RNA is a simple intermediate between DNA and protein [Mattick, 2001]. The biological complexity of higher organisms renders in these RNA species that orchestrate all fundamental cell processes, rather than in the number of protein-coding genes.

Non-coding RNAs can be divided into two major classes based on transcript size: small ncRNAs (e.g., miRNAs, siRNAs, or piRNAs), and long ncRNAs (e.g., long intergenic or intronic ncRNAs, pseudogens, or transcribed ultraconserved regions). Of this class of ncRNAs, microRNAs (miRNA) have captured the spotlight in the past decade. These miRNA are phylogenetically conserved, single-stranded RNAs of 19–25 nucleotides, mostly transcribed from intragenic or intergenic regions by RNA polymerase II into primary transcripts, termed primary miRNAs [Bartel, 2004]. The pri-miRNAs are then processed to a smaller, hairpin intermediates, called precursor miRNA (pre-miRNAs), by Drosha RNase III endonuclease and exported to the cytoplasm by Exportin 5. In the cytoplasm, the pre-miRNAs are further cleaved by Dicer, also an RNase III endonuclease, resulting in mature double-stranded miRNAs. After strand separation, the mature miRNA is incorporated in the RNA-induced silencing complex (RISC), whereas the other strand commonly undergoes degradation. The RISC complex contains the proteins necessary for the degradation and/or silencing of mRNA targets, such as argonautes, helicases, deadenylases, and methyl-transferases [Di Leva et al., 2006]. For target recognition and incorporation into the RISC, the mature miRNAs are essential. As perfect complementarity is required only between the positions 2–8 from the 5′ miRNA (seed sequence) with the 3′-untranslated region (UTR) of their target mRNA for efficient silencing, each miRNA can potentially target a large number of mRNAs, and each mRNA can be targeted by more than one miRNA [Bartel, 2004]. Thus, miRNAs can function in cancer cells as tumor suppressor or as oncogenes, or in some cases, both, rendering them the capability of reprogramming molecular pathways and networks in cancer (Fig. 1).

It is then not surprising that these small ncRNAs have emerged as appealing therapeutic targets and diagnosis and prognosis tools.

miRNAs AND CANCER

A plethora of studies linked by now the abnormal expression of these ncRNAs to the pathogenesis of several human diseases, including solid and hematopoietic tumors. miRNA frequent location at amplified, deleted, or translocated chromosomal regions (fragile sites), further supports their role in cancer development [Calin et al., 2004]. It was the discovery by Calin et al. [2002] that miR15a/16-1

