Chapter 16
Therapeutic Potentials of Noncoding RNAs: Targeted Delivery of ncRNAs in Cancer Cells

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Abstract Knowledge of multiple actions of short noncoding RNAs (ncRNAs) has truly allowed for viewing DNA, RNA, and protein in novel ways. The ncRNAs are an attractive new class of therapeutics, especially against undruggable targets for the treatment of cancer and other diseases. Despite the potential of ncRNAs in cancer therapy, many challenges remain, including rapid degradation and clearance, poor cellular uptake, off-target effects, and immunogenicity. Rational design, chemical modifications, and delivery carriers offer significant opportunities to overcome these challenges. In this chapter, the development of ncRNAs as cancer therapeutics from early stages to clinical trials and strategies for ncRNA-targeted delivery to cancer cells will be introduced.

Keywords RNA interference • Small interfering RNA (siRNA) • MicroRNA (miRNA) • Cancer therapeutics • Delivery system

16.1 Introduction

Cancer is a genetic disease resulting from the dysregulation of the gene networks that maintain normal cellular identity, growth, and differentiation. A key development in unraveling the complex genetics of cancer may be the shift in focus from exclusively investigating the protein-coding components of the genome to considering the role of variation in regulatory elements [1]. Cancer in particular has been a major focus of noncoding RNA, especially microRNA research over the past decade, and many studies have demonstrated the importance of microRNAs in

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E. Song (ed.), *The Long and Short Non-coding RNAs in Cancer Biology*, Advances in Experimental Medicine and Biology 927,
DOI 10.1007/978-981-10-1498-7_16

cancer biology through controlling the expression of their target mRNAs to facilitate tumor growth, invasion, angiogenesis, and immune evasion. Additionally, tumor microRNA profiles can define relevant subtypes, patient survival, and treatment response [2].

MicroRNA dysregulation in cancer was first reported in 2002 when a cluster of two microRNAs (miR-15 and miR-16) was identified at 13q14.3, a region frequently deleted in chronic lymphocytic leukemia (CLL) [3]. This miRNA deletion was correlated with higher expression of the antiapoptotic target B-cell lymphoma 2 (BCL2). MicroRNAs have since been documented in roles in all of the cancer hallmarks including sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis, reprogramming of energy metabolism, and evading immune destruction [4].

MicroRNA is dysregulated through the following mechanisms: genetic alterations, epigenetic mechanisms, miRNA suppression by oncogenic transcription factors (such as Myc and KRAS), and miRNA downregulation by loss of tumor-suppressor transcription factors. MicroRNA-155 overexpression is associated with many cancer types including hematopoietic cancers, breast, lung, and colon cancer [5]. Overexpression of miR-155 is implicated in facilitating tumor cell growth and invasion and has attracted considerable interest as a putative therapeutic target [6]. MicroRNA-21 was the first miRNA to be coined an oncomiR due to the rather universal overexpression of this miRNA in cancer [7]. Studies in miR-21 knockout mice have demonstrated reduced lung tumor burden following activation of a mutant KrasG12D allele, and, in accordance, a miR-21 transgene resulted in increased tumor outgrowth [8]. MicroRNA-34a is a tumor-suppressor microRNA downstream of p53. Its replacement in cancer cells antagonizes key hallmarks including self-renewal, migratory potential, and chemoresistance [9].

Along with miRNA, another type of small regulatory ncRNA known as exogenous small interfering RNA is also involved in gene regulation and genome defense and shares components of the cellular pathways of RNA interference (RNAi). Small interfering RNAs (siRNAs) are 20–28-nt-long RNA molecules that can specifically cleave mRNA through a cytoplasmic pathway known as RNA interference (RNAi). Due to its special advantages such as unique specificity, unlimited range of targets, and high efficiency, siRNA has emerged as a powerful tool for cancer therapeutic gene silencing since its initial discovery in 1998 [10]. Due to the special mechanism of siRNA, it has four advantages as a potential cancer therapeutic strategy compared with traditional chemotherapy. The first is its high degree of safety. siRNA acts on the posttranslational stage of gene expression. Thus, it does not interact with DNA and avoids the mutation and teratogenicity risks of gene therapy. The second advantage of using siRNA is its high efficacy. In a single cancer cell, siRNA can cause dramatic suppression of gene expression with just several copies. Compared to other small molecule drugs or antibody-based drugs, the greatest advantages of siRNA are the unrestricted choice of targets and specificity determined by the principle of complementary base pairing. This strategy also benefits from rapid devel-

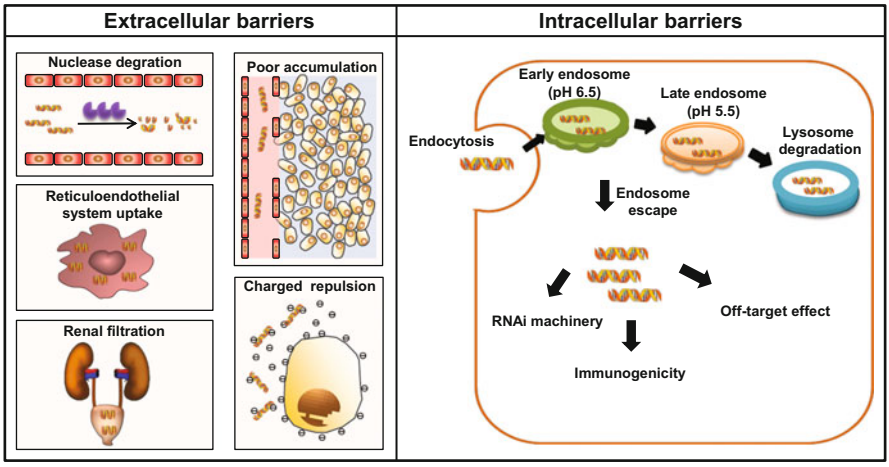

Fig. 16.1 Barriers encountered by ncRNAs following systemic administration

opments in molecular biology and whole-genome sequencing. In addition, comprehensive nucleotide sequence databases have been established, including human genomic databases, cDNA databases, and disease gene databases, which have laid a solid foundation for siRNA drug development. The basic strategy of a siRNA drug is to treat cancer by silencing the specific cancer-promoting gene with a rationally designed siRNA.

The miRNA-based therapeutics could include anti-miRNA antisense oligodeoxyribonucleotide (also known as antagomirs), and miRNA replacement therapy with synthetic miRNA or miRNA mimics [11]. Along with synthetic siRNAs, ncRNA-based therapeutics are usually short nucleotides (~20 nt), and there are multiple challenges for ncRNA-based therapeutics in vivo (see Fig. 16.1), such as off-target effects, delivery barriers, and immunogenicity [12].

MicroRNAs bind and block translation of their target mRNAs having partial complementary sites typically located in the 3′-UTR, which may cause some off-target effects associated with miRNA-based therapeutics [13]. Studies have shown that siRNAs may also silence an unknown number of unintended genes. There are two mechanisms suggested to explain this off-target effect. First, siRNAs can tolerate several mismatches at the mRNA target and retain their ability to silence targets with imperfect complementarity [14]. The second mechanism involves the promiscuous entry of siRNAs into endogenous miRNA machinery [15]. MicroRNAs recognize targets with perfect complementarity to their "seed regions" composed of 2–8 nucleotides. Complementarity of the remaining nucleotides has less importance for recognition. Because siRNAs are very nearly identical to the related class of miRNAs, they can recognize mRNAs with their seed region and lead to degradation of an unpredictable number of mRNAs in a miRNA-like manner.

