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review

RNA Interference as a New Tool in Therapeutics

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Summary

The basic principle of RNA interference, a possible new therapeutic tool, involves destruction of messenger RNA upon interaction with homologous double-stranded RNA present in the cell cytoplasm. Studies have shown that both viral and non-viral small interfering RNA delivery methods and delivery of chemically synthesized small interfering RNAs to the cell can provide selective gene suppression through this mechanism, both *in vitro* and *in vivo*. Before becoming a functional therapeutic tool, there are a number of problems concerning RNA interference that should be solved. Major problems involve off--target effects, insertional mutagenesis and malignant transformation, as well as problems of delivery methods and reduction of toxicity.

Key words: RNA interference, small interfering RNA, off-target effects, therapeutics

Introduction

Using genomic information as a target for drug therapy has always been and still is a great challenge to pharmaceutical companies. The main problem, however, is how to determine the gene products functionally involved in the pathology of a certain disease and how many genes are involved. Application of RNA interference (RNAi) in solving this problem started after the discovery of an evolutionary conserved mechanism of posttranscriptional gene silencing by double-stranded RNA (dsRNA) in a range of eukaryotic organisms: plants, *Neurospora crassa*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and mammals (*1*).

Mechanism of RNAi

In mammalian cells, naturally occurring posttranscriptional gene silencing is mainly mediated by microRNA (miRNA). Double-stranded miRNAs are processed into short dsRNAs through a cascade of biochemical events involving the cytoplasmic ribonuclease III (RNase III)-like protein Dicer and the RNA-induced silencing complex (RISC). Dicer enables the formation of small RNA duplexes, 19–25 base pairs long, with characteristic 3′-dinucleotide overhangs, a 5′-monophosphate and a 3′--hydroxyl group, but requires the cooperation of dsRNA binding proteins to complete its function. Homologues of this protein can be found in *Saccharomyces pombe*, *C. elegans*, *D. melanogaster*, plants, and mammals. A product of Dicer, small dsRNA, consists of two strands: the guide or antisense strand, which is complementary to the target sequence and will trigger its recognition by RISC, and the passenger or sense strand, which provides stability to dsRNA. To choose which of the two strands will be given the function of the guide strand, the dsRNA product of Dicer cleavage is handed off to Argonaute 2 protein (Ago2), a Dicer-interacting protein and part of RISC, which then cleaves the passenger strand, dissociating it from the RISC. In the same way, a small interfering RNA (siRNA) duplex of exogenous origin is incorporated into the RISC, whereupon an ATP-dependent helicase unwinds the duplex, enabling one of the strands to independently recognize messenger RNAs (mRNAs). RISC carries out the final gene silencing step. Ago2 is its catalytic or slicer component. Two different domains in

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Ago2, Piwi domain (named after the Piwi protein in *D. melanogaster*) and Piwi-Argonaute-Zwille (PAZ) domain, bind the 5′ and 3′ ends of the guide strand. The 5′ end bound to the Piwi domain determines target recognition and the site of cleavage. Nucleotides 2–8 of the guide strand, known as the seed sequence, initiate pairing with the target mRNA. Ago2 cleaves the mRNA between the nucleotides complementary to bases 10–11 of the guide strand. The generated 5′ and 3′ mRNA fragments are then degraded by different exonucleases (Fig. 1). The 3′ end of the guide strand bound to the PAZ domain of Ago2 plays no essential role in target recognition but provides a helical geometry required for the catalytic step. The presence of mismatches in the 3′ half beyond nucleotide 11 slows down the rate of mRNA cleavage (*1–5*).

Naturally Occurring miRNA

Naturally occurring miRNAs are synthesized in the nucleus from miRNA genes, which are transcribed by RNA polymerase II into long RNA, known as primary miRNA (pri-miRNA). Pri-miRNA contains an 80-nucleotide long hairpin flanked by single-stranded RNA sequences of a few hundred nucleotides. Pri-miRNAs are recognized by a complex formed by the DiGeorge syndrome critical region 8 (DGCR8) protein and Drosha in the nucleus. DGCR8 is a dsRNA-binding protein that helps identify the cleavage site, whereas Drosha is a RNase III that cleaves the pri-miRNA to generate a 70--nucleotide long hairpin RNA known as miRNA precursor. Drosha products have typically 2 or 3 nucleotide 3′ overhangs that facilitate the transport of miRNA precursors to the cytoplasm where they are further processed by Dicer.

