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siRNAs: their potential as therapeutic agents – Part II. Methods of delivery

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RNA interference (RNAi) is a sequence-specific mechanism to control the expression of target genes. This technique has proven potentials both *in vivo* and *in vitro*. The main hurdle for using RNAi-based therapy is the effective delivery of RNAi-based drugs to the target cells or tissues *in vivo*. The aspects of off-target effects, delivery methods, induction of immune response and dose determination for delivery should, however, be considered carefully. If these challenges associated with siRNA can be met, then the potentials of RNAi could be exploited to the full for the development of therapeutic tools and drugs.

Introduction

RNA interference (RNAi) is a highly conserved process of post-transcriptional gene silencing triggered by double stranded RNA (dsRNA). In the process of RNAi, long double stranded RNA (dsRNA) is cleaved by the enzyme Dicer. Once processed by dicer, one of the strands in the small RNA duplex is incorporated into the RNA-induced silencing complex (RISC) and binds to the target mRNA by complementary base pairing, which subsequently suppresses gene expression either by cleavage or by translational repression. As a result siRNAs have been widely used in the field of functional genomics to study gene function *in vitro*. siRNA delivery methodologies need to be standardized in order to establish them as a potential therapeutic tool. siRNA delivery, therefore, still represents a significant challenge. Appropriate siRNA delivery technologies may play a significant role in reducing off-target and toxicity related effects.

siRNA delivery approaches

Naked siRNAs do not freely diffuse across the cell membrane, because of their relatively large molecular weight and polyanionic nature. Therefore, a delivery system is required to facilitate siRNA access to its intracellular site of action. Different strategies have been reported for the *in vivo* and *in vitro* delivery of siRNAs. Broadly, we can categorize them into viral and nonviral delivery methods.

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Nonviral delivery approaches

Naked siRNA/shRNA/dsRNA delivery approaches

Nonviral delivery methods can involve the use of unmodified, chemically synthesized siRNAs, chemically modified siRNAs/shRNAs, *in vitro* transcribed siRNAs and siRNA/shRNAs/dsRNAs expressed by plasmid based vectors. Delivery can be achieved using liposomes, lipids, protein–antibody conjugates and peptides and so on. Routes of administration can be classified into local and systemic. Local delivery approaches require lower amounts of siRNA. This avoids unwanted delivery to other organs and decreased elimination through renal filtration [1]. Intratumoral injection of siRNAs targeting the survivin gene into bladder cancer xenografts inhibited tumor growth in mice [2]. Direct application of siRNA to the eye has been used to target VEGF after laser-induced choroidal neovascularization in a mouse model [3]. Intrathecal/intraventricular administration of appropriate siRNAs has been demonstrated to downregulate the serotonin transporter [4] and the pain-related cation channel, P2X3, in rat models [5]. Zhang *et al.* demonstrated the intranasal administration of siRNAs against heme oxygenase-1 in mouse lung. This study suggests that siRNA delivery can be done without viral vectors [6]. Similar studies showed that the intranasal administration of siRNA against para-influenza and respiratory syncytial virus (RSV) also provided protection in mice [7].

Systemic delivery of siRNA is another approach for delivering siRNAs. Systemic delivery can be performed in several ways by using siRNAs encapsulated in liposomes, lipoplexes and cationic lipids; injection of unmodified and chemically modified siRNAs and by hydrodynamic injection or electropulsation [1]. Hydrodynamic injection has been successful in targeting caspase-8 to

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prevent Fas-mediated apoptosis and attenuation of acute liver damage [8] and in targeting FAS in fulminant hepatitis to protect the mice from liver failure and fibrosis [9].

siRNAs can be used *in vitro* or delivered *in vivo* in their natural form or after incorporating chemical modifications. Naked siRNAs can be transfected into cells *in vitro* to induce RNAi. Different transfection methods have their own advantages and limitations. The induction of innate immune response has not been reported for siRNA delivered through electroporation, albeit in only a few cases. Liposome-mediated transfection of the same siRNAs, however, led to the induction of an immune response [10,11]. Delivery through nucleofection has been found to be more efficient than electroporation and other transfection methods. shRNAs can be produced through *in vitro* transcription by using phage polymerase and can be used either *in vitro* or *in vivo*. The loop sequences of shRNA also contribute in enhancing the efficiency of shRNAs [12,13]. shRNAs act as substrates for dicer and gives rise to siRNAs.

siRNAs or dsRNAs can be delivered by soaking or immersing the hosts in a solution of siRNAs or dsRNAs. This was first demonstrated in the case of *Caenorhabditis elegans* [14,15] and subsequently in *Entamoeba histolytica* [16] and many other insects, such as ticks [17], honey bees [18] and western corn rootworm (WCR), *Diabrotica virgifera virgifera LeConte* [19]. *Drosophila melanogaster* has been reported to take up siRNA/dsRNAs by soaking during specific stages of its development like early embryonic stages [20]. Some cell lines, like *Drosophila* S2 cells, can take up siRNAs/dsRNAs by soaking [21,22] but this type of uptake is limited to relatively few cell lines.

The use of a pool of siRNAs against a single target might be useful in only a few cases. siRNA pools can be produced enzymatically by dicing dsRNAs produced by *in vitro* transcription using an *Escherichia coli* endoribonuclease (RNase-III) or recombinant dicer [23,24]. These enzymatic siRNAs (esiRNAs) can be delivered to cells to produce efficient silencing [25,26]. siRNA pools show lesser off-target activity than individual siRNAs because when an siRNA pool is used to silence a target gene, the concentration of individual siRNAs against specific targets is reduced and, hence, the off-target activity of each siRNA will also be reduced [27].

