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MINI-REVIEW

Therapeutic potentials of short interfering RNAs

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Abstract Short interfering RNA (siRNA) is one of the members of the family of RNA interference (RNAi). Coupled with the RNA-induced silencing complex (RISC), siRNA is able to trigger the cleavage of target RNAs which serve as a defensive system against pathogens. Meanwhile, siRNA in gene silencing opens a new avenue for the treatment of various diseases. SiRNA can effectively inhibit viral infection and replication and suppress tumorigenesis and various inflammation-associated diseases and cardiovascular diseases by inactivation of viral genes and downregulation of oncogene expression. Recently, endogenous siRNAs (endo-siRNAs) were discovered in the reproductive cells of animals which may be associated with regulation of cell division. Structural modification of siRNA enhances the delivery, specificity and efficacy and bioavailability to the target cells. There are at least five categories of siRNA delivery systems including viral vectors, lipid-based nanoparticles, peptide-based nanoparticles, polymer-based nanoparticles and inorganic small molecules like metal ions, silica and carbon. Sufficient preclinical and clinical studies supported that siRNA may be a potential medicine for targeted therapy of various diseases in the near future.

Keywords siRNA · Drug conjugation · Site-specific delivery · Targeted therapy

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Introduction

RNA interference (RNAi) molecules are a group of non-coding RNAs that are involved in different regulatory systems. The first discovered RNAi, lin-4, came from research on *Caenorhabditis elegans* in 1993. RNAi is composed of several categories of RNA, such as microRNA (miRNA), short interfering RNA (siRNA) and PiwiRNA (PiRNA) (He and Hannon 2004; Meister 2011b). In 1993, various molecular biology research teams discovered its powerful potential in molecular diagnosis and targeted therapy. Numerous findings have since been published in various international journals. Timmons reviewed and proposed that there are short RNAs and long RNAs found in a plant species *Nicotiana benthamiana* with different regulatory mechanisms in gene silencing (Timmons 2002). Khvorova, Reynolds and Jayasena, through the thermostability of RNAi synthesis, discovered that the RNA-inducing silencing complex (RISC) is responsible for cleaving double-stranded siRNA (dssiRNA) or miRNA (dsmiRNA) into a single-stranded siRNA (sssiRNA) or miRNA (ssmiRNA) (Khvorova et al. 2003). These two ssRNAs are bound to RISC and form an ssRNA-RISC complex. The ssRNA serves as an effective RNAi component to capture target RNA. He, Hannon, Carthew and Sontheimer summarized the previous findings and presented the biosynthesis and molecular mechanism of gene silencing of miRNA and siRNA (Carthew and Sontheimer 2009; He and Hannon 2004) (Fig. 1).

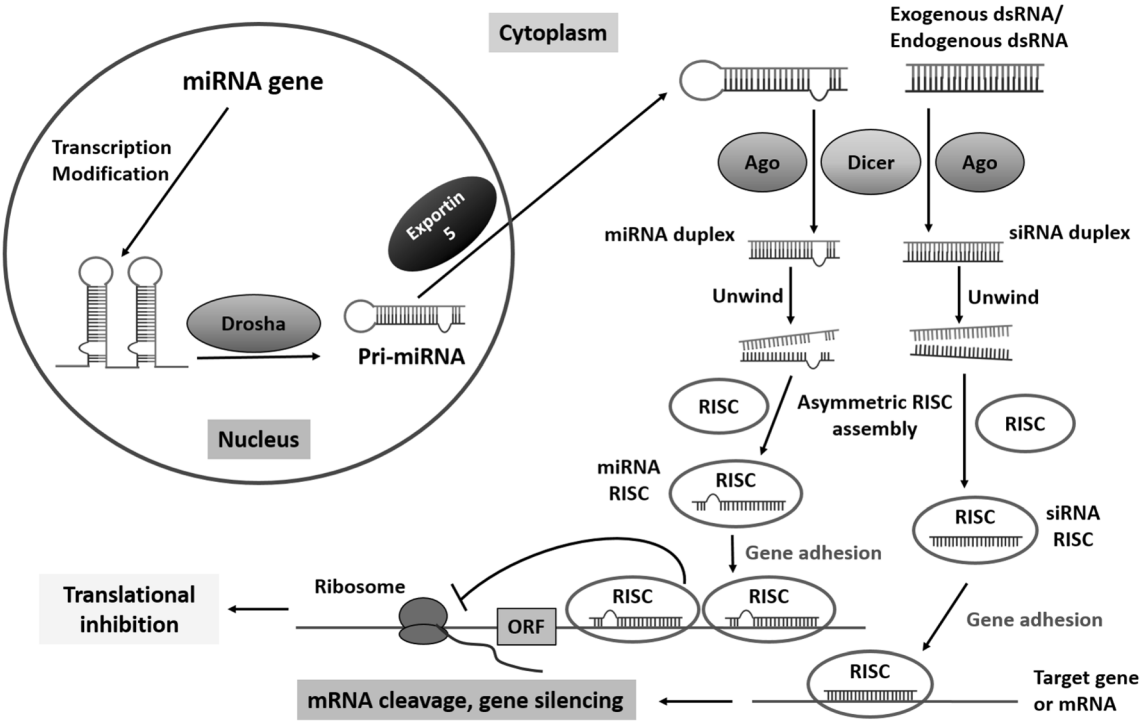

Fig. 1 Biosynthetic pathway of miRNA and siRNA. Majority of miRNA precursors are generated from the nucleus while siRNA could come from both exogenous and endogenous sources. Packaging of functional RNA unit is similar which includes sequential reactions driven by Dicer, argonaute (Ago) and RNA-induced silencing complex (RISC). *ORF* open reading frame

MiRNA is a group of RNAi transcribed by RNA polymerase II and modified by Drosha in the nucleus of the cell. It cooperates with RISC target mRNAs or other alien RNAs for gene suppression and blockage of the further transcription or serves as a cleavage machine for RNA degradation (He and Hannon 2004; Meister 2011b). Multiple research and biotechnological modifications to miRNA therapeutic applications have been developed. Some miRNAs have proceeded to testing for use in diagnostic tools and disease treatment under the animal trial tests (Oom et al. 2014).

SiRNA is generally described as an exogenous RNAi. Long and complementary double-stranded RNAs (dsRNAs) are imported from the outer environment into the cytoplasm of a cell. Ago protein binds to Dicer (RNase III) and generates a complex. Ago protein guides Dicer to find siRNAs. Dicer cleaves dsRNA into the siRNA duplex with 20 to 25 nucleotides. The siRNA duplex has a two-nucleotide overhang at both 3′ ends. The duplex unwinds, and a strand with the less thermostable 5′ end enters Ago protein. Dicer, Ago and single-stranded siDNA assemble and form short interfering RNA-induced silencing complex (siRISC). SiRISC uses siRNA fragments as a detector to identify and bind target RNAs. The target RNA is cleaved by Ago protein into fragments. Through the RNA decay pathway, 5′ to 3′ exoribonuclease Xrn1 and 3′ to 5′ exosome degrade RNA fragments into nucleotides (Carthew and Sontheimer 2009; He and Hannon 2004; Meister 2011a; Meister 2011b). SiRNAs have been developed for drug testing for targeted therapy against viruses, tumours and other gene-dependent diseases (Aigner 2006; Tiemann and Rossi 2009).

Biological functions and applications

Reproductive regulation

Early discovery of siRNA indicated that siRNA was from the external environment. The latest reviews suggested that ancient siRNA could be incorporated into the host genome and be generated by activation (Claycomb 2014). Stein et al. recently conducted an experiment on the function of endogenous siRNA (endo-siRNA) in oogenesis in mice. In a knock-in mouse model of Ago2 (Ago2ADH), miRNA was suppressed while endo-siRNA expression remained at a high level. Endo-siRNA was discovered with an important regulatory function in oocyte synthesis. The study suggested that endo-siRNA may be associated with meiosis I in oocyte division. Inhibition of endo-siRNAs causes disruption of meiotic maturation. The consequence of meiotic disruption inhibits spindle synthesis and chromosome alignment in oogenesis and could be one of the reasons for female infertility (Stein et al. 2015).

Spermatogenesis in mammals is known to be controlled by miRNA and piRNA while the role of siRNA remains unclear.

In 2011, it was discovered that endo-siRNA is essential for male germ cell development. The experiment demonstrated that endo-siRNA-T40 targeted Kif17 mRNA and endo-siRNA-T19 targeted Spata1 and Syt11 (Song et al. 2011). Mouse models with inactivated Dicer1 or Dgcr8 male germ cells exhibit a low expression of siRNA. Reduction of endo-siRNAs could trigger meiotic progression and spermatocyte apoptosis and inhibit spermatozoal maturation. The phenotype of the experimental mice included reduced size of the testes and suppression of mature spermatozoa resulting in male infertility. Coupled with miRNA, the physiological effect on infertility would be more serious (He et al. 2009; Zimmermann et al. 2014). A recent review by Hilz et al. suggested that siRNA could be associated with chromatin dynamic regulation. It could also play a role in recovering from a double-strand break (DSB) of DNA during the leptotene/zygotene stage in spermatogenesis (Hilz et al. 2016).

Testicular endo-siRNAs target for post-transcriptional modification. The majority of siRNAs are mRNA regulators (92%) while the transcriptional product of pseudogenes (about 3%), retrotransposons (about 1%) and other non-coding RNAs (~4%) are also the target of siRNA in spermatogenesis. On the other hand, endo-siRNAs of oogenesis suggest an association of retrotransposons and transcripts for protein synthesis (Yadav and Kotaja 2014). SiRNA may be critical in AGO2-dependent oocyte maturation. Some scientists suggested that siRNA could regulate retrotransposon activities in oogenesis while further verification is required (Hilz et al. 2016). It is possible to introduce control factors that trigger the generation of endo-siRNA. By remodelling of siRNA, it can be used for a future treatment plan for RNAi-induced infertility. However, verifications in aspects of basic sciences like target specificity, efficacy and biosafety should be determined before it is applied for development into therapeutics.

Combating viral infection

Influenza A virus is one of the negative sense ssRNA viruses. Due to the high frequency of mutations and reassortment of influenza A virus, traditional target therapies are not sufficient to control its dispersion. siRNA could control viral infection, gene replication and assembly and packaging of viral proteins and genetic materials like the genes of non-structural nucleoprotein (NP), polymerases (PA and PB) and matrix protein (M1 and M2) (Bennink and Palmore 2004; Ge et al. 2003). M2 protein is important for viral replication and viral component as it is one of the regulators of coding genes. Iwatsuki-Horimoto et al. suggested that M2 protein is essential for viral assembly and replication (Iwatsuki-Horimoto et al. 2006). Alvarado-Facundo et al. illustrated that M2 contributes to hemagglutinin fusion in H1N1 influenza virus (Alvarado-Facundo et al. 2015). An experiment testing the action of M2 gene-specific siRNA (siM2) against M2 demonstrated that siM2 attenuates M2 gene expression in H1N1 and H5N1 influenza A viruses and the inactivation is displayed in a long-term manner (Sui et al. 2009).