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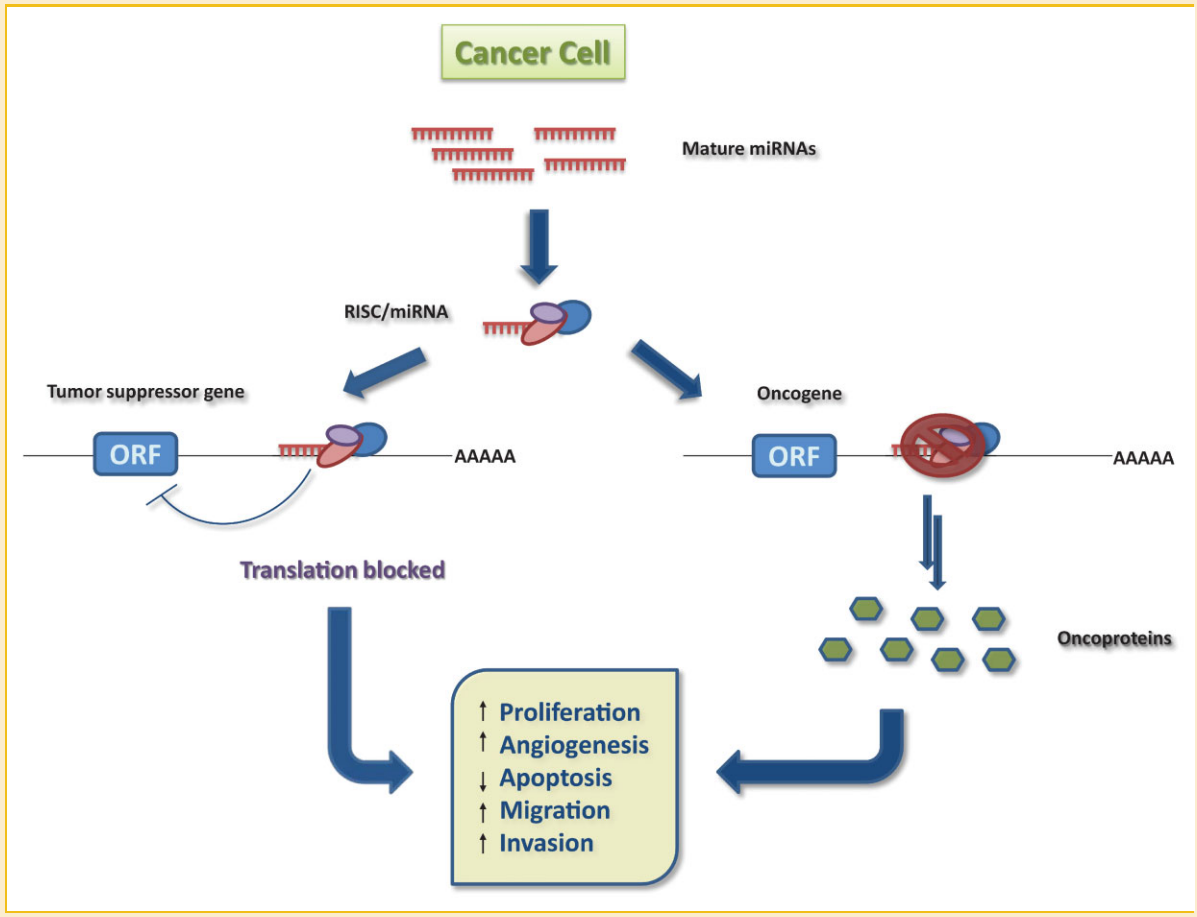

Fig. 1. miRNAs as oncogenes and tumor suppressors.

are located in 13q14, a region frequently either deleted or downregulated in chronic lymphocytic leukemia (CLL) patients, that provided the first link of miRNAs to cancer. Expression of miR15a/16-1 was inversely correlated to the levels of the anti-apoptotic protein, BCL-2 in CLL, supporting the previous findings [Cimmino et al., 2005]. Furthermore, Klein et al. [2010] have recently reported that miR-15a/16-1 knock-out mice develop CLL-like diseases and lymphomas. The miR-29 and miR-181 were also reported to be downregulated in CLL and to target TCL1, a gene overexpressed in 25–35% of CLL cases [Pekarsky et al., 2006], whereas, in hepatocellular carcinoma (HCC), these miRNAs exhibited opposite expression levels. While miR-29 is down-regulated and regulating apoptosis through a mitochondrial pathway that involves MCL-1 and BCL-2 [Xiong et al., 2010], miR-181 upregulation by TGFbeta promotes carcinogenesis by targeting TIMP3 and enhanced resistance to anticancer drug Doxorubicin [Wang et al., 2010a]. Moreover, Ji et al. [2009] found high expression of miR-181 in EpCAM-positive hepatic cancer stem cells, and determined that inhibition results in cell differentiation and suppression of tumorigenicity.

The miR-17/92a cluster, also know as oncomir-1, is among the most potent oncogenic miRNAs, carrying out pleiotropic functions during malignant transformation. O'Donnell et al. [2005] reported that transcription of this cluster is directly transactivated by MYC, a transcription factor frequently hyperactive in cancer cells. MYC transgenic mice developed lymphomas more rapidly when infected with murine hematopoietic stem cells with a retrovirus carrying miR-17/92a cluster [He et al., 2005]. Ventura et al. [2008] showed that miR-17/92a knock-out mice die shortly after birth of lung hypoplasia and ventricular septal defect. Moreover, it was recently demonstrated that miR-19 is the key oncogenic component of the cluster, promoting cell survival by repressing PTEN and activating the AKT-mTOR pathway [Olive et al., 2009].