Plasmid-based delivery approaches

An alternative to gene silencing by unmodified or modified siRNAs is plasmid-mediated expression of shRNAs or siRNAs. Plasmids encoding shRNAs or siRNAs with both sense and antisense sequences remain separated either by a loop-like sequence or intron and can be expressed under the control of promoters of choice.

Promoters can be RNA polymerase II (pol-II)-dependent or RNA polymerase III (pol-III)-dependent. Pol-III promoters, such as U6 are expressed constitutively in almost all cells and their transcription initiates at a precise position and ends precisely with an overhang of uridine residues. A stretch of more than four thymidines can be used as a termination signal. These can be very helpful in expressing short stretches like shRNAs by avoiding unwanted nucleotides at their termini. $tRNA^{val}$ and H1 promoters are the other pol-III promoters, which are weaker than U6. Because overexpression of siRNAs/shRNAs results in undesired toxic effects, the promoters have to be chosen carefully according to their strength of expression in order to avoid toxic effects. There are many well-characterized pol-II promoters that can be used to express shRNAs. Such promoters provide the advantage of regulating RNAi temporally and/or spatially, depending on their individual properties. Inducible promoters such as tetracycline-inducible promoter and metallothionine promoter are used to induce and control the process of RNAi, but pol-II promoter products do not terminate at precise positions and, hence, transcripts will have many unwanted nucleotides at their termini, which might interfere in inducing sequence-specific RNAi. These unwanted sequences in the transcripts can even make the target sequences unrecognizable by the RNAi machinery [28]. Peng *et al.* developed a system that combines the advantages of two systems (the precision of T7-RNA polymerase and regulation of expression by pol-II promoters). They made a plasmid construct that expresses shRNAs under T7-RNA polymerase promoter and T7-RNA polymerase expression was controlled by a pol-II promoter. So the T7-RNA polymerase can be regulated by the pol-II promoter, resulting in regulated shRNA expression in the cell [29]. One of the bottlenecks in these strategies is the efficiency of delivery, which differs from cell to cell and is highly variable under different experimental conditions.

RNAi can be executed by delivering dsRNAs through bacteria. Bacteria can express dsRNAs under the control of the T7 RNA polymerase promoter and, when they are ingested by host cell or organisms, the dsRNAs expressed in the bacteria can trigger RNAi in the respective cell or organism. The first report on the use of bacteria for dsRNA delivery was reported in *C. elegans*. *C. elegans*, which normally feed on bacteria, were effectively targeted by using *E. coli* expressing dsRNAs against *unc-22*, *fem-1* and *GFP* [15] but the efficiency of silencing was found to be variable for different genes [30]. Similarly, RNAi was induced in other nematodes *Trichostrongylus colubriformis* [31] and *Paramecium* [32]. Lactic acid bacteria *Lactobacillus casei* have also been used for the delivery of shRNAs/dsRNAs into host cells [33].

Trans-kingdom RNAi (tkRNAi) is another way of eliciting RNAi by using bacteria. This is achieved by using a plasmid called *Trans*-kingdom RNAi plasmid (TRIP). The TRIP plasmid expresses shRNAs by a T7 RNA polymerase promoter. The same plasmid also encodes the *Inv* (Invasin) gene derived from *Yersinia pseudotuberculosis* [34]. This gene localizes on the surface of bacteria encoding it and can interact with β-integrins present in host cells. This interaction leads to the endocytosis of bacteria expressing *invasin*. This TRIP plasmid also encodes another gene called *hly* gene encoding *listeriolysin O*, derived from *Listeria monocytogenes* [35,36]. This gene product (listeriolysin O) is released into the endosome of the host cell during the lysis of bacteria. The dsRNA expressed under T7 RNA polymerase promoter in the bacteria is released into the cytoplasm after the disruption of endosomal membrane. This dsRNA then acts as a trigger to RNAi. This strategy was used to knock down the genes in cell lines [37]. The oral delivery of TRIP-containing bacteria could induce the RNAi locally in the intestinal epithelial cells in mice.

Apart from these nonpathogenic bacteria, many other infectious bacteria have also been used to elicit RNAi in the host cells and this phenomenon is called as bacteria-mediated RNAi (bmRNAi), for example *Salmonella typhimurium*. These infectious bacteria can deliver plasmid encoding shRNAs into host cells. Production of shRNAs in the host cell will be dependent on the transcription machinery of the host cells. So the plasmid vectors used for bmRNAi contain expression cassettes compatible with the

host transcription machinery. The bacteria used in such cases were attenuated. Attenuated *S. typhimurium* gets accumulated in tumors with very high affinity and distributed in all other tissues. So this strategy was used to demonstrate the utility of *bmRNAi* using *S. typhimurium* in specifically eliciting tumor-specific RNAi [38].

Viral delivery of siRNAs

The main advantage of using virus-mediated siRNA/shRNA delivery system is that this can be used in cells that are difficult to be transfected by other methods and even can be used in nondividing cells. The viral vectors transduce cells naturally and show a very high transduction efficiency compared to transfection or any other nonviral methods. Viruses can be made less or nonvirulent by using genetic engineering techniques. The most widely used viral vectors for shRNA delivery include Adenovirus, Adeno associated virus (AAV), Lentivirus, Retrovirus, Herpes and Baculovirus vectors. The host range of these viruses can be increased by pseudotyping with envelope proteins of other viruses.

Adenoviral vectors (AdV)

Adenoviral vectors (AdV) are used mainly because of their ability to be transduced into a broad range of dividing and nondividing cells. Compared to other viral vectors, the use of first and second generation AdV has been limited, as a result of their immunogenicity and low packaging capacity (approximately 7.5 kb). The second generation vectors, which have deletions in the E2-E4 locus are comparatively less immunogenic and show prolonged expression of recombinant genes [39–41]. Third generation AdV were produced by deleting all the viral genes. This avoided the expression of viral genes, resulting in lesser immunogenicity and also increased the packaging ability of AdVs [42]. A wide variety of promoters based on pol-II, pol-III [43] and inducible promoters [44] can be used in AdV to express shRNAs.

