

# RNA Interference Technologies and Therapeutics

## From Basic Research to Products

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### Abstract

RNA interference (RNAi) is a natural cellular process that regulates gene expression by a highly precise mechanism of sequence-directed gene silencing at the stage of translation by degrading specific messenger RNAs or blocking translation. In recent years, the use of RNAi for therapeutic applications has gained considerable momentum. It has been suggested that most of the novel disease-associated targets that have been identified are not 'druggable' with conventional approaches. However, any disease-causing gene and any cell type or tissue can potentially be targeted with RNAi.

This review focuses on the current knowledge of RNAi mechanisms and the safety issues associated with its potential use in a therapeutic setting. Some of the most important aspects to consider when working towards the application of RNAi-based products in a clinical setting have been related to achieving high efficacies and enhanced stability profiles through a careful design of the nucleic acid sequence and the introduction of chemical modifications, but most of all, to developing improved delivery systems, both viral and non-viral. These new delivery systems allow for these products to reach the desired target cells, tissues or organs in a highly specific manner and after administration of the lowest possible doses. Various routes of application and target locations are currently being addressed in order to develop effective delivery systems for different targets and pathologies, including infectious pathologies, genetic pathologies and diseases associated with dysregulation of endogenous microRNAs. As with any new technology, several challenges and important aspects to be considered have risen on the road to clinical intervention, e.g. correct design of preclinical toxicology studies, regulatory concerns, and intellectual property protection. The main advantages related to the use of RNAi-based products in a clinical setting, and the latest clinical and preclinical studies using these compounds, are reviewed.

RNA interference (RNAi) is a naturally occurring regulatory mechanism of most eukaryotic cells that uses small double-stranded RNA (dsRNA) molecules to direct homology-dependent gene silencing. Its discovery by Fire and Mello in the worm *Caenorhabditis elegans*<sup>[1]</sup> resulted in these investigators being awarded the Nobel Prize in 2006. Shortly after its first description, RNAi was also shown to occur in mammalian cells, not through long dsRNAs but by means of double-stranded small interfering RNAs (siRNAs) 21 nucleotides long.<sup>[2]</sup> Since the discovery of the RNAi mechanism, there has been an explosion of research to uncover new compounds that can selectively alter gene expression as a new way to treat human disease by addressing targets that are otherwise 'undruggable' with traditional pharmaceutical approaches involving small molecules or proteins. In this review, we provide an overview of the mechanism of action of RNAi and discuss how to maximize its potency and minimize its adverse effects in therapeutic applications. We also review *in vivo* delivery strategies and stabilizing modifications. Finally, we revisit the barriers that need to be overcome in regards to use of RNAi in clinical applications and its current development as a new class of therapeutic agent.

## 1. Mechanism of RNA Interference (RNAi)

According to current knowledge, the mechanism of RNAi is initiated when long dsRNAs are processed by an RNase III-like protein known as Dicer. The protein Dicer typically contains an N-terminal RNA helicase domain, an RNA-binding so-called Piwi/Argonaute/Zwille (PAZ) domain, two RNase III domains and a dsRNA binding domain (dsRBD),<sup>[3]</sup> and its activity leads to the processing of the long dsRNA into 21–24 nucleotide double-stranded siRNAs with two base 3' overhangs and a 5' phosphate and 3' hydroxyl group. The resulting siRNA

duplexes are then incorporated into the effector complex known as the RNA-induced silencing complex (RISC), where the antisense or guide strand of the siRNA guides the RISC to recognize and cleave target messenger RNA (mRNA) sequences<sup>[2]</sup> upon adenosine triphosphate (ATP)-dependent unwinding of the double-stranded siRNA molecule through an RNA helicase activity.<sup>[4]</sup> The catalytic activity of RISC, which leads to mRNA degradation, is mediated by the endonuclease Argonaute 2 (AGO2).<sup>[5,6]</sup> AGO2 belongs to the highly conserved Argonaute family of proteins. Argonaute proteins are ~100 kDa highly basic proteins that contain two common domains, namely the PIWI and PAZ domains.<sup>[7]</sup> The PIWI domain is crucial for the interaction with Dicer and contains the nuclease activity responsible for the cleavage of mRNAs.<sup>[6]</sup> AGO2 uses one strand of the siRNA duplex as a guide to find mRNAs containing complementary sequences, and cleaves the phosphodiester backbone between bases 10 and 11 relative to the 5' end of the guide strand.<sup>[2]</sup> An important step during the activation of RISC is the cleavage of the sense or passenger strand by AGO2, removing this strand from the complex.<sup>[8]</sup> Crystallography studies analyzing the interaction between the siRNA guide strand and the PIWI domain reveal that it is only nucleotides 2–8 that constitute a 'seed sequence' that directs target mRNA recognition by RISC.<sup>[9]</sup> Once the mRNA has been cleaved, and because of the presence of unprotected RNA ends in the fragments, the mRNA is further cleaved and degraded by intracellular nucleases and is no longer translated into proteins,<sup>[10]</sup> while the RISC is recycled for subsequent rounds.<sup>[11]</sup> This constitutes a catalytic process leading to the selective reduction of specific mRNA molecules and of the corresponding proteins. It is possible to exploit this native mechanism for gene silencing with the purpose of regulating any gene(s) of choice by directly delivering siRNA effectors into

the cells or tissues, where they will activate RISC and produce a potent and specific silencing of the targeted mRNA.

Post-transcriptional gene silencing (PTGS) can be induced not only by siRNA through sequence-specific cleavage of perfectly complementary mRNA but also, according to recent discoveries, by other endogenous post-transcriptional regulatory mechanisms. One of these mechanisms is that mediated by microRNAs (miRNAs), which are functional, naturally occurring small non-coding RNAs that require only partial complementary targets to bind to their target mRNAs through their 3' untranslated regions (3' UTRs).<sup>[12,13]</sup> miRNAs act as guide sequences to regulate the expression of multiple genes that are often functionally related. Furthermore, the translation of many mRNAs is regulated by multiple different miRNAs. They are critical factors in coordinating the development, differentiation, and functions of cells and tissues and it is estimated that there are hundreds of these molecules in humans. There are approximately 500 miRNAs that have been identified in the human genome and they are believed to regulate the expression of up to 30% of all human genes by preventing translation of mRNAs into proteins.

miRNAs arise from class II RNA polymerase transcripts, termed primary miRNA (pri-miRNA), that vary in length from a few hundred bases up to tens of kilobases and have significant secondary structures. These pri-miRNAs are then recognized by the microprocessor complex, consisting of the proteins Drosha and DGCR8 (DiGeorge syndrome critical region gene 8), which cleaves the pri-miRNA into ~70 nucleotide hairpin containing a 2-nucleotide overhang on its 3' end.<sup>[14]</sup> This precursor (pre)-miRNA is then exported from the nucleus to the cytoplasm by the protein exportin 5 (Exp5),<sup>[15]</sup> where it is processed by a Dicer-containing complex to ~21–25 nucleotide imperfect dsRNA duplexes that constitute the mature miRNAs.<sup>[16,17]</sup> Once processed by this Dicer complex, consisting of Dicer, the HIV transactivating response RNA-binding protein TRBP,<sup>[18]</sup> and the protein activator of the interferon-induced protein kinase, PACT,<sup>[19,20]</sup> the miRNA duplex is assembled into the RISC;<sup>[21]</sup> however, because the miRNA duplexes are almost always asymmetric and not completely complementary, they do not have an antisense stretch of nucleotides as happens with siRNAs. The mechanism of selection of one strand above the other is not completely clear, but once one strand has been loaded into the RISC, imperfect sequence complementarity between both strands of miRNA might prevent AGO2 from cleaving the passenger strand,<sup>[21]</sup> which is instead unwound and discarded. The remaining strand then guides the RISC to the 3' UTRs of the mRNAs, leading to the repression of protein expression by a number of mechanisms,<sup>[22,23]</sup> often accom-

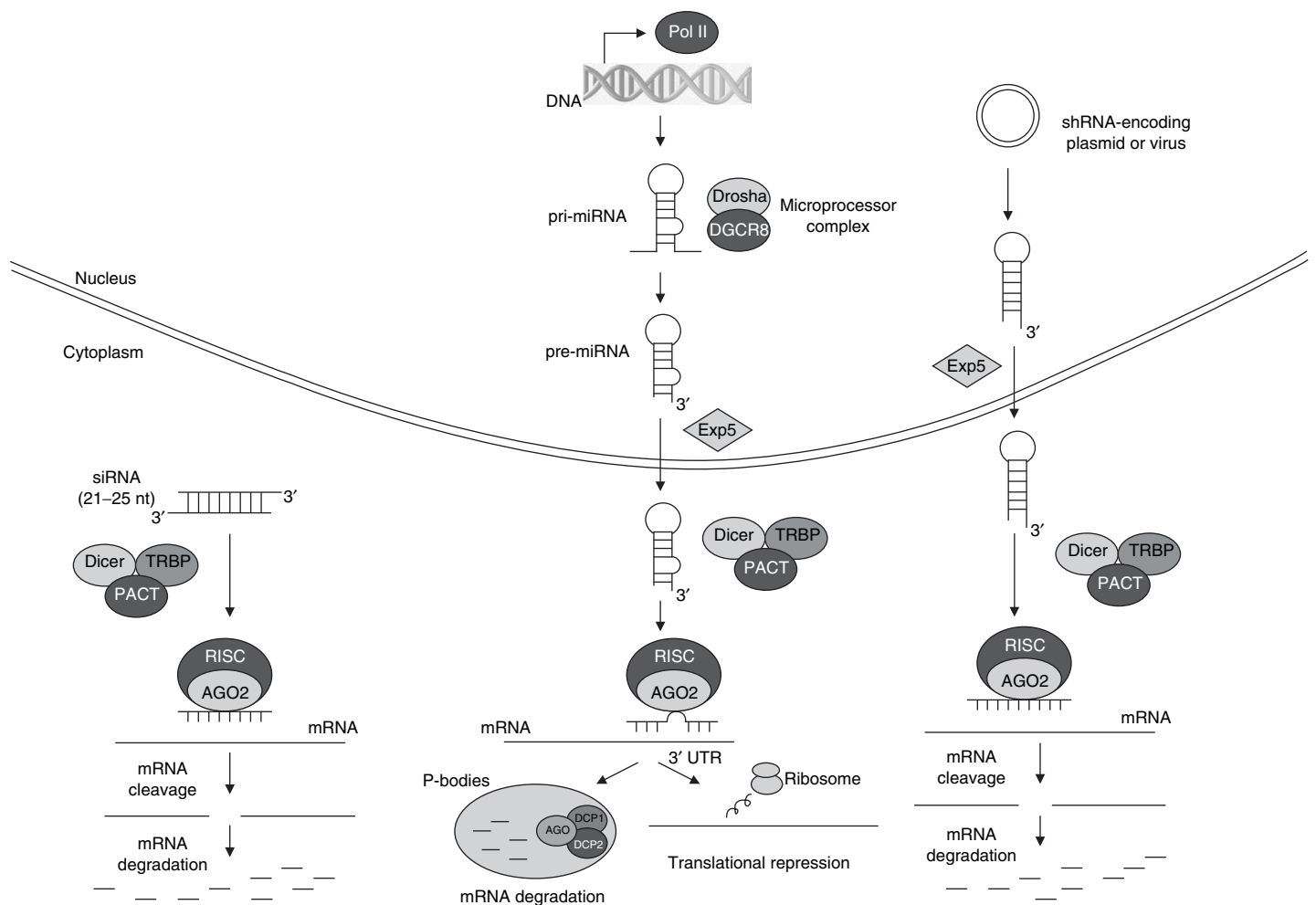
panied by mRNA degradation in cytoplasmic compartments known as processing bodies or P-bodies.<sup>[24]</sup> When miRNAs share complete sequence complementarity with their target sequence they instead direct their cleavage by RISC activity.<sup>[25]</sup> A specific stretch of the mature miRNA, which includes the first 2–8 nucleotides from its 5' end,<sup>[26]</sup> must have complete complementarity with the target in order to obtain effective silencing, whereas mismatched nucleotides in the 3' end are better tolerated.

