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CHAPTER 14

Strategies for Safe and Targeted Delivery of MicroRNA Therapeutics

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14.1 Introduction

From the elucidation that microRNAs (miRNAs) are involved in numerous biological processes to the knowledge that many are reduced in expression in human diseases such as cancer,[1–5] it is only logical that the next step is to explore if these 22-nucleotide entities can be utilized as therapeutic agents. Indeed, miRNAs are recognized as key modulators of cellular processes often dysregulated in disease.[6,7]

Unbeknown to the scientists involved at the time, the miRNA field began with the discovery of *lin-4*, a small RNA involved in heterochronic signaling in *Caenorhabditis elegans*.[8] Mutated in worms with a defect in developmental

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timing, *lin-4* does not encode a protein-coding gene but instead produces a small RNA. This small RNA binds to the 3′ untranslated region of a messenger RNA (mRNA), *lin-14* negatively regulating its translation.[8] Seven years later, a second miRNA was discovered, also in *C. elegans*. In this case, worms mutated for this miRNA exhibited a lethal phenotype, thus the mapped gene was given the name *lethal-7* (*let-7*). Like *lin-4*, *let-7* is also involved in the heterochronic pathway in *C. elegans* and, extraordinarily, is evolutionarily conserved in all metazoans,[9] including humans.[10] Currently more than 2500 miRNAs have been predicted in humans (miRBase v22).[11]

Determined through genomic sequencing, expression profiling, and functional studies, miRNAs are intimately involved in multiple cellular processes such as cell differentiation and growth, apoptosis, neurogenesis, and immune response.[12,13] Because of their contribution to nearly every biological process, it is not surprising that miRNA dysregulation alters normal cellular homeostasis, influencing various disease states. The first example of miRNAs dysregulation in disease was determined after careful examination of a region of chromosome 13q14 that is deleted in more than half of B-cell chronic lymphocytic leukemias (B-CLLs).[14] In this case, the only genes within the 13q14 region that showed consistent involvement in B-CLL were *mir-15a* and *mir-16-1*, two miRNA-encoding genes contained within a single miRNA cluster. Loss of miR-15a and miR-16-1, which function as tumor suppressors, resulted in elevations in MCL1, BCL-2, and JUN, proteins involved in cell proliferation and anti-apoptosis. As such, when miR-15a and miR-16-1 are restored in leukemic mouse models a significant decrease in tumor growth and volume occurs.[15] This extraordinary finding, followed by others, found that miRNAs are globally downregulated in tumor tissues compared to normal tissues, as opposed to being overexpressed.[16] Many of these so-called downregulated miRNAs are located in fragile regions of the genome often deleted in various cancers.[17] This evidence is highly suggestive that dysregulation of miRNAs is not only involved in disease pathogenesis, but that restoration of some of these depleted miRNAs may have therapeutic relevance.[18]

Some of the more frequently reduced miRNAs in human carcinogenesis belong to the *let-7* family of miRNAs. The *let-7* family consists of 13 family members that primarily function as tumor suppressors through repressing the translation of multiple oncogenes including *KRAS*, *MYC*, and *HMGA2*,[19] maintaining cellular differentiation and preventing uncontrollable cellular proliferation. Thus, loss of mature *let-7* predisposes the cells to a tumorigenic fate. There are multiple mechanisms that can contribute to reduced *let-7* levels including chromosomal deletion, transcriptional defects, or impaired *let-7* biogenesis,[20] the latter of which can reduce the levels of multiple *let-7* family members at the same time. Attempts to restore *let-7* by increasing the pools in various cancer models have been quite successful. For example, systemic delivery of mature *let-7* into mouse models with constitutively activated Kras ($Kras^{G12D/+}$) prevented lung tumor formation if administered at the

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same time as *Kras*$^{G12D/+}$ activation.[21] Importantly, *let-7* administration post *Kras*$^{G12D/+}$ activation decreased the volume of already formed lung tumors.[22]

Similar to the *let-7* family, the miR-34 family is typically downregulated in various malignancies. This is perhaps not surprising, since all the three *mir-34* family members are transcriptionally induced by the most mutated gene in human cancers, namely p53.[23–27] The bulk of p53 mutations occur in two hot-spots that alter either the DNA binding domain[28] of p53 or its conformation,[29–31] impairing p53 function. As a transcription factor, wild-type p53 regulates the expression of various protein-coding genes, and in 2007 was determined to be a major transcriptional regulator of the miR-34 family of miRNAs.[23–27] The miR-34 family consist of three isoforms: miR-34a, miR-34b, and miR-34c, which function as tumor suppressors. Loss of any isoform of miR-34 allows the target mRNAs, such as *CDK4*,[32] *BCL-2*,[33] and *MET*[26] to be expressed, leading to sustained cell growth, anti-apoptotic responses, and metastasis. Unsurprisingly, restoration of miR-34 in various murine tumor models slows tumor growth, including tumors in the lung,[34–36] prostate,[1] and liver.[37] These results, among many others, supported the advancement of miR-34a as the first miRNA mimic to enter clinical trials in 2013.[38]

Although miRNAs are globally lost in cancer, a select few upregulated miRNAs act as prominent oncogenes that tumors can become addicted to; so-called "oncomiRs". For example, the oncomiR miR-21, located in a region of chromosome 17 (17q23), is amplified in multiple tumor types.[17] Indeed, miR-21 is the most upregulated miRNA in carcinogenesis.[39] Importantly, miR-21 not only serves as a biomarker of disease, it directly contributes to disease onset and maintenance. As an oncogenic miRNA, overexpression of miR-21 promotes cell proliferation, anti-apoptosis, metastasis, and chemoresistance by inhibiting tumor-suppressive genes such as *PTEN*,[40] *PDCD4*,[41,42] *and BCL-2*.[43] Through indirect mechanisms, miR-21 also negatively regulates p53.[44] By reducing the levels of these critical tumor suppressors, miR-21 initiates a tumorigenic cascade that cells become addicted to.[45] This phenomenon, referred to as oncomiR addiction, is what makes miR-21 inhibitors attractive therapeutics. Inhibiting miR-21 using an antagomiR (a chemically engineered oligonucleotide used to sequester miRNAs) reduces cell growth and induces apoptosis in glioblastoma,[46,47] breast cancer,[42] and colorectal cancer cells.[48] Inhibiting miR-21 activity also sensitizes various tumors to chemotherapeutics, thereby expanding the clinical utility of miR-21 inhibitors.[49–52]

In addition to miR-21, miR-155 is also classified as an oncomiR. miR-155, an important regulator of immune cell development, located in the B-cell integration cluster on chromosome 21q23, is overexpressed in lymphoma.[53] Functionally, miR-155 has multiple targets, including PU.1,[54] AID,[55] C/EBPβ,[56] and SHIP1.[57] SHIP1 is a negative regulator of myeloid cell proliferation and survival, thus reduced SHIP1 through miR-155 targeting blocks these critical functions, leading to sustained growth. Inhibition of miR-155 with antagomiRs increases SHIP1 expression and reduces tumor burden in mouse

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models of lymphoma,[58] further supporting the use of antagomiRs as miRNA-based therapeutics.