When planning therapeutic RNAi experiments, several features of the nuclear processing of miRNA have to be taken into consideration. One of the prospects in RNA therapeutics design is to establish specific structural requirements that imitate endogenous pri-miRNA in order to ensure their recognition by DGCR8 protein and processing by Drosha. Inadequate design and there-

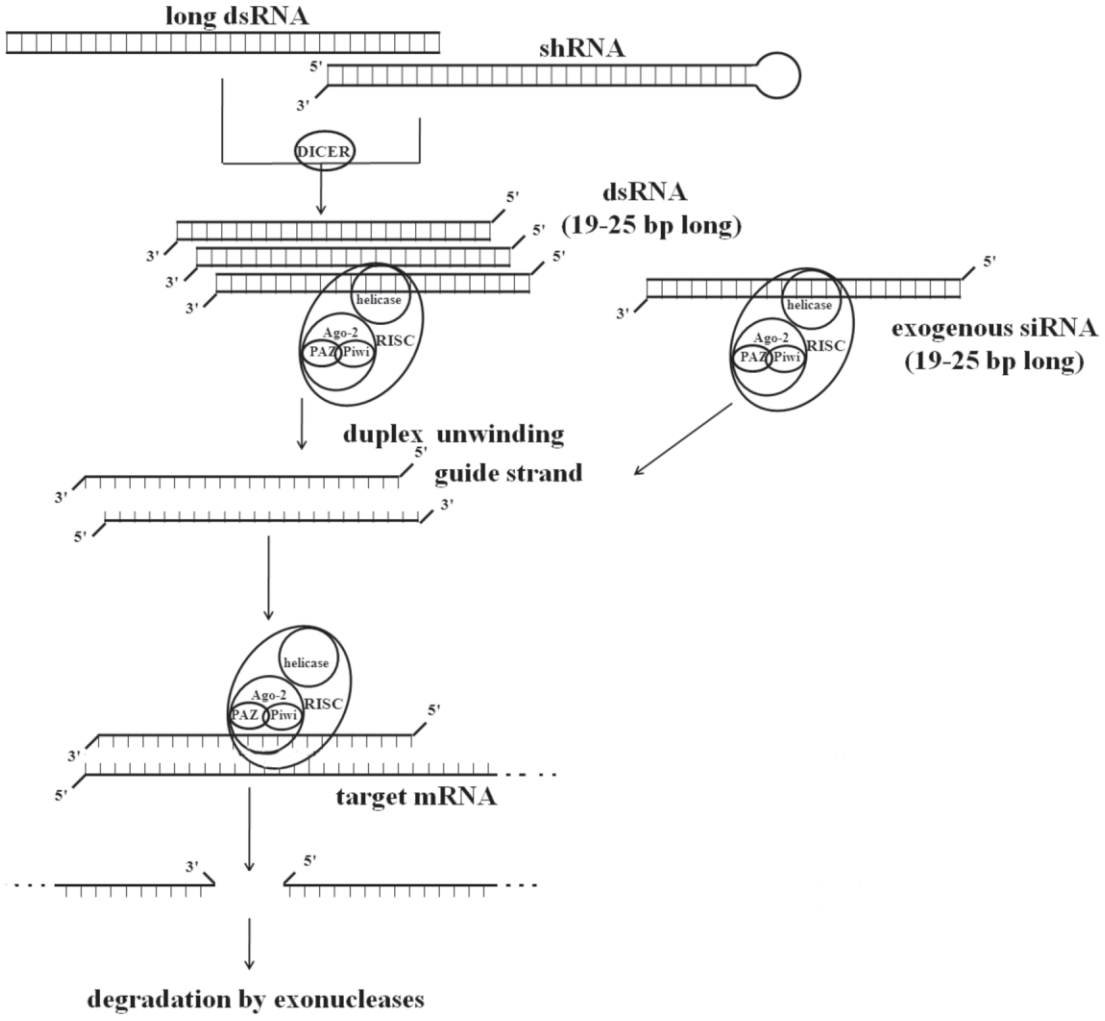

Fig. 1. RNAi can be achieved with any long dsRNA of endogenous (miRNA) or exogenous origin (shRNA). Long dsRNAs are processed into short dsRNAs through the activity of Dicer, an endoribonuclease in the RNase III family. Small RNA duplexes 19–25 base pairs long, as well as exogenously introduced siRNAs, are further incorporated into the RNA-induced silencing complex (RISC), whereupon an ATP-dependent helicase unwinds the duplex. Domains in Argonaute 2 protein (Ago2), Piwi protein and Piwi-Argonaute-Zwille (PAZ) domain bind the 5′ and 3′ ends of the guide strand and enable binding to the target mRNA and its cleavage. The 5′ and 3′ mRNA fragments generated are then degraded by different exonucleases

by inefficient nuclear processing of a hairpin would end in nuclear accumulation of non-processed transcripts and ineffective silencing of the target (*2*,*6*,*7*).

Antisense Molecules *vs.* RNAi Technology

The first major experiments in RNA gene therapy were performed with single-stranded antisense molecules (oligodeoxynucleotides). Although the final result, the degradation of target mRNA, is identical, the mechanism of gene silencing by antisense molecules and siRNAs or short hairpin RNAs (shRNAs) in mammalian cells is different (*8*). Antisense molecules are synthetic single-stranded oligonucleotides that are usually composed of DNA sequences capable of Watson-Crick base pairing to a complementary sequence in the target mRNA. Translation of target mRNA into protein is either blocked by steric interruption of binding to the ribosome, or by cleavage of the mRNA through the activity of endonucleases RNase H or RNase L (*9*,*10*). Parallel delivery of antisense oligodeoxynucleotides and siRNA, both targeted to the coding region of a gene, showed greater stability as well as efficiency in the case of siRNA.

