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Small Interfering RNAs: Heralding a New Era in Gene Therapy

Maro Bujak[1], Ivana Ratkaj[2], Mirela Baus Loncar[1], Radan Spaventi[3] and Sandra Kraljevic Pavelic[2]
[1]*Rudjer Boskovic Institute, Division of Molecular Medicine, Laboratory for Systems Biomedicine, Zagreb,*
[2]*Department of Biotechnology, University of Rijeka, Rijeka,*
[3]*Galapagos Research Centre, Zagreb,*
Croatia

1. Introduction

Last decades have witnessed a tremendous expansion in knowledge and availability of the genome sequence, which was of great importance for advancements in the field of gene therapy. This led to improved strategies based on use of nucleic acids with sequences complementary to specific target genes in treatment of many diseases. Especially, advancements have been achieved in discovery and use of diverse RNA molecules other than messenger RNAs (mRNAs), transfer RNAs (tRNAs), or ribosomal RNAs (rRNAs). Such RNA molecules, known as non-coding RNAs (ncRNAs), serve diverse biological roles some of which are still elusive (Gesteland 2006). Generally, the ncRNA molecule is functional even when it does not encode for a protein. Recent evidence provided by many projects including the Encode project (The Encyclopedia Of DNA Elements) suggests that larger part of the genomes of mammals and other complex organisms is transcribed into ncRNAs. These ncRNAs are transcribed from both exon and intron DNA regions, and include small interfering RNAs (siRNAs), micro RNAs (miRNAs) and small nucleolar RNAs (snoRNAs), while many of such molecules remain yet to be discovered. A vast amount of evidence demonstrates that ncRNAs play essential roles in cellular physiology. Some biological processes known to be regulated by ncRNAs include transcriptional regulation of genes, gene silencing, messenger RNA stability and translation, development, proliferation, haematopoiesis, apoptosis, protein translocation and chromosome replication (Bühler 2007, Mattick 2006, Lee 1993).

There is no doubt that RNA regulatory networks are critical for determining our most complex traits, and they play an important role in disease pathogenesis as well. The specific disease phenotypes might indeed result from deficiency of one or more specific ncRNA instead from protein structural defects, as is usually expected. A challenge for the future might thus be to map the whole cells/organisms complement of ncRNAs and to understand their biological role. Up to now, the use of ncRNAs as a research tool has greatly improved gene therapy approaches for various diseases (Gallaso 2010), but also substantially improved drug discovery and target validation. In this book chapter, we will therefore focus

on the use of a particular approach, namely RNAi for improved gene silencing for both, therapeutic approaches and identification of new therapeutic targets.

2. RNA interference

RNA interference (RNAi) is an evolutionary conserved cellular defence mechanism that protects plants and vertebrates from viruses and transposable genetic elements, but is also involved in direct development and gene expression in general (Lecellier 2004,Vastenhouw 2004, Meister 2004). Two types of ncRNA molecules – micro RNA (miRNA) and small interfering RNA (siRNA) are involved in the RNAi mechanism through binding to mRNA molecules. Through this process, either increase or decrease of mRNA activity or repression of translation occurs (Hannon 2002). Small interfering RNAs are 20-25 nucleotides long double-stranded RNA molecules, that play a variety of biological roles. The most notable one is its involvement in the RNAi pathway, where it interferes with the expression of a specific gene (Devi 2006, Elbashir 2001a). siRNA may also be involved in RNAi-related pathways, such as shaping the chromatin structure. Similarly, miRNAs are short non-coding, 19-22 nucleotides long, functional RNA molecules that play important regulatory roles by sequence-specific base pairing on the 3′ untranslated region (3′-UTR) of target messenger mRNAs, promoting mRNA degradation or inhibiting translation (Bartel 2004). RNAi is thus a post-transcriptional gene silencing mechanism employed to silence an endogenous gene, e.g. by the introduction of a homologous dsRNA. The selective and rapid degradation of the transcript ensured in the RNAi pathways makes it a valuable laboratory technique in biotechnology and medicine for controlled silencing of genes. For that purpose, synthetic dsRNA are usually introduced into cells to suppress expression of specific genes of interest (Elbashir 2002).

The RNAi pathway is initiated by the Dicer enzyme, which cleaves long double-stranded RNA (dsRNA) molecules (500-1000 nucleotides) into short siRNA fragments of ~20 nucleotides or pre-miRNAs into mature miRNA (Figure 1) (Elbashir 2001b). While miRNAs have incomplete base pairing to a target and inhibit the translation of many different mRNAs with similar sequences, siRNAs have perfect complementarity and induce mRNA cleavage only in a single, specific target (Pillai 2007). Interestingly, about one-third of human protein-coding genes are controlled by miRNAs (Du 2005), while siRNAs participate in chromosome dynamics and formation of heterochromatin (Mattick 2005). Exogenous siRNAs may be derived from experimentally introduced double-stranded RNAs (dsRNAs) or viral RNAs (Fire 1998). Endogenous siRNA (endo-siRNA) precursors are derived from repetitive sequences ,transposons, sense–antisense pairs or long stem-loop structures (Babiarz 2008; Watanabe 2008). RNAi interference can be exerted through naturally occurring antisense transcripts (NATs) that are complementary to other RNA transcripts (Osato 2007). They are involved in alternative splicing, genomic imprinting, and X-chromosome inactivation as well (Zhang 2004). Based on the locus of their transcription, NATs can be divided into two groups, namely cis-NATs and trans-NATs. Cis-NATs are transcribed from the same genomic locus as their target, but from the opposite DNA strand, therefore forming a perfect match with their targets (Wang 2005). So far, five orientations have been identified, among which the so-called ‘head to head’ orientation where both transcripts align their 5' ends is considered to be the most common (Lavorgna 2004). On the other hand, trans-NATs are transcribed on different genome locations and are complementary to multiple transcripts resulting, however, in a number of mismatches