RNAi is a mechanism involved in the innate immune response to protect cells from invasion by nucleic acids of pathogens such as viruses and bacteria. Several studies have demonstrated that ncRNAs themselves can activate innate immunity by inducing interferon expression, even at low concentrations [16]. Protein kinase R (PKR) and Toll-like receptor (TLR) 3 signaling pathways may be involved in sequence-independent immune activation by siRNAs. Some sequence motifs, such as 5′-UGUGU-3′ [17] or 5′-GUCCUUCAA-3′ [18], secondary structures, and uridine content of the sequence have been identified as important factors for immune activation by these pathways. However, the exact rules of sequence-dependent immune activation are not yet known. Hence, potential therapeutic siRNAs must be tested for an immune response prior to clinical applications.

The systemic delivery and in vivo application of ncRNAs are further hampered by many additional anatomical and physiological defensive barriers presented by the human body, which must be overcome for ncRNAs to reach their sites of action. The ncRNAs are easily filtered from the glomerulus and rapidly excreted from the kidney [19]. Together with rapid excretion kinetics, the susceptibility to degradation by nucleases is a major problem leading to the short half-life (15 min to 1 h) of ncRNAs in plasma, potentially limiting the use of noncoding RNA drugs administrated by intravenous injection [20]. In addition to circulating nucleases and renal clearance, another major barrier to effective in vivo delivery of noncoding RNA drugs is the clearance by the reticuloendothelial system (RES) [21]. The RES is composed of phagocytic cells, including circulating monocytes and tissue macrophages, whose physiological function is to clear the body of foreign pathogens, remove cellular debris that is generated during tissue remodeling, and clear cells that have undergone apoptosis. Phagocytic cells of the RES, particularly the abundant Kupffer cells in the liver and splenic macrophages, also detect and phagocytose noncoding RNAs, as well as nanoparticle carriers that may be used to enhance their delivery. The unfavorable physicochemical properties such as negative charge, large molecule weight, and size complicate passive diffusion of noncoding RNAs through the cell membrane, which makes their cellular uptake to be one of the major hurdles [22]. Once the noncoding RNA drugs are transferred into the tumor cells, the intracellular release which is always associated with endosomal escape is the crucial challenge of efficient gene delivery [23]. The intracellular trafficking of ncRNA delivered by different reagents generally begins in early endosomes. These early endosomes subsequently fuse with sorting endosomes, which in turn transfer their contents into late endosomes. The endosomal compartments of cells are significantly acidic (pH 5.0 ~ 6.2), while the cytosol or intracellular space is neutral. Endosomes are then relocated to lysosomes, which are further acidified (pH ~4.5) and contain various nucleases that promote the degradation of ncRNA. The intracellular stability is another barrier for ncRNA therapeutics.

16.2 Preclinical and Clinical Development of ncRNA-Based Therapeutics

16.2.1 Preclinical Development of ncRNA-Based Therapeutics

To overcome the abovementioned challenges, many efforts have made in preclinical studies of ncRNA-based therapeutics. The rational design enables improvement of efficacy, specificity, and off-target profiles of siRNAs. The backbone length, secondary structures, and nucleotide sequences of siRNAs have effects on these properties, and several rules have been formulated for the rational design of siRNAs: 2-nt overhangs at each 3′-end (typically UU or TT) are important for recognition of siRNAs by the RNAi machinery [24]; the GC content of the sequence determines the thermodynamic stability of siRNAs and should ideally be between 30 and 70 % [25]; the target sequences are generally chosen 75–100 bases downstream of the start codon to avoid nucleotide sequences occupied by regulatory or translational proteins and exon–exon junctions [26]; and inclusion or exclusion of specific nucleotides at particular positions (e.g., A/U at positions 10 and 19, a G/C at position 1) is also considered important for the specificity and efficacy of designed siRNAs [27].

Chemical modifications at the sequence or structural level can help alleviate major obstacles for therapeutic use of siRNAs [28]. A variety of chemical modifications of siRNA have been developed to improve the nuclease stability of siRNAs: modification of the 2′-position of the ribose (such as 2′-*O*-methyl, 2′-OMe) can decrease susceptibility of internucleotide phosphate linkages to nuclease cleavage and increase the stability of the duplex [29]; 2′-fluoro (2′-F) modifications are known to increase nuclease resistance without causing a significant compromise in efficiency [30]; modification with locked nucleic acids (LNAs) is another strategy to increase stability and nuclease resistance [31]; and another alternative strategy to increase stability while retaining potency is the substitution of DNA bases into siRNAs [32]. The replacement of the guide-strand seed region by deoxynucleotides, placing a single 2′-OMe residue at position +2 of the guide strand, selective placement of LNA residues, and modification of the 5′-phosphate group were commonly used chemical modifications to reduce off-target effects [33]. siRNA-induced immune activation can be limited by the replacement of uridines with their 2′-F-, 2′-deoxy-, or 2′-OMe-modified counterparts. The 2′-OMe-modified siRNAs inhibit production of TNF-alpha induced by their unmodified immunostimulatory counterparts even at very low concentrations. To minimize stability issues and reduce off-target effects, these chemical modifications are likely to be transferrable to miRNAs due to their similar structures.

Rational design strategies and chemical modifications have substantially improved some of the problems involved with ncRNA-based therapeutics. However, poor cellular uptake remains an important issue that requires the use of carriers to facilitate ncRNA uptake into the cells. Viral vectors have the advantages in terms of gene transfer efficiency as a result of optimized receptor-mediated internalization,

efficient cytosolic release, directed and fast intracellular transport toward target compartments, and immediate disassembly [34]. However, the need for long circulation in the blood and the accumulation in the target site in addition to safety concerns including carcinogenesis, immunogenicity, and broad tissue tropism limit the application of viral vectors [35] and have motivated the exploration of nonviral vectors such as nanocarriers. Nanocarriers are small particles (ranging from 1 to 300 nm) that can carry and deliver drugs, oligonucleotides, peptides, or desired cargos to target tissues. Various nanocarriers have been used for ncRNA delivery in biomedical applications. Based on surface charge, size and hydrophobicity, they have unique tissue biodistribution, toxicity, and tumor cell uptake profiles [36]. The nanomaterials used in the fabrication process, such as natural or synthetic lipids (e.g., liposomes, micelles) and polymers (e.g., chitosan, polylactic-co-glycolic acid, polylactic acid, polyethylenimine), determine the attributes of the resulting carrier [37]. Recently, it has been reported that direct conjugation of small drug molecules, aptamers, lipids, peptides, proteins, or polymers to ncRNA can improve the in vivo pharmacokinetic behavior of ncRNAs. Such ncRNA bioconjugates, either with or without forming nanocomplexes with cationic carriers, can significantly enhance biological half-life with a concomitant increase of delivery efficiency to the target tissue while maintaining sufficient gene-silencing activity [38].

16.2.2 Clinical Development of ncRNA-Based Therapeutics

After validation using in vivo models, siRNA-based therapies were introduced into clinical trials. Since the discovery of RNAi, there have been more than 50 clinical trials involving 26 different siRNAs. Although many of the earlier studies have not reached the clinical stage due to safety concerns and poor efficacy, ncRNA-based therapeutics are still being pursued (see Table 16.1).

