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EXPERT REVIEW

microRNA Replacement Therapy for Cancer

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ABSTRACT microRNA are small noncoding RNAs that translationally repress their target messenger RNAs. Many microRNAs are expressed at reduced levels in tumors. microRNAs with reduced expression in cancer often regulate oncogenes, resulting in enhanced tumor growth. One therapeutic option is to restore microRNA levels in the tumor to that of the non-diseased tissue. This is possible by delivering microRNA to the tumor in the form of an oligonucleotide mimic or by expressing the microRNA in the cancer using a gene vector. This article surveys the field of oligonucleotide mimics and gene vector approaches to restore microRNA levels in tumors and reviews the literature on experimental and pre-clinical studies that have used these approaches to treat cancer.

KEY WORDS microRNA mimetic · microRNA mimic · therapeutic microRNAs · cancer treatment

INTRODUCTION

Since the discovery of microRNAs (miRNAs) in mammals ten years ago, much effort has been placed on not only understanding the mechanisms of miRNA function (1), but also on translational applications to benefit patients (2). miRNAs have been implicated in a variety of diseases including cardiovasculature (3), neurological (4), diabetes (5) and cancer (6). Two applications with the greatest translational potential are the use of miRNAs as biomarkers or as therapeutic targets.

In terms of a therapeutic, antisense oligos may be used to inhibit the activity of miRNAs that are increased in disease (7). In situations with reduced miRNA expression, therapy is possible by restoring miRNA levels to that of the normal state. This is achievable by three different approaches: miRNA may be dosed to the patient in the form of a miRNA oligo mimic (Table I), the miRNA gene may be introduced into the patient via a DNA vector (Table II), or small molecules may be used to reverse epigenetic silencing of miRNA (Fig. 1). Many excellent reviews have been written on these subjects, including basic biology of miRNAs (1,8), the use of miRNAs as biomarkers (9), technologies to inhibit miRNA activity (2), and small molecule drugs to inhibit/mimic the effects of miRNAs in disease (10). The purpose of this review article is to summarize the literature on oligonucleotide and DNA vector approaches that restore miRNA expression in cancer.

miRNA BASICS

miRNAs represent a class of small, noncoding RNAs that are expressed in all multicellular organisms. There are presently 1,048 human and 672 mouse miRNAs. miRNA genes are often located within introns of coding or noncoding genes and have also been identified in exons and intergenic regions (11). Endogenous miRNAs are initially transcribed by RNA polymerase II into a long primary transcript or pri-miRNA. The pri-miRNA is processed to a ~75 nt pre-miRNA by the ribonucleoprotein complex Drosha/DGCR8 (Fig. 1). Both the pri- and

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Table I Oligonucleotide Mimic Approaches to Restore miRNA Levels in Cancer

miRNA	Cancer	*In vitro*	*In vivo*	Reference	Targets of miR
Let-7	Liver	y	n	Lan, *et al.*, 2011 (65)	c-Myc, p16INK4A
Let-7	Laryngeal	y	n	Long, X.B *et al.*, 2009 (61)	RAS, c-Myc
Let-7	Colon Ca	y	n	Akao, Y. *et al.*, 2006 (62)	
Let-7	Pancreas	y	y	Torrisani, J. *et al.*, 2009 (60)	K-ras, MAPK
27a	Breast	y	n	Zhu, H. *et al.*, 2008 (100)	MDR-1
29	Liver	y	y	Xiong, Y. *et al.*, 2010 (67)	bcl-s, mcl-1
29b	AML	y	n	Garzon, R. *et al.*, 2009 (68)	mcl-1, CXXC6, CDK6
34a	Lung	y	y	Trang, P. *et al.*, 2010, 2011 (66, 115); Wiggins, J.F. *et al.*, 2010 (74)	bcl-2
34	Pancreas	y	y	Ji,Q. *et al.*, 2009 (71)	bcl-2
34	Gastric	y	n	Ji,Q. *et al.*, 2008 (70)	bcl-2
34	Prostate	y	n	Kojima,K. *et al.*, 2010 (72); Liu,C. *et al.*, 2011 (73)	SIRT1, Bcl2, HuR, CD44
101	Liver	y	y	Su,H. *et al.*, 2009 (69)	Mcl-1
122	Liver	y	y	Bai,S. *et al.*, 2009 (84)	ADAM10, SRF, Igf1R
124	Glioma	y	n	Silber,J. *et al.*, 2008 (41)	CDK6
137	Glioma	y	n	Silber,J. *et al.*, 2008 (41)	CDK6
143	Colon Ca	y	y	Kitade,Y. *et al.*, 2010 (53)	FLS-1, YES, STAT-1
145	Colon Ca	y	y	Kitade,Y. *et al.*, 2010 (53)	ERK-2
199a-3p	Liver	y	n	Henry,J.C. *et al.*, 2010 (57)	CD44
199a/b-3p	Liver	y	n	Hou,J. *et al.*, 2011 (83)	PAK4
203	Bladder	y	n	Bo,J. *et al.*, 2011 (96)	bcl-w
326	Breast	y	n	Liang,Z. *et al.*, 2010 (101)	MRP-1/ABCC1
449	Prostate	y	n	Noonan,E.J. *et al.*, 2009, 2010 (103, 104)	Cyclin D1, HDAC1j
451	Breast	y	n	Kovalchuk,O. *et al.*, 2008 (102); Zhu,H. *et al.*, 2008 (100)	MDR-1
451	Glioma	y	n	Nan,Y. *et al.*, 2010 (105)	PI3K/AKT pathway
520 h	Pancreas	y	n	Wang,F. *et al.*, 2010 (106)	ABCG2