Although many studies report promising anti-cancer effects following the delivery of miRNAs or antagomiRs into cancer cells in culture and in mouse models there are critical hurdles that still need to be addressed and overcome.[59] The importance of efficiently delivering intact miRNAs and antagomiRs to the diseased tissue without promoting toxicity in normal cells is crucial if miRNA-based agents are to be advanced as therapeutics in humans. In this review, we discuss the strengths and limitations of conventional chemotherapeutics and targeted therapeutics, the challenges and advances in developing miRNAs as effective therapeutics, and the strategies being developed to deliver miRNAs to obtain clinical utility.

14.2 Limitations of Conventional Cancer Therapeutics and the Advent of "Therapeutic-miRNAs"

The development and discovery of new drugs has impacted the lives of many patients. Many of these new drugs are designed to target disease-associated molecules. Indeed, our understanding of the genetic and molecular mechanisms of cancer has improved tremendously over the past few decades; however, safe and targeted drug development is slow to progress, with high failure rates.[60] Major reasons why current cancer therapeutics fail can be classified into the following: (1) toxicity from the drug or its carrier; (2) lack of specificity in targeting cancer cells; (3) development of acquired resistance to the drug; and (4) difficulty in targeting sites of metastatic disease that are often inaccessible to the agent.

Expectedly, many of the targeted agents currently on the market target proteins such as enzymes or receptors. For example, tyrosine kinases, which are crucial mediators of cell growth, cell proliferation, anti-apoptosis, metastasis, and angiogenesis are often constitutively activated in various tumors due to overexpression and/or mutation, and thus, have been investigated as effective therapeutic targets.[61] The majority of drugs used to target tyrosine kinases either compete with the substrate adenosine triphosphate (ATP) or block the interaction of the ligand with the receptor, both impairing downstream signaling. Epidermal growth factor receptor (EGFR), a receptor tyrosine kinase, is notorious for being overexpressed and/or mutated, primarily in lung cancer[62] and glioblastoma.[63] In wild-type cells, two EGFRs homodimerize allowing ATP to bind, which results in *trans*-phosphorylation of the two receptors. Once the receptors are phosphorylated, signaling proteins are recruited, leading to transient activation of downstream RAS and PI3K pathways. In cancer, due to overexpression and/or mutation, the receptor is constitutively activated promoting sustained stimulation of the downstream signaling proteins and subsequent tumor formation. Erlotinib (Tarceva), an EGFR inhibitor, binds to the ATP binding site of the receptor with higher

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affinity than ATP, preventing downstream signaling from occurring.[64–66] Erlotinib, approved by the United States Food and Drug Administration (FDA) in 2004 as a treatment for cancer, has been effective in lung cancer patients and is currently under investigation for efficacy in other cancers. Similar FDA-approved targeted agents have been developed and approved for targeting other tyrosine kinases, such as imatinib (Gleevec) targeting BCR-ABL, used for treating leukemia[67] and sunitinib (Sutent) which targets both the vascular endothelial growth factor receptor and the platelet-derived growth factor receptor for use in renal cell carcinoma.[68,69] Despite the positive effects of tyrosine kinase inhibitors (TKIs) in the clinic, a critical problem is target-cell specificity. These drugs target not only diseased cells, but also normal cells. Likewise, many of the TKIs accumulate in the bone marrow and gastrointestinal tract, where cells are rapidly dividing and depend on these pro-growth pathways.[43] An additional concern is the development of acquired resistance. Cancer cells that have initially responded to EGFR TKI can acquire a second mutation in EGFR, leading to drug resistance.[70] Beyond secondary mutations in the target, cells challenged with drugs that block one signaling pathway can respond through alternative by-pass signaling tracks that can compensate or overcome the targeted cancer treatment. A recent study has shown that TKI drug resistance is also tied to epithelial-to-mesenchymal transition (EMT). Cells that undergo EMT gain the ability to invade, migrate, and proliferate. Examining response to erlotinib, resistance was significantly more abundant in mesenchymal cells than in epithelial cells.[71] Therefore, cells that migrate to other tissue sites may acquire erlotinib resistance at a greater frequency. Due to these concerns, and many others (for a thorough review on drug resistance in cancer please refer to the review by Holohan *et al.*[72]), alternative novel therapeutic approaches are being investigated.

Apart from tyrosine kinases, for the past few decades there have been countless attempts to target RAS. RAS is a GTPase which cycles between its guanosine triphosphate (GTP)-bound active state and guanosine diphosphate (GDP)-bound inactive state. Guanine nucleotide exchange factors exchange GDP for GTP, promoting the active state, whereas GTPase-activating proteins (GAPs) induce hydrolysis of GTP, promoting the inactivate state.[73] Mutations in RAS disrupt the interaction with GAPs, leading to constitutive activation and stimulation of downstream signaling pathways. Sustained activation of RAS promotes cellular proliferation, survival, and invasion.[74] Unfortunately, RAS is considered "undruggable" due to its smooth surface, lack of a deep binding pocket, and the high binding affinity it has for GTP, which hampers the ability of a drug to act as a competitive inhibitor at a concentration that would be physiologically achievable.[75] Researchers have continued to search for new methods to target undruggable RAS. Included among them are targeting downstream pathways and the use of synthetic lethal approaches.[76] One recently reported method is based on altering the localization of RAS to endomembranes. Delocalization prevents lipid modifications on RAS, blocking the translocation of RAS to the plasma membrane, which is essential for RAS activity. The target,

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prenyl-binding protein phosphodiesterase δ (PDEδ), directly alters the localization and activation of RAS, facilitating its diffusion in the cytosol. A small molecule inhibitor that disrupts the PDEδ–RAS interaction blocks RAS signaling in pancreatic cancer cells and xenografts.[77] While this study provides a new method for reducing RAS activity, the challenge with targeting undruggable targets such as RAS has been realized by the National Cancer Institute and is reflected in an initiative put forth in 2013, the RAS Initiative (www.cancer.gov/research/key-initiatives/ras).

Due to lack of specificity in targeting cancer cells, development of drug resistance, and unidentifiable drug target sites on the protein structure for some targets, current drugs are limited in their use. Therefore, alternative and/or additional approaches are necessary. For many of these targets, inhibiting the production of the protein through transcriptional or translational block may represent an effective way to target diseases that are not treatable by current drugs.

miRNAs hold great therapeutic promise for these undruggable targets, including RAS, due to their ability to target the messenger RNA and reduce expression of the protein product.[78] The main advantage of using miRNAs as a replacement therapeutic is that miRNAs are small and thus presumably easy to deliver. Furthermore, because non-diseased cells are expected to still harbor the miRNA, it is probable that toxicity due to unintentional delivery to non-diseased cells would be modest in comparison to toxicity from small molecules. Moreover, miRNAs target multiple genes, and many of the genes targeted by a single miRNA can modulate several target genes in a single pathway, or in complementary pathways leading to an enhanced response.[36,79] To identify the critical miRNAs that when altered in cancer promote or maintain disease, various transcriptomic and functional studies have been conducted. In a recent study, the transcriptome of 15 of the most common tumor subtypes was analyzed.[80] The study identified differential expression of 147 genes and 95 miRNAs that were shared in most tumor types. Importantly, 36 paired interactions between the miRNA and the reported targeted transcript were deregulated together. This result indicates that a single dysregulated miRNA can alter the expression of its target genes, and that these altered mRNA–miRNA interactions are conserved in cancer pathways between various tumors.[80] Therefore, balancing the levels of miRNAs that are dysregulated in cancer, through miRNA-based therapeutic approaches, can potentially impact multiple major signaling pathways in carcinogenesis.