Wu et al., Zhou et al. and Zhang et al. discovered that siRNA suppresses the gene expression of NS1 and PA and PB as well as NP in H5N1 avian influenza A virus. Those genes are essential for viral gene replication and viral protein assembly (Wu et al. 2008; Zhang et al. 2009; Zhou et al. 2008). Hale et al. (2008) found that NS1 protein may target interferon (IFN) synthesis. Further studies illustrated that siRNA such as 5′-triphosphate-siRNA (p-mNP1496-siRNA) triggers the pathway of IFN lambda-1 response (IFN-j1/IL-29) against viral components and activates the expression of retinoic acid-inducible gene I protein (RIG-I) by restoring ubiquitination of RIG-I. Activated RIG-1 may cooperate with interferon gamma-inducible protein 16 (IFI16) to promote the pathway of stimulation of interferon genes-tank-binding kinase 1-interferon regulatory factor (STING-TBK1-IRF3) for non-specific immune response against viral infection and replication in the host (Lin et al. 2012; Rajsbaum et al. 2012; Sui et al. 2014).

Besides influenza viruses, siRNAs have potential for treatment to combat chronic hepatitis B virus (HBV) and respiratory syncytial virus (RSV) infections. Dane particles produced by viral gene could be transported from hepatocytes to the blood stream. It is found that the delivery of these particles is associated with coagulation factor VII (F7). It demonstrated that cholesterol-conjugated siRNA (chol-siRNA) functionally affects the dispersion of HBV after infection by a polymer-based dynamic polyconjugate (DPC) technique. *N*-Acetylgalactosamine-conjugated melittin-like peptide (NAG-MLP) was introduced together with chol-siRNA targeting F7. Reduction of F7 helped to control the viral transmission to the blood stream (Woodell et al. 2013). In addition, 5′-triphosphate-siRNA could trigger the synthesis of RIG-I-dependent type I interferon that may be useful to suppress HBV replication (Chen et al. 2013). ALN-RSV01 siRNA was effective for inhibition of respiratory syncytial virus (RSV) replication and infection in vitro and in vivo. A large human clinical trial test revealed its anti-viral action. No evidence showed that severe side effects were generated after ALN-RSV01 consumption (DeVincenzo et al. 2008).

Cancer therapy

The molecular basis of cancer therapy usually targets on the inhibition of anti-apoptotic factors, activation of apoptotic factors and suppression of cancer cell proliferation and metastasis. Recent findings support that siRNAs play roles in this area. Mammalian target of rapamycin complex 1 (mTORC1) is a protein kinase with high expression rate in most types of cancers. Its function is to regulate the rate of cell proliferation and autophagy. mTORC1 is driven by S6 phosphorylation. By

genome-wide siRNA screening, it was discovered that siRNA inhibits gene expression of S6 phosphorylation and suppresses the production of mTORC1 for cancer progression (Papageorgiou et al. 2015). Ribophorin II (RPN2) siRNA targeting RPN2 (Fujita et al. 2015b) and EZH2 siRNA targeting EZH2 (Zhou et al. 2015) could be functional for the treatment of non-small cell lung cancer and gastric cancer respectively. SiRNA enhances apoptosis and inhibits proliferation in liver cancer by targeting NAE inhibitor MLN4924 and cleaving NOXA mRNA which codes for proapoptotic protein (Chen et al. 2015). Mizutani et al. suggested that siRNA inhibits sphingosine kinase 2 (SPHK2) expression that indirectly reduces cell proliferation in colon cancer by blocking the activities of SPHK2 for promotion of cell growth and survival. FLICE (FADD-like IL-1β-converting enzyme) inhibitory protein (c-FLIP) is an analogue of caspase-8 and caspase-10 that suppresses programmed cell death (Mizutani et al. 2015). SiRNA inhibits c-FLIP and reveals TRAIL-induced apoptosis of oestrogenic sarcoma (Zhang et al. 2015).

In combined schemes, siRNA targeting CD44 signalling and TrkA tyrosine kinase suppression in conjunction with a targeted therapeutic drug lestaurtinib inhibits tumour metastasis (Aubert et al. 2015). siRNAs, combined with multiple miRNAs or chemotherapeutic drugs, also effectively suppress Kirsten rat sarcoma (Kras) viral oncogene homologue and MAPK signalling pathway and thus enhance apoptosis of lung adenocarcinoma (Xue et al. 2014).

Other findings and applications

Spleen tyrosine kinase (Syk) is a protein found in leukocytes of the immune system. Syk is able to bind to the high-affinity IgE (FcεRI) receptor complex located on mast cells, basophils and eosinophils and activates IgE-associated inflammation by phosphorylation of an inflammatory complex. Scientists later discovered a Syk siRNA which inhibits the production of Syk and prevents the further signalling transduction for allergy-associated inflammation (Sanderson et al. 2010).

Carbohydrate sulfotransferase 15 (CHST15) triggers the synthesis of chondroitin sulphate E (CS-E). However, CS-E promotes formation of proinflammatory cytokines which causes chronic heart failure (CHF). SiRNA suppresses CHST15 expression and reduces cardiac fibrosis and hypertrophy in mice (Watanabe et al. 2015).

For years, siRNAs were found from exogenous sources. There was a new discovery that endogenous siRNAs (endo-siRNAs) found in *Drosophila*, mouse, *C. elegans* and *Arabidopsis* associated with gene regulatory pathways direct Ago proteins to the RISC precursor and form a functional protein (Claycomb 2014). A group of trans-acting siRNAs (ta-siRNA) could be used for gene labelling. It was suggested that 12 ta-siRNAs target DRAXIN and ATCAY genes in human brain cells (Liu et al. 2015). It could be a future target for endogenous gene regulation.

Development of delivery systems

SiRNA structural enhancement and delivery innovation

Preclinical analysis for siRNA to be used as therapeutics is a primary stage of new drug development. Success of an in vitro test does not guarantee the same achievement in vivo. Also, tests that have been verified in animal models can fail in human trials. Therefore, studies regarding siRNA modification and innovation are necessary for further improvement. In consideration of drug development, efficacy is the first important criterion. Bioavailability of active drugs to the target location is also critical for drug tests especially for the in vivo milieu. Therefore, scientists started to develop a variety of siRNA nanoparticles and siRNA-conjugate molecules for delivery. SiRNA delivery innovation focuses on three main strategies: enhancing cellular uptake, preventing degradation by endosomes and lysosomes before arrival at the target site and reducing immune responses, especially for innate immunity (Lee et al. 2016).

Target specificity is an important point for a new-generation treatment. Original siRNA is generated in the external environment, and some are synthesized in the cells. The generated siRNA serves as a ready-to-use molecule coupled with the RISC complex that directs target RNA for degradation. However, it is difficult to directly deliver siRNA by injection or oral consumption because naked siRNA may be easily degraded by nucleosomes. Therefore, delivery of siRNA to the target site is one of the challenges. Recently, scientists found new pathways for drug delivery. The method is to encapsulate siRNA into a liposome or a nanoparticle. The lipid coat or nanoparticle protects the drug, enabling it to travel along the blood circulation until siRNA enters the target cells. Further modification could be achieved by addition of pilot agents onto the surface of the particles and guide the particles with siRNA to the target site. Another possible agent for drug delivery is a modified virus like retrovirus. The modified virus usually contains the target drug while the genes for viral development are eliminated. However, one of the challenges to deliver siRNA through a virus is to select a suitable viral transporter for a specific cell type without promoting a severe immune response (Tiemann and Rossi 2009; Videira et al. 2014).

Structural modification is another important study for pharmacological application. It is an integrated science subject in biochemistry, biotechnology and molecular biology, material sciences. Structural modification aims to increase bioavailability of the drugs or functioning compounds in the host and enhance the effectiveness and specificity by active site

remodelling and regulate transportation targeting the specific location (Yan et al. 2012). Goldberg, in the study on targeted therapy of ovarian cancer, described several models for enhancing the delivery of siRNA. The first model type is encapsulation of siRNA: stable nucleic acid particle (SNALP) and cyclodextrin polymer. SNALP is an artificial lipid-bilayer micelle that consists of cationic lipids, neutral lipids and cholesterol as a core and short polyethylene glycol (PEG) molecules attached on the outer membrane. The intermembrane space and outer membrane are both hydrophilic. SiRNAs are stored inside the SNALP. Cyclodextrin is a polymer of polysaccharides which contain a hydrophilic porin-like pocket inside the cyclodextrin for storage of siRNA. The second type is attachment of large linear lipid molecules: cholesterol-siRNA conjugate, various lipidoids (such as 98N12·5(1) and C12·200), polyethylenimine, etc. The molecules in this group are usually linked with PEG and lipidoids additionally attached to cholesterol. As a result, siRNA can be protected by these highly hydrophobic molecules free from nuclease degradation or other inhibitory agents. In advanced development, a multifunctional delivery complex is generated which has considered the strength of the cell penetrating ability. The macromolecule is composed of LyP-1 on the outer layer (a cyclic compound for tumour cell penetration), multiple polycationic and amphipathic cell penetration peptides (CPP) linked with LyP-1, N-myristoylation as a core linked with CPP and cargo siRNA that is functional for enhancing hydrophobic interactions and increasing affinity to the cell membrane of the target (Goldberg 2013). Guo, Evans and O'Driscoll in their review of targeted therapy in prostate cancer (Guo et al. 2013) and Fujita, Kuwano and Ochiya in their review of targeted therapy in lung cancer (Fujita et al. 2015a) have also presented the efficiency of the new delivery systems by structural modification. Examples of siRNA carriers are listed in Table 1.

Viral carriers for siRNA delivery

Since the discovery of attenuated virus vaccine, modification of the viral protein coat has been studied for many years. Scientists tried to use some viral carriers because they can bring drug components and penetrate the target cell membrane. From the beginning of the development of siRNA delivery, investigators have tried to develop a viral coated siRNA system by attenuated lentivirus or bacteriophages. First, the lentivirus and bacteriophages are attenuated by removing disease-associated genes. Genes for lentiviral coat are modified as a vector. For lentivirus A, lentivirus vector ligates with siRNA sequence and forms a small plasmid and is then loaded into a mouse by intravenous injection. For siRNA-bacteriophage technology, siRNAs may either be conjugated with phage protein or can be stored in the phage coat. The phages are usually modified and ligated with target cell surface protein for cell-specific recognition (Bedi et al. 2011; Karimi et al. 2016; Xiong et al. 2016).

Viral particle is one of the early developed drug transporters in molecular medicine. However, due to the potential hazards of viral proteins and in view of the difficulty encountered in viral manipulation, there were many siRNA delivery studies redirected to non-viral systems. It has been suggested that these complex delivery molecules release active siRNAs inside the cells by adjustment of the cellular environment or protein activity.