Table II Gene Vector Approaches to Restore miRNA Levels in Cancer

microRNA	Cancer	*In vivo*	Plasmid	Transfection	Reference
miR-128-1	Glioma	n	pMIF-cGFP-Zeo	Lentivirus	Godlewski *et al*, 2008 (110)
miR-145	Breast	n	pCDH-CMV-MCS-EF1-copGFP	Lentivirus	Sachdeva *et al*, 2010 (111)
miR-145	NSCLC	n	pLemiR	Lentivirus	Chen *et al*, 2010 (112)
miR-122	HCC	n	pPGK-GFP	Lentivirus	Tsai *et al*, 2009 (40)
miR-1/206	Rhabdomyosarcoma	n	pCDH-CMV- MCS-EF1-copGFP	Lentivirus	Yan *et al*, 2009 (113)
Let-7a	NSCLC	y	Construct not specified	Lentivirus	Trang *et al*, 2010 (115)
miR-126	NSCLC	n	pLEGFP-N1	Lipid	Sun *et al*, 2009
miR-126	Gastric	n	pSilencer	Lipid	Feng *et al*, 2010 (117)
miR-451	NSCLC	n	pcDNA-GW/EmGFP-miR	Lipid	Bian *et al*, 2011 (118)
miR-17-92	Prostate	n	psiREN-RetroQ	Lipid	Zhang *et al*, 2009 (119)
Let-7a	Prostate	n	pcDNA3-GFP	Lipid	Dong *et al*, 2010 (120)
miR-96	Pancreas	n	pcDNA6.2-GW/EmGFP	Lipid	Yu *et al*, 2010 (121)
miR-205	Melanoma	n	pEP	Lipid	Dar *et al*, 2011 (122)
miR-26	HCC	y	scAAV.miR26a.eGFP	Adenovirus	Kota *et al*, 2009
miR-101	Gastric	y	pBHGlox∆E1,3Cre/ pDC316	Adenovirus	Wang *et al*, 2010 (106)

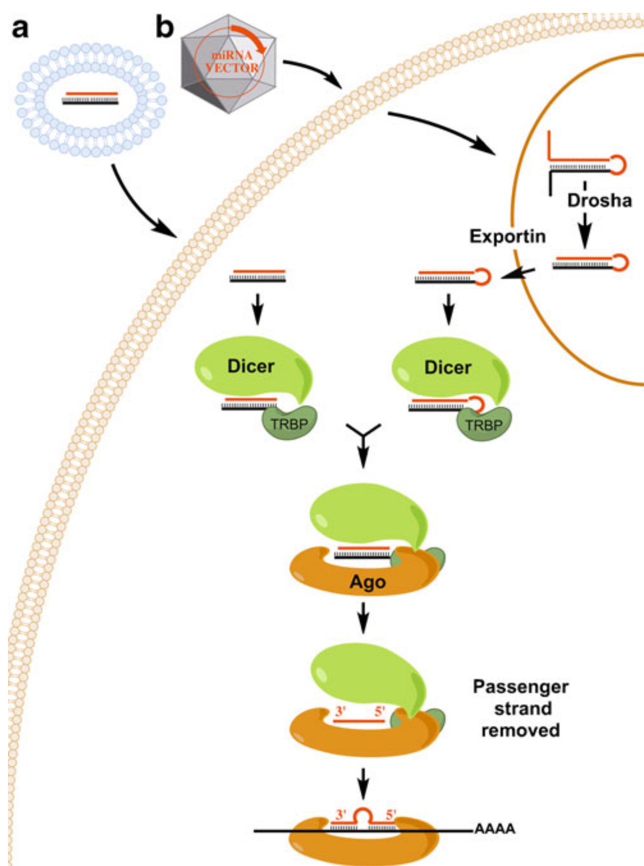

Fig. 1 miRNA mimic loading and processing by miRISC. Mature miRNA may be delivered to cells either by formulating an oligonucleotide mimic with lipid (**a**) or by gene delivery using a virus or other vector (**b**). miRNA genes delivered by a vector are processed by Drosha and exported to the cytoplasm, and the loop of the pre-miRNA is removed by Dicer. The passenger strand of the duplex miRNA that is loaded into miRISC, either from gene processing or introduction of the mimic, will be degraded by argonaute (Ago). MiRISC will then scan RNA until the miRNA binds to one of its target messenger RNAs, resulting in translational repression.

pre-miRNA contain the characteristic hairpin structure. Following cytoplasmic transport by exportin 5, the pre-miRNA is loaded into the Dicer/TRBP complex, which removes the loop of the hairpin. The duplex miRNA, that lacks a hairpin loop, is loaded into the processing complex at this point (Fig. 1). Formation of the miRISC complex, including argonaute, occurs shortly thereafter. Similar to siRNA, the miRNA guide strand is defined by the base pair stabilities at the 5′ end of the duplex (12). The strand with the poorer 5′ end stability is removed by a bypass mechanism that may include a helicase (13). The complex then scans messenger RNA to locate the miRNA's target. Binding of the mature miRNA typically occurs in the 3′ UTR; however, miRNA binding sequences have been identified in 5′ UTRs (14,15) coding regions of mRNAs (16) and noncoding RNAs (17). Binding of the miRNA to the 3′ UTR of mRNA will result in translational repression of the messenger RNA.