14.3 miRNA Therapeutic Strategies

Due to advances in RNA sequencing, profiling miRNA expression levels in normal and tumorigenic samples has become routine. Indeed, aberrant expression of miRNAs is often associated with numerous disease states,[16] and in many cases changes in miRNA abundance can directly contribute to various hallmarks of cancer.[81] These early findings have paved the way

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for the development of novel miRNA-based therapeutic options to alter the levels of dysregulated miRNA. Depending on how the miRNA expression level in the disease state changes, two different strategies for miRNA-based therapeutics have been developed: (1) miRNA inhibition—for cases where the miRNA is found to be overexpressed in the disease state; or (2) miRNA restoration—for cases where the miRNA is downregulated in the disease state (Figure 14.1A).

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14.3.1 Antagonizing Overexpressed miRNAs

When a causal miRNA is overexpressed in the disease state, miRNA inhibition therapy is used to block miRNA function. This very common strategy has been achieved using a variety of mechanisms that collectively sequester the overly abundant miRNA. Antisense oligonucleotides (ASO) are synthetic short single-stranded RNA or DNA molecules that bind to the mature miRNA sequence *via* complementary base pairing, thus sequestering the miRNA away from the endogenous targets allowing target-gene translation. Initial attempts to introduce naked ASOs systemically were ineffective due to rapid degradation by nucleases in the bloodstream and poor uptake by the target tissue and cells.[82,83] However, chemical modifications were incorporated into the design of the ASO to improve stability and cell permeability.[84] The first oligonucleotide modification that was incorporated to prevent RNA ASOs from degradation by nucleases was 2′-*O*-methyl of the ribose sugar.[85] The addition of the 2′-*O*-methyl group provides nuclease resistance and improved binding to the miRNA target. Alternative modifications such as, 2′-*O*-methoxyethyl,[86] 2′-fluoro,[87] 2′-4′-methylene (locked nucleic acids, LNAs),[88] phosphorothioate linkages,[89] and peptide nucleic acids[90] also provide resistance to nucleases and improve overall binding affinity (Figure 14.1B). Moreover, addition of a cholesterol to the 3′ end of the ASO increases cellular delivery kinetics *in vivo*. Cholesterol promotes uptake of the ASO by facilitating ASO binding to cell surface membrane receptors.[91] ASOs synthesized with such modifications have been effective against hepatitis C virus (HCV) infection, which causes liver inflammation. Long-term illnesses associated with HCV infection can lead to liver cancer. In HCV-infected cells, miR-122 is elevated, and through binding to the 5′ non-coding region of the viral genome miR-122 stimulates viral replication.[92] Thus, silencing miR-122 through ASO targeting was hypothesized as a valid treatment option. Miravirsen (SPC3649), developed by Santaris Pharma, is a LNA that does just that. ASO inhibition of miR-122 results in reduced HCV RNA with no signs of side effects in mice and primates,[93–95] nor were there any reported short- or long-term side effects in human patients.[96] Miravirsen is currently in phase II clinical trials (ClinicalTrials.gov identifier NCT01872936).[97]

Although ASOs are currently the most clinically relevant method for miRNA sequestration, other technologies have been used in pre-clinical studies as proof of concept with the intent for future development; for example, DNA

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plasmids that encode multiple complimentary miRNA binding sites in tandem. These so-called miRNA sponges, which can be transfected into cells, sequester miRNAs preventing miRNA–target gene engagement.[98] Recent studies suggest that some competing endogenous RNAs (ceRNAs) such as pseudogenes,[99] long non-coding RNAs[100] or circular RNAs[101] act as endogenous sponges sequestering miRNAs leading to upregulation of miRNA target genes. Studies attempting to adopt some of these endogenous ceRNAs as therapeutics to inhibit miRNA activity are of great interest.

Not all attempts to inhibit miRNA activity are based on base pairing between the miRNA and the inhibitor. Small molecule inhibitors are also used to inhibit miRNA expression and function. These compounds primarily inhibit the miRNA biogenesis pathway or disrupt the interaction between the miRNA and its target mRNA,[102] although other small molecules have been identified that inhibit transcription of miRNAs, miR-21 or miR-122 being two examples of miRNAs that fall into this class.[103,104]

14.3.2 Restoring Downregulated miRNAs

Conversely, when a miRNA is lost or downregulated in the disease state, miRNA restoration therapy supplements miRNA levels, leading to a therapeutic effect. While miRNAs levels can be enriched through delivery of DNA-encoding miRNA vectors,[105] restoration is typically done through introducing synthetic oligonucleotides that have the same mature miRNA sequence and a fully complementary passenger strand, generating a miRNA duplex referred to as mimic.[106] Similar to ASOs, miRNA mimics are chemically modified to prevent nuclease degradation and enhance their binding affinity. However, to maintain activity, careful consideration of the composition of the RNA bases and various modifications must be taken into account. For example, duplex RNAs composed entirely of 2′-*O*-methyl modifications tend to over-wind, while RNAs duplexes composed solely of 2′-fluoro modified ribose bases are under-wound. Similarly, a duplex composed of all LNAs has decreased helical winding and an enlarged helical pitch.[107] To overcome the helical constraints, short interfering RNAs (siRNAs) have been developed where 2′-*O*-methyl residues are alternated with 2′-fluoro residues. Additionally, some of the phosphodiester groups on the ends of the two strands have been replaced with phosphorothioate internucleotide linkages to reduce the activity of extra- and intra-cellular nucleases. While these modified bases and linkages lead to reduced innate immunity, increased stability, and roughly a 100-fold increase in tissue distribution,[108] displacement of the passenger strand from the guide strand poses an issue. This rate-limiting effect is alleviated *via* shortening of a fully-modified passenger strand to 15 nucleotides, reducing the thermal melt of the duplex.[108] Several other siRNA chemistries have been developed by Alnylam Pharmaceuticals,[109] many of which show exceptional stability and targeting, and reduced off-target effects relative to unmodified