Commercially available systems and other therapeutic initiatives aimed at mimicking the mechanism of RNAi make use of DNA vector constructs or viral particles coding for long-term and stable short hairpin RNAs (shRNAs) expression that are transcribed from RNA polymerase II or III promoters *in vivo* or shRNAs that are synthesized exogenously and transfected into cells. The double-stranded region of shRNAs is formed through a hairpin structure and intramolecular hybridization that resembles that of miRNA precursors.<sup>[27,28]</sup> These shRNAs molecules are recognized by Dicer, leading to the formation of siRNAs homologous to the target mRNA. The main difference with siRNAs is that while these mediate only transient silencing because their concentrations in the cytoplasm are diluted over time with successive cell divisions, shRNAs mediate a very potent and stable silencing effect for as long as their transcription takes place. On the other hand, the obvious problems with this approach are the same ones encountered with gene therapy and those related to the expression of long exogenous RNAs. shRNAs also enter the endogenous silencing pathway at an earlier stage than siRNAs, having a higher chance of saturating the natural miRNA natural pathways.<sup>[29]</sup> Recent studies have sought to address this issue by showing that it is possible to avoid at least some of the safety concerns by seeking localized expression of shRNAs using vectors harboring tissue-specific polymerase II promoters with improved tolerability.<sup>[30]</sup> Nevertheless, most current efforts rather lean towards the therapeutic use of synthetic siRNAs. All the mechanisms of action described above for siRNAs, miRNAs, and shRNAs are summarized, in figure 1.

## 2. The Safety Issue

### 2.1 Stimulation of Innate Immune Responses

In humans, survival upon infection largely depends on the ability of the immune system to detect pathogens and mount an appropriate protective immune response.<sup>[31,32]</sup> Many immune cells have the ability to sense the presence



**Fig. 1.** Mechanism of RNA interference in mammalian cells. RNA interference is an intracellular mechanism triggered through small RNAs that include small interfering RNAs (siRNAs), microRNAs (miRNAs) and short hairpin RNAs (shRNAs). The siRNA pathway begins when double-stranded RNAs (dsRNAs) are trimmed down by the Dicer complex into siRNAs. Alternatively, synthetic siRNAs can be introduced directly into the cell cytoplasm. These siRNAs are incorporated into the RNA-induced silencing complex (RISC), where they are unwound. If the siRNA has perfect sequence complementarity, the Argonaute 2 protein (AGO2) present in RISC cleaves the passenger (sense) strand so that active RISC containing the guide (antisense) strand can recognize target sites on the messenger RNA (mRNA) to direct mRNA cleavage. This cleavage is performed by the catalytic domain of AGO2. The miRNA pathway starts when primary miRNA (pri-miRNAs) are transcribed from RNA polymerase II (Pol II) promoters, forming hairpin-shaped structures. These are processed by the Drosha-containing microprocessor complex, giving rise to precursor miRNAs (pre-miRNAs), which are also stem-like structures with a 2-nucleotide 3' overhang. Pre-miRNAs are transported into the cytoplasm by exportin 5 (Exp5), where they are processed by a Dicer containing complex to ~21–25 nucleotide (nt) imperfect dsRNA duplexes that constitute the mature miRNAs. Once the miRNA duplex is processed, the guide sequence is loaded into RISC and then mediates binding to the target sequence in the 3' untranslated region (UTR) of cellular mRNAs. If the miRNA guide sequence is fully complementary to its target site, it triggers site-specific cleavage and degradation of the mRNA through the catalytic domain of AGO2. On the other hand, if the base pairing is incomplete but fully complementary in the seed region (nucleotides 2–8 of the miRNA), repression of protein expression occurs, often accompanied by mRNA degradation in cytoplasmic processing (P)-bodies. Mimicking the miRNA mechanism, synthetic DNA vector constructs or viral particles code for stable shRNAs, which are transcribed from an RNA polymerase II/III promoter and form hairpin-like structures. These shRNAs are transported into the cytoplasm by Exp5 and recognized by Dicer, leading to the formation of siRNAs homologous to the target mRNA and, subsequently, to mRNA degradation. **DCP** = mRNA decapping protein; **DGCR8** = DiGeorge syndrome critical region gene 8; **PACT** = protein activator of interferon-induced protein kinase; **TRBP** = TAR RNA binding protein.

of microbial organisms through several families of pattern recognition receptors (PRRs), which mediate the recognition of conserved microbial structures known as pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide, peptidoglycan, flagellin, capsular structures,

bacterial DNA and viral RNAs, and glycoproteins. Activation of the innate immune response is normally used to fight viral infections and leads to the production of type I interferons, downregulation of gene expression and induction of apoptosis.

It is well documented that dsRNAs longer than 30 nucleotides can trigger potent immune responses. However, siRNAs largely circumvent this problem as they seem to be too small to induce cellular toxicity. Nevertheless, this does not always appear to be true. Two pathways can lead to the activation of immune responses: one involves recognition by cytosolic RNA-binding proteins such as the serine/threonine protein kinase R (PKR), the helicase retinoic acid-inducible protein I (RIG-I), and melanoma differentiation-associated protein 5 (MDA5),<sup>[33]</sup> and the other includes three members of the Toll-like receptor (TLR) family (TLR3, TLR7, and TLR8).<sup>[34]</sup> Detection of RNA molecules can also be triggered in a sequence-specific manner and recognition seems to be cell specific.<sup>[35,36]</sup> The work to identify immunostimulatory motifs and the mechanisms of interferon responses to foreign pathogens and nucleic acids can be of great relevance in the design of synthetic siRNAs so that unwanted activation of the immune system can be prevented. For example, plasmacytoid dendritic cells can be activated via their endosomal TLRs by a specific GU-rich region, so called 'danger motifs', such as 5'-UGUGU-3' and 5'-GUCCUCAA-3'.<sup>[34,37,38]</sup> This is reminiscent of the immunostimulatory cytosine-phosphate-guanine (CpG) motifs in antisense oligonucleotides (ODNs) that give 'danger signals' to the cells via their TLR9 receptors. Therefore, even though such warning signals can be beneficial under certain situations, they should be avoided in order to achieve safe therapeutic use of RNAi. It has also been shown that *in vitro* T7-transcribed siRNAs potentially induce interferon responses due to the presence of a 5' triphosphate moiety.<sup>[39]</sup> Several reports have shown that the presence of 2'-O-methyl, 2'-F and phosphorothioate backbone modifications within the siRNAs can be used to avoid their binding to TLRs and prevent cytokine induction while maintaining silencing activity.<sup>[34,40,41]</sup>

To minimize these adverse effects, siRNAs could be transfected into human primary cells with a full repertoire for immune stimulation that results in discarding those that elicit interferon responses,<sup>[34,38]</sup> followed by careful *in vivo* analysis to gain knowledge of their immune stimulatory properties.

## 2.2 Off-Target Effects

RNAi is highly specific as a result of Watson-Crick base pairing interactions. Nevertheless, a number of studies have demonstrated that siRNAs induce gene expression changes in a wide range of seemingly unrelated genes.<sup>[42-45]</sup> Although microarray studies have shown that changes in off-target mRNAs are usually <2-fold, this may result in substantial changes in protein expression if miRNA pathways are acti-

vated. The rules defining miRNA targets are still not fully understood, so it is difficult to predict when any given siRNA will elicit off-target silencing. Very limited sequence homology at the 3' end UTRs of the off-target genes might suffice to induce gene silencing. Target specificity of miRNA depends on a 7-nucleotide region called the 'seed region' or 'seed sequence', comprising nucleotides 2–8 from the 5' end of the guide strand of the miRNA.<sup>[46]</sup> Thus, such small sequence homology is enough to trigger off-target effects (OTEs) and the chances of finding 7-nucleotide complementary regions in the entire human transcriptome are far greater than would have been desirable.

Careful comparison of candidate guide strand sequence with the entire transcriptome, attempting to avoid long stretches of homology, might reduce the risk but it has been estimated that approximately 83% of the possible 21mers within the coding sequences of the genome are unique,<sup>[47]</sup> leaving one out of every five 21-nucleotide long siRNAs to display some homology compared with a given mRNA. Snove and Holen<sup>[48]</sup> performed an independent investigation of 360 published siRNA sequences and found that almost 75% of these oligonucleotides had the potential to trigger unwanted OTEs. They suggested that use of inappropriate programs, such as basic local alignment and search tools (BLAST), to design effective oligonucleotides leads to abundant OTEs because a precise homologous stretch of six or seven base pairs is necessary for detection through BLAST. This would eliminate the detection of very closely related sequences of 5–10 base pairs that could have one or two mismatches, enough to allow hybridization to off-target sequences. Additionally, nonspecific OTEs are not limited to the guide strand and can also be triggered by the passenger strand if it incorporates into RISC and binds to mRNAs bearing total or partial sequence homology.<sup>[43]</sup> RISC incorporation favors the strand with the least tightly bound 5' end, such that it becomes the active strand. Strand selection can in fact be manipulated by designing siRNAs destabilized at the 5' end of the guide strand, e.g. by making a single nucleotide substitution at the end of the duplex to alter the relative binding of the ends, thereby promoting incorporation of this strand and not the passenger into RISC and minimizing potential OTEs.<sup>[49-51]</sup> A comparison of the effectiveness of siRNAs using different delivery methods has also shown that many OTEs largely depend on the lipid-based transfection reagent more than on the siRNA itself.<sup>[52]</sup>

## 2.3 Saturation of Endogenous Pathways

Bioactive drugs that rely on cellular processes to exert their functions face the risk of saturating endogenous pathways. This

may be the case with RNAi-based drugs. shRNAs and siRNAs are very similar to miRNA precursors before and after Dicer processing, respectively, and rely on endogenous miRNA machinery to achieve target silencing. Therefore, miRNA pathways might become saturated by high doses of exogenous RNAs. One of the ways adenoviruses avoid potential host RNAi antiviral activity is by expressing large quantities of a non-coding RNA stem-loop that interferes with transport from the nucleus to the cytoplasm by binding to the nuclear karyopherin Exp5, thereby inhibiting transport and subsequent processing of cellular pre-miRNAs.<sup>[53]</sup> Similar to this process, some reports have described that *in vivo* adeno-associated virus-encoded overexpression of liver-directed shRNAs can saturate Exp5. This results in inhibition of endogenous pre-miRNA nuclear export and, ultimately, causes death.<sup>[29]</sup> Strong expression of shRNAs has also been shown to induce cytotoxicity in primary lymphocytes, whereas the same shRNA expressed using a weaker promoter presents no toxic effects<sup>[54]</sup> and robust levels of antisense RNAs emerging from shRNA expression systems cause toxicity in the mouse brain, regardless of the sequence.<sup>[55]</sup> The export function mediated by Exp5 is not required for the activity of synthetic siRNAs;<sup>[56]</sup> however, a recent report has shown that synthetic siRNAs and expressed shRNAs compete against each other and with endogenous miRNAs for transport and incorporation into RISC and that TRBP is one of the sensors for selection and incorporation of the guide sequence of interfering RNAs.<sup>[57]</sup>

Thus, a number of factors that alter endogenous cellular processes can result in toxicity and it is therefore crucial to work at the lowest possible concentrations to mitigate the potential of adverse effects. These factors may also set limits to the number of different siRNAs that can be used simultaneously in one target cell.