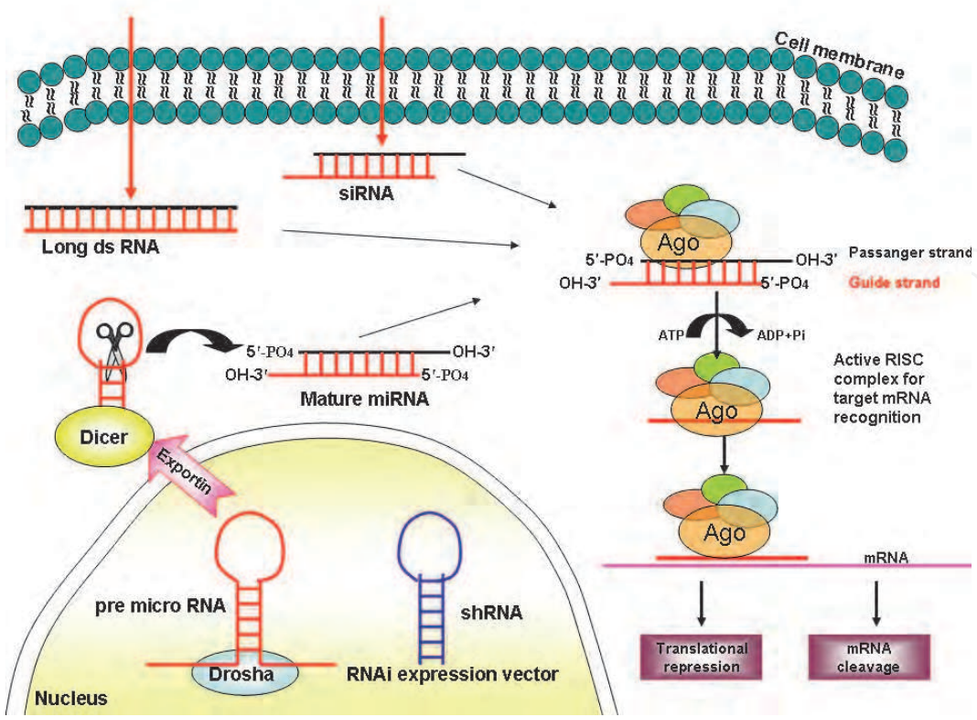

Fig. 1. Pre-micro RNA and shRNA are expressed in the nucleus, exported to the cytoplasm and processed into a mature form by the 'Dicer' enzyme. It is possible to exogenously introduce synthetic RNAi constructs directly into the cytoplasm and to specifically silence the target gene. Long double-stranded RNA (dsRNA) and hairpin structures are cut into smaller strands, namely interfering siRNA by Dicer, leaving ~2nt overhangs at the 3′ end and phosphate group at the 5′ end. The guided strand is incorporated into the RNA-induced silencing complex (RISC), while the passenger strand is discarded. Active RISC complex uses the guide strand to cleave complementary target, which causes mRNA degradation and translational repression. The same RISC complex may carry out several cleavage cycles. Long primary transcripts of miRNA genes (pri-miRNA) are cleaved by Drosha to produce a stem-loop structured precursor, pre-microRNA (pre-miRNA). Subsequently, it leaves the nucleus through the nuclear pores and enters the cytoplasm, where is being processed by Dicer. Mature ds miRNA is loaded onto the RISC. Only one strand is successfully incorporated into the RISC, while the other is eliminated. Interaction between miRNA and target RNA is characterized by imperfect base pairing. Namely, the guide miRNA strands usually form bulge structures due to mismatches with its target sequence. Consequently, there is no perfect complementarity between base pairs. In this way, miRNA together with the RISC induce repression of protein translation (Jackson 2003; Bartel 2004).

(Carmichael 2003). miRNAs are typical representatives of trans-NATs involved in transcriptional silencing, translation repression, deadenylation and heterochromatin formation. miRNA genes are found in introns of non-coding or coding genes and in exons of non-coding genes.

Both miRNAs and siRNAs molecules have two strands, one named the 'passenger strand' and the other called the 'guide strand'. The passenger strand is the one to be degraded, while the guide strand further incorporates into the RNA-induced silencing complex (RISC)

(Lee 2004) in an ATP-independent process performed directly by the protein components of the RISC (Leucschner 2006; Gregory 2005). This complex contains the Argonautes (Ago) proteins that cleave the passenger strand and liberate the guide strand from the siRNA duplex (Liu 2004; Meister 2004). Activated RISC is then capable of cleaving target mRNAs. The guide strand recognizes homologous sequence of the target mRNA. When mRNA is associated with the guide strand (template) in the RISC complex, it is cleaved by the Ago proteins (Matranga 2005, Leuschner 2006). In this process, template siRNA remains intact and serves for subsequent cycles of mRNA cleavage. The mRNAs cleaved by the RISC are degraded by cellular exonucleases. In this way, the translation of mRNA is ceased (Hall 2005).

The third group of interfering RNA molecules is comprised of Piwi-interacting RNAs (piRNAs) that are processed from single-stranded RNA precursors transcribed from intergenic repetitive elements, transposons or large piRNA clusters. They are associated with the Piwi subfamily proteins, and therefore do not depend upon Dicer. piRNAs are highly abundant in germ cells and at least some of them are involved in transposon silencing through heterochromatin formation or RNA destabilization (Vagin 2006). The precise mechanisms and the functions of most piRNAs are still unknown.

