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Progress in MicroRNA Delivery

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Abstract

MicroRNAs (miRNAs) are non-coding endogenous RNAs that direct post-transcriptional regulation of gene expression by several mechanisms. Activity is primarily through binding to the 3' untranslated regions (UTRs) of messenger RNAs (mRNA) resulting in degradation and translation repression. Unlike other small-RNAs, miRNAs do not require perfect base pairing, and thus, can regulate a network of broad, yet specific, genes. Although we have only just begun to gain insights into the full range of biologic functions of miRNA, their involvement in the onset and progression of disease has generated significant interest for therapeutic development. Mounting evidence suggests that miRNA-based therapies, either restoring or repressing miRNAs expression and activity, hold great promise. However, despite the early promise and exciting potential, critical hurdles often involving delivery of miRNA-targeting agents remain to be overcome before transition to clinical applications. Limitations that may be overcome by delivery include, but are not limited to, poor *in vivo* stability, inappropriate biodistribution, disruption and saturation of endogenous RNA machinery, and untoward side effects. Both viral vectors and nonviral delivery systems can be developed to circumvent these challenges. Viral vectors are efficient delivery agents but toxicity and immunogenicity limit their clinical usage. Herein, we review the recent advances in the mechanisms and strategies of nonviral miRNA delivery systems and provide a perspective on the future of miRNA-based therapeutics.

Keywords

microRNA; miRNA therapeutics; small RNA delivery; human disease; nonviral delivery

I. Discovery and Action of miRNAs

MicroRNAs (miRNAs) are one of a growing number of non-coding RNA molecules that act within a cell [1, 2]. Noncoding RNAs include miRNA, small interfering RNA (siRNA), ribozymes, among others [3]. Both miRNA and siRNA interact with messenger RNA (mRNA), typically marking the mRNA for degradation as will be described in this review. Ribozymes are RNA molecules that bind to mRNA (or other RNAs) and catalyze the

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degradation of the RNA with no enzyme [4]. Ribozymes are the only RNAs in this class known to act independent of proteins as catalysts to exert their action. Herein, we focus on microRNAs as a novel class of therapeutic molecules with an emphasis on the methods used for delivery.

Discovered little more than a decade ago [5, 6], miRNAs, small (20–24 nt) single stranded endogenous RNAs, are a class of post-transcriptional gene regulators. Since miRNA activity was confirmed in animals [7, 8] and humans [9, 10], miRNAs have attracted significant attention due to their critical regulatory impact on biological functions in development, cell proliferation, differentiation, apoptosis, and metabolism [11–15]. The primary miRNA precursor, pri-miRNA, is first transcribed as capped, polyadenylated RNA strands that form double stranded stem-loop structures (Figure 1). These pri-miRNAs are processed in the nucleus by DGCR8 and ribonuclease Drosha into 70 to 100 nt long hairpin structures, called pre-miRNAs. Pre-miRNAs are then exported to the cytoplasm by Exportin-5 and further processed by the RNAse Dicer into approximately 22 nt double-stranded miRNA duplexes [16–18]. The miRNA duplexes enter the miRNA-induced silencing complex (miRISC) and the Argonaute protein in the miRISC unwinds the mature strand from the passenger strand [19]. The mature strand is retained in the miRISC while the passenger strand is released and subsequently degraded.

The majority of miRNA activities [11] are proposed to be through interactions with the miRISC complex (Figure 2). Guided by the mature miRNA strand, miRISC binds target mRNAs, and thereby induces translational repression by blocking mRNA translation and degrading the mRNA [20, 21]. This binding is typically to the 3’ untranslated region (UTR) of mRNA; however, 5’-UTR binding and action is also reported [20, 21]. Much like siRNA and siRISC recognition of mRNA, miRISC recognition of mRNA can be through perfect base pairing of the miRNA with the mRNA strand. But, the binding of miRISC to target mRNAs does not require perfect pairing, which is one factor allowing one miRNA strand to recognize an array of mRNA. Although this promiscuity is thought to be a unique feature of miRNA compared to siRNA (Table 1), there is significant overlap in the mechanism of miRISC and siRISC function [22]. There is such overlap that miRNA delivery, particularly when double-stranded approximately 21 nt sequences are delivered, that the terminology si(mi)RNA or simply siRNA is recommended by some [23]. Although the similarity in at least one mechanism of action may be true, we propose the difference between siRNA and miRNA to be the intent to knock down an individual mRNA with siRNA and an array of mRNAs with miRNA. In that, the delivery may converge, but the broad action desired is the primary difference between the two types of small non-coding RNA.

The miRISC complex binding to mRNA can either repress or promote translation, although the latter appears to be rare [24, 25]. Degradation of mRNA occurs primarily when prefect base-pairing is present and deadenylation and destabilization of mRNA that is not perfectly base-paired appears to predominate. Even when mRNA is not degraded, initiation may be repressed directly or the mRNA sequestered in regions of the cell (e.g., P-bodies) where low protein production takes place. If initiation takes place, the ribosomes stall during translation and may drop off the mRNA strand resulting in truncation of the protein. Also, proteases are recruited that degrade the protein as translation is underway. Through a combination of these mechanisms, miRNA has significant effect on the proteins produced by cells (Figure 2).

Extracellular action is directed through exosome formation [26] and nuclear actions have been proposed [27–35]. The small RNAs have been implicated in direct interactions with DNA, particularly promoter regions, or alterations of the chromatin structure thus altering final mRNA production. Much still remains to be learned about miRNA biosynthesis and

actions, but sufficient knowledge has been gained particularly in the expression of miRNAs in varying tissues and diseases. As we start to understand how altered miRNA expression can be associated with or even cause diseases in humans, including cancer, central nervous system (CNS) disorders, infectious diseases, cardiovascular diseases, metabolic disorders, and autoimmune diseases [36–40], new opportunities for developing novel therapeutics targeting miRNA dysregulation will emerge. To achieve the clinical reality, an understanding of how miRNA therapeutics can act is needed.

II. MicroRNA Therapeutic Approaches

Using high-throughput techniques including miRNA microarrays, unique miRNAs expression profiles are being confirmed to mediate critical pathogenic processes in human disease [38, 41, 42]. These findings are rapidly being translated into recommendations targeting miRNA dysregulation. To translate these discoveries to viable therapies, appropriate, stable molecules must be designed and chemically modified poly(nucleic acids) are typically utilized for miRNA therapy. In addition to stabilization, chemical modifications can also be used to augment the specific binding of miRNA to the miRISC complex [43, 44]. The current generation of miRNA therapeutics all utilize one or more of the chemical modifications developed over the last 20 years.

a. Chemical Modification to RNA for miRNA Therapy

Naked RNA (Figure 3) has a very short half-life in blood for several reasons, primarily being degraded by the abundant ribonucleases present in blood stream [45]. Thus, the first generation antisense phosphodiester oligodeoxynucleotides (ODNs), without further chemical modification, were proven to be ineffective in therapeutic applications [46]. The stability of the antisense sequences is augmented using chemically modified oligonucleotides [47, 48]. Chemical modifications (Figure 3) include phosphorothioate containing oligonucleotides [49], 2′-O-methyl- (2’-O-Me) or 2′-O-methoxyethyl-oligonucleotides (2’-O-MOE) [50], locked nucleic acid (LNA) oligonucleotides [51], peptide nucleic acids (PNA) [52], fluorine derivatives (FANA and 2’-F) and other chemical modifications [53]. Many of these modifications have evolved previous to the discovery of miRNA first with antisense therapies in the 1980s and 1990s [54] then with siRNA therapies [55–57].

Phosphorothioate ODNs were developed attempting to overcome the stability issue, in which one of the non-bridging oxygen in the phosphate group is replaced by sulfur [58]. This modification dramatically increases nuclease resistance for parenteral administration, eliciting sufficient RNAse H activation for the target RNA cleavage and desired binding to cellular and serum proteins for uptake, absorption and distribution [49, 59]. However, limitations include relatively short *in vivo* half-life, low binding affinity to RNA, and nonspecific inhibition of cell growth still hinders the application of phosphorothioate ODNs for therapeutic intervention [60].

The introduction of 2’-O-methyl group to the ribose moiety in a phosphorothioate oligoribonucleotides increases binding stability and reduces the non-specific inhibitory effects on cell growth [50]. Similarly, 2′-O-methoxyethyl modification markedly protects phosphorothioate oligoribonucleotides from nuclease degradation and will likely slow down oligoribonucleotides clearance from tissues and cells. The greater stability brought in by 2′-O-methoxyethyl modification and the phosphorothioate backbone should allow for less frequent dosing and may avoid the need for continuous infusions [49].