Usually, adenovirus-mediated expression is short-lived because it does not integrate into the genome. To increase the duration of expression, the first generation AdV were engineered to have ITRs of AAVs. These hybrid viruses can replicate like AdV and get integrated into the cellular genome. This integration results in the persistent expression of genes (or shRNAs) cloned between ITRs [45]. Stable integration of transgenes has also been demonstrated by using Retrotransposon–AdV hybrid vectors [46] and hybrid AdV–AAV vectors. This seems to be a feasible strategy for the stable expression of shRNAs with prolonged effects on RNAi. The major concerns for using AdV are their immunogenicity and the duration of silencing effect.

Adeno associated virus (AAV)

AAV, having a single stranded DNA genome of 4681 nt, belong to the class *Parvoviridae*. AAVs are one of the most commonly found viruses in the human population. Nearly 80% of the human population is seropositive for AAVs [47]. The essential parts of their genome are inverted repeat regions (ITRs) of ~145 nt. The presence of ITRs *in cis* is essential for packaging of the genome. DNA of approximately 4.5 kb in size, capable of expressing shRNAs, can be packaged in this vector under the control of pol-II or pol-III promoters. The packaging can be done in different variants or serotypes of AAVs (cross-packaging) to use them repeatedly and to avoid the induction of humoral immune responses [48]. AAVs and the cross-packaged AAVs can transduce a broad range of tissue types [49]. They can transduce into dividing and nondividing cells with very high efficiency.

AAVs are probably the safest of the available viral delivery methods because they have not been found to be associated with malignancy and they do not induce immune response like AdV [50]. Another important feature of AAVs is their low rates of random integration into the host genome, which is potentially a major concern. Hybrid Herpes virus type 1–AAV vectors [51] and Hybrid Baculovirus–AAV vectors [52] have been used in site-specific integration of genes. Although this strategy has not been applied yet in the expression of shRNAs, this can be used to minimize their random integration into the host genome. Persistent expression of shRNAs helps in the long-term silencing of genes in animals. Moreover, the addition of modifications in AAVs helps in tissue-specific delivery [53], inducible expression of shRNAs [54] and combinatorial knockdown of genes [55]. AAVs have been efficiently used to specifically knock down genes in the mouse mid brain and their effect lasted for about 50 days [56]. AAVs have been used for knocking down genes of different viruses such as: HIV-1 [57], dengue virus [58] and hepatitis B virus [59]. Careful selection of promoters and siRNA sequences, along with delivery of optimal quantity of the virus are the important parameters to get efficient RNAi.

Retroviral vectors

Most widely used retroviral vectors are the derivatives of murine leukemia virus (MLV). The advantage of these vectors is their ability to integrate into the host genome, which results in a long-lasting expression of genes or shRNAs. One important factor to be considered here is that the integration of retroviral vectors takes place in a random fashion, which could lead to many undesirable effects. Another limitation of these viruses is that they can only transduce into dividing cells [60]. Their host range is restricted because they enter cells by binding to specific receptors, but their host range can be broadened by pseudotyping them with other viral protein like *env* of Gibbon ape leukemia virus (GALV), or feline endogenous retrovirus (RD114) [61]. Retroviral vectors have been used effectively in knocking down many genes, including *p53*, in a wide variety of cells [62]. Retroviral vectors can be used to express shRNAs by pol-II- or pol-III-based vectors.

Lentiviral vectors

Lentiviral vectors are capable of transducing both dividing and nondividing cells but the efficiency of transduction is highest in dividing cells. Unlike the above-mentioned retroviral vectors, lentiviral vectors can enter the nuclei of nondividing cells. The lentiviral vectors can transduce in only a very limited cell types, but pseudotyped lentiviruses can transduce efficiently into a wide variety of cell lines and tissues [63] including liver, muscle [64], neurons, brain [65], stem cells [66] and hematopoietic stem cells [67]. This approach has been used extensively for the inhibition of HIV [68,69]. The most widely used lentiviral vectors are HIV-based, for which biosafety is the major concern. The new generation of lentiviral vectors has reduced risks because they are available in the form of replication-defective vectors and self-inactivating vectors [70,71]. Lentiviral vectors are convenient tools to induce RNAi.

shRNA libraries covering large part of the genome of human and mouse have been made by using lentiviral vectors [72–74].

Other viral vectors

Like herpes virus vectors [75–77], baculoviral vectors have also been used for the expression of shRNAs by transducing into cells. Herpes viruses are usually infectious to cells, but upon deletion of some of the immediate early expressing genes these viruses can still replicate although their toxicity is significantly reduced [78].

Baculoviruses are insect viruses and do not infect mammals but they can transduce into mammalian cells with the help of their envelope protein. This feature of baculoviruses has been well exploited to deliver DNA into cells. A baculovirus-mediated siRNA delivery approach has been successfully used to inhibit viral replication of porcine arteirvirus [79] hepatitis C virus [80] and hepatitis B virus [81] in cell lines. They have also been reported to transduce efficiently into primary cells [82].

