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RNAi therapeutics: Principles, prospects and challenges ☆

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Abstract

RNA interference (RNAi) was discovered less than a decade ago and already there are human clinical trials in progress or planned. A major advantage of RNAi versus other antisense based approaches for therapeutic applications is that it utilizes cellular machinery that efficiently allows targeting of complementary transcripts, often resulting in highly potent down-regulation of gene expression. Despite the excitement about this remarkable biological process for sequence specific gene regulation, there are a number of hurdles and concerns that must be overcome prior to making RNAi a real therapeutic modality, which include off-target effects, triggering of type I interferon responses, and effective delivery *in vivo*. This review discusses mechanistic aspects of RNAi, the potential problem areas and solutions and therapeutic applications. It is anticipated that RNAi will be a major therapeutic modality within the next several years, and clearly warrants intense investigation to fully understand the mechanisms involved.

Keywords: siRNA; shRNA; RNAi; RNA interference; Antisense; miRNA; RISC

Contents

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1. Introduction to RNAi

RNA interference (RNAi) is a regulatory mechanism of most eukaryotic cells that uses small double stranded RNA (dsRNA) molecules to direct homology-dependent control of gene activity [1]. Known as small interfering RNAs (siRNA) these ~21–22 bp long dsRNA molecules have a characteristic 2 nucleotide 3′ overhang that allows them to be recognized by the enzymatic machinery of RNAi that eventually leads to homology-dependent degradation of the target mRNA (Fig. 1). In mammalian cells siRNAs are produced from cleavage of longer dsRNA precursors by the RNaseIII endonuclease Dicer [2]. Dicer is complexed with the TAR-RNA binding protein (TRBP) and hands off the siRNAs to the RNA-induced silencing complex (RISC), which contains the "slicing" protein Argonaute 2 that cleaves the target mRNA molecules between bases 10 and 11 relative to the 5′ end of the antisense siRNA strand. The core components of RISC are the Argonaute (Ago) family members, and in humans only Ago-2 possesses an active catalytic domain for cleavage activity [3,4]. While siRNAs loaded into RISC are double-stranded, Ago-2 cleaves and releases the "passenger" strand leading to an activated form of RISC with a single-stranded "guide" RNA molecule that directs the specificity of the target recognition by intermolecular base pairing [5]. Rules that govern selectivity of strand loading into RISC are based upon differential thermodynamic stabilities of the ends of the siRNAs [6,7]. The less thermodynamically stable end is favored for unwinding of the 5′ end of the guide strand which binds to the PIWI domain of Ago-2. Messenger RNA molecules with perfect or near-perfect complementarity to the guide RNA are recognized and cleaved by Ago-2. Partial complementarity between an siRNA and target mRNA may in certain cases repress translation or destabilize the transcripts if the binding mimics microRNA (miRNA) interactions with target sites. MicroRNAs are endogenous substrates for the RNAi machinery. The are initially expressed as long primary transcripts (pri-miRNAs), which are processed within the nucleus into 60–70 bp hairpins by the microprocessor complex which consists of Drosha-DGCR8 [8,9]. The loop is removed by further processing in the cytoplasm by the RNAse III Dicer and one of the two strands is loaded into RISC in the cytoplasm. The mature miRNAs share only partial complementarity with sequences in the 3′UTR of target mRNAs. The primary mechanism of action of miRNAs is translational repression, although this can be accompanied by message degradation [10].

Importantly, it is possible to exploit this native gene silencing pathway for regulation of gene(s) of choice. If the siRNA effector is delivered to the cell it will "activate" RISC, resulting in potent and specific silencing of the targeted mRNA. Because of the potency and selectivity of RNAi, it has become the method of choice for silencing specific gene expression in mammalian cells. Control of disease-associated genes makes RNAi an attractive choice for future therapeutics. Basically every human disease caused by activity from one or a few genes should be amenable for RNAi-based intervention. This list includes cancer, autoimmune diseases, dominant genetic disorders and viral infections. Moreover, since miRNAs may work as both tumor suppressors and oncogenes, endogenous miRNAs may also become therapeutic targets (review by [11]).