ALTERED miRNA EXPRESSION IN CANCER

Shortly after the discovery of miRNAs in mammals, a number of miRNA profiling studies were undertaken to determine the miRNA expression in cancer (18–22). The results of these profiling studies identified two important facts. First, miRNAs were differentially expressed in cancer (18,22–24), and second, miRNAs produced a unique expression signature for a given tumor type (18,22). The repercussion of these profiling studies suggested that miRNA signatures could be used as biomarkers of disease. It also raised the possibility of miRNA-based therapeutics by restoring miRNA levels to their natural state. This is achievable by inhibiting miRNAs that are over-expressed in cancer with antisense oligonucleotides (7) or by replenishing cellular miRNA levels using miRNA mimics in cancers with reduced miRNA expression (25).

Differential miRNA expression in cancer fits the cancer progression paradigm. miRNAs that are over-expressed in cancer typically target tumor suppressors or suppressors of metastases. Examples include miR-21, miR-17-92, and miR-221 targeting of *PTEN* (26,27), and *CDKN1C/p57* (28), respectively. miRNAs that are under-expressed in the tumor typically regulate oncogenes. A list of miRNAs that are reduced in cancer and their respective target genes are presented in Table I. Much has been learned about the causes of differential miRNA expression in cancer. miRNA genes are often located at fragile genomic sites (29); therefore, gene duplication or deletion will result in altered expression levels of the miRNA. miRNAs are also epigenetically regulated; promoter methylation and histone acetylation/methylation interferes with gene transcription (30). Alterations in mature miRNA in the neoplasia may also be due to differences in precursor biogenesis (31).

Are miRNAs predominately over-expressed or under-expressed in cancer? Early reports suggested a general down-regulation of miRNAs in solid tumors (18). A global reduction in mature miRNA levels due to Dicer knockout enhanced tumorigenesis in mice (32). Despite these findings, it became clear that a statement regarding the global reduction of miRNAs in cancer cannot be made, as numerous studies reported oncogenic miRNAs with increased expression in cancer. These include the miR-17-92 polycistron (33), miR-21 (19,26,34), miR-155 (35), miR-221 (36), and miR-181 (37).

Many instances exist of reduced miRNA expression in cancer (Table I); each of these miRNAs represents a potential therapeutic. One advantage of using miRNAs as therapeutics is that miRNAs by their nature target entire pathways, one miRNA may target multiple messenger RNAs, and one messenger RNA may be targeted by multiple miRNAs. For example, miR-21 has been found to target multiple components of the p53 and transforming

growth factor-β (TGF-β) pathways in glioblastoma cells (38).

REDUCED LEVELS OF DIFFERENTIATION ENHANCING miRNAS IN CANCER

Early *in situ* hybridization studies of zebra fish showed that certain miRNAs are expressed or enriched in specific tissues (39). These results were later confirmed in miRNA profiling studies. Examples include miR-122a in liver (40), miR-124/-128 in brain (41), and miR-1/-133a in skeletal muscle (42). A consistent phenomenon is observed with respect to these tissue-specific miRNAs in cancer; their expression levels are predominately reduced in the tumor compared to the cancer's tissue of origin. It has been hypothesized that the tissue-specific miRNAs maintain the differentiated state of the organ. The expression patterns of tissue-specific/enriched miRNAs follow a similar trend during development, differentiation, and cancer. Their expression levels are low during development, increase to a maximum level following differentiation to the adult state, and then are reduced in cancer (Fig. 2).

High levels of the tissue-specific miRNAs suppress genes that would otherwise cause that tissue to de-differentiate. An excellent example is the suppression of *REST/SCP1* by the brain-specific miR-124 (27). REST (NRSF) and its co-repressor SCP1 transcriptionally inhibit the expression of neuronal genes in non-neuronal tissues by binding to the RE1 repressor element. MiR-124 targets *SCP1*, and there is an RE1 site in the miR-124 promoter. In neuronal tissues with high miR-124 expression, SCP1 is suppressed by miR-124; low levels of REST/SCP1 maintain high transcription of miR-124. This results in the expression of a variety of neurological genes in neurological tissue. However, in non-neuronal tissues, low levels of miR-124 result in high levels of REST/SCP1, which not only suppress miR-9, but also non-neuronal genes. Reduced expression of miR-124 has been implicated in glioblastoma multiforme (43). Restoring miR-124 levels with miRNA mimics could conceivably reverse the malignant phenotype by restoring miRNA levels in neuronal tumors to that of the more differentiated state (Fig. 2).

miRNA OLIGONUCLEOTIDE MIMIC DESIGN

miRNA oligonucleotide mimics are RNA duplexes consisting of the guide strand that is identical to the mature miRNA sequence and is designed to "mimic" the function of the endogenous miRNA. The other strand of the duplex (passenger strand) is partially or fully complementary to the guide strand. The use of synthetic miRNA oligonucleotides *in vivo* has several shortcomings that must be overcome to be effective. These include susceptibility to nuclease degradation, activation of the innate immune system, off-target effects, and poor cellular uptake (44). Chemical modifications are made to the oligonucleotide to reduce degradation. The 2′ OH on the ribose ring serves as a nucleophile to attack the phosphodiester bond and is thus often substituted. Common means to enhance stability of small RNA molecules such as antisense or siRNA include 2′ O-methyl (45–49), O-2′-methoxyethyl (50), fluorine (51), or a combination thereof (52). Some of these modifications have been used to stabilize miRNA mimics. A 3′ cholesterol, typically added to the passenger strand, enhances oligonucleotide permeability and targeting to the liver. Oligonucleotides may be modified with placement of non-