Further utilizing modifications at the 2’-oxygen, LNAs are RNA analogs with the ribose moiety chemically locked by a bridge connecting the 2'-oxygen and 4'-carbon in a RNA-

mimicking N-type (C3′-endo) conformation [61]. Miravirsen or SPC3649 (Santaris Pharma, Horsholm, Denmark), an LNA-based therapeutic used to treat hepatitis C virus (HCV) infection, progressed to Phase II clinical trials in 2010. Miravirsen efficiently suppresses HCV genotype 1a and 1b infections when administered to chimpanzees, with no evidence of apparent viral resistance or side effects [62]. The promising Miravirsen clinical results where a dose-dependent decrease in HCV RNA without evidence of resistance [63] was observed suggests utilization of the LNA chemistry to develop miRNA therapeutics for the treatment of other diseases.

Peptide nucleic acids (PNA) are uncharged oligonucleotides analogues in which the sugar–phosphodiester backbone of DNA/RNA has been replaced by an achiral structure consisting of N-(2-aminoethyl)-glycine units. PNAs exhibit high specificity and stability without generating unwanted toxicity [64]. Most importantly, PNAs frequently do not require assistance of delivery from transfection reagents due to the lack of charge. In addition, cell penetrating peptides can be linked with PNAs to enhance delivery using standard peptide chemistry [43, 65]. Naked PNA-based anti-miR-155 specifically inhibited miR-155 in cultured B cells as well as in mice. However, very high dosage (50 mg PNA/kg/day for 2 days in mice) was required for efficiency and the untargeted mode of delivery poses a challenge for systemic administration [64].

Depending on the expression status of the target miRNA, miRNA therapeutic approaches can be separated into two categories: (1) miRNA inhibition therapy when the miRNA is overexpressed and (2) miRNA replacement therapy when the miRNA is repressed. These methods can be accomplished with small RNAs directly delivered (Figure 4) or by more conventional gene therapy (Table 1) approaches where plasmids or virus are delivered to express the therapeutic molecules. The gene therapy approach will not be further discussed as the limits of delivery are equivalent to those of other gene therapy approaches and the activity are similar to the systems described herein.

b. MiRNA Inhibition Therapy

When an upregulated miRNA contributes to disease pathology, miRNA inhibition therapy can be used to block the miRNAs repression of protein expression (Figure 4). Several methods have been used to inhibit miRNA repression of protein expression, but each involves the disruption of the miRISC complex. The most straightforward method utilizes oligonucleotides complementary to the miRNA mature strand, anti-miRNA oligonucleotides (AMOs), to inhibit interactions between miRISC proteins and the miRNA or miRISC and its target mRNAs. This approach mimic the idea of anti-sense DNA and RNA toward the mRNA strand preventing translation of the mRNA [66, 67]; however, the AMO is designed to bind the miRNA (not the mRNA), prevent degradation of the mRNA, and allow the mRNA to be translated. In order to achieve effective inhibition, chemical modifications are often used to enhance selective hybridization with the endogenous miRNAs.

Similar to AMO therapy, miRNA sponges seek to occupy the mature miRNA, but instead of delivering short sequences complementary to the miRNA mature strand, DNA sequences, typically plasmids, expressing miRNA target mRNA in high copy number are transiently transfected in cells. This approach has recently been reported as similar to natural phenomena occurring in mammalian cells where circular RNA acts as a miRNA sponge inhibiting activity [68]. Those transcripts contain multiple binding sites to the miRNA of interest and saturate the miRISC complex repressing the activity toward natural mRNA [23]. Using this approach, miR-9 sponge, previously identified as having metastasis-promoting function in breast cancer cells, inhibited breast cancer metastasis formation [69]. However, due to limited control and homogeneity of transcripts expression, miRNA sponges could

lead to serious unwanted side effect and thus is better suited for studying miRNAs function in cell culture assay *in vitro*.

In contrast to occupying the miRISC complex with decoys, miRNA masks were designed to complement the miRISC binding sites in the 3' UTR of the target mRNA [70]. In zebrafish, this gene-specific, miRNA-interfering strategy prevented the miR-430 repression of transforming growth factor-β signaling [71]. The most significant advantage of this strategy lies in that signaling pathways regulated by a miRNA can be selectively blocked by choice of sequence, but this strategy also requires the clear profile of miRNA target genes. Further, the miRNA masks are designed to have specificity for specific mRNA, and thus lose the ability to return protein production for many proteins other than the specific protein that's mRNA is masked.

c. MicroRNA Replacement Therapy

MiRNA replacement therapy supplements the lowered level of miRNAs with oligonucleotide mimics containing the same sequence as the mature endogenous miRNA, known as miRNA mimics. In order to achieve the same biological function as the naturally produced miRNA, mimics should possess the ability to enter the RISC complex and affect miRNA target mRNAs. Theoretically speaking, a single strand RNA molecule containing the same sequence, as the mature miRNA would work as a miRNA mimic. However, double stranded miRNA mimics composed of a guide strand and a passenger strand have 100 to 1000 fold higher potency compared with single stranded miRNA mimics [72]. The guide strand contains a sequence identical to the mature miRNA and the passenger strand sequence is complementary to the mature miRNA. For example, intratumoral injection of cholesterol-conjugated miR-99a mimics significantly inhibited tumor growth in hepatocellular carcinoma-bearing nude mice [73]. In addition to the miRNA mimics having identical sequence as the endogenous mature miRNA, synthetic miRNA precursor mimics with longer sequence ranging from just a few additional nucleotides to full length pri-miRNA have been proposed [74]. Since pri-miRNA is processed in the nucleus, significantly different strategies would be mandated for nuclear targeting of pri-miRNA that would not be necessary for pre-miRNA or mature miRNA. In any case, choosing the appropriate materials to augment the delivery of the miRNA appears to be one of the most important factors influencing the activity of the miRNA.

III. Synthetic Materials for miRNA and anti-miRNA Oligonucleotide Delivery

Synthetic materials have demonstrated potential as effective carriers for DNA and siRNA [75–79]. Under most circumstances, synthetic materials are cationic and condense negatively charged poly(nucleic acid)s through electrostatic interactions. Synthetic delivery systems have considerable advantages over viral-based vector due to the control of their molecular composition, simplified manufacturing, modification and analysis, tolerance for cargo sizes, and relatively lower immunogenicity [80]. However, synthetic systems have relatively lower efficiency compared to viral vectors. Relative efficiency has been greatly improved by modifying particle size and surface properties to achieve specific biodistribution *in vivo*. Further, conjugating small molecule ligands to polymeric carrier targeting diseased cell surface specific receptors has been widely used to augment cell uptake in appropriate cells.

The administration of miRNA expressing vectors, *i.e*. DNA sequences that would be transcribed into pre-miRNA, falls within the purview of DNA delivery, which requires not only nuclear localization but also further processing by RNA machinery. In addition to the general barriers to miRNA delivery, delivery of miRNA expressing DNA resulting in high levels of expression which can saturate exportin-5 resulting in off-target effects [81].

Although this too is miRNA delivery (some would consider this the only type of miRNA delivery), we focus our discussion to research that used synthetic miRNA molecules, such as pre-miR mimics, mature miRNA mimics, and AMO [82, 83].

a. Lipid-based Delivery System

Liposomes are one of the most commonly used transfection reagents *in vitro*; however, safe and efficacious delivery *in vivo* is rarely achieved due to toxicity, nonspecific uptake, and unwanted immune response [84]. Much of the nonspecific response and toxicity is directly linked to the positive charge on the surface of the particles necessary for the binding of oligonucleotides. Currently, there are several commercially available lipid-based delivery systems (Table 2) and most investigations validating miRNA targets *in vitro* have used such systems. In recent years, significant effort has been dedicated to modifying the composition and chemical structure of liposomes, and some success has been achieved in delivering siRNAs [85].