Potential of RNAi

The promise and successes showed by RNAi in basic research have drawn the attention of researchers to exploit this tool for therapy. Approaches like antisense, aptamers and ribozymes raised some hopes, but, over the past two decades, have not really delivered on their promise. The emergence of RNAi rekindled the hopes of producing drugs designed to address the perturbations in specific genes. Currently many pharmaceutical companies, like Silence Therapeutics, Quark Biotech, Pfizer, Alnylam, Benitec and Trans-Derm are undertaking RNAi-based drug trials targeting various diseases and disorders such as age-related maculodegeneration (AMD), choroidal neovascularization, HIV infection, RSV infection, pachyonychia congenital chronic HBV infection among many others [83]. siRNA delivery is one of the major obstacles in the development of siRNA-based therapeutic tools in clinical settings. siRNAs transiently silence gene expression, because their intracellular concentration decreases due to their intrinsic half-life and also due to cell division. In contrast to siRNAs, the shRNAs mediate prolonged knockdown of their target transcripts. Such prolonged expression of siRNAs/shRNAs is an important requirement for efficient long-term silencing in a clinical setting against viral pathogens [84]. The siRNAs can be delivered through nonselective or selective systemic delivery-based methods. The nonselective form of systemic delivery can be used, however, for only a few types of tissues. The nonsystemic delivery methods require large amount of siRNA for therapeutic gene silencing; however, the selective systemic delivery requires much smaller amounts of siRNAs, because of selective targeting. Effective siRNA delivery depends on the various factors such as stability and activity of siRNAs, cellular uptake, biological distribution, specific targeting and clearance from the body.

Application of nonviral methods and its potential in therapeutics

The nonviral delivery methods are preferred over viral delivery methods to avoid adverse effects generated by vectors. Reich *et al.* reported sequence-specific silencing effects and reduction in age-related macular degeneration (AMD) by administering siRNAs through intra-vitreal injection against vascular endothelial growth factor (VEGF) [3]. The siRNA targeting of *VEGF receptor-1* to inhibit ocular neovascularization in mice has been successful [85]. siRNAs suspended in saline or lung surfactants were administered intranasally in nonhuman primate model of SARS and inhibited viral replication in the lung [86]. siRNAs complexed with lipoplex (Transit-TKO) have been administered intranasally to rhesus macaques and are found to suppress both RSV and para-influenza virus [7]. To organs like central nervous system (CNS) siRNAs could not be delivered efficiently by systemic delivery approaches. So localized delivery approaches like direct injection of siRNAs into CNS have been employed [4,5,87,88]. The uptake of siRNA was found to be improved by complexing them with liposomal carriers. Local electroporation of siRNAs directly into the hippocampal region has been effective in reducing the levels of metabotropic glutamate receptor-2 (mGluR2) and the COX1 gene [89].

Intramuscular and intratumoral deliveries of siRNAs have also been tried to some reasonable effect. The delivery of siRNAs through intradermal injections has been tried into mouse foot pad [90–92]. Intravaginal application of siRNAs targeting HSV2 protected mice from lethal infection [93].

These direct application methods are suitable for cells that can directly uptake siRNAs or to those cells whose process of uptake can be facilitated by conjugation with liposomal complexes. The differential uptake of siRNAs by different cells has been observed *in vivo* and this leads to inconsistent gene suppression. It is not advisable to use higher doses of siRNA in such cases because higher dose of siRNAs can induce cell death. Therefore, effective strategies are required for the improved delivery of siRNAs into different cell types.

Conjugating siRNAs with lipophilic substances, aptamers, peptides and antibodies increase the efficiency of uptake, which can improve the distribution of the delivered siRNAs into cells or organs to some extent. Soutschek *et al.* [94] knocked down *Apo-B* gene in mice by using cholesterol-conjugated siRNAs and showed the reduction in the level of cholesterol in the blood. This was the first *in vivo* verification of siRNA efficacy to use systemic delivery. This approach has been further improved by complexing the cholesterol-conjugated siRNAs with lipoproteins [95]. The siRNAs delivered systemically using this approach were preferentially taken up by liver and jejunum.

Aptamers conjugated to siRNAs have been used for the delivery of siRNAs to specific cells, based on their affinity to particular cell surface proteins [96,97]. The cell-specific targeting of neuronal cell was achieved by conjugating siRNAs to rabies virus glycoprotein via a nonameric arginine linker [98]. Molecules like transferring, folate and RGD peptides have been used to coat nanoparticles to deliver siRNAs [99]. Many peptides can bind to siRNAs noncovalently, like MPG (27 residues), penetrin, chloesteryl-oligoarginine and these peptides have also been used for *in vivo* delivery [100–102]. Polymers, like polyethylene glycol (PEG), atellocollagen, chitosan or polyethylenimine (PEI) and cyclodextrin form cationic complexes with siRNAs and have been used for *in vivo* delivery. These complexes have been administered intranasally [103,104] or by the intravenous route [105]. Intraperitoneal injection of PEI-complexed siRNAs targeting the human epidermal growth factor receptor 2 (HER2) resulted in the downregulation of HER2 expression along with significant antitumorigenic effects

in subcutaneous ovarian xenograft-bearing mice [106]. Ge *et al.* used intravenous injection of PEI-complexed siRNAs targeting conserved regions of influenza virus genes to prevent lethal influenza infection in mice [107].

Song *et al.* used a heavy chain antibody fragment (Fab fragment) fused to 'protamine' and conjugated with siRNAs against HIV-1 Env protein. They were successful in specifically targeting HIV-1 *env* expressing cells both *in vitro* and *in vivo* [108]. Santel *et al.* have demonstrated that siRNAs formulated in liposomes can be delivered to tumor endothelial cells [109]. Judge *et al.* successfully knocked down *Apo-B* by using Stable Nucleic Acid Lipid Particles (SNALPs) in mice and primates [110,111]. Liposomes of dioleoyl phospatidylcholine (DOPC) and 2-dioleoyl-3-trimethylammonium propane (DOTAP) have also been used for *in vivo* delivery of siRNAs. Lipoplexes like Lipofectamine2000, Transit-TKO have been used *in vivo* or for local delivery of siRNAs [112]. Aouadi *et al.* recently demonstrated the *in vivo* suppression of TNF-α in macrophages of peritoneum, spleen, liver and lung in mice model by delivering siRNAs encapsulated in beta-1,3-D-glucan (GeRPs) through oral delivery [113].