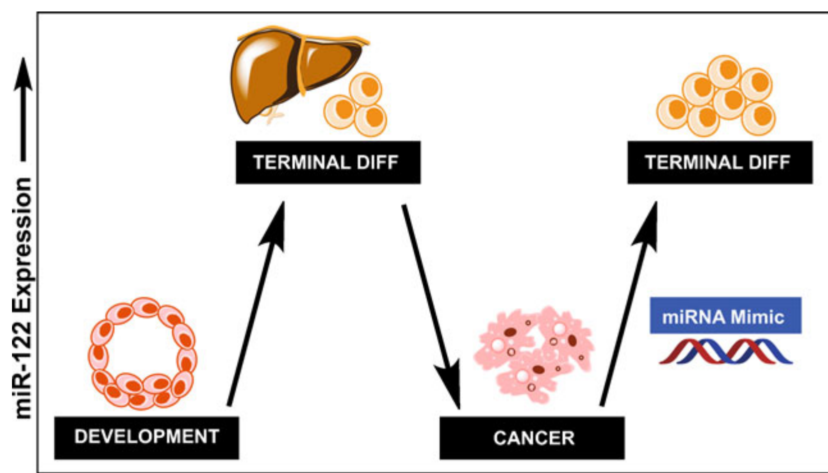

Fig. 2 Tissue-specific miRNA expression during differentiation and cancer. Expression of many tissue-specific miRNAs follow a similar pattern during differentiation or cancer development. An example for miR-122 is shown. During development, miR-122 levels rise until they reach a maximum level upon terminal differentiation of the liver. During tumorigenesis, miR-122 levels are reduced, and the liver takes on a more undifferentiated phenotype. Using miRNA mimics, it is possible to restore the miR-122 levels to that of the terminally differentiated state, reversing the cancer phenotype.

nucleotide bases at the 3′end to reduce degradation (44). Aromatic compounds (3′-benzene-pyridine) added to the 3′ end of miRNA mimics enhanced their activity and reduced degradation compared to a commercially available mimic (53). Designing one's own chemically modified miRNA mimics to be synthesized by an oligonucleotide provider is a challenge because little has been published in this area and chemically modified off-the-shelf miRNA mimics from many of the suppliers use proprietary design and modifications.

We have evaluated miRNA mimics designed with optimized chemistry to ensure proper miRISC loading, passenger strand degradation, and activity (M. Behlke, personal communication). The passenger strand was modified with 2′ OMe RNA and 5′ SpC3 to protect the duplex from degradation and to block immune activation (54–56) (Fig. 3). The guide strand contains three, 2′ OMe modifications at the 3′ end and has a 2 nucleotide 3′ overhang to assist with miRISC loading and degradation of the passenger strand. Designs containing a single 3′-overhang combined with a blunt-ended DNA base strongly direct loading into proteins that have a PAZ domain such as the Dicer and Ago families. Following unwinding, the remaining passenger strand will exist in a non-protein-bound single-stranded form that will be degraded by general nucleases. If the same passenger strand sequence were stabilized using locked nucleic acid or phosphorothioate linkages, it would be resistant to general nucleases and may cause off-target effects. The optimized chemistry (Fig. 3) included the reverse compliment of the guide strand rather than the natural guide strand sequence that would contain bulges in the stem portion of the hairpin. miRNA mimics designed to have a guide strand hybridized to the reverse compliment passenger strand have improved stability (and thereby increased potency) following transfection. It could be argued that introduction of an unnatural

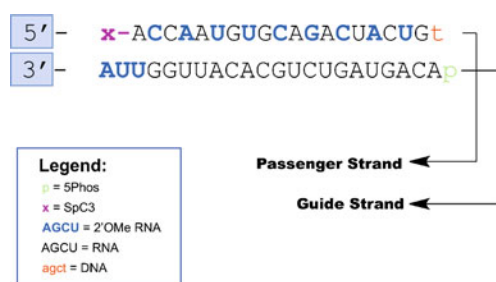

Fig. 3 Optimal miRNA mimic design, showing chemical modifications and other design parameters necessary for optimal miRNA mimic activity. The passenger strand is more heavily modified with 2′ OMe substitutions than the guide strand to protect the duplex and to block immune activation. To maintain maximal activity, the guide strand is less modified. To ensure proper loading into miRISC and passenger strand degradation, the passenger has a 3′ blunt end while the guide strand has a 3′ 2 nt overhang.

guide strand to the cell could produce off -target effects. However, if properly designed (as mentioned above), then the passenger strand will not enter RISC, will be rapidly degraded, and will not produce off-target effects.

To ensure proper loading into miRISC, and thus optimal biological activity, miRNA mimics should be duplex rather than single-stranded (2,44). We compared the antiproliferative activity of single or duplex miRNA mimics modified as shown in Fig. 3. When single-stranded guide or passenger strands of miR-199a-3p were transfected into SNU-449 hepatoma cells, no change in proliferation was observed. In comparison, the duplex miR-199a-3p mimics reduced cell proliferation in the cells to the same extent as the commercially available pre-miR-199a-3p oligonucleotide (57). These data emphasize the importance of using duplex mimic to ensure optimal activity.