2. Barriers to RNAi-based therapies

Various RNAi therapy approaches *in vivo* are hampered by unwanted side effects such as induction of immune response and toxicity, including the activation of Toll-like receptors (TLRs), type I interferon responses and competition with the endogenous RNAi pathway components (Marques 2005). Several reports have shown that chemical modifications of siRNA can attenuate immune reaction by abrogating interferon (IFN) and cytokine induction (Judge 2005, Sioud 2005, 2006). Family of Toll-like receptor proteins (TLRs) are known to be involved in the recognition of pathogen molecules such as viral dsRNAs, and are central to the activation of immune cell response. TLRs recognise siRNAs in a sequence-dependent manner in the endosome prior to the siRNAs cytoplasm internalization. In particular, the so-called 'off-target' effects of siRNAs are widely recognized as an issue associated with the use of siRNAs (Jackson 2003). Off-target effect is undesired down-regulation of non-targeted transcripts, either by miRNAs or siRNAs. This phenomenon mainly occurs due to lack of complementarity between siRNAs and target mRNAs. RNAi machinery tolerates single mutations located in the centre of siRNA molecules without losing the gene silencing ability. In this manner, some siRNAs have the ability to silence other genes besides complementary target genes. These problems may, however, be partially overcome by the use of computer algorithms in combination with the experimental validation procedures that ensure optimized siRNA sequences complementary to the target mRNA inducing minimal immune responses.

Additionally, silencing 'off-target' genes other than interferon-induced pathway represents nowadays the major problem in designing effective siRNA approaches, which impedes the clinical usage of RNAi (Jackson 2003, Persengiev 2004, Birmingham 2006). Indeed, cross-hybridization of interfering RNA molecules may partially match the sequence of non-target genes and consequently knockdown these genes. miRNAs require only a small match at the 5' end of the anti-sense strand as to induce such "off-target" effect while similarly, the insertion of the sense siRNA strand into the RISC complex instead of the anti-sense strand should significantly contribute to unwanted gene silencing as well (Jackson 2003). Finally,

"off-target" effects may occur due to the seed-sequence-dependent binding, where "off-targeted" genes contain matches between the seed region of siRNA and their sequences in the 3'UTR (Jackson 2006a). Increase of the RNAi specificity has, however, been achieved by minimizing sense strand incorporation into activated RISC and selective thermodynamic stabilization of the sense strand 5' ends by incorporation of locked nucleic acids (LNA) (Schwarz 2003, Elmen 2005).

Though the siRNA macromolecules have strong negative anionic charge deriving from the phosphates on their surface that enables spontaneous passage across the negatively charged cell membrane, a variety of biological barriers should be overcome for *in vivo* delivery. These barriers include filtration, phagocytosis and degradation in the bloodstream, passage across the vascular endothelial barrier, diffusion through the extracellular matrix, uptake into the cell, escape from the endosome and unpackage and release of siRNA to the RNA interference (RNAi) machinery (Whitehead 2009).

For example, naked siRNAs are relatively unstable in blood and serum in its native form, though more stable in comparison to single-stranded RNAs (Whitehead 2009). What happens to siRNAs when entering blood is rapid degradation by ribonucleases, a rapid renal excretion and non-specific uptake by the reticuloendothelial system. According to studies in rats that received naked siRNA intravenously, a rather short half-life of 6 min and a clearance of 17.6 mL/min was documented (Soutschek 2004). Poor pharmacokinetic properties of siRNA arise from endogenous RNAses degradation and rapid elimination by kidney filtration due to small molecular masses (~7 kDa) (Soutschek 2004).

Recently, even a novel elimination pathway for siRNAs *in vivo* has been identified, where liver-enriched siRNA is secreted into the gallbladder and then excreted into the intestine (Huang 2011). After their delivery into the bloodstream, siRNAs are subjected to rapid clearance from blood through liver accumulation and renal filtration, but up until now, it has been believed that the siRNAs elimination could be carried out only by the renal system. Unpredictable biological stability and cellular uptake of siRNAs may be partially surmounted by chemically modifying the siRNA structure including backbone, base and sugar modifications without affecting gene silencing.

If however, administered siRNAs survive in the plasma, they encounter a problem of extravagation through the tight vascular endothelial junctions (Juliano 2009). Interestingly, transport of macromolecules across tumour endothelium was found to be more efficient than transport across normal endothelium that was leaky and had discontinuous vascular structures with poor lymphatic drainage (Jang 2003). Additionally, siRNA diffuses through the extracellular matrix, a dense network of collagen and carbohydrates surrounding a cell (Zamečnik 2003), and it finally reaches its last destination - the cytoplasm of the target cell. Here, siRNAs incorporate into RNAi machinery and encounter target mRNAs. At this point, endosomes represent a natural barrier to internalisation and subsequent degradation of siRNAs (Boussif 1995, Oliveira 2007). However, the use of acid-responsive delivery carriers may improve escape of siRNA from endosomes, as the endosome environment is naturally mildly acidic. In addition, fusogenic peptides that undergo acid-triggered conformational changes may also accelerate endosomal escape of nucleic acids, and are liberated from carriers in the last stage of delivery (Medina-Kauwe 2005, Cho 2003).

3. Chemical modifications

Delivery of siRNAs in their unmodified form has several advantages over chemically modified forms ensuring maximal efficiency (maximized RNAi per siRNA molecule) and avoiding potentially inefficient and time/labour-consuming modification process.