Until 2009, however, only a few of the antisense oligonucleotides entered clinical trials as therapeutic opportunities. One of them, ISIS 301012 (mipomersen), a human specific apolipoprotein B-100 (apoB-100) antisense inhibitor, is currently in Phase 3 clinical development for treating hypercholesteremia. ApoB-100 is a protein component of atherogenic lipids and triglycerides, including LDL cholesterol, synthesized and packaged into lipoprotein particles in the liver of different species. LDL cholesterol is one of the risk factors of coronary heart disease and atherosclerosis and therefore a good target for antisense inhibition. Administration of ISIS 301012 produced remarkable reductions in mRNA and liver proteins, resulting in a decrease of serum apoB-100, LDL cholesterol and total cholesterol. Delivery was tested on multiple animal species, including mice, hamsters, rabbits and monkeys, before antisense inhibitor was applied in treating human hypercholesteremia (*11*).

To date, the only antisense oligonucleotide that has been approved by the U.S. Food and Drug Administration (FDA) for use in therapeutics is Vitravene (fomivirsen sodium intravitreal injectable), designed to treat cytomegalovirus (CMV) infections in CMV retinitis, the most common opportunistic infections in patients with acquired immunodeficiency syndrome (AIDS). Vitravene is a 21-base synthetic phosphorothioate oligonucleotide designed to be complementary to mRNA that encodes CMV proteins for the major immediate-early region (IE2). Binding to this location leads to specific inhibition of gene expression of essential viral proteins, but only in the eye into which it is injected. Vitravene is not designed for treating CMV anywhere else in the body (*12*,*13*).