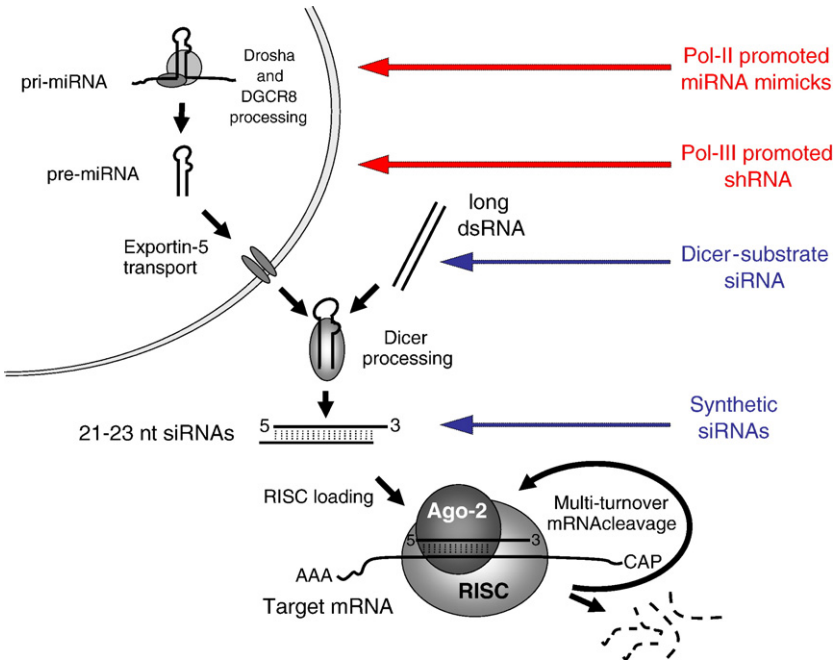

Fig. 1. The left hand side shows a schematic diagram of the mammalian RNAi pathway and formation of small interfering RNAs (siRNA) that mediates homology-dependent target mRNA degradation in the cytoplasm though the RNA-induced silencing complex (RISC). The right hand side shows different entry point for artificial DNA-based (marked in red) or RNA-based (marked in blue) siRNA drugs that enters and activates RISC for gene silencing. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

While RNAi has been a tremendous resource for studying mammalian gene function without the laborious use of gene knockout techniques, the next frontier is to harness this powerful technology for therapeutic purposes. Initial results have been very promising and a number of pharmaceutical companies are already focusing on commercialization of various disease-specific strategies or technological platforms. However, recent research has made it clear that important safety issues need to be addressed before RNAi-based drugs are ready for clinical use.

2. Advantages RNA interference

Prior to the discovery of RNAi in 1998, nucleic acid-based antisense technologies for sequence-specific inhibition of gene expression were pursued for a number of years. The major advantages of all antisense strategies are the specificity by which target versus non-target discrimination can be controlled via the specificity of Watson–Crick base pairing interactions, and the almost unrestricted choice of targets. In the most simplified scenario only the mRNA sequence is sufficient to design antisense drugs. Theoretically, all disease associated genes should be amenable to antisense mediated suppression. The unrestricted potential of RNAi has encouraged strategies for large scale silencing of virtually all annotated protein encoding genes in the human genome. These studies are currently yielding valuable information on gene function and pathway analysis. The high specificity may even allow targeting of disease-specific alleles that differ from the normal allele by only one or few nucleotide substitutions. This is clearly useful for targeting dominant mutants as would the case for some oncogenes.

Compared to other antisense strategies such as antisense DNA oligonucletides and ribozymes, RNAi is much more potent [12]. Of note, single stranded small antisense RNAs may also be loaded into RISC and guide target mRNA cleavage, however with a much reduced efficiency [13]. Importantly, the higher potency of RNAi means that the effector molecules may function at much lower concentrations than antisense oligos or ribozymes. This is an important factor in a therapeutic setting.

Notably, efficacy (usually measured as half-maximal inhibition levels or IC_{50} values) for individual target sites varies widely among siRNAs. Important criteria for siRNA efficacy include thermodynamic end stability [6], target mRNA accessibility [14], structural features [15] and additional position specific determinants [16,17]. To date the most important siRNA design rule is differential end stability (or asymmetry) which is consistent with what is observed for miRNA strand selection [7]. Still, our knowledge of siRNA and design target selection is far from complete and identifying "hyper functional" siRNAs that are functional in subnanomolar amounts remains an elusive problem.

3. Two basic strategies: siRNA versus shRNA

RNAi can be triggered by two different pathways: 1) a RNA-based approach where the effector siRNAs are delivered to target cells as preformed 21 base duplexes; or 2) a DNA-based strategy in which the siRNA effectors are produced by intracellular processing of longer RNA hairpin transcripts (Fig. 1). The latter approach is primarily based on nuclear synthesis of short hairpin RNAs (shRNAs) that are transported to the cytoplasm via the miRNA machinery and are processed into siRNAs by Dicer. While direct use of siRNA effectors is simple and result in potent gene silencing, their effect is transient. In a clinical setting this would usually mean that repeated treatments would have to be administered, and these are large and costly drugs. DNA-based RNAi drugs on the other hand have the potential of being stably introduced when used in a gene-therapy setting, allowing in principle, a single treatment of viral vector delivered shRNA genes (Fig. 2).