Nevertheless, the use of chemical modifications was found to reduce cleavage of RNA duplexes by nucleases, scale down the activation of innate immune response, lower the incidence of off-target effects, and improve pharmacodynamics (Behlke 2008). For example, phosphorothioate (PS) linkage is one of the simplest modifications of the siRNA backbone. Studies showed that toxicity and loss of silencing activity could pose a hurdle when phosphorothioate-modified siRNAs are employed (Manoharan 2004, Mahato 2005). A better alternative to backbone modification is the boranophosphonate linkage, which is more effective at silencing than phosphorothioate siRNAs, and is 10 times more nuclease resistant in comparison with unmodified siRNAs. Furthermore, boranophosphate siRNAs are more potent than unmodified siRNAs, and act through the standard RNAi pathway (Hall 2004).
Another chemical modification of interest is ribose ring-like modification of RNA at 2′-position of the ribose ring. These modifications include 2′-O-methyl (2′-OMe), 2′ deoxy-2′-fluoro modifications and locked nucleic acid. They increase siRNA stability against endonucleases and reduce immune response activation (Chiu 2003). In addition, 2′-OMe modifications at specific positions within the siRNA region reduce the number of off-target transcripts and the magnitude of their regulation without significantly affecting silencing of the intended targets (Jackson 2006b). Interestingly, 2′-OMe modifications reduce the hybridisation free energy that compensates for somewhat weaker base pairing (Inoue 1987, Lesnik 1993). It was proved that 2′-OMe modifications greatly prolonged siRNA half-live in the plasma (Chiu and Rana 2003), but a number of siRNAs currently used in clinics had been designed prior to findings on 2′-OMe modification benefits to siRNA application *in vivo*. Further on, ribose modification or locked nucleic acid (LNA) also protracts the functional half-life of siRNA *in vivo* by two different mechanisms: 1) enhancing the protection of RNA from degradation by enzymes, and 2) stabilizing the siRNA duplex structure indispensable for silencing activity (Elmen 2005). Such modified RNA nucleotide is modified *via* a methylene bridge connecting the 2′ oxygen with the 4′ carbon of the ribose ring (Bondensgaard 2000, Braasch 2001), which produces a locked ribose conformation known to increase the hybridization properties of oligonucleotides (Kaur 2006). LNA is highly compatible with the siRNA intracellular machinery and preserves the molecule integrity (Braasch 2003, Elmen 2005). There is, however, a possibility that production of non-natural molecules might occur upon degradation of chemically modified siRNAs, as these RNAs may produce unsafe metabolites or trigger unwanted effects.

4. siRNA delivery systems

Obstacles to efficient delivery of siRNA *in vivo* might be overcome by diverse approaches aimed at increasing cellular uptake, protecting from enzymatic degradation, bypassing the immune recognition and improving the pharmacokinetics properties. These delivery systems, namely bioconjugation, complex formation with lipids and polymers, viral vectors, encapsulation into lipid particles and non-pathogenic bacteria vector are designed to specifically localize siRNA in desired tissue, which minimizes side effects and decreases the concentrations of siRNA required for efficient gene scilencing *in vivo*.

4.1 Bioconjugation

Conjugation of siRNAs with lipids and polymers increases thermodynamic stability, protects siRNAs' strands from nucleases and improves the biodistribution and

pharmacokinetic profiles of siRNAs along with their targeting to specific cell types (Cheng 2006, Lorenz 2004, Soutschek 2004, Wolfrum 2007, DiFiglia 2007, Mahat 1999; Schepers 2005). Therefore, conjugation of siRNA with lipids either enhances the uptake *via* receptor-mediated endocytosis, or increases penetration across the cell membrane, as demonstrated by the studies where cholesterol-conjugated siRNAs were effectively delivered to cells in cell culture, liver and other organs (Cheng 2006). Indeed, cholesterol conjugation increases hydrophobicity and cellular association of nucleic acids (Lorenz 2004), and conjugation of cholesterol with anti-ApoB siRNAs efficiently lowers the level of ApoB mRNA in the mice liver and jejunum leading to decline in the blood cholesterol level (Soutschek 2004). Similar approach was successfully applied to deliver siRNAs in murine vaginal mucosal tissue for prevention and inhibition of potentially lethal herpes simplex type 2 infections. It seems that cholesterol-siRNA conjugates incorporate into circulating lipoprotein particles, and are efficiently internalized by hepatocytes *via* a receptor-mediated process. Pre-binding of cholesterol-siRNA conjugates to lipoparticles dramatically improves silencing efficiency in mice and distribution of lipoparticle cholesterol-siRNA conjugate in various tissues (Wolfrum 2007). Intrastriatal injection of cholesterol-siRNA conjugates silenced mutant huntingtin gene in a transgenic mouse model for Huntington's disease, attenuating neuronal pathology as well as delaying the abnormal behavioural phenotype (DiFiglia 2007).

Furthermore, siRNAs may be conjugated to peptides termed protein transduction domains (PTDs). The latter have the ability to translocate across the cell membrane and therefore to efficiently deliver siRNAs into cells. PTDs consist of short amino acid sequences with stretches that have positively charged amino acids arginine and lysine, which facilitate their translocation through the plasma membrane. Such amphipathic molecules interact with negatively charged head groups of the plasma membrane *via* their positive amino acid residues. siRNA is finally released in the cytoplasm upon reduction of the disulfide bond. The uptake of peptides-siRNA conjugates is rapid, effective and occurs without the need for specific receptors, which provides an important role for these conjugates in siRNAs delivery into all kinds of mammalian cells *in vivo* (Mahat 1999, Schepers 2005).