### 3. Efficacy and Stability

The efficacy of siRNAs for individual targets normally depends on different factors, such as thermodynamic stability,<sup>[50]</sup> structural features,<sup>[58]</sup> target mRNA accessibility,<sup>[59]</sup> and additional position-specific determinants.<sup>[51,60]</sup> Systematic studies of targeting efficacies have shown that optimal siRNAs should be between 19 and 25 nucleotides long, should have 3' symmetric dinucleotide overhangs, low guanine-cytosine content (between 30% and 52%)<sup>[51]</sup> and specific nucleotides at certain positions. For example, features that increase siRNA efficacy are the presence of an adenine or uracil in position 1, adenosine in position 3, a uracil in positions 7 and 11, a guanine in position

13, a uracil or adenine in position 10 (this is the site for RISC mediated cleavage), a guanine in position 21 and/or the absence of guanines or cytosine at position 19 of the sense strand (see Dykxhoorn and Lieberman<sup>[61]</sup> for a full review of the topic). In general, enrichment in adenosines and uracils along the first 6–7 base pairs of the sequence, and consequently, weak hydrogen bonding, allows the RISC to easily unravel the double-stranded duplex and load the guide strand.<sup>[62]</sup>

siRNA duplexes should also be thermodynamically flexible at their 3' end, i.e. at positions 15–19 of the sense strand. This correlates with their silencing efficacy, such that the presence of at least one adenosine-uracil pair in this region would decrease the internal stability and increase the silencing efficacy. In contrast, internal repeats or palindrome sequences decrease the silencing potential of the siRNAs.

Another consideration that needs to be taken into account when designing a siRNA sequence is the nature of the target sequence. Under certain circumstances it will be preferable to include all the splice variants and isoforms for the design of the siRNA, whereas in other instances they should be specifically left out. Similarly, attention should be paid to choice of sequences within the coding region of the target gene sequence, as gene silencing is an exclusively cytoplasmic process.<sup>[63]</sup> The 'good news' is that, taking all these established criteria into account, RNAi allows for almost unrestricted choice of targets. Computer-based algorithms can help in the design of optimal siRNA sequences for any given gene, and will consider properties such as thermodynamic values, sequence asymmetry, and polymorphisms that contribute to RNA duplex stability. Nevertheless, any theoretically optimal siRNA will require extensive testing to achieve high silencing efficacy without any adverse effects.

Regarding the issue of enhanced stability, several chemical modifications have been described that can increase the half-life of siRNAs, including the introduction of phosphorothioate bonds.<sup>[64,65]</sup>

### 4. Delivery

One of the major problems in the development of RNAi-based therapies is the delivery of these molecules to the desired target cells, tissues or organs. Most small-molecule drugs are able to reach their targets because they can passively diffuse into and out of the cells through the cell membrane. Larger and more complex molecules, such as ODNs and siRNAs, are more restricted in terms of their ability to pass through tissue barriers. *In vitro* experiments have established methods to

efficiently transfer siRNAs across the plasma membrane and into the cytoplasm. However, delivering siRNAs to animal tissues *in vivo* is much more complicated because of the dense and complex tissue microenvironments. The different routes of application and the target location are essential points to be considered when developing effective delivery systems.

The high therapeutic potential of siRNAs and their application in clinical settings is currently limited due to the lack of efficient delivery systems. Clinically acceptable siRNA delivery systems should be carefully designed to improve the stability of siRNAs after *in vivo* administration.<sup>[66]</sup> Additionally, siRNAs will only be able to induce RNAi efficiently when they reach their target organ/tissues, their target cells within that organ, and the correct intracellular compartment within those cells. Considering the various barriers in the organism, this poses one of the most important hurdles for *in vivo* application of RNAi, but several other problems such as enzymatic stability, cellular uptake through biological membranes, enhancement of endosomal and lysosomal escape within the cell, pharmacokinetic behavior, safety, target specificity and potential for OTEs and immunostimulation will also need to be addressed.<sup>[67]</sup>

#### 4.1 Viral versus Non-Viral Delivery Methods

Viral siRNA delivery has been used to specifically down-regulate the expression of genes of pathological relevance, especially for chronic diseases in which long-term gene silencing is desired, e.g. neurodegenerative disorders, cancer, heart failure and HIV infections. This can be achieved using gene therapy approaches in which a short hairpin RNA expression cassette is stably integrated into the host cell genome or expressed episomally. Retroviral, adenoviral and adeno-associated and herpes viral shRNA delivery systems have been successfully used to silence genes both *in vitro* and *in vivo*. Stable integration can be mediated by lentivirus-based vectors that are well suited for these applications since they are able to transduce both dividing and non-dividing cells.<sup>[68]</sup> Singer and colleagues<sup>[69]</sup> demonstrated how intracranially injected lentiviral vectors expressing shRNAs targeting  $\beta$ -site amyloid precursor protein cleaving enzyme 1 (BACE-1) significantly reduced amyloid production, the main cause of neurodegeneration in Alzheimer's disease. More transient expression without genome integration can be provided by adenoviruses, resulting in short-term gene silencing. Adeno-associated viruses have been used for the delivery of specific siRNAs targeting mutant ataxin-1 in a spinocerebellar ataxia-1 (SCA1) mouse model, showing that reduction of the expression of ataxin-1 could improve motor coordination and restore cerebellar morphology.<sup>[70]</sup> Sabbioni

and co-workers<sup>[71]</sup> use herpes simplex virus type I (HSV-1)-based vectors for siRNA delivery into mammalian cells. Using human polyomavirus BK (BKV)-transformed cells as a model system, the ability of amplicon vectors to inhibit the expression of BKV-T-antigen has been demonstrated. The use of these amplicon vectors is highly efficient for the delivery of siRNA molecules and their ability to deliver multiple copies may constitute a useful tool in the development of novel therapies. Suckau and colleagues<sup>[72]</sup> used a rat model of transaortic banding to demonstrate how an RNAi-based therapy can be used to rescue heart failure and restore cardiac function. After intravenous injection of an adeno-associated virus vector (rAAV9-shPLB) targeting phospholamban, a key regulator of cardiac  $\text{Ca}^{2+}$  homeostasis, cardiac phospholamban protein was reduced to 25%. Suppression of sarcoplasmic reticulum  $\text{Ca}^{2+}$  ATPase was rescued in the heart failure groups. Moreover, rAAV9-shPLB displayed high affinity for the myocardium, low affinity for the liver and other organs and no hepatotoxicity or miRNA deregulation.

In summary, viral delivery systems have the advantage of achieving high transfection efficiencies due to the inherent ability of viruses to transport genetic material into cells. However, viral systems have a limited loading capacity, i.e. the genetic material is rather difficult to produce in large scale. Additionally, viral systems pose severe safety risks because of their oncogenic potential via insertional mutagenesis,<sup>[73,74]</sup> their inflammatory and immunogenic effects,<sup>[75,76]</sup> and the difficulties in controlling the timing and dose of interference. Therefore, it would be essential to develop improved viral vectors that could target specific cell types or tissues after systemic *in vivo* applications in order to minimize the toxicities associated with treatment.

Because of the important safety issues described above, non-viral delivery strategies have been more widely used. These display important benefits over delivery with viral vectors. Most notable are their potential lack of immunogenicity, low frequencies of integration and relatively simple large scale production. These systems can also be designed to incorporate a variety of nucleic acids and be easily modified with different ligands to achieve specific cell type targeting. This targeting would allow a reduction in the concentration of or a decrease in the number of doses needed to obtain therapeutic effects, thereby reducing costs and the possibility of adverse effects and toxicity.

#### 4.2 Local Delivery

As mentioned, siRNAs are generally not taken up by mammalian cells, including those that actively sample their

environment. However, certain tissues and cells in the lungs, mucosal environments, eyes, and even the central nervous system have been shown to efficiently take up siRNAs in the absence of transfection reagents.<sup>[77-81]</sup> Clinical programs are underway that use direct intravitreal injection for the treatment of age-related macular degeneration and intranasal administration for pulmonary viral infection. Different groups demonstrated that siRNA injected either intravitreally or subretinally into monkey and rat eyes efficiently silenced vascular endothelial growth factor (VEGF) expression and accordingly reduced ocular neovascularization.<sup>[82,83]</sup> Based on these results, anti-VEGF siRNAs have been one of the first to be tested in a clinical setting. In general, the advantages of local administration include the fact that siRNAs might require only a simple formulation and, therefore, be easier to produce and administer. Additionally, site-specific delivery might facilitate a localized effect and is likely to require a lower dose of the siRNAs for them to carry out their therapeutic function, exerting minimal systemic effects and reducing the risks of OTEs. Thus, whenever possible, local delivery of siRNAs is likely to be the most cost-efficient strategy *in vivo*.

### 4.3 Systemic Delivery

Systemic delivery strategies might become necessary depending on the distribution of the target gene and its accessibility. Several non-viral delivery systems have been developed based on various nanoparticulate systems, including liposomes, lipids, polymers and peptides. The resulting complexes can provide the siRNAs with protection from attack by extracellular nucleases and allow an easy cellular uptake via the endocytic pathway.<sup>[66]</sup> Some of these systems will be described below.

#### 4.3.1 Bioconjugation

Since siRNA is a double helix formed by two complementary strands, there are four terminal ends for potential conjugation sites. Beyond the enhancement of siRNA stability, conjugation reactions have been performed to increase the uptake of chemically modified or unmodified siRNA molecules, thus serving as a delivery vehicle. There are several conjugation strategies that result in an increase in thermodynamic and nuclease stability as well as an improvement in biodistribution and pharmacokinetic profiles.