TKM-PLK1 (solid lipid-based stable nucleic acid lipid particles, SNALP-carried siRNA), targeting polo-kinase-1, was tested in solid tumors with liver involvement by Tekmira Pharmaceuticals. The drug was well tolerated in phase I of the trial. Currently, two distinct phase II trials are recruiting participants to determine the safety and efficacy in hepatocellular carcinoma or neuroendocrine tumors and adrenocortical carcinoma. Alnylam Pharmaceuticals develops ALN-VSP02, with two distinct siRNAs targeting kinesin spindle protein (KSP) and VEGF, in a partnership with Tekmira for the use of SNALP as carrier. In phase I, ALN-VSP02 was well tolerated, and an anti-VEGF effect was observed in patients with advanced solid tumors with liver involvement. An extension study was then initiated in patients who responded to therapy in phase I, in order to collect long-term safety data. The siRNA siG12D, which targeted mutant KRAS (KRASG12D), was designed by Silenseed Ltd. for pancreatic ductal adenocarcinoma [39]. siG12D was encapsulated in a biodegradable polymer local drug eluter (LODER) for controlled and prolonged delivery. A phase II study to assess the efficacy of siG12D LODER in combination with gemcitabine or FOLFIRINOX chemotherapy was announced in

Table 16.1 ncRNA-based drug in clinical trials

Drug	Target	Delivery system	Disease	Phase	Status	Company	ClinicalTrials.gov identifier
Bevasiranib	VEGF	Naked siRNA	Macular degeneration	I	Completed	Opko Health, Inc.	NCT00306904
				II	Completed		NCT00306904
				III	Terminated		NCT00499590
AGN211745	VEGFR1	Naked siRNA	Age-related macular degeneration, choroidal neovascularization	I/II	Completed	Allergan	NCT00363714
SYL040012	β2-AR	Naked siRNA	Glaucoma, ocular hypertension	I	Completed	Sylentis, S.A.	NCT00990743
				I/II	Completed		NCT01227291
ALN-RSV01	RSV-N gene	Naked siRNA	RSV infections	IIb	Completed	Alnylam Pharmaceuticals	NCT01065935
DCR-MYC	Myc	LNP	Hepatocellular carcinoma	Ib/II	Recruiting	Dicerna Pharmaceuticals	NCT02314052
siRNA-EphA2-DOPC	EphA2	LNP	Advanced cancers	I	Recruiting	MD Anderson Cancer Center	NCT01591356
Atu027	PKN3	Cationic lipoplex	Pancreatic ductal carcinoma	Ib/IIa	Active	Silence Therapeutics GmbH	NCT01808638
PRO-040201	ApoB	SNALP	Hypercholesterolemia	I	Terminated	Tekmira Pharmaceuticals	NCT00927459
TKM-080301	PLK1	SNALP	Solid tumors with liver involvement	I	Completed	National Cancer Institute	NCT01437007
				I/II	Completed		NCT01262235
			Neuroendocrine tumors			Tekmira Pharmaceuticals	
			Adrenocortical carcinoma				
			Hepatocellular carcinoma				
				IIa	Recruiting		NCT02191878

(continued)

Table 16.1 (continued)

Drug	Target	Delivery system	Disease	Phase	Status	Company	ClinicalTrials.gov identifier
CALAA-01	RRM2	Cyclodextrin	Solid tumor	I	Terminated	Calando Pharmaceuticals	NCT00689065
siG12D LODER	KRAS G12D	LODER polymer	Pancreatic ductal adenocarcinoma, pancreatic cancer	I	Completed	Silenseed Ltd.	NCT01188785
				II	Not yet recruiting		NCT01676259
APN401	E3 ubiquitin ligase Cbl-b	Mononuclear cells	Melanoma, pancreatic cancer, renal cell cancer	I	Recruiting	Wake Forest University	NCT02166255
ALN-TTR02	TTR	LNP	TTR-mediated amyloidosis	III	Recruiting	Alnylam Pharmaceuticals	NCT01960348
ALN-TTRSC	TTR	siRNA–GalNAc	TTR-mediated FAC	III	Recruiting	Alnylam Pharmaceuticals	NCT02319005
ALN-VSP02	KSP and VEGF	LNP	Solid tumors	I	Completed	Alnylam Pharmaceuticals	NCT01158079
MRX34	miR-34a	LNP	Liver cancer	I	Recruiting	Mirna Therapeutics, Inc.	NCT01829971
Miravirsen	miR-122	LNA	HCV	II	Completed	Santaris Pharma A/S	NCT01200420
			HCV with no response to PEGylated interferon alpha	II	Active		NCT01727934

Abbreviation

VEGF vascular endothelial growth factor, *VEGFR 1* vascular endothelial growth factor 1, *β2-AR* β2 adrenergic receptor, *RSV-N* respiratory syncytial virus nucleocapsid gene, *EphA2* ephrin type-A receptor 2, *ApoB* apolipoprotein B, *LNP* lipid nanoparticle, *PLK1* polo-kinase-1, *LODER* local drug eluter, *SNALP* stable nucleic acid lipid particle, *RRM2* M2 subunit of ribonucleotide reductase, *KRASG12D* K-ras G12D mutant, *FAC* familial amyloidotic cardiomyopathy, *KSP* kinesin spindle protein, *TTR* transthyretin, *HCV* hepatitis C virus

patients with unresectable, locally advanced pancreatic cancer. Silence Therapeutics designed AtuPLEX, which was a cationic lipoplex with negatively charged nucleic acids. Atu027, a siRNA targeting protein kinase N3 (PKN3) carried in AtuPLEX, was shown to cause stabilization or regression of disease with no dose-dependent toxicities in patients with advanced solid tumors. A phase Ib/IIa trial is currently being conducted to evaluate the safety and activity of Atu027 in combination with standard gemcitabine treatment in patients with advanced or metastatic pancreatic adenocarcinoma [40]. Dicerna Pharmaceuticals announced two distinct trials in 2014 for DCR-MYC, an LNP carrying siRNA against MYC for hepatocellular carcinoma and solid tumors, multiple myeloma, or non-Hodgkin lymphoma. Lastly, a phase I clinical trial is underway with siRNA-EphA2-DOPC in patients with ovarian cancer (OC) at the MD Anderson Cancer Center.

As to miRNA-based therapeutics, there are two drugs in clinical trials that may shed light on the clinical application. Miravirsen (or SPC3649) is an LNA-modified oligonucleotide designed to inhibit miR-122 developed by Danish firm Santaris Pharma [41]. Miravirsen has gone through two phase I clinical trials, successfully demonstrating that the drug is safe even in humans (NCT00688012, NCT00979927) and one phase IIa clinical trial (NCT01200420). This phase IIa trial enrolled 38 patients with treatment-naïve chronic HCV infection to monitor safety, tolerability, pharmacokinetics, and efficacy on HCV viral titer. Multiple dosage of miravirsen administered subcutaneously to patients gave promising outcomes with a mean reduction of HCV RNA levels by two to three logarithmic levels. Further, almost half of the patients treated by the highest dose displayed undetectable levels of HCV RNA within 4 weeks. As for miRNA replacement therapy, MRX34, a miR-34a mimic compound, will probably be the first miRNA replacement compound to reach clinical stages [42]. miR-34a represents one of the most documented tumor suppression-associated miRNAs, being a transcriptional product of the transcription factor and genome guardian p53. Mirna Therapeutics has developed custom nanoparticle liposomes to increase stability, enhance delivery, and prevent immune response effects, and the upcoming clinical trial in phase I is recruiting patients with non-respectable primary liver cancer or metastatic cancer such as melanoma with liver involvement.

ncRNAs serve as therapeutic drugs for cancer treatment, while effective strategies for short ncRNA delivery into cancer cells in vivo are being extensively explored. The recent strategies to deliver ncRNAs as therapeutic molecules for cancer treatment will be introduced in the following section (see Table 16.2).