Lipid-based nanoparticles for siRNA delivery

The lipid-based delivery system (LBDS) is the technique that packages cell-like liposomes with long chain and complex lipids of different physical appearances. For example, in a stable nucleic acid-lipid particle (SNALP), cationic lipid 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA), 1,2-distear-oyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol are assembled to form a double-bilayer liposome. High-density polyethylene glycol (PEG) is a cross-layer lipid to prevent phagocytosis. Target siRNAs are stored inside the SNALP. LBDs have been developed for decades, and some projects have entered phase I to phase II clinical trials for targeting various cancers, hypercholesterolemia, virus infection and amyloidosis (Ho et al. 2016).

Zatsepin, Kotelevtsev and Koteliansky reviewed that LBDS for siRNA delivery includes three major groups of complex lipids: cationic lipids and lipidoids such as DLin-MC3-DMA and C12-200 lipidoid, phospholipids such as dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and DSPC, and PEG lipids like 3-*N*-[(ω-methoxypoly(ethyleneglycol)2000)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA) and 2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (mPEG-DSPE). Those siRNAs should have minor structural rearrangements to replace one of the oxygens of the phosphate group with sulphur in each siRNA ribonucleotide in order to link up the siRNA sequence and the lipid backbone. Lipid-siRNA nanoparticles have been used for clinical trials from phase I to phase III against various diseases like viral infection, tumourigenesis, lipid accumulation diseases and type 2 diabetes (Zatsepin et al. 2016).

Peptide-based nanoparticle for siRNA delivery

Peptide-based nanoparticle is one of the mature delivery techniques for siRNA. Typical peptides used in siRNA-peptide conjugation are short peptides with considerations of specificity to target cell surface ligands, cell penetration ability and prevention of degradation by endosomal/lysosomal activity. Peptide-based nanoparticles usually are resistant to immune responses and stable until they reach the target cells. Some

Table 1 Examples for the design of siRNA delivery carriers

Type of carrier	Base of nanoparticle packaging	Characteristics	Reference
Viral coat	Lentivirus vector	Cell penetrating ability	Xiong et al. 2016
Viral coat	Fusion proteins with bacteriophage	Cell penetrating ability, surface protein recognition	Bedi et al. 2011; Karimi et al. 2016
Lipid nanoparticle	DLinDMA-DSPC-cholesterol double bilayers with PEG on surface	Cell endocytosis entry, prevention of phagocytosis	Ho et al. 2016
Lipid nanoparticle	Lipidoids for core packaging: DLin-MC3-DMA, C12-200	Cell endocytosis entry, breakdown protection	Zatsepin et al. 2016
Lipid nanoparticle	Modified phospholipids for core packaging: DOPC, DOTAP	Cell endocytosis entry, breakdown protection	Zatsepin et al. 2016
Lipid nanoparticle	PEG-lipid complex for packaging: PEG-C-DMA, mPEG-DSPE	Cell endocytosis entry, prevention of phagocytosis	Zatsepin et al. 2016
Peptide nanoparticle	Hydrophilic CPPs: Tat, arginine oligomer	Cell penetrating ability	Lee et al. 2016; Tai and Gao 2016
Peptide nanoparticle	Amphiphilic CPPs: bipartite peptides such as MPGΔNLS, MPG-8, MPGα, BPrPp, Pep-1 α-helical peptides such as penetratin, transportan, CADY and KALA	Cell penetrating ability, intracellular environment-dependent proteolysis for drug release	Lee et al. 2016; Tai and Gao 2016
Peptide nanoparticle	Ligand targeting CPP-homing peptide: A1-Tat, RVG-R9	Surface protein recognition	Lee et al. 2016; Tai and Gao 2016
Peptide nanoparticle	Oligo glutamate peptide end-non-polar peptide-oligo arginine peptide end	Special degradation by intracellular enzymes like MMP-2	Lee et al. 2016; Tai and Gao 2016
Peptide nanoparticle	Conformational changes: HA2, INF7, E5, E5WYG, C6M1, GALA, EB1, Endo-Porter	Packaging difference for endosome disruption	Lee et al. 2016; Tai and Gao 2016
Peptide nanoparticle	Chemical structural changes: melittin	Packaging difference for endosome disruption	Lee et al. 2016; Tai and Gao 2016
Peptide nanoparticle	Proton-buffering peptide: H3K8b and PepFect 6	Control of cellular proton to inhibit endosome events	Tai and Gao 2016
Peptide nanoparticle	Biologically modified aptamers: αvβ3 integrin, PSMA, HIV-1 envelope protein (gp120), transferrin receptor-specific FB4	Cell penetrating ability, surface protein recognition	Lee et al. 2016
Peptide nanoparticle	Antibody conjugates (ligand binding): CD20-dependent mAb: ibritumomab, tositumomab, ofatumumab, obinutuzumab CD30-dependent mAb: brentuximab CD3- and CD19-bi-specific antibody: blinatumomab CD33-dependent mAb: gemtuzumab CD52-dependent mAb: alemtuzumab	Surface protein recognition	Tushir-Singh 2016
Peptide nanoparticle	Small functional antibody conjugates: Fab-protamine, scFv-dR9, mAb-R9	Surface protein recognition, cell penetration ability	Tai and Gao 2016
Polymer nanoparticle	Polymer core with siRNA core linker: PEI complex core with potential linkers	pH- and enzyme-dependent breakdown for drug release, surface protein recognition	Kim et al. 2016a, b

Table 1 (continued)

Type of carrier	Base of nanoparticle packaging	Characteristics	Reference
	Potential linkers include 4-methoxybenzyl acetate, hydrazone, β-thiopropionate, phosphoramidate, orthoester, citraconic amide		
Polymer nanoparticle	PEI polymer core with ε-caprolactone	Cells dissolved for siRNA release but being resistant in the blood stream	Espana-Serrano and Chougule 2016
Polymer nanoparticle	PEG-peptide core	MMP cleavage for siRNA release	Kim et al. 2016a, b
Polymer nanoparticle	PE4K-A13e0.33C6/PE4K-A13e0.33-C10	Surface protein recognition	Nguyen et al. 2012
Polymer nanoparticle	Branched PEI with HAI peptide	Surface protein recognition	Takanashi et al. 2015
Polymer nanoparticle	PAMAM-PEG-b-P(PrMA-co-MAA) polyion complex micelle	pH-dependent breakdown for drug release	Ho et al. 2016
Polymer nanoparticle	Cyclodextrin polymers by AD-PEG and Tf-AD-PEG condensation	Cells dissolve siRNA release but bring resistance in the blood stream	Ho et al. 2016
Small-molecule nanoparticle	*N*-Acetylgalactosamine	Surface protein recognition	Ho et al. 2016
Small-molecule nanoparticle	Gadolinium(III) tetramer-gold nanocarrier with other small inorganic particles	Cells dissolve for siRNA release but bring resistance in the blood stream	Ho et al. 2016
Small-molecule nanoparticle	(Au0)25-G5NH2-(PEG-RGD)10-mPE-G10/ (Au0)25-PEI-(PEG-RGD)10-mPE-G10	NP can pass through the blood-brain barrier to the brain	Kong et al. 2016; Kong et al. 2017
Small-molecule nanoparticle	Branched PEI-mesoporous silica with particles of stimuli activation for siRNA release	Cells dissolve for siRNA release but bring resistance in the blood stream	Prabhakar et al. 2016
Special complex	Therapeutic siRNA-conjugate hydrogel: PEI-CCA/DEX-HEMA hydrogel	Prolong controlled release of drugs	Krebs et al. 2009; Nguyen et al. 2013

advanced peptides may even be sensitive to pH changes in the intracellular microenvironment or can be digested by cellular enzymes for siRNA release (Tai and Gao 2016).

Categorized by peptide function, siRNA-conjugated peptides include cell-penetrating peptides (CPPs) and endosome disruption peptides (EDPs). CPPs usually are small peptides with a polar amino acid sequence which can be recognized by cell surface proteins. There are four groups of CPPs, namely, hydrophilic CPPs, amphiphilic CPPs, ligand targeting CPPs and enzyme-activated CPPs. Hydrophilic CPPs include Tat and arginine oligomers which are small peptides with less than 20 amino acids. Amphiphilic CPPs are, as the name implies, peptides with both a polar peptide head and a non-polar peptide head. Amphiphilic CPPs can be subdivided into bipartite peptides like MPGΔNLS, MPG-8, MPGα, BPrPp and Pep-1 and helical peptides like penetratin, transportan, CADY and KALA. Ligand-targeting CPP is an arginine-enriched peptide that can recognize cell surface receptors which include antibody conjugates and homing peptides like A1-Tat and RVG-R9. Enzyme-activated CPPs include an acidic oligo glutamate end and basic oligo arginine with a non-polar central amino acid sequence which is recognized by cellular enzymes like matrix metalloproteinase-2 (Lee et al. 2016; Tai and Gao 2016).

EDPs focus on peptide conformational or chemical structural changes or proton buffering to keep a higher pH to avoid endosome clearance activity. Some peptides may even trigger endosomolysis by light. EDP peptides with conformational changes include HA2, INF7, E5, E5WYG, C6M1, GALA, EB1 and Endo-Porter. Melittin is one of the peptides with chemical structural change. H3K8b and PepFect 6 are functional to proton buffering. TatU1A-Alexa Fluor 546 is a typical peptide to promote photo-induced endosome breakdown. Some multifunctional peptides are peptides coated with DNA- or RNA-binding peptides that may direct siRNA to the target genes. There are some other functional peptides like lytic peptides (e.g. bacterial toxin and INF7) and simple cell-receptor binding peptides (e.g. RGD, LHRH, GRP and IGF1) which are under investigation (Lee et al. 2016; Tai and Gao 2016).

Aptamer is a complex peptide macromolecule which is composed of peptides as a core with modifications of helical structure and forming a stem-and-loop structure. Materials of aptamer peptides could be a short modified peptide or rearranged protein from functional proteins or viruses. DNA can also be a functional material in aptamer technology. αvβ3 integrin aptamer, prostate-specific membrane antigen (PSMA) aptamer, HIV-1 envelope protein (gp120) aptamer and transferrin receptor-specific FB4 aptamer are typical examples of chimeric aptamers from biologically synthetic sources. Nucleolin aptamer and mucin1 aptamer are examples of aptamers generated by DNA modification (Lee et al. 2016).