Similar, miR-21 has an integral role in tumor pathogenesis, and extensive studies indicate its involvement in all know processes of cancer. It is overexpressed in most solid tumors with a wide range of targets. In lung cancer, it was demonstrated that overexpression of miR-21 increased K-RAS tumorigenesis in vivo [Hatley et al., 2010a], while in glioblastoma, Chan et al. [2005] identified miR-21 as an anti-apoptotic factor. Recently, Liu et al. [2011] presented in their study on a prostate cancer cell line, that miR-21 induces carcinogenesis by targeting PTEN, which leads to AKT and ERK1/2 signaling pathways activation, and thereby enhancing HIF-1α and VEGF expression.

Frank Slack's group confirmed the large body of in vitro evidence nominating miR-21 as a powerful oncogene, in a *NesCre8*,

mir-21$^{LSL\text{-}Tetoff}$ mouse model. They showed that overexpression of miR-21 can lead to a pre-B malignant lymphoid-like phenotype, while inactivation of the oncomiR resulted in regression of the tumors [Medina et al., 2010]. Similar, Hatley et al. [2010b] used a miR-21 knock-in and a knock-out KRAS^{LA2} mouse model of non-small-cell lung cancer (NSCLC) to evaluate the role of miRNA in tumor development. Their findings not only confirmed miR-21 as a tumor promoter, but also identified as an important regulator of the Ras/MEK/ERK pathway and apoptosis by targeting APAF1, PDCD4, RhoB, and FASLG.

In contrast, members of the miR-34 family function as tumor-suppressor downstream of the p53 pathway, and dysregulation of their expression occurs in various types of cancer. Of the three members of the family, miR-34a, which is expressed at higher levels than miR-34b/c, resides in 1p36 which is commonly deleted in neuroblastomas and its epigenetic inactivation was identified in cell lines derived from some of the most common tumors (breast, lung, colon, kidney, bladder, pancreatic cancer, and melanoma) [Lodygin et al., 2008]. In human colon cancer cells, Tazawa et al. [2007] reported suppression of cell proliferation and senescence-like growth arrest through modulation of E2F pathway, when miR-34a was induced, and reduction of tumor growth in vivo. The effect miR-34 on apoptosis in lung cancer was recently determined by Duan et al. [2010]; the data revealed the implication of miR-34a in the apoptotic network generated by PRIMA-1 (p53-dependent reactivation and induction of massive apoptosis).

The let-7 family, with 13 members located on nine different chromosomes, is widely viewed as the longest family of tumor-suppressor miRNA. Consistent with this activity, the expression of let-7 family members is downregulated in many cancer types when compared to normal tissue and during tumor progression and indicates a poor survival. This direct targeting of RAS by let-7 was confirmed in NSCLC, where it was demonstrated in a mouse model that let-7g inhibited tumor growth via suppression of RAS [Kumar et al., 2008]. While in ovarian cancer, Ratner et al. [2010] reported a variant in the KRAS 3′-UTR that interferes with let-7 binding, increasing the risk of developing the disease. In breast cancer, let-7g was identified as a possible prognostic marker, its diminished expression was associated with lymph node metastasis and poor survival in breast cancer patients. The same study showed that abrogation of let-7g expression in otherwise non-metastatic mammary carcinoma cells elicits rapid metastasis from the orthotopic location, through preferential targets, GAB2 and FN1, and consequent activation of p44/42 MAPK and specific matrix metalloproteinases [Qian et al., 2011].

MICRORNAs INVOLVEMENT IN CHEMORESISTANCE

Chemotherapy is the main strategy for cancer treatment; however it can fail in eliminating all malignant cells due to drug resistance. Resistance to therapy can be classified into two categories: intrinsic—the factors that would make the therapy ineffective exist prior to administration; and acquired—the tumors are not initially resistant to a particular drug, but develop resistance during the course of the treatment.