Pre-miR-133b containing DOTMA:cholesterol:TPGS lipoplexes were prepared by adding pre-miR-133b to the empty liposomes. The *in vitro* transfection efficiency and *in vivo* biodistribution of lipoplex formulations were compared with siPORT NeoFX transfection agent. *In vitro*, the lipoplexes transfected pre-miR-133b more efficiently than siPORT NeoFX, a commercially available lipid-based agent, in A549 non-small cell lung cancer cells. Approximately 30% of Cy5 labeled oligodeoxynucleotides lipoplexes accumulated in the lung, which was 50-fold higher than the NeoFX formulation. The mature miR-133b level in lungs following i.v. administration of pre-miR-133b containing lipoplexes was approximately 52-fold higher than untreated mice [88]. However, no further therapeutic effect on inhibiting tumor growth was reported. Using DDAB:cholesterol:TPGS based lipoplexes, head and neck squamous cell carcinoma was treated with systemic administration of pre-miR-107 lipoplexes. Tumor growth was reduced by 45.2% compared to lipoplexes containing pre-miR-control [93]. Although effective, the inclusion of charged lipids on the surface of the liposomes suggest that increased clearance of the liposomes would be observed. Additionally, limited information is presented on the stability of the interaction following injection into biologic fluids where the high salt concentration and proteins may greatly alter the ionic complexes.

Polycationic liposome-hyaluronic acid (LPH) nanoparticles have also been described by several investigators [112]. As applied to miRNA delivery, a tumor targeting GC4 single-chain antibody fragment modified LPH (scFv-LPH) nanoparticles systemically co-delivered siRNA and miR-34a into experimental lung metastasis of murine B16F10 melanoma. The scFv-LPH nanoparticles encapsulating combined siRNAs against c-Myc, MDM2, and VEGF and miR-34a decreased the metastasis tumor growth to about 20% of the untreated control. When treated with scFv-LPH nanoparticles only containing combined siRNAs or miR-34a, the reduction was about 30 and 50% of the untreated control, suggesting the effects are through different mechanisms. The advantage of such a system lies in the potential to deliver siRNA and/or miRNA together to simultaneously target several different oncogenic pathways [94]. Using the same liposome-polycation-hyaluronic acid nanoparticle conjugated with cyclic RGD, an integrin-binding tripeptide (cRGD-LPH), delivered anti-miR-296 efficiently interrupting blood vessel formulation and endothelial cell migration in human umbilical vein endothelial cells by specific uptake through $\alpha_v\beta_3$ integrin. Anti-miR-296 containing cRGD-LPH nanoparticles exhibited the most significant inhibition of cell proliferation and microvessel formation in matrigel plugs subcutaneously implanted in mice [95]. Even though these targeted miRNA therapeutic delivery systems showed enhanced activity in tissue culture and mouse models, they do not guarantee the same result in human beings. Like most targeted delivery systems, the accumulation of the particles is

limited by the flow of particles into the diseased tissue, which typically is limited to less than 5% of the total particles administered. The targeting moieties only allow particles to be taken up by cells they reach and can not generally increase the amount of particles reaching the diseased cells.

To overcome disruption of the ionic interactions that stabilize most liposome-miRNA interactions, dicetyl phosphate-tetraethylenepentamine-based polycation liposomes (TEPA-PCL) were combined with cholesterol-grafted miR-92a, an angiogenesis regulating miRNA, for cancer anti-angiogenesis therapy. The miR-92a/TEPA-PCL complex showed significant inhibition on endothelial cell tube formation at 200 nM *in vitro* [113]. Although complicating the production and design of the therapeutic molecules, combinations of delivery strategies hold the greatest promise to overcoming the current limitations of the delivery platforms that are available.

Even though cationic lipids are the most widely used for lipid-based system, their *in vivo* application is frequently limited by toxicity [84]. Aiming to overcome this hurdle, a neutral lipid emulsion (NLE), MaxSuppressor *in vivo* RNALancerII (BIOO Scientific, Inc.) was developed. MiR-34a and Let-*7* were delivered with a NLE to treat non-small cell lung cancer (NSCLC). Unlike cationic lipids, NLE preferentially accumulated in the lungs rather than the liver. The treatment with miR-34a or Let-*7* significantly decreased lung tumor burden to approximately 40% of the mice treated with miRNA controls and the expression level of miR-34a and let-7 in lungs was also significantly higher than groups treated with miRNA mimic controls [90]. These findings demonstrate the potential of developing miRNAs therapy formulations with NLE as novel therapies for lung cancer patients. These particles, however, are limited in the diseases for which they could be applied as they rely on the passive accumulation in the lung. Strategies to overcome this limitation should be sought, but few methods have overcome the passive accumulation of particles in the liver, lungs, or spleen.

In addition to being used directly as delivery carriers, lipids were also used as modification agents using a dendrimer-like structure of lysine with lipid surfaces, referred to as iNOP-7. The delivery of apoB siRNA with iNOP-7 efficiently silenced apoB mRNA, decreased apoB protein levels in liver and plasma, and lowered total plasma cholesterol [114]. Similarly, anti-miR-122, delivered with iNOP-7 (2 mg/kg) administered on three consecutive days, effectively silenced 83.2+3.2% of miR-122 expression, which was accompanied by regulating miR-122 target gene mRNA expression in liver and lowering of plasma cholesterol [96].

There is no doubt lipid-based materials will be one of the most important candidates for further exploitation of miRNA therapeutics formulation. As one of the most widely studied material for pharmaceutical delivery, the knowledge accumulated forms a solid foundation for any further exploration. However, lipid-based systems are not the only option and other alternative materials might be needed for specific disease and offer unique advantages.

b. Polyethylenimine (PEI)-based Delivery System

Polyethylenimine is one of the most widely used and studied polymers for gene delivery. In the physiological milieu, PEI is positively charged due to protonation of the amine groups and thus can be used to condense nucleic acids. Cationic polyplexes formed by PEI and nucleic acids typically retain a net positive charge (ζ-potential) promoting interactions with negatively charged polysaccharides on the cell surface. Once interacting with the cell surface, it is proposed that the complexes undergo endocytosis. The polyplexes escape from the endosome through so called ‘proton sponge’ effect where PEI causes influx of protons and water and endosome swelling and eventually disruption to release the polyplexes to

cytoplasm [115]. Using PEI, both DNA and siRNA have been successfully delivered in animal models *in vivo*, as has been comprehensively reviewed elsewhere [80, 99, 116]. Many of the same limitations that have limited the translation of PEI to the clinic are also present for miRNA delivery although PEI remains in the pipeline for translation [117, 118] due to the augmented activity compared to many other polymeric systems.

Using a polyurethane-short branch polyethylenimine (PU-PEI) as a carrier, miR-145 was delivered to treat cancer stem cell (CSC) derived lung adenocarcinoma (LAC). The LAC-CSC xenograft tumors did not respond to the combination of ionizing radiation (IR) and cisplatin during the 30-day experimental course; however, PU-PEI-bound miR-145 delivery moderately reduced tumor growth. Most importantly, the miR145 delivery combined with IR and cisplatin led to significant tumor growth inhibition [100]. When administered to orthotopic CSC-derived glioblastoma tumors, intra-cranially delivered PU-PEI-miR-145 significantly suppressed tumorigenesis. When used in combination with radiotherapy and temozolomide, synergistic effects and improved survival rate were achieved [119]. The significant inhibitory effect of PU-PEI-miR-145 on lung adenocarcinoma and glioblastoma CSC-induced tumors demonstrated the potential of miRNA therapy in overcoming tumor chemoradioresistance, preventing cancer relapse and achieving cancer eradication.

Beyond traditional delivery approaches, PEI-based systems have been modified for transport across the blood-brain barrier (BBB). The BBB is the most significant physiologic obstructions of systemic drug or gene delivery to the brain parenchyma and central nervous system (CNS) [120]. Using a short peptide derived from rabies virus glycoprotein (RVG), the PEI-RVG bound specifically to nicotinic acetylcholine receptors on neuronal cells. RVG was coupled to PEI via disulfide bond (RVG-SSPEI) to deliver miR-124a, a neuron-specific miRNA that can potentially promote neurogenesis [101, 121]. To overcome the size limitation of PEI vector transport across the BBB, mannitol was used to permeablized the BBB. After administered, much higher accumulation of miR-124a in the brain was observed in the RVG-mediated SSPEI delivery group compared to that in the miR-124a/SSPEI group by tracking the Cy5.5 labeled miR-124a [101]. However, the functional activities of miR-124a to promoting neurogenesis were not tested. The modification of PEI using RVG decreased the toxicity associated with PEI and achieved remarkable targeted delivery to neuronal cells. The RVG-SSPEI could be a useful system to deliver miRNA therapeutics for the treatment of brain diseases. Although this system did show greater accumulation in the brain, the limitation of using permeabilizing agents limits the utility. Combination of delivery strategies that each improve the activity of the miRNA have great potential, but the complexity of the systems at times can counterbalance the improvements [122].