3.1. Delivery and stability of siRNA

The major bottleneck in the development of siRNA therapies is the delivery of these macromolecules to the desired cell type, tissue or organ. siRNAs do not readily cross the cellular membrane because of their negative charge and size. Cellular delivery of chemically synthesized or *in vitro* transcribed siRNAs is usually achieved by cationic liposome based strategies. The disadvantage of lipid based delivery schemes *in vivo* is the rapid liver clearance and lack of target tissue specificity. Cationic polymer and lipid-based siRNA complexes have been used for systemic delivery in mice [18,19]. Liposomes and duplexed siRNAs are complexed *in vitro* and the resulting siRNA containing vesicles are taken up by cells via the endosomal pathway. siRNAs are released into the cytoplasm where they associate with RISC. Typically, transiently transfected cells in cultures show gene silencing for three to five days. However, non-dividing cells may show sustained silencing for several weeks [20–22]. Optimization of the *in vivo* stability of siRNAs has been accomplished by chemically modifying the RNA backbone, and several strategies are now available for improved *in vivo* stability that may eventually reduce the requirement for high dosage (reviewed in [23]). These include 2′F, 2′O–Me and 2′H substitutions in the RNA backbone, all of which increase serum stability. Importantly, selective modifications of the backbone does not seem to reduce RNAi efficiency [24]. Until recently most trials have relied on duplexed 21-mer RNA species, but current research indicate that longer (25–27 mer) RNA species that undergoe intracellular processing by Dicer may increase the potency by channelling the duplexes through Dicer where the siRNAs are handed off to RISC [25–27] thereby further reducing the required concentrations of siRNAs for achieving a therapeutic effect.

Other *in vivo* delivery approaches for siRNAs include conjugation of cholesterol to the siRNA sense strand [28], antibody-protamine fusions that bind siRNAs [29], cyclodextrin nanoparticles [30] and aptamer-siRNA conjugates [31]. Each of these approaches resulted in tissue or cell type specific targeting, thereby expanding the therapeutic potential for *in vivo* siRNA delivery.

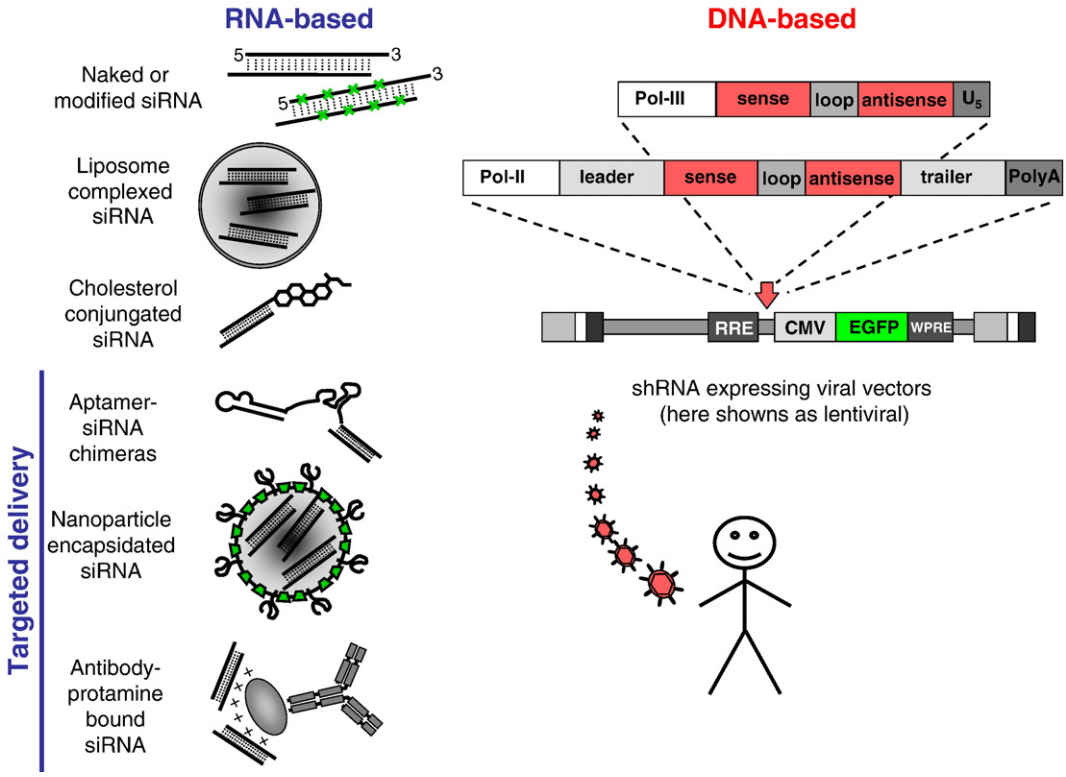

Fig. 2. The left hand side shows various non-targeted or targeted *in vivo* delivery strategies for RNA-based siRNA drugs. The right hand side shows a schematic diagram of either a DNA-based pol-III or pol-II promoted shRNA expression cassettes. These shRNA expression units can by delivered by viral or non-viral methods to the target tissue, as illustrated by gene transfer using lentiviral vector technology.

3.2. Delivery and expression of short hairpin RNAs

Soon after the realization that synthetic siRNA could be introduced into mammalian cells and trigger RNAi without activating type 1 interferon responses, researchers discovered that DNA-based expression cassettes could be generated that express short hairpins (shRNA) or separate sense and antisense 21 mers from Pol III promoters [32–35]. These promoters have well defined transcriptional start sites and simple terminators. Consequently the have become the most popular choice for DNA-based gene silencing in molecular biology. The minimal shRNA expression system includes a PolIII-promoter, directly followed by at least 19 nucleotides of sense (or antisense) target sequence, a 4–10 base loop, the complementary antisense (or sense) target sequence and finally a stretch of at least four to six U's as a terminator. Alternatively, a dual promoter system with individual expression of the two RNA strands can be used. The separately expressed strands hybridize and produce functional siRNAs [34,36]. The duplex RNAs produced by either system are substrates for nuclear export by the exportin-5 pathway. The hairpins are further processed by Dicer to yield functional siRNA duplexes whereas the siRNAs enter RISC directly.