RNAi with siRNA and shRNA

Specific and effective silencing of genes by RNAi can be achieved in two ways: by using siRNA, up to 25 nucleotides long, and by using shRNA. However, one has to be cautious because unlike in *C. elegans* and *D. melanogaster*, where gene silencing can be achieved using long (>500 bp) dsRNA without any adverse effect, dsRNA of >30 bp can trigger a γ-interferon response in mammalian systems. The interferon response, which is otherwise an important defence mechanism against viral infection, can result in global repression of translation.

Some other problems, regardless of immunological responses and specificity, need to be resolved when using RNAi *in vivo*. For example, in contrast to *C. elegans*, where RNAi effects are stable, long lasting, and are passed on to the offspring, gene silencing by transfected siRNA duplexes in mammalian cells is temporary. Gene silencing is dependent on the number of siRNA molecules introduced into cells and the number of duplexes per cell decreases as cells divide. For this reason, the silencing of targeted genes by synthetic siRNA is usually limited to 2 weeks, or even only 3–7 days in rapidly dividing cells. In most diseases, especially cancer, which is characterized by fast and uncontrollable division of cells, persistent suppression of the targeted gene would be necessary to prevent disease progression. The use of siRNA would therefore require repeated transfection of cells with siRNA. To avoid this problem, a vector-based system for the introduction and stable expression of siRNA in target cells has been developed. These DNA vectors contain an RNA polymerase III promoter and a transcription termination site. After their transcription, by cellular machinery, the RNAs fold in the shape of shRNAs with a stem-loop structure, which are later cleaved by Dicer to produce 19–25 nucleotide siRNAs. As the DNA template for shRNA does not have the structural characteristics of pri-miRNA, it does not undergo Drosha processing in the nucleus. Once they are processed by Dicer and incorporated into RISC in the cytoplasm, 19–25 nucleotide siRNAs lead to target cleavage, the target being mRNA (Fig. 1) (*10*,*14*,*15*). As siRNAs have become more widely used, the basic structure of effective siRNAs has been defined as a duplex with a 2-nucleotide overhang on the 3′ ends in compliance with miRNA processing. It has also become evident that the effectiveness of siRNA silencing is sequence specific.

Design, Stability and Delivery of siRNA

Using a bioinformatics programme, the potential target for RNAi should be identified on the basis of its role in the disease process. Another prerequisite is that its suppression should not be harmful. Once a specific gene has been selected, a targeting sequence has to be identified to achieve effective silencing with minimal off-target effects. First, it is important to select a sequence that is unique to our targeted gene by using the Basic Local Alignment Search Tool (BLAST). Genes that contain significant matches with the selected region should be monitored to verify that they are not being silenced. Another tip is to select sequences displaying a thermodynamic profile that favours incorporation of the guide strand into the RISC (*16*,*17*). Most researchers screen a minimum of 4 or 5 different target sequences for each gene before choosing the most effective one. However, even sequences that display efficacy in screening experiments might lead to unexpected toxicity *in vivo*, suggesting that more than one therapeutic con-

struct should be selected for therapeutic trials in animal models. That is why two or three different sequences for siRNA target site are synthesized; the most specific and effective siRNA sequence must be validated by measuring the levels of the target mRNA or protein *in vitro* (*2*,*18*).

siRNA should be generally designed in such a way as to be fully complementary to its target mRNA. First, a few parameters affecting the siRNA or shRNA function have to be examined in the design process. The guanine-cytosine (GC) content of a siRNA duplex is a parameter that might correlate with siRNA functionality. Too high GC content may slow down duplex unwinding and might be associated with a prohibitive secondary structure of the target mRNA. Guanine-rich RNA sequences can also form Hoogsteen-paired quartets of G residues, or the so-called 'tetrads', which cause difficulties during RNA synthesis and purification. Too low GC content, on the other hand, may reduce the efficiency of target mRNA recognition and hybridization. Analysis of the biochemistry and mechanisms of RNAi revealed that base pair mismatches introduced at the 5′ end of siRNA improved gene silencing. Also, duplex end asymmetry, in the form of differences in adenine-uracil (AU) content between the three terminal nucleotides of the 3′ and 5′ ends, rather than duplex properties, correlated most strongly and highly significantly (p=0.005) with siRNA functionality (*16*,*17*).