Antibody-siRNA conjugates form a special group of peptide conjugates. In an antibody-siRNA conjugate (Ab-siRNA), fragment antigen-binding (Fab) regions, variable regions of an antibody, serve as the target specific ligands for cell surface proteins. Fragment crystallisable (Fc) region, the constant region, is functional to ligate with target drugs, therapeutic siRNAs. When the recognition site of Fab regions is attached to the target receptors, it will trigger cell endocytosis and bring the target siRNAs to enter the cells. Regarding some antibody-siRNA conjugates in cancer treatment like leukaemia, the antibody itself may be one kind of therapeutic drug to promote antibody-dependent cell cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) along the tumour cells and siRNA is able to complete oncogene silencing. Tushir-Singh reviewed that there were at least 10 possible monoclonal antibodies available for target drug delivery. Rituximab targets cluster of differentiation 20 (CD20) on the cell-specific non-Hodgkin lymphoma (NHL) and follicular lymphoma (FL). Ibritumomab, tositumomab, ofatumumab and obinutuzumab target on CD20 in NHL and chronic lymphocytic leukaemia (CLL). Brentuximab can recognize CD30 in both Hodgkin lymphoma (HL) and systemic anaplastic large-cell lymphoma (sALCL). Nivolumab targets programmed cell death protein 1 (PD-1) in HL. Blinatumomab is a bi-specific antibody that targets CD3 and CD19 respectively in acute lymphoblastic leukaemia (ALL). Gemtuzumab targeting CD33 in acute myeloid leukaemia (AML) and alemtuzumab targeting CD52 in CLL and cutaneous T-cell lymphoma (CTCL) could be the potential carriers of siRNA while they are off-market because of safety concerns (Tushir-Singh 2016). Antibody conjugates may also be designed with cell penetrating ability by keeping small functional peptides. Typical examples include Fab-protamine, scFv-dR9 and mAb-R9 (Tai and Gao 2016). Antibody-siRNA conjugates can be considered as an alternative choice for therapeutic siRNA delivery.

Polymer-based nanoparticles for siRNA delivery

Polymer-based delivery system (PBDS) is another potential drug driver for siRNA. PBDS is derived from an idea similar to LBDS. Multiple complex organic compounds polymerize as a spherical micelle structure and stores therapeutic siRNAs inside the micelle. The designed molecules are usually sensitive to the changes of cellular environment so that the target drug will only be released inside the cells.

Kim et al. have prepared a brief summary of the ideal design of polymeric nanoparticles in cancer therapy. In the core centre, the environment should be hydrophobic to augment siRNA stability, high cell-specific recognition and a response to signalling protein attachment and pH changes, but resistant to endosome/lysosome activity. Hence, it was suggested that block copolymers with PEI and secondary or tertiary amines could be suitable for the core layer. In the intermediate layer, it could be a hydrophobic and endosome-

resistant, coated with glutathione, peptide in response to matrix metalloproteinase (MMP). The advanced vehicles were suggested to be dissociated by changes in the microenvironment of the cells like decrease in pH or in the presence of MMP and release of therapeutic siRNAs from the core linkers. There are six potential linkers including 4-methoxybenzyl acetate, hydrazone, β-thiopropionate, phosphoramidate, orthoester and citraconic amide for acidic pH-dependent dissociation. PEG peptide is one of the reasonable linker particles for MMP cleavage. The co-polymer complex with carbon chains, thiol groups and short peptides was suggested for this layer. The surface layer is usually coated with ligands and peptides with a layer of hydrophilic monomers that target the highly expressed receptors on the tumour cell surface (Kim et al. 2016b).

Polyamidoamine dendrimer (PAMAM) condensed with a polymer polyethylene glycol-b-polypropyl methacrylate-co-methacrylic acid (PEG-b-P(PrMA-co-MAA) with siRNA attachment can form large polymers called polyion complex micelles (PICM). It was suggested that PICM are dissolved inside the cells by changes of cellular pH while remaining stable throughout the blood circulation. Cyclodextrin polymer (CDP) is another polymer consisting of adamantine-PEG (AD-PEG) and cellular transferrin-AD-PEG (Tf-AD-PEG) which is a driver designed for anti-cancer siRNAs. This nanoparticle was suggested to be stable during travelling in the blood stream while it may dissolve in the cells. Transferrin will recognize the transferrin receptor on the cancers and increase uptake of the drug (Ho et al. 2016).

Small-molecule nanoparticles for siRNA delivery

Besides, there are some delivery agents with simpler structures compared to complex spherical nanoparticles LBDS, PBDS or peptide systems including carbohydrate derivative-modified long-chain compounds and inorganic metal ions or carbon molecules. *N*-Acetylgalactosamine (GalNAc) is one of the candidates that may be conjugated with siRNA via a small organic phosphate compound. The reason to choose GalNAc is that it can recognize the GalNAc receptor and bring the therapeutic siRNAs to target cells such as cancerous hepatocytes. Inorganic nanoparticles can be simple or complex conjugate structures. The mechanism of siRNA inorganic nanoparticles relies on the condensation of three key components i.e. therapeutic siRNAs, linker molecules to link up organic siRNA and inorganic particles, and the core inorganic particles. For example, in a siRNA-gadolinium(III) tetramer-gold (siRNA-Gd tetramer-Au) nanoparticle, a 13-nm-diameter gold particle with a nucleotide linker becomes a core molecule. siRNA is attached with multiple Gd tetramers and condenses with the linker of the gold particle. Current studies are focused on the following inorganic nanoparticles: gadolinium(III) tetramer-gold (Gd tetramer-Au), iron oxide, calcium phosphate (CaP) complex nanoparticle, mesoporous silica, carbon molecules with graphene nano carbon sheets or nano diamonds (Ho et al. 2016).

Small inorganic nanoparticles have great potential in drug development, especially design of some selective entrances like the blood-brain barrier. Kong et al. designed a gold nanoparticle-based delivery complex for therapeutic siRNA targeting brain cancer cells. The gold nanoparticle ((Au0)25-G5NH2-(PEG-RGD)10-mPEG10) was coated with carboxyl group condensed modified polyethylene glycol (mPEG-COOH) and carboxyl group condensed mPEG with arginine-glycine-aspartic (RGD, from the corresponding amino acid symbols) ligand (RGD-mPEG-COOH). RGD is the ligand for the RGD receptor on the cancer cells. Two sets of siRNAs were fixed on the mPEG-COOH molecules targeting cancer enhancer vascular endothelial growth factor (VEGF) and anti-apoptotic B-cell lymphoma 2 (Bcl-2) (Kong et al. 2016). They further designed an advanced gold nanoparticle entrapped with PEI (Au PENP, (Au0)25-PEI-(PEG-RGD)10-mPEG10) with Bcl-2 siRNA for anti-cancer drug. It was discovered that this modified PEI-gold nanoparticle could pass through the blood-brain barrier and enter glioblastoma resulting in around one-quarter reduction of Bcl-2 expression (Kong et al. 2017).

In addition, Prabhakar et al. developed a siRNA-PEI-mesoporous silica nanoparticle (siRNA-PEI-MSN) for delivery. Mesoporous silica nanoparticle is a nanoparticle with three components, namely, an MSN core, an attached branched PEI on the MSN surface and a disulphide linker molecule with a positive charge. siRNAs are adsorbed by disulphide linker while it can be released by activation of stimuli such as dithiothreitol and glutathione (Prabhakar et al. 2016).

siRNA-NP in practice: bioavailability and clinical significance

Kim et al. reviewed the half-lives of different siRNAs and siRNA nanoparticles in vivo for cancer therapy. Naked siRNAs were found to have an in vivo half-life of about 3 to 10 min. Lipid- or liposome-dependent siRNA delivery such as ALN-VSP02 and Atu027 could have a half-life within 2 h. Polymer-based nanoparticles such as PEG-poly{*N*-[*N*-(2-aminoethyl)-2-aminoethyl] aspartamide} (PEG-PAsp(DET)), PEG-NP CALAA-01, cationic lipofection complex and hyaluronic acid-coated silica NP had an in vivo half-life of as diverse as 10 min, around 30 min, 3 to 4 h and 18 h, respectively. The redox reaction-dependent polyion complex micelle was 20 min while those of two pH-dependent polymeric NPs were 1 and 5 h respectively. The half-life of multiple layers of polymers with ligand-bound NP was 4 min in the first phase but 27 h in the second phase. The half-lives of ionic particles like two kinds of gold NPs were about 30 and

1 min in the first phase and 5 h in the second phase (Kim et al. 2016b).

Barata, Sood and Hong also reviewed that one project of naked siRNA for brain cancer had undergone phase I test. Three lipid NP projects including Atu027 NP and ALN-VSP02 NP for solid tumours were in phase I clinical trial. Another three lipid NP projects including DCR-MYC for advanced cancers, TKM 080301 for neuroendocrine/adrenal cancer and Atu027for pancreatic carcinoma had proceeded to phase I to phase II clinical trial. A polymeric NP project for siGD12-LODER-NP in pancreatic cancer was in phase I to II test while the CALAA-1 NP project was terminated because of frequent fatigue and fever along patients. The majority of the lipid and polymer NPs might cause fatigue, nausea and vomiting, and some may even develop nanoparticle-induced inflammatory cytokine elevation (ICE). One death, one G3 thrombocytopenia case and G3 hypokalaemia incidents and 10% ICE were reported for ALN-VSP02 (Barata et al. 2016).

Other alternatives for siRNA delivery

Previous studies showed that the half-life of therapeutic siRNA in clinical trials was relatively short. In addition, a high dose of siRNA could cause some side effects to patients. Therefore, some researchers tried to develop siRNA drugs in a controlled release manner. Krebs, Jeon and Alsberg first introduced a model as chitosan or polyethylenimine (PEI)-condensed calcium cross linked alginate (CCA) hydrogels for siRNA delivery. The hydrogel was able to protect siRNA and gradually released siRNA to the target gene. Later, the same research team provided an improved hydrogel model which was constructed by photo-cross linked dextran hydroxyethyl methacrylate (DEX-HEMA) ligated with siRNA-linear PEI-glycidyl methacrylate (siRNA-LPEI-GMA). The DEX-HEMA hydrogel trended to gradually degrade and release therapeutic siRNA as long as 9 days or more. Hydrogel could be considered as a choice for a prolonged treatment scheme (Krebs et al. 2009; Nguyen et al. 2013). Wang and Burdick reviewed that sustained siRNA/siRNA-conjugate hydrogels have been applied for various preclinical therapeutic studies on cancer, inflammation, bone degeneration and cardiovascular diseases (Wang and Burdick 2017).

Road to therapeutics: update of research on siRNA-based therapeutic strategies

Aided by the innovation of advanced siRNA delivery systems, a plethora of studies on siRNA-based therapeutics has been developed in the past decade. There are some studies focused on naked siRNA therapy and viral NP-capped siRNA while the bulk of the studies targets on complex NP siRNA delivery systems. The following paragraphs illustrate siRNA in therapeutic applications.