4.2 Complex formation with lipids and polymers

Bioconjugation substantially improves delivery of siRNA, but still fails to ensure reversible binding of siRNAs for controlled release of siRNAs into target cells, protection of siRNAs from nuclease degradation and serum binding during transit through the circulation, escape from endosomal compartment, biocompatibility as to escape hosts immune response, and resistance to liver and kidney rapid clearance.

Cationic polymers interact with siRNAs spontaneously and self-assemble in a process induced upon electrostatic interactions that results in formation of nanoparticles known as polyplexes. The efficiency of siRNA polyplexes to silence genes of interest depends on several factors such as capability to bind cellular membranes, cellular uptake rate and escape from endosomes.

Several cationic polymers have been widely investigated as siRNA carriers *in vitro* and *in vivo* (Mahato 1997), and their design has been optimized in the cell cultures (Friend 1996, Xu 1996).

Cationic polymers spontaneously form complexes with nucleic acids due to electrostatic interactions between positively charged amine groups of the polycations and negatively charged phosphate groups of the nucleic acids. These interactions enhance the uptake of

cationic polymers by cells and increase transfection efficiency (Han 2000). Among cationic polymers employed for gene delivery, polyethylenimine (PEI) is one of the most common ones in siRNA delivery *in vitro* and *in vivo*. PEIs of various molecular weights, degrees of branching and other modifications have been largely used for transfection of siRNAs in different cell lines and live animals. For instance, siRNA targeted towards the HER2 growth receptor was delivered intraperitoneally to subcutaneous tumours as siRNA/PEI complex, and significantly reduced tumour growth. Moreover, pain receptors for N-methyl-D-aspartate were effectively knocked down in rats by specific PEI/siRNA delivered intrathecally (Tan 2005). PEIs should thus play an important role for non-viral siRNA delivery *in vivo*, if toxicity and limited biodegradability issues are appropriately addressed.

On the other hand, cationic lipids are constructed by protonable polyamines linked to dialkyl or cholesterol anchors, and represent one of the most widely used strategies for *in vivo* delivery of siRNA (Whitehead 2009). Physicochemical properties of lipid/nucleic acid complexes (nanoparticles) are influenced by the relative proportions of each component, structure of the cationic lipids head group, co-lipid molar and charge ratio, particle size of complexes, and liposome size (Mahato 1998, Spagnou 2004). Electrostatic interactions between siRNA and cationic liposomes may provoke relatively uncontrolled interaction processes giving rise either to the excessive size of the formed lipid/siRNA complex and its poor stability, or to incomplete encapsulation of siRNA molecules posing a risk of their potential enzymatic or physical degradation prior to delivery into the cells (Spagnou 2004, Keller 2005).

Still, cationic lipids complexed with siRNAs of interest were successfully used in nonhuman primates (Akinc 2008, Frank-Kamenetsky 2008), and are currently being evaluated in several clinical trials.

Still, some shortcomings of using the lipid-siRNA biocunjugates remain. Major obstacles refer to the plasma stability for intravenous applications (Mahato 1998,1999, Keller 2005), where they interact with serum proteins, lipoproteins, heparin and glycosaminoglycans in the extracellular matrix precipitating the aggregation or release of nucleic acids from the complexes before reaching the target cell. Cationic lipids activate the complement system resulting in rapid clearance by macrophages (Mahato 1997).

However, polyethylene glycol (PEG) coating of liposomal carriers (Lia 2005) substantially lowers their interaction with serum proteins and with the proteins of the complement system thus improving the complexes circulation time. It is now widely accepted that PEGylation-aided stabilization of the lipid/nucleic acid complexes leads to the reduction in macrophage clearance.

Cationic lipids represent a convenient and flexible method for siRNA delivery. Indeed, various approaches to designing cationic lipid structure and liposome composition have been successfully developed in combination with diverse reliable methods for their preparation. This ensures increased *in vivo* efficiency tailored for different models and diseases.

Recently, a promising siRNA delivery carrier, namely stable nucleic acid lipid particles - SNALPs, has been described (Zimmermann 2006). SNALPs consist of a lipid bilayer containing a mixture of cationic and fusogenic lipids that enable cellular uptake and endosomal release of siRNAs. These particles are additionally coated with the polyethylene glycol-lipid (PEGylated lipid) conjugate that provides neutral hydrophilic exterior and stabilizes the particle during formulation. The silencing effect of SNALP-conjugated siRNAs is more potent (>100-fold) than that of systemic administration of cholesterol-conjugated

siRNAs targeted against ApoB in mice. Another study confirming higher potency of SNALP-conjugated siRNAs was performed in mice (Morrissey 2005). Chemically modified siRNAs against hepatitis B virus (HBV) were conjugated with SNALPs and administered intravenously into mice carrying replicating HBV. The results confirmed improved efficacy and longer half-life of siRNA encapsulated in SNALPs in the plasma and liver compared to unformulated siRNA (Morrissey 2005).

Another newly described delivery vehicle for siRNAs is the liposome-siRNA-peptide complex (LSPCs) that showed a potential in therapy of neurodegenerative disorders (Pulford 2010). For that purpose, intravenous injections were used for transvascular delivery of siRNA complexed with LSPCs across the blood-brain barrier to the brain. The LSPCs complex consisted of a modified peptide from the rabies virus glycoprotein that acts as a ligand for acetylcholine receptors (AchR), a small peptide that links siRNA with modified peptide and liposomal nanoparticle. This complex effectively delivered siRNA to neuronal cells expressing AchR in brain. Furthermore, LSPCs' liposomes increased the stability of siRNA/peptide complex in serum during vascular transport. This approach proved promising in the treatment of prion diseases as well. For example, LSPCs coupled with the prion protein (PrP) siRNA were shown to significantly suppress cellular prion protein PrP^{C} expression and to eliminate misfolded protease-resistant isoform of the cellular prion protein PrP^{RES} in the AchR-expressing cells *in vitro* (Pulford 2010). Similarly, LSPCs injected intravenously in mice efficiently bypassed serum degradation and the PrP siRNAs were delivered to AchR- and PrP^{C}- expressing neurons in brain. Still, these promising results need to be proved for the future human siRNA therapy and possible beneficialy effects in case of prion disease, neurodegenerative disorders such as Alzheimer's disease or viral encephalitis.

At last, it is worth to mention that it has become possible recently to quantitatively estimate the disassembling ratio of nanoparticles complexes with nucleic acids in complex biological media such as serum (Buyers 2009). The measurement is performed by the use of fluorescence fluctuation spectroscopy (FES) that quantifies nanomolar concentrations of released siRNA. First measurements showed that the gene silencing efficacy of siRNA polyplexes in the serum depends on the serum concentrations. These findings will aid in the development of siRNAs polyplexes and other nanoparticle nucleic acid as delivery systems.

4.3 Viral and non-pathogenic bacterial vectors

It is well-known that siRNA-mediated gene silencing is usually transient in cell culture and lasts for only a couple of days. Such short-term knockdown is not sufficient for studying phenotypic effects that require longer duration of knockdown of the target protein. Moreover, transient transfection of siRNA varies in efficiency between different cell types, but the key to resolving this problem is stable expression of RNAi effector molecules from plasmids or viral vectors (Amarzguioui 2005). There are several viral vectors used therein: double-stranded adeno-associated viruses (AAV), lentiviral vectors and adenoviruses (Brummelkamp 2002a, Zufferey 1998, Andersson 2005, Yoo 2007). However, the most commonly used approach involves RNA polymerase III-mediated transcription of short hairpin structures (shRNA) with a stem of 19–29 bp and a short loop of 4–10 nt. Besides, siRNAs may be introduced by viral vectors and transcribed from separate expression units, from either the same or two separate plasmids. Finally, the effector molecules may be expressed as a chimera of siRNA and miRNA (Figure 2).

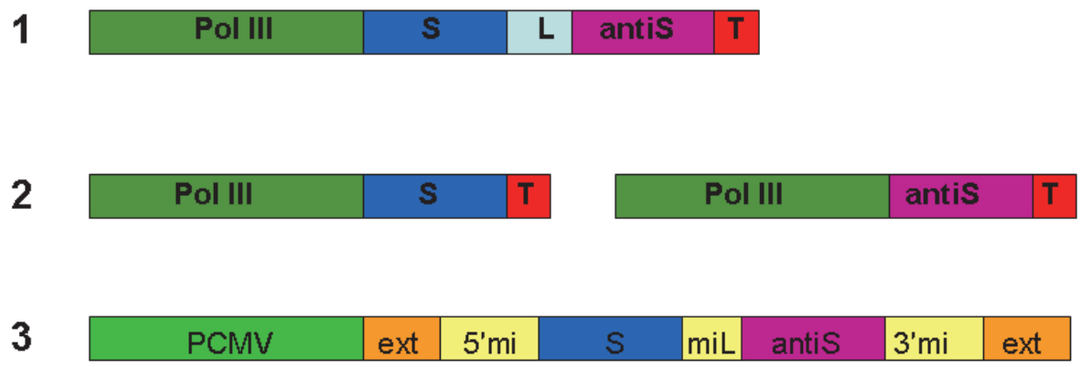

Fig. 2. Construction of expression cassettes for 1) shRNA and 2) siRNA 3) miRNA. PIII: pol III promoter, PCMV: pol II promoter S: siRNA sense strand, antiS: siRNA antisense strand, L: loop, T: terminator, 5′mi: 5′ pri-miRNA sequence, 3′mi: 3′ pri-miRNA, ext: extraneous transcript sequences. Correct excision of the siRNA from the heterologous transcript is directed by 50mi and 30mi sequences.

AAV vectors are the safest and thus most promising viral gene delivery vehicles known to date (Grimm 2003). The wild-type AAV viruses are non-pathogenic in humans, persistently infect a large variety of dividing and non-dividing cells, and do not integrate into chromosomes. Despite these advantages, their clinical application is restricted due to their potential in some mutagenic and/or oncogenic transformations and host immune responses, and high production costs.

Non-pathogenic bacteria may also be used as delivery vectors. For example, transkingdom RNAi (*tk*RNAi) uses non-pathogenic bacteria to produce and deliver therapeutic short hairpin RNA (shRNA) encoding plasmid DNA into target cells for precise gene silencing (Krühn 2009). Plasmid or TRIP vector contains shRNA of interest and is controlled by bacteriophage T7 promoter. TRIP vector also contains the *Inv* locus from *Yersinia pseudotuberculosis* that encodes invasin, which helps bacteria to enter into β-1 integrin-positive mammalian cells. Listeriolysin O, an additional product of TRIP vector coded by the HlyA gene, makes it possible for shRNA to escape from entry vesicles. TRIP vectors are introduced into competent non-pathogenic *Escherichia coli* strains BL21(DE3). This technique showed very good results in silencing catenin-β1 in human colon cancer cells *in vitro* as well as *in vivo* (Xiang 2006). It is also suitable for targeting the multidrug resistance (MDR)-mediating drug extrusion pump ABCB1 (MDR/P-gp) in multidrug resistant cancer cells, but it is not yet as good as conventional siRNA (Nieth 2003, Stein 2008) and virally delivered shRNAs (Kaszubiak 2007). With additional ongoing improvements, *tk*RNAi may become a powerful tool for delivery of RNAi effectors for the reversal of cancer MDR in future.

5. Targeted siRNA

Considerable effort has been invested in targeted siRNA delivery *in vivo*. For that purpose, important requirements must be fulfilled including stability, prolonged circulation in the body, high accessibility to target tissues, specific binding to target cells, active endocytosis in the cell and siRNA activity in the target cells. Only then, one can expect maximized delivery and optimal concentration in the target tissue. Targeted siRNA design may also prevent

non-specific siRNA distribution. Ligands that recognize cell-specific receptors expressed by the target cells can be conjugated to polymers and cationic lipids in order to promote specific cellular uptake *via* receptor-mediated endocytosis (Dubey 2004, Lu 2005). Folate receptor is one of the most popular target molecules in cancer-specific gene and drug delivery (Gosselin 2002). Folic acid is essential for rapid cell growth, thus many cancer cells over-express folate receptors. They have binding sites for FA and monoclonal antibodies. FA is convenient for conjugation with liposomal and polymeric siRNA carriers with or without the polyethylene glycol spacer. In the study presented by Kim et al. (Kim 2006), FA-conjugated polyethylenimine enhanced gene silencing *via* receptor-mediated endocytosis. Another group of receptors that are potential targets for efficient siRNA delivery are integrins and transferrin. The arginine-glycine-aspartic acid (RGD) motif has been used for target delivery of drugs and genes because of its ability to bind to integrins expressed on the activated endothelial cells found in tumour vasculature (Schiffelers 2004, Kim 2004). In addition, cyclodexstrin-based polycation delivery system can be used to target metastatic tumours (Hu-Lieskovan 2005). Aptamers can be used for site-specific delivery of siRNA, as they possess high affinity and specificity for their target. Prostate-specific membrane antigen (PSMA)-specific aptamers can be internalized into PSMA expressing-like prostate cancer cells (Hicke 2000, Pestourie 2005). Antigen-conjugated siRNA carriers are an alternative (Park 2002, Mamot 2005). HER-2 siRNA-carrying liposomes decorated with transferrin receptor-specific antibody fragments silenced the HER-2 gene in xenograft tumours in mice, significantly inhibiting tumour growth (Pirollo 2007).

6. Local and systemic delivery

The administration of siRNA can be local or systemic depending on the types of target tissues and cells. siRNA can be directly applied to some organs like eye or skin, as well as muscle *via* local delivery. Systemic siRNA delivery is the only way for metastatic and haematological cancer cells. Local delivery has several advantages, such as low effective doses, simple formulation, low risk of inducing systemic side effects and facilitated site-specific delivery (Dykxhoorn 2003). Local injections of siRNA into the eye were used in initial clinical trials for age-related macular degeneration (Oh 2009). Moreover, intranasal siRNA administration for pulmonary delivery and direct injection into the central nervous system were also tested in clinical trials (Howard 2006, Bitko 2005, Zhang 2004). Systemic delivery by intravenous (i.v.), intraperitoneal (i.p.) or oral administration is convenient for target sites that are not readily accessible. This especially refers to metastatic tumours. Thus, for example, Yano et al. (Yano 2004) showed that human bcl-2 oncogene targeting siRNA complexed with cationic liposomes injected i.v. inhibited tumour growth in a mouse liver metastasis model.. Another research carried out by Morrissey et al. (Morrissey 2005) revealed efficient and persistent antiviral activity after injection of siRNA encapsulated in lipid vesicle into the hepatitis B virus mouse model. Moreover, in systemic delivery, siRNA must maintain active form in circulation and be able to reach target tissues after passing through multiple barrier organs.

siRNA technology is a promising application of naturally occurring processes in the human body. There is evidence that mature miRNAs, mRNA and signal peptides are loaded into exosomes (Thery 2002), small membrane-bound particles derived from the endocytic compartment that are secreted and act as intercellular mediators of biological information

(Graner 2009). Barr virus (EBV)-infected cells secrete exosomes containing EBV-miRNA that are transferred to uninfected neighbouring cells (T-cells) in the peripheral blood of patients helping to spread the virus (Rechavi 2009). Cancer cells can affect function of immune system *via* exosomes by inhibiting functions of T cells and natural killer cells (Zhang 2011), thus avoiding immunosurveillance. The fact that mast cells-derived exosomes can carry mRNAs for more that 1300 genes and more than 100 miRNAs (Zhang 2011) clearly demonstrates the potential of this intercellular genetic exchange mechanism as a target in treatment of various diseases. Knowledge of this process will be highly beneficial in terms of siRNA therapy application.

7. RNAi as a research tool

Knocking down the genes of interest by using siRNAs has turned out to be an important laboratory tool for large-scale RNAi screens, especially in the field of medical research. There are several methods for siRNA generation. Direct chemical synthesis is an obvious choice for creating siRNA library, but this could be a rather expensive option for most researchers, so that the only large-scale synthetic siRNA library was made for Novartis by Qiagen and Dhamarcon. Vector-based approach has lower cost enabling not only high transfection efficiency and delivery of siRNA expression cassettes but also the selection of transfected cells. The basic idea is to use pol III promoters followed by DNA coding for shRNA that structurally resembles miRNA (Brummelkamp 2002, Miyagishi and Taira 2002, Sui 2002, Xia 2002, Yu 2002). It is possible as well to use dual Pol III promoters (Chen 2005, Zheng 2004) or even two tandem Pol III promoters (Lee 2002), which is less popular method due to its more laborious construction. Some other promoters like T7 and CMV can be used for constructing siRNA vectors (Xia 2002, Holle 2004). Bacteriophage T7 promoter is not functional in mammalian cells. CMV promoter, on the other hand, is RNA polymerase II promoter, which is stronger promoter than Pol III resulting in more transcribes from the same vector that are capped at the 5'-end and tailed at the 3'-end with a long poly (A) sequence. These modifications are well-tolerated, indicating that such approach might be used for *in vivo* research purposes. If lentivirus and retrovirus are used, it is possible to make stable knockdown cells as a consequence of genome integration. So far, three large-scale siRNA libraries have been constructed, two for academic research (Paddison 2004, Berns 2004, Michiels 2002) and one for industrial sector by Galapagos, with more libraries covering a whole mammalian genome on the horizon. siRNA libraries are usually designed to explore and study target genes central to important biological pathways, which is important for development of novel therapeutic options. Because disease pathogenesis is driven by the alteration in multiple genes and/or pathways, it is expected that modulation of gene activity by siRNA might produce a therapeutic benefit. Thus, Galapagos library targeted over 4,900 human druggable transcripts like G-protein-coupled receptors, ion channels and nuclear hormone receptors. Bernards and colleagues constructed human RNAi library (the 'NKi library') covering 7,914 human genes (Michiels 2002). Genes implicated in cancer and other diseases, as well as genes coding for major cellular pathways like cell cycle, transcription regulation, stress signalling, proteolysis and metabolism are included in the library. However, a rather robust method in the laboratory environment turned out to pose quite a lot of technical challenges when used for treatment *in vivo*. For example, siRNAs are large molecules (~13kDa) with phosphodiester backbone bearing strong negative anionic charge that hampers diffusion through the anionic cell membrane surface. Until nowadays,

numerous delivery strategies have been developed to circumvent this problem, some of them being successfully employed for introduction of siRNAs into cells *in vitro* and *in vivo*. These systems are based on the use of diverse compounds or materials and viruses complexed to siRNAs, *e.g.*. chitosan-based siRNA nanoparticle delivery (Howard 2006), adenovirus-mediated siRNA delivery (Uchida 2004), antibody-mediated delivery of siRNAs *via* cell-surface receptors (Song 2005), or bioconjugation (Cheng 2006). Improved siRNA delivery (Whitehead 2009) resulted in efficient silencing of disease-associated genes, including allelic variants in tissue culture and animal models (De Paula 2007) that fostered interest in developing RNAi-based reagents for clinical applications, *e.g.*. cancer treatment, viral infections, autoimmune diseases and neurodegenerative diseases. However, blood stability, targeted delivery, poor intracellular uptake and non-specific immune stimulation are major bottlenecks in modern approaches to delivery of RNAi reagents in clinics. On the other hand, low siRNA production costs (Hall 2005) in comparison to antibodies and other therapeutic proteins make them appealing novel drugs. siRNAs possess favourable pharmacokinetic properties, can be delivered to a wide range of organs, and are increasingly considered as a basis for development of next generation targeted drugs.

Diverse RNAs may be also useful to mimic or antagonize miRNAs that are central to regulation of oncogenic or tumor suppressor pathways (Chen 2005). For example, Nohata et al. (Nohata 2011) observed that restoration of *miR-1* in cancer cells inhibits cell proliferation, invasion and migration, supporting the hypothesis that *miR-1* functions as a tumour suppressor in head and neck squamous cell carcinoma (HNSCC). Furthermore, transgelin 2 (TAGLN2), a potential oncogene, is directly regulated by *miR-1*. Silencing of *TAGLN2* significantly inhibited cell proliferation and invasion in HNSCC cells (Nohata 2011).

Recent clinical trials using siRNAs to cure age-related macular degeneration (Bevasiranib by Opko Health, Inc., Miami, USA; phase III) and respiratory syncytial virus infection (ALN-RSV01 by Alnylan, Cambridge, USA; phase II) have proved the therapeutic potential of RNAi pathways. In other studies with siRNA employed for treatment of disease *in vivo*, multiple non-specific effects were also observed. One of them occurs due to delivery of siRNA into target cells by lipid-mediated transfection, resulting in combined transfection and siRNA-specific effects (Fedorov 2005). Furthermore, common non-specific effect is the interferon-induced response (Sledz, 2003). For example, in patients with blinding choroidal neovascularisation receiving intravenous injections of siRNA, targeting vascular endothelial growth factor to block angiogenesis induced strong immune system response (Kleinman 2008). These common issues might be adequately addressed by careful optimization of concentration, delivery method and siRNA design. Nonetheless, proof of concept for RNAi-mediated specific gene silencing efficacy in humans was recently reported in a clinical trial of melanoma (Davis 2010). Nanoparticle-mediated siRNA delivery was employed for treatment of melanoma patients. Intracellular localized nanoparticles were detected in all tumour biopsies obtained upon treatment in amounts that correlated with dose levels of administered nanoparticles. Furthermore, a reduction of specific messenger RNA - M2 subunit of ribonucleotide reductase (RRM2) and the protein (RRM2) levels were observed as well (Davis 2010).

In conclusion, implementation of siRNA in clinical applications for treatment of disease through RNAi will be beneficial for such disorders that exert the symptoms *via* dominant-negative or gain-of-function mechanism. Here, we clearly foresee the challenge of inducing endogenous degradation of mutant RNAs while leaving wild-type transcripts unaffected.

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The aim of this book is to cover key aspects of existing problems in the field of development and future perspectives in gene therapy. Contributions consist of basic and translational research, as well as clinical experiences, and they outline functional mechanisms, predictive approaches, patient-related studies and upcoming challenges in this stimulating but also controversial field of gene therapy research. This source will make our doctors become comfortable with the common problems of gene therapy and inspire others to delve a bit more deeply into a topic of interest.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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