Most PolIII based promoters are quite strong and give robust and long term silencing in cell culture systems. However, strong expression may turn out to be less favourable in therapeutic settings as the endogenous RNAi pathway may be saturated (see below). One way to modulate expression is the use of inducible systems. Inducible systems should in theory be tightly controlled by the inducer with prompt, dose-dependent and reversible regulation of transcription. Several PolIII-based systems have been engineered to respond to either tetracycline or ecdysone. While tetracycline analogs such as doxycycline have been applied for *in vivo* use in mice [37], ecdysone has so far been limited to cell culture systems [38]. Inducible transcription systems for use in mammalian cells are known to be somewhat leaky in the uninduced state. New technologies such as the RheoSwitch system from New England Biolabs may eventually improve inducible systems to achieve tighter control of gene expression. Alternatively, non-reversible genetic switches can be devised with the Cre-loxP system. However, this requires ectopic expression of the Cre recombinase protein which complicates the use.

Since 2002 a number of different promoters have been successfully applied for cellular synthesis of shRNAs. Polymerase II-based systems, including "standard" viral promoters such as the CMV promoter, produce 5′ capped and 3′ polyadenylated transcripts but often have less well defined transcriptional start sites compared to Pol III promoters. One important problem with this is that shRNAs need to initiate with the first base of the shRNA for proper processing and work best when the shRNA is followed by a minimal, rather than a complete poly A signal [39]. Longer transcripts which use miRNA scaffolds, such as miR30, can in fact be more readily produced using a Pol II promoter [40]. These miRNA mimics may enhance RNAi via interactions with the miRNA processing machinery. Kim and colleagues

suggest that the DGCR8 component of the Microprocessor recognizes the junction of the single stranded and double stranded regions in pri-miRNA transcripts, and cuts about one helical turn into the stem loop structure [41]. Thus, the sequences flanking the miRNAs are clearly important for correct miRNA processing. Several, PolII/miRNA-based systems have been developed and recently, a number of more advanced polycistronic or inducible systems have been published [42–44] and their applicability *in vivo* awaits further studies. Use of Pol-II promoted shRNAs offers several advantages. Inducible systems are well characterized and have been applied successfully *in vivo* and tissue-specific promoters that respond to various combinations of transcription factors allow temporal and spatial directed expression in the organism.

Like siRNA delivery DNA-based shRNAs face the problem that negatively charged nucleic acids do not readily pass the cellular membrane without facilitating carriers. Direct plasmid delivery can be achieved using high-pressure tail vein injections in mice. However, this strategy is limited to the liver and due to transient heart congestions it is not suitable for clinical use. Vector-based shRNA systems usually rely on delivery via viral vectors. A number of platforms exist including the popular adenovirus- and adeno-associated virus-(AAV) derived vectors that provide an efficient delivery vehicle for transient shRNA expression. Retroviruses on the other hand allow the researcher to stably silence target genes since the virus-encoded proteins mediate integration into the host cell chromosomal DNA. Retroviral vectors that have found clinical interest are either based murine leukemia virus (MLV) or lentivirus (e.g. HIV, FIV or EIAV). Notably, lentiviral vectors represent an efficient system for both somatic and germ-line transduction because of their ability to transduce non-dividing cells. Long term silencing would be particularly useful for RNAi treatment of chronic infections such as HIV, HBV and HCV.

4. Limitations of RNAs

4.1. Competition with endogenous RNAs

Bioactive drugs that rely on cellular processing to exert their action face the risk of saturating such pathways and hence perturb the natural system. Ectopically introduced RNAi triggers behave no different since siRNA/shRNA relies on the endogenous microRNA machinery in order to achieve potent target silencing. Although our understanding of the natural role of RNAi in mammalian cells have expanded tremendously with the discovery of miRNAs, other facets of this highly conserved biological system likely awaits to be unravelled. Yet, studies of miRNAs clearly suggest that they play important role for balancing gene activity [45,46]. Examples include the implication of miRNAs as oncogenes [47] or as tumor suppressor genes [48,49]. An intact RNAi machinery is clearly essential for mammalian cells as suggested by the early embryonic lethality of Dicer knockouts [50].