c. Dendrimers

Dendrimers have attracted significant attention in gene delivery due to their defined architecture and a high ratio of surface moieties to molecular volume [123]. Dendrimers have been successfully used to deliver DNA and RNA; however, few applications to miRNA delivery have been reported. Co-delivery of anti-miR-21 and 5-fluorouracil (5-FU) to U251 glioblastoma cells using poly(amidoamine) (PAMAM) dendrimer increased apoptosis of U251 cells markedly. Migration of tumor cells was decreased compared with cells that were only treated with 5-FU [103]. Anti-miR-21 delivered by PAMAM increased the chemosensitivity of human glioblastoma cells to taxol [102]. Although not *in vivo*, this suggests that dendrimers may be amenable for *in vivo* miRNA delivery. Dendrimers are capable of binding miRNA and aiding in the entry into cells, but the entry is non-specific in nature. The alternate mechanism of cell entry that should not be overlooked as this has the potential to protect miRNA by avoiding the endosomal and lysosomal compartments [124].

d. Poly(lactide-co-glycolide) (PLGA) Particles

The majority of the previously described polymers focus on the ability to condense miRNA and non-specifically enter the cell while poly(lactide-co-glycolide) (PLGA) focus on these factors but also allow the release of miRNA over time. PLGAs are a family of water-insoluble polymers that have been widely used in biomedical applications over the last half-century [125]. PLGA polymers can be readily formed into microparticles and nanoparticles, which entrap biologically active molecules [126, 127]. PLGA particles overcome several limitations facing current miRNA therapy because they can protect nucleic acids from degradation, achieve high loading capacities, and afford multiple surface modifications so as to generate potentially favorable pharmacodynamics [128]. Successful delivery of DNA and RNA have been achieved and some ongoing clinical trials showed great potential [75].

Using a miR-155 Cre-lox*P* tetracycline-controlled knockin mouse model, pre-B-cell tumors were dependent on high miR-155 expression where withdrawal of miR-155 using doxycycline caused rapid tumor regression. Systemic delivery of anti-miR-155 peptide nucleic acids (PNAs) using PLGA polymer nanoparticles exhibited enhanced delivery efficiency and achieved therapeutic effect. The surface of the nanoparticles was modified with penetratin, a cell-penetrating peptide [105]. Although their previous studies have shown that PLGA NPs modified with nona-arginine can effectively deliver anti-miR-155 PNAs to KB cells and achieve therapeutic miRNA inhibition [104], pre-B cells preferentially took up nanoparticles coated with penetratin (ANTP-NP). The ANTP NPs preferentially accumulated in tumor tissue compared with other organs and tissues through enhanced permeability and retention effect (EPR) [129]. The pre-B cell tumors had an approximately 50% decrease in growth relative to control-treated tumors after systemic delivery of 1.5 mg/kg anti-miR-155 PNAs loaded in ANTP-NP for 5 days, which was approximately 25-fold less than naked anti-miR dosage needed [105]. There was need, in this case, to protect the PLGA particle using steric stabilization, i.e. PEGylation, and also add a cell penetration enhancer. PLGA particles are typically non-specifically cleared and the PEGylation diminished the ability of the particles to enter cells. This type of particle is readily adaptable, but still does not have significantly more than 5% accumulation in the diseased organ due to passive accumulation.

One of the greatest advantages of PLGA polymers is their adaptability and ability to load multiple cargos. MRI-detectable nanoparticles were synthesized using PLGA and loaded with perfluoro-1,5-crown ether (PFCE) for ^{19}F-MRI tracking. These PLGA particles also contained protamine sulfate for miRNA condensation. The nanoparticles accumulated in the endosomal and lysosomal compartments and efficiently released miR-132, inducing potent pro-survival activity in endothelial cells (ECs) transplanted *in vivo*. There was also a strong enhancement blood perfusion in ischemic limbs relatively to control. Important to the future development of miRNA is the assertion by the authors that RNA degradation occurs in or around the endosomal and lysosomal compartments [130, 131]. If this proves true, much of the design criteria for miRNA will alter. The fact that Ago2 resides in the membrane of the endolysosomal compartment could explain the better performance of NP170-PFCE formulation [132]. The future development of miRNA (and possibly siRNA) will rely on the rational design of systems that take advantage of the complex biology of the disease and molecule being delivered.

e. Other Nonviral MiRNA Therapeutics Delivery System

In addition to synthetic polymeric material as delivery system, naturally occurring polymers, such as chitosan, protamine, atelocollagen, and peptides derived from protein translocation domains have also attracted significant attention [133, 134]. However, their application is usually limited by immunogenicity [135]. Atelocollagen exhibits limited immunogenic

response because it is purified from pepsin-treated type I collagen, during which antigenicity conferring telopeptide is removed [136]. Similar to other materials used for gene delivery, atelocollagen is positively charged and can condensate oligonucleotides by forming nanoparticles through electrostatic interactions. Due to its successful application in delivering DNA and siRNA [137], several research groups attempted to use it for miRNA therapeutics administration. MiR-34a/atelocollagen complexes administered intratumorally into the subcutaneous spaces around human colon cancer mice xenograft suppressed tumor cell growth *in vivo* [106]. Using the same approach, an LNA miR-135b inhibitor was administered subcutaneously to human lymphoma mice xenograft and tumor growth was significantly inhibited comparing with the negative control groups at 13 days post tumor inoculation [107]. In addition, miR-16/atelocollagen complexes were delivered systemically to a prostate cancer mouse xenograft model. Atelocollagen not only delivered miR-16 to bone tissue but also inhibited metastatic tumor growth in bone tissues without causing noticeable side effects [108]. The choice of natural materials may have great advantages–specifically in terms of biologic acceptance and ability to mimic natural processes–if the properties allow sufficient efficiency.

Using another biologically derived molecule, protamine, miR-29b was delivered to human mesenchymal stem cell (hMSC). Complexes formed were around 34 nm with a zeta potential +27mV. The miR-29b/protamine complexes were readily taken up by hMSC post transfection while lipoplexes exhibited very little transfection. In addition, the inhibition of miR-29b/protamine complexes on cell proliferation was almost negligible, which is a critical factor for transfecting stem cells since the cell number is a critical factor for their use as therapeutics. MiR-29b/protamine complexes were not only able to induce the expression of osteoblast differentiation markers, their expression level also showed a dose response to increasing concentration of miR-29b/protamine formulation [138]. Using biologically derived proteins may allow the direct use of human molecules to deliver miRNA particularly when human proteins can be utilized and have significantly decreased toxicity.

Inorganic materials have also been exploited to produce nanoparticles. The inorganic materials usually have monodispersed particle size and good optical properties. As a model miRNA, miR-130b was coupled to gold nanoparticles through oligo(ethylene glycol) thiol (miRNA-AuNPs). The miRNA-AuNPs own good surface stability since the miRNA did not show exchange activity with a highly charged surfactant when assessed by gel electrophoresis. The Cy5-labeled miRNA-AuNPs was found to localize inside the cells with confocal microscopy and the cell morphology was not significantly changed after transfection. MiRNA-AuNPs mediated efficient knockdown in functional luciferase assay compared with the controls [139]. Another gold nanoparticles based miRNA delivery system first functionalized AuNPs with cystamine and modified the miRNA-AuNPs with thiol-polyethylene glycol post miRNA loading, which demonstrated high payload, low cytotoxicity and robust miRNA transfection capacity [140]. These systems, as with many of the non-viral systems, have not been assessed *in vivo*. There is potential for delivery using these systems, but there are also questions that need to be answered concerning toxicity and clearance.

Similarly, silica-based nanoparticles are stable and inert with biodegradable and non-toxic properties [141]. Silica particles release the cargo through hydrolysis of the silica network. Taking advantage of neuroblastoma's property of expressing high level of cell surface antigen disialoganglioside (GD_2), GD_2-targeting silica nanoparticles were designed to deliver miR-34a into neuroblastoma tumors. Anti-GD_2 nanoparticles containing miR-34a resulted in a significant up-regulation of miR-34a in the neuroblastoma cell lines, but not in HEK293. At day 14, 17 and 20 post-tumor establishment, anti-GD_2-miR-34a-NP or anti-GD_2-miR-scramble-NP were administered to $NB1691^{luc}$ or $SK\text{-}N\text{-}AS^{luc}$ mice. Tumor sizes

in NB1691^{luc} and SK-N-AS^{luc} mice were monitored by bioluminescent imaging on days 18, 21 and 25 and a significant reduction in the bioluminescent intensity of the tumors was observed in mice treated with anti-GD_2-miR-34a-NPs relative to negative control. Investigation of the molecular mechanism of the tumor growth inhibition effect revealed that delivery of miR-34a using anti-GD_2-NPs caused statistically significant increase in apoptosis and mediated negative impact on vascularization through downregulation of MYCN protein and upregulation of tissue inhibitor metallopeptidase 2 precursor (TIMP2) protein [110].