EXPERIMENTAL AND PRE-CLINICAL EVALUATION OF miRNA MIMICS TO TREAT CANCER

The *let-7* miRNA mimic has been successfully tested in a wide variety of cancers. *Let-7* is under-expressed in many solid tumors, including lung (29,58), liver (59), pancreas (60), laryngeal (61), and colon (62). Validated *let-7* targets include *RAS* (58), *HMGA2* (63), *MYC* (64), and $p16^{INK4A}$ (65). In hepatocellular carcinoma, *let-7g* mimics decreased cell proliferation *in vitro* (65). Mimics of *let-7* inhibited proliferation, induced apoptosis, and caused a synergistic effect with cisplatin on laryngeal cancer cell lines (61). *In vitro* growth suppression in colon cancer cell lines was accomplished with *let-7* mimics (62). Finally, *let-7* has been demonstrated to be effective in an *in vivo* mouse model of lung cancer. The lung cancer tumors of orthotopic and transgenic mice were inhibited by intravenous *let-7* formulated as a neutral lipid emulsion (66). While decreased proliferation was seen in cell cultures treated with *let-7* mimics, growth of pancreatic xenografts was not altered following intra-tumoral injection (60).

MiR-29b was first shown to be down-regulated in breast cancer (23) and has been subsequently shown to be down-regulated in several solid tumors and blood cancers (67,68). Its utility as a therapeutic agent has been investigated in hepatocelluar carcinoma (67) and acute myeloid leukemia (68). The miR-29 family (miR-29a/b/c) is significantly down-regulated in hepatocelluar carcinoma (67,69). Through targeting of the apoptosis mitochondrial pathway genes *Mcl-1* and *Bcl-2*, miR-29 mimics induced apoptosis and increased the chemosensitivity in hepatocelluar carcinoma cell lines (67). Hepatocelluar carcinoma cell lines transfected with miR-29 mimic and then implanted into

nude mice resulted in a decreased capacity for tumor formation compared to control (67). Through the same *Mcl-1* target, miR-29b mimics induced apoptosis in acute myeloid leukemia patient samples in culture and decreased tumor xenografts following intra-tumoral injection (68).

MiR-34 is a therapeutic miRNA with great potential and is among the most published of all miRNA mimics. MiR-34 is reduced in many cancers, including gastric (70), pancreas (71), prostate (72,73), and lung (66,74). The *p53* tumor suppressor induces the transcription of miR-34 (75,76). MiR-34 is involved with the apoptosis pathway by targeting proteins such as *SIRT1* (72,77) and Bcl-2 (70,72, 76). HuR, a transcription factor of *Bcl-2* and *SIRT1*, is also under control of miR-34a, demonstrating apoptosis regulation at several points throughout the pathway (72). *Notch1* and *HMGA2*, genes involved with cancer progression and self-renewal, are reported targets of miR-34a (70). CD44, a marker and regulator of cancer stem cells in many types of neoplasia, is also a miR-34 target (73). MiR-34 mimics have successfully modulated cancer stem cells in prostate (73), pancreas (71), and gastric cancers (78) by modulating *CD44, Notch/BCl-2* and *BCl-2*, respectively.

In mouse xenograft models of prostate cancer, cells transfected with miR-34a mimics had reduced tumor formation on initial and secondary transplantation, but had decreased levels of miR-34 on successive treatments due to degradation of the oligonucleotide (73). Mimics of miR-34 induced apoptosis and sensitized pancreatic cancer cell lines to gemicitabine and radiation. Sorted cancer stem cells derived from MiaPaCa-2 pancreatic cells had reduced tumorsphere formation with restoration of miR-34a levels by the mimic. Finally, pancreas cells transfected with miR-34a mimic and then implanted into mice had reduced tumor formation (71). Similarly, mimics of miR-34 once transfected into the gastric cancer cell line, Kato III, decreased cell growth, inhibited progression through the cell cycle, increased apoptosis, and inhibit tumorsphere formation (70). Prostate cancer cells resistant to paclitaxel were sensitized by miR-34a mimic oligonucleotide (72). Finally, two extensive studies examined the ability of miR-34a to be delivered systemically to treat mouse models of lung cancer. MiR-34 formulated into a lipid-based delivery vehicle when administered at an intravenous dose of 5 mg/kg, decreased the growth of xenograft tumors in mice (79). MiR-34a packaged in a novel neutral lipid emulsion vehicle at a 1 mg/kg dose, reduced tumor burden by 60% compared to control in *K-ras* autochthonous non-small cell lung cancer mouse models (66).

MiR-101 is down-regulated by approximately five-fold in hepatocellular carcinoma compared to non-cancerous liver tissue (69). Both *in vitro* and *in vivo* evaluation of hepatocellular carcinoma cell lines transfected with miR-101 successfully decreased cell proliferation and tumor burden. The anti-tumor effects of this miRNA were due to targeting *Mcl-2* and allowing of cells to go through apoptosis (69).

Another miRNA mimic studied as a potential therapy for hepatocellular carcinoma is miR-122. This miRNA is the most abundant, specific mRNA in liver and is reduced in human and murine hepatocellular carcinomas (80–82). MiR-122 expression is reduced by 3- to 30-fold in 25–50% of hepatocellular carcinoma cases (82,83). Three oncogenic proteins have been identified as targets for miR-122 in hepatocellular carcinoma. These include *ADAM10*, a protein involved with migration, invasion, cell adhesion, and cell fusion; *SRF*, a transcription factor; and *Igf1R*, a receptor tyrosine kinase (84). All of these genes are up regulated in hepatocellular carcinoma. *In vitro* work on several hepatocellular carcinoma cell lines demonstrated a decrease in proliferation, invasion, and colony formation with the use of miR-122 mimics (85).

In vitro studies on glioblastoma multiforme cells with miR-124 and miR-137 oligo mimics have been conducted. Human glioblastoma tissues were evaluated and had a 57- and 63-fold decrease in miR-124 and miR-137, respectively, compared to benign tissue (43). Oligonucleotides of both miRNAs decreased glioblastoma cell line proliferation and caused glioblastoma cancer stem cells to differentiate. *CDK6*, a regulator of differentiation and progression through the cell cycle was found to be a target of both miRNAs (43).