##### Lipid Conjugation

Conjugation with lipids may enhance siRNA uptake via receptor-mediated endocytosis or by increased membrane

permeability of the otherwise negatively charged RNA. Cholesterol has been covalently conjugated to siRNA for systemic delivery by Soutscheck and co-workers.<sup>[84]</sup> This study reports the conjugation of cholesterol to the sense strand 3' terminus of an apolipoprotein B (ApoB) siRNA via a pyrrolidone linkage. The cholesterol-siRNA conjugate could induce intracellular RNAi without any significant loss of gene silencing activity compared with the unconjugated version. In addition, the conjugate exhibited significantly higher cellular transfer efficiency in cultured cells without the aid of any transfectant agent. Significant silencing of *APOB* gene, which encodes a protein essential for cholesterol metabolism, was observed in the liver and jejunum after intravenous administration of the cholesterol-siRNA conjugate *in vivo*. Silencing of the *APOB* gene resulted in decreased plasma ApoB protein levels and, consequently, in a reduction in total cholesterol levels. Conjugation to cholesterol also improved siRNA pharmacokinetic behavior *in vivo*: conjugates showed an elimination half-life ( $t_{1/2}$ ) of 95 minutes and plasma clearance (CL) of 0.5 mL/min, whereas unconjugated siRNAs had a  $t_{1/2}$  of 6 minutes and a CL of 17.6 mL/min.<sup>[84]</sup> Other studies have also shown that conjugation of nucleic acids with cholesterol enhances cellular uptake in human liver cells without the use of any transfection reagent *in vitro*<sup>[85,86]</sup> and good hepatic deposition after systemic administration *in vivo*.<sup>[86]</sup>

Another lipophile-siRNA conjugate,  $\alpha$ -tocopherol (vitamin E)-siRNA has been used for systemic siRNA delivery to the liver.<sup>[87]</sup> Lipophilic vitamin E was covalently conjugated to the 5' terminus of the antisense strand of a 27/29-mer siRNA, which was partially modified with 2'-O-methylated riboses and phosphorothioate linkages. After intracellular delivery, the 27/29-mer siRNA is processed by the action of Dicer to generate 21/21-mer siRNAs, simultaneously releasing the vitamin E moiety. Intravenous administration of the conjugate achieved a significant reduction in the target protein (ApoB) in the liver without any induction of inflammatory interferons.<sup>[66]</sup>

##### Peptide Conjugation

Cell penetrating peptides (CPPs), also referred to as membrane permeant peptides (MPPs) or protein transduction domains (PTDs), offer an alternative to the traditional methods of siRNA delivery. CPPs are short amino acid sequences that are able to interact with the plasma membrane in a way that leads to highly efficient uptake into the cytoplasm. These protein domains consist mainly of positively charged amino acids, such as arginine and lysine, responsible for translocating the CPPs through the plasma membrane. Cellular uptake occurs in a receptor-independent fashion and by an energy-independent



mechanism. The only common feature of these peptides appears to be that they are amphipathic molecules and net positively charged at physiological pH, and therefore interact with the negatively charged head groups of the plasma membrane. CPP-conjugated siRNAs can enter different cell types with very high efficiency and rapid uptake kinetics.<sup>[88]</sup>

The idea of using peptides as carriers was first suggested 20 years ago, when it was shown that the HIV-1 transactivating protein tat is taken up by mammalian cells. Since these first reports, a large number of naturally occurring and engineered CPPs have been discovered, e.g. penetratin,<sup>[89]</sup> transportan,<sup>[90]</sup> TP10,<sup>[91]</sup> oligoarginine,<sup>[92]</sup> model amphipathic peptide (MAP),<sup>[93]</sup> MPG (a bipartite amphipathic peptide derived from the fusion peptide domain of HIV-1 gp41 protein and the nuclear localization sequence of SV40 large T antigen)<sup>[94]</sup> and MPG $\alpha$ .<sup>[95]</sup> Simeoni and co-workers<sup>[96]</sup> have described a new peptide-based gene delivery system. They were the first to non-covalently complex siRNAs with the MPG peptide, which is a bipartite amphipathic peptide derived from both the fusion peptide domain of HIV-1 glycoprotein 41 protein and the nuclear localization signal (NLS) of simian virus 40 large T antigen. At a 1:10 ratio of negative nucleic acid to positive peptide charges, a decrease in luciferase activity of about 80% was found in HeLa or Cos-7 cells. The investigators showed that cell entry is independent of the endosomal pathway and that the NLS of the MPG peptide is involved both in electrostatic interactions with the nucleic acid and in nuclear targeting.<sup>[96]</sup> Davidson and co-workers<sup>[97]</sup> showed a remarkably strong RNAi effect in primary neuronal cells using a penetratin-coupled siRNA against several endogenous proteins. The observed downregulation of the target proteins after peptide-mediated siRNA delivery was found to be far more effective than that mediated by lipofectamine 2000.

#### Polyethylene Glycol Conjugation

Polyethylene glycol (PEG) is a biocompatible, hydrophilic and non-ionic polymer that can be conjugated to siRNAs via a reducible disulfide linkage. The PEG-siRNA conjugate can be further complexed with cationic polymers or peptides as core-condensing agents to form colloidal nanoparticles, called polyelectrolyte complex (PEC) micelles. The negatively charged siRNA segment remains completely buried inside the electrolyte core by the addition of the core-forming polycation with resultant charge neutralization, while the hydrophilic PEG segment surrounds the charged polyelectrolyte core. The siRNA found in PEG-siRNA conjugates is much more stable than its naked counterpart when incubated in the presence of

50% serum, lasting up to 16 hours without a significant loss of integrity.<sup>[98]</sup> The PEG-siRNA/polyethylenimine (PEI) PEC micelles have been used for local and systemic treatment of tumors in animal models. A siRNA targeting VEGF that was selected for antiangiogenic cancer therapy achieved a significant retardation in tumor growth in mice after intravenous administration of the PEC micelles *in vivo*.<sup>[66]</sup>

#### 4.3.2 Complex Formation

##### Cationic Lipids

Initially introduced as DNA transfection reagents, many cationic liposomes have been tested for *in vitro* and *in vivo* transfection of nucleic acids. The flexibility in the design of cationic lipid structures and liposome composition, combined with the diversity of methods for their preparation and *in vivo* efficiency, have promoted the notion that cationic lipids can be efficiently used for human gene transfer. Nucleic acids, including siRNAs, are able to electrostatically interact with cationic liposome-forming particles. However, in contrast to large DNA plasmid molecules, the considerably smaller siRNAs cannot condense into particles of nanomeric dimensions.<sup>[99]</sup> Additionally, electrostatic interactions between siRNAs and cationic liposomes pose two potential problems: (i) a relatively uncontrolled interaction process, leading to lipid-siRNA complexes of excessive size and poor stability; and (ii) incomplete encapsulation of the siRNA molecules, thereby exposing the siRNAs to potential enzymatic or physical degradation.<sup>[88]</sup> Several liposomal systems have been developed over the years. Zhang and colleagues<sup>[100]</sup> successfully delivered siRNAs into lung tumor cells by loading siRNAs into liposomes bearing arginine octamer (R8) attached to the liposome surface. The R8 liposomes containing siRNAs showed high stability and protection of incorporated siRNA, achieving very high transfection efficiency in lung tumor cells. Another new liposomal system was able to deliver siRNA into different cell lines (HeLa and human umbilical vein endothelial cells [HUVEC]) at very low concentrations. These siRNA-containing liposomes were able to silence protein kinase 3 (PKN3) expression in a concentration-dependent manner and improve the cellular uptake of siRNAs escaping from the endosomal/lysosomal pathways.<sup>[101]</sup> Another example of liposomes used for delivery are stable nucleic acid lipids particles (SNALPs). In an *in vivo* mouse model of hepatitis B virus (HBV) infection, stabilized siRNAs incorporated into specialized liposomes to form SNALPs had a longer  $t_{1/2}$  in plasma and liver compared with unformulated siRNAs, and when injected into mice, were associated with a reduction in serum HBV DNA. This effect was dose-dependent and persisted for 1 week

after dosing. Likewise, treatment of guinea pigs with a pool of SNALP-formulated siRNAs targeting polymerase (L) gene of Zaire species of ebola virus (EBOV) completely protected the animals from death when administered shortly before EBOV challenge.<sup>[67]</sup>

#### Cationic Polymers

As with liposomes, the charged nature of siRNAs allows their complexation with various cationic polymers based on electrostatic interactions. Polymers used for delivery can be divided into two main categories: (i) those of synthetic origin, such as dendrimers, polyethylenimine (PEI), and poly-L-lysine (PLL); and (ii) those of natural origin that are biodegradable and more easily degraded and excreted from the body, such as atelocollagen (ATCOL), gelatine, chitosan, and cyclodextrin.<sup>[102]</sup>

#### Dendrimers

Dendrimers consist of a central core molecule out of which multiple arms of branched polymers project. The core molecule is referred to as 'generation 0' and each successive repeat unit along all branches forms the next generation, i.e. 'generation 1', 'generation 2', and so on until the terminating generation. Successive branches are created using a stepwise synthesis that allows particle size to be precisely controlled and, with each step of branch synthesis, the number of branches increases exponentially, causing an increase in polymer density.<sup>[102]</sup> This pattern creates a physically protected void within the macromolecule, which has chemical properties that differ from those of the surface and that can be exploited to host nucleic acids. The most well studied molecules are those based on ethylene diamine or ammonia cores with polyamidoamine (PAMAM) dendrites or those based on butylenediamide cores and polypropylenimine dendrites. Solubility of PAMAM dendrimers can be enhanced by partial acetylation of the reactive amino groups, which can then be conjugated with various targeting entities, such as folic acid to target receptors on specific cancer cells.<sup>[103]</sup> Although PAMAM dendrimers have been largely applied for delivery of plasmids and ODNs<sup>[104,105]</sup> and some studies have reported the use of monoclonal antibody-targeted dendrimers to target specific cells,<sup>[106]</sup> the latter strategy has not yet been shown to be effective for siRNA delivery. Kang and co-workers<sup>[107]</sup> reported the use of PAMAM dendrimers for delivery of siRNAs targeting multidrug resistance 1 protein (MDR-1) in NIH-3T3 MDR cells, but this strategy was not effective.

#### Polyethylenimine

PEIs with different molecular weights, degrees of branching and other modifications have been largely used for transfection

of siRNAs in different cell lines and animal models.<sup>[108,109]</sup> PEI consists of linear or branched alkyl chains interspersed with amines. The densely cationic nature of pure PEI can cause the polymer to be cytotoxic. Linear PEI usually shows higher transfection efficiencies and lower cytotoxicities than branched PEI. Grzelinski and colleagues<sup>[109]</sup> showed that the complexation of unmodified siRNAs with PEI leads to the formation of structures that condense around and completely cover siRNAs. These investigators reported that delivery of siRNAs against the growth factor pleiotropin complexed with PEI was able to generate antitumoral effects in an orthotopic mouse glioblastoma model with U87 cells growing intracranially. Urban-Klein and co-workers<sup>[108]</sup> showed that non-covalent complexation of synthetic siRNAs with low-molecular-weight PEI efficiently stabilizes siRNA and delivers it into cells where it can display full bioactivity at nontoxic concentrations. PEI polymers can be complexed to other molecules such as PEG and peptides, contributing to the PEI complex stabilization. Kim and collaborators<sup>[110]</sup> conjugated a prostate cancer-binding peptide with PEI via a PEG linker to deliver a VEGF small interfering molecule to human prostate carcinoma (PC)-3 cells. They reported an enhanced gene-silencing activity that was maintained even under serum conditions. Another example is the anti-VEGF siRNA/PEI-hyaluronic acid (HA) that achieved inhibition of tumor growth using the HA receptor-mediated endocytosis in tumor cells *in vivo*. These complexes can be successfully applied as specific antiangiogenic therapeutics for the treatment of diseases in tissues with HA receptors, such as liver and kidney.<sup>[111]</sup>

#### Poly-L-Lysine

The linear polypeptide PLL is able to effectively complex with nucleic acids using its many positively charged amino groups to form stable polyplexes. PLL alone is highly cytotoxic, but charge shielding of PLL with PEG mitigates the toxicity to make it a useful *in vivo* therapeutic gene-delivery agent.<sup>[102]</sup> As with PEI polymers, PLL can be complexed with other molecules being part of a micelle.

