Songklanakarin J. Sci. Technol.
34 (3), 293-301, May - Jun. 2012

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY
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Review Article

Overview of RNA interference therapeutics

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Received 21 December 2010; Accepted 13 May 2012

Abstract

Since the term RNA interference (RNAi) was coined in 1998, much knowledge about RNAi has accumulated. Presently, RNAi is known as a power tool for studying gene function as well as a potential therapeutic molecular technique for a wide range of disorders. This review discusses potential problem areas such as off-target effects, *in vivo* delivery and RNAi saturation, and also indicates solutions including the current stage of RNAi therapeutics.

Keywords: RNA inference, siRNA, off-target effects, delivery, RNAi therapeutics

1. Introduction to RNA Interference

RNA interference (RNAi) is a specific mechanism for controlling down-regulation of gene expression. It is evolutionally conserved in plants, *Caenorhabditis elegans*, *Drosophila melanogaster*, non-mammalian vertebrates and mammals (Bosher and Labouesse, 2000; Naqvi, *et al*., 2009; Poethig *et al*., 2006; Ramadan *et al*., 2007). The RNAi process is initiated by short double-stranded RNAs (dsRNAs) that lead to the sequence-specific inhibition of their homologous genes (Figure 1). These short dsRNAs (21-25 nucleotides in length) are normally produced in cells from cleavage of longer dsRNA precursors by the ribonuclease III (RNase III) family member Dicer (Zhang *et al*., 2004) and incorporated into a multi-component nuclease complex known as the RNA-induced silencing complexes (RISC), which has the splicing protein Argonaute-2 (Ago-2) (Hammond *et al*., 2000). Then, the single stranded RNA derived from the short dsRNA acts as a guide sequence (the antisense strand) directing the complex to the specific target mRNA by intermolecular base pairing, where a RISC-associated endoribonuclease silences the target mRNA (Bartel, 2004; Khvorova *et al*., 2003; Schwarz *et al*., 2003)

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In eukaryotic cells, two major types of short dsRNAs are present in the RNAi pathway, namely small interfering RNAs (siRNAs) and microRNAs (miRNAs). In more detail, siRNAs have a characteristic two nucleotides 3′ overhang, which are processed from larger dsRNAs by Dicer. The siRNAs are incorporated into RISC and the sense strand of the siRNA is removed in an ATP-dependent manner. The antisense strand of the siRNA perfectly pairs with its target mRNA, where RISC mediates endonucleolytic cleavage and subsequent degradation of the target RNA (Elbashir *et al*., 2001; Fire *et al*., 1998; Parrish *et al*., 2000) (Figure 1). On the other hand, miRNAs are initially processed from long primary transcripts (pri-miRNA) within the nucleus into 60-70 base-paired hairpins known as precursor miRNAs (pre-miRNAs) by the microprocessor complex, which consists of Drosha-DGCR8 (Han *et al*., 2004; Lee *et al*., 2003). Following processing by Drosha, the pre-miRNA is exported to the cytoplasm by the Ran-GTP dependent cargo transporter Exportin-5 (Bollman, *et al*., 2003). In the cytoplasm the pre-miRNA is processed by Dicer into the mature miRNA, which is incorporated into RISC. In much the same way that siRNA functions, the mature miRNA guides the complex to the target mRNA for translational repression or message degradation (Beverley, 2003). Notably, typical miRNAs are not perfectly matched to their mRNA targets and exert silencing through translational suppression (Ambros *et al*., 2003; Nelson *et al*., 2004) (Figure 1).