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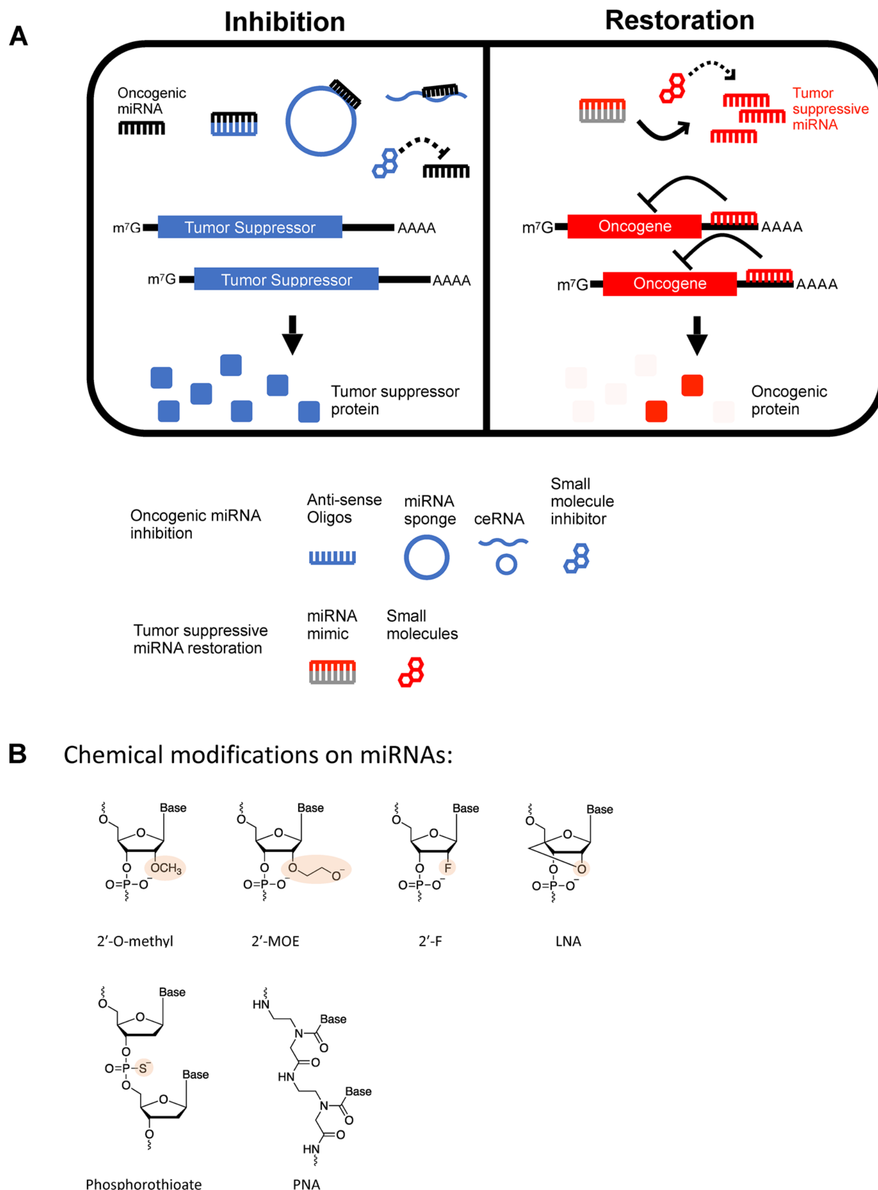

Figure 14.1 Therapeutic strategies for altering miRNA activity. (A) Two major strategies for miRNA-based therapeutics are being developed. Left: When oncogenic miRNAs are upregulated in the disease state, miRNA inhibition is performed using anti-sense oligonucleotides (ASOs), miRNA sponges, competing endogenous RNAs (ceRNAs: pseudogenes, long non-coding RNAs, circular RNAs), or small molecule inhibitors. Sequestration of oncogenic miRNAs promotes the expression of tumor suppressive proteins (blue squares). Right: When tumor-suppressive miRNAs are downregulated in the disease state, miRNA

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siRNAs.[110] Importantly, while these modifications and shorter passenger strand design have been efficacious for siRNA delivery, attempts to modify miRNA mimics in a similar manner have had mixed results, requiring more careful engineering.

A minimally modified miR-34a mimic entered clinical trials; in this case the mature strand was not modified while the passenger strand contained modifications that facilitated loading of the mature strand into the RNA-induced silencing complex (RISC). As the first miRNA to enter into clinical trial, miR-34a had promising initial results, including prolonged confirmed partial response lasting 48 weeks in a patient with hepatocellular carcinoma.[111] Unfortunately, as dose escalation increased, several severe immune-related adverse events occurred, resulting in termination of the phase I study in 2016. Additional miRNAs have since entered into the clinic. Therapeutic delivery of miR-29b mimics (MRG-201), developed by miRagen Therapeutics, was tested in patients with cutaneous fibrosis and had promising effect. Previous studies from the van Rooij lab observed that restoration of miR-29b levels during pulmonary fibrosis progression decreased collagen expression and blocked pulmonary fibrosis.[112] Currently, MRG-201 has completed phase I clinical trials (ClinicalTrials.gov identifier NCT02603224); however, the future of MRG-201 is not certain at this time. MiRagen has other miRNA-based drugs in the pipeline, although the remaining candidates are all miRNA inhibitors: MRG-106, which inhibits miR-155 and is being developed for hematological malignancies; MRG-107, also a miR-155 inhibitor, although the target population in this case is patients with amyotrophic lateral sclerosis; and MRG-110*, an antagomiR to miR-92a which is predicted to enhance blood vessel growth to accelerate the healing process.

With regard to enhancing the cellular abundance of miRNA, direct administration of the miRNA mimics is the obvious mechanism; however, due to challenges with miRNA delivery (see subsequent sections), small molecules are being used to restore miRNA expression. Compounds that can activate specific transcription factors or inhibit negative regulators of miRNA transcription or biogenesis can increase the cytosolic levels of miRNA. For example, compounds that inhibit the expression of *let-7* targets using a cell-based assay were identified by the Lowry lab. In this study, three compounds with a similar structure targeted and inhibited phosphodiesterase. Inhibition of phosphodiesterase stimulated cAMP levels and activated transcription factor

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restoration is performed using miRNA mimics or small molecules to induce expression and/or restore biogenesis. Increased levels of the tumor-suppressive miRNA reduce the expression of oncogenic proteins (red squares). (B) Chemical modifications of miRNA mimics and ASOs improve stability, cell permeability, and binding affinity to mRNA. Modifications are made at the 2′ position on the sugar ring such as, 2′-*O*-methyl, 2′-*O*-methyoxyethyl (2′-MOE), 2′-fluoro (2′-F), and locked nucleic acid (LNA). Additional modifications are made on the RNA backbone: phosphorothioate and peptide nucleic acid (PNA).

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CREB. Activation of CREB led to a reduction of the *let-7* target genes *HMGA-2* and *MYC*.[113] An additional study by the Gregory lab identified compounds that selectively block a negative regulator of *let-7* biogenesis. LIN28, an RNA-binding protein, negatively regulates *let-7* biogenesis through recruitment of the 3′ terminal uridylyl transferases, TUT4 and TUT7. Once recruited, the TUTases add an oligo (U)-tail to pre-*let-7*. The uridylated pre-*let-7* is then targeted for degradation preventing processing to the mature tumor suppressive form. Using a biochemical assay that monitors TUT4 activity, six compounds were identified as TUT4 inhibitors impairing the TUT4-pre-*let-7* interaction *in vitro* as well as in cells.[114] Modulating miRNA levels *via* small molecules may not necessarily be the most efficient approach yet as there are anticipated challenges that need to be overcome, such as permeability of the small molecule, and potential off-target effects. Nonetheless, these studies shed light on some of the basic biology of miRNA regulation, and perhaps, in the future, may lead to miRNA-based small molecule therapeutics.

While there is substantial evidence to suggest that modulating the levels of certain miRNAs can alter the pathology of disease, the biggest challenge in the field is achieving safe, specific, and efficient delivery of the miRNAs to the target tissue. Modification of the oligos have prevented degradation and increased binding efficacy as described; however, still due to delivery vehicle associated toxicity, poor transfection efficiency, systemic clearance, and non-specific biodistribution safe and targeted delivery strategies are essential to realize the full potential of miRNA therapeutics.[115] Initially, viral vectors were used to deliver DNA encoding miRNA precursors into cells, which following transcription are subject to processing by the endogenous machinery. Viruses such as lentivirus, adenovirus, and adeno-associated viruses (AAV) were modified by genetically engineering viral capsid protein to increase the binding affinity between the virus and the cancer cell receptor for specific targeting. In the context of hepatocellular carcinoma, miR-26a is dramatically reduced in expression, which contributes to disease.[116] To revert the cancerous phenotype, modified AAV was used to deliver the gene encoding *mir-26a* specifically to the liver.[117] Exogenous miR-26a resulted in tumor regression by promoting cell cycle arrest and apoptosis without toxicity.[118] However, despite efficient delivery using viruses there are some concerns. Viruses can trigger an immune response, which can reduce the efficiency of delivery. In addition, lentiviruses, which integrate their DNA into the host genome, can activate or inhibit genes depending on the integration site. Lastly, high-quality viruses are difficult to scale, limiting their application. Although virus-based delivery is efficient for delivering miRNAs and for establishing proof of concept, due to the concerns described, improvements are necessary to utilize viruses in the clinic. Thus, other non-viral delivery vehicles are receiving more attention since they tend to be safer, and easier to manufacture and scale up for miRNA therapy.