#### Atelocollagen

ATCOL was the first biomaterial introduced as a gene delivery system. It is generated through pepsin treatment of type I collagen of calf dermis, which removes the telopeptide immunogenic N and C terminal ends, reducing immunogenicity.<sup>[112]</sup> The size of the complexes formed between ATCOL and negatively charged nucleic acid molecules is determined by the ratio between the two components. The complexation with ATCOL has been shown to protect siRNAs and allow their *in vivo* delivery. Intratumoral injection of ATCOL/siRNA complexes

targeting the growth factor VEGF was performed in a prostate carcinoma xenograft model and resulted in decreased tumor growth and angiogenesis.<sup>[67,113]</sup> Suppression of tumor growth was also observed upon downregulation of the proteinase-activated receptor-2 (PAR-2) in Panc1 pancreatic carcinoma xenografts or in HPV18E6 and E7 cervical xenografts.<sup>[114]</sup> Additionally ATCOL-mediated local or systemic application of a siRNA targeting myostatin, a negative regulator of skeletal muscle growth, caused a marked increase in muscle mass within a few weeks after application in skeletal muscles of normal or diseased mice, implying that ATCOL-mediated application of siRNAs could be a powerful tool to treat diseases such as muscular atrophy.

#### **Gelatin**

Gelatin consists of a denatured collagen that has shown great promise both *in vitro* and *in vivo* as a delivery vehicle. Cationized gelatin nanoparticles are relatively simple to produce when compared with synthetic polymers and have been shown to display a transfection efficiency *in vitro* of approximately one order of magnitude less than PEI but to show 4-fold less cytotoxicity as well.<sup>[115]</sup> Cationized gelatin has been used to mediate vector-based RNAi in a murine model of obstructive nephropathy after intraureteral delivery.<sup>[115]</sup> Administration of a plasmid encoding siRNAs against the transforming growth factor- $\beta$  receptor gene resulted in a reduction of collagen content and fibrotic tissue in the kidney interstitium for up to 10 days after administration. Gelatin has proven to be an effective mediator of DNA vector-based RNAi in a NRS-1 squamous cell carcinoma murine xenograft model.<sup>[102]</sup> A vector encoding siRNA against VEGF was complexed to cationized gelatin microspheres and administered *in vivo*, showing effective knockdown, suppressed tumor growth and reduced vascularity.

#### **Chitosan**

Chitosan is a positively charged, natural, biodegradable polymer that shows high biocompatibility and low toxicity and immunogenicity. It is obtained by deacetylation of chitin resulting in a biodegradable polysaccharide composed of two subunits, D-glucosamine and N-acetyl-D-glucosamine. The physicochemical properties and the targeting efficacies of the chitosan-siRNA nanoparticles depend on the molecular weight and degree of deacetylation of the chitosan. Howard and colleagues<sup>[116]</sup> showed that chitosan-siRNA particles led to enhanced green fluorescent protein (EGFP) knockdown in bronchiole epithelial cells of transgenic mice after nasal administration of the complexes. Tan and collaborators<sup>[117]</sup> generated chitosan nanoparticles with encapsulated fluorescent quantum dots to deliver human epidermal growth factor 2

(HER2)/neu siRNA. Targeted delivery of HER2 siRNA to HER2 over-expressing cancer cells was shown to be specific when chitosan/quantum dot particles were surface-labeled with HER2 antibodies.

#### **Cyclodextrin**

Cyclodextrins are cyclic oligomers of glucose with an amphipathic structure, having a central hydrophobic cavity and a hydrophilic exterior that makes them water soluble.<sup>[102]</sup> These cyclic oligomers are used as carriers for small organic molecules, displaying high biocompatibility and low toxicity. Cyclodextrin-containing polycations (CDP) functionalized with transferrin to achieve preferential uptake into transferrin receptor-expressing tumor cells were employed in a murine model of Ewing's sarcoma, targeting the Ewing's sarcoma-Friend leukemia virus integration 1 (EWS-FLI1) gene product.<sup>[118]</sup> Similarly, a tumor growth reduction in Neuro2A tumor xenografts was reported after targeting of ribonucleotide reductase subunit 2 (RRM2). Non-targeted nanoparticles were significantly less efficient upon intravenous injection compared with their functionalized counterparts.<sup>[67,119]</sup> Recently published results have shown the first targeted delivery of synthetic siRNA in humans via a self-assembling cyclodextrin-based nanoparticle.<sup>[120]</sup> This study reported targeted *in vivo* delivery of a siRNA against RRM2, reducing the proliferative activity of a broad spectrum of human, mouse, rat, and monkey cancer types. Another RRM2 targeted nanoparticle formulation is CALAA-01, which in addition to the specific siRNA contains CDPs, PEG as a steric stabilization agent and transferrin. This four-component formulation is self-assembled into nanoparticles and administered intravenously to patients, in whom the nanoparticles circulate and localize to the tumors. The CDP contains organic groups that are protonated around pH=6. This chemical-sensing mechanism triggers a number of processes of escape mechanisms from endocytic vesicles and releases the nucleic acid into the cytoplasm. Each of the components of the formulation is small enough to be cleared from the body via the kidney after the nanoparticle has disassembled into its individual parts. CALAA-01 was used to treat the first patients in a phase I clinical trial in May 2008. The trial was a safety study treating adults with solid tumors who were refractory to standard-of-care therapies.<sup>[120]</sup>

### 4.4 Targeted Delivery

An important consideration to take into account for the therapeutic application of RNAi is the dosage of siRNA needed to achieve efficient silencing. An important disadvantage of systemic delivery systems is the large amount of siRNA that

needs to be administrated to achieve efficient *in vivo* gene silencing at the target site. Therefore, strategies that would facilitate cell-type-specific delivery could allow a reduction of the amount of siRNA needed and/or the number of doses to be administered. Selective ligands that bind cell-specific receptors expressed by target cells can be conjugated to polymers and cationic lipids in order to promote specific cell uptake via receptor-mediated endocytosis. This ligand-targeted delivery can be accomplished by direct attachment of the ligand to the siRNA moiety or by incorporation into the siRNA complexing formulation.<sup>[121]</sup> Several groups have exploited this specific delivery strategy. Schiffelers and collaborators<sup>[122]</sup> developed a tumor-selective delivery system where siRNA was complexed with PEGylated PEI and an arginine-glycine-aspartic acid (RGD) peptide was attached to the distal end of the PEG to target integrins expressed on the tumor neovasculature. This PEG-PEI-RGD system was used to deliver siRNAs inhibiting VEGF receptor 2 (VEGF-R2), improving serum stability compared with unformulated siRNAs. Alternatively, Kim and co-workers<sup>[123]</sup> developed an approach by which siRNAs silencing green fluorescent protein (GFP) were complexed in PEI-PEG nanoparticles functionalized with folate. These nanoparticles were efficiently targeted to GFP-transfected human epidermal carcinoma cells overexpressing the folate receptor, which is abundant in many cancers and frequently used for targeted drug delivery. Mannose receptors and mannose-related receptors are highly expressed in dendritic cells and can be used to target these cells.<sup>[124]</sup> Transferrin receptor, typically upregulated on cancer cells, has also been used to target siRNA-cyclodextrin-containing polycations to transferrin receptor-expressing tumor cells in the lungs,<sup>[118]</sup> in gastric cancers<sup>[125]</sup> and in Ewing's sarcoma.<sup>[120]</sup> Alternatively, aptamer-siRNA chimeric RNAs have been shown to be capable of cell-type-specific binding and delivery. McNamara and co-workers<sup>[126]</sup> have used an aptamer against prostate-specific membrane antigen (PSMA), a cell surface receptor overexpressed in prostate cancer cells and tumor vascular endothelium, to downregulate polo-like kinase 1 (PLK-1) and B-cell lymphoma-2 (BCL-2) in a xenograft model of prostate cancer, inducing tumor growth inhibition and tumor regression.

## 5. Targets Addressable by RNAi

### 5.1 Infectious Pathologies

#### 5.1.1 Viruses

Since the first report on RNAi-mediated inhibition of respiratory syncytial virus (RSV) in 2001,<sup>[127]</sup> several *in vivo*

proof-of-concept studies have shown that this technology will likely be a viable therapeutic alternative in the future. The limitations of current therapies for many viral infections and the availability of the genome sequence for many pathogenic viruses open the field for novel RNAi-based antiviral therapies. Viral genes that are essential for virus replication and host genes that are essential for virus entry or that play an essential role in the virus life cycle constitute attractive targets. Silencing of genes relevant to viral infection, such as HIV,<sup>[128]</sup> hepatitis,<sup>[129]</sup> and severe acute respiratory syndrome (SARS)-associated coronavirus,<sup>[130]</sup> has been achieved using RNAi.

HIV is the perfect example of a virus where immediate intervention is needed. Although significant success has been achieved with current antiviral therapies, their toxicity, complexity, cost, and, mostly, the appearance of drug resistances call for novel methods of intervention. It has been previously shown that silencing of the primary HIV receptor, chemokine (C-C motif) receptor 5 (CCR5), using siRNA results in the prevention of viral entry in human peripheral blood lymphocytes<sup>[131]</sup> and primary hematopoietic cells.<sup>[132]</sup> Since then, most HIV viral transcripts have been effectively targeted using RNAi. However, targeting single sequences might be hampered by viral escape mechanisms in the same way as other failed monotherapies. Alternative strategies can be developed to avoid this genetic variability of HIV that can lead to a decreased therapeutic effect of the RNAi-based agents. Several versatile strategies involving multiple RNAi effectors or other gene expression inhibitors, called combinatorial RNAi (coRNAi), have been suggested, e.g. targeting a single region of viral DNA with multiple shRNAs expressed from individual promoters or as concatemers from one promoter. Other strategies have been targeting several regions of viral DNA, or both viral and host cellular DNA, with constructs expressing multiple shRNAs from one or separate promoters, expressing a long hairpin RNA from one promoter to silence numerous regions on a single target, or using coRNAi vectors co-expressing shRNAs with other nucleic acid-based inhibitors such as aptamers, ribozymes, trans-acting response element (TAR) decoys or therapeutic proteins.<sup>[133]</sup> An example of this kind of therapeutic strategy is Benitec's RNAi-based anti-HIV candidate.<sup>[68]</sup> Their product mediates DNA-directed RNAi (ddRNAi) using a lentiviral vector containing three genes for an shRNA targeting the tat-rev exon of HI, an anti-CCR5 ribozyme and a nucleolar-localizing TAR decoy. The therapy is delivered to mobilized hematopoietic stem cells. Once stem cells are circulating peripherally, they are collected, isolated and genetically modified with the lentiviral vector expressing the therapeutic products. Patients undergo full chemoablation, the regenerative cells of

both the bone marrow and lymphoma cells are killed, and the treated stem cells are infused back into their bloodstream.