Although siRNA molecules appear to be more resistant to nuclease degradation than antisense molecules, some serum nucleases can degrade siRNAs. As a result, many research groups have investigated the use of chemical modifications that improve stability and protect against nuclease degradation. Several groups showed that boranophosphate-modified siRNAs were 10 times more nuclease resistant than unmodified siRNAs (*19*). Along with successful siRNA design, efficient delivery is another essential characteristic of gene silencing in mammalian systems. Chemically synthesized siRNAs, viral or non-viral vectors expressing shRNA, can be delivered locally or systematically to silence the target gene *in vivo*. Chemical modifying of siRNAs renders them resistant to RNAse digestion and extends their half-life. Modifications reported in the literature include capping the 5′ end, modifying the ribose sugars or substituting phosphorothioates, 2′-*O*-methyl-RNA nucleotides at the 5′ end and four methylated monomers at the 3′ end. Complexing siRNA or shRNA expressing plasmids with cationic polymers or peptides or incorporating siRNAs into liposomes and nanoparticles have been proposed to extend the half-life of siRNA (*3*,*15*,*20*).

Delivery of siRNA or shRNA can also be achieved by using viral vectors. Thus far, retroviral, lentiviral and adenoviral vector systems for RNA delivery have been developed. Retroviral vectors are based on the murine stem cell virus or Moloney murine leukaemia virus and permit a stable introduction of shRNA into dividing cells. Lentiviral vectors are derived from the human immunodeficiency virus (HIV)-1 and can infect both dividing and nondividing postmitotic cells. Adenoviral vectors based on adeno-associated viruses can infect both dividing and nondividing cells and since they integrate a site specifically into the AASV1 region of chromosome 19, they are safer than retroviral or lentiviral vectors, which are associated with insertional mutagenesis (*1*,*21*). The disadvantages of adenoviral vectors are the lack of tissue tropism and the dose-limiting hepatoxicity (*2*).

In general, two routes of *in vivo* siRNA and shRNA delivery can be distinguished: local (intranasal, intravitreous, intratracheal, intracerebral, intramuscular, intratumoral) or systematic (intravenous, intraperitoneal) (*22*). Both RNA types can be delivered to the cell using either route. The choice of using one or the other means of delivery depends on the targeted tissues as well as on the disease. Electroporation and topical gels are techniques commonly used for local siRNA delivery; the former is most commonly used for administration into liver and brain. Electroporation has also been used to deliver siRNA into the kidneys, eyes, muscles and skin of rodents. In a study done by Akaneya *et al.* (*23*) electroporation was performed using two needle electrodes inserted *in vivo* into a restricted brain region of a rat. Although this method, like other local delivery methods, brings a benefit in controlling 'off-target tissue' effects, it has one major disadvantage: the use of high voltage in attempting to increase uptake efficiency.

Topical gels, as a means of siRNA delivery to cells, could open the way for dermatological applications, as well as the treatment of cervical cancer (*1*,*24*).

The first systemic delivery of siRNA *in vivo* was carried out using the hydrodynamic technique of inserting naked siRNA in a large volume of physiological solution under high pressure into the tail vein of mice (*18*,*25*). A number of different approaches have been developed for the *in vivo* delivery of siRNA, all with the same aim: to improve cellular uptake and site-specific delivery.

Compared to local delivery methods, systemic delivery has the same purpose, to reach specific disease tissues, and to overcome other problems, such as potential serum degradation.