To more specifically target the disease sites, mediate effective therapeutic effect, and contain traceable label for monitoring drug distribution, magnetic particles (MP) have also been proposed [142]. Ultra-small magnetic nanoparticles surface modified with RGD and LNA oligonucleotides were designed as an imaging-capable particle that can alleviate breast cancer metastasis through downregulating miR-10b level. Both MRI and infrared fluorescence optical imaging indicated that the particles preferentially accumulated in the primary tumor and lymph nodes comparing with muscles. In orthotopic MDA-MB-231-*luc*-D3H2LN tumors, particles prevented breast cancer metastasis to lymph nodes when the treatment was initiated before detectable metastasis formation. Surprisingly, even though the particles did not inhibit primary tumor growth, an inhibitory effect on the growth of lymph node metastases when the treatment was given post lymph node metastases formation was observed. It was proposed that miR-10b plays a context-dependent role in breast tumorigenesis, namely miR-10b controlled metastasis but not tumor growth in the primary tumor while in the lymph nodes metastases the miRNA extended to regulate tumor proliferation [111]. This finding indicates the possibility of effectively treating disseminated cancer by not only specifically targeting to the tumor metastases but also monitor the process by noninvasive imaging approach. The elucidation of potential context-dependent role of miRNAs in oncogenesis opens new door for developing anti-metastatic therapies.

V. Future Outlook: Challenges and Opportunities

Over the last several years, miRNA research has made amazingly rapid progress. But despite this, there is a belief that “RNAi is dead” [143] partly due to the departure from or reduction in emphasis on siRNA and miRNA by the pharmaceutical industry [144]. Few siRNA therapeutics and only two candidate miRNAs, SPC3649 (Santaris Pharma, Horsholm, Denmark) [145–151] a miR-122 antisense locked nucleic acid, and MRX34 (Mirna Therapeutics, Inc.) [152] a liposomal miR-34 mimic, have reached clinical trials. There is significant risk for investment by the pharmaceutical companies that is due to the biologically challenging therapeutic molecules, the expense of making and scaling up the therapeutics, and the unproven delivery systems with clinical approval challenges [153]. The field is still in the infancy and will take some time to mature. The costs and challenges will only become more reasonable. Of all of the scientific (Table 3) and commercial challenges, the greatest challenge to translating siRNA and miRNA is delivery [143].

It is now widely recognized that designing miRNA specific for a disease target is not the bottleneck for RNA therapeutics, but the design of appropriate delivery systems that will reach pharmacologic targets *in vivo* is the key to miRNA development. The development of RNAi therapeutics has to be considered in terms of which cell/tissue types available delivery technologies can address. A sing system will not be the answer for all diseases. The examples are liver-directed gene knockdown [145, 146, 152], bladder cancer-directed miRNA therapy [117, 118], and endothelial cell-directed knockdown using Silence Therapeutics’ cationic AtuPLEX lipoplex technology [155] which each reach organs that are known to take up particles or where particles can be directly in contact with the diseased tissue. Much progress has been gained in the area of disease targeted drug delivery, but even

with these advances there are significant limits to the amount of drug that accumulates in the diseased organs [156].

Many of the parameters necessary to control the delivery of any particulate system are well established. To improve the residence time in the circulation, a material should be greater than the renal filtration limit [157–159], but it should be excreted when the payload is released [160]. While in the bloodstream, particles must avoid the uptake by phagocytic cells [161, 162]. The charge on a particle, typically positive for RNA condensation, has dramatic influence on the cellular uptake. Most transfection reagents are highly cationic and taken up through non-specific means, but this also leads to rapid clearance. Competing with these issues is the need to cross the endothelial layer to reach the diseased tissue [163–167], when not administered for local response. Even when the endothelial barrier is traversed, the local extracellular matrix yields a substantial resistance to penetration into the tissue [163–167]. The systems described within this review address each of these issues in different ways.

The inherent properties of miRNA are a significant challenge to delivery. Molecular stability before reaching the patient is a concern and this is often ignored. This is coupled with the cost and complexity of the production of the molecules and delivery systems. Once the miRNAs are administered, unprotected miRNAs are rapidly degraded by serum nucleases in bodily fluids. While chemical modifications protect miRNA, these molecules can impair specificity and potentially introducing off-target effects [57, 168]. This is compounded by the clearance of particulate systems even when they adequately protect the miRNA from degradation. When particles reach cells, the miRNA must separate for action to be possible. The limited separation of miRNA from the carrier has forced increased dose of miRNA to be active. Many current systems are focusing on using targeted drug delivery strategies to increase the amount of the drug that reaches the site, but targeting has a limited effect at increasing the total amount of drug that reaches the target organ [169].

The chemical and physical properties are not the only challenges as biologic properties of miRNA present difficulties that must be comprehended to properly deliver the molecules. The property of miRNA being able to regulate a network of genes without perfect pairing also imposes the challenge of consistency and predictability of miRNA therapeutics. Moreover, the presence of exogenous artificial miRNAs brings the risk of saturation of RNA processing machinery and cause untoward side effect [81, 170]. Most importantly, the expression level and function of miRNAs vary not only between normal and diseased tissues and organs but also between disease stages [171, 172]. For example, a total of 28 epigenetically regulated miRNAs were identified as being associated with poor patient survival when under expressed in neuroblastoma, among which miR-340 induced either differentiation or apoptosis in a cell context dependent manner [173]. This feature is challenging because it requires miRNA therapeutics to achieve both temporal and spatial control over biodistribution *in vivo*.

Even when active, small RNAs can have nonspecific effects that must be examined. Interferon response can occur following activation interferon stimulated genes [174–177]. When this happens, it leads to non-specific inhibition of protein synthesis coupled with non-specific degradation of endogenous mRNA. Therefore, in addition to non-targeting RNA controls, one must rule out the possible interferon response when validating the small RNA. This can be achieved by determining the expression levels of several key genes involved in the interferon response [178–181]. These responses can be controlled possibly by appropriate delivery and design of the therapeutics.

To begin to validate the technologies, diseases with limited alternatives for treatment have been chosen. This is exemplified by the fact that only two oligonucleotide-based therapeutics, Vitravene® and Macugen®, have reached the market and both are for intravitreal administration, chosen due to the inherent difficulty to deliver and dire need for therapy. These products, although successful clinically, are not considered to be successes commercially. Part of the commercial challenge has come from the entry of competing products that are less expensive and similar if not more effective. The commercial success is also limited by the expense of the products themselves. There is much room to improve the commercial success of the products as we learn from the current generation of miRNA therapeutics and delivery systems. As the cost of production and scale-up of the products decreases, there will be success in this field, both commercial and clinical. During the design of new miRNA delivery system, the feasibility and cost associated with production for clinical translation should be considered.

As we develop delivery system, we must focus on choosing diseases that have high need for new, and potentially expensive, therapies. Unique miRNA expression profiles have been identified in untreatable or poorly treated diseases, such as cardiovascular disease, neurological disorders, and autoimmune disease [37, 39, 182]. An array of miRNAs were found to function as gene regulators in Alzheimer's disease by targeting the mRNAs of amyloid precursor protein (APP), β- and γ-secretase, of which the latter two are responsible for the cleavage of APP and the generation of amyloid-beta peptide, a significant target for treating Alzheimer's disease [183]. Cordes and colleagues demonstrated that miR-145 was necessary and sufficient to induce multipotent stem cells differentiation into vascular smooth muscle cells [184]. This important finding raises the possibility of suppressing smooth muscle hyperplasia observed in vascular injury and atherosclerosis using miR-145 replacement therapy. The important roles played by miRNAs in diseases for which conventional medicine offers no effective solution indicates that focusing miRNA therapeutics development on those diseases is worth the effort and warrants further study.

The complexity and heterogeneity of signaling pathways involved in many diseases, including tumor initiation, progression, metastasis and chemo/radio resistance accounts for the difficulty of developing effective medicine [185]. The discovery of miRNAs might be the critical piece of the cancer research puzzle since one miRNAs can regulate a broad network of genes involved in different signaling pathways. This fact is both a significant advantage for miRNA compared to siRNA and a biologic challenge as untoward effects may be augmented by the broad activity. Proper design of the AMO and miRNA mimic sequences [186, 187] is key to the ability to selectively alter the necessary networks. This fact is also one of the greatest challenges due to the fact that the prediction of side effects may be difficult.