Most hepatitis B and C virus infections progress to chronic liver disease when the infections show poor responses to current therapies, and affected patients eventually develop cirrhosis and require liver transplants or develop liver cancer.<sup>[134]</sup> In hepatitis C, siRNA silencing led to a 98% reduction in detectable virus in infected cells.<sup>[135]</sup> Other studies have shown that by silencing a surface antigen region of hepatitis B virus using a siRNA, viral transcripts, viral antigens and viral genomic DNA were significantly reduced *in vivo*.<sup>[136]</sup>

Another example of a viral disease that could be tackled with an RNAi-based therapy is influenza A infection. New therapies for this infection are required every year, as these viruses change their viral determinants, giving rise to new virulent strains. Influenza A virus genome contains eight pieces of a segmented negative-sense RNA that codes for a total of 11 proteins: hemagglutinin (HA), neuroaminidase (NA), nucleoprotein (NP), M1, M2, NS1, NS2, PA, PB1, PB1-F2 and PB2. Separation of the genome into these segments of viral RNA allows for reassortment of viral RNA if more than one type of influenza virus infects the cells. The resulting rapid change in viral genetics produces antigenic shifts. These changes allow the virus to infect new host species. The WHO and the US Centers for Disease Control and Prevention have expressed major concerns about the potential for this virus to mutate and to be responsible for a global pandemic. In humans, current vaccines and existing antiviral agents may not protect against newly emerging strains of influenza. Additionally, most flu vaccines are manufactured using chicken egg-based systems, which return low yields and are not amenable to scaling up and would be inadequate to fight a pandemic flu. Despite the high mutation and recombination rate, new chemically synthesized and easily scalable RNAi-based drugs could be designed, and multiple specific siRNAs targeting the most conserved regions required for viral replication could be used with antiviral activity across multiple strains of flu.<sup>[137]</sup> Several pharmaceutical companies, such as Alnylam Pharmaceuticals and Novartis, are collaborating to develop RNAi-based drugs targeting pandemic influenza infections.

RSV is ubiquitous in the environment and is the common cause of bronchiolitis-associated hospitalization of children and immunocompromised adults. Monick and co-workers<sup>[138]</sup> showed that activation of extracellular signal-regulated kinase (ERK) via epidermal growth factor receptor (EGFR) occurs after an RSV infection, leading to pronounced inflammation and prolonged survival of infected cells.<sup>[138]</sup> Targeting EGFR with a specific siRNA resulted in apoptosis and resolution of inflammation. Additionally, Kong and colleagues<sup>[139]</sup> showed

that mice intranasally treated with siRNA nanoparticles targeting the viral NS1 gene before and after infection with RSV showed substantially decreased virus titers in the lungs and decreased inflammation and airway reactivity relative to controls.

Similarly, many other viral infections can be tackled using an RNAi-based approach. It is possible to induce apoptosis in primary patient tumor samples by targeting the E6 gene of human papillomavirus.<sup>[140]</sup> In the case of SARS, it has recently been shown that SARS-associated coronavirus replication can be efficiently inhibited using siRNAs against two viral polymerases.<sup>[130,141]</sup> Other remarkable examples of pathogens responsible for important human diseases and mortality are flaviviruses, including Dengue virus, Japanese encephalitis virus, yellow fever virus and West Nile Virus. There is currently no specific therapy available for any flavivirus infection and there are commercial vaccines for only three flaviviruses. All this evidence supports the idea that RNAi can be successfully used to combat viral diseases.

### 5.1.2 Bacteria

In contrast to viruses, bacteria are not generally amenable to silencing by siRNA because they replicate mostly outside the host cell. However, it might still be possible to reduce morbidity and mortality from life-threatening bacterial infections by silencing host genes involved in aspects of the immune response that lead to adverse consequences. For example, reducing the expression of proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), lessened septic shock in mice treated with lipopolysaccharide without jeopardizing the development of protective immunity.<sup>[142]</sup> Similarly, silencing host genes involved in mediation of bacterial invasion constitutes an interesting approach, e.g. silencing of caveolin-2 in murine lung epithelial cells inhibited invasion of *Pseudomonas aeruginosa* (the major pulmonary pathogen in cystic fibrosis patients) by a lipid raft-dependent mechanism.<sup>[143]</sup> Mycobacterial infections are very difficult to treat because of their characteristic extremely hard cell wall and because they are naturally resistant to all antibacterials that work by destroying cell walls. Moreover, these pathogens elude sterilizing immunity by residing in the intracellular compartment of host cells, where they are protected from microbicidal attacks. Therefore, mycobacterial infections such as tuberculosis are perfect candidates for treatment with RNAi-based therapeutics targeting host genes involved in mycobacterial invasion and growth inside the cells. The feasibility of using antisense therapies to treat *Mycobacterium tuberculosis* infection was proven in a study in which phosphorothioate-modified antisense oligodeoxyribonucleotides against glutamine synthetase transcripts were used.<sup>[144]</sup>

This enzyme is associated with mycobacterial pathogenicity and with the formation of poly-L-glutamate/glutamine cell wall structures. Therefore, reducing the activity of this enzyme would have a great impact on bacterial replication. One recent study also showed inhibition of mycobacterial growth by reduction of the lysosomal enzyme  $\beta$ -hexosaminidase, which is a peptidoglycan hydrolase that facilitates mycobacterial-induced secretion of lysosomes at the macrophage plasma membrane.<sup>[145]</sup>

### 5.1.3 Parasites

Protozoan parasites cause diseases of considerable medical and veterinary importance throughout Africa, Asia, and the Americas. The first report of RNAi in protozoan parasites was made in 1998 in Elisabetta Ullu's laboratory, where investigators found that dsRNA could induce sequence-specific mRNA degradation in *Trypanosoma brucei*.<sup>[146]</sup> Since then, RNAi has not only provided an invaluable tool for the study of *T. brucei* biology, but has additionally been tested as a therapeutic tool against *T. brucei* infection *in vivo*.<sup>[147,148]</sup> In a similar manner, *T. congolense*, the causative agent of Nagana disease in cattle, has also been shown to possess RNAi machinery.<sup>[149]</sup> The widespread resistance of common anti-malarial drugs is showing the needs of efficacious and innovative drugs and vaccines to fight *Plasmodium* parasites. During the latter part of the 20th century, there was an alarming increase in the number of cases of malaria reported in the Indian subcontinent, Southeast Asia, and South America.<sup>[150]</sup> Chloroquine remains the gold standard treatment for malaria today. However, chloroquine resistance is a growing concern.<sup>[151]</sup> The causative agent of disease, *Plasmodium falciparum*, is a member of the intracellular protozoan phylum *Apicomplexa*. While RNA-like silencing has been reported in *Plasmodium* parasites, it remains controversial whether this phenomenon actually takes place in this organism. Kumar and co-workers<sup>[152]</sup> silenced a serine-threonine protein phosphatase-1 (PP1) in the parasite and showed that this enzyme plays an essential role in its life cycle, therefore offering a potential target for drug development. The downside to this approach is that PP1 is highly conserved throughout evolution. Therefore, toxic side effects can be expected unless specific variants of the protein are identified.<sup>[153]</sup> Another protozoan parasite, *Entamoeba histolytica* causes human amebiasis. Once inside its host, the parasite invades the intestinal mucosa, causing dysentery, and travels through the circulatory system to the liver, where it causes development of abscesses. Vayssie and colleagues<sup>[154]</sup> achieved a specific and efficient silencing of  $\gamma$ -tubulin mRNA, resulting in loss of the parasites' highly organized microtubule array. These

results showed that  $\gamma$ -tubulin is essential for microtubule nucleation and cycling of the parasite. Importantly, the protein primary amino acid sequence is homologous (46%) but not identical to its human homolog. Therefore, specific siRNAs may be developed to destroy the parasite's  $\gamma$ -tubulin while leaving the host's counterpart untouched.<sup>[153]</sup>

## 5.2 Genetic Pathologies Associated with Mutations

The discovery of new disease-causing mutations in the genome identifies a number of possible therapeutic targets. Sequence aberrations can potentially be used to selectively target mutated transcripts associated with disease. RNAi has enormous potential for the treatment of many genetic and acquired diseases.

The use of RNAi-based therapeutics is especially appealing, as RNAi can be used to reduce the levels of toxic gain-in-function proteins, to inhibit the expression of disease-associated alleles without suppression of expression of wild-type alleles, and to target single-base mutation diseases, missense mutation diseases, single nucleotide polymorphisms and dysregulation of splicing process mutations associated with some genetic disorders. RNAi can also be used to modulate the expression of proteins not normally accessible by more traditional pharmaceutical approaches, e.g. those that lack ligand-binding domains or those that share a high degree of structural homology, both of which are difficult to target as individuals.<sup>[155]</sup>

### 5.2.1 Cis-Acting Mutations that Disrupt Splicing Processes

A large number of exonic mutations that result in aberrant splicing have been documented and could be good targets for a siRNA-based therapy.<sup>[156,157]</sup> A striking example of the detrimental effect that mutations in exonic splicing signals can have is the nucleotide substitution in the massive (2.4 million base pairs) dystrophin gene. Duchenne muscular dystrophy (DMD) is a severe progressive neuromuscular disorder caused by several different mutations, usually loss-of-function mutations. While >65% of DMD mutations are genomic deletions, a large number of exonic and intronic point mutations can cause the disease through aberrant splicing that abolishes the production of the functional protein. Dystrophin is positioned at the cytoplasmic side of the skeletal muscle sarcolemma, where it communicates signals between the extracellular matrix and the cellular contractile apparatus and stabilizes the cell membrane. A particularly revealing T  $\rightarrow$  A substitution in exon 31 not only creates a premature termination codon, but also introduces an exonic splicing silencer that binds to heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), resulting in partial

skipping.<sup>[158]</sup> Interestingly, mRNAs coding for one spectrin-like repeat but retaining the correct reading frame produce a partially functional protein, explaining why an individual harboring this mutation has a milder form of the disease.<sup>[159]</sup> Recent studies show a novel strategy for the treatment of this pathology employing steric block methylphosphorothioate antisense oligoribonucleotides (2'-O-MeAO)<sup>[160]</sup> or morpholino oligomers conjugated with a dendrimeric octoguanidine (Vivo-Morpholinos).<sup>[161]</sup> These studies show how a local administration of a specific 2'-O-MeAO in the dystrophic mouse model bearing a nonsense mutation or a Vivo-Morpholino systemic delivery, respectively, can effectively skip the mutated exon in the dystrophin gene, creating a shorter but in-frame transcript that is translated and leads to near-normal dystrophin expression in both skeletal and cardiac muscles.