16.3 Conjugate Delivery Systems

One strategy for improving the function of ncRNAs in vivo is to make dramatic changes to conjugate the ncRNAs to small molecules or peptides which are designed to increase binding to proteins or cellular uptake. The concept behind ncRNA conjugates is simple. One part of the conjugate is siRNA or miRNA, which provides

Table 16.2 Current strategies for ncRNA in vivo delivery

Delivery system	Target gene	Indications	Route	Ref.
Cholesterol conjugate	ApoB	Hypercholesterolemia	Intravenous injection	[43, 44]
Cholesterol conjugate	Let-7a	Hepatocellular carcinoma	Intravenous injection	[45]
R-tocopherol conjugate	ApoB	Hypercholesterolemia	Intravenous injection	[46]
PTD-DRBD conjugate	EGFR and Akt20	Glioblastoma	Intracerebral injection	[48]
RVG-9R peptide conjugate	FvE	Viral encephalitis	Intravenous injection	[97]
TRA conjugate	Luciferase	Brain	Intravenous injection	[51]
scFvCD7-9R complex	CCR5	HIV	Intravenous injection	[52]
F105-P complex	c-Myc/MDM2/VEGF	Melanoma	Intravenous injection	[53]
F5-P complex	PLK1	Breast cancer	Intravenous injection	[54]
A10 aptamer–siRNA chimera	PLK1	Prostate cancer	Intratumoral injection	[59]
A10 aptamer–siRNA chimera	PLK1	Prostate cancer	Intraperitoneal injection	[60]
GL21.T aptamer–let-7 g chimera	Let-7 g	Lung adenocarcinoma	Intravenous injection	[64]
Neutral liposome	EphA2	Ovarian cancer	Intraperitoneal injection	[69]
Neutral liposome	PAR-1	Melanoma	Intravenous injection	[70]
Cationic liposome	TNFα	Sepsis	Intraperitoneal injection	[71]
Cationic liposome	MCL-1	Non-small cell lung cancer	Intravenous injection	[72]
Cationic liposome	c-Myc/MDM2/VEGF	Melanoma	Intravenous injection	[74]
SNALP	HBsAg	Hepatitis B virus	Intravenous injection	[81]
SNALP	ApoB	Hypercholesterolemia	Intravenous injection	[82]

(continued)

Table 16.2 (continued)

Delivery system	Target gene	Indications	Route	Ref.
SNALP	PLK1/KSP	Hepatocellular carcinoma	Intravenous injection	[83]
CDP	EWS-FLI1	Metastatic Ewing's sarcoma	Intravenous injection	[89]
CC9-PC	miR-34a	Pancreatic cancer model	Intravenous injection	[92]
LMW-PEI	HER-2	Ovarian carcinoma	Intravenous injection	[93]
PEI	miR145	Lung adenocarcinoma	Intravenous injection	[96]
PEI	miR-145	Glioblastomas	Intracranial injection	[97]
RVG–SSPEI	miR-124a	Brain	Intravenous injection	[99]
PLGA	ERK2	Infectious disease	Vaginal instillation	[103]
PEG-PLA	PLK1	Breast cancer	Intravenous injection	[104]
PEG-PLA	GATA2	Non-small cell lung cancer	Intravenous injection	[105]
PAMAM	TAT/REV	HIV	Intravenous injection	[110]
PEG-PAMAM	GFP	GFP-transgenic mouse model	Intramuscular injection	[113]
Amphiphilic PAMAM	Hsp27	Prostate cancer	Intravenous injection	[114]
PPI	EGFR	Glioblastoma	Convection-enhanced delivery	[116]

Abbreviation
ApoB apolipoprotein B, *PTD-DRBD* peptide transduction domains and double-stranded RNA-binding domain, *EGFR* epidermal growth factor receptor, *RVG-9R* rabies virus glycoprotein-conjugated oligo-9-arginine, *TRA* transferrin receptor antibody, *scFvCD7-9R* CD7-specific single-chain antibody-conjugated oligo-9-arginine, *CCR5* C-C chemokine receptor type 5, *F105-P* Fab antibody (F105) fragment directed against HIV-1 envelope fused to protamine, *MDM2* mouse double minute 2 homolog, *VEGF* vascular endothelial growth factor, *F5-P* Fab antibody (F5) fragment directed against Her2 fused to protamine, *A10 aptamer* aptamer against the extracellular domain of the prostate-specific membrane antigens, *GL21.T aptamer* anti-Axl receptor inhibitory aptamer, *PAR-1* protease-activated receptor-1, *TNFα* tumor necrosis factor alpha, *MCL1* myeloid cell leukemia 1 protein, *HBsAg* hepatitis B surface antigen, *SNALP* stable nucleic acid lipid particle, *CDP* cyclodextrin polymer, *CC9-PC* CC9 peptide-conjugated β-cyclodextrin-polyethylenimine, *LMW-PEI* low molecular weight polyethylenimine, *PLGA* poly(lactide-co-glycolide), *PEG-PLA* polyethylene glycol–polylactic acid, *PAMMA* polyamidoamine, *GATA2* GATA-binding protein 2, *GFP* green fluorescent protein, *PPI* poly(propylenimine)

specificity for the target mRNA sequence. The other part of the conjugate is a molecule optimized for improving biodistribution, cellular uptake, or other in vivo properties. A major strength of the approach is that the two portions of the conjugate can be developed as separate modules and then coupled to create hybrid molecules that combine the strengths of the two parts. A weakness is that the synthesis of novel conjugates is complicated by the need to couple a molecule to the ncRNAs, making a large and complex ncRNA even larger and more complex. The ncRNA bioconjugates could be lipophile–ncRNA conjugates, peptide–ncRNA conjugates, antibody–ncRNA conjugates, and aptamer–ncRNA conjugates.

16.3.1 Lipophile Conjugates

Cholesterol was covalently conjugated to siRNA for systemic delivery [43]. It was conjugated to the 3′-terminus of the sense strand of siRNA via a pyrrolidone linkage. The cholesterol-modified siRNAs could silence an endogenous gene encoding apolipoprotein B (ApoB) after intravenous injection in mice. The administration of chemically modified siRNAs resulted in silencing of the ApoB messenger RNA in the liver and jejunum, decreased plasma levels of ApoB protein, and reduced total cholesterol levels [43]. In addition to the chol–siRNA conjugate, a series of lipophilic siRNA conjugates, including siRNA conjugates with bile acids and lipids, were synthesized by Wolfrum et al. [44]. The degree of hydrophobicity, which directly related to the length of the alkyl chain, seemed to be a major determinant for the affinity of siRNA–fatty acid conjugates to lipoproteins. The siRNA conjugates with higher affinity to lipoproteins (i.e., the ones with longer fatty acid chains) showed enhanced gene-silencing capabilities, suggesting that lipoproteins may facilitate the cellular uptake of the conjugates. When systemically administered, chol–siRNA bound to HDL demonstrated fivefold higher cleavage of the target RNA transcript (ApoB) in mice, compared to unbound chol–siRNA at the same concentration. Liu et al. [45] have recently demonstrated antitumor efficacy of cholesterol-conjugated let-7a mimics (Chol-let-7a) in vitro and in vivo and verified for the first time that Chol-let-7a effectively carries let-7a to orthotopic tumors in the liver and successfully inhibits tumor growth in a preclinical model when delivered systemically. The results show that Chol-let-7a downregulates all three human Ras at transcriptional and translational levels and primarily functions in the cytoplasm, thus, suggesting that the use of cholesterol-conjugated miRNAs is a promising tool for HCC systemic therapy. Another lipophile–siRNA conjugate, R-tocopherol (vitamin E)-siRNA, was introduced for systemic siRNA delivery to the liver [46]. The α-tocopherol was covalently bound to the antisense strand of 27/29-mer siRNA at the 5′-end (Toc-siRNA). The 27/29-mer Toc-siRNA was designed to be cleaved by Dicer, producing a mature form of 21/21-mer siRNA after releasing α-tocopherol. Using this new vector, intravenous injection of 2 mg/kg of Toc-siRNA, targeting apolipoprotein B (ApoB), achieved efficient reduction of endogenous ApoB messenger RNA (mRNA) in the liver.