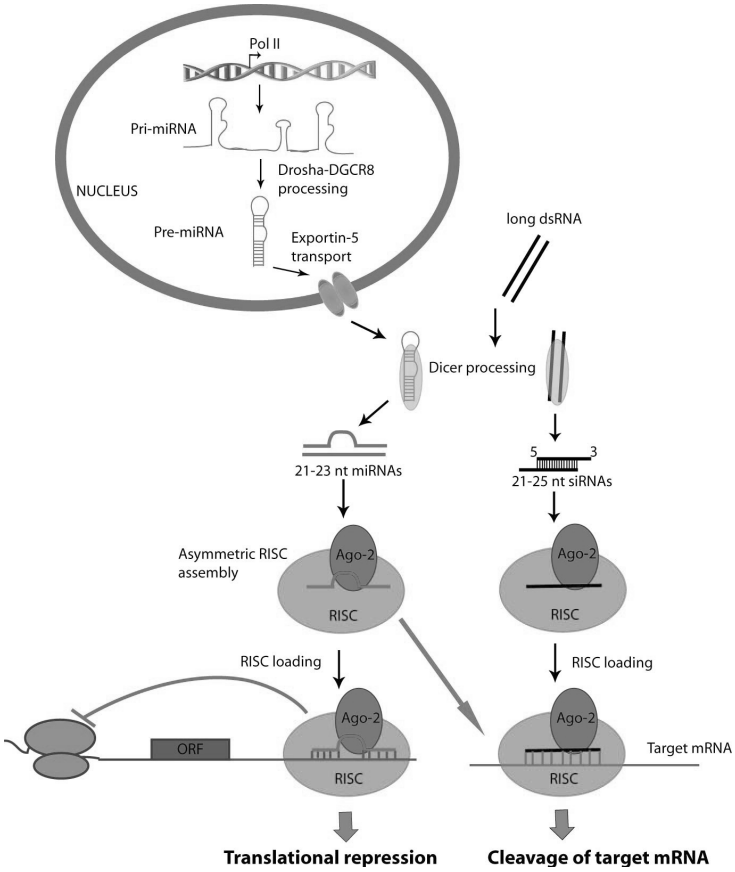

Figure 1. RNAi mechanism. The left hand side demonstrates of the mammalian miRNA pathway. The right hand side shows the pathway of synthetic siRNAs in mammalian cells (modified from Panjaworayan and Brown, 2011).

Currently, RNAi has become a powerful tool for reverse functional genomics. In addition, it has been employed as a potential molecular therapeutic method for combating a wide range of disorders including autoimmune diseases, metabolic disorders, viral infections, neurological diseases and cancer in many studies using mammalian tissue culture system or the mouse model. For example, the expression of mutant p53 or RAS genes that are observed in most tumour cells was specifically silenced in SW480 human colon cancer cells by siRNAs without affecting the wild type genes (Kawasaki and Taira, 2003). Target of siRNAs to human telomerase RNA inhibited telomerase activity in variety of human cancer cell lines (Kosciolek *et al*., 2003). Several pioneering studies have demonstrated great possibilities for using siRNAs for treating serious viral diseases that caused by HIV and HCV (Jakobsen *et al*., 2009; Lee *et al*., 2002; Wilson *et al*., 2003).

In addition, recent studies indicated that some miRNAs are linked to several human diseases including viral and metabolic diseases. Therefore, inhibition or interference of miRNAs function could potentially be a new therapeutic approach (McBride *et al*., 2008).

Despite the numerous successful studies of RNAi on inhibition of specific genes in the mammalian tissue culture system, inefficient delivery system, poor intracellular uptake and off-target effects have impeded RNAi therapeutics. Albeit clinical trials with RNAi have now begun, challenges such as off-target effects, toxicity and the need for safe and efficient delivery methods have to be overcome before using RNAi for gene therapy.

2. RNAi and Gene Therapy

In medical sciences, the term gene therapy has been indicated for more than 10 years. Several innovative therapeutic modalities have been investigated including numerous potent drugs such as anti-sense, ribozymes and the use of regulatory elements for reversing malignant phenotypes (Pan *et al*., 2009; Quon and Kassner, 2009; Schmidt, 2009). Clinical studies of current gene therapy have experienced significant obstacles such as the unexpected frequency of major side effects, inefficient gene delivery method and the high cost of the therapy (Aagaard and Rossi, 2007; Li and Shen, 2009).