### 5.2.2 Single-Base and Missense Mutations

A siRNA therapeutic approach has been applied in a number of contexts, including dominantly inherited and untreatable single-base mutations that lead to neurodegenerative diseases, such as Alzheimer's disease (AD). In recent studies by Miller et al.,<sup>[162]</sup> amyloid precursor protein (APP) and tau genes were chosen as candidate RNAi targets because of their central role in inherited and acquired forms of age-related dementia. AD is characterized by two major pathological hallmarks: senile plaques, which contain  $\beta$ -amyloid ( $A\beta$ ) derived from cleavage of APP, and neurofibrillary tangles, which contain filamentous tau proteins. It is well known that  $A\beta$  production plays an essential role in the pathogenesis of all forms of AD, both in sporadic and inherited forms. Mutations in three genes known to cause familial AD, the genes encoding APP, presenilin 1 and presenilin 2, act dominantly to enhance the production of neurotoxic  $A\beta$ . The best studied AD mutations are the well characterized V337M mutation in the microtubule-associated protein tau (*MAPT*) gene and the Swedish double mutation in APPsw, in which two consecutive missense changes alter adjacent amino acids near the  $\beta$ -secretase cleavage site. Both have been used as target models for the design of allele-specific siRNAs. These siRNAs displayed successful and optimal allele-specific silencing against mutant tau and APP alleles.<sup>[162]</sup> Other single-nucleotide APP substitutions followed by amino acid substitutions (V717I, V717L, V717G) have been successfully targeted by a forked-siRNA (F-siRNA) approach, showing a high allele-specific gene silencing.<sup>[163]</sup> Mutations within and downstream of the alternatively spliced exon 10 of the *MAPT* gene encoding the tau protein, disrupting the 1:1 ratio of mRNAs that include or exclude this exon, have also been found. Exon 10 encodes the fourth of four repeated microtubule-

binding domains (R) and disruption of the balance between the four repeat microtubule binding domain (4R-tau) and the three repeat microtubule binding domain (3R-tau) isoforms results in hyperphosphorylation and aggregation of tau proteins in neurofibrillary tangles that are hallmarks of several neurodegenerative diseases such as AD. Numerous mutations within and around *MAPT* exon 10 disrupt exonic and intronic splicing elements and cause the inherited neuropathological disorder frontotemporal dementia with parkinsonism linked to chromosome 17 (FTPD-17), demonstrating a direct relationship between aberrant expression due to alternative splicing disruption and neuropathology.<sup>[164]</sup> Miller and collaborators<sup>[165]</sup> successfully targeted four missense tau mutations that are responsible for FTPD-17. They employed a series of 21-24 nucleotide siRNAs designed against *MAPT* G272V, P301L, V337M and R406W mutations. Their results demonstrate how siRNAs can be engineered to silence expression of disease alleles differing from wild-type alleles by as little as a single nucleotide.

### 5.2.3 Single Nucleotide Polymorphisms

A dominantly inherited and untreatable neurodegenerative disease is the polyglutamine (poly Q) neurodegeneration in Machado-Joseph disease spinocerebellar ataxia type 3 (MJD/SCA3). This poly Q neurodegenerative disorder consists of at least nine diseases caused by CAG-repeat expansions that encode poly Q in the diseased protein. Poly Q expansion confers a dominant toxic property on the mutant protein that is associated with aberrant accumulation of the disease protein in neurons. To selectively inactivate the mutant allele, Miller and co-workers<sup>[165]</sup> took advantage of a single nucleotide polymorphism (SNP) in the *MJD1* gene, a G to C transition immediately 3' to the CAG repeat (G987C). This SNP is in linkage disequilibrium with the disease-causing expansion, in most families segregating perfectly with the disease allele. Worldwide, 70% of disease chromosomes carry the C variant. To optimize differential suppression, siRNAs containing a centrally placed mismatch were designed. Central mismatches might discriminate between wild type and mutant alleles. Designed siRNAs placing the C of the SNP at position 10 (siC10) preceded by the final three triplets in the CAG repeat caused allele-specific suppression of the mutant protein.<sup>[165]</sup> Other RNAi approaches are showing promise for the treatment of several other neurodegenerative diseases, including Huntington's disease, spinocerebellar ataxia and amyotrophic lateral sclerosis (ALS).<sup>[70,166]</sup>

Another SNP-associated pathology is sickle cell anemia. This pathology is an inheritable blood disease caused by a single

nucleotide mutation in the human  $\beta$ -globin (*HBB*) gene (codon 6 GAG→GTG [Glu→Val]), creating a hemoglobin variant that polymerizes upon deoxygenation to produce long fibers and distorts red blood cell shape, leading to a reduction in red blood cell flexibility that impairs their transit through the microvasculature. Individuals homozygous for this mutant  $\beta$ -globin allele (*HBB* Glu6Val) have severe clinical symptoms. The abnormal hemoglobin produced by this gene, termed sickle cell hemoglobin or  $\beta$ S-globin, can polymerize under low oxygen tension and change red blood cell rheology and shape, potentially resulting in vaso-occlusive crisis, infarction, and organ damage. A significant reduction in mouse adult  $\beta$ -globin mRNA by  $\beta$ S-globin-specific siRNA has been reported in murine erythroleukemia cells.<sup>[167]</sup>

#### 5.2.4 Protein Fusions

Several tumorigenic processes emerge as a result of molecular alterations, point mutations and translocations that lead to the generation of a novel chimeric fusion protein. These proteins are the consequence of the in-frame joining of two genes to generate unique fusion proteins with a novel function.<sup>[168]</sup> The mixed-lineage leukaemia (MLL) family of oncogenic fusion proteins represents one class of such deregulated transcriptional regulators in acute leukemia.<sup>[169]</sup> Other examples are EWS-FLI1 fusion proteins in Ewing's sarcoma, EWS-Wilms tumor 1 (WT1) in small round cell desmoplastic tumor, EWS-activating transcription factor 1 (ATF1) in clear cell sarcoma,<sup>[170]</sup> EWS-myxoid chondrosarcoma (CHN) in extra-skeletal myxoid chondrosarcoma<sup>[171]</sup> and FUS-CHOP in round cell myxoid liposarcoma.<sup>[172]</sup> All these fusion proteins could be excellent addressable targets for RNAi-based therapies because they are present only in cancer cells and not in normal tissues. siRNAs could be directly designed against the junction point in the fusion protein transcript, inhibiting the synthesis of the fusion protein. There are several groups that have applied RNAi technology *in vitro*<sup>[118,173]</sup> and *in vivo*<sup>[174]</sup> as a new approach to the treatment of these types of cancers with junction oncogenes.

### 5.3 MicroRNAs

miRNAs are short non-protein coding RNAs ~22 nucleotides in length that are known to alter gene expression at a post-transcriptional level. They are integral components of the genetic program that account for approximately 1–5% of the predicted transcripts in plants, worms and vertebrates, and their genes are localized in the introns of protein-coding genes

or in the non-coding regions of the genome.<sup>[175]</sup> Interest in the role of miRNAs in the regulation of fundamental biological processes emerged rapidly following the discovery of the first miRNA, lin-14, in *C. elegans* a decade ago.<sup>[14]</sup> Distinct classes of miRNAs have been identified as key regulators of gene activation and suppression and they are highly specific for different tissues and developmental stages. Their functions have been appreciated in fundamental biological processes and cellular functions, such as cell proliferation, stem cell division, cell differentiation, stress response, apoptosis, immunity and transcriptional regulation. miRNA sequences are freely available to all through the web interface at <http://microrna.sanger.ac.uk/>.<sup>[176]</sup> Actually, more than 8000 unique precursor and mature miRNAs have been identified and tabulated in the last miRNA base registry (release 12.0), including those in primates, rodents, birds, fish, worms, flies, plants, and viruses. Given the remarkable spectrum of biological pathways regulated, at least in part, by miRNAs, it is not surprising that abnormal miRNA signatures have been identified in disease states and that they may be valuable diagnostic and/or prognostic markers for disease. Furthermore, modulation of their activity may be of therapeutic benefit.<sup>[177]</sup> Recent use of synthetic analogs of these small RNA molecules, termed 'antagomirs', has shown that genes of interest can be specifically targeted.

Multiple steps in the miRNA processing pathway could be targeted to achieve inhibition of miRNA production or function. Targeted degradation of the pri-miRNA transcripts in the nucleus with an RNase H-based antisense oligonucleotide (ASO) may be feasible and could constitute an interesting approach to inhibition of the production of multiple miRNAs from polycistronic pri-miRNA transcripts. RNase H recognizes RNA-DNA duplexes, cleaving the RNA strand. siRNAs targeting the pri-miRNA are not likely to be effective, as siRNAs loaded into the RISC work primarily in the cytoplasm, where the pri-miRNA substrate is inaccessible. Alternatively, targeting the loop of the hairpin with an RNAase H ASO or siRNA may be possible. Indeed, several investigators have developed chemically engineered single- and double-stranded oligonucleotides as specific silencers of miRNA expression.<sup>[178,179]</sup> Krutzfeldt and co-workers<sup>[178]</sup> designed a cholesterol-conjugated single-stranded RNA molecule of 21–23 nucleotides complementary to the mature miRNA miR-122. They specifically silenced miR-122 in the liver, lungs, intestine, heart, skin, and bone marrow for more than a week after a single intravenous injection. As a result, up- and downregulation of hundreds of genes regulated by this miRNA was achieved. Davis and colleagues<sup>[179]</sup> extended these



studies and highlighted how inhibition of miRNAs may be a possible therapeutic approach to the treatment of disease. They reported inhibition of miR-122 in both normal and high-fat-fed mice with a 2'-O-methoxyethyl (2'-MOE) phosphorothioate modified antisense oligonucleotide for over 5 weeks. A significant reduction in hepatic sterol and fatty-acid synthesis rates and stimulation of hepatic fatty-acid oxidation was observed. These results suggest that miR-122 is a key regulator of cholesterol and fatty-acid metabolism and that it could be a therapeutic target for metabolic and cardiovascular diseases.<sup>[179]</sup> Additionally, miR-122 contributes to the liver tropism of hepatitis C virus (HCV), accelerating binding of ribosomes to the viral RNA and thereby stimulating HCV translation. The involvement of miR-122 in HCV infection reveals how this viral factor is a potential target for RNAi and miRNA-based antiviral strategy and a possibly very important and novel therapy for HCV infection.<sup>[180]</sup> Several pharmaceutical companies, such as Regulus Therapeutics and Santaris Pharma, are developing a portfolio that is built on miRNA biology, with different miRNAs being targeted. This is evidence that a new kind of therapeutic field is emerging.

## 6. Advantages of RNAi-based Therapeutics

Prior to the discovery of RNAi in 1998, nucleic acid-based antisense technologies for sequence-specific inhibition of gene expression had been used for a number of years. These antisense-based approaches presented the great advantage of allowing gene silencing of virtually any target with very high selectivity and specificity through Watson-Crick base pairing. With the completion of the Human Genome Project in 2003 and the identification of approximately 20 000–25 000 genes in human DNA, the possibilities of therapeutic intervention became enormous as, theoretically, any disease-associated gene should be amenable to antisense-mediated suppression.