16.3.2 Peptide Conjugates

Cell-penetrating peptides (CPPs) are another conjugate materials used for siRNA transfection efficacy improvement. A well-known CPP is the TAT trans-activator protein from human immunodeficiency virus type 1 (HIV-1). TAT has been conjugated to the 3′-terminus of the antisense strand of a siRNA using a heterobifunctional cross-linker (HBFC), such as sulfosuccinimidyl-4-(p-maleimidophenyl) butyrate [47]. The extent of cellular uptake showed a direct relationship with the amount of conjugate used for the transfection and the time elapsed after transfection. An alternative peptide–siRNA complexation approach utilizes a recombinant fusion of the HIV Tat protein PTD with a double-stranded RNA-binding domain (DRBD) that binds to siRNA and neutralizes its negative charges. The PTD-DRBD peptide vector has shown excellent cellular delivery of siRNA into various primary and transformed cells. PTD-DRBD has been used to package two siRNAs simultaneously (against EGFR and Akt20) to induce tumor-specific apoptosis in a glioblastoma model after intracerebral injection and to also substantially increase mouse survival [48]. Stearylated peptide vectors have also been used successfully to deliver siRNA. A TP10-derived lipopeptide (PF6) was designed to aid endosomal release through the attachment of four pH titratable trifluoromethylquinoline moieties to a lysine side chain of TP10. It was shown to form nanoparticles with siRNA and knockdown HPRT1 mRNA production in a range of cell types as well as in the kidney, lung, and liver of mice upon tail vein infusion at 1 mg/kg [49]. Most of the anti-miR oligonucleotide types utilize 2′-OMe, LNA, or 2′-fluoro analogues usually as mixmers of more than one analogue type or with DNA. In vivo applications all utilize PS linkages. Some naked oligonucleotide analogues may have the ability not only to enter cells through endocytosis but also to efficiently block miRNA activity without the need for any enhancement of transfection by peptides [50].

16.3.3 Antibody Conjugates

The targeted delivery of different therapeutic ncRNA formulations to desired tissues/cells may be a prerequisite for the clinical use of the drugs. Antibody-mediated targeted drug delivery systems have attracted much attention due to their superior stability and high specificity. Xia et al. have delivered siRNAs to the brain in vivo with the combined use of a receptor-specific monoclonal antibody delivery system and avidin–biotin technology. The siRNA was mono-biotinylated on the terminus of the sense strand, in parallel with the production of a conjugate of the targeting MAb and streptavidin. Following the formation of intracranial tumors, the rats were treated with a single intravenous injection of 270 mg/kg of biotinylated siRNA attached to a transferrin receptor antibody via a biotin–streptavidin linker. The intravenous administration of the siRNA caused a 69–81 % decrease in luciferase gene expression in the intracranial brain cancer in vivo [51]. Kumar et al. have used a

CD7-specific single-chain antibody conjugated to oligo-9-arginine peptide (scFvCD7-9R) for T cell-specific siRNA delivery in NOD/SCIDIL2rγ$^{-/-}$ mice reconstituted with human lymphocytes (Hu–PBL) or CD34+ hematopoietic stem cells (Hu–HSC). In HIV-infected Hu–PBL mice, treatment with anti-CCR5 (viral co-receptor) and antiviral siRNAs complexed to scFvCD7-9R controlled viral replication and prevented the disease-associated CD4 T cell loss. This treatment also suppressed endogenous virus and restored CD4 T cell counts in mice reconstituted with HIV+ peripheral blood mononuclear cells. Moreover, scFvCD7-9R could deliver antiviral siRNAs to naive T cells in Hu–HSC mice and effectively suppress viremia in infected mice [52].

The delivery of siRNA by targeting the single-chain variable fragment (scFv) on the cell surface is rapid. scFv can accurately identify intracellular and extracellular antigens and achieve the precise positioning. Song et al. have designed a protamine–antibody fusion protein to deliver siRNA to HIV-infected or envelope-transfected cells. The fusion protein (F105-P) was designed with the protamine-coding sequence linked to the C-terminus of the heavy-chain Fab fragment of an HIV-1 envelope antibody. siRNAs bound to F105-P induced silencing only in cells expressing HIV-1 envelope. Additionally, siRNAs targeted against the HIV-1 capsid gene *gag* inhibited HIV replication in hard-to-transfect, HIV-infected primary T cells. Intratumoral or intravenous injection of F105-P-complexed siRNAs into mice targeted HIV envelope-expressing B16 melanoma cells, instead of normal tissue or envelope-negative B16 cells. Injection of F105-P with siRNAs targeting c-myc, MDM2, and VEGF inhibited envelope-expressing subcutaneous B16 tumors. Furthermore, an ErbB2 single-chain antibody fused with protamine delivered siRNAs specifically into ErbB2-expressing cancer cells [53]. Yao et al. have used a protamine peptide fused to a scFv that binds ERBB2 (F5-P) to specifically deliver a siRNA targeting PLK1 into ERBB2 (also known as HER2)-expressing breast cancer cells. F5-P-mediated delivery of PLK1 siRNAs effectively reduced PLK1 expression and proliferation and increased apoptosis of ERBB2+ breast cancer cell lines and primary breast cancer cells in vitro. F5-P was also capable of delivering PLK1 siRNAs to ERBB2^{+} cell lines or primary breast tumor cells grown as xenografts in nude mice. In these models, tail vein injection of PLK1 siRNAs in a complex with F5-P twice a week for 4 weeks significantly slowed tumor growth (followed for 7 weeks). Meanwhile, ERBB2^{-} tumors were insensitive to this treatment [54].

16.3.4 Aptamer Conjugates

Aptamers are short, structured, single-stranded RNA or DNA ligands that bind to target molecules with high specificity and affinity. Since their discovery in the 1980s, aptamers have been generated that target the extracellular domain of transmembrane receptors overexpressed in tumors, thus becoming (along with monoclonal antibodies) ideal tools for the specific recognition of cancer cell surfaces. Aptamers are generated from high-complexity pools through a combinatorial

process named systematic evolution of ligands by exponential enrichment (SELEX) to tightly bind to their proper targets [55]. The use of aptamers offers the possibility to overcome insertional mutagenesis and immunogenicity of viral vectors [56] and possible limited effectiveness and toxicity of nanoparticles, enabling the specific accumulation of ncRNAs in target tumor cells in a safe and effective manner.

Aptamers against the extracellular domain of the prostate-specific membrane antigens (PSMAs), A9 and A10 aptamers [57], have been extensively characterized for siRNA delivery by developing different approaches based either on noncovalent or covalent conjugation. Chu et al. [58] have developed a multivalent RNA aptamer–siRNA chimeric structure in which two biotinylated anti-PSMA aptamers (A9) are linked to two biotinylated anti-lamin A/C siRNAs using streptavidin as a connector. To enhance siRNA release after internalization, a reducible disulfide linker was designed between the sense strand of the siRNA and the biotin group. By using such a streptavidin connector, this RNA aptamer–streptavidin–siRNA conjugate was efficiently internalized by the PSMA-positive LNCaP cells and mediated a rapid inhibition of gene expression. McNamara et al. [59] have described the first-generation aptamer–siRNA chimera. An aptamer that specifically bound to PSMA was covalently linked to the passenger strands of siRNAs, followed by annealing of the guide strands of the siRNAs to the passenger strands to create a functional siRNA duplex. The chimeras mediated targeted silencing in prostate cancer cells expressing PSMA and efficiently promoted cell death. When the chimera was injected intratumorally, the tumor volume in a xenograft mouse model of prostate cancer was decreased. Subsequently, Dassie et al. [60] have optimized the aptamer–siRNA chimera for systemic administration, leading to second-generation chimeras. They reduced the aptamer portion of the chimera, designed a 2-nt (UU) overhang at the 3′-end of the siRNA duplex and swapped the passenger and guide strands of the siRNA. They also appended a PEG (MW = 20 kDa) onto the siRNA passenger. As a result of these modifications, the optimized second-generation, aptamer–siRNA chimeras displayed a clear regression of PSMA-expressing tumors in nude mice following intraperitoneal administration. Several other aptamers against cell surface proteins overexpressed on cancer cells have been used for siRNA delivery. For example, aptamers specific for Her-2-positive breast cancer cells were covalently conjugated to BCL-2 siRNA, generating a chimera able to sensitize cells to chemotherapy [61].