Since shRNA and siRNA resembles miRNA precursors before and after Dicer processing, respectively, all component of the miRNA pathway might be clogged by high doses of ectopic RNA. This was recently realized in a key publication from Mark Kays lab. In their work Grimm et al. [51] observed fatality in mice that had received high doses of liver-directed AAV-encoded shRNAs. Nearly half of the 49 shRNA tested resulted in dose-dependent and liver damage and high doses killed the recipient mice within 2 months. Morbidity was associated with reduced expression of liver-specific miRNA. Competitions assays showed that RNAi efficacy *in vivo* is enhanced by co-expression of the nuclear export component Exportin 5, which suggest that the liver toxicity might stem form saturation of the miRNA pathway. Work by Cullen labs [52] have also shown that nuclear export is a potential rate-limiting step, since over-expression of shRNAs inhibit miRNA function and overexpression of Exportin 5 reverses this effect. We have *in vitro* and *in vivo* evidence that combining low level expression of the same shRNA using a combination of the pol III U6 promoter and the pol II U1 promoter results in more effective RNAi than high level expression from a single U6 promoter. This is due to the saturation of the Exportin 5 pathway by U6 transcribed shRNAs. The U1 shRNAs are largely exported through the Crm 1 export pathway, so combining the U1 and U6 promoters results in more available siRNAs for entry into RISC (D. Castanotto, J. Heidel, M. Davis and J. Rossi, unpublished observations). Clearly, other factors of the RNAi apparatus could be susceptible to saturation and call for further work with animal model systems. The important message from this work is that vector-based shRNA expression systems should opt for controllable or moderate promoter systems in conjunction with siRNAs that are efficient at low doses.

4.2. Stimulation of innate immune responses

Until the groundbreaking discovery by Elbashir et al. [53] it was believed that double stranded RNA would induce innate responses in mammalian cells that would lead to shut down of cellular protein synthesis and eventually cell death. However, mammalian cells could apparently be transfected with dsRNA less than 30 nucleotides without inducing cellular toxicity. Long dsRNA on the other hand is known rapidly induce both interferon responses by binding to double-stranded-RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase-RNase L system or several Toll-like receptors (TLRs); all evolutionary conserved mechanism aimed at combating invading viral pathogens. Yet, last year a key publication demonstrated that certain siRNA sequence motifs invoked TLR7-dependent immune stimulation [54]. A particular sequence motif (5′-GUCCUUCAA-3′) seems to be recognized by TLR7 in the endosomal compartment of plasmacytoid dendritic cells and activate immune responses. A number of subsequent papers has since characterized a number of so-called “danger motifs”, GU-rich regions that has been shown to stimulate innate immune responses and lead to secretion of inflammatory cytokines in a cell-type and sequence-specific manner. This is reminiscent of the immunostimulatory CpG motifs in antisense oligonucleotides that signals “danger” of hostile nucleic acids to the cell via TLR9. While induction of the immune system could

be beneficial in some clinical application, these results have raised some concerns for the safe therapeutic use of RNAi. Since siRNA-mediated immune induction seem to rely on endosome located TLR receptors (TLR7 and TLR8 in particular [55]), the mode of delivery and hence compartmentalization of the siRNA greatly influences cellular responses. While knowledge of immune stimulatory properties calls for research to proceed to animal models, *in vitro* use of human primary cells with a full repertoire for immune stimulation are also needed. We have recently shown that human primary CD34+ hematopoietic cells can successfully be stably transduced with polII-promoted shRNAs without causing immune stimulation using sequences that otherwise potently induce interferon responses in the shape of lipid-delivered siRNA. Moreover, an *in vivo* model using 2′-F, 2′-O-methyl and 2′-H backbone modified siRNA against HBV have demonstrated that cytokine induction can be abrogated while maintaining silencing activity [56]. Despite these promising result great care and thorough testing are clearly needed before proceeding to clinical use.

Notably, use of longer (27–29 bp) and more potent Dicer substrates siRNAs [25,27], should be carefully weighed with the risk of increased immune stimulation. Recently, Dharmacon published a report that even short 23 nt long siRNAs may affect cell viability and invoke interferon responses in cell culture assays [57]. Importantly, the length threshold seems to vary among cell types which make it hard to foresee the outcome of Dicer substrate siRNAs *in vivo*. It has also been shown that T7-transcribed siRNAs potently induce interferon responses due to the presence of a 5′ triphosphate moiety [58] and earlier reports using T7-transcribed siRNA should thus be viewed with extra scrutiny.