Many miRNAs have been identified as key oncogenic targets in different kinds of cancer, including cancer stem cells [70, 188–193]. Those findings suggest that therapeutic intervention of supplementing a tumor suppressor miRNA or inhibiting an oncomiR may indeed induce multiple antitumor effects by simultaneously interfering with several oncogenic pathways, thus preventing tumor cells from activating escape mechanisms. An additional challenge, however, is the need to efficiently elicit the response in all diseased cells, particularly for cancer. When a small number of cells evade treatment, the disease will progress. Improvements in the delivery will greatly diminish this concern, but appropriate choice of miRNA may also minimize this concern. By selecting miRNAs that will increase extracellular signaling molecules, treated cells will influence cells within the vicinity by these paracrine factors.

For cells to respond to a miRNA therapeutic, separation from the delivery system and subcellular localization of miRNA to miRISC complexes will be necessary. The majority of the activity of miRNA is related to miRISC formation. Much like has been discussed for siRNA [194], the fact that inactive and active RISC has been localized to the endosome may have great advantage for delivery. The location of RISC and possibility that activity is localized to endosomes [130, 131, 195] suggests that escape from the endosome may be unnecessary, and if escape is necessary, the localization to the endosomal membrane may increase activity. This is counter to the idea that P-bodies are the primary locations for miRISC activity where P-body localization would be necessary for augmented intracellular activity. A thorough understanding of the biology behind the activity of the therapeutic can be used to guide the design of the delivery system for optimal effectiveness to be achieved. As with any therapeutic approach, the interaction between the therapeutic molecule and delivery system is paramount to the activity of the final therapy.

The efficiency of miRNA-based therapeutic molecules rely largely on the capacity of delivery agent to protect the oligonucleotides against serum degradation, accumulate to the diseased organ or tissue via active targeting, and elicit powerful therapeutic effect without causing unwanted side effects. The delivery of miRNA is limited to parenteral or local injection due to the lack of enteral uptake of RNA although other routes are being examined [196]. Depending upon the disease, the amount of miRNA reaching a target organ (or organs) is limited. The chemical challenges to stabilizing RNAs have been overcome by modification and complexation, but the stability of the RNAs does not improve cellular uptake and escape. The vast majority of delivered RNA never will enter a cell, let alone the target cells. Each of the systems described overcomes some of the numerous challenges, but much improvement is possible.

VII. Conclusion

Recent developments in the understanding of miRNA have suggested their therapeutic potential. But, the understanding of the biological genesis and activity will be key to translation of this promising therapeutic class of molecules. Many of the challenges of delivering miRNA are similar, if not equivalent to siRNA and DNA, but some differences exist. The primary differences exist in the specificity of mRNAs that are repressed and the possibility to have promotion of expression directly through the miRNA. Significant progress has been made in confirming the ability to deliver miRNA, but substantial improvements will be necessary. Delivery systems will continue to be the key to bringing miRNA to the clinic.

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Abbreviations and Acronyms

AMO	anti-miRNA oligonucleotides
APP	amyloid precursor protein
BBB	blood-brain barrier
bp	base pairs

CNS	central nervous system
CSC	cancer stem cell
DDAB	dimethyldioctadecylammonium bromide
DGCR8	DiGeorge syndrome critical region gene
DNA	deoxyribonucleic acid
DOTMA	1,2-Di-O-octadecenyl-3-trimethylammonium propane
FANA	fluorine derivatives nucleic acid
GC4	phage identified internalizing scFvs that target tumor sphere cells
HCV	hepatitis C virus
iNOP	nanotransporter interfering nanoparticle-7
IR	ionizing radiation
LAC	lung adenocarcinoma
LNA	locked nucleic acid
LPH	liposome-hyaluronic acid
2’-Me	2’-methyl
miRNA	microRNA
2’-MOE	2’-methoxyethyl
NLE	neutral lipid emulsion
NSCLC	non-small cell lung cancer
nt	nucleotides
ODN	oligodeoxynucleotides
PEI	polyethyleneimine
PNA	peptide nucleic acids
PU	polyurethane
RNA	ribonucleic acid
RISC	RNA induced silencing complex
scFv	single-chain variable fragment
siRNA	small interfering RNA
TEPA-PCL	dicetyl phosphate-tetraethylenepentamine-based polycation liposomes
TPGS	D-alpha-tocopheryl polyethylene glycol 1000 succinate
UTR	untranslated regions
VEGF	vascular endothelial growth factor

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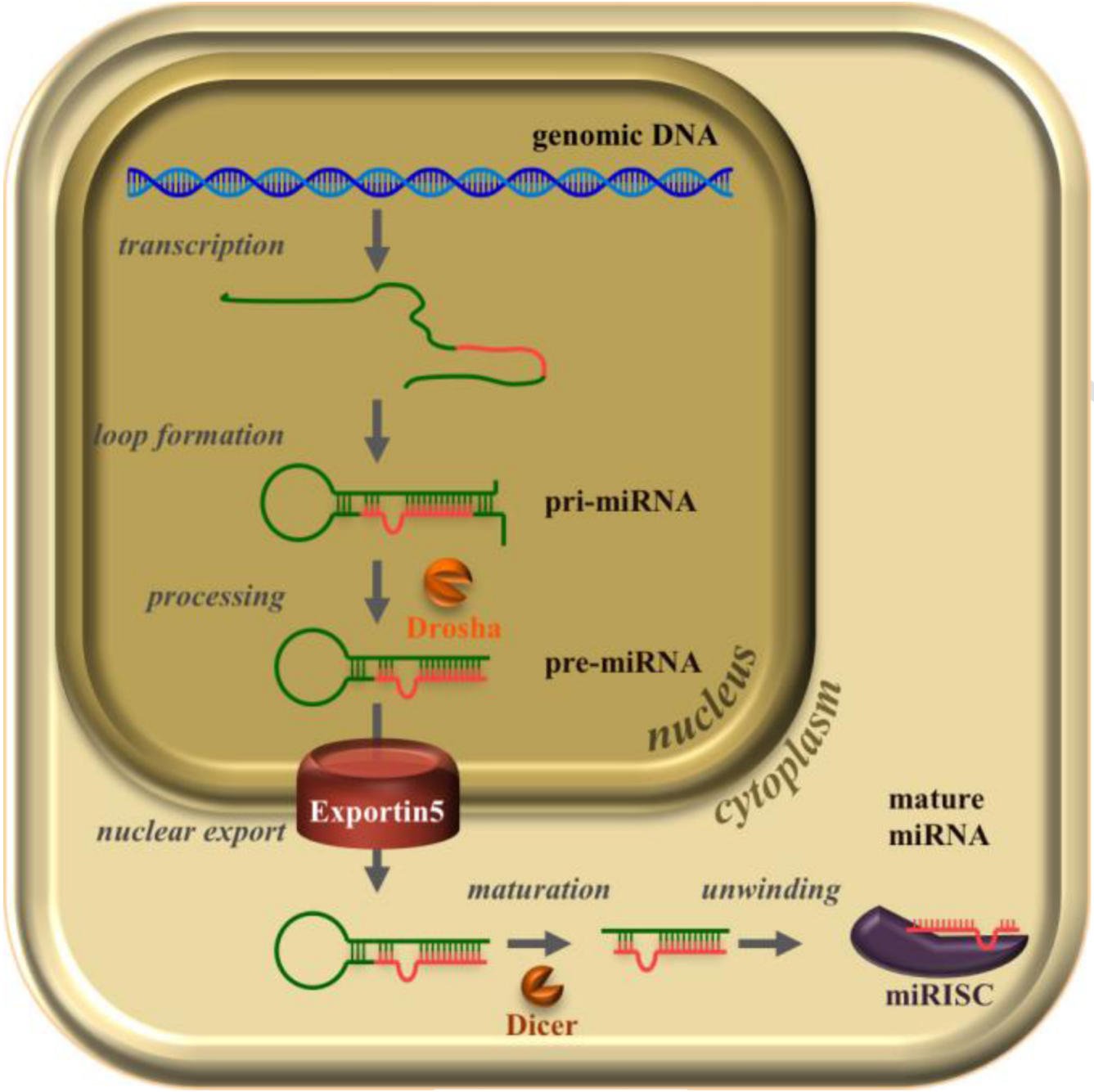

Figure 1. Schematic representation of the biogenesis of miRNA

From genomic DNA, RNA is transcribed either as (1) an independent miRNA sequence or (2) an intron that is removed from mRNA. Following loop formation, the pri-miRNA is processed by DROSHA into pre-miRNA. The pre-miRNA is exported from the nucleus by Exportin5 before maturation by Dicer. Upon loading into the RISC complex, the miRNA duplex is unwound and the mature strand (red strand) retained within the miRISC complex. The passenger strand (green strand) is degraded.