Given the progress in the design of aptamer-based strategies for siRNA delivery, the use of aptamers as delivery moieties for microRNAs has recently been explored. A second-generation aptamer against PSMA (A10-3.2) was conjugated to a polyamidoamine (PAMAM)-based microRNA (miR-15a and miR-16-1) using PEG as a spacer. The construct demonstrated selective delivery of the miRNA moiety into LNCaP (PSMA-positive) prostate cancer cells, inducing cell death in vitro [62]. Hao et al. used the same aptamer as recognition ligand in an atelocollagen (ATE)-based microRNA (miRNA, miR-15a and miR-16-1) vector to target prostate cancer bone metastasis. The anticancer efficacy of miRNA/ATE-APT was superior to that of other treatments in a human PCa bone metastasis mice model [63]. Esposito et al. have recently combined the anti-Axl receptor inhibitory aptamer named GL21.T

with the tumor-suppressor let-7g miRNA. The conjugate combined the miRNA activity with the aptamer function (Axl signaling inhibition), resulting in an effective inhibition of cell migration and survival in vitro and of tumor growth in vivo [64]. The selective delivery of anti-miRs to target cancer cells is still in its infancy; nevertheless, the development of aptamer-mediated approaches represents a concrete possibility to achieve this goal.

16.4 Lipid-Based Delivery Systems

Liposomes are one of the most commonly used transfection reagents in vitro. Usually, liposomes are formed in an aqueous environment, in which a lipid bilayer forms a sphere with an aqueous core. For example, one set of polar head groups can create the outer surface of the nanocomplex, while another set of polar head groups faces the interior hydrophilic core, which houses the nucleic acid payload [12]. Liposomes can be created using single or multiple types of synthetic or natural lipids, which allows for additional flexibility when optimizing the physical and chemical properties of the nanoparticle [65]. Almost 50 years after the discovery of liposomes, the US FDA has approved 13 liposome-based products for human use, and a large number of liposomal products are in different phases of clinical trials [66].

Liposomes offer several advantages as a ncRNA delivery system due to their ability to (1) prevent degradation of the payload, (2) accumulate preferentially in tumor tissues (passive targeting/delivery) and deliver high concentrations of the payload, (3) specifically target to tumor cells and the microenvironment with high-affinity ligands (active targeting), and (4) provide safe and effective systemic delivery platforms in animals and humans depending on the lipid content [67].

However, safe and efficacious delivery in vivo is rarely achieved due to toxicity, nonspecific uptake, and unwanted immune response [68]. 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. In recent years, a significant effort has been dedicated to modifying the composition and chemical structure of liposomes for pharmaceutical drug delivery. For robust and successful ncRNA delivery with lipid-based systems, optimization of lipid composition, drug-to-lipid ratio, particle size, charge, surface-targeting moieties, payload encapsulation efficiency, and the manufacturing process are required.

16.4.1 Cationic Liposomes

Cationic liposomes have been traditionally the most commonly used nonviral delivery systems for oligonucleotides, including plasmid DNA, antisense oligos, and ncRNAs. Cationic lipids, such as 1,2-dioleoyl-3-trimethylammonium-propane

(DOTAP) and *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethyl-ammonium methyl sulfate (DOTMA), can form complexes or lipoplexes with negatively charged siRNA or miRNA to form nanoparticles by electrostatic interaction, providing high in vitro transfection efficiency [69, 70]. Sorensen et al. have used cationic DOTAP liposomes to deliver siTNF-α, and the lethal reaction to LPS injection in a mouse model of sepsis is suppressed [71]. To maintain an overall positive surface charge for adsorption through the cell membrane and to reduce the possible clearance caused by positive charge, the N/P (nitrogen-to-phosphate) ratio usually ranges from 2 to 3. Pre-miR-133b contains DOTMA–cholesterol. In the previous study, 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. The mature miR-133b level in lungs following *i.v.* administration of pre-miR-133b-containing lipoplexes was approximately 52-fold higher than that in untreated mice [72]. Polycationic liposome–hyaluronic acid (LPH) nanoparticles have also been described by several investigators [73]. 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 approximately 20 % of the untreated control. When treated with scFv-LPH nanoparticles containing only combined siRNAs or miR-34a, the reduction was approximately 30 and 50 %, respectively, of the untreated control, suggesting that the effects were mediated 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 [74].

Cationic liposomes, while efficiently taking up and condensing ncRNAs, have had limited success for in vivo gene downregulation, perhaps because of their stable intracellular nature and resultant failure to release siRNA or miRNA contents [75]. In addition, toxicity of cationic lipids is the major issue following systemic administration preventing them from being a major candidate for ncRNA delivery. The use of cationic liposomes in in vivo mouse models elicits dose-dependent toxicity and pulmonary inflammation, hepatotoxicity, and a systemic interferon type I response, which is attributed in part to the activation of TRL4 [68]. Cationic lipids also activate the complement system and cause their rapid clearance by macrophages of the RES. It has been demonstrated that cationic lipids are highly toxic to macrophages and other immune cells (ED50 < 50 nm/L) [76]. Different lengths of hydrocarbon chains can also influence the cytotoxicity of cationic lipids [77]. Toxicity of cationic lipids is linked to induction of reactive oxygen species (ROS) and increased intracellular calcium levels. In addition, DOTAP-based particles accumulate near the vasculature and are preferentially taken up by the liver and spleen, limiting their effectiveness in systemic or antitumor therapy [69]. Overall, although cationic lipid-based delivery systems offer some advantages as an ncRNA

delivery system, potential toxicities need to be addressed before their translation in clinical trials. Careful selection of lipids and formulation strategies may help reduce the potential toxicities.

16.4.2 Neutral Liposomes

Because the surface charge of all biological membranes is negative, electronegative or neutral liposomes are more biocompatible than cationic liposomes and have superior pharmacokinetics in general. DOPC (1,2-dioleoylsn-glycero-3-phosphatidylcholine) is a kind of neutral lipid which has been used to improve the siRNA entrapment efficiency. In 2005, Landen et al. developed the oncoprotein EphA2 targeting DOPC-encapsulated siRNA liposomes, which was highly effective in reducing EphA2 expression 48 h after the administration of a single dose in an orthotopic model of ovarian carcinoma [69]. Currently, the EphA2 targeting DOPC-encapsulated siRNA liposome (siRNA-EphA2-DOPC) is in a phase I clinical trial initiated by the MD Anderson Cancer Center. As for miRNA, miR-34a and let-7 were delivered with a type of neutral liposome to treat non-small cell lung cancer (NSCLC). The treatment with miR-34a or let-7 significantly decreased the 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 [78]. These findings demonstrate the potential of developing ncRNA therapy formulations with neutral liposomes as novel therapies for lung cancer patients.

Coating liposomes with lipid-anchored PEG can reduce particle size, prevent aggregation during storage, increase circulatory half-life, and reduce uptake by the reticuloendothelial system (RES), such as red blood cells and macrophages [79]. However, using PEG is not always advantageous because the steric effect and charge effect of PEG block the interaction between the liposome and the endosomal membrane and prevent the liposome from escaping the endosome. Many studies have been performed to improve the efficacy of PEGylated nanoparticles, including rationally designed PEG length and density or incorporation of pH-sensitive bonds linking PEG to the liposome. How to achieve the best outcome with modulation of PEG length and density remains controversial. However, pH-sensitive modified PEG with ionic interactions, such as the HEMA–histidine–methacrylic acid-modified PEG liposome, has been shown to be effective. At neutral pH, the PEG copolymer has a net negative charge, whereas the liposomal core consisting of DOPE and cholesterol has a net positive charge. In the endosome, imidazole and methacrylic acid residues become protonated, and the net charge of the PEG becomes positive, which results in PEG release and positively charged liposomal membrane exposure, after which the liposome can fuse with the endosome and escape successfully [80].