4.3. Suppression of off targets

The almost ideal specificity of RNAi has shown not to hold entirely true in reality. Genome-wide monitoring gene activity by microarray technology has clearly demonstrated that siRNA treated cells shows off-target silencing of a large number of genes [59]. Silencing of off targets is clearly unwanted as the cellular consequence of altered gene activity is unknown and at large unpredictable. Studies of transfected cells *in vitro* show that one third of randomly chosen siRNA affect cell viability and may indicate that off-targeting results in a toxic phenotype [60]. Initial analysis demonstrated that as little as 11 nucleotides match between target and siRNA could result in off-target knockdown [59]. Recent work has shed more light on off targeting, and suggests that a majority of experimentally verified off targets have a 6–7 nucleotide match to the siRNA in the so-called “seed” region [61–64]. Surprisingly, the transfection agent itself may also influence expression profiles independent of the siRNA [65]. Studies of miRNA-target mRNA pairs have also demonstrated the importance of position 2–8 in the microRNA as the seed site for target recognition [66,67]. Position one of a miRNA has no importance in target selection [67]. This fits with structural modelling of Argonaut-siRNA interaction which suggests that 5′ phosphate is bound in the PIWI domain and does not pair with the mRNA target [68,69]. Thus off targets are preferentially silenced if they encompass hexamer regions in the 3′ UTR that mimic miRNA binding sites [61]. Since, miRNA may influence target gene expression via a combination of inhibition of translation, endonucleolytic cleavage or mRNA decay mechanisms, experimental determination of off targeting that rely solely on measuring mRNA may likely miss out on genes that are affected by translational inhibition rather than mRNA instability. However, in terms of predicting off targets the picture is far from complete as a large number of genes with good seed match remain unaffected [62]. It is also possible that not only mRNA but also miRNA expression profiles of a given cell will influence off targeting. Importantly, off targeting remains a critical issue for therapeutic applications of RNAi and empirical tests for siRNAs with tolerable levels of off targeting are required. Hopefully, novel protein array technology will provide a better picture of siRNA effects on cellular protein expression profiles and provide a better way of screening siRNA. Identification of very potent or hyper functional siRNA will help resolve unwanted off targeting since these siRNAs works at subnanomolar concentration. Recent research suggests that siRNAs with a 2′ O-Me modification at the second base can significantly reduce off targeting without compromising the degree of target silencing [70]. Likewise intelligent design of siRNA that improves strand selectivity should disfavour loading of the passenger strand which has been shown to cause off targeting.

Differential thermodynamically end stability favors incorporation of the strand with a low 5′ pairing energy, and purposive mismatches in the passenger strand may greatly improve cleavage efficiency [6]. Targeting from the passenger strand can readily be experimentally addressed by designing artificial sense and antisense targets that allow a fast readout such as the commercially available psiCheck vectors system (Promega). Improvement of strand loading may be restricted in the cases where the target site is fixed (as when targeting SNP alleles), and may require additional changes in the siRNA in order to allow efficient discrimination between wildtype and mutant transcript. Simplified, only the 5′ end of the guide strand binds to the targets mRNA while the 3′ end plays a minor role in target recognitions [71]. However, efficient cleavage requires near-full complementarity in the 3′ end of the guide strand and more than three mismatched significantly reduces cleavage kinetics [72]. However, central mismatches at or flanking nucleotide position 10 and 11 abolish RNAi as they are located at the scissile phosphate bond.

5. Applications of RNAi *in vivo*

Recent progress in the potential therapeutic applications of siRNAs is owing largely to major breakthroughs in delivery. Systemic delivery of therapeutic amounts of anti-ApoB siRNAs in Chimpanzees was recently accomplished by the use of bi-layer liposomes [73]. These important proofs of principle studies demonstrated that it is safe to systemically delivery therapeutically effective doses of siRNAs to primates, paving the way for other future systemic applications of RNAi. Listed

below are some potential diseases that may be therapeutic targets for RNAi in future years.

5.1. Genetic diseases

A potential therapuetic application for RNAi is the treatment of genetic diseases. A very promising lead in this direction has been provided by preliminary studies demonstrating that single nucleotide polymorphisms in mutant allele transcripts can be used as selective targets for RNAi [74,75]. Disease causing polyglutamine proteins encoded by CAG repeat containing transcripts found in several neurological diseases present especially challenging targets because CAG repeats are common to many normal transcripts as well, and cannot be selectively targeted by siRNAs. Alternatively, single nucleotide polymorphisms are very often found in mutant allele transcripts, and represent potential selective targets. The challenge is to find a siRNA/SNP combination that is highly selective. This has been accomplished by systematic analyses of siRNAs in which the polymorphic nucleotide is complementary to the mid region of the siRNA. In certain examples, the siRNAs direct selective degradation of only the mutant transcripts, leaving the wild type transcripts intact despite having only a single mismatch with the wild type sequence [74,75]. An additional application of siRNAs targeting a SNP was reported by Ding et al. [76] in studies of amyotrophic lateral sclerosis (ALS) caused by mutations in the Cu, Zn superoxide dismutase (SOD1) gene. Schwarz *et al.* recently tested siRNAs systematically for their ability to discriminate wildtype from mutant alleles for the SOD1 and the huntingtin (HTT) gene. Their findings support the notion that single nucleotide polymorphisms may indeed suffice to make mutant specific siRNAs if mismatches are rationally placed [77]. Particular purine–purine mismatches at positions 10 and 16 relative to 5′ end of the guide strand provide selectivity [77]. Since the wild-type SOD1 performs important functions it is important to be selectively eliminate expression of only the mutant allelic transcript. Many SOD1 mutations are single nucleotide changes. These investigators were able to achieve selective degradation of a mutant allele encoding SOD1 thereby providing a potential therapeutic application for the treatment of ALS.

Since delivery of siRNAs and viral vectors expressing siRNAs to affected regions of the brain is technically feasible [78], the promise of clinical use of RNAi for treatment of degenerative, neurological diseases may approach reality very rapidly.