Thus far, atelocollagen has been shown to be the best harmless biomaterial for *in vivo* siRNA delivery. Atelocollagen is obtained from type I collagen of calf dermis by pepsin treatment. At the N- and C-terminals of collagen molecules there is an amino acid sequence called telopeptide; it contains most of collagen antigenicity. Atelocollagen obtained by pepsin treatment is low in immunogenicity because it is telopeptide-free. The surface of atelocollagen molecules is positively charged; molecules can therefore bind electrostatically with negatively charged nucleic acid molecules. Furthermore, it is believed that siRNA complexed with atelocollagen is resistant to nucleases and is transduced efficiently into the cells, thereby allowing long-term silencing (*18*). Chitosan is another material used for siRNA delivery. It is a naturally occurring cationic polysaccharide, biocompatible, noninflammatory, nontoxic and biodegradable (*4*). Alternatively, polyethylenimine (PEI) is used because of its ability to form non-covalent interpolyelectrolyte complexes with DNA or RNA. During investigations, siRNAs complexed with PEI are protected against degradation in the presence of nucleases but retain full biological activity (*20*). PEI, as a substance foreign to the living cell, bears a certain level of toxicity and this has to be taken into consideration (*10*). In some studies, however, siRNAs

have been conjugated with derivates of cholesterol, lithocholic or lauric acid and all these biological substances seemed to stabilize siRNAs in a way that they facilitate their binding to human serum albumin and increase their uptake by the liver (*26*). Also, cell-penetrating peptides covalently or noncovalently attached to siRNA molecules have provided an efficient uptake mechanism of complexes through the process of internalization, which is somehow different from endocytosis (*27*).

Nonspecific Effects of Using RNA Interference

Off-target activity is the main problem of RNAi technology. This is due to the fact that siRNA can target more than one mRNA molecule because it can tolerate several mismatches when pairing. While a single mismatch between siRNA and its desired target will only reduce specific silencing efficiency, the same siRNA may still be able to downregulate the expression of non-targeted genes that contain regions of partial complementarity. Another important consideration is the case when, experimentally, a restricted portion of a particular mRNA is targeted by RNAi; siRNAs corresponding to other portions of mRNA can be detected as well. This phenomenon is called transitive RNAi and has been observed in *C. elegans* and in plants. In *C. elegans*, these secondary siRNAs are homologous to sequences upstream of those targeted by the primary trigger. However, besides off-target downregulations, upregulations have also been observed *in vitro* (*4,28*).

Possible Use of RNA Interference

One of the most exciting opportunities offered by RNAi is the ability to identify all the genes required for certain physiological processes using the genome-wide RNAi screens. Cancer, as a condition characterized by a dense network of signal-transducing pathways, is in the centre of interest in RNAi usage. A major problem is that oncogenes and oncosuppressors involved in cancer do not contribute equally to carcinogenesis, making it difficult to identify the key genes whose blockage would lead to tumour cell death. RNAi technology is the most prospective tool that could help uncover the role of functional genes in cancer development and could also serve as potential therapeutic strategy because of its high efficacy and specificity in the down-regulating gene expression. However, in most cancers, blockage of a single gene does not suffice to eliminate the disease and usually multiple pathways are supposed to be inhibited simultaneously in order to prevent malignant proliferation. Target genes most widely investigated by researchers are receptors associated with certain mitogenic pathways known to be involved in malignancy. More relevant examples are the protein tyrosine kinase (PTK), a member of the receptor tyrosine kinase pathway, and the adenomatosis polyposis coli (APC) protein, a member of the Wnt signalling pathway (*1,29*). Among other cancer genes, genes that encode multidrug-resistance (MDR) protein, telomerase, and an integral membrane protein named B-cell lymphoma 2 (Bcl-2) protein are further examples of genes that are not mutated in cancer but are overexpressed in a variety of cancers and are representative targets for silencing. Thus far, initial *in vitro* studies have demonstrated effective silencing of a wide variety of mutated oncogenes, such as Kirsten ras (K-Ras), which encodes proteins with GTP-ase activity, human epidermal growth factor-like receptor 2 (Her2/neu), and bcr-abl oncogene that causes Philadelphia-chromosome-positive (Ph+) leukaemia (*1,18,25*). RNAi can also be directed against several genes involved in cell cycle control in order to promote tumour cell apoptosis by preventing cell division. Major attempts at RNA silencing have also been made on the retinoblastoma tumour suppressor (RB) and p53 protein. p53 is a transcription factor considered to be involved in the control of the cell cycle (cell proliferation, cell division, apoptosis). It is mutated in almost one half of cancers. Cellular senescence as well as tumour-host interactions are other processes that can be targeted through RNAi.