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14.4 Non-viral miRNA Delivery Systems for Cancer Therapy

Development and design of an appropriate delivery system for miRNA mimics is as important as selecting therapeutically relevant miRNAs. An ideal delivery system should be (1) biocompatible with minimum toxicity or immune response; (2) stable in circulation, but should be excreted after the payload is released; (3) efficient in loading and releasing the payloads into or out of the delivery system; (4) selective in targeting disease cell; (5) easy to penetrate the endothelial layer to reach the target tissue; and (6) cost-effective and easy to scale up. There has been ongoing research to address each of these criteria, through the development and testing of various delivery platforms (Figure 14.2). This section gives an overview of the main delivery vehicles that have been tested and developed, including lipid-based vehicles, polymer-based vehicles, inorganic material-based vehicles, and a class of novel vehicles. Due to the amount of effort that has been conducted in the cancer field, a majority of the review is dedicated to studies delivering miRNAs to tumor tissue. However, many of the same strategies can be applied to other disease models.

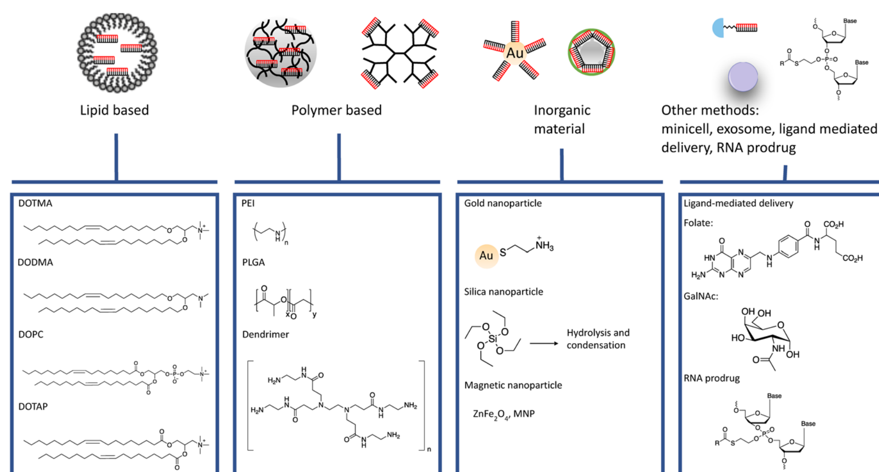

Figure 14.2 Delivery systems for miRNA therapeutics. Modified anti-sense oligonucleotides (ASOs) or miRNA mimics are encapsulated in multiple delivery vehicles to efficiently deliver miRNAs. The lipid composition is modified to create lipid vesicles with different properties using DOTMA, DODMA, DOPC, and DOTAP. Different polymer-based systems such as polyethylenimine (PEI), polylactic-*co*-glycolic acid (PLGA), and dendrimers were also developed to deliver miRNAs. Lately, inorganic gold, silica, and magnet nanoparticles have been developed as promising tools for miRNA delivery. Due to toxicity associated with some of the delivery vehicles, novel methods have been developed such as mini cells, exosomes, FolamiRs, and RNA prodrugs.

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14.4.1 Lipid-based Delivery

The most well-studied vehicles used for delivering miRNAs are lipid-based vesicles. A lipid vesicle is composed of a lipid bilayer and a hydrophilic core that incorporates the payload, in this case a miRNA mimic. Cationic lipids, which facilitate packaging of negatively charged miRNAs, are typically used. The positively charged lipid–miRNA complexes interact with the negatively charged cell membrane and undergo endocytosis. However, cationic lipids often promote immunomodulatory activity. To alleviate this, the lipid composition can be modified to create lipid vesicles with different surface charges. Surface charges also help facilitate endosomal escape and stability in circulation. Although there have been many successful studies using lipid vesicles for delivery, there are some drawbacks that have prevented lipid-based miRNA delivery platforms from successfully making their way through clinical trials. These include, but are not limited to, toxicity, non-specific uptake, immune responses, and endosomal sequestration. Improvements in modifying the lipid composition and structure, and adding unique moieties to the complexes have begun to overcome some of these challenges, increasing efficacy and reducing toxicity.

A cationic lipid composed of *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA), cholesterol, and polyethylene glycol (PEG) has been developed as a vehicle to deliver miRNA for cancer therapy. Cholesterol facilitates the release of miRNA into the cells and reduces cytotoxicity by neutralizing some of the positive charges of DOTMA.[119] Addition of PEGylation helps to dampen the immune response and increases stability during circulation. Delivery of miR-122 mimics using DOTMA–cholesterol, with the addition of a helper lipid, oleic acid, effectively delivered miR-122 to hepatocellular carcinoma (HCC) cells *in vivo*. Delivery was more robust than commercially available liposomal transfection reagents.[120] This DOTMA–cholesterol complex, loaded with precursor miR-133b, was also tested in non-small cell lung cancer (NSCLC). When miR-133b is processed into its mature form, mature miR-133b functions as a tumor suppressor by targeting the anti-apoptotic gene *MCL-1*. Delivery of miR-133b using this delivery vehicle achieved better accumulation in the lung tissue compared to commercial siPORT NeoFX transfection reagent.[121] The same group also evaluated delivery of the tumor suppressor miR-29b to lung tumors *in vivo* using DOTMA–cholesterol as a vehicle. Delivery reduced xenograft lung tumor growth through targeting *CDK6*, *DMNT3B*, *and MCL-1*.[122]

DOTMA cationic lipids have permanently charged tertiary amine head group whereas, the 2-dioleyloxy-*N,N*-dimethyl-3-aminopropane (DODMA) lipids have a protonatable tertiary amine head group. DODMA allows the lipid to be ionizable depending on the pH. At physiological pH the lipid has a neutral charge, but in more acidic environments the amphoteric lipid is converted into a positively charged, cationic lipid. When DODMA, in complex with miRNA, is delivered to the cell, the complex undergoes endocytosis and is trafficked to endosomes. The acidic endosomal environment alters the

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charge of DODMA–miRNA from neutral to positive, resulting in fusion with the anionic endosomal membrane. This allows the encapsulated miRNA to be released into the cytoplasm. Using this method, miR-122 was delivered to HCC cells, resulting in a significant reduction in tumor burden *in vivo* by targeting *Adam10* and *Srf* genes.[123]