There are a number of mechanisms known to be involved in anticancer drug resistance, such as increased expression of target proteins, alteration of drug target, increased repair of DNA damage, reduced apoptosis, failure of the drug to reach or enter the target cell, ejection of the drug from the cell, drug-induced karyotypic changes, or altered metabolism of the drug [Gottesman, 2002]. Genes often involved in these processes were shown to be affected by miRNA pathways [Wu and Xiao, 2009]. Several recent findings strongly support the miRNA involvement in chemoresistance.

Zhao et al. [2008] reported that miR-221 and miR-222 modulate the ERα status in breast cancer cell lines. Overexpression of the miRNAs in ERα-positive cell lines resulted in decreased mRNA levels of the receptor and induced resistance to Tamoxifen, whereas downregulation of the miRs in a ERα-negative cell line had the opposite effects.

The increased expression of the already mentioned, miR-21, has been shown to generate chemoresistance through two pathways: downregulating programmed cell death 4 (PDCD4), which leads to increased expression of inhibitors of apoptosis proteins (IAP) and multidrug resistance 1 (MDR1)/P-glycoprotein [Bourguignon et al., 2009] observed in breast cancer, and repression of tumor-suppressor PTEN in NSCLC [Zhang et al., 2010a].

In a different study, in a Doxorubicin resistant cancer cell line, expression of miR-451 was inversely correlated to expression of MDR1, and, more importantly, increasing levels of miR-451 led to higher cell sensitivity to Doxorubicin [Kovalchuk et al., 2008]. Fujita et al. [2008] found miR-34a to be downregulated in drug-resistant prostate cancer cells, and ectopic expression of the miRNA resulted in increased sensitivity to Camptothecin. Recently, Port et al. [2011] reported miR-371/373 cluster as a promising target for explaining the cisplatin resistance in germ cell tumor cell lines. By upregulation, the cluster prevents p53-driven cellular senescence through several target genes (NEO1 and LATS2), leading to cell proliferation.

Several more examples of miRNAs involved in chemoresistance are summarized in Table I.

STRATEGIES FOR TARGETING miRNAs

In light of the potential that lays in miRNAs (Fig. 2), it is not surprising that several approaches to employ miRNAs as therapeutic targets have been developed. Mainly there are two strategies to target miRNA expression: by blocking the expression of an oncomiR or re-expression of a tumor-suppressor miRNA, or by targeting the genes involved in their transcription and processing (Fig. 3).

ANTI-miRNA OLIGONUCLEOTIDES (AMOs)

Artificially reducing the expression level of miRNAs by using synthetic nucleotides represents a new application of antisense technology. Anti-miRNA oligonucleotides (AMOs) are designed to bind specifically to the miRNA "seed region" and sterically block their mechanism of action. Chemical modifications can be used to alter the properties of these synthetic oligonucleotides by conferring

TABLE I. MicroRNAs Involved in Chemoresistance

miRNA	Target or pathway	Tumor type	Mechanism of resistance to therapy	Refs.
miR-146a	BRCA1	Breast cancer	Increased proliferation and resistance to cisplatin	Pogribny et al. [2010]
miR-200c	ZEB1	NSCLC	Suppression of EMT program and restoring sensitivity to cisplatin and cetuximab	Ceppi et al. [2010]
miR-221/222	EGFR/ErbB2 pathway Wnt/β-catenin pathway	Breast cancer	Activation of the pathways supports estrogen-independent cell growth and fulvestrant resistance	Ao et al. [2011]
miR-9*	SOX2	Glioblastoma	ID4 renders GSC resistance to doxorubicin through the ID4-miR-9*-SOX2-ABCC3/ABCC6 regulatory pathway	Jeon et al. [2001]
miR-21	PTEN and PI3K/Akt pathway	Leukemia	Activation of the PI3K/Akt pathway by decrease in the PTEN protein level induces resistance to daunorubicin	Bai et al. [2011]
miR-181a	Bim	Non-Hodgkin lymphoma	Downregulation of the Bim-apoptosis pathway	Lwin et al. [2010]
miR-34a	SIRT1, BCL-2	Prostate cancer	SIRT1 and BCL-2 reduction of expression levels induces sensitivity to paclitaxel	Kojima et al. [2010]
miR-143	ERK5/NF-kβ pathway	Colon cancer	Increases sensitivity to 5-fluorouracil by downregulation the ERK5/NF-kβ pathway	Borralho et al. [2009]