Kumar *et al.* [114] used a nonviral method (single chain antibody (scFv) against the T cell receptor CD7) for the systemic delivery of antiviral siRNA into T cells. CD7 is surface protein present on the majority of T cells. The binding of antibody to CD7 results in rapid internalization of the CD7 receptor. The targeting of CD7 did not adversely affect the T cells. They used a combination of siRNAs targeting the cellular CCR5 and conserved target sequences of HIV *vif* and *tat* genes. CCR5 was targeted to block viral entry and viral genes (*vif* and *tat*) were targeted to block viral replication [114].

Application of viral delivery methods and its potential in therapeutics

Lentiviral vectors have been used to suppress mutant genes and rescue phenotype in many neurodegenerative disorders. An *et al.* used them to express shRNAs, under the control of the H1 promoter, against the CCR5 receptor in CD34+ hematopoietic stem/progenitor cells. The CCR5-suppressed cells were re-transplanted into rhesus monkeys [115] and the expression of CCR5 was found to be suppressed for over 14 months, making the rhesus monkeys less susceptible to infection by simian immunodeficiency virus [115]. Many researchers have used viral delivery methods to deliver shRNAs to brain [116,117] and muscle [118]. Lentiviral vectors have been used to express multiple shRNAs simultaneously. Such a combinatorial approach to expressing more than one shRNA targeting different sequences is more effective than individual shRNAs [119]. A recent report by Alves *et al.* showed that a human ataxin-3 mutant, differing by just one SNP, could be targeted by lentiviral vectors; the treatment produced a very promising recovery of phenotype in a rat model of Machado-Joseph disease [120]. This is the first report of a gene differing by one nucleotide that can be targeted by lentiviral vectors *in vivo*. Such reports demonstrate a very promising future for viral vectors in RNAi-based therapy although the lentiviral vectors showed some adverse effects, caused by insertional mutagenesis [121]. AdV have also shown toxic effects and lethality in mice because of the saturation of exportin-5 or by the induction of an interferon response [122,123]. Still toxicity remains a major issue of concern. The viral coat proteins which facilitate membrane fusion and cell entry have been utilized to make vesicles called virosomes. These vesicles contain hemagglutinin (HA), a glycoprotein of influenza virus, in the membrane, which helps in the internalization of these vesicles by endosomal uptake in the cells. These vesicles can be used as a carrier to carry siRNA–lipid complexes. de Jonge *et al.* used virosomes to deliver siRNAs *in vivo* into the peritoneal cavity of mice [124]. Virosomes have been delivered into cells through different routes, such as the intramuscular, subcutaneous and intranasal routes [125,126].

Conclusion

Recently, reports have shown that the success of siRNA-based therapy in some of the clinical cases was due to the induction of toll-like receptors by the siRNAs, but not due to their RNAi-dependent activity [127,128]. Therefore, the inclusion of proper controls and examination of the ability of siRNAs to induce immune responses are highly important aspects for the functional studies of siRNA. In spite of these hurdles, RNAi remains a useful, novel therapeutic tool. RNAi could be useful in cases that were otherwise thought to be undruggable and for other diseases where a combination of therapies including RNAi, might give better outcome. siRNA-based therapeutic tools can represent a realistic alternative for the treatment of various diseases. Further studies are required to develop the efficient delivery and regulated expression of siRNAs, to utilize their potentials in therapeutics.

Conflict of interest statement

There are no financial interests associated with this manuscript between the authors. Authors would like to apologize to those investigators, whose work could not be cited due to the limitation of space.

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References

1 Aigner, A. (2007) Nonviral in vivo delivery of therapeutic small interfering RNAs. *Curr. Opin. Mol. Ther.* 9, 345–352

2 Hou, J.Q. *et al.* (2006) Effect of small interfering RNA targeting survivin gene on biological behaviour of bladder cancer. *Chin. Med. J. (Engl.)* 119, 1734–1739

3 Reich, S.J. *et al.* (2003) Small interfering RNA (siRNA) targeting VEGF effectively inhibits ocular neovascularization in a mouse model. *Mol. Vis.* 9, 210–216

4 Thakker, D.R. *et al.* (2005) siRNA-mediated knockdown of the serotonin transporter in the adult mouse brain. *Mol. Psychiatry* 10, 782–789 714