Therapeutic siRNAs for heart disease

Atherosclerosis is one of the common cardiovascular diseases in an advanced society. It is a disease of blockage of aortic blood vessels caused by accumulation of lipid plaques. Current studies discovered that peritoneal macrophages and circulating monocytes, members of innate immunity, contribute to atherosclerosis through enhancing expression of sialic acid-binding Ig-like lectin 1 (Siglec-1). Xiong et al. developed a Siglec-1 siRNA shSiglec-1 with lentivirus vector as a carrier (Lv-shSiglec-1) for the treatment of atherosclerosis in an apolipoprotein E (APOE)-deficient mouse model. The reason for using an APOE-negative model is to eliminate any positive effect on clearance of blood lipids that disrupt the results from shSiglec-1. It was found that Lv-shSiglec-1 could inhibit the expression of Siglec-1 resulting in downregulation of monocyte chemoattractant protein 1 (MCP-1)/chemokine (C-X-C motif) ligand 2 (CXCL2) activity in aortic blood and C-C chemokine receptor type 2 (CCR2)/C-X-C motif chemokine receptor 2 (CXCR2) activity in monocytes. ShSiglec-1 may also prevent oxidized low-density lipoprotein accumulation by peritoneal macrophages via the correspondent receptor oxidized low-density lipoprotein receptor 1 (ox-LDLR1) (Xiong et al. 2016).

Ischemic heart disease (IHD) in the population is a key health issue in an advanced society. It is one of the three killer diseases around the world. Current treatments rely on surgical methods like coronary artery bypass surgery or percutaneous coronary intervention. Typical findings suggested that downregulation of vascular endothelial growth factor (VEGF) and elevated Src homology region 2 domain-containing tyrosine phosphatase-1 (SHP-1) may enhance the pathological progress of IHD. Recently, Kim et al. introduced IHD treatment by a polymeric carrier with siRNA of SHP-1 (siSHP-1) gene for anti-apoptosis and plasmid DNA of hypoxia-inducible VEGF expression vector (pDNA of pHI-VEGF) for angiogenesis. The polymeric carrier consists of deoxycholic acid-PEI (DA-PEI) with cationic particles that interact with multiple siSHP-1 molecules through primary electrostatic interaction followed by pHI-VEGF pDNA packaging by secondary electrostatic interaction to make a stable bifunctional DA-PEI/siSHP-1/pHI-VEGF nanoparticle to attenuate cardiomyocyte apoptosis and enhance cardiac microvessel formation to fight against ischemic heart diseases (Kim et al. 2016a).

Therapeutic siRNA for eye diseases

Patients who completed extracapsular cataract surgery may have a higher risk of developing visual diseases due to

posterior capsule opacification (PCO) caused by lens epithelial cell (LEC) proliferation. LEC generation was found to be associated with elevated mammalian target of rapamycin (mTOR) followed by heightened p70 ribosomal protein S6 kinase (p70S6K) and protein kinase B (PKB or Akt). It was discovered that siRNA of mTOR (simTOR) could be effective in mTOR/p70S6K/Akt inhibition in a human LEC B3 cell model which was created by cell transfection. They also proved that simTOR could suppress the expression of mTOR complex 1 (mTORC1) and mTORC2 and further transforming growth factor-β (TGF-β)-induced epithelial-to-mesenchymal transition (EMT) (Zhang et al. 2016).

Age-related macular degeneration (AMD) is another common eye disease in the population especially in the elderly. AMD is developed by the gradual degeneration of light sensory cells and angiogenesis located on macula resulting in visual disability. Vascular endothelial growth factor A (VEGF-A) was known as the pathological initiator. Nguyen et al. reported that siRNA PF-04523655 with ranibizumab could attenuate the pathological progression (Nguyen et al. 2012). Takanashi et al. further discovered that small synthetic RNAs nkRNA and PnkRNA could inhibit AMD-associated angiogenesis via the Toll-like receptor 3-independent pathway (Takanashi et al. 2015). Recent findings revealed that poly(ADP-ribose) polymerase-1 (PARP-1) was the key factor in retinal cell apoptosis which could be downregulated by the corresponding siRNA and the enzyme inhibitor olaparib (Jang et al. 2017).

Therapeutic siRNAs for cancers

To enhance the efficacy of the original drug compound, structural modification of the drug or combined therapy by multiple entries of tumour inhibitors can be performed. Co-administration of siRNA and other anti-tumour drugs like tumour initiation and proliferation suppressors, cell migration blockers, invasion and metastasis inhibitors and other enhancers of tumour progression could be one of the therapeutic plans for consideration.

Efficacy of single administration of therapeutic siRNA was relatively low in in vivo animal tests, and hence, some scientists have developed a delivery polymer for siRNA. The polymer backbone was a polycondensation molecule of trimethylolpropane allyl ether (TPAE) with an amino thiol side chain and suberoyl chloride (diACl-C8) linked with an alkyl thiol side chain. PE4K-A13e0.33C6 and PE4K-A13e0.33C10 were the test examples in this study. siRNA linked with the above PE4K polymers to form a siRNA ployplex nanoparticle (siRNA PNP) covered with PEG2000 DMG or amphiphilic pluronic block copolymers (F-68 and F-127). PEG2000 DMG and amphiphilic pluronic block copolymers are functional in reducing non-specific protein interactions and promoting stability of the siRNA PNPs. It was discovered that the delivery performance of aerosol inhalation to the lung tumour xenograft of test mice was better than intravenous injection. It is because the majority of siRNA PNPs through intravenous injection accumulated in the liver rather than in the lungs (Yan et al. 2017). Xie et al. found that siRNA-B-PEI condensed with a short transferrin binding peptide HAIYPRH (HAI peptide) might increase the affinity of binding between the drug complex and its target over-expressed transferrin receptors in non-small cell lung carcinoma cells (Xie et al. 2016b).

Bedi et al. demonstrated a cell-specific siRNA-phage protein delivery system in breast cancer cells. Phage proteins isolated from phage and ligated with glyceraldehyde-3-phosphate dehydrogenase siRNA (si-GAPDH) particle and formed a si-GAPDH-nanophage complex. An in vitro experiment employing Si-GAPDH-nanophages showed that it could penetrate into breast cancer cells and suppress GAPDH expression. It was suggested that this technique may be useful for siRNA-based oncogene silencing in breast cancer (Bedi et al. 2013).

Cluster of differentiation 73 (CD73, also known as ecto-5′-nucleotidase) is one of the inflammatory factors produced by regulatory T (Treg) cells which is a key component for the initiation of various diseases such as viral infection pathology, autoimmune diseases, and cancer development by adjustment of a series of interleukins (ILs) and interferons (IFNs) and ADP/ATP-dependent cellular activity (Antonioli et al. 2013). SiRNA of CD73 (siCD73) could be the solution for CD73-dependent pathogenesis. SiCD73 may be able to suppress the synthesis of CD73 and cause low activity in Treg cells, myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages in the 4T1 breast cancer-bearing mouse model. The master inhibition may reduce the level of various inflammatory factors IFN-γ, IL-17 and IL-10 in breast tumourigenesis and suppress matrix metalloproteinases 2 (MMP 2) and MMP 9 implicated in lung metastasis. It was discovered that chitosan lactate nanoparticles could enhance siRNA delivery and effectively deliver siCD73 to the breast cancer cells (Jadidi-Niaragh et al. 2017).

Vascular endothelial growth factor (VEGF) and the downstream signalling pathway phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) are the major tumourigenesis effectors in non-small-cell lung cancer (NSCLC) that promote cell proliferation, angiogenesis, cell migration and metastasis. Espana-Serrano and Chougule found that co-administration of siRNA-VEGF for silencing VEGF gene expression and PI3K/Akt/mTOR suppressor PF-04691502 in vitro were functional in counteracting tumour progression. They further discovered that moderate doses of PF-04691502 from 70 to 500 nmol/l with a fixed dose (50 nmol/l) of siRNA-VEGF could be the most effective combined drug for NSCLC treatment (Espana-Serrano and Chougule 2016). Meanwhile, there was another finding on

siRNA-VEGF delivery for cancer treatment. Exosome nanovesicles originate from brain endothelia cell. Yang et al. discovered that these nanovesicles could be used for carrying siRNA-VEGF across the blood-brain barrier and bring the siRNA across the brain cancer cells in an in vivo zebrafish model (Yang et al. 2016).

Integrin-β6 is one of the tumour development factors in colon cancer which is a multifunctional component in cancer cell growth, invasion and metastasis. Initially, it was found that siRNA of integrin-β6 (siIntegrin-β6) could inhibit colon cancer progression and metastasis and trigger apoptosis of cancer cells while the delivery level should be further improved. Liu et al. developed integrin-β6-targeted immunoliposomes carrying siIntegrin-β6 for cancer treatment. It was proved that siIntegrin-β6 immunoliposomes could effectively recognize colon cancer cells and deliver the target siRNAs to the cells resulting in cancer growth retardation and metastasis inhibition (Liu et al. 2016). Other than the siRNA immunoliposome carrier, Xie et al. designed another polyethylenimine (PEI)-based nanoparticle, namely, ε-caprolactone-modified PEI nanoparticles (CL-PEINPs) for colon cancer therapy in vivo. CL-PEINPs are prepared by condensing the PEI backbone with ε-caprolactone and are formed as macromolecules. In mice, addition of CL may help protect siRNA during travelling in blood. SiRNA targets genes for tumourigenesis stored inside the nanoparticles. In a luciferase reporting experiment, it was discovered that CL-PEINPs were stable in the serum and arrived at the target HCT-116 colon tumour xenograft in the mouse colon (Xie et al. 2016a).

In the beginning of drug development, the efficacy of a drug is the most important criterion. Therefore, the drug test usually relies on a single drug test. Recent research also disclosed that siRNAs and current chemotherapeutic drugs could be stored in the same nanoparticle for dual anti-tumour treatment. SiRNAs for inhibition of anti-apoptotic factors, blockage of tumour cell surface receptors for signalling transduction and tumour cell survival factors combined with current chemotherapeutic drugs like doxorubicin (DOX), paclitaxel (PTX), docetaxel and cisplatin may be employed to fight against chemo-resistant breast carcinoma and lung cancers (Jones and Merkel 2016).

Selective targeted specific combination therapy has been introduced for many years. The plan of combination therapy usually targets one key pathological molecule by exploiting a specific suppressor as a base, and multiple inhibitors targeting upstream and downstream effectors for the key molecule will be co-administered accordingly. Recent research findings reported by Aliabadi et al. suggested combination therapy with multiple inhibitors targeting signalling cascades for cancers. They identified some relatively effective inhibitory siRNAs of signalling enhancers like Bcl-2 family apoptosis regulator, myeloid cell leukaemia 1 (Mcl-1), Janus kinase 2 (JAK2), signal transducer and activator of transcription 3 (STAT3) and JUN expression. A combination of siRNAs for the four proteins and four individual siRNAs of these proteins was tested for cancer cell viability. The preliminary test results showed that combined siRNAs performed better in co-silencing of the gene expression of these four proteins. Although single siRNA administration could effectively suppress one specific signalling molecule, some other tumour enhancers were elevated. It was suggested that these elevated enhancers could be activated by alternative pathways in the cells (Aliabadi et al. 2016).