NSCLC, non-small-cell lung cancer; ID4, inhibitor of differentiation 4; GSC, glioma stem cells; ABC, ATP-binding cassette transporters.

increasing binding affinity, nuclease resistance, aiding in cellular uptake and altering the ability to trigger an immune response [Lennox and Behlke, 2011].

Meister et al. [2004] employed 2′-*O*-methyl (2′OMe) RNA oligonucleotides to regulate the expression of miR-21 in HeLa cells. The advantages that the 2′OMe RNA chemistry offers regard a higher binding affinity for the duplex formation with RNA targets, and higher nuclease resistance compared to DNA oligonucleotides. Endo- and exonuclease degradation proved to be problematic and to overcome this impediment several phosphorothioate (PS) bonds were used to link nucleotides at each end of the molecule. Krutzfeldt et al. [2005] were the first to introduce this modification; a cholesterol group was attached to the 3′-end to assist in vivo delivery and the new molecule was named "antagomir." The miR-16 and miR-122 antagomirs were administrated to mice and showed good efficiency in lowering the expression levels of the miRNAs and consequentially of their gene targets [Krutzfeldt et al., 2005, 2007]. However, PS linkages reduce binding affinity and could compromise the potential of 2′OMe. The 2′-*O*-methyoxyethyl (2′MOE) modification improved the nuclease resistance and increased binding affinity, as shown in an in vivo model targeting miR-122 [Esau et al., 2006].

Besides the AMOs with chemical modifications of the ribose nucleic acid backbone, other non-chemical backbones have been described, such as peptide nucleic acids (PNAs) or phosphorodiamidate morpholino oligonucleotides (PMOs), which rendered good results in both in vitro [Fabani and Gait, 2008] and in vivo [Fabani et al., 2010] studies.

LOCKED NUCLEIC ACIDS (LNA)

Locked nucleic acids (LNAs) are generally considered to be RNA mimics in which the ribose sugar moiety is locked by an oxymethylene bridge connecting the 2′-C- and 4′-C-atoms which conformationally restricts LNA monomers into an N-type conformation [Koshkin et al., 1998]. This modification provides stabilization against nucleases, higher binding affinity, and low toxicity in biological systems, making LNAs a versatile tool not only in anticancer therapy. The first to report miRNA inhibition by LNA-antimiR was Orom et al. [2006], who knocked down *bantam* miRNA

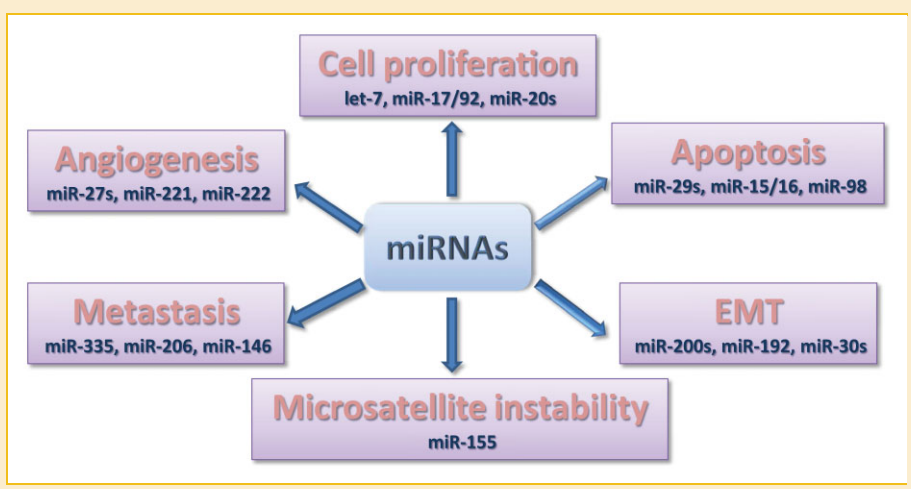

Fig. 2. miRNAs as key regulators of tumor initiation and progression.