Although cationic lipids are effectively internalized into cells, they are rapidly cleared from circulation due to their net positive charge. To improve biodistribution in target tissues, neutral lipids were used as an alternative for generating lipid vehicles. When miR-34a was encapsulated in a neutral lipid emulsion (NLE; MaxSuppressor™ *In Vivo* RNA-LANCEr II, BIOO Scientific, Inc.) composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), squalene oil, polysorbate 20, and an antioxidant, it effectively accumulated in the target lung tissue and reduced tumor burden in mouse models of NSCLC.[34,37] Other studies show therapeutic effects with miRNAs encapsulated in the same NLE. For example, delivery of miR-221/222 inhibitors using NLE promoted anti-proliferative effects in human multiple myeloma both in cells and *in vivo* by upregulating *p27Kip1*, *PUMA*, and *PTEN*.[124] In addition, NLE-delivery of miR-182 antagomiRs increased *Bcat2*, *Foxo3*, *and Adcy6* expression to regulate hypertrophy *in vivo*.[125]

Although use of neutral lipid delivery vehicles results in reduced toxicity and improved biodistribution compared to cationic lipid vehicles, there are challenges with inefficient delivery of the miRNAs into cells and loading of the miRNAs into the lipid vehicles. Therefore, to overcome these issues liposome-polycation-hyaluronic acid (LPH) was developed.[126] The hyaluronic acid-based biopolymer complexed with miRNAs and protamine improved miRNA loading and formed an anionic complex. This anionic complex is then encapsulated in cationic lipids *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTAP), cholesterol, and PEG, to neutralize some of the positive charges, reducing delivery-associated toxicity. To improve targeting, and thus specificity, additional modifications were added to this complex. For example, LPH with the addition of a GC4 single-chain variable fragment (scFv) efficiently targeted lung metastasis in the B16F10 murine melanoma model. The GC4 scFv is a monoclonal antibody that was initially selected and isolated from a phage antibody library to target the CD133 epitope on B16F10 melanoma cells. Delivery of miR-34a using the GC4-targeted nanoparticles resulted in a reduction in lung metastasis as well as primary lung tumors *in vivo*.[126] Similarly, modification of LPH with cyclic pentapeptide c(Arg-Gly-Asp-D-Phe-Lys) (cRGD) targets $\alpha_v\beta_3$ integrin-positive angiogenic endothelial cells. Delivery of anti-miR-296 using cRGD–LPH inhibited angiogenesis through enhancing the miR-296 target hepatocyte growth factor-regulated tyrosine kinase substrate (HSG) expression. Elevated HSG promoted growth factor receptor degradation to ultimately diminish blood tube formation and endothelial cell migration in cells and *in vivo*.[127,128]

Improvements in lipid-based vehicles have resulted in their advancement into clinical trials. A pH-dependent lipid vehicle in complex with miR-34a, called MRX34, delivered the first miRNA mimic to human patients in a phase I study (Mirna Therapeutics).[129] MRX34 is composed of a double-stranded

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miR-34a mimic encapsulated in a lipid carrier called a Smarticle. The Smarticle, composed of amphoteric lipids, has an overall anionic charge at physiological pH, while at lower pH conditions, such as those that occur in the tumor environment, the lipids become cationic, facilitating uptake into the tumor cells. Initially, MRX34 was effective in multiple *in vivo* mouse models promoting its transition into a phase I clinical trial for patients with primary liver cancer or with primary cancers that metastasize to the liver. However, due to severe immune-related side effects resulting in the death of three patients, the clinical trial was terminated.[111] Whether the toxicity was due to miR-34a or the vehicle has not yet been reported.

Lipid vehicles have been developed as an effective delivery system for miRNA. However, despite the protected miRNAs stability in circulation and modifications of the lipids that help to reduce toxicity, further improvements are needed to translate lipid-based delivery systems into the clinic. In the meantime, other delivery methods have been developed to advance miRNA delivery with unique features.

14.4.2 Polymer-based Delivery

Synthetic or natural polymers can be tailored in size and charge to efficiently deliver miRNAs. Polyethylenimines (PEIs) are the most widely used cationic polymer for miRNA delivery. Due to the high cationic charge density, the PEI and miRNA complex retain a net positive charge. Thus, the complex targets and interacts with the negatively charged polysaccharides on the cell surface, promoting efficient endocytosis. Once the complex is inside the cell, the pH buffer effect facilitates endosomal swelling through the influx of protons and water acting as a so-called "proton sponge", ultimately leading to endosomal bursting. Release of the miRNAs into the cytoplasm facilitates miRNA-mediated target gene silencing.[130] Examples of miRNAs delivered by PEI include miR-145 and miR-33a, which were administered either locally or systemically. Local delivery of miR-145 or systemically delivered miR-33a reduced cell proliferation, increased apoptosis, and reduced tumor growth in mouse models of colon carcinoma.[131] Additionally, delivery of PEI-encapsulated miR-145 in combination with chemoradiotherapy and cisplatin-enhanced therapeutic efficacy and improved survival in xenograft mouse models of lung cancer.[132]

Although there are multiple benefits to using PEI, there are also critical limitations, such as a high charge density of the particle and poor particle biodegradation that contribute to toxicity. To circumvent this, modifications of PEIs have been made to reduce toxicity and enhance polymer biocompatibility and biodegradability. For example, by adding a disulfide linkage to the PEI the polymer complex can be reduced by enzymes such as glutathione reductase as it enters the cell forming smaller and less toxic PEI polymer fragments.[133] Further refinement of PEIs can lead to enhanced uptake in specific tissues. For example, conjugation of PEI to a polyarginine (R11)

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peptide enhances PEI uptake by prostate cancer cells.[134] These polyarginine-disulfide-linked PEIs efficiently delivered miR-145 to prostate tumor cells, reducing tumor growth and increasing animal survival.[135]

Polylactic-*co*-glycolic acid (PLGA) has been well characterized and widely studied due to its safe, biodegradable, and biocompatible properties. PLGA is composed of two copolymers: poly-lactic acid and poly-glycolic acid. The ratio of the two copolymers changes the particle's overall physical properties. These properties alter the degradation kinetics of the molecule and release of the payload. For example, increasing the content of glycolic acid, which is slightly more hydrophilic than lactic acid, increases the hydrolysis rate of the particle leading to faster release of the payload. To increase circulation time and retention of the particles in the tumor tissue, multiple surface modifications have been added.[136] Such surface-modified particles have successfully co-delivered anti-miR-21 and anti-miR-10b, resulting in reduced tumor growth in a breast cancer xenograft model.[137] To improve targeting, PLGA particles have also been coated with a cell-penetrating peptide, penetratin, which was used to efficiently deliver anti-miR-155 to lymphocytes, delaying tumor growth in a mouse model of lymphoma.[58] A key advantage of using PLGA as a delivery vehicle is the high loading efficiency of PLGA particles. Taking advantage of the high-loading capacity of PLGA particles and efficient cellular uptake of PEI particles, delivery using copolymers containing both PLGA and PEI was attempted. Combining the hydrophobic and neutral properties of PLGA with the cationic PEI, this micelle-like copolymer was loaded with the chemotherapeutic drug doxorubicin along with miR-542-3p. Use of this hybrid particle efficiently promoted apoptosis and cytotoxicity in breast cancer cells.[138]

Another polymer-based carrier that has been developed is composed of dendrimers. Dendrimers are highly branched polymers with multiple surface moieties. These particles typically contain cationic amine groups on their branches which facilitate association with negatively charged miRNAs. Similar to PEIs, dendrimers act as proton sponges enabling endosomal burst and release of miRNA once inside of the cells. Dendrimer vehicles are highly efficient in delivering miRNAs into cells; however, due to their charge-related toxicity there have only been a few successful deliveries reported. For example, poly(amidoamine) (PAMAM) dendrimers have been generated and loaded with anti-miR-21 and the chemotherapeutic 5-fluorouracil (5-FU). Delivery of this complex to glioblastoma cells increased chemosensitivity of 5-FU and decreased migration of tumor cells.[139,140] Similarly, a triple helix RNA molecule containing miR-205 and anti-miR-221 was conjugated to PAMAM G5 dendrimer. Delivery of this complex to a triple-negative breast cancer mouse model resulted in reduced tumor size and increased survival.[141]