MiR-143 and miR-145 represent potential therapies in colorectal cancer. Both miRNAs are reduced in multiple cancers, most notably gastric and colon cancers (86,87). The oncogenes *FLI1* (88), *YES*, and *STAT1*(89) are confirmed targets of miR-145, whereas Erk5, the transcriptional activator of *MYC*, is the target of miR-143 (90). In a study that examined the therapeutic potential of both miRNAs, modified oligos produced by the investigators were compared to commercially available miRNA mimics (53). The miR-143 and −145 mimics containing altered passenger strands and benzene-pyridine backbones had a greater decrease in proliferation in cancer cell lines. A head-to-head comparison *in vivo* was not done, but this new miR-143 oligonucleotide demonstrated decreased tumor growth in a xenograft mouse model (53).

Another important miRNA in hepatocellular carcinoma is miR-199a-3p. Numerous studies have shown that miR-199a-3p was reduced in hepatocellular carcinoma compared to normal/adjacent benign liver (20,91–93). More recently, RNA deep sequencing was used to demonstrate that miR-199a-3p was one of the most abundant miRNAs in HCC (making up ~5% of the entire miRNome in liver) (83). Importantly, miR-199a-3p was reduced by ~95% in hepatocellular carcinoma compared to adjacent benign or normal liver; making it the most significantly

reduced miRNAs in hepatocellular carcinoma (83). MiR-199a-3p targets a number of important oncogenes in cancer including *MET* (hepatocyte growth factor receptor) (91,94), *CD44* (57), mammalian target of rapamycin (*mTOR*) (91), *PAK4* (83) and *ERK2* (94). MiR-199a-3p was even shown to regulate HCV replication, a predisposing factor in hepatocellular carcinoma (95). Introduction of miR-199a-3p mimic to cancer cell lines *in vitro* or to mouse xenografts *in vivo* caused a general reduction in viability, proliferation, colony formation, or tumor growth (83,91–95).

A single study has evaluated the role of miR-203 as a potential therapy for bladder cancer (96). This was an interesting choice as a prior publication showed the miRNA was up-regulated in bladder cancer (97), but down-regulated in gastric, colon, and endomentrial cancers (98,99). The authors once again evaluated the miR-203 levels in tumor and benign samples and demonstrated a decrease in the miRNA. With this finding, they showed that bladder cancer cells had decreased proliferation due to increased apoptosis with the introduction of miR-203 mimic. One validated target includes *BCL2L2* (96).

Three miRNAs were evaluated to treat breast cancer by increasing susceptibility to standard chemotherapy agents, including miR-27a (100), miR-326 (101), and miR-451 (100,102). MiR-326 has also as been evaluated as a potential therapy in this cancer (101). MiR-27a and miR-451, both down-regulated in breast cancer, target the *MDR-1* protein, a member of the ABC transporter family that confer chemotherapy resistance (100,102). Transfection with oligonucleotide mimics of both miRNAs sensitized resistant, breast cancer cell lines to doxorubicin (100,102). MiR-326 was reduced in breast cancer tissues and targeted another ABC transporter family member, *MRP-1*. The addition of the miR-326 to breast cancer cell lines also induced susceptibility to doxorubicin (101). miRNA may fill a necessary niche in breast cancer and perhaps other cancers in reducing multi-drug resistance that is seen in many long-term, relapsed survivors.

Another miRNA therapy targeting prostate cancer has been conducted with miR-449a (103,104). MiR-449a may function as a tumor suppressor in prostate cancer (104). In prostate cancer cell lines, growth arrest was obtained with the introduction of this miRNA. Rb phosphorylation genes *cyclin D1* and *HDAC1* along with tumor promoter *p27* were all targets of miR-449a in this disease (103,104).

MiR-451 regulates the *PI3K/AKT* pathway, induces apoptosis, and reduces proliferation and invasion of glioblastoma cell lines (105). Finally, miR-520h has been investigated in pancreatic cell lines. Demonstrated to target *ABCG2*, a known breast cancer resistance gene, pancreatic cells had decreased invasion and migration and side populations, but no change in proliferation. Interestingly, no chemosensitivity studies were conducted (106)

All of the previously mentioned studies were performed by re-introducing mimics of the identical sequence of the endogenous miRNA. At least one lab has taken a different approach. They have identified candidate proteins that are over-expressed in cancer and created completely synthetic, double-stranded miRNA molecules to target the messenger RNA of these proteins (107,108). Using this approach, they increased apoptosis in a pancreas cell lines with miRNA molecules designed to target glioma-associated antigen 1 (108).

EXPERIMENTAL AND PRE-CLINICAL EVALUATION OF miRNAS EXPRESSED FROM GENE VECTORS TO TREAT CANCER

This review thus far has focused on synthetic oligonucleotide miRNA mimics as potential therapeutics for cancer. Many groups have employed different approaches for increasing tumor suppressor miRNA levels, including DNA plasmid delivery of the pri-miRNA with or with out the assistance of viral delivery. A recent review by Sibley *et al.* outlines the mechanistic details of these approaches (109). miRNA up-regulation through DNA plasmids is commonly seen in the recent scientific literature.