For RNAi, the specificity of mechanism, large scale silencing and a natural defence mechanism are attractive criteria for being a good molecular therapeutic method (Aagaard and Rossi, 2007; Ebbesen *et al*., 2008; Inoue *et al*.,

2006). The concept of successful RNAi therapeutics is based on 3 main conditions: (i) lack of toxicity, (ii) specificity and (iii) efficacy (usually measured as half-maximal inhibition levels or IC_{50} values) in *vitro* and *in vivo* (Ichim *et al*., 2004; Takeshita and Ochiya, 2006; Vorhies and Nemunaitis, 2007). The major challenge in RNAi gene therapy is the delivery of siRNAs or miRNAs to the desired cell type, tissue or organ.

2.1 Delivery of RNAi

Most of reviews classify delivery of RNAi based on the delivery systems: viral and non-viral methods. As these two systems are selectively used for molecules that are carried to trigger RNAi pathways, this review article describes two basic strategies used to activate RNAi pathways: (i) a RNA based - approach by delivery of synthetic 21 base siRNA duplex (Figure 1) and (ii) a DNA-based method in which the active siRNAs are generated from longer RNA hairpin transcripts that are transported to the cytoplasm via the miRNA machinery and are processed into active siRNAs by Dicer (Figure 2).

1) Delivery of siRNA duplexes

Cellular delivery of synthetic siRNA duplexes is usually achieved by cationic liposome - based strategies. Liposome and synthetic siRNAs are complexed *in vitro* and taken up by cells via the endosomal pathway, Then, siRNAs are released into the cytoplasm where they associate with RISC (Sioud and Sorensen, 2003). Although this approach is considered to be passive and its lack the ability to target specific cells or tissue, it provides a safer delivery compared to intravenous injection or local administration of naked siRNA. Naked siRNA delivery for therapeutic purposes is ineffective due to the instability of siRNA, low bioactivity and high dosage requirement of siRNA. Moreover, naked siRNA is incapable of crossing the blood-brain barrier.

The transient transfection of liposome-siRNA complex typically shows effects of gene silencing for 3-5 days in culture cells whereas the effects could be sustained for several weeks in non-dividing cells (Omi *et al*., 2004; Song *et al*., 2003). As mentioned above, the disadvantage of this approach *in vivo* is the rapid liver clearance and lack of target specificity (Jones, 2009; Sioud and Sorensen, 2003). To improve *in vivo* stability of siRNA duplex, backbone of siRNA is chemically modified and linked to molecules such as 2′F, 2′O-Me and 2H. As a result, such molecules show an improved stability of siRNAs in serum and do not reduce RNAi efficiency (Shiraishi *et al*., 2008; Watts *et al*., 2007). Alternatively, *in vivo* delivery methods for siRNAs include the use of Atelocollagen (Minakuchi *et al*., 2004), conjugation of cholesterol to the siRNA sense strand (Han *et al*., 2008), binding of antibody-protamine fusion to siRNAs (Song *et al*., 2005), aptamer-siRNA conjugates (McNamara *et al*., 2006), and cyclodextrin nanoparticles (Hu-Lieskovan *et al*., 2005). The approaches target siRNAs to specific tissue or cell type and could potentially be translated into clinical studies.

2) Delivery of short hairpin RNAs (shRNAs)

Since production of synthetic siRNA duplex is costly, DNA-based expression cassettes are alternatively used to generate the functional siRNA in cells. Like siRNA duplex