In addition to synthetic polymers, natural polymers are also being developed and used as delivery vehicles for miRNAs. Chitosan is a natural cationic polymer composed of glucosamine and *N*-acetylglucosamine

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residues.[142] The biocompatibility, biodegradability, and low toxicity of chitosan makes it an attractive delivery vehicle. The addition of hyaluronic acid enhanced biocompatibility as well as targeting to tumor cells expressing high levels of CD44 on their surface.[143] When miR-34a and doxorubicin were co-encapsulated in hyaluronic acid–chitosan complexes, breast cancer cells expressing high CD44 selectively took up the particle and delivered miR-34a promoted anti-tumor activity through silencing anti-apoptotic *BCL-2* and suppressed cell migration through targeting the Notch signaling pathway.[144]

14.4.3 Inorganic Material-based Delivery

Lately, inorganic materials such as gold, silica, and magnetic-based nanoparticles have been developed as miRNA delivery vehicles. The advantage of inorganic-based delivery systems is that they are biocompatible, non-toxic, and non-immunogenic. For example, gold nanoparticles (AuNPs) have unique physical and chemical properties such as shape, surface area, biocompatibility, and low cytotoxicity that aids in effective delivery of miRNAs.[145–147] Specifically, the coating on gold nanoparticles are a mix of hydrophobic and hydrophilic components that can bind to scavenger receptors on the cell surface leading to efficient uptake *via* endocytosis. In addition, cysteamine-functionalized AuNP-S-PEG nanoparticles also have high miRNA loading and release capacities, and low toxicity. This is exemplified in a study that delivered two miRNAs with different functions. Using AuNP-S-PEG nanoparticles, delivery of tumor suppressor miR-31 reduced cell proliferation in neuroblastoma cell lines, whereas delivery of oncogenic miR-1323 increased cell proliferation in ovarian cancer cell lines.[148]

Silica-based nanoparticles are stable, biodegradable, and non-toxic, making these nanoparticles an attractive system for miRNA delivery. Taking advantage of neuroblastoma cells, which express high levels of antigen disialoganglioside (GD_2) on their cell surface, systemic delivery of silica nanoparticles conjugated with an anti-GD_2 antibody specifically targeted neuroblastoma cells. Delivery of these nanoparticles containing miR-34a to a tumor-bearing mouse model resulted in tumor growth inhibition through the induction of apoptosis and reduction in vascularization.[149]

Magnetic nanoparticles are an additional inorganic particle used to deliver miRNAs. The imaging capability of these magnetic nanoparticles through magnetic resonance imaging allows for visualization of the nanoparticle in the target tissues. Breast cancer mouse models treated with LNA anti-miR-10b encapsulated in ultra-small magnetic particles modified with integrin binding peptide RGD prevented breast cancer metastasis.[150] Similarly, magnetic zinc-doped iron oxide nanoparticles ($ZnFe_2O_4$) complexed with the tumor suppressor *let-7* and coated with PEI efficiently delivered the miRNA into glioblastoma cancer cell. Once delivered into the cells, and under magnetic hyperthermia, the cancer cells became sensitized to the treatment and apoptosis was enhanced.[151]

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The use of inorganic materials for miRNA delivery does have some disadvantages. In general, inorganic nanoparticles lack cargo protection and are thus targeted for endosomal entrapment. In addition, other than the gold nanoparticle, most inorganic nanoparticles have low loading efficiency. Although these studies are expanding the potential vehicles that can be used to deliver miRNAs, marked improvements are needed before these vehicles would have clinical utility.

14.4.4 Novel Delivery Methods

Other novel approaches currently being developed to deliver miRNAs include the use of enucleated non-living nano-sized bacterially derived cells called mini cells or EnGenelC Delivery Vehicle (EDV). These mini cells have been modified to selectively target cells overexpressing EGFR through coating the nanoparticle with anti-EGFR antibodies. A phase I clinical trial of EDV nanoparticles containing miR-16 mimic for the treatment of malignant pleural mesothelioma and NSCLC was completed in early 2017 (ClinicalTrials.gov identifier NCT02369198).[152–154] The results of the study have yet to be released.

Delivery of miRNAs has also been achieved using endogenous miRNA delivery vehicles such as exosomes. Exosomes are 50–100 nm extracellular vesicles that aid in cell-to-cell communication through the transfer of bioactive materials such as lipids, proteins, and nucleic acids, including miRNAs. These miRNA-containing exosomes are transported to the recipient cells leading to miRNA-directed targeting in the recipient. Utilizing this mechanism, modified exosomes that express a peptide that specifically targets upregulated EGFR on cancer cells has been developed. These modified exosomes, loaded with *let-7a* mimics were delivered to a breast cancer xenograft model reducing tumor growth.[155] However, in order to advance the clinical utility of exosomes some critical hurdles need to be resolved, such as reducing unwanted accumulation of the exosomes in the liver and increasing their low miRNA loading efficiency.

While all these approaches rely on a protective delivery vehicle, the idea of abandoning the protective vehicle can prevent many of the vehicle-associated problems such as toxicity and immune response. Indeed, a vehicle-free method for delivering miRNAs has been developed. Our group has generated folate-conjugated miRNAs (also called FolamiRs) that can specifically target cancer cells expressing high levels of the folate receptor on the cell surface,[156] a feature common to some hematological malignancies, and lung,[157] breast,[158] ovarian,[159] and other solid tumors. These cancer cells internalize the bound FolamiR *via* endocytosis, ultimately releasing some of the miRNA cargo into the cytosol to induce gene silencing.[160] Therapeutic effects of miR-34a-conjugated FolamiRs were observed in a breast cancer xenograft model and in a genetically engineered model of NSCLC.[156] Although the results are promising, endosomal entrapment of the FolamiR molecules is a rate-limiting step, a feature common to most of the delivery vehicles. Various

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methods to promote endosomal escape and enhance efficiency of delivered miRNAs are being evaluated.[161]

Additional targeting ligands that bind to other surface receptors have been developed for RNA delivery. Using the *N*-acetylgalactosamine (GalNAc) ligand, an RNA prodrug was efficiently delivered to the liver, which highly expresses the GalNAc receptor asialoglycoprotein (ASGPR). The RNA prodrug contains a neutral phosphotriester backbone which allows the RNA to enter the cell unimpaired by the typical negative charge that prevents RNA uptake.[162] Once inside, the RNA prodrug converts to its native negative charged phosphodiester backbone by cytoplasmic thioesterases, restoring its activity. The RNA prodrug displayed cellular stability and low immune response *in vivo*.[163] In addition, an siRNA targeting transthyretin (TTR) was developed by Alnylam Pharmaceuticals for the treatment of TTR-mediated amyloidosis. When mutated, TTR is unable to carry thyroid hormones and retinol binding proteins from the liver, resulting in accumulation of amyloid protein in the tissue, subsequently leading to peripheral neuropathy and cardiomyopathy.[164] A TTR siRNA conjugated to GalNAc was efficiently delivered to the liver and knocked down both mutant and wild-type TTR.[165] This treatment, also called ALN-TTR02 or Patisiran is currently in phase III clinical trials (ClinicalTrials.gov identifier NCT01617967). While these later examples highlight siRNA-mediated delivery, based on the success of folate-conjugated miRNAs, it is expected that additional ligand-mediated miRNA delivery vehicles will be developed and validated in the near future.