Compared with other antisense strategies, however, such as antisense DNA oligonucleotides and ribozymes, RNAi is considerably more potent,<sup>[181]</sup> which means it may function at much lower concentrations. In fact, it may require only a few molecules of dsRNA to cause gene silencing. Additionally, the potency of some chemically modified siRNAs and miRNAs may be even higher. This has broad implications in a clinical setting, as it would mean less frequent and/or lower doses, increasing patient compliance and decreasing the risk of adverse effects. siRNA are also natural cellular components, meaning cells should have the capacity to handle breakdown products,

thus reducing their toxicity. Development of siRNA-based drugs also requires a shorter pharmacological development than that of conventional drugs (2–3 vs 4–6 years since proof of concept). siRNA compounds are made from strands of RNA that are manufactured by an RNA synthesizer, which greatly facilitates large-scale production. Being produced by a chemical synthesis process, they do not fall into the category of biologics, which has several important advantages from a regulatory point of view.

On the other hand, delivery of siRNAs is technically difficult to achieve. Although advances have been made in this area, much work still remains to be done before the full therapeutic potential of these applications can be fully exploited. In a clinical intervention program, RNAi has the limitation that it can only be used to treat pathologies caused by the expression or overexpression of a given protein or by the presence of exogenous organisms, as its mechanism works through suppression of protein expression, i.e. RNAi will only confer a therapeutic advantage in situations in which amelioration of the disease can be achieved by loss-of-function. Furthermore, even though any gene target could potentially be silenced using RNAi, in practice some genes are harder to target than others. Newly designed algorithms will need to be developed in order to address this issue. Finally, the off-target effects of siRNAs targeting unwanted genes could lead to safety issues in the clinical situation.

## 7. The Road to Clinical Intervention

Different strategies of gene expression inhibition have been used in many therapeutic applications, among which is the treatment of ocular diseases. Indeed, intraocular delivery of nucleic acids has attracted much interest in recent years.

The first antisense drug to achieve marketing clearance was fomivirsen (Vitravene™), developed by Isis Pharmaceuticals (Carlsbad, CA, USA). This drug was approved in 1998 for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS, which if left untreated, can lead to blindness.<sup>[182]</sup> Its worldwide commercial rights were licensed to Novartis Ophthalmics (formerly CibaVision). Fomivirsen is 21 nucleotides long and blocks the translation of viral mRNA by binding to a coding segment of a key CMV gene<sup>[183,184]</sup> that is administered as an intraocular injection. Antisense-mediated reduction of CMV proteins limits viral replication and inhibits adsorption of CMV to host cells, possibly by interaction with virions that may prevent adsorption or uncoating.<sup>[184,185]</sup> Its approval was important to antisense technology because it demonstrated that

antisense drugs were effective in the treatment of local disease, that they could gain marketing approval by regulatory agencies around the world and that antisense drugs could be manufactured for commercial use. Although this drug changed the course of the disease, it can hardly be considered as commercially successful; it is offered only on a limited basis and sales have been extremely small as the number of HIV-infected individuals with CMV retinitis has declined with the appearance of new anti-HIV drugs (particularly protease inhibitors and combination treatment regimens). In addition, fomivirsen was shown to induce ocular inflammation as one of its major adverse effects.

The first siRNA drug, bevasiranib (Opko Health, Miami, FL, USA; formerly Cand5 from Acuity Pharmaceuticals), is a siRNA that shuts down the genes that produce VEGF, which stimulates blood vessel overgrowth and regulates blood vessel permeability. A phase II study demonstrated the safety of the product and that a single dose of bevasiranib safely and significantly reduced both neovascularization and vessel leakage, in a dose-dependent manner, for >5 weeks in patients with wet age-related macular degeneration (AMD). Recently, Opko Health decided to terminate its phase III trial of bevasiranib for the treatment of wet AMD following a review of preliminary trial data that showed that, although bevasiranib showed activity when used in conjunction with ranibizumab, the trial was unlikely to meet its primary endpoint.

AGN-745 (Allergan, Irvine, CA, USA; formerly Sirna-027 from Sirna Therapeutics/Merck) is a chemically modified siRNA against VEGF receptor-1 (VEGFR-1). VEGFR-1 is found primarily on vascular endothelial cells and is stimulated by both VEGF and placental growth factor (PGF), resulting in the growth of new blood vessels. By targeting VEGFR-1, AGN-745 is designed to shut down activation of pathologic angiogenesis initiated by both VEGF and PGF. AGN-745 showed promising results in a phase I study that evaluated safety, tolerability and biological effect of single-ascending doses of AGN-745 in patients with AMD but Allergan has recently halted its development after the drug failed to meet a key efficacy endpoint in a phase II study. Although AGN-745 had initially held promise as the second RNAi drug to enter human testing, Allergan's decision to shelve the drug was not entirely unexpected in light of a recent report on the immunostimulatory nature of siRNAs<sup>[186]</sup> in which Jayakrishna Ambati's group showed that siRNAs at least 21 nucleotides in length, including AGN-745 and bevasiranib, suppressed neovascularization in mouse models of choroidal and dermal neovascularization, regardless of their sequence and targets, by triggering TLR3. However, even if AGN-745 or bevasiranib had proven effective in the clinic, the drugs were expected to

face difficulties in attempts to share the AMD market with ranibizumab, given the strong safety and efficacy record of the latter. Indeed, Alnylam Pharmaceuticals had been developing its own VEGF-targeting siRNA drug, ALN-VEG01, but in fall 2005, the company dropped this program in light of the growing competition and decided to focus its efforts on RSV. Despite the loss of these three drug candidates for AMD, it appears that the target, rather than the indication, may have been the main issue. The real value of siRNAs is tackling targets that are not accessible through antibodies or small molecules; aiming at VEGF loses the real benefit of a siRNA approach.

Meanwhile, Quark Pharmaceuticals continues to move forward with its own RNAi-based AMD treatment. PF-4523655 (RTP801i-14) is a synthetic, siRNA molecule designed to inhibit the expression of Quark Pharmaceutical's proprietary target, RTP801. The product candidate is licensed to Pfizer on an exclusive worldwide basis. Results from a phase I/II trial showed that PF-4523655 was well tolerated in patients with wet AMD. Following successful completion of phase I clinical studies, PF-4523655 is currently in two phase II studies for diabetic macular degeneration and AMD.

The lungs are also a prime target for RNAi-based therapeutics because inhaled drugs can reach the respiratory system directly. Alnylam Pharmaceuticals is developing ALN-RSV01, a nasal formulation aimed at the treatment of RSV infection. RSV is highly contagious and causes infections in both the upper and lower respiratory tracts. Infection typically results in cold-like symptoms but can lead to more serious respiratory illnesses and, in extreme cases, death. ALN-RSV01 was designed to target the nucleocapsid 'N' gene of the RSV genome, a gene that is required for RSV replication, thereby reducing the virus' ability to reproduce. In early 2008, Alnylam Pharmaceuticals Inc. presented data from the phase II GEMINI study, which showed a statistically significant decrease in infection rate in adults experimentally infected with RSV, demonstrating that ALN-RSV01 has antiviral activity in a disease setting.<sup>[187]</sup> In ongoing expanded phase II clinical trial is aiming to further extend understanding of the safety, tolerability, and antiviral activity of ALN-RSV01 in naturally infected patients.

Acute renal failure (ARF), also known as acute kidney injury (AKI), is a rapid loss of renal function due to damage to the kidneys that can lead to death and for which there currently is no effective therapy. Phase II clinical trials are underway to evaluate the efficacy of AKIi-5 for the treatment of ARF. AKIi-5, developed by Quark Pharmaceuticals (Fremont, CA, USA), targets the tumor protein p53 (*TP53*) gene, thus delaying apoptosis of damaged kidney tubular cells and enabling the activation of natural repair mechanisms.

Therefore, RNAi-mediated therapeutics, such as those mentioned above and those included in table I, are currently being tested for the treatment of several diseases relating to the aberrant expression of gene expression. In addition, these and other pharmaceutical companies summarized in table II are developing preclinical programs for the use of RNAi-based compounds to treat a wide range of diseases. RNAi programs are carefully moving forward and RNAi-based therapies are establishing themselves as a novel class of therapeutic intervention.

## 8. Challenges Ahead

There are several challenges that need to be addressed if a siRNA-based therapy is to be widely used, e.g. siRNA design, improvement of siRNA stability and the development of good delivery systems. Unmodified naked siRNAs are rapidly degraded by endo- and exonucleases, resulting in short half-lives *in vivo*. As discussed in section 3, chemical modifications can be introduced in the RNA structure to enhance their biological stability without adversely affecting gene-silencing activity. However, good delivery systems are considered by many to be the most important remaining hurdles to the widespread therapeutic use of RNAi-based compounds. Selection and formulation of siRNAs with appropriate biocompatible and 'genocompatible' delivery systems is necessary to improve biological stability, targeted cell uptake and the pharmacokinetics of siRNAs in the organism.<sup>[188]</sup> Delivery of a drug to its target is as important as the efficacy of the drug itself. Failure to reach the target means failure to provide a therapeutic effect. Multifunctional delivery systems must be implemented to accomplish many tasks at the right place and at the right time. This integration of numerous functions in a delivery system successfully improves the therapeutic efficacy of siRNA-based drugs. Different design criteria must be considered. A complete delivery system must be adaptable, meaning it should be able to be modified in response to new mechanistic information, yielding bioactive molecules with easily modifiable surfaces, well defined structure and controllable sizes, thereby ensuring reproducible pharmacokinetics and pharmacodynamics. A good delivery system should also facilitate enhanced penetration and retention of siRNAs into specific cells and/or tissues. All of the delivery formulation components must be scalable at current good manufacturing practice (cGMP) conditions to facilitate large-scale production. It would also be necessary to develop non-immunostimulatory formulations that would enable repeated dosing if necessary. The formulation should additionally allow complete siRNA encapsulation to protect it

from nucleases and recognition by TLRs on the cell surface. Moreover, all system components should be non-toxic and of such a size that would allow them to be cleared via the kidneys when the delivery systems are disassembled. Finally, an optimal delivery system should be achieved without compromising siRNA gene-silencing activity and specificity.

Another major issue to be studied for the use of oligonucleotides in the clinic is toxicity. Toxicity by oligonucleotide-based therapeutics can be induced through hybridization in two ways: excessive silencing of the intended gene product or reduction in expression of an unintended target by an antisense mechanism. The first type of toxicity can usually be avoided by selecting gene products that are not critical for survival and, in any case, can usually be easily detected at an early preclinical stage. The second type of toxicity is based on the low probability of the short RNAi sequence having a perfect or almost perfect match with more than one site in the genome. This eventuality can be minimized by performing complete BLAST searches for matches at other sites of the transcriptome and selecting unique sequences for the drug candidates. Nevertheless, the potential for unintended OTEs is probably greater than initially expected. As mentioned in section 2.2, in any siRNA two different strands are being administered, the sense and antisense, and both are potentially able to elicit unwanted effects. Additionally, since a perfect match is needed only in the so-called seed region, this allows for many more potential binding sites than if full complementarity was required. Therefore, these issues should be fully addressed with appropriate and complete toxicity studies.

To add a further level of complexity, many and perhaps most of the toxicities elicited by oligonucleotides are related to hybridization-independent effects. These class effects are related to the chemical characteristics of the compounds, and many are known to be related to interactions of the oligonucleotides with proteins, inducing toxicities such as prolongation of the activated partial thromboplastin time, activation of complement and immunostimulation. Class effects can also be sequence dependent, inducing stimulation of innate immunity (reviewