Expert Opinion

On the delivery of small interfering RNAs into mammalian cells

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RNA interference is becoming the technique of choice for analysing gene function and drug target validation. In this process, sequence-specific gene inhibition is initiated by small RNA duplexes, known as small interfering RNAs (siRNAs). The possibility that exogenously delivered siRNAs or endogenously expressed hairpin siRNAs can cause the destruction of specific target mRNA *in vitro* and in animal models has been demonstrated. However, the key challenges for the development of siRNAs as human therapeutics is largely dependent on the development of suitable delivery agents and improved siRNA specificity. This review highlights recent advances in siRNA delivery, as well as challenging problems related to immune stimulation.

Keywords: cationic liposome, innate immunity, RNA interference, siRNA, Toll-like receptor

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1. Introduction

RNA interference (RNAi) is a conserved biological process in which double-stranded RNA suppresses the expression of a target gene by triggering the specific degradation of the complementary mRNA sequence [1]. Initial studies in *Caenorhabditis elegans* identified RNAi as a sequence-specific post-transcriptional gene silencing mechanism similar to those described in plants [2]. The resulting active RNAs are ~ 21 – 25 nucleotides long, and are now referred to as small interfering RNAs (siRNAs) [3].

Despite the presence of RNAi in several organisms (*Drosophila*, certain parasitic *protozoa* and plants), researchers have been pessimistic about applying RNAi to mammalian cells because of the induction of the IFN response genes by double-stranded RNAs (> 30 nucleotides), leading to the unspecific inhibition of protein synthesis [4]. However, the seminal discovery by Tuschl and colleagues that siRNAs, normally generated from long dsRNA during RNAi, can function in a potent and sequence-specific manner to block gene expression in mammalian cells has paved the way for functional genomics [5]. RNAi involves a multistep process in which siRNA duplexes are incorporated into an RNAi targeting complex known as RNA-induced silencing complex (RISC) that uses the antisense siRNA strand to recognise the targeted mRNA, which is cleaved and further degraded by nucleases (**Figure 1**). The evolutionary conservation of the enzymatic machinery involved in RNAi reflects the importance of a second class of noncoding RNAs, known as microRNAs (miRNAs) [6]. These tiny RNAs are genome encoded as primary transcripts (pri-miRNAs), which are processed in the nucleus by Drosha, an RNase III, into pre-miRNAs (60 – 80 nucleotides). Subsequent to transport to the cytoplasm and processing by Dicer, mature miRNAs (22 nucleotides) are incorporated into the ribonucleoprotein complex that can direct either mRNA cleavage (RNAi) or translation arrest (**Figure 2**). Based on several studies, miRNAs are likely to be critical for normal development and tissue physiology in mammals [6]. Since the discovery of RNAi in somatic mammalian cells [5], a number of studies have demonstrated that

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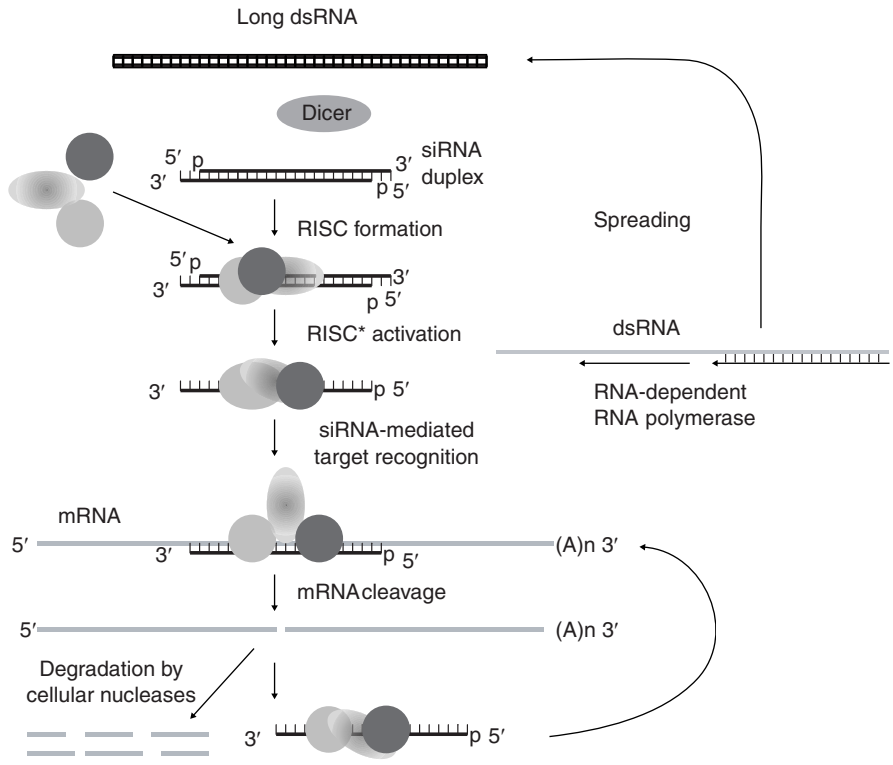

Figure 1. Gene silencing by siRNA. Double-stranded RNA is processed by the Dicer enzyme into 22 nucleotide siRNAs, which are then incorporated into a multiprotein complex termed the RISC. After unwinding by a helicase and activation of the complex (RISC*), the antisense strand guides the active RISC* to its site on mRNA, which is then cleaved by a nuclease. Cleaved mRNA is degraded by cellular nucleases. Amplification of silencing via RNA-dependent RNA polymerase occurs at various stages of the RNAi pathway and has been documented in plants and other organisms such as *Caenorhaditis elegans*.

dsRNA: Double-stranded RNA; RISC: RNA-induced silencing complex; RNAi: RNA interference; siRNA: Small interfering RNA.

siRNAs can be used to silence any gene of interest [7-8]. Gene silencing via RNAi offers several significant advantages over the existing ribozyme- and antisense oligonucleotide-based techniques [9], including ease of selection of suitable target sequences and efficacy. However, there are still some drawbacks associated with the former technologies, including delivery to the target cell, stability and activation of innate immunity.

2. Delivery

In general, siRNA delivery into living cells can be achieved by exogenous application of synthetic siRNAs or via an endogenous approach that relies on the expression of short hairpin RNAs (shRNAs) from plasmid or viral vectors (**Figure 3**). The choice of the delivery method is based on a practical determination.

2.1 Exogenous delivery

In the absence of a carrier agent, the anionic nature of nucleic acids hinders their passive diffusion through the lipophilic cell membrane. Even small nucleic acid molecules, such as antisense oligonucleotides and siRNAs, cannot generally pass through in sufficient amount to exhibit biological activities *in vitro*. To overcome this barrier, a number of methods have been developed to introduce DNA or RNA into human cells. Two of the most used delivery methods for siRNAs are cationic lipid-mediated transfection and electroporation [10,11]. Because of their inherent positive charge, cationic lipids have a high affinity for most negatively charged membranes and can be used virtually with any cell type. Moreover, lipid-based transfection of siRNAs is compatible with high-throughput analysis, as the reagents can be easily prepared and applied into experimental wells.

In the past, several varieties of liposomes have been developed and tested *in vitro* and *in vivo* [10-14]. Most of these liposomes are monocationic, polycationic or cationic polymers such as polyethylenimine (PEI) [15]. Cationic lipid-based liposomes are easy to prepare, reasonably cheap and nonimmunogenic. Using lipofectamine, several studies reported gene knockdown by siRNA of 40 – 99% depending on the target and cell lines used [7,8]. In contrast to electroporation, cationic liposomes deliver nucleic acids into cells predominantly via the endocytic pathway. Once inside the target cell, additional requirement for transfection include the need for nucleic acids, such as siRNAs, to escape the endosomes and subsequently the liposomes. Thus, efficacy is strongly dependent not only on their absorption by the cells, but also on the

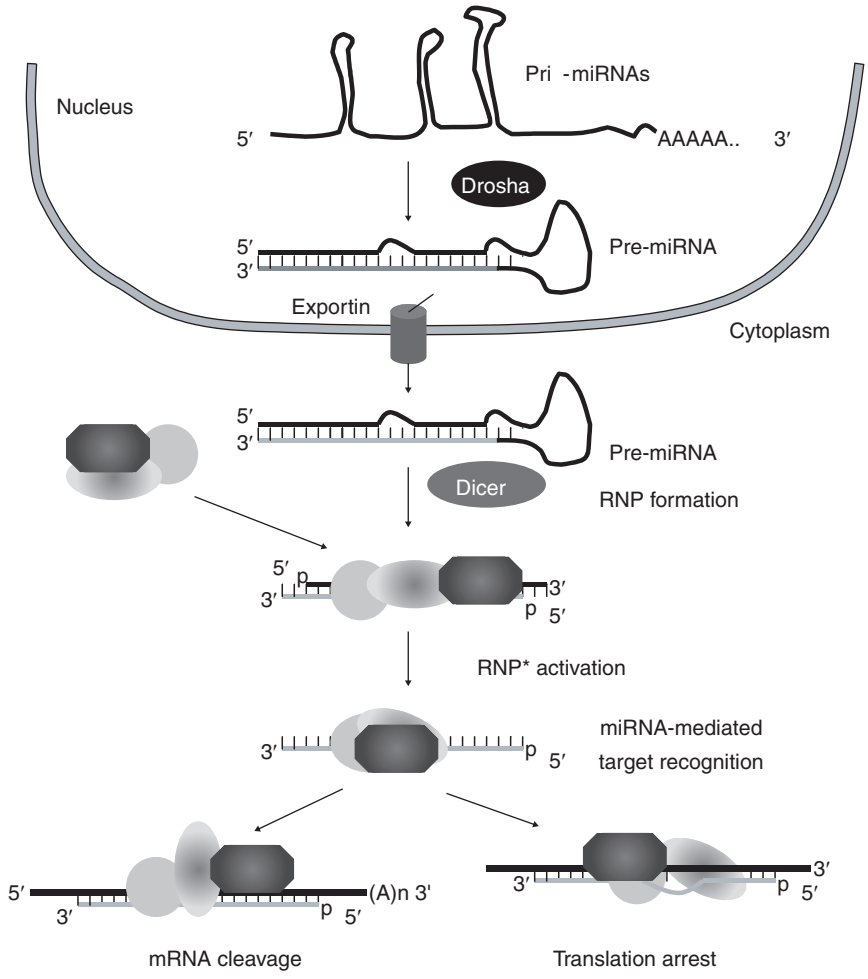

Figure 2. Gene regulation by miRNAs. miRNAs are derived from genome transcribed primary transcripts (pri-miRNAs) that are predicted to form multiple stems and hairpin structures. Pri-miRNAs are processed by the ribonuclease Drosha to 70-nucleotide pre-miRNAs that are transported to the cytoplasm by exportin. Subsequently they are transported into the cytoplasm, where Dicer processes the pre-miRNAs into mature 22-nucleotide miRNAs, which are then incorporated into a RNP complex that can direct either RNA cleavage (perfect complementarity with mRNA) or translation arrest (mismatches with target mRNA). Most of the proteins involved in miRNA processing and activity are also involved in RNAi.
miRNA: MicroRNA; RNP: Ribonucleoprotein; RNAi: RNA interference.

ability of lipids to cross intracellular barriers, and entry of the siRNA molecules into the RNAi pathway (**Figure 4**).

To achieve the release of liposome contents, liposome modification with various pH-sensitive polymers have been developed [14,16]; for example, dioleoylphosphatidyl ethanolamine (DOPE) forms a stable lipid bilayer at physiological pH 7, whereas at acidic pH 5, the hexagonal-II structure destabilises the endosomal membranes, leading to cytoplasmic delivery. Although the development of pH-sensitive liposomes has frequently been associated with the incorporation of DOPE in the liposome formulation, other strategies have also been explored, such as anionic pH-sensitive liposomes composed of diolein/cholesteryl hemisuccinate [14]. Furthermore, the inclusion of fusogenic peptides into the liposome formulations also improved cytoplasmic delivery. It should be noted that fusogenic properties of the liposomes do not always correlate with their efficacy in mediating intracellular delivery. Other chemicals (mostly polymers) have also been investigated for enhancing siRNA delivery, including PEI and its conjugates [17,18].

Commercially available cationic lipids have achieved promising results in siRNA delivery *in vitro*, their *in vivo* application has been hampered by their rapid elimination from the blood and the capture of the lipid preparations by cells of the reticuloendothelial system, primarily in the liver. At present, the role of various factors affecting the interaction of cationic lipids with blood cells remains unclear. Recently, smaller liposomes with a more stable lipid composition and protection from the reticuloendothelial system by surface coating with polyethylene glycol (PEG) have been developed [19]. The PEGylation reduces liposome recognition by opsonation and, therefore, subsequent blood clearance. Although, PEG

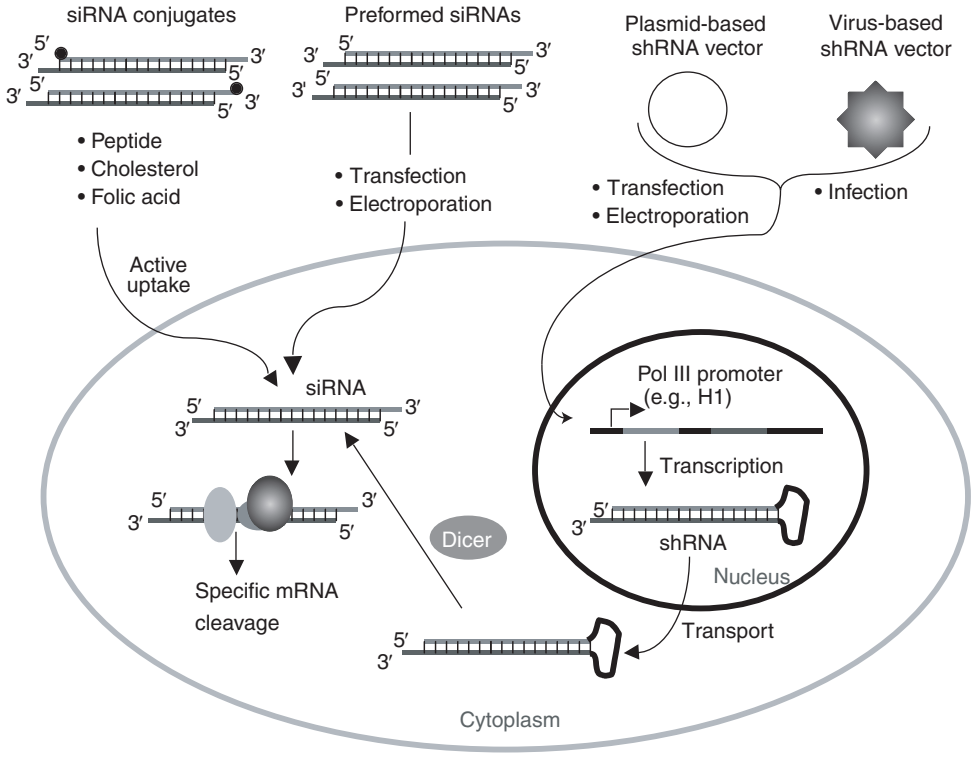

Figure 3. Basic delivery strategies. Synthetic siRNA duplexes and shRNA expressed from integrated pol III promoter in plasmid vectors can be delivered via either lipids or the electroporation method, whereas conjugate siRNAs can be added directly to cells. shRNAs expressed from integrated pol III promoter plasmid vectors in viral vectors (e.g., adenovirus, lentivirus and retrovirus) are delivered via infection with viral particles.
shRNA: Small hairpin RNA; siRNA: Small interfering RNA; Pol: Polymerase.

remains the main agent for steric protection of the liposomes, some studies reported on the use of other polymers that can be used to prepare long-circulating liposomes [20]. Generally, the use of liposomes of appropriate size (< 150 nm) can also contribute to the increase of the circulation time.

Despite the limitations mentioned above, various classes of lipid carriers have been used to deliver anticancer drugs, plasmids, antisense oligonucleotides, ribozymes and siRNAs *in vivo*. In addition to lipid formulations, a new strategy of nucleic acid delivery has recently emerged, which is based on the use of cell-penetrating peptides (CPPs) and protein transduction domains (PTDs). CPPs are defined as short peptides (5 – 30 amino acids) capable of forming complexes with nucleic acids and exhibiting membrane fusion and permeabilisation activities at neutral pH [21]. In particular, the synthetic MPG peptide developed by Divita *et al* was shown to deliver oligonucleotides such as siRNAs directly to the cytoplasm [22]. This short peptide (27 residues) contains a hydrophobic domain, derived from the fusion sequence of HIV glycoprotein 41, and a hydrophilic domain, derived from the nuclear localisation sequence of simian virus 40 T antigen. Promising results were obtained with peptides derived from several proteins known to enter various cells when added to the media [23,24]. Although the mechanisms of cellular entry are not understood, the penetratin (PEN; DNA binding domain of the *Drosophila melanogaster* transcription factor antennapedia) and Tat peptides translocate through the plasma membrane even at 4 °C and have been shown to mediate the delivery of siRNAs to multiple cell types [25].

Another approach takes advantage of the binding ability of certain proteins to nucleic acids and the internalisation of the formed complexes by cells. In this respect, Puebla *et al.* [26] showed that siRNA can be delivered to Hela cells via the use of the human tone protein H1F4. Minakuchi *et al.* used theatelocollagen, a highly purified pepsin-treated type I collagen from calf dermis, to deliver siRNAs to mammalian cells [27]. Although the use of peptides and proteins for siRNA delivery is a promising strategy, its application in humans has been largely hampered by their short half-life in the circulation. In addition, non-self peptides may activate the immune system.

2.1.1 In vivo delivery of synthetic small interfering RNAs

Current strategies for siRNA delivery into animals are based on several techniques using either naked siRNA or siRNA formulated with cationic lipids. Among these techniques, the hydrodynamics method (rapid injection via tail vein) has been applied to deliver naked siRNAs to major organs, including the liver, kidney, lung and heart, with delivery efficiencies of 30 – 40% [28-31]. Indeed McCaffrey *et al.* [28]

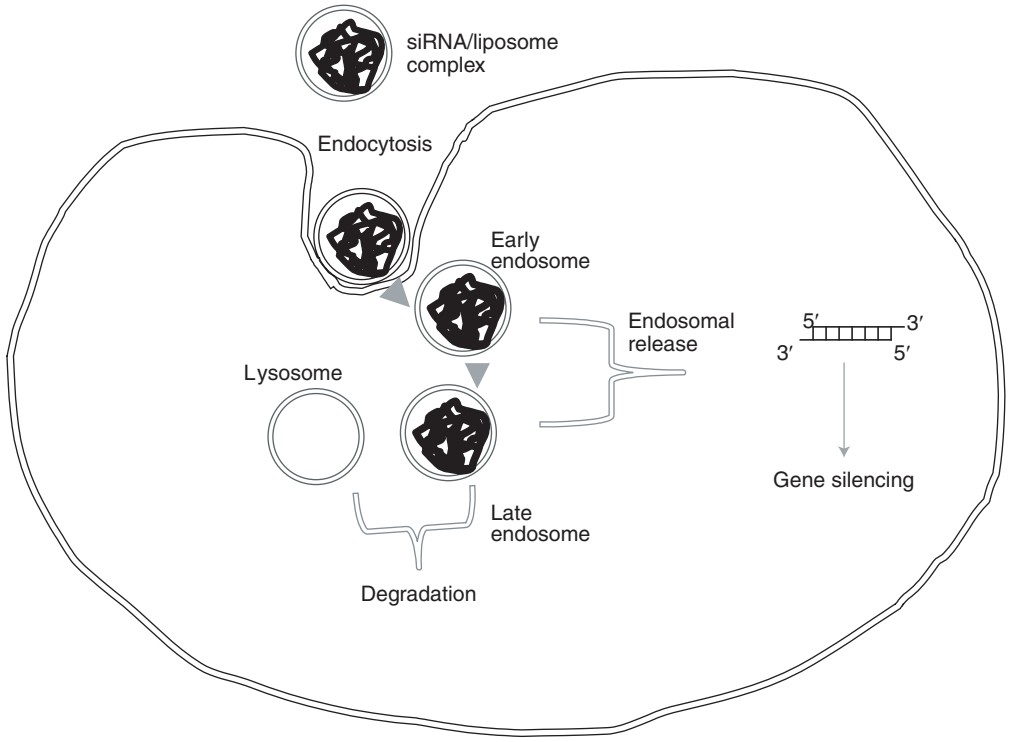

Figure 4. Mechanisms for cell entry of nucleic acid–liposome complexes. A complex of cationic lipid binds to the plasma membrane electrostatically and is internalised via endocytosis. Endosomes can fuse with lysosomes, leading to degradation of siRNAs. Certain fractions of nucleic acids, such as siRNAs, are released into the cytosol, leading to the activation of RNAi.
RNAi: RNA interference; siRNA: Small interfering RNA.

and Lewis *et al.* [29] reported RNAi-mediated transgene expression of an exogenous luciferase gene or hepatitis B virus mRNA by the hydrodynamics technique. Similarly, Song *et al.* [30] and Zender *et al.* [31] showed that mice treated with siRNAs against the gene encoding the Fas receptor or caspace, respectively, were less susceptible to liver damage induced experimentally. Although the hydrodynamics-based technique enables nucleic acids to circumvent one of the most important intracellular obstacles (e.g., passage via the cellular membrane and avoidance of endosomal or lysosomal degradation), a key constraint to this technique is that delivery tends to be restricted to highly vascularised tissues such as the liver, spleen or kidneys. In addition, this technique cannot be applied to humans.

In order to develop an alternative method that can induce RNAi effectively *in vivo*, experiments were initiated with cationic lipids [32]. To evaluate the ability of *N*-[1-(2,3-dioleoyloxyl)propyl]-*NNN*-trimethylammoniummethyl sulfate to deliver siRNA, the intravenous injection using a conventional volume (≥ 200 µl for a 20 – 25 g mouse) has been assessed. Examination of liver tissue showed a substantial amount of siRNAs around the vessels 6 h after injection [32]. Coadministration of an anti-green fluorescent protein (GFP) siRNA and a GFP-encoding plasmid inhibited GFP expression in several organs, such as the liver and spleen [33]. Having demonstrated that RNAi can function in whole adult mice, the next step was to investigate the possibility of silencing endogenous targets via the intraperiotoneal delivery of anti-TNF-α siRNAs. Injection of siRNAs inhibited *TNF-α* gene expression and delayed the onset of septic shock induced by lipopolysaccharide (LPS) injection [33]. Of the 12 recently investigated siRNAs, an siRNA targeting the mRNA site 5′-CCAACGGCAUGGAUCUCAA-3′ exhibited the greatest protective effect. **Figure 5** shows the *in vivo* inhibition of TNF-α expression by siRNA following intraperitoneal delivery. This example represents a proof of principle to apply siRNA technology to diseases associated with infections and target validation *in vivo*. Notably, severe sepsis and septic shock are life threatening complications of infections and the most common cause of death in intensive care units. The data also highlights the potential of using lipid carriers as delivery method for siRNA *in vivo*. In this respect, several studies reported on the use of cationic lipids to successfully silence gene expression in animals; for example, siRNA complexed with oligofectamine inhibited *β-catenin* gene expression and suppressed tumour growth in nude mice when delivered either via intravenous or intraperitoneal injections [34]. Administration of siRNAs via intraperitoneal delivery in complexes with PEI reduced influenza virus production in lungs of infected mice [35]. Interestingly, either given before or after virus infection, siRNAs exhibited similar effects on virus replication. Using via intraperitoneal delivery, Flynn and colleagues demonstrated that anti-IL-12 siRNA complexed with oligofectamine

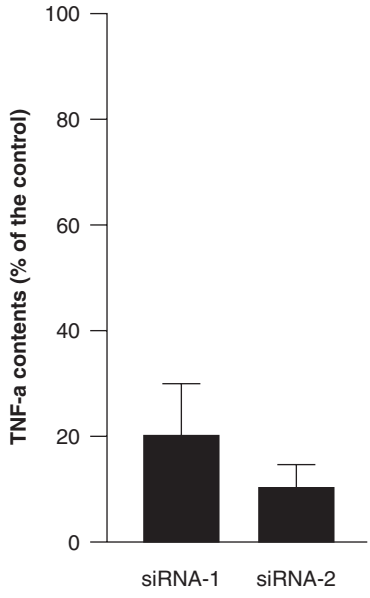

Figure 5. A representative example of *in vivo* modulation of TNF-α production by siRNAs. Mice were injected intraperitoneally with siRNA/liposome complexes (siRNA 50 μg, DOTAP 100 μg/mouse). After 18 h, the mice were intraperitoneally challenged with lipopolysaccharide 20 μg and euthinasised 8 h later. The peritoneal cavities were washed with PBS 2 ml and the TNF-α contents within the lavage fluids were measured by ELISA. The data are expressed as percentage of control animals that received a siRNA against the green fluorescent protein. Two active siRNAs were tested (siRNA-1; 5′-GGCCUUCCUACCUUCAGACTT-3′ and siRNA-2; 5′-CCAACGGCAUGGAUCUCAATT-3′). Only the sequence of the sense strand is shown. The sequence of siRNA-1 corresponds to that of site 3, which was mistakably indicated as site 5 in previous work [33].

DOTAP: *N*-[1-(2,3-dioleoyloxyl)propyl]-*NNN*-trimethylammoniummethyl sulfate; ELISA: Enzyme-linked immunosorbent assay; PBS: Phosphate-buffered saline; siRNA: Small interfering RNA.

can inhibit LPS-induced IL-12 expression in mice [36]. Similarly, infection by respiratory viruses was prevented by intranasally delivered siRNA complexed with the transfection agent TransIT-TKO® [37]. Taken together, the data show that it is possible to use lipid carriers to specifically target gene expression *in vivo*.

Typically, when using bolus injections intravenously, the highest concentration is achieved in highly vascularised tissues such as the liver and kidneys. Considering the potential of concentration-dependent side effects and the need to deliver siRNA to other organs, such as the brain, various delivery routes have been explored. As mentioned above, intranasal siRNA administration, complexed with the transfection reagent TransIT-TKO, reduced infection by respiratory syncytial virus and parainfluenza virus [37]. Subretinal and intravitreal injections of siRNA targeting vascular endothelial growth factor (VEGF) reduced angiogenesis [38]. Intranasal delivery to specifically target the lung has also been successful [39]. Intrathecal infusion of siRNAs has been administrated in rats, targeting the pain-related cation channel P2X3, and significant inhibition was obtained [40]. Intratumoural delivery of siRNAs against the colony-stimulating factor-1 or its receptor, c-fms, suppressed the growth of human mammary tumour xenografts in nude mice [41]. Intracranial delivery of anti-VEGF and protein kinase C-α siRNAs, complexed with the commercially available cationic polymer (PEI) from Aldrich, inhibited tumour growth *in vivo* (Sørensen and Sioud, unpublished data). Taken together, these data indicate that several tissues are amenable to RNAi.

2.1.2 *Activity of naked small interfering RNAs*

Based on studies with antisense oligonucleotides, it appears that *in vitro* 'naked' oligonucleotides are poorly internalised into cells; high concentration is required to observe antisense effects. Thus, delivery agents are required for the *in vitro* activity of antisense oligonucleotides; however, *in vivo* activity is obtained with naked oligonucleotides, and the addition of delivery agents have not shown to increase their efficacy. Similar to antisense oligonucleotides, 'naked' siRNA exhibited no significant activity *in vitro*. In addition, several reports indicated that administration of naked siRNA, even chemically stabilised, does not seem to induce RNAi *in vivo* [42]. However, a number of recent reports showed success with naked siRNAs in animal studies; for example, systemic intraperitoneal delivery of naked anti-VEGF siRNA (125 μg/kg/day) inhibited tumour growth in nude mice [43]. Intranasal administration of naked siRNAs also exhibited a substantial reduction of viral infection [37]. Intracerebroventricular infusion of siRNA via osmotic minipumps in the dorsal third ventricle produced a significant knockdown of GFP that was overexpressed in the mouse genome [44]. These findings are important as transfection reagents may themselves have adverse effects and/or render certain siRNA sequences more immunostimulatory (see Section 4).

2.1.3 *Improving small interfering RNA activity and biodistribution via chemical modifications*

One approach to addressing the challenge of siRNA systemic delivery includes stabilising modifications. The variety of chemical modifications that has been developed for antisense oligonucleotides and ribozymes can, in principle, be incorporated into the siRNAs. However, it is important that the silencing activity of the siRNA is not affected by modifications. Several chemical 2′-ribose and backbone modifications have been incorporated into siRNA without reducing their ability to block gene expression. In particular, siRNAs that have been modified with 2′-fluoro pyrimidines exhibited enhanced activity in cell culture as compared with 2′-OH containing siRNAs [45,46]. Interestingly, 2′-fluoro-modified siRNA also exhibited activity in mice delivered via the hydrodynamic transfection method [47]. By coupling these stabilised molecules to the appropriate conjugates, such as specific lipid formulation or

peptides, both delivery and stability challenges may be addressed. With regards to siRNA stability, a recent study indicated that once siRNAs are delivered into cells, modifications that enhance their stability exhibited no significant influence on siRNA activity or persistence [47]. These observations are consistent with those of Song *et al.* who reported a significant persistent suppression of Fas in liver cells by unmodified siRNAs (≤ 10 days) [30]. Although further studies are needed, the reported results may indicate that the RISC proteins do increase the siRNA stability *in vivo* [48]. Initial biodistribution studies indicated that phosphorothioate and phosphodiester RNA are distributed similarly and that phosphorothioate modification may improve tissue accumulation [49]. However, whatever the route of administration, intravenous or intraperitoneral injection, both modifications led to the accumulation of siRNAs in the liver and kidneys.

2.2 Endogenous delivery

2.2.1 Plasmid vectors

One of the drawbacks of synthetic siRNAs is the transient effect of gene silencing [5]. In contrast to plants and some organisms such as *Caenorhabditis elegans*, mammalian cells lack mechanisms that amplify silencing and ensure spreading (**Figure 1**). Consequently, the number of molecules introduced into a cell limits gene silencing induced by exogenous delivered siRNAs. Even if effective *in vivo* delivery is developed, the repeated delivery of synthetic siRNAs is needed to treat, for example, chronic diseases. To circumvent this problem, several groups have developed expression strategies for siRNA production in mammalian cells. One approach for silencing relies on the expression of transcripts that form shRNAs that can be processed by Dicer to produce siRNAs [50]. Most of the cloning strategies use the pol III promoter U6 or H1 to express the shRNAs [51,52]. The advantages of using pol III promoters to create shRNAs are that their transcription initiation and termination sites are well defined and highly conserved. Moreover, they are active in most, if not all, mammalian cells. In addition to pol III promoters, siRNA expressed from the modified cytomegalovirus promoter with minimal polymerase A (polyA) sequence exhibited *in vitro* and *in vivo* activity [53]. Artificially designed miRNAs targeting specific genes have been expressed in cells, and shown to induce mRNA cleavage [54]. Expression of a long nuclear-restricted dsRNA in cells and mice has also been shown to silence gene expression effectively [55]. Although these plasmids provide significant advantages over synthetic siRNAs, their delivery into cells is typically achieved via lipid-mediated transfection and, therefore, they suffer from the same issues as synthetic siRNAs.

2.2.2 Viral vectors

To overcome some of the problems related to plasmid vectors (e.g., low transfection efficiency), and to obtain efficient and long-lived gene silencing using RNAi, particularly *in vivo*, several groups have incorporated the siRNA expression cassettes into a variety of viral vectors [56,57]. Several types of viral vectors are commercially available at present for shRNA delivery and expression. Using adenoviral-based siRNA delivery approach, Uprichard and colleagues showed that hepatitis B virus replication *in vivo* can be cleared by siRNA targeting viral RNA for several days [58]. Effective delivery of siRNAs was also obtained with adenonovirus-associated viruses (AAVs), the only known mammalian virus that is capable of site-specific integration in human cells, thus minimising the chance of a mutagenic effect of the integrated virus [59]. AAV-mediated delivery of antityrosine hydroxylase siRNAs into mouse midbrain neurons reduced the expression of its target protein [60]. Interestingly, mice with reduced enzyme activity exhibited an attenuated locomotor response to amphetamine. In addition, Xia and colleagues showed that AAV-delivered siRNA against ataxin-1 can improve the behaviour of mice with spinocerebellar ataxia, a neurodegenerative disease [61]. Gene silencing has also been achieved with retrovirus- and lentiviral-based vectors [57,62,63]. One of the advantages of using retroviral vectors is their ability to integrate into the genome and maintain expression for long periods of time. Notably, sustained transgene expression in most clinical settings has only been achieved through the use of retroviral vectors such as the murine leukaemia virus [59]. Recombinant lentiviral vectors can also infect a broader host range including both dividing and non-diving cells. Furthermore, they can provide long-term gene expression because they can integrate their DNA into the host genome. Primary effusion lymphoma (PEL) was found to be associated with Kaposi sarcoma herpes virus. To inhibit PEL growth in a murine model, Godfrey *et al.* targeted the viral cyclin and FADD-like IL-1β-converting enzyme mRNAs using a lentiviral vector [63]. Interestingly, most mice remain ascites-free after treatment, indicating the amenability of this cancer to lentiviral-delivered siRNAs. Based on these results, viral-based delivery could be successfully used clinically in patients.

Whatever the nature of the expression vector used, it is clear that for efficient silencing to be achieved, the shRNA transcripts must be efficiently transported from the nucleus to the cytoplasm, be processed by Dicer to a siRNA capable of associating with RISC and triggering target mRNA cleavage. Depending on the tissues and/or cell types, all cloning strategies require significant optimisation to design expression vectors that deliver the appropriate amount of siRNAs for gene inactivation.

2.3 Targeting small interfering RNA to specific tissues and/or cell types

Ideally, an effective drug must be active only in the targeted cells. As the delivery systems mentioned above can enter all cell types, specificity must be built into the delivery agents or the expressed siRNAs. Cellular specificity can be achieved by the use of ligands (e.g., peptides, antibodies) that recognise cell-specific receptors. Ligand-directed liposomes showed success in targeting genes to tumours. Several

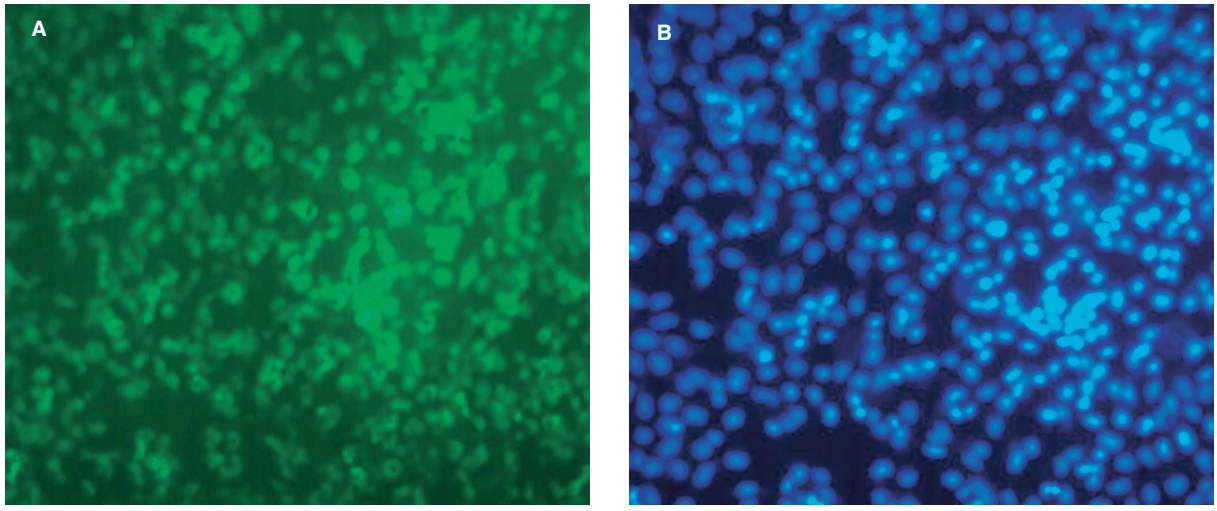

Figure 6. Folate-mediated targeting of siRNA tumour cells. The sense strand of a siRNA targeting the proteine kinase was synthesised with 5′-FITC and 3′ amino group. To prepare the conjugate with folate, 3′-amino RNA 30 nmol was mixed with folic acid 300 nmol and sulfo-NHS 450 nmol in 0.2 ml 0.02 M phosphate buffer at pH 7.4. The reaction mixture was stirred at room temperature for 5 h and then the conjugate was separated from free folate by gel filtration on sephadex G25. The double-stranded siRNA was obtained by mixing equal amount of sense and antisense strands. MCF-7 cells were incubated with folate–siRNA conjugate (200 nM) for 12 h. Subsequently, the cells were incubated with Hoechst for 5 min, fixed with ethanol, washed with PBS and then analysed by a fluorescent microscope. **A.** Cellular uptake of the folate-siRNA conjugate, **B.** Hoechst nuclear staining.
FITC: Fluorescein-5-isothiocyanate; NHS: *N*-hydroxysuccinimide; PBS: Phosphate buffered saline; siRNA: Small interfering RNA.

monoclonal antibodies have been shown to deliver liposomes to many target cells [64]. Targeting tumour cells with folate-modified liposomes has been reported. Indeed, liposomal daunorubicin, as well as doxorubicin, have been delivered into various tumour cells via folate receptors, which are frequently overexpressed in a range of tumour cells [65,66]. A similar approach was applied to the delivery of therapeutics via transferin receptors [67]. Coupling a folic acid to antisense oligonucleotides led to selective delivery to tumour cells [67]. Recently, the isolation of breast cancer-targeting peptides by panning a phage display library on intact cells has been reported, and it has been shown that the selected peptides can deliver antisense oligonucleotide in a cell-specific fashion [68]. To target tumour neovasculature expressing integrins, siRNA targeting the VEGF receptor-2 was delivered with sterically stabilised nanoparticle with an exposed Arg-Gly-Asp peptide and sequence-specific inhibition was obtained in tumours [18].

Despite the successful data obtained with siRNA in amimals, efficient and controlled delivery of siRNAs to target cells is crucial for success in humans. One strategy to achieve this objective is to either use targeting liposomes or to directly conjugate siRNA to cell-specific ligands, in particular small molecules. Early studies indicated that conjugation to either the 5′- or the 3′-end of the sense strand had no inhibitory effect on the gene silencing. Although specific delivery was not the main objective of the study, conjugation of siRNAs with lipophilic molecules such cholesterol increased their cellular uptake [69]. In addition, intravenous injections of the siRNA-cholesterol conjugates into mice resulted in uptake into several tissues such as the liver, lung and kidneys. In contrast to the unconjugated siRNA, the cholesterol-conjugated siRNA inhibited its target apolipoprotein-B, resulting in reduced blood cholesterol level. In these experiments, animals have received a large doses of siRNAs (50 mg/kg, for three consecutive days), a concentration that is extremely high when compared with either clinically accepted doses or to that obtained with systemic delivery of anti-TNF-α siRNA formulated with cationic liposomes (**Figure 5**). When covalently linked to siRNAs, penetratin and transportan peptides also facilitated siRNA delivery into human cells [25].

In order to improve the delivery of siRNA into tumour cells, a folic acid was coupled to siRNA. Preliminary results showed significant uptake of folate-conjugate siRNA by the breast cancer cell line MCF-7 (**Figure 6**). Thus, it will be interesting to determine if such preferential delivery translates into improved siRNA therapeutic activity *in vivo*. Using folic acid as a ligand for targeted siRNA has several advantages compared with peptide-based targeting, including a lack of immunogenicity, high affinity, low molecular weight and folate conjugates are efficiently internalised into cells via folate receptors.

Although viral delivery of shRNA is an attractive option for siRNA-based gene therapy, the immunogenicity and cytotoxicity have limited the clinical usefulness of this gene delivery method. To overcome the problem related to cytotoxicity, adenovirus vectors have been retargeted by either genetic or non-genetic means [59]. In this respect, targeting peptides have been functionally incorporated into several coat proteins [70,71]. Inserting an Arg-Gly-Asp motif that

binds to integrins facilitated vector uptake by tumour cells. These modified viruses have not been used for the delivery of siRNA but one can speculate that the combination of these two technologies will generate new opportunities in ligand-directed targeting of siRNAs.

3. Regulation of small interfering RNA activity

Whereas constitutive expression of siRNAs has provided important insights into the analysis of gene function and target validation in animals, regulation of siRNA expression should further extend the application of RNAi. Different methods can be used to ensure that RNAi is tissue- and/or time-specific [73-76]. Regarding tissue targeting, one approach is to place the vector under a tissue specific promoter. In this respect, several cancer-specific promoters, including promoters for genes encoding prostate-specific antigen (PSA), telomerase and survivin, have been characterised and used in gene therapy. In addition to being tissue-specific, the PSA promoter is androgen responsive. Recently, Song *et al.* reported on the expression of siRNA from either a plasmid or lentiviral-based vector under the control of the PSA promoter [77]. Reduced gene expression was achieved in a tissue-specific and hormone-dependent manner. In respect to timing, one approach to this is to place the expression of shRNA under the control of an inducible promoter. This has been accomplished by the incorporation of tetracycline or ecdysone responding sequences into the pol III promoter [75-78]. In another approach, Rossi and colleagues developed a target-dependent siRNA-inducible system using a pol II promoter, in which the target virus (HIV) specifically triggers the expression of the siRNA [79]; therefore, only infected cells will express the siRNA. Other promising results were obtained with the Cre-driven recombination of modified pol III promoters. Indeed, several studies demonstrated that gene silencing could be induced or inhibited by Cre-driven recombination of modified promoters [80-82].

4. Off-target effects and cellular response to small interfering RNAs

Although siRNA-mediated gene knockdown was originally reported to be highly specific, several recent studies have shown that siRNA can silence several genes in addition to the gene intended [83,84]. Interestingly, even stretches of cross-hybridisation as small as 11 nucleotides between a siRNA and mRNA can trigger gene silencing [83]. Because siRNAs can function as miRNAs, it is more likely that some non-target genes will be inhibited by any given siRNA. As these sequence-dependent off-target effects cannot be predicted from the siRNA sequences, several different siRNAs should be used. Furthermore, an effective siRNA should be titrated and must be used at the lowest effective concentration in order to minimise these potential off-target effects [84]. It is worth noting that these effects are not observed with siRNA generated by Dicer, indicating the presence of a proofreading activity that protects other genes being affected.

In addition to cross-hybridisation, however, cells can recognise siRNAs as pathogen-associated molecular patterns and divert specific RNAi-mediated targeting to a response that is proinflammatory. In contrast to early work by Tuschl and colleagues, it has been noted that certain siRNA sequences can induce immune responses in freshly isolate blood cells, leading to TNF-α and IL-6 production [32]. Moreover, siRNAs induced type I IFN response via the activation of double-stranded RNA activated protein kinase [85]. A recent study has indicated the involvement of toll-like receptor 3 in siRNA activation of RNA-dependent protein kinase [86]. Long double-stranded RNA-mediated TLR3-signalling has been shown to lead to activation of the immune system, and induction of type I IFN [87]. Interestingly, the activation of the inflammatory and IFN responses by either double- or single-stranded siRNAs in adherent peripheral blood mononuclear cells was found to require endosomal acidification. Within the endosomes, siRNAs activate the immune system through a much more specific and restricted class of receptors, in particular TLR8 [88]. Several double-stranded siRNAs did not induce inflammatory cytokines and IFN responses; however, their single-stranded sense or antisense did. This observation would support the hypothesis that within the endomosal compartment, certain double-stranded siRNAs may dissociate and activate the TLR8 [88]. Although the full characterisation of the stimulatory motifs needs further investigation, the data indicate that GU nucleotides are involved in siRNA recognition by TLR8. In accordance with these findings, a recent study showed sequence-dependent stimulation of the immune system by siRNAs [89]. Taken together, the data illustrate the importance of developing a delivery system that can bypass the endosome compartments. Alternatively, chemical modifications may block the recognition of siRNAs by endosomal TLR8 and activation of the immune response. However, if we view the induction of inflammatory cytokines as a beneficial mediator in cancer and infectious diseases, immunostimulatory siRNAs could emerge as a viable agent to knockdown specific genes and activate innate and acquired immunity against tumour cells.

5. Expert opinion and conclusions

As with any new compound, issues of delivery, distribution and clearance are major obstacles before siRNAs can be adapted to clinical trials. After entering the systemic circulation, either via intravascular injection or via other routes of administration, carrier vesicles are expected to encounter blood cells and a range of serum proteins. The design of a delivery system that minimises such nonspecific interactions would improve the therapeutic activities of siRNAs. Although some success was achieved with naked siRNAs, the use of chemical modifications should facilitate the design of siRNAs that can be administered as simple saline solutions. In this

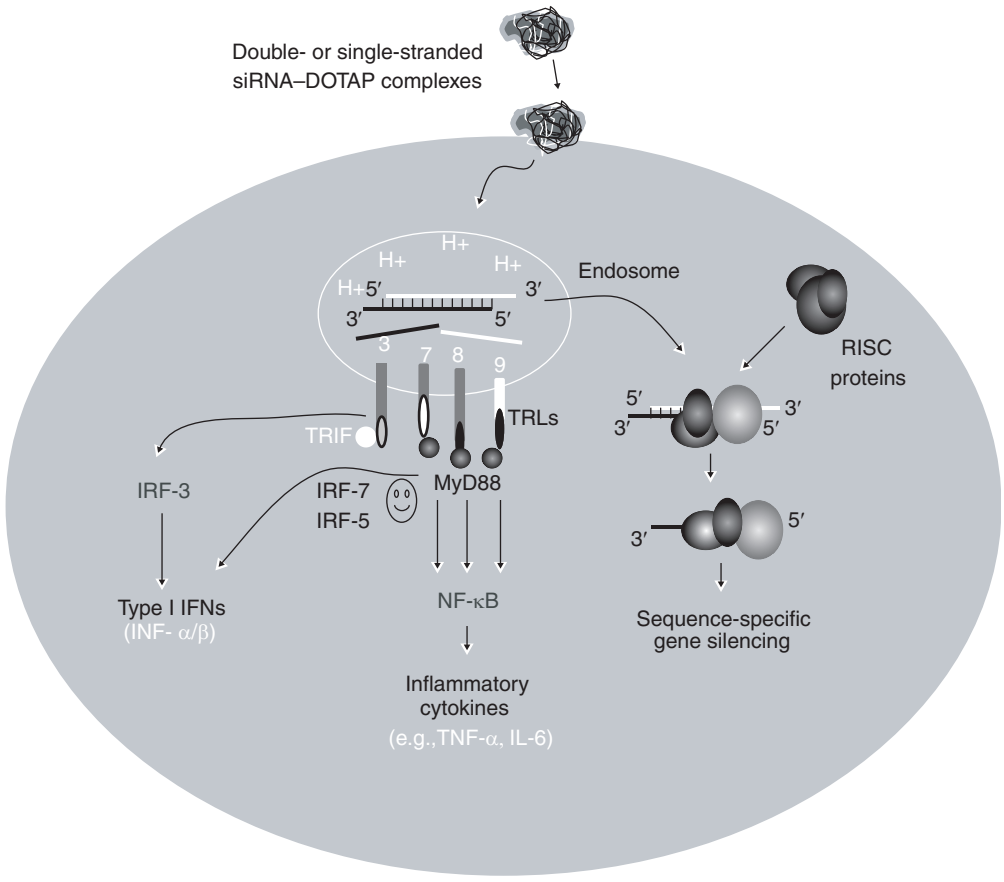

Figure 7. Schematic illustration of intracellular siRNA signalling. Cationic lipid siRNA complexes are internalised via endocytosis. Within the endosomal compartments, RNA molecules can bind to Toll-like receptors, in particular TLR7 and 8, which can activate the transcription factor NF-κB. This will lead to the induction of NF-κB responding genes such as TNF-α and IL-6. Analysis of global gene expression in peripheral blood mononuclear cells and monocytes transfected with a single-stranded siRNA revealed a significant upregulation of the IFN regulatory factor 7, which is responsible for the induction of type I IFNs. Notably, IFN-α production by either double- or single-stranded siRNAs in PBMC requires endosomal localisation and acidification, as was strongly inhibited by chloroquine or bafilomycin [88].

DOTAP: *N*-[1-(2,3-dioleoyloxyl)propyl]-*NNN*-trimethylammoniummethyl sulfate; IRF: IFN redulatory factor 7; RISC: RNA-induced silencing complex; siRNA: Small interfering RNA; TRIF: Toll-like receptor domain containing adaptor inducing IFNβ; TLR: Toll-like receptor.

respect, cholesterol–siRNA [42] and folate–siRNA conjugates (**Figure 6**) represent the first step toward the development of pharmaceutical siRNAs that can be taken up by cells. In addition, specific targeting of siRNAs to particular tissues and/or cell types should also facilitate the clinical applications of siRNAs and reduce their unwanted side effects. This issue could be addressed by using specific liposomes and/or small ligands that can bind to cell-specific receptors. Placing the expression of shRNA under tissue-specific promoters is a second option that should be further investigated. Notably, lessons learned in developing delivery strategies and clinical trials with other forms of nucleic acids, such as ribozymes and antisense oligonucleotides, will serve as a starting point for improving the therapeutic applications of siRNAs.

Comparable to antisense oligonucleotides, siRNAs were found to activate the immune system via TLRs in particular TLR8 (**Figure 7**). As has been previously suggested, there is a need to examine the immunostimulatory effects of any potential therapeutic siRNA in human blood cells prior to clinical applications [7,32]. Lessons learned from the antisense field with regard to chemical modification strategies to enhance stability and reduce the immune stimulation are likely to lead to improved siRNA design. With regards to siRNA application in humans, non-viral vectors have important safety advantages over viral approaches, including their reduced pathogenicity and capacity for insertional mutagenesis, as well as their low cost and ease of production. The development of effective non-viral delivery, such as direct derivatisation of siRNA with folate, should facilitate the clinical application of siRNAs.

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