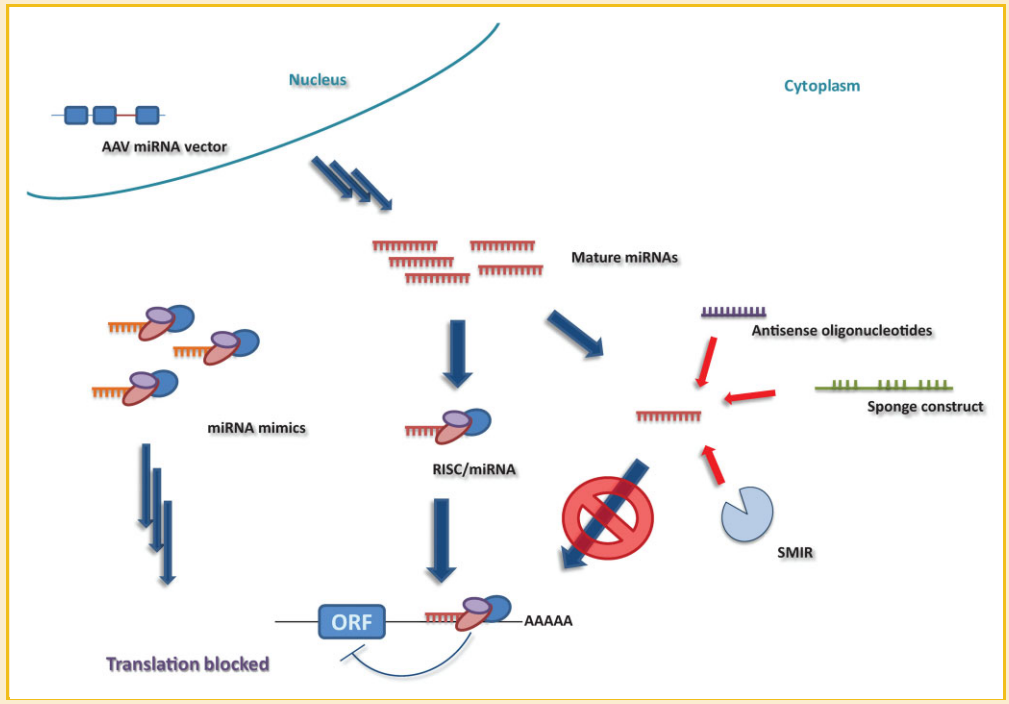

Fig. 3. Strategies of targeting miRNAs.

in *Drosophila* cells. Following, Fabani and Gait [2008] described a miR-122 LNA/2′OMe mixer AMO, containing 10 LNA bases and 13 2′OMe bases with PO backbone, which displayed increased potency. Furthermore, Elmen et al. [2008a] used in their study a similar AMO but fully PS modified to suppress miR-122 in mice.

In a preclinical trial on non-human primates (African green monkeys), Santarias Pharma (Horsholm, Denmark) employed an optimized version of the anti-miR-122 LNA/DNA-PS compound, and was able to denote a 40% decrease in total plasma cholesterol. The effect could be detected for 3 months after the last dose [Elmen et al., 2008b].

As inhibition of entire miRNA families may be of interest in some cases, short LNA, which bind solely to the seed sequence, have been developed recently [Obad et al., 2011]. These tiny compounds efficaciously downregulated the expression levels of miR-221/222 and let-7 families in cell cultures.

SMALL-MOLECULE INHIBITORS (SMIRs)

The use of heterocyclic derivatives to modulate the expression of miRNAs may be closer to reality as anticipated. The complex three-dimensional structure of the miRNAs allows formation of defined pockets suitable for binding of these small-molecules, leading to disrupture of their biological function [Thomas and Hergenrother, 2008].