Among the most important targets in this area are the vascular endothelial growth factor (VEGF) and the VEGF-receptor, which have a major role in angiogenesis (*26*). In January 2009, Alnylam Pharmaceuticals received a clearance from FDA to start the first clinical trial of an RNAi drug targeted against the gene for kinesin spindle protein (KSP), the protein essential for tumour spreading, and the previously mentioned gene for VEGF, which enables tumour growth by helping formation of blood vessels. This new candidate, called ALN-VSP, has been designed to treat primary liver tumours; due to its encapsulation in lipid nanoparticles, it is capable of circulating throughout the body after being administered *via* intravenous infusion.

Another example of VEGF targeting is bevasiranib, a drug molecule already in Phase 3 clinical trials in patients with age-related macular degeneration (AMD). AMD is caused by the abnormal growth of blood vessels behind the retina leading to loss of vision (*27*). In Phase 1 and 2 clinical trials, bevasiranib, after being administered directly into the eye (intravitreal injection), did not affect the patient systemically, but locally inhibited the overgrowth of blood vessels that would otherwise lead to vision loss.

Other attractive targets for the RNAi technique are diseases caused by viruses and bacteria. In the case of viruses, genes essential for virus replication, for the assembly of viral particles and host genes, and viral genes important for virus-host interactions are good targets for siRNAs. These are usually surface antigens responsible for virus protein recognition and entrance into the cell. For example, in the case of HIV, virus silencing can be achieved through the primary HIV receptor chemokine (C-C motif) receptor 5 (CCR5) by siRNA targeted against mRNA transcribed from its gene (*1,30*). In the case of human papilloma virus (HPV), it is possible to induce apoptosis of tumour cells in primary patient tumour samples by targeting the E6 gene of HPV. E6 protein is a transcriptional activator essential for malignant transformation as well as maintenance of a malignant tumour phenotype. Thus, when silenced with anti-E6 siRNA, the future development of a tumour cell is prevented (*31,32*). Many other animal viruses, especially respiratory viruses, are considered to be targets for RNAi ther-

apy. This is because epithelial cells of the respiratory tract have been proven to be good uptakers of siRNA even without transfection, which would enable an easy way of their administration through inhalation (*31*,*33*), though some delivery problems concerning lung surfactants remain to be solved (*34*). In contrast to viruses, bacteria are not generally subject to silencing by siRNA because they mainly replicate outside the host cell and lack the necessary machinery. However, it might be possible to reduce life-threatening bacterial infections which lead to adverse consequences by silencing the host genes involved in those aspects of the immune response or by silencing the host genes involved in mediating bacterial invasion (*1*).

RNAi could also be used for studies of different immunological diseases. Possible targets in this field are molecules expressed on the surface of immune cells (CDs, cytokine and chemokine receptors, adhesion molecules), cytokines and chemokines, and intracellular signal transducing proteins. Examples also include transforming growth factor beta (TGF-β) and the nuclear transcription factor kappa B (NF-κB) (*2*,*3*).

Conclusions

RNAi-based gene therapy has great potentials in the treatment of cancer and infectious diseases, as well as genetic diseases. RNAi has a variety of important functions in plants and animals, such as a defence mechanism to protect the genome from viruses and other foreign nucleic acids. The critical issue of using RNAi for therapeutic purposes is the fact that exogenous manipulation could undesirably interfere with the function of endogenous RNAi – antiviral protection. Efficiency of the therapeutic use of RNAi in cancer relies on the development of safe and efficacious delivery systems that introduce siRNA and shRNA expression vectors into target tumour cells without disturbing cell homeostasis. The RNAi approach will hopefully replace the more toxic traditional treatment modalities and lead to better tolerated but more effective anticancer therapeutics. In general, the efficiency of the therapeutic use of RNAi relies on the conditions such as lack of toxicity, specificity of silencing effects and efficacy of silencing *in vitro* and *in vivo*.

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