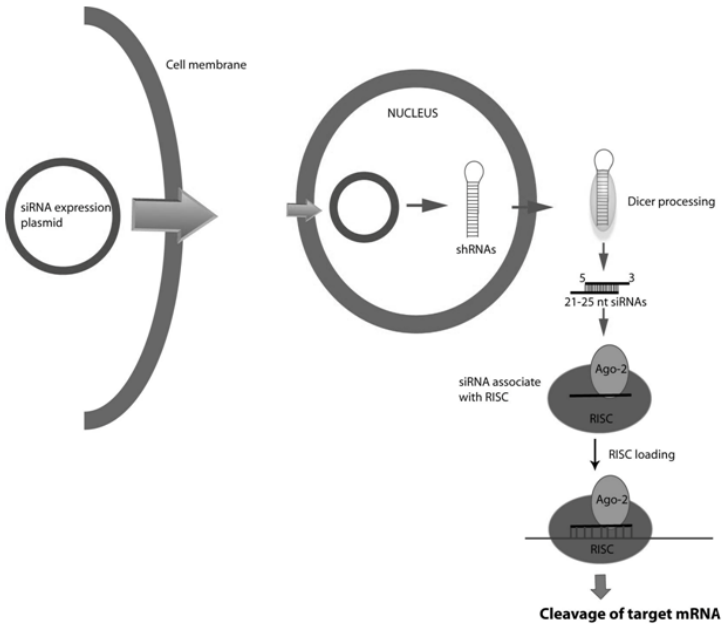

Figure 2. Schematic diagram indicates induction of RNAi pathway by DNA based expression vectors designed to express short hairpin RNAs (shRNAs)

delivery, DNA-based shRNA plasmids do not readily cross the cellular membrane because of their negative charge. Therefore, they require facilitating carriers such as cationic liposome, Atelocollagen or viral vectors. Presently, there are a number of viral vectors available. Each type of viral vector has specific characteristics that need to be determined for the specific therapeutic target. The adenovirus and adeno-associated virus (AAV) derived vectors provide an efficient delivery vehicle for transient shRNA expression (Gao *et al*., 2004). Particularly, the Ad-gutless vector is used for liver directed systemic delivery with prolonged silencing effects (Hosono *et al*., 2004) while a conditionally replicating adenovirus (CRAd) is designed to replicate and kill tumour cells specifically (Carette *et al*., 2004). Retroviruses on the other hand provide a major advantage to incorporate the transgenic siRNAs into the host cell genome. Potential retroviral vectors used for RNAi therapeutics are Moloney murine leukaemia virus (Mo-MuLV) and lentivirus such as human immunodeficiency virus (HIV), feline immunodeficiency virus (FIV) or equine infectious anaemia virus (EIAV) (Poeschla, 2003). Several studies indicated that the Mo-MuLV and lentivirus based-vectors are efficient delivery system, which can significantly silence expression of target genes in a specific manner (Amendola *et al*., 2009; Frka *et al*., 2009; Sun and Rossi, 2009; Ye *et al*., 2009).

Despite the high transfection efficiency demonstrated by viral systems, this approach raises safety concerns for human use because of the associated oncogenic potential and immunological complications.

Additional examples of recent RNAi mediated approaches against viral infection, cancer and metabolic diseases are summarized in Table 1.

2.2 Off-target effects

Despite the specific mechanism of RNAi, some studies reported that siRNAs can have off-target effects. For example, strings of dsRNAs (pri-siRNAs/pri-miRNAs) can trigger non-specific cellular innate immune response such as the interferon response. Cullen (2006) demonstrated that longer dsRNAs (more than 30 nucleotides in length) could induce interferon response by binding to double-stranded-RNA-activated protein kinase (PKR), 2′, 5′-oligoadenylate synthetase-RNase L system or several Toll-like receptors (TLRs) (Hornung *et al*., 2005). Therefore, the use of longer dsRNAs raises concern over the risk of increased immune stimulation. Analysis of interferon response can be done by checking the level of expression of an interferon-response gene such as oligoadenylate synthase-1 (OAS1) (Bridge *et al*., 2003; Fish and Kruithof, 2004). In addition, saturation of the RNAi machinery due to high concentration of shRNA transfection is reported to cause toxic non-specific effects. Competition assays showed that over-expression of shRNA inhibited miRNA function and saturated the Exportin 5 pathway. Therefore, it is important to transfect the minimum amount of the siRNA duplex to eliminate the off-target effects (Cullen, 2006).