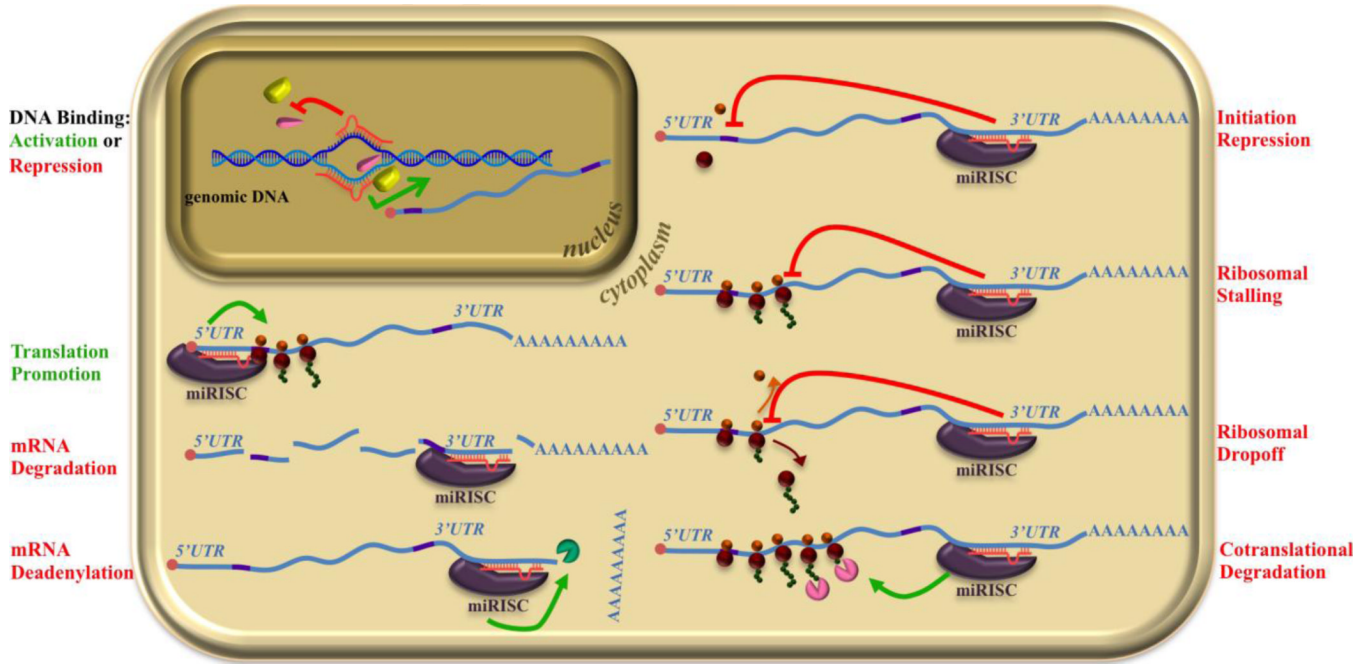

Figure 2. Mechanisms of natural miRNA-mRNA action

The roles of miRNA in protein production have been proposed to be through many routes. The majority of the actions inhibit protein production, but several modes of promotion of protein production have also been proposed. Binding of miRNA to DNA may promote transcription directly or through the recruitment of other factors. In addition, translation can be promoted by 5'UTR binding. Inhibitory action is likely through a combination of the mechanisms shown which include (1) direct mRNA degradation, (2) deadenylation, (3) initiation repression, (4) ribosomal stalling, (5) ribosomal drop-off, (6) co-translational degradation, and (7) translation repression following DNA binding.

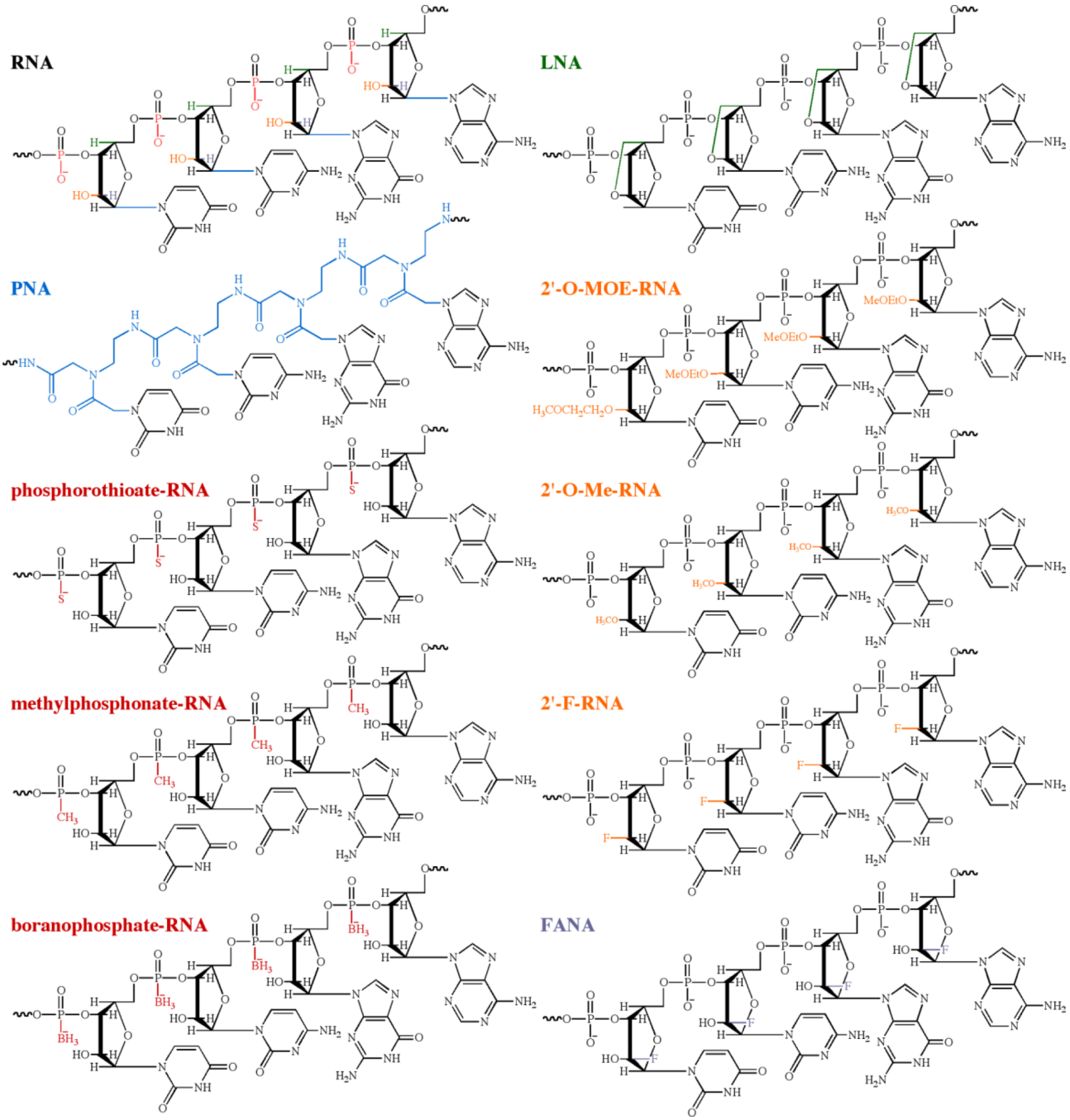

Figure 3. Chemistry and structure of miRNA therapeutic molecules

RNA molecules (RNA) has several bonds that have been modified for stability including the alpha oxygen of phosphate (red), 4' hydrogen (green), 2' hydroxide (orange), 4' hydrogen (purple), and the 1' amide linkage to the base (blue). Peptide nucleic acids (PNAs) substitute a peptide bond at the 1' amide linkage to the base. Phosphorothioates, methylphosphonates, and boranophosphates substitute a sulfur, methyl, and a borano group, respectively, for the alpha-oxygen of the phosphate. Locked nucleic acids (LNAs) add a secondary linkage between the 4' carbon and the 2' hydroxide while 2'-O-(2-methoxyethyl)-(2'-O-MOE), 2'-O-methyl-(2'-O-Me), and 2'-fluoro-(2'-F) RNAs substitutes less reactive groups for the 2' hydroxyl group.