5 Dorn, G. *et al.* (2004) siRNA relieves chronic neuropathic pain. *Nucleic Acids Res.* 32, e49
6 Zhang, X. *et al.* (2004) Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis. *J. Biol. Chem.* 279, 10677–106847
7 Bitko, V. *et al.* (2005) Inhibition of respiratory viruses by nasally administered siRNA. *Nat. Med.* 11, 50–55
8 Zender, L. *et al.* (2003) Caspase 8 small interfering RNA prevents acute liver failure in mice. *Proc. Natl. Acad. Sci. U. S. A.* 100, 7797–7802
9 Song, E. *et al.* (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. *Nat. Med.* 9, 347–351
10 Sioud, M. (2006) Single-stranded small interfering RNA are more immunostimulatory than their double-stranded counterparts: a central role for 2′-hydroxyl uridines in immune responses. *Eur. J. Immunol.* 36, 1222–1230
11 Sioud, M. (2005) Induction of inflammatory cytokines and interferon responses by double-stranded and single-stranded siRNAs is sequence-dependent and requires endosomal localization. *J. Mol. Biol.* 348, 1079–1090
12 Brummelkamp, T.R. *et al.* (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550–553
13 Zeng, Y. *et al.* (2005) Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. *EMBO J.* 24, 138–148
14 Tabara, H. *et al.* (1998) RNAi in *C. elegans*: soaking in the genome sequence. *Science* 282, 430–431
15 Timmons, L. and Fire, A. (1998) Specific interference by ingested dsRNA. *Nature* 395, 854
16 Vayssie, L. *et al.* (2004) Double-stranded RNA mediates homology-dependent gene silencing of gamma-tubulin in the human parasite *Entamoeba histolytica*. *Mol. Biochem. Parasitol.* 138, 21–28
17 Soares, C.A. *et al.* (2005) Capillary feeding of specific dsRNA induces silencing of the isac gene in nymphal *Ixodes scapularis* ticks. *Insect. Mol. Biol.* 14, 443–452
18 Patel, A. *et al.* (2007) The making of a queen: TOR pathway is a key player in diphenic caste development. *PLoS ONE* 2, e509
19 Baum, J.A. *et al.* (2007) Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–1326
20 Eaton, B.A. *et al.* (2002) Dynactin is necessary for synapse stabilization. *Neuron* 34, 729–741
21 Clemens, J.C. *et al.* (2000) Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc. Natl. Acad. Sci. U. S. A.* 97, 6499–6503
22 Lum, L. *et al.* (2003) Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. *Science* 299, 2039–2045
23 Yang, D. *et al.* (2002) Short RNA duplexes produced by hydrolysis with *Escherichia coli* RNase III mediate effective RNA interference in mammalian cells. *Proc. Natl. Acad. Sci. U. S. A.* 99, 9942–9947
24 Myers, J.W. *et al.* (2003) Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. *Nat. Biotechnol.* 21, 324–328
25 Kittler, R. *et al.* (2004) An endoribonuclease-prepared siRNA screen in human cells identifies genes essential for cell division. *Nature* 432, 1036–1040
26 Virieux, C. (1966) The outcome of Hodgkin's disease associated with pregnancy. A study of 9 cases. *Rev. Med. Suisse Romande* 86, 821–839
27 Kittler, R. and Buchholz, F. (2005) Functional genomic analysis of cell division by endoribonuclease-prepared siRNAs. *Cell Cycle* 4, 564–567
28 Paddison, P.J. *et al.* (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 16, 948–958
29 Peng, Y. *et al.* (2007) shRNA driven by Pol II/T7 dual-promoter system effectively induce cell-specific RNA interference in mammalian cells. *Biochem. Biophys. Res. Commun.* 360, 496–500
30 Kamath, R.S. *et al.* (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* 2 RESEARCH0002
31 Issa, Z. *et al.* (2005) Development of methods for RNA interference in the sheep gastrointestinal parasite, *Trichostrongylus colubriformis*. *Int. J. Parasitol.* 35, 935–940
32 Galvani, A. and Sperling, L. (2002) RNA interference by feeding in Paramecium. *Trends Genet.* 18, 11–12
33 Kuwahara, A. *et al.* (2007) Delivery of dsRNA with lactic acid bacteria for RNA interference. *Nucleic Acids Symp. Ser. (Oxf.)* 51, 413–414
34 Isberg, R.R. *et al.* (1987) Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. *Cell* 50 (5), 769–778
35 Courvalin, P. *et al.* (1995) Gene transfer from bacteria to mammalian cells. *C. R. Acad. Sci. III* 318, 1207–1212
36 Grillot-Courvalin, C. *et al.* (1998) Functional gene transfer from intracellular bacteria to mammalian cells. *Nat. Biotechnol.* 16, 862–866
37 Xiang, S. *et al.* (2006) Short hairpin RNA-expressing bacteria elicit RNA interference in mammals. *Nat. Biotechnol.* 24, 697–702