3. Clinical Trials for RNAi Therapies

The process of new drug development begins with extensive pre-clinical studies, which involve *in vitro* and *in vivo* experiments for obtaining pharmacokinetic information including efficacy and toxicity of the new drug. Pre-clinical studies of RNAi therapeutics have been widely conducted against cancer using the mouse model. For example, siRNAs were used to silence the colorectal cancer-associated gene beta-catenin, the oncogene H-ras to inhibit tumour growth of human ovarian cancer, the oncogenic K-ras to inhibit cancer cells (Brummelkamp *et al*., 2002; Liu *et al*., 2007). Results from pre-clinical studies showed that the siRNAs were sufficiently selective as they only silenced expression of tumour genes. The results therefore hold promise for further RNAi therapeutic development.

According to the U.S. National Institutes of Health (NIH), clinical trials for new drugs can be classified into five types based on purpose of the trials: prevention trials, screening trials, diagnosis trials, treatment trials and quality of life trials (The US National Institutes of Health, 2007). They are conducted following four phases. Phase I trials treat a small group of people (20-80) for determining a safe dosage range and identifying side effects. Phase II trials treat a larger group of people (100-300) for evaluating efficacy and safety. Phase III trials study a large group of people (1,000-3,000) to verify the effectiveness of the drug and compare its effects with current conventional drugs. Phase IV trials consist of post-approval studies involving safety surveillance such as risk-benefit analysis and optimal usage (National Cancer Institute). The process of new drug development will normally proceed through all four phases over many years. Clinical trials for RNAi therapies have already begun and they belong to the category of treatment trials.

The first application of RNAi therapy is for age-related macular degeneration (AMD) using siRNAs to inhibit the vascular endothelial growth factor (VEGF) pathway that causes abnormal growth of blood vessels behind the retina. This treatment is designed to be administered directly to the eye (Takeshita and Ochiya, 2006). In addition, the RNAi therapies have been extended to investigate infectious disease from viruses such as hepatitis C (HCV), HIV and Rous sarcoma virus (RSV). Examples of current trials for RNAi therapy is summarised in Table 2.

Subsequently, the results of these trails will address whether RNAi therapeutics cause unpredictable side effects.

4. Conclusion

The RNAi pathway has emerged as a powerful tool for the study of gene function as well as a new promising therapeutic approach. Despite challenges such as off-target effects, toxicity and the need for safe delivery methods, RNAi therapeutics appears to hold promise in the treatment of dis-

Table 1. RNAi used for inhibiting specific genes that link to cancer or human disorders