5.2. Viral diseases

The first demonstration of RNAi efficacy *in vivo* involved hydrodynamic co-delivery of a hepatitis B replicon and a pol III expression unit encoding an anti-hepatitis B virus (HBV) shRNA in mice [79]. These studies demonstrated that that a significant knockdown (99%) of the HBV core antigen in liver hepatocytes could be achieved by the expressed shRNA providing an important proof of principle for future antiviral applications of RNAi in the liver.

Hepatitis C (HSV) is a virus that infects an estimated 3% of the world's population. HCV is a major cause of chronic liver disease, which can lead to the development of liver cirrhosis and hepatocellular carcinoma, and is the leading cause of liver transplantation in the United States. The HCV genome is a positive-strand RNA molecule with a single open reading frame encoding a polyprotein that is processed post-translationally to produce at least ten proteins. The only therapy currently available uses combined interferon (IFN) and ribavirin. While improvements have been made in the treatment regimens, response is often poor, particularly with some HCV subtypes.

Subgenomic and full-length HCV replicons that replicate and express HCV proteins in stably transfected human hepatoma cell-derived Huh-7 cells have been used to study viral replication and the effects of various antiviral drugs [80–85]. Several groups have tested the efficacy of siRNA mediated inhibition of replicon function *in vitro* using these replicon systems [86–88]. Small inhibitory RNAs targeting the internal ribosome entry site (IRES) and non-structural protein NS3 andNS5b encoding mRNAs were shown to inhibit HCV replicon function in cell culture [87]. Furthermore, anti-HCV siRNAs were shown to "cure" Huh-7.5 cells bearing persistently replicating HCV replicons [86]. McCaffrey et al. used hydrodynamic tail vein injections to demonstrate that both synthetic and Pol III promoter expressed anti-HCV siRNAs directed efficient cleavage of HCV sequences in a HCV-luciferase fusion construct *in vivo* in mouse hepatocytes [89].

A different *in vivo* study used siRNAs to treat fulminant hepatitis induced by an agonistic Fas-specific antibody in mice. Anti-Fas siRNAs were hydrodynamically injected into the antibody treated mice resulting in 82% of the treated mice surviving for 10 days of observation, whereas all control mice died within 3 days [90]. Importantly, mice already suffering from auto-immune hepatitis also improved after Fas siRNA treatment. Thus, it may be feasible to use siRNAs to ammeliorate the severity of certain diseases by targeting the infammatory response pathways rather than the infectious agent.

Delivery of the siRNAs or vectors that carry siRNA expression cassettes is the major challenge for treatment of HCV. The method of delivery used in a number of *in vivo* studies, hydrodynamic intravenous injection, is not feasible for the treatment of human hepatitis. Delivery is a problem that must be confronted for any therapeutic application of RNAi. A recent report demonstrates that it is feasible to introduce genetic material into hepatocytes using catheters or even localized hydrodynamic procedures [91]. Whether or not siRNAs can be delivered in larger mammals by such procedures is yet to be determined, but remains an enticing possibility.

HIV was the first infectious agent targeted by RNAi perhaps owing to the fact that the life cycle of HIV is well understood as is its pattern of gene expression. Synthetic and expressed siRNAs have been used to target a number of early and late HIV-encoded RNAs including the TAR element [92], tat [34]; [93,94], rev [34,93], gag [95,96], env [96], vif [92], nef [92] and reverse transcriptase [94]. Cellular cofactors, such as NFκβ [94], the HIV receptor CD4 [95] and co-receptors CXCR4 and CCR5 [97] have also been successfully downregulated by RNAi

resulting in an inhibition of HIV replication. Moreover, inhibition of HIV replication has been achieved in numerous human cell lines and primary cells including T lymphocytes and hematopoietic stem cell derived macrophages [34,93,95,97–100].

Despite the success of *in vitro* RNAi-mediated inhibition of HIV-1, for future clinical applications, targeting the virus directly represents a substantial challenge since the high viral mutation rate will certainly lead to escape mutants [101]. RNAi-mediated downregulation of cellular co-factors required for HIV infection is an attractive alternative or complementary approach. One such target that holds particular promise is the macrophage-tropic CCR5 co-receptor. This cellular target is non-essential for normal immune function, and individuals homozygous for a 32 bp deletion in this gene show resistance to HIV infection, and individuals who are heterozygous for this deletion have delayed progression to AIDS [102,103]. Taking these facts into account, Qin et al. [104] used a lentiviral vector to transduce a Pol III expressed anti-CCR5 shRNA in human lymphocytes. Downregulation of CCR5 resulted in a somewhat modest, but nevertheless significant 3 to 7 fold reduction in viral infectivity relative to controls. Despite this downregulation, the CCR5 shRNA treated cells were still susceptible to infection by T-tropic CXCR4 utilizing virus, emphasizing the need to combine shRNAs against co-receptors and the virus to achieve potent inhibition of HIV-1. Since CXCR4 is essential for engraftment of hematopoietic stem cells in a marrow environment [105,106], this is not a good choice for a hematopoietic stem cell based therapy nor is the CD4 receptor. Thus, viral targets will need to be included in any successful gene therapy strategy using RNAi. These targets should be sequences that are highly conserved throughout the various clades.