14.5 Conclusion

Over the past 20 years miRNAs have emerged as important regulators of nearly every cellular process and proved to be promising entities for cancer therapeutics in pre-clinical models. Delivery of miRNA mimics to restore relevant miRNA levels or ASOs to sequester oncogenic miRNAs has led to appropriate therapeutic consequences. However, a major challenge to be met while using ASOs and miRNA mimics is to ensure their safe and targeted delivery. To accomplish this, two major factors need to be addressed: (1) the chemical and biological properties of the miRNA mimic or ASO need to be understood and improved upon; and (2) safe and effective targeted delivery strategies need to be developed, validated, and put into practice.

Previous efforts to identify specific RNA modifications that improve activity, stability, and reduce toxicity were attempted using a large-scale chemical screen.[166] In this initial screen, LNA-modified siRNAs were determined to be compatible with loading into the RISC and siRNAs synthesized with LNAs had improved targeting activity. In addition, increased stability of the RNA was found to occur with only a few modified bases rather than full modification. However, more recent reports suggest that fully modified siRNAs are more effective.[108] The discrepancy is likely due to the types of modifications and the position of the modified bases in the RNA duplex. Moreover, to alleviate the increased melting temperature of fully modified duplexes, which may

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have impaired activity in the earlier study, Khvorova and co-workers shortened the antisense strand to 15 nucleotides to facilitate easier strand displacement. Despite these attempts some chemical modifications that may be useful for improving activity, stability, and reducing toxicity may lead to unwanted effects such as impaired specificity and off-target effects due to sustained binding to lower-affinity targets.[167]

Indeed, the chemical properties of the delivered miRNA can significantly alter the efficacy of the miRNA; however, one cannot overlook similar challenges related to the biological properties of miRNAs. For example, one of the key features of miRNAs is that one miRNA can downregulate multiple genes that regulate diverse signaling pathways. At times, the concerted regulation of multiple pathways is an advantage—assuming the pathways converge on a desired phenotype. However, due to the broad activity of some miRNAs, therapeutic doses may lead to unwanted silencing of low-affinity targets. The solution is to selectively regulate pathways that contribute to the particular disease progression, while not affecting other pathways or wild-type genes, whose expression may be critical to certain cells. Through computational analysis, artificially generated miRNAs can be engineered that selectively target genes with single nucleotide mutations that contribute to the tumorigenic phenotype without affecting the wild-type gene.[168] This method can be expanded to designing miRNAs that selectively target genes only involved in pathology of the disease while eliminating targeting of irrelevant genes, thus preventing side-effects associated with unwanted targeting. An algorithm that may be useful for predicting such designer miRNAs is miRBooking.[169]

Although use of current miRNA delivery systems has resulted in improvements in reducing vehicle-associated toxicity and immune response, while enhancing biocompatibility, transfection efficiency, and endosomal escape, targeted delivery of miRNAs *in vivo* is still a challenge. Due to the negative charge of the miRNA, the vast majority of the delivery vehicles are engineered using cationic lipids or polymers for packaging. However, the positive charge density of the delivery vehicles can promote rapid clearance in the bloodstream and cell toxicity. To overcome this, new biocompatible materials and/or novel strategies to remove or minimize the charge will be essential. For example, neutralizing the charge of the delivery vehicle through the use of neutral lipids, polymers, or masking the charge through conjugating PEG molecules to the vehicle has reduced cationic-related toxicity. Similarly, the use of uncharged RNA pro-drugs accomplishes the same, albeit in the absence of a delivery vehicle. Together with a targeting agent, tissue specific delivery can improve biodistribution and reduce the delivered dose, further preventing delivery-associated toxicity. Many of the targeting agents currently being tested include antibodies (scFV, GD_2), peptides (cRGD, R11, penetratin), or ligands (hyaluronic acid, folate, GalNAc). The miRNA delivery methods that have been tested *in vivo* are summarized in Table 14.1.

While many of these individual challenges related to miRNA delivery are being tackled, the future involves combining these novel chemistries into a single agent. The next decade will likely be met with additional

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Table 14.1 Summary of miRNA therapeutics tested *in vivo*.[a]

Delivery system	Targeted miRNA	Therapeutic strategy	Model	Administration	Reference
Lipid-based delivery					
DOTMA-cholesterol	miR-133b, miR-29b	Replacement	Lung cancer	Systemic	Wu *et al.*, 2013[122]
DODMA	miR-122	Replacement	Liver cancer	Intratumoral	Hsu *et al.*, 2013[123]
Neutral lipid emulsion	miR-34a	Replacement	Lung cancer	Systemic, intratumoral	Wiggins *et al.*, 2010[34] Trang *et al.*, 2011[37]
	miR-221/222	Inhibition	Multiple myeloma	Intratumoral	Di Martino *et al.*, 2013[124]
	miR-182	Inhibition	Myocardial hypertrophy	Systemic	Li *et al.*, 2016[125]
LPH-GC4	miR-34a	Replacement	Melanoma lung metastasis	Systemic	Chen *et al.*, 2010[126]
LPH-cRGD	miR-296	Inhibition	Angiogenesis	Subcutaneous	Liu *et al.*, 2011[127]
Polymer-based delivery					
PEI	miR-145 and miR-33a	Replacement	Colon cancer	Systemic, intratumoral	Ibrahim *et al.*, 2011[131]
	miR-145	Replacement	Lung cancer	Systemic, subcutaneous	Chiou *et al.*, 2012[132]
	miR-145	Replacement	Prostate	Systemic	Zhang *et al.*, 2015[135]
PLGA	miR-21, miR-10b	Inhibition	Breast cancer	Systemic	Devulapally *et al.*, 2015[137]
	miR-155	Inhibition	Lymphoma	Intratumoral	Babar *et al.*, 2012[58]
Dendrimer	miR-205, miR-221	Replacement and inhibition	Breast cancer	Intratumoral	Conde *et al.*, 2015[141]
Inorganic material-based delivery					
Silica-based	miR-34a	Replacement	Neuroblastoma	Systemic	Tivnan *et al.*, 2012[149]
Magnetic	miR-10b	Inhibition	Breast cancer	Systemic	Yigit *et al.*, 2013[150]
Novel delivery					
EDV nanocell (mini cell)	miR-16	Replacement	Lung cancer	Systemic	Taylor *et al.*, 2015[154]
Exosome	Let-7a	Replacement	Breast cancer	Systemic	Ohno *et al.*, 2013[155]
FolamiR (ligand-mediated)	miR-34a	Replacement	Breast cancer Lung cancer	Systemic	Orellana *et al.*, 2017[156]

[a]cRGD: cyclic (Arg-Gly-Asp-D-Phe-Lys); EDV: EnGenelC Delivery Vehicle; LPH: liposome-polycation-hyaluronic acid; PEI: polyethylenimines PLGA: polylactic-*co*-glycolic acid.

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interdisciplinary approaches between chemists, biologists, engineers, and clinicians to develop these all-encompassing vehicles, to validate them in pre-clinical models, and successfully transition them into human patients. It is highly expected that the next decade will reveal the true promise of miRNA-based therapeutics as this major obstacle – delivery – is overcome.

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