16.4.3 Stable Nucleic Acid Lipid Particles (SNALPs)

To date, 12 clinically tested siRNA-based therapeutics have been administered by the *i.v.* route. All but one of these siRNAs has been carried by synthetic carriers, mostly SNALPs, which are a type of lipid nanoparticle that encapsulates siRNAs and delivers them to their target cells. SNALPs are microscopic particles approximately 120 nm in diameter. They have been used to deliver siRNAs therapeutically to mammals in vivo. In SNALPs, the siRNA is surrounded by a lipid bilayer containing a mixture of cationic and fusogenic lipids, coated with diffusible polyethylene glycol [81]. With enhanced permeability and retention due to prolonged circulation time in the blood, SNALPs are highly bioavailable, which leads to the accumulation of SNALPs at the sites of vascular leakage, especially at cancer growth sites. After accumulation, SNALPs are easily endocytosed by cancer cells and deliver the siRNAs into cells successfully. SNALPs have been used for the treatment of many diseases, including hepatitis B viral infection, dyslipidemia, and Ebola (Zaire) [12, 82]. Judge et al. described the preclinical development of chemically modified siRNAs targeting the essential cell-cycle proteins polo-like kinase 1 (PLK1) and kinesin spindle protein (KSP) in mice. The siRNAs formulated in stable nucleic acid lipid particles (SNALPs) displayed potent antitumor efficacy in both hepatic and subcutaneous tumor models. This was correlated with target gene silencing following a single intravenous administration that was sufficient to cause extensive mitotic disruption and tumor cell apoptosis. Their siRNA formulations induced no measurable immune response, minimizing the potential for nonspecific effects. Additionally, RNAi-specific mRNA cleavage products were found in tumor cells, and their presence correlated with the duration of target mRNA silencing. Histological biomarkers confirmed that RNAi-mediated gene silencing effectively inhibited the target's biological activity [83].

Another lipid-like delivery system is lipoid nanoparticles, which are comprised of cholesterol and PEG-modified lipids specific for siRNA delivery [84]. To improve SNALP-mediated delivery, a new class of lipid-like delivery molecules was described, termed lipidoids, as delivery agents for RNAi therapeutics [85]. Chemical methods were developed to allow the rapid synthesis of a large library of over 1200 structurally diverse lipidoids. From this library, they identified lipidoids that facilitated high levels of specific silencing of endogenous gene transcripts when formulated with either double-stranded small interfering RNA (siRNA) or single-stranded antisense 2-O-methyl (2′-OMe) oligoribonucleotides targeting microRNA (miRNA). The safety and efficacy of lipidoids were evaluated in three animal models: mice, rats, and nonhuman primates. One of the most potential lipidoid drugs was the lipidoid-based siRNA formulation $98N_{12}$-5, which led to a 75–90 % reduction in ApoB or FVII factor expression in hepatocytes in nonhuman primates and mice. In addition, mice injected intraperitoneally with thioglycollate as a sterile inflammation stimulus followed by injection of $98N_{12}$-5-formulated siCD45 showed a 65 % reduction of CD45 protein expression in the peritoneal macrophage population. In the end, the potential of $98N_{12}$-5 to facilitate the delivery of anti-miRs was

tested. The results demonstrated that $98N_{12}$-5-formulated anti-miR122 dosed at 5 mg/kg on three consecutive days in mice resulted in greater miR-122 repression than the cholesterol-conjugated version of the same oligoribonucleotide (antagomir122) dosed at 80 mg/kg on three consecutive days.

16.5 Polymer-Based Delivery Systems

Polymer-mediated delivery systems, usually called polymeric nanoparticles, are solid, biodegradable, colloidal systems which have been widely studied as drug vesicles. According to the material used, polymeric nanoparticles are classified into two major categories, natural polymers and synthetic polymers. Natural polymers for siRNA delivery include cyclodextrin and chitosan [86]. Of the synthetic polymers, polyethylenimine (PEI), poly(lactide-co-glycolide) (PLGA), and dendrimers have been intensively investigated [87].

16.5.1 Cyclodextrin

Cyclodextrins are natural polymers generated during the bacterial digestion of cellulose, and they possess defined geometric (~70 nm) and cationic structural characteristics that offer advantages for cationic siRNA and miRNA payloads to form inclusion complexes. Additionally, each cyclodextrin molecule may contain covalently bound polyethylene glycol (PEG), which acts to stabilize the nanoparticle and avoid nonspecific interaction with blood and extracellular elements under physiological conditions [88]. It was first introduced for the delivery of plasmid DNA in 1999 and later optimized for siRNA delivery. Less than a decade later, cyclodextrin polymer (CDP)-based nanoparticles were moved into clinical trials for siRNA delivery. The cyclodextrin-containing polycation system was developed for the targeted delivery of siRNA [89]. This system consists of a cyclodextrin-containing polymer, PEG for stability, and human transferrin as the targeting ligand for binding to transferrin receptors, which are often overexpressed on cancer cells. This targeted nanoparticle system, called CALLA-01, which targets the M2 subunit of ribonucleotide reductase (R2) to inhibit tumor growth was developed for the first siRNA phase I trial by Calando Pharmaceuticals (Pasadena, CA, USA) [90].

The β-cyclodextrin-PEI (PEI-CD) carrier was developed for delivery of the tumor-suppressor miR-34a mimic to pancreatic cancer cells. The PEI-CD nanoparticles were conjugated with CC9, a specific tumor-homing and tumor-penetrating bifunctional peptide via its CRGDK motif, which binds to neuropilin-1 (NRP-1) [91]. This delivery system could greatly upregulate the miR-34a level in the PANC-1 cell line and substantially inhibit the target gene expressions such as E2F3, Bcl-2, c-myc, and cyclin D1, inducing cell cycle arrest and apoptosis and suppressing migration. More importantly, the in vivo evaluation of the antitumor activity

indicated that the delivery of miR-34a significantly inhibited tumor growth and induced cancer cell apoptosis [92].

16.5.2 Polyethylenimine (PEI)

PEI, a commonly used cationic polymeric drug carrier with high transfection efficiency, has been widely investigated for siRNA and miRNA delivery. PEI's high charge density enables the formation of small and compact structures with nucleic acid delivery, facilitating endosomal escape via the proton sponge effect. The PEI–siRNA/miRNA complexes protect siRNA/miRNA from nuclease degradation, resulting in prolonged half-life. In addition, complete encapsulation of siRNA/miRNA prevents off-target effects such as immune activation by a Toll-like receptor-dependent mechanism. Polyethylenimine (PEI) has been used successfully for nucleic acid delivery under both in vitro and in vivo conditions. Urban–Klein et al. have showed that the noncovalent complexation of synthetic siRNAs with low molecular weight PEI efficiently stabilizes siRNAs and delivers siRNAs into cells where they display full bioactivity at completely nontoxic concentrations. More importantly, in a subcutaneous mouse tumor model, the systemic (intraperitoneal, *i.p.*) administration of complexed, but not of naked, siRNAs leads to the delivery of intact siRNAs into the tumors [93]. High molecular weight PEIs provide high transfection efficiency but also have high toxicity, while low molecular weight PEIs are more biocompatible and are much less efficient. A type of micelle-like nanoparticle (MNP) has been reported that is based on the combination of a covalent conjugate between a phospholipid and low molecular weight PEI (1.8 kDa) with PEG-stabilized liposomes as the outer layers [94]. MNPs have been shown to have the capacity for siRNA delivery and gene silencing with improved biocompatibility properties. The MNP delivery system was further utilized in silencing P-gp to overcome doxorubicin resistance in MCF-7 human breast cancer cells. The presence of P-gp on the surface of resistant cells decreased after treating cells with MNP-loaded siRNAs targeting MDR-1, which effectively inhibited the drug efflux activity [94]. PEI has also been used to construct ligand-targeted, sterically stabilized nanoparticles for systemic siRNA delivery. The PEGylated nanoparticles were conjugated with an Arg–Gly–Asp peptide ligand attached at the distal end of PEG to target integrin-expressing tumor neovasculature. The resulting nanoparticles, upon intravenous administration to tumor-bearing mice, successfully delivered siRNAs in a tumor-selective manner, inhibited vascular endothelial growth factor (VEGF) receptor-2 expression, inhibited tumor angiogenesis, and slowed tumor growth [95].