Therapeutic siRNAs for inflammation-associated diseases

For colon cancer therapy, some research studies have been focused on one of the initiators in cancer development, i.e. inflammatory diseases. Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease are a group of diseases arising as a consequence of the pathogenesis of bowels with chronic and progressive inflammation which could be associated with an altered colon microenvironment due to imbalance of gut bacteria, and alteration of gene expression in the colon epithelial cells and immune response cascades. Although the actual pathology remains unclear, IBD could be considered as one of the initiation factors of colon tumourigenesis. Previous research has proved that inhibition of various proinflammatory factors alleviates IBD development. SiRNAs targeting mRNAs of major inflammatory initiation factors such as tumour necrosis factor-α (TNF-α), cyclin D1 (CyD1), CD98, mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4, also known as MAPK/ERK kinase kinase kinase 4) and Krüppel-like factor 4 (Klf4) were effective in IBD-related gene silencing treatment while a good delivery carrier should be introduced (Guo et al. 2016).

Guo, Jiang and Gui reviewed some in vitro and in vivo experiments on siRNA nanoparticles in C57BL/6 or BALB/c mice or mice bearing IBD symptoms including polysaccharide-derived, lipid-coated, polylactide (PLA)-derived, calcium phosphate/poly(D,L-lactic-co-glycolic acid) (CaP/PLGA)-based, nanoparticles-in-microsphere oral system (NiMOS)-based, thioketal-based and PEI-coated nanoparticles (NPs). The efficacy experiments showed that these siRNA nanoparticles could attenuate inflammation. SiRNA-lipid-coated nanoparticles and neutral liposome-hyaluronan–integrin-mAb stored with siRNAs of CyD1 were functional in inhibiting TNF-α, IFN-γ, IL-2 and IL-12 for inflammation progression. SiRNA-polysaccharide-derived nanoparticles like modified chitosan-urocanic acid-PEG-single chain CD98 (mC-UAC-PEG-scCD98) with CD98 siRNAs, galactosylated trimethyl chitosan-cysteine (GTCC) with Map4k4 siRNA and TNF-α siRNAs, SC12-cyclodextrin-click-propylamine with TNF-α siRNAs (siTNF-α) and β-

1,3-D-glucan with siTNF-α were delivered into colon epithelial cells and silence their targets. PLA with siTNF-α or Klf4 protein siRNA and PLA-PEG-maleimide-Ab with siTNF-α were also considered as the candidate anti-inflammatory drugs. CaP/PLGA-based CaP/PLGA/PEI NPs, thioketal-based poly(1,4-phenyleneacetone dimethylene thioketal) (PPADT) NPs and PEI-based poly (*N*,*N*′-bioreducible cystamine bisacrylamide-branched-polyethylenimine-PEG-mannose (p(CBA-B-PEI)-PEG-Man)) NPs condensed with siTNF-α were effectively transported into colon cells and inhibited TNF-α expression. Primary data from in vivo siRNA-NiMOS tests showed that it could enter colon cells and attenuate TNF-α and corresponding proinflammatory factors (Guo et al. 2016).

Reactive astrocytosis (also known as astrogliosis) and corresponding glial scar are the common signals for spinal cord injury. Elevated growth of astrocytes may inhibit axonal regeneration resulting in malfunction in signal transduction. It was discovered that xylosyltransferase-1 (XT1) and chondroitin sulphate proteoglycans (CSPGs) could be the keys for initiating reactive astrocytosis. Abu-Rub et al. suggested a siRNA of xylosyltransferase-1 (siXT1) with a polymeric delivery system. To prepare siRNA-polymer, polymerization of 2-dimethylaminoethyl methacrylate and ethylene glycol dimethacrylate (PDE) was first generated to form a cyclized complex polymer. The cyclized complex polymer was ligated with xylosyltransferase-1 SiRNA (siXT1) to synthesize a complex called siXT1-PDE which may inhibit XT1 gene expression. Experimental results showed that 65% of XT1 expression activity was attenuated, ensuing in the elimination of spinal cord injury. SiXT1-PDE was considered as one of candidate drugs for neurodegenerative diseases (Abu-Rub et al. 2016).

Asthma is a disease driven by over-excited immune response of T helper 2 (Th2) cells that causes inflammation in the airway. Previous findings proved that siRNAs from inhibition of interleukins (e.g. IL-5 and IL-13) and Th2 transcription factor GATA-3 could attenuate inflammation in the airway. Xie et al. designed a siRNA-transferrin-PEI complex polymer and found that it could successfully enter Th2 cells. However, inhibition of interleukin was not significant. One of the possible reasons was that the majority of siRNA was not released from the complex. The research team planned to improve the complex polymer by replacing the PEI core and conduct a GATA-3 siRNA animal test (Xie et al. 2016c).

Progress update of siRNAs in clinical trials

Rudloff et al. from the US National Institutes of Health Clinical Center conducted a phase I clinical trial siRNA with a lipid-based nanoparticle (LNP), TKM-080301, on various metastatic cancers originating from hepatic cancer (Identification number: NCT01437007). TKM-080301 targeted to silence the expression of oncogene polo-like kinase-1 (PLK1). Hepatic arterial infusion (HAI) for therapeutic siRNA was the original method with relatively low efficacy. Therefore, Rudloff et al. used LNP delivery for therapeutic siRNA to test drug efficacy. This clinical trial was a project with non-randomized allocation, non-masking and single-group assignment. The design of the clinical trial included the following: patients with unresectable cancers with hepatic metastases or with primary hepatic cancers will administrate TKM-080301 via HAI. Patients initially took 4 mg/m^2 drug, and the administration period was once every fortnight up to a 12-dose completion. Evaluation was made every 6 weeks. Patients with resectable tumours were offered the treatment. Test would be terminated if the patients show cancer progression (Kim et al. 2016b) (ClinicalTrials.gov. 2016).

Vaishnaw et al. from Alnylam Pharmaceuticals held a phase I clinical trial of intravenously (IV) administered ALN-VSP02 for patients with hepatic-associated advanced solid tumours to study the safety, stability and bioavailability of certain siRNA drugs (NCT00882180). This project was a trial with non-randomized allocation, open-label and single-group assignment. Intravenous administration of one dose every fortnight was carried out up to the 16th week. Plasma and urine from patients were collected to study pharmacokinetics and pharmacodynamics in 8 weeks. Assessment of antitumor and antiangiogenic activity was conducted throughout the 16-week treatment period (Kim et al. 2016b; USNIH 2017).

O'Reilly et al., Golan et al. and Silenseed Ltd. prepared to operate the phase II clinical trial of siG12D-LODER/chemotherapy combined treatment for the patients with unresectable locally advanced pancreatic cancer (LAPC) including pancreatic ductal adenocarcinoma and pancreatic carcinoma (NCT01676259). This study is a randomized, open-label study with parallel assignment. The design of the clinical study is shown as follows: 80 patients with unresectable LAPC will plan to recruit and they will be separated into two arms. One arm will receive a single dose of 2.8 mg of siG12D-LODER (eight points in total with 0.35 mg target drug per position) by site-specific implantation with gemcitabine/nab-paclitaxel chemotherapy. The patients of another arm will receive gemcitabine/nab-paclitaxel chemotherapy only during the treatment period. A phase I clinical trial on 15 patients with the abovementioned condition has been completed. It was discovered that dose-escalating siG12D-LODER did not develop dose-limiting toxicity (DLT) but it exhibited high safety and tolerability (Kim et al. 2016b; USNIH 2017).

Sorensen et al. from Alnylam Pharmaceuticals completed patient recruitment of a phase I clinical study of ALN-AT3SC in patients with haemophilia A and haemophilia B and healthy participants (NCT02035605). This study is a randomized, participant-masked study with parallel assignment which aims to study the pharmacokinetics and pharmacodynamics of

ALN-AT3SC through blood plasma testing. The patients will be separated into groups according to their health condition (i.e. haemophilia A, haemophilia B and healthy participants). One arm of patients receive dose-escalating ALN-AT3SC through subcutaneous injection, and another arm receive administrations of sterile normal saline (0.9% NaCl). Primary data processing will be completed in mid-2017 (Lee et al. 2016; USNIH 2017).

Strumberg et al. conducted an intergraded project on phase I and phase II clinical trials of Atu027 nanoparticle and chemotherapeutic drug gemcitabine combination therapy in advanced or metastatic pancreatic cancer (NCT01808638). This study design was a randomized and open-label project with parallel assignment. The patients were separated into two arms for a 28-day experiment. Arm 1 patients were treated with Atu027 and gemcitabine once a week on day 1, day 8 and day 15, but there was no administration in the fourth week. Arm 2 patients were treated with Atu027 and gemcitabine twice a week followed by additional gemcitabine treatment for 28 days. Treatment would be terminated if drug toxicity was in danger level or disease progression developed during the consecutive 28-day cycles (Lee et al. 2016; USNIH 2017).

Gollob et al. from Alnylam Pharmaceuticals completed a phase III clinical trial of ALN-TTR02 (also called patisiran) in transthyretin (TTR)-driven amyloidosis (NCT01960348). It was a randomized, double-blind study with parallel assignment. Recruited 225 patients were separated into two groups. The first group would receive administration of ALN-TTR02 via IV infusion, but the second group as a placebo group took sterile normal saline (0.9% NaCl). There is an on-going comparative study on ALN-TTR02 treatment in phase III clinical trials (NCT02510261) held by Alnylam Pharmaceuticals. It is a non-masking project with single-group assignment for patients who had been administered this drug before. Patients would be administered ALN-TTR02 via IV infusion (Lee et al. 2016; USNIH 2017).

Kim et al. reviewed that there was another completed siRNA lipid-based nanoparticle (LNP) in the phase I clinical trial for silencing oncogene expression (identification number: NCT00938574). One siRNA LNP in phase Ib to II clinical trials was scheduled for patient recruitment who had liver cancer (NCT02314052). Another siRNA LNP, siRNA-EphA2-DOPC (NCT01591356), was planned to recruit patients for the phase I clinical trial. Moreover, two ex vivo transfections of therapeutic siRNAs with dendritic cell carrier and peripheral blood mononuclear cell APN401 carrier for metastatic melanoma (NCT00672542) and solid tumour (NCT02166255) respectively were in the phase I clinical trial (Kim et al. 2016b).

In addition, Lee et al. reviewed clinical trials using naked siRNA particles, siRNA conjugates, siRNA-lipid NPs and siRNA cell transfection. For naked siRNA, there are three projects (NCT00554359, NCT00802347 and NCT00716014) in the phase I clinical trial targeting p53 gene and K6a promoter respectively and six projects (NCT02341560, NCT01965106, NCT01445899, NCT02455999, NCT02250612 and NCT01065935) in the phase II clinical trial targeting caspase-2, RTP801, TrpV1, ADRB2 and RSV N expression respectively. There is another project (NCT01814839) on siRNA conjugate targeting AT gene and TTR gene in phase I. Additionally, one project on siRNA conjugate ARC-520 for HBV DNA inhibition has entered the phase II clinical trial. For, lipid-based siRNA NPs, there are four projects (NCT01262235, NCT01158079, NCT01437059 and NCT01148953) in phase I to phase II clinical trials for PLK1, KSP/VEGF, PCSK9 and TTR gene silencing (Lee et al. 2016).