However, the identification of such molecules can be challenging. One approach is efficient screening of chemical libraries. Gumireddy et al. [2008] discovered diazobenzene and its derivatives as inhibitors of pri-miR-21 formation, by applying this method. Complementary sequences to miR-21 were cloned into a luciferase reporter gene, and the construct was transfected into HeLa cells, resulting in low luciferase activity ascribable to the high levels of miR-21. When cells were treated with diazobenzene, a 250% increase in the intensity was observed.

Another approach suggested by Zhang et al. [2010b] is to use an integrated drug discovery platform that can provide the 3D structure of the miRNA and perform molecular docking-based virtual high-throughput screening (vHTS), identifying potential hits based on RNA-compatible scoring functions.

The advantages of small-molecule inhibitors (SMIR), such as cost-efficiency and their pharmacokinetic and pharmacodynamic properties, will push these molecules to the top of anti-cancer drug research, if specific hits will be identified and confirmed.

miRNA SPONGES OR DECOYS

The microRNA sponges or decoys represent transcripts that contain multiple tandem binding sites for miRNAs and are transcribed from mammalian expression vectors, such as adenovirus, lentivirus, or retrovirus. The binding site of a sponge is complementary to the seed sequence of the miRNA, therefore making it possible to target entire families [Ebert and Sharp, 2010]. Sponges can inhibit miRNA function at least as effectively as chemically modified AMOs, but have the advantage of being stably integrated into the host's genome.

Valastyan et al. [2009] identified miR-31 as strongly down-regulated in aggressive metastatic cancer and using a retroviral eGFP sponge demonstrated in an in vivo model, that loss of miR-31 leads to lung metastasis and 10 times more lesions. A similar approach was taken by Ma et al. [2010] to show the miR-10b promotes metastasis in breast cancer.

With the recent development of the technology, transgenic vertebrates expressing sponges are a work in progress.

NANOPARTICLES

The nanotechnology platforms have been primarily used for siRNA-therapeutics and it has only recently expended to the delivery of miRNA into target cells. Nanocarriers (1–1,000 nm) are usually made from biodegradable nanomaterials, such as natural or synthetic lipids [e.g., liposomes, micelles or solid lipid nanoparticles (SLN)] and polymers (e.g., poly lactic co-glycolic acid, polyethylenimin, or atellocollagen) or iron oxide magnetic nanoparticles. The safety, biodistribution and uptake of these particles by cells and tissues vary according to size, surface charge, and hydrophobicity [Ozpolat et al., 2010].

Chen et al. [2010] developed a liposome-polycation-hyaluronic acid (LPH) nanoparticle modified with a tumor-targeting single-chain antibody fragment (scFv) for the delivery of miR-34a into a murine model of metastatic melanoma. The authors observed a significant downregulation of survivin expression in the metastatic tumor and reduction of tumor load in the lung.

Aside from delivering the actual miRNAs, nanoparticles can be suitable vehicles for carrying oligonucleotides, as they can protect the oligonucleotides against endo- and exonucleases. Two different groups have recently exploited this benefit in their studies. One group produced an AMO-CLO (cationic lipid-binded oligonucleotide-loaded SLN) which efficiently targeted miR-21 in an in vitro model of lung cancer, subsequently decreasing proliferation, migration, and invasion of tumor cells [Shi et al., 2011].

The other group reported antiangiogenesis activity and suppressed cell migration in human umbilical vein endothelial cells (HUVEC) by employing a PEGylated LPH nanoparticle functionalized with cyclic RGD peptide for delivery of anti-miR-296 AMO into $\alpha_v\beta_3$ integrin-positive endothelial cells. The same results were presented in vivo using Matrigel plug assay [Liu et al., 2010].

miRNA MIMICS AND ADENOVIRUS-ASSOCIATED VECTORS (AAV)

A competent way of restoring expression of tumor-suppressor miRNAs is by introducing miRNA mimics. A miRNA mimic has the same sequence as the depleted, naturally occurring miRNA, and is therefore expected to have the same mRNA targets, making non-specific, off-target effects unlikely [Bader et al., 2010].

miRNA mimics have been used in various in vitro studies resulting in induced cell death or blocked proliferation. In vivo data using miRNA mimics was provided by Takeshita et al. [2010] in a prostate cancer metastasis model. The miR-16 chemically modified precursor and complexed with atelocollagen, was administrated into the tail vein of mice-bearing bone metastasis from prostate cancer cells. Significant inhibition of tumor growth by restoration of the miRNA expression was reported at the end of the experiment.