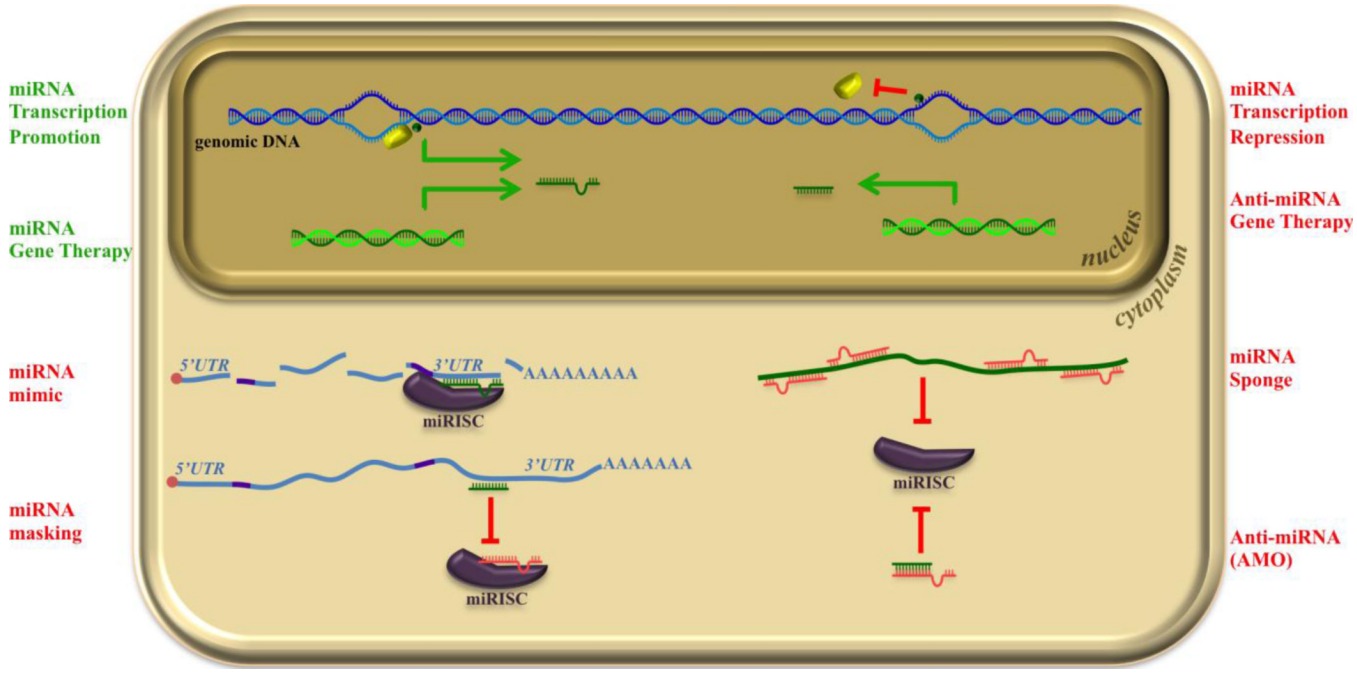

Figure 4. Therapeutic Strategies for miRNA activity

Delivered DNA or RNA (green) can act to augment, mimic miRNA activity, or prevent activity of miRNA. MicroRNA can be produced by plasmid DNA (miRNA gene therapy) that is introduced into the cell, or by introducing agents to increase by translation promoters or cofactors, or delivered directly (in the forms of pri−, pre−, or mature with or without passenger), i.e. miRNA mimic. To prevent the activity of expressed miRNAs, masks that compete with the binding site on mRNA inhibit miRNA binding with mRNA.

In addition, binding of miRNA with the RISC complex may be inhibited with a sponge or a single miRNA binding anti-miRNA. Finally, translation may be directly repressed at the DNA level.

Table 1

General properties of commonly described poly(nucleic acid) therapeutics.[†]

Property	miRNA	siRNA	ribozyme	antisense RNA/DNA	gene therapy
Size	~20 bp	~20 bp	≥10 bp	≥20 bp	>1 kbp
DNA or RNA	RNA	RNA	RNA	RNA/DNA	DNA
Structure	Single or double stranded	Double stranded	Single stranded	Single stranded	Double stranded
Proteins directly affected	many	one	one	one	one
Protein production change	downregulated or upregulated	downregulated	downregulated	downregulated	upregulated

[†]Although these general properties are typically true, there are exceptions to the many of the attributes for specific cases.

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Table 2

Representative recent nonviral gene delivery systems for miRNA therapeutics.

Material	Disease model	Targeted miRNA	Therapeutic Approaches	Route	Refs
Naked oligonucleotides					
N/A	Metabolic disease	miR-122	Inhibition	Systemic	[61, 62, 86]
Aptamer-ologonucleotide	Ovarian cancer	let-7i	Replacement	Systemic	[87]
Lipid-based delivery system: commercially available					
siPORT	Non-small cell lung cancer cells; Lymphoma	miR-133b; miR-34a	Replacement	Systemic	[88] [89]
MaxSuppressor	Non-small cell lung cancer	MiR-34a /Let-*7*	Replacement	Systemic	[90]
LipoTrust	Colorectal tumors	miR-143 /miR-145	Replacement	Intratumoral /systemic	[91]
Lipid-based delivery system: newly explored					
$98N_{12}$-5	Fat and cholesterol metabolism	miR-122	Inhibition	Systemic	[92]
DOTMA:cholesterol: TPGS lipoplexes	Non-small cell lung cancer cells	miR-133b	Replacement	Systemic	[88]
DDAB:cholesterol:TPGS lipoplexes	Head and neck squamous cell carcinoma	miR-107	Replacement	Systemic	[93]
GC4 scFv-targeted LPH nanoparticles	Murine B16F10 melanoma	miR-34a	Replacement	Systemic	[94]
cRGD-targeted LPH nanoparticles	Antiangiogenesis	miR-296	Inhibition	Subcutaneous	[95]
iNOP-7	Fat and cholesterol metabolism	miR-122	Inhibition	Systemic	[96]

Material	Disease model	Targeted miRNA	Therapeutic Approaches	Route	Refs
DC-6-140-DOPE-Choleterol liposomes	Lung cancer	miR-7	Replacement	Systemic	[97]
Solid lipid nanoparticles	Lung cancer	miR-34a	Replacement	Systemic	[98]
Polyethyleneimine (PEI)					
PEI	Colon carcinoma	miR-145/ miR-33a	Replacement	Intratumoral /systemic	[99]
Polyurethane-short branch PEI	Lung adenocarcinoma ; glioblastoma	miR145	Replacement	Systemic; intracranial	[100]
RVG-coupled PEI	Neuron diseases	miR-124a	Replacement	Systemic	[101]
Dendrimers					
Poly(amidoamine)	Glioblastoma	miR-21	Inhibition	Cell culture	[102, 103]
Poly(lactide-co-glycolide) (PLGA)					
Nonaarginine-modified PLGA nanoparticles	KB cells	miR-155	Replacement	Cell culture	[104]
Penetratin-modified PLGA nanoparticles	Pre-B cell lymphoma	miR-155	Replacement	Systemic	[105]
Natural polymers					
Atelocollagen	Colorectal cancer; Lymphoma; Prostate;	miR-34a; miR-135b; miR-16	Replacement; Inhibition; Replacement	Intratumoral; intratumoral; systemic;	[106–108]
Exosomes					
exosome	Breast Cancer	let-7a	Replacement	Systemic	[109]
Inorganic materials					
Disialoganglioside-targeting silica nanoparticles	Neuroblastoma	miR-34a	Replacement	Systemic	[110]

Material	Disease model	Targeted miRNA	Therapeutic Approaches	Route	Refs
RGD modified ultrasmall magnetic nanoparticles	Breast cancer	miR-10b	Inhibition	Systemic	[111]

Table 3

Barriers and solutions to miRNA delivery (Adapted from Scholz and Wagner [154]).

Barrier/Step to Administration	Solutions
administration	local administration i.v. administration\
degradation and elimination	local administration condensation to carrier control of • particle size • particle shape • particle charge • steric stabilization chemical modification
disease accumulation	local administration targeting particle size
cellular entry	targeting ligands non-specific interactions cell penetrating moieties
endosomal/lysosomal	endosomal escape • lytic lipids • fusogenic peptides • fusogenic polymers • osmotic lysis endosomal arrest/lysosomal inhibition MVB targeting
intracellular localization	MVB/P-body targeting for RISC activity