Delivery of siRNAs or shRNA encoding genetic units to HIV infected cells is also a challenging problem. The target cells are primarily T lymphocytes, monocytes and macrophages. Since synthetic siRNAs will not persist for long periods in cells, delivery would have to be done repetitively for years to effectively treat the infection. Systemic delivery of siRNAs to T lymphocytes is a major barrier and probably not feasible. The other approach is to utilize viral vectors to deliver anti-HIV encoding shRNA genes. With viral vectors, systemic delivery is for now not feasible since the immunogenicity of the vectors themselves would preclude the use of multiple injections. Therefore T-cell isolation from patients followed by transduction, expansion of the transduced cells and re-infusion is the preferred path. There is an ongoing clinical trial utilizing T lymphocytes from HIV infected individuals that are transduced *ex vivo* with a lentiviral vector that encodes an anti-HIV antisense RNA. The transduced cells are subsequently expanded and reinfused into patients [107,108]. This type of therapeutic approach is certainly applicable to vectors harboring genes that encode siRNAs. A different approach is to transduce isolated hematopoietic progenitor or stem cells with vectors harboring the therapeutic genes. This approach has the advantage that all the hematopoietic cells capable of being infected by the virus are derived from a multipotent stem cell that can be transduced with an appropriate viral vector. Hematopoietic stem cells are mobilized from the patients and transduced *ex vivo* prior to reinfusion. Two clinical trials utilizing retroviral vector transduced ribozymes in hematopoietic stem cells have already been conducted, demonstrating the feasibility of this approach [109,110]. Since RNAi is more potent than ribozyme or antisense approaches, movement of this technology to a human clinical trial for HIV-1 treatment is certain to take place within the next year or two.

5.3. Cancer

The use of RNAi for cancer therapeutics could revolutionize treatment of this devastating disease. The challenges for cancer are not dissimilar to those faced for other diseases, and include finding good targets, delivery and minimizing toxicity. Many oncologic targets have been reported in the literature, far too many to summarize here. Perhaps the most significant work utilized transferin containing nanoparticles to target Ewing's sarcoma cells in a mouse xenograph model [30]. This study demonstrated the feasibility of using non-lipid based nano particles for the targeted delivery of siRNAs in a cancer model, and provides a powerful proof of principle for systemic delivery of siRNAs to a metastatic cancer.

Mouse xenograft models have been reported to be effective in limiting tumor growth in a number of studies (reviewed in [111]). A few recent examples include adenoviral or retroviral delivery of shRNAs targeting Hec1 that promoted efficacy on adenocarcinoma-induced tumor growth [112], aptamer-siRNA chimeric RNAs that resulted in tumor regression by specific delivery to prostate cancer cells by aptamer binding to surface expressed tumor cell marker (PSMA) [31], atelocollagen-complexed siRNA [113] that effectively silenced Vascular endothelial growth factor (VEGF) and limited angiogenesis and tumor growth *in vivo*, and transferrin receptor targeted cyclodextrin nanoparticles that delivered anti-Ews-Fli1 siRNAs to human Ewing's tumors, effectively blocking metastasis [30]. Finally, miRNA is a new class of targets that have yet unexploited therapeutic avenues that may hold value for cancer treatment (reviewed in [11]). MicroRNAs are known to be important regulators of cell differentiation and may function as oncogenes [47] or tumor suppressors [48,49]. They are amenable to down modulation via antisense based mechanisms [114] and ectopic expression could rescue de-regulated miRNAs as shown by studies in *C. elegans* [48].

Interestingly, RNAi may also be exploited to silence pathways that facilitate the effects of traditional cancer drugs. This includes targeting of the multidrug resistance gene (MDR1) for re-sensitization to chemotherapy [115] and silencing of double-strand break repair enzymes for enhanced effects of radio- and chemotherapy [116].

6. Conclusions

The discovery of RNA interference less than a decade ago was a turning point for molecular biology. RNAi has become a powerful tool for studies of gene function in mammals. It provides the researcher with the ability to silence virtually any gene with artificial triggers of RNAi and utilizing the cellular

machinery for efficient targeting of complementary transcripts. Proof-of-principle studies *in vivo* have clearly demonstrated that both viral and non-viral delivery methods can provide selective and potent target gene suppression without any clear toxic effects. Translational research using breakthroughs in basic RNAi research has taken place with an unprecedented speed and already there are several RNAi based human clinical trials in progress. Despite excitement from a large number of animal model studies, including systemic delivery to non-human primates [73], there are a number of hurdles and concerns that must be overcome before RNAi will be harnessed as a new therapeutic modality. These include off-target effects, triggering of type I interferon responses, competition with cellular RNAi components and effective delivery *in vivo*. As with all biological discoveries, understanding of the mechanism is paramount to effective applications in human disease. Given the pace of new findings and discoveries of applications, we anticipate that RNAi will be a major therapeutic modality within the next several years. Hopefully RNAi will revolutionize the treatment of human disease in the same way that it has revolutionized basic research.

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