An alternative to miRNA mimics are the adenovirus-associated vectors (AAV). They present the advantage of efficiently transducting the target cells, without integrating into the genome. The advances made with self-complementary AAV vectors and the availability of AAV serotypes for improved transduction of specific target tissues has nominated them for being ideally suited for therapeutic gene delivery [Wu et al., 2006]. With he use of AAV vectors, Kota et al. [2009] were the first to provide the evidence that restoring the expression of a tumor-suppressor miRNA can stop cancer progression in vivo. The miR-26a was packed into a AAV vector system and injected into the tail vein of tet-o-MYC/LAP-tTA mice, leading to suppression of tumorigenicity by repressing proliferation and inducing apoptosis.

These studies conclude that restoring expression of tumor-suppressor miRNAs seems to be a promising perspective in cancer therapy.

FUTURE PERSPECTIVES—OTHER NON-CODING RNAs AS THERAPEUTIC TARGETS

Originally thought to be just ''transcriptional noise,'' these lnRNAs are emerging as new, essential players in the cancer paradigm, with roles in both oncogenic and tumor-suppressive pathways. Various studies have shed light into their potential functions, and revealed their involvement in high-order chromosomal dynamics, telomere biology, and subcellular structural organization [Amaral and Mattick, 2008]. One of their major roles seems to be the regulation of neighboring protein-coding genes and recent findings suggest that lncRNA can act as natural ''miRNA sponges'' to reduce miRNA levels [Cesana et al., 2011].

Among the better characterized lncRNAs that have been associated with cancer biology, is HOTAIR (HOX antisense intergenic RNA), involved in metastasis. The lncRNA, located in the mammalian HOXC locus on chromosome 12q13.13, was found to be highly upregulated in primary, as well as metastatic breast tumors and its elevated levels were correlated with both metastasis and poor survival rate [Gupta et al., 2010].

Also associated with metastasis and poor prognosis for patients with NSCLC [Ji et al., 2003] is MALAT1 (metastasis-associated lung adenocarcinoma transcript 1), residing at 11q13.1, which has been found to harbor chromosomal translocation breakpoints linked to cancer [Rajaram et al., 2007]. Other in vitro studies, have implicated MALAT1 in the regulation of the invasive potential of cancer cells, in cervical [Guo et al., 2010] and lung [Tano et al., 2010] cancer.

As mentioned before, lncRNAs can act as decoys for miRNAs and highly upregulated in liver cancer (HULC) is one of them. Transcribed from chromosome 6p24.3, with extremely high expression levels in liver cancer [Panzitt et al., 2007], seems to function as a miRNA sponge, for miR-372, of which one function is the translational repression of PRKACB, a kinase targeting cAMP response element binding protein (CREB). A feedback loop was also found, the activated CREB protein is able to promote HULC transcription by maintaining and open chromatin structure at the HULC promoter [Wang et al., 2010b].

Nevertheless, a more comprehensive understanding of their mechanism of action will provide novel approaches of regulating genes, including mimetics to compete with binding sites for miRNAs, chromatin remodelers or DNA. Furthermore, their cancer type-specific expression can be used to reduce the risk of affecting normal tissue during transgene-mediated therapy or it may potentially correlate with patient response to therapy.

As discussed in this review, there are well-founded arguments for exploiting ncRNAs as therapeutic targets and the studies conducted so far show promising results. However, it must be admitted that our yet limited understanding of the biology and function of these ncRNAs burdens the clinical translation of these new strategies and further studies are necessary to improve our ability to utilize their full potential.

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