Target genes	RNAi expression system	Delivery methods	Models	Effects/ Duration	References
Enhancer of zeste homolog 2 (EZH2) in tumorigenesis and liver metastasis of pancreatic cancer	Pol III promoter-shRNA plasmid expressing vectors	Lentiviral expression system	- Human pancreatic cancer SW1990 and PANC-1 cells. - Athymic nude mice.	RNAi-mediated knockdown of EZH2 expression can inhibit tumour growth in the mouse model. Duration: 45 days	(Chen *et al*., 2010)
Human apurinic or apyrimidinic endonuclease/redox factor-1 gene (APE1/Ref-1) associated with human pancreatic cancer	Chemically synthesized small interfering RNA (siRNA)	Lipofectamine 2000 (Invitrogen)	Human pancreatic cancer, SW1990 cells	Down regulation of APE1/Ref-1 gene expression significantly sensitize the SW1990 cells to gemcitabine and enhance cell apoptosis Duration: 3 days	(Xiong *et al*., 2010)
C-MYC gene associated with gastric tumour	A vector-based siRNA system	Liposome reagent	Human gastric cancer cell SGC7901 and the gastric cell line HFE145	Down regulation of C-MYC can restrain the growth and proliferation of gastric cancer cells Duration: 7 days	(Zhang *et al*., 2010)
Bcl-2 gene associated with tumour cells	Human telomerase RT promoter expressing mi-Bcl2	Lipofectamine 2000, Invitrogen)	Tumour Lung cell lines A549,Hela-S3 and HepG2	Down regulation of Bcl-2 and induction of apoptosis Duration: 7 days	(Zhang *et al*., 2009)
Insulin-like growth factor-I receptor (IGF-IR) in colon cancer	ShRNA plasmid expressing vectors	Liposome (FuGene6, Roche)	Human colon cancer cell line SW480	Reduction of IGF-IR and inhibition of tumour growth Duration: 2 days	(Yavari *et al*., 2009)
Amyloid precursor protein (APP) in Swedish variants	Short-hairpin RNA (shRNA)	Recombinant adeno-associated virus	Transgenic mouse model	Reduction of soluble Ab peptide Duration: 35 days	(Rodriguez-Lebron *et al*., 2009)
Influenza M2 gene	H1-promoter-driven shRNA cassettes	Recombinant Lentiviral vectors	Madin-Darby cannie kidney and Human embryonic kidney 293T cells	Inhibition of viral replication. Duration: 3 days	(Sui *et al*., 2009)
NS5-1, NS5-2, E NS1 genes in Yellow Fever Virus	H1-promoter shRNA expression plasmid	Lipofectamine 2000, Invitrogen)	Vero E6 cells and mouse model	Inhibition of YFP replication Duration: 10 days	(Pacca *et al*., 2009)
Anti-hepatitis B virus pre-miR DNA	Pol II cassettes encoding primary (pri)-miR-31	Lipofectamine 2000 (Invitrogen) and injection	HuH-7 cells and mouse model	Inhibition of HBV replication Duration: 5 days	(Ely *et al*., 2008)

ease along with more conventional approaches.

Acknowledgement

NP is funded by the Research Grant for New Scholar (co-funded by TRF and CHE, Grant Number: MRG5380104); The Kasetsart University Research and Development Institute Grant (Grant number: 45.53) and ScRF Grant from Faculty of Science, Kasetsart University (Grant number: ScRF-E13/ 2553)

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Table 2. Example of current trials for RNAi therapy (The US. National Institutes of Health, 2012)

Condition	Target	Drug/ intervention	Sponsor	Status
Age-related macular degeneration; choroidal neovascularization	Vascular endothelial growth factor receptor-1 (VEGFR-1)	AGN211745, modified siRNA Sirna duplex - Single intravitreal injection	Therapeutics, Inc	Completed phase I, II
Pachyonychia Congenita (keratin disorder)	Pathogenic mutation in keratin K6a	TD101, siRNA duplex - Injection into a callus on the bottom of patient's feet	Pachyonychia Congenita project	Completed Phase 1
Cancer/Solid tumour	M2 subunit of ribonucleotide reductase (R2)	CALAA-01, targeted nanocomplex that contains anti-R2 siRNA - Administration	Calando Pharmaceuticals	Active phase I
Diabetic Mascular Edema	VEGF	Bevasirnanib (or Cand5), modified siRNA duplex with 2 deoxyribose at the 3' end	Opko Helaths, Inc.	Completed phase II
Advanced cancer Metastatic cancer Solid tumour	Stahmin1/oncoprotein 18 (STMN1)	Pbi-shRNA^{TM} STMN1 LP, shRNA expression plasmid complex with bilamella invaginated vesicle - A single intratumoral injection	Gradalis, Inc	Active phase I
HCV- infection	HCV gene	SPC3649, mir-122 - Administration 5 weekly dose	Santaris Pharma A/S	Completed phase I
HIV-1 infection	HIV Tat protein, HIV TAR RNA, human CCR5	pHIV7-shl-TAR-CCR5RZ, lentivirus vector-expressed RNAi in autologous T- cells of HIV-patents	City of Hope Medical Centre	Terminated phase 0
RSV	RSV nucleocapsid	ALN-RSV01, modified siRNA duplex - Administation by nebulization once daily for 3 days	Alnylam Parmaceuticals	Completed phase II

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