38 Zhang, L. *et al.* (2007) Intratumoral delivery and suppression of prostate tumor growth by attenuated *Salmonella enterica* serovar *typhimurium* carrying plasmid-based small interfering RNAs. *Cancer Res.* 67, 5859–5864
39 Gao, G.P. *et al.* (1996) Biology of adenovirus vectors with E1 and E4 deletions for liver-directed gene therapy. *J. Virol.* 70, 8934–8943
40 Armentano, D. *et al.* (1997) Effect of the E4 region on the persistence of transgene expression from adenovirus vectors. *J. Virol.* 71, 2408–2416
41 O'Neal, W.K. *et al.* (2000) Toxicity associated with repeated administration of first-generation adenovirus vectors does not occur with a helper-dependent vector. *Mol. Med.* 6, 179–195
42 Schiedner, G. *et al.* (1998) Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat. Genet.* 18, 180–183
43 Shen, C. *et al.* (2003) Gene silencing by adenovirus-delivered siRNA. *FEBS Lett.* 539, 111–114
44 Hosono, T. *et al.* (2004) Adenovirus vector-mediated doxycycline-inducible RNA interference. *Hum Gene Ther.* 15, 813–819
45 Lieber, A. *et al.* (1999) Integrating adenovirus–adeno-associated virus hybrid vectors devoid of all viral genes. *J. Virol.* 73, 9314–9324
46 Soifer, H.S. and Kasahara, N. (2004) Retrotransposon–adenovirus hybrid vectors: efficient delivery and stable integration of transgenes via a two-stage mechanism. *Curr. Gene Ther.* 4, 373–384
47 Erles, K. *et al.* (1999) Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J. Med. Virol.* 59, 406–411
48 Grimm, D. and Kay, M.A. (2003) From virus evolution to vector revolution: use of naturally occurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. *Curr. Gene Ther.* 3, 281–304
49 Grimm, D. (2002) Production methods for gene transfer vectors based on adeno-associated virus serotypes. *Methods* 28, 146–157
50 Stilwell, J.L. and Samulski, R.J. (2004) Role of viral vectors and virion shells in cellular gene expression. *Mol. Ther.* 9, 337–346
51 Heister, T. *et al.* (2002) Herpes simplex virus type 1/adeno-associated virus hybrid vectors mediate site-specific integration at the adeno-associated virus preintegration site, AAVS1, on human chromosome 19. *J. Virol.* 76, 7163–7173
52 Palombo, F. *et al.* (1998) Site-specific integration in mammalian cells mediated by a new hybrid baculovirus–adeno-associated virus vector. *J. Virol.* 7, 5025–5034
53 Girod, A. *et al.* (1999) Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat. Med.* 5, 1438
54 Fitzsimons, H.L. *et al.* (2001) Insulators coupled to a minimal bidirectional tet cassette for tight regulation of rAAV-mediated gene transfer in the mammalian brain. *Gene Ther.* 8, 1675–1681
55 Yu, J.Y. *et al.* (2003) Simultaneous inhibition of GSK3alpha and GSK3beta using hairpin siRNA expression vectors. *Mol. Ther.* 7, 228–236
56 Hommel, J.D. *et al.* (2003) Local gene knockdown in the brain using viral-mediated RNA interference. *Nat. Med.* 9, 1539–1544
57 Boden, D. *et al.* (2004) Efficient gene transfer of HIV-1-specific short hairpin RNA into human lymphocytic cells using recombinant adeno-associated virus vectors. *Mol. Ther.* 9, 396–402
58 Zhang, W. *et al.* (2004) Attenuation of dengue virus infection by adeno-associated virus-mediated siRNA delivery. *Genet Vaccines Ther.* 2, 8
59 Chen, C.C. *et al.* (2007) Long-term inhibition of hepatitis B virus in transgenic mice by double-stranded adeno-associated virus 8-delivered short hairpin RNA. *Gene Ther.* 14, 11–19
60 Barquinero, J. *et al.* (2004) Retroviral vectors: new applications for an old tool. *Gene Ther.* 11 (Suppl 1), S3–9
61 van der Loo, J.C. *et al.* (2002) Optimization of gene transfer into primitive human hematopoietic cells of granulocyte-colony stimulating factor-mobilized peripheral blood using low-dose cytokines and comparison of a gibbon ape leukemia virus versus an RD114-pseudotyped retroviral vector. *Hum. Gene Ther.* 13, 1317–1330
62 Barton, G.M. and Medzhitov, R. (2002) Retroviral delivery of small interfering RNA into primary cells. *Proc. Natl. Acad. Sci. U. S. A.* 99, 14943–14945
63 Cronin, J. *et al.* (2005) Altering the tropism of lentiviral vectors through pseudotyping. *Curr. Gene Ther.* 5, 387–398
64 Kafri, T. *et al.* (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat. Genet.* 17, 314–317
65 Naldini, L. *et al.* (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc. Natl. Acad. Sci. U. S. A.* 93, 11382–11388
66 Rubinson, D.A. *et al.* (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat. Genet.* 33, 401–406