Pri-miR-128-1 expression was stably induced in U251MG, U87MG, and OHG02 glioma cell lines using a lentivirus delivery system. Briefly, the 82 bp fragment containing the hsa-miR-128-1 plus ~200 bp upstream and downstream from the site were amplified from U87MG genomic DNA and cloned into the pMIF-cGFP-Zeo lentiviral vector. This DNA vector expresses the pri-miRNA through a pol-II CMV viral promoter and can be packaged into lentiviral particles by co-transfection with the pPACKF1 packaging vector into 293T cell lines (110).

MiR-128-1 was shown by microarray and qPCR to be down-regulated in the tumor when compared to adjacent disease free tissue. Restoration of miR-128-1 levels by DNA lentiviral plasmid showed reduced proliferation of the stably transfected cell lines when compared to the empty plasmid control. The transfected U87MG cell lines were also implanted into the flanks of nude mice to assess tumor formation. When compared to the empty vector control cells, miR-128-1-expressing cells formed tumors that were 50% smaller. This tumor suppressing quality of miR-128-1 is attributed to its targeting of the stem cell renewal factor *Bmi-1*. Regulation of the 3′ UTR of *Bmi-1* through a single miR-128 binding site was shown in addition to five-fold down-regulation in expression of the *Bmi-1* gene in the glioma cell lines transfected with pre-miR-128-1 oligonucleotide or pri-miR-128-1 lentiviral vector (110).

MiR-145 is also down-regulated in cancer and has been shown to inhibit cellular growth by targeting *MYC* (111).

Sachdeva *et al.* showed ectopic expression of miR-145 in breast cancer cells suppressed invasion as measured by Matrigel™ chamber assays. Expression was achieved by using the pCDH-CMV-MCS-EF1-copGFP lentiviral DNA plasmid. This expression lentivector is under the direction of the EF1 promoter and contains the GFP reporter gene. Like pMIF-cGFP-Zeo, pCDH-CMV-MCS-EF1-copGFP can be packaged into lentiviral particles by co-transfection with the pPACKF1 packaging vector. *In vivo* metastasis studies were also conducted by tail vein injection of MDA-MB-231 or LM2-4142 cells. Cells were either infected with the miR-145-expressing construct or an empty construct. Animals injected with miR-145 expressing cells showed 72% less nodules. These *in vivo* results further support the role of miR-145 as a suppressor of metastasis. Direct targeting of *MUC1* by miR-145 is also shown, and *MUC1* is demonstrated to promote invasiveness. The authors therefore propose targeting of *MUC1* by miR-145 as the mechanism of metastasis suppression (111).

MiR-145 is also shown to have a tumor suppressor role in lung cancer by targeting *MYC*. In a study focusing on non-small-cell lung cancer, single-stranded DNA oligonucleotides with restriction enzyme overhangs of pre-miR-145 were hybridized and ligated into the digested pLemiR vector. Lentiviral particles containing the expression vector were then created using the Trans-Lentiviral GIPZ packing system. The over-expression of miR-145 in cell lines decreased proliferation and ability to form colonies *in vitro* (112).

Many other miRNAs have been ectopically expressed in cell lines with the help of lentivirus delivery. The partial primary transcript of miR-122 was restored in hepatocellular carcinoma cell lines reducing cell invasion. miRNA restored cells were also implanted in nude mice, and tumor size was shown to be significantly reduced (40). For miRNA-1 and miRNA-206, a lentivirus system was used for expression restoration in rhabdomyosarcoma cells and transplanted into nude mice. Again, tumor volumes were decreased in cells infected with miR-1 or miR-206, as compared with negative control (113).

An *in vivo* delivery of a miRNA construct has been attempted for non-small-cell lung cancer. Esquela-Kerscher *et al.*, showed the ability of *let-7a* miRNA mimics to decrease tumor size and tumor initiation *in vivo* (114). In a more recent study, levels of *let-7a* were up-regulated in a transgenic murine model by lentiviral infection. An unidentified lentiviral construct containing the *let-7a* sequence was purchased from Select Biosciences and packaged. Nasal inoculations were performed for lentiviral infection. Tumor-containing $Kras^{G12D}$ transgenic mice were used in order to explore the therapeutic effect of *let-7a*. Reduced tumor burden was observed in the *let-7a* lentivirus-treated group when compared to the lenti-control (75% reduction in tumor area). Ki67 staining also showed decreased proliferation in $Kras^{G12D}$ tumors treated with *let-7a* lentivirus when compared to the control (115).

Another tumor suppressor miRNA reported in non-small-cell lung cancer is miR-126. MiR-126 has been shown to inhibit NSCLC cellular proliferation when up-regulated. Levels of miR-126 were induced in A549 cells by lipid transfection of a pLEGFP-N1-miR-126 DNA plasmid. This approach varied from the ones cited above, as it used Lipofectamine 2000 for transfection of the DNA expression vector and not a lentivirus delivery system. MiR-126 over-expressing A549 cells showed induced EGFL7 levels and decreased cell proliferation. The miR-126 over-expressing cells were also implanted into nude mice to test tumor formation. Tumors produced by A549/miR-126 cells were significantly smaller than tumors formed by A549 cells and A549/empty control cells (116). MiR-126 has also been identified as a tumor suppressor in gastric cancer. A miR-126 expression vector was constructed (pSilencer-miR-126) and stably transfected into SGC-7901 cells. Transfection was also performed with lipid delivery. As in the lung, miR-126-expressing cells produced smaller tumors when compared to the control and showed less proliferation by Ki67 staining (117).