Meanwhile, according to the updated information from ClinicalTrials.gov, seven new therapeutic siRNA clinical trials were initiated in the recent 2 years. Coleman et al. started their phase I clinical trial on EphA2-targeted siRNA delivery by neutral liposome (NCT01591356). Triozzi et al. operated the phase II trial on siRNA-transfected APN401 cells which are a type of peripheral blood mononuclear cells targeting non-surgery available cancers (NCT03087591). Paduch et al. held a project on the small RNA pathway (including siRNA) in gametogenesis to determine detailed functions of small RNA in gametogenesis and infertility (NCT02864329). The pharmaceutical company Sylentis, S. A., continues the phase III clinical trial on siRNA SYL1001 for the patients with moderate to severe dry eye disease (NCT03108664). The Medicines Company operates the siRNA ALN-PCSSC phase II trial for the treatment of homozygous familial hypercholesterolemia (NCT02963311). Alnylam Pharmaceuticals started ALN-GO1 phase I and phase II trials for the treatment of primary hyperoxaluria type I (NCT02706886). Li et al. operated a trial on JAK2 inhibiting siRNA targeting alveolar macrophages for treating chronic beryllium disease (NCT02596347) (USNIH 2017).

Discussion on improvement and further development of siRNA therapeutics

siRNA-based nanoparticle as a therapeutic drug contains high efficacy to target the position contributed by multiple conjugation availability to other ligand particles or antigens that recognize the target cells. The conjugation availability may also be adapted to other therapeutic drugs to develop combined therapy on a specific disease. According to the previous studies, siRNA has low likelihood to develop adverse events because of its high turnover rate in vivo. This technology would also be available in establishing personalized medicine because the level of the therapeutic siRNA can be adjusted and administered according to the elevated or suppressed pathological factors. The most distinctive advantage of

siRNA is that it can regulate the pathological pathway as early as on a gene expression level. Suppression of generation of pathological elements helps attenuate the severity of a certain disease. siRNA targets on genetic level control which is critical to treat some diseases with a long developmental period like inflammation and tumourigenesis, neurodegenerative diseases and viral infection.

However, siRNAs still involve some technological barriers. For siRNA, as a new investigational drug, studies on the certain pharmacokinetics and pharmacodynamics were only conducted in a short period of time. Some unknown side effects could be generated because there are still some pathways that are not fully elucidated and the clinical assessment of long-term administration of siRNA therapeutics has not been completed. Off-target effects happen when different organs or tissues share similar genes. siRNA, as a short fragment, could develop non-specific binding towards different tissues. Therefore, cell-specific ligand or antigen-coated siRNA nanoparticles may help guide the drug onto the target site. siRNA on the one hand has high turnover rate while it has low bioavailability in vivo in recent researches; degradation and neutralization begin within minutes to hours from different delivery agents. There is a chance that siRNA may be released from the protective particles before arrival of the target tissue. Although siRNA conjugation and encapsulation can enhance the bioavailability of siRNA, the cost of the certain technology is extremely high. This technology can support siRNA therapeutics in targeted therapy development while it cannot serve as a public medicine in mass production unless massive cost reduction is available.

Short sequences have a problem in target recognition that may potentially generate an off-target effect. Off-target effect describes the situation in which the RNAi molecules can recognize multiple gene sequences or even mismatch with non-interest genes (Tiemann and Rossi 2009). This problem could form an obstacle and pose a risk for anti-tumour and anti-viral treatments. For example, in tumour cells, most of the genomic materials are the same as those of the normal cells and hence the expressed products are similar. The differences between cancer cells and normal cells are usually about an abnormal rate of expression of several target molecules or accumulation of non-functional or truncated products in cancer cells. To prevent or minimize the off-target effect, additional markers for target cell labelling could be a solution to increase the specificity. For example, there are distinctive signal differences between cancer cells and normal cells for the number of growth factor receptors on the cell surface. In the early stage of tumour development, the expression of growth factor receptors is highly active which helps increase the rate of proliferation. It is possible to raise a marker or structurally modified monoclonal antibody targeting the cells with highly expressed growth factor receptors. These markers are attached on the siRNA particles that serve as pilot agents to the target site (Guo et al. 2013; Karagiannis and El-Osta 2005; Witsch et al. 2010).

Considering delivery systems, the viral coat is one of the earliest delivery systems for drugs. The viral coat is made by proteins which may easily be digested by cellular enzymes and release the target drugs. However, antigens on the viral protein coat could be the potential sources to trigger unexpected immunological effects. In the above situation, there were several non-viral systems developed for siRNA delivery. The siRNA conjugate targets on developing a compound with a small molecular weight. The chemical conjugates usually are simple organic compounds that target to stabilize the siRNA structure, and partial conjugates may also become an assistant drug for the corresponding disease. However, this technique could be useful in vitro but can still be challenging in vivo. It is because the siRNA conjugate could be disrupted by some of the defensive systems such as endosomes and lysosomes. Therefore, there are three parallel next-generation designs in siRNA encapsulation technologies including lipid nanoparticles, peptide nanoparticles and polymeric nanoparticles. During their development, these three technologies have been considered to increase the stability, bioavailability and escape from immunosurveillance by different combinations of lipids, peptides and polymers.

Lipid nanoparticles could be more focused on the siRNA protection and increase drug stability and bioavailability by the lipid bilayer delivery envelope. Lipid nanoparticles compared to other materials may more easily penetrate the target cells. The distinctive characteristic of peptide nanoparticles is that the design of nanoparticles highly focuses on drug specificity and efficacy. Elevated cancer cells, for instance, express large amounts of growth factor receptors and proliferative enhancers on the cell surface. Peptide nanoparticles allow embedment of receptor-specific ligands which target the cell surface receptor. This may help the drug nanoparticles recognize the target cells. Meanwhile, some peptide nanoparticles are adapted from the challenge of enzyme digestion which may protect the core therapeutic siRNA. Polymeric nanoparticle is a nanoparticle with a combination of inorganic substances and organic compounds. The design of polymeric nanoparticles involves many of the advantages from lipid nanoparticles and peptide nanoparticles. Polymeric nanoparticles may be able to develop a strong protective coat, and they are available to conjugate with target-specific ligands that provide high bioavailability and specificity onto the target. Partial polymeric nanoparticles may change their conformation and packaging in different pH values. Some may avoid enzymatic destruction during delivery but release siRNA through target enzyme digestion.

Contributed from continuous research in siRNA delivery, the new design of nanoparticles could combine the functions of peptide nanoparticles and polymeric nanoparticles which have been under consideration. Recent reviews suggested

polymeric nanoparticles as a core to store siRNA with ligation of functional peptides to detect the target receptors. According to the latest reviews of in vivo experiments and clinical trials from Barata, Sood and Hong, siRNA delivery still requires substantial improvement in bioavailability. It was reported that a majority of the siRNA nanoparticles could be retained inside the body for less than 12 h. However, a short retaining period may greatly affect the functional drug deliverance to the target cells. Hence, it is suggested that improvement of stability is critical for new particle design by introducing some building blocks with surface charge balance and enzyme-resistant ability. In addition, reduction of particle size and precise control release of siRNA are the other two keys for new design.

SiRNA could be useful for curing diseases with a normal gene mutation rate. However, the characteristics of rapid gene mutation, recombination and reassortment of genetic materials in various influenza viruses present a formidable challenge in therapeutic application (Bennink and Palmore 2004). Delivery system and structural enhancement of siRNA-based drugs for influenza virus A have been reviewed and modified (Ge et al. 2004; Lin et al. 2012). It can be seen that there is hope for controlling outbreaks of viral infections. Synthesis of a new drug or treatment for patients is a long and complicated process. Multiple verifications of the therapeutic products should be performed before use. It may be difficult to develop rapid-acting therapeutic measures targeting virus-infected cells unless conserved gene sequences and patterns of reassortment systems of viruses are clearly identified. Development of siRNA treatment for influenza viruses for co-inhibition of viral activity as well as reduction of disease severity has been suggested.

It may be easy to make a good hypothesis on medical application while the actual practice may in fact be challenging. Currently, there is supporting biological evidence that siRNA is suitable for in vitro molecular analysis owing to its high specificity and accuracy of its conjugates. From the perspective of a clinical study, drug safety and pharmaceutical certainty should be first considered. Any lack of clarity or some uncertainties may be a safety concern regarding the clinical study. There were a sufficient number of accidents cautioning us that a drug with the ability of cellular signalling modification may stand a chance of eliciting adverse events. It was disclosed that trastuzumab, a monoclonal antibody designed for breast cancer treatment, might exhibit cardiotoxicity by blockage of the human epidermal growth receptor 2 (HER2) signalling pathway. It was because HER2 protein, despite promoting breast cancer development, also contributes to cardioprotective pathways. Therefore, alteration of the HER2 signalling pathway could increase the risk of cardiovascular diseases (Onitilo et al. 2014; Sandoo et al. 2014; Sandoo et al. 2015). In fact, some other HER2 receptor antagonists such as ado-trastuzumab emtansine, pertuzumab and lapatinib may also have cytotoxic effects when high doses are administered (Palumbo and Bubalo 2016). Therefore, any adverse events in clinical trials should be properly recorded and examined in order to tackle the problems involved and further to improve the drug formulation. To develop a new treatment, medical tests must be conducted under control in various conditions, i.e. drugs should pass several tests for validation of efficacy, toxicity in animal experiments in human gene-induced mice and possibility of induction of gene mutation or alteration, and overall performance using a human cell line. If siRNA-based drugs can pass through preliminary tests, human trials could be considered. In consideration of the database, patient anamnesis, diagnostic results and personalized medical treatment schemes will be developed in the future. Development of sufficient cell-based models for drug efficacy and toxicity tests is also an advantage for drug development before preclinical study.

To promote precise and personalized medicine, development of pathological-genetic mega databases and rapid diagnosis methods are two fundamental tools. It is more appropriate to elucidate RNAi functions in cellular physiology and pathology before it enters clinical trial tests. Another potential difficulty is about the regional and rational differences in lncRNA profiling. Human genome project and the associated epigenetic studies have proved that people from the Western, Asian, African and Latin American countries may have differences in gene expression appearance as well as epigenetic discrepancies. Their RNA balance as well as certain pathological protein levels could be different, and the hallmark molecules could also be different. Therefore, it is important to focus more on the development of a mega database for gene expression profiling and RNA profiling in regional and rational differences. It helps medical specialists to ascertain a suitable treatment plan for different groups of people around the world. The profiling database also contributes to pharmaceutical production on a mass scale. Personal data such as patients' anamnesis and genetic data are suggested to be included in the local medical system record that may serve as a good reference for a personalized treatment plan. As a result, all of the data in the database can be summarized and provide a more effective and target-specific therapy for each patient. Meanwhile, a high-throughput screening for expression-specific or cell regulator genes was suggested for further investigations because it is beneficial to siRNA development (Zhang 2011). Following the above research progress, tailor-made siRNA will be available for precise and personalized medicine in the foreseeable future.