67 Uchida, N. *et al.* (1998) HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 95, 11939–11944
68 Li, M.J. *et al.* (2003) Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. *Mol. Ther.* 8, 196–206
69 Qin, X.F. *et al.* (2003) Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc. Natl. Acad. Sci. U. S. A.* 100, 183–188
70 Zufferey, R. *et al.* (1998) Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* 72, 9873–9880
71 Abbas-Terki, T. *et al.* (2002) Lentiviral-mediated RNA interference. *Hum. Gene Ther.* 13, 2197–2201
72 Root, D.E. *et al.* (2006) Genome-scale loss-of-function screening with a lentiviral RNAi library. *Nat. Methods* 3, 715–719
73 Paddison, P.J. *et al.* (2004) A resource for large-scale RNA-interference-based screens in mammals. *Nature* 428, 427–431
74 Moffat, J. *et al.* (2006) A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* 124, 1283–1298
75 Sabbioni, S. *et al.* (2007) Use of herpes simplex virus type 1-based amplicon vector for delivery of small interfering RNA. *Gene Ther.* 14, 459–464
76 Lambeth, L.S. *et al.* (2009) Targeting Marek's disease virus by RNA interference delivered from a herpes virus vaccine. *Vaccine* 27, 298–306
77 Saydam, O. *et al.* (2005) Herpes simplex virus 1 amplicon vector-mediated siRNA targeting epidermal growth factor receptor inhibits growth of human glioma cells in vivo. *Mol. Ther.* 12, 803–812
78 Samaniego, L.A. *et al.* (1998) Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. *J. Virol.* 72, 3307–3320
79 Lu, L. *et al.* (2006) Suppression of porcine arterivirus replication by baculovirus-delivered shRNA targeting nucleoprotein. *Biochem. Biophys. Res. Commun.* 340, 1178–1183
80 Suzuki, H. *et al.* (2008) Suppression of hepatitis C virus replication by baculovirus vector-mediated short-hairpin RNA expression. *FEBS Lett.* 582, 3085–3089
81 Starkey, J.L. *et al.* (2009) Hepatitis B virus (HBV)-specific short hairpin RNA is capable of reducing the formation of HBV covalently closed circular (CCC) DNA but has no effect on established CCC DNA in vitro. *J. Gen. Virol.* 90 (Pt 1), 115–126
82 Nicholson, L.J. *et al.* (2005) RNA interference mediated in human primary cells via recombinant baculoviral vectors. *Mol. Ther.* 11, 638–644
83 Nguyen, T. *et al.* (2008) RNAi therapeutics: an update on delivery. *Curr. Opin. Mol. Ther.* 10, 158–167
84 Singh, S.K. (2008) RNA interference and its therapeutic potential against HIV infection. *Expert Opin. Biol. Ther.* 8, 449–461
85 Shen, J. *et al.* (2006) Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1. *Gene Ther.* 13, 225–234
86 Li, B.J. *et al.* (2005) Using siRNA in prophylactic and therapeutic regimens against SARS coronavirus in Rhesus macaque. *Nat. Med.* 11, 944–951
87 Luo, M.C. *et al.* (2005) An efficient intrathecal delivery of small interfering RNA to the spinal cord and peripheral neurons. *Mol. Pain* 1, 29
88 Makimura, H. *et al.* (2002) Reducing hypothalamic AGRP by RNA interference increases metabolic rate and decreases body weight without influencing food intake. *BMC Neurosci.* 3, 18
89 Akaneya, Y. *et al.* (2005) RNAi-induced gene silencing by local electroporation in targeting brain region. *J. Neurophysiol.* 93, 594–602
90 Wang, Q. *et al.* (2007) Delivery and inhibition of reporter genes by small interfering RNAs in a mouse skin model. *J. Invest. Dermatol.* 127, 2577–2584
91 Hickerson, R.P. *et al.* (2008) Single-nucleotide-specific siRNA targeting in a dominant-negative skin model. *J. Invest. Dermatol.* 128, 594–605
92 Smith, F.J. *et al.* (2008) Development of therapeutic siRNAs for pachyonychia congenita. *J. Invest. Dermatol.* 128, 50–58
93 Palliser, D. *et al.* (2006) An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* 439, 89–94
94 Soutschek, J. *et al.* (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173–178
95 Wolfrum, C. *et al.* (2007) Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nat. Biotechnol.* 25, 1149–1157
96 McNamara, J.O., 2nd *et al.* (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat. Biotechnol.* 24, 1005–1015
97 Chu, T.C. *et al.* (2006) Aptamer mediated siRNA delivery. *Nucleic Acids Res.* 34, e73
98 Kumar, P. *et al.* (2007) Transvascular delivery of small interfering RNA to the central nervous system. *Nature* 448, 39–43
99 Hu-Lieskovan, S. *et al.* (2005) Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res.* 65, 8984–8992
100 Zatsepin, T.S. *et al.* (2005) Conjugates of oligonucleotides and analogues with cell penetrating peptides as gene silencing agents. *Curr. Pharm. Des.* 11, 3639–3654
101 Simeoni, F. *et al.* (2003) Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. *Nucleic Acids Res.* 31, 2717–2724
102 Kim, W.J. *et al.* (2006) Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. *Mol. Ther.* 14, 343–350
103 Howard, K.A. *et al.* (2006) RNA interference in vitro and in vivo using a novel chitosan/siRNA nanoparticle system. *Mol. Ther.* 14, 476–484
104 Heidel, J.D. *et al.* (2007) Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proc. Natl. Acad. Sci. U. S. A.* 104, 5715–5721
105 Pille, J.Y. *et al.* (2006) Intravenous delivery of anti-RhoA small interfering RNA loaded in nanoparticles of chitosan in mice: safety and efficacy in xenografted aggressive breast cancer. *Hum. Gene Ther.* 17, 1019–1026
106 Urban-Klein, B. *et al.* (2005) RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene Ther.* 12, 461–466
107 Ge, Q. *et al.* (2004) Inhibition of influenza virus production in virus-infected mice by RNA interference. *Proc. Natl. Acad. Sci. U. S. A.* 101, 8676–8681
108 Song, E. *et al.* (2005) Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* 23, 709–717
109 Santel, A. *et al.* (2006) A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium. *Gene Ther.* 13, 1222–1234
110 Judge, A.D. *et al.* (2005) Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.* 23, 457–462
111 Zimmermann, T.S. *et al.* (2006) RNAi-mediated gene silencing in non-human primates. *Nature* 441, 111–114
112 Nakamura, H. *et al.* (2004) RNA interference targeting transforming growth factor-beta type II receptor suppresses ocular inflammation and fibrosis. *Mol. Vis.* 10, 703–711
113 Aouadi, M. *et al.* (2009) Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation. *Nature* 458, 1180–1184
114 Kumar, P. *et al.* (2008) T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* 134, 577–586
115 An, D.S. *et al.* (2007) Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. *Proc. Natl. Acad. Sci. U. S. A.* 104, 13110–13115
116 Van den Haute, C. *et al.* (2003) Lentiviral vector-mediated delivery of short hairpin RNA results in persistent knockdown of gene expression in mouse brain. *Hum. Gene Ther.* 14, 1799–1807
117 Harper, S.Q. *et al.* (2005) RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. *Proc. Natl. Acad. Sci. U. S. A.* 102, 5820–5825
118 Ralph, G.S. *et al.* (2005) Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat. Med.* 11, 429–433
119 ter Brake, O. *et al.* (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol. Ther.* 16, 557–564
120 Alves, S. *et al.* (2008) Allele-specific RNA silencing of mutant ataxin-3 mediates neuroprotection in a rat model of Machado-Joseph disease. *PLoS ONE* 3, e3341
121 Hacein-Bey-Abina, S. *et al.* (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N. Engl. J. Med.* 348, 255–256
122 Grimm, D. *et al.* (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441, 537–541
123 Witting, S.R. *et al.* (2008) Helper-dependent adenovirus-mediated short hairpin RNA expression in the liver activates the interferon response. *J. Biol. Chem.* 283, 2120–2128
124 de Jonge, J. *et al.* (2006) Reconstituted influenza virus envelopes as an efficient carrier system for cellular delivery of small-interfering RNAs. *Gene Ther.* 13, 400–411
125 Arkema, A. *et al.* (2000) Induction of cytotoxic T lymphocyte activity by fusion-active peptide-containing virosomes. *Vaccine* 18, 1327–1333
126 Bungener, L. *et al.* (2002) Virosome-mediated delivery of protein antigens to dendritic cells. *Vaccine* 20, 2287–2295
127 Robbins, M. *et al.* (2008) Misinterpreting the therapeutic effects of small interfering RNA caused by immune stimulation. *Hum. Gene Ther.* 19, 991–999
128 Kleinman, M.E. *et al.* (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* 452, 591–597