Polyethylenimine has also been utilized for the delivery of miRNAs. 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

miR-145 delivery combined with IR and cisplatin led to significant tumor growth inhibition [96]. When administered to orthotopic CSC-derived glioblastoma tumors, intracranially delivered PU–PEI-miR-145 significantly suppressed tumorigenesis. When used in combination with radiotherapy and temozolomide, synergistic effects and improved survival rates were achieved [97]. 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 obstruction of systemic drug or gene delivery to the brain parenchyma and central nervous system (CNS) [98]. 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 bonds (RVG–SSPEI) to deliver miR-124a, a neuron-specific miRNA that could potentially promote neurogenesis [99, 100]. To overcome the size limitation of PEI vector transport across the BBB, mannitol was used to permeabilize the BBB. After administration, a 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 as determined by tracking the Cy5.5-labeled miR-124a. However, the functional activities of miR-124a in 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 use of permeabilizing agents limits the utility. The combination of delivery strategies that improve the activity of the miRNA has great potential. However, the complexity of the systems at times can counterbalance the improvements [92].

16.5.3 Poly(lactide-co-glycolide) (PLGA)

Poly(D,L-lactide) (PLA) and poly(lactide-co-glycolide) (PLGA) have also demonstrated the potential for sustained nucleic acid delivery. The advantages of PLGA- or PLA-based siRNA delivery include high stability, facile cellular uptake by endocytosis, ability to target specific tissues or organs by adsorption or ligand binding, biodegradability, low toxicity, sustained release characteristics, and multiple surface modifications [101, 102]. In 2009, Saltzman and coworkers reported that PLGA nanoparticles could be densely loaded with siRNA in the presence of spermidine and, when applied topically to the vaginal mucosa, led to efficient and sustained gene silencing [103]. Yang et al. reported a cationic lipid-assisted polymeric nanoparticle system with a stealthy property for efficient siRNA encapsulation and delivery, which was fabricated with poly(ethylene glycol)-b-poly(D,L-lactide), siRNA, and a cationic lipid, using a double emulsion-solvent evaporation technique.

By incorporation of the cationic lipid, the encapsulation efficiency of siRNA into the nanoparticles was greater than 90%. Systemic delivery of specific siRNA by nanoparticles significantly inhibited luciferase expression in an orthotopic murine liver cancer model and suppressed tumor growth in an MDA-MB-435s murine xenograft model, suggesting its therapeutic promise in disease treatment [104]. Using the same cationic lipid-assisted polymeric nanoparticle system, GATA2 siRNA was delivered to non-small cell lung cancer (NSCLC) harboring oncogenic KRAS mutations and successfully inhibited tumor growth in a mouse model [105].

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 polymeric nanoparticles exhibited enhanced delivery efficiency and achieved therapeutic effects. The surface of the nanoparticles was modified with penetratin, a cell-penetrating peptide [6]. 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 the naked anti-miR dosage needed. There was a need, in this case, to protect the PLGA particle using steric stabilization (i.e., PEGylation) and also add a cell penetration enhancer. PLGA particles were typically nonspecifically 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. Important to the future development of miRNAs is the assertion by the authors that RNA degradation occurs in or around the endosomal and lysosomal compartments [106, 107]. If this proves true, much of the design criteria for miRNAs will be altered. The fact that Ago2 resides in the membrane of the endolysosomal compartment can explain the better performance of NP170-PFCE formulation [108]. 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.

16.5.4 Dendrimers

Dendrimers are synthetic, highly branched monodisperse and usually highly symmetric, spherical macromolecules with three-dimensional nanometric structures. The unique structural features such as tunable structure and molecular size, large number of accessible terminal functional groups, and ability to encapsulate cargos add to their potential as drug carriers [109]. Polycationic dendrimers such as poly(amidoamine) (PAMAM) and poly(propylenimine) (PPI) dendrimers have been studied for siRNA delivery in recent years. PAMAM dendrimers have become the most used dendrimer-based carriers for gene delivery because of the ease of synthesis and commercial availability. Rossi JJ's group has reported on the generation 5 (G5) dendrimer for functional delivery of siRNAs that inhibit HIV infection

and replication by targeting HIV genes *tat* and *rev* and host dependency factors CD4 and transportin-3 (TNPO3). The G5 dendrimer–siRNA complexes demonstrated effective inhibition of HIV-1 replication in T lymphocytes in vitro and in a humanized mouse model [110]. However, PAMAMs were demonstrated to be cytotoxic, predominately related to apoptosis mediated by mitochondrial dysfunction [111]. Cytotoxicity could be reduced by various modifications without compromising gene silencing. Surface-modified and cationic PAMAM dendrimers showed very low cytotoxicity, even at high concentrations and efficiently penetrated cancer cells in vitro and in vivo [112–114]. PPI dendrimers were also used to formulate siRNA nanoparticles, and these nanoparticles showed efficient gene silencing [115]. Dendrimer-conjugated magnetofluorescent nanoworms (dendriworms) were developed to achieve siRNA delivery in a transgenic murine model of glioblastoma [116]. Dendriworms were well tolerated after 7 days of convection-enhanced delivery to the mouse brain, and in an EGFR-driven transgenic model of glioblastoma, anti-EGFR dendriworms led to specific and significant suppression of EGFR expression. For targeted delivery, dendrimers could be easily conjugated with one or multiple targeting ligands. For example, the 9-mer luteinizing hormone-releasing hormone (LHRH) peptide was conjugated to PAMAM dendrimers, whose internal amino group was quaternalized for siRNA loading [117]. The cellular uptake was observed to be dependent on the targeting peptide. Similarly, a 53-mer epidermal growth factor (EGF) peptide was grafted with generation 4 (G4) PAMAM dendrimers for siRNA delivery [118]. Few applications of miRNA delivery with dendrimers 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 [119]. Although not in vivo, this suggests that dendrimers may be amenable for in vivo miRNA delivery. Dendrimers are capable of binding miRNAs and aiding in the entry into cells. However, the entry is nonspecific in nature. This alternate mechanism of cell entry should not be overlooked due to the potential to protect miRNAs by avoiding the endosomal and lysosomal compartments [120].

16.6 Summary

In this chapter, the therapeutic potentials and delivery strategies of noncoding RNAs have been introduced. The main challenges facing ncRNA-based cancer therapeutics, including off-target effects, immunogenicity, and the most difficult delivery barriers, have been described in detail. Rational design, chemical modification, and nanoparticle delivery carriers offer significant opportunities to overcome these challenges. Multiple approaches to the delivery of ncRNAs as therapeutic molecules for cancer treatment and ncRNA-based clinical trials are systemically summarized. Improvements in delivery strategies hold great potential to make the translational process of ncRNA-based drugs faster and more effective for cancer clinical applications.

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