A study by Bian *et al.* evaluated whether restoration of miR-451 levels in cancer cell lines affected their sensitivity to chemotherapy drug cisplatin. The precursor sequence of miR-451 was cloned into the pcDNA-GW/EmGFP-miR vector, and the construct was transfected by lipid delivery. Up-regulation of miR-451 in the cell lines showed a decrease in growth and induction of apoptosis as well as increased sensitivity to cisplatin. Implantation of miR-451-expressing A549 cells led to significantly smaller tumors after cisplatin treatment compared to cells transfected with an empty plasmid. Apoptotic rate was also higher in miR-451-expressing tumors (118). Lipid delivery was again used in miR-17-3p and let7-a constructs in prostate cancer, miR-96 in pancreatic cancer, and miR-205 in melanoma. All studies showed decreased cellular proliferation and reduction of tumor size when over-expressing cells were implanted in nude mice (119–122).

A last approach that has been applied is transfection of miRNA through adenovirus infection. In this study, a self-complementary adeno-associated virus (scAAV) vector was constructed to expresses miR-26a under the regulation of the EF1α promoter. MiR-26, a miRNA that is normally expressed at high levels in many tissues, has reduced expression in hepatocellular carcinoma. The therapeutic potential of this miRNA was investigated by restoring expression levels in tumor containing tet-o-MYC; LAP-tTA mice. Doxycycline treatment (to induce tumor formation) started at week 4 and cAAV8.miR-26a.eGFP treatment started at week 11. Animals

were sacrificed at week 14, and tumor burden was assessed. Six out of 8 animals treated with a control AAV showed aggressive tumor formation, while 8 out 10 animals treated with cAAV8.miR-26a.eGFP were protected from tumors (123).

In another study, adenovirus vectors were constructed using the Ad Max Cre-lox-based system. This system requires a genomic vector and a shuttle vector to be co-transfected into 293T cells in order to produce the recombinant viral vector. A 328-bp fragment carrying pri-miR-101 was used to generate the adenovirus encoding miR-101 (Ad-miR-101). Human gastric cancer cells were infected with Ad-miR-101 to access miRNA expression restoration *in vitro*. Ad-miR-101 significantly inhibited proliferation in one of the cell lines, MKN-45, and decreased migration and invasion in all four cell lines studied (BGC-823, SGC-7901, MKN-45 and AGS). *In vivo* studies were also undertaken for miRNA-101 with a MKN-45 xenograft model. Once tumor formation was seen, mice were infected with the Ad-miR-101 adenovirus. Mice treated with Ad-miR-101 showed reduction in tumor size when compared to the adenovirus control (124).

COMPARISON OF miRNA DELIVERY METHODS: OLIGO MIMICS *VERSUS* GENE VECTORS

Contrasting oligonucleotide and gene vector delivery as applied to miRNA mimics is no different than comparing these delivery methods for siRNA or antisense. miRNA oligo mimics are straightforward to synthesize, and introduction into cells using lipid reagents or electroporation is easily achievable in most *in vitro* situations. miRNA oligo mimics are becoming more widely used *in vivo* and will be more quickly moved into clinical trials than vector-based miRNA mimic delivery. *In vivo* studies of oligo mimics in animals (53,60,67,74,115) and several clinical trials are underway to evaluate their efficacy in humans. Disadvantages of oligo miRNA mimics include the high cost for synthesis and purification and rapid clearance following transfection. Expression of miRNA mimics from plasmid vectors have the advantages of longer expression than transfection of oligo mimics and reduced likelihood of off target effects as the guide and passenger strand of the mimic are completely natural to the cell. While more complex systems to engineer, viral vectors are less costly than oligo mimics for routine experimentation. The limitations of miRNA vectors for delivery in clinical applications include insertion of genetic material into the specific location, introduction of an immune response, and transient expression of the gene.

CONCLUSION AND PERSPECTIVE

Restoring miRNA levels in tumors represents a powerful therapeutic option for cancer. Since miRNAs target multiple genes/pathways, re-establishing the expression of a single miRNA to that of the non-diseased tissue will produce a more pervasive therapeutic effect compared to drugs that obey the one-drug-one-target paradigm. This is particularly attractive in the field of oncology, as many miRNAs with reduced expression in cancer target messenger RNAs of genes that are oncogenic or maintain a state of de-differentiation (Table I). Toxicity may be minimized as miRNA replacement therapy gives back gene products that are already present in the normal tissue.

miRNA replacement therapy is not without considerable challenges. Both the oligo mimic and gene vector approaches discussed here have limitations that will need to be overcome before these therapies are widely used in patients. Another option that was not discussed here is to use small molecules to reverse the block that is responsible for reducing miRNA expression in cancer. Caution must be taken here as well so that drug treatment does not profoundly impact gene expression. Reversing an epigenetic block using DNA demethylating agents, for example, will not only restore the expression of many miRNAs but also many protein coding genes. A more focused approach may be to target factors that are responsible for reducing the expression of specific miRNAs. Levels of let-7 were restored in K562 cells by shRNA knock down of Lin28, a protein that is responsible for reduced biogenesis of the miRNA (125). Small molecules that restore let-7 levels by interfering with the interaction between Lin28 and let-7 may make ideal anti-cancer drugs.

While a number of obstacles need to be overcome in order for miRNA replacement therapy to become routine, one must recall that miRNAs were discovered in mammals only 10 years ago. Only time will tell if the rapid pace of discovery that has led to a better understanding of the genetics and function of miRNA will be translated into applications such as miRNA replacement therapy for cancer and other diseases. The authors also note that during the final preparation of this manuscript, an excellent article on the role of novel nucleobase and 2′-ribose modifications on immunostimulation of miR-122 mimic was published (126).

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