Dr. Sauvage, Dr. Stoddart and Dr. Feringa were the 2016 Nobel Laureates in Chemistry who invented the methodology of design and synthesis using molecular machines. Their research illustrated the uses of transition metals and complex organic compounds and electron transfer of molecules (Peplow 2015; Stoddart 2009). It could be suggested that controlled release or sequential release of a

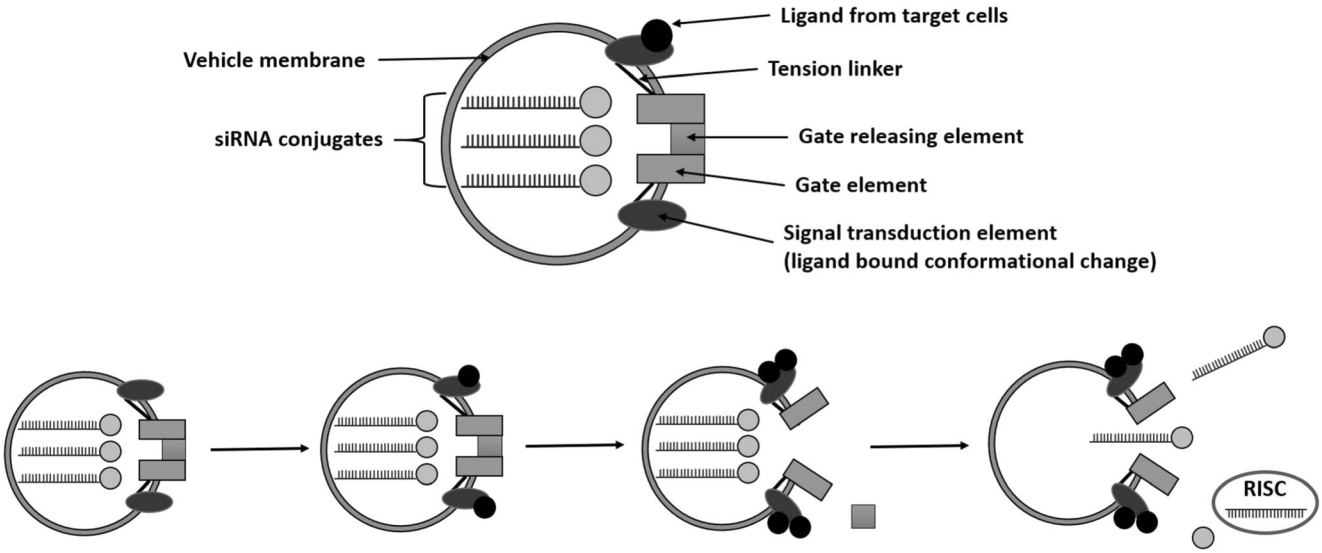

Fig. 2 Proposed mechanism of sequential release of therapeutic siRNA. The pathological element serves as a ligand. When the concentration of pathological element increases, more ligands will bind to the signal transduction element and trigger conformational changes. Since the signal transduction element is linked with the gate element by a tension linker, the movement of the signal transduction element will change the shape of the gate elements. The linkage of the gate elements and a gate-releasing element will break and open the vehicle gate. Therapeutic siRNA conjugates can be released into target cells

drug to the target regions can be contributed by cooperation of molecular machinery, for example, sensory molecular machine design for intercellular/intracellular detection of some pathological hallmarks. An intracellular ligand-small molecule-dependent drug release vesicle can be designed with the following components: a simple lipid or peptide vesicle with target intracellular ligands attached, small chemical molecule as a signal and function activator, small molecule-dependent gate protein and therapeutic siRNAs. Pathological particles in the cells such as inflammatory cytokines will serve as the ligands for ligand-small molecule-dependent drug release vesicles. During disease progression, accumulation of these particles may increase the binding affinity of the corresponding ligand receptor. When the released small molecule signals add on the vesicle gate and reach the threshold level, the gate will open and release therapeutic siRNA or other drugs to the target genes (Fig. 2). These models are also applicable for development of intercellular drug release machines.

Combined therapy is considered to be a future therapeutic trend. Some previous studies introduced mix-mode medicines for therapeutics. The combination could be molecules in the same category (intrasystem) with similar functions or different drugs targeting multiple locations (intersystem). An intrasystem-combined therapy involves a combination of drugs in the same category such as five different siRNAs against viral infection and growth by targeting the glycoproteins, polymerase and assembly molecules. An intersystem-combined therapy aims to develop a sequential treatment plan by applying different methods.

Lau et al. demonstrated siRNA against HIV-1 integrase 10 years ago. It was discovered that short hairpin RNA (shRNA) could inhibit HIV-1 integrase in four regions along the multimerization domain and catalytic domain (Lau et al. 2007). Later, there was a host of chemical drugs available for inactivation of functional HIV proteins: HIV-1 ribonuclease H, reverse transcriptase and integrase. Therefore, co-administration of siRNA/miRNA/shRNA and chemical drugs for inhibiting the expression of these functional genes could be an advantage for new development of HIV therapy (Bernatchez et al. 2015; Corona et al. 2014; Hu and Kuritzkes 2014; Lau et al. 2007). With inspiration from a classic anti-HIV-combined therapy, siRNA-based therapy could involve a combination of drugs against different targets. In hepatocellular carcinoma driven by hepatitis B virus (HBV), there are several defensive mechanisms like prevention of coagulation and attachment of virus, inhibition of viral replication and blockage of mature virus assembly. It is possible to develop a combined drug with multiple targets.

Ovarian cancer is one of the top lethal cancer types in the human population. More than a decade ago, overexpressed oestrogen receptor α (ERα) is the initiator of ovarian cancer development. Apigenin (4′,5,7-trihydroxyflavone)-induced oestrogen receptor β (ERβ) can inhibit tumourigenesis (Mak et al. 2006). Further, it was discovered that overexpressed ERα could downregulate miR-486-5p expression but enhance the gene expression of olfactomedin 4 (OLFM4) resulting in cell proliferation, invasion and metastasis in the ovary. They demonstrated that injection of miR-486-5p could attenuate cancer progression (Ma et al. 2016). It

is suggested that siRNA targeting OLFM4 may also contribute to suppression of ERα-induced cancer development. Faratian et al. found that co-administration of trastuzumab and pertuzumab could be effective in terminating HER2-induced and ERα-induced ovarian cancer (Faratian et al. 2011). G-protein-coupled oestrogen receptor 1 (GPER) is a functional receptor in ovarian cancer cell proliferation via tubulin polymerization. A synthesized GPER agonist G-1 (1-[4-(6-bromobenzo[1,3]dioxol-5yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone) could inhibit GPER activity by disrupting tubulin polymerization (Wang et al. 2013). Moreover, Leung et al. proved that focal adhesion kinase (FAK), cAMP response element-binding protein (CREB) and troponin C1 (TNNC1) signalling cascades were the effectors of metastasis of ovarian cancer through overexpression of microfibrillar-associated protein 5 (MFAP5). This process was suggested as a calcium ion-dependent pathway. They found that siRNA of MFAP5 could silence its expression (Leung et al. 2014). It is believed that silencing gene expression of the FAK/CREB/TNNC1 pathway by the corresponding siRNA and miRNA could also contribute to cancer attenuation.

Inspired by the development of siRNA delivery technology, a pH-dependent or cellular enzyme-dependent therapeutic polymer nanoparticle with multiple drugs can be designed for a sequential and stage-dependent therapeutic scheme. The drug polymer nanoparticle for ovarian cancer consists of apigenin, trastuzumab and pertuzumab and receptor agonist G-1 which can be used for oestrogen receptor functional blockers; cancerous calcium ion channel blockers with FAK/CREB/TNNC1 siRNAs and MFAP5 siRNA for preventing cancer cell metastasis; and co-administration of OLFM4 siRNA and miR-486-5p which is for OLFM4-dependent cell proliferation and metastasis. The prolonged controlled release mechanism of the drug-storing capsule can also be an advantage if the corresponding design is available.

Ionov et al. (2015) and Dzmitruk et al. (2015) illustrated the efficiency of siRNA-combined therapy for cancer treatment. Xue et al. (2014) demonstrated the application of a combination of siRNA, miRNA and chemotherapy in lung cancer. It is suggested that short-term radiotherapy and chemotherapy are used to minimize the growth of tumour cells on the surface. Then markers like monoclonal antibody or modified siRNA will be used to label the cancer stem cells. SiRNAs with other target therapeutic drugs could be co-administered to eradicate all cancer stem cells and the daughter cells.

Inactivation of key effectors in cancers can be considered as the first-line treatment. PI3K/Akt and extracellular signal-regulated kinase (ERK) pathways are the key enhancers of tumourigenesis in most of the cancers. It was discovered that 4E-binding protein 1 (4E-BP1), which is a translational inhibitor, was proved to be the upstream activator of the above oncogenic pathways (She et al. 2010). It is hypothesised that co-administration siRNAs and chemical drugs against 4E-BP1, PI3K/Akt and ERK expression may be useful for inhibiting cancer initiation.

It was discovered that siRNA is associated with reproductive regulation. Some studies suggested that siRNA is essential in germ cell development during meiotic division. It may be hypothesised that some infertility cases are associated with malfunction in siRNA homeostasis. Further studies in reproduction-driven siRNAs seem to be worthwhile for tackling the problem of reproductive dysfunction (He et al. 2009; Zimmermann et al. 2014). There is a small clinical trial for studying regulatory siRNA, gametogenesis and infertility (NCT02864329) launched in early 2017.

Conclusion

Based on the experimental evidences, siRNA-based therapy is highly applicable for treatment of various inflammatory disorders, cancers and various diseases with gene dysregulation. Although the majority of siRNA clinical trials are at an early stage, preclinical experiments have revealed the power of siRNA in targeted therapy. Continuous improvement in structural modification will further enhance the pharmacological availability to develop new target therapies. New development of delivery systems will enhance the siRNA efficacy and bioavailability in vivo. In addition, the discovery of endo-siRNA controlling spermatogenesis and oogenesis is another valuable study. However, it will still necessitate further studies for antiviral treatments as species with a high mutation rate like influenza viruses could be a barrier for siRNA-based therapy. There is a long way for siRNA to be developed into a drug for human therapy. However, its immense potentials may allow us further insight and reveal a new pathway for human-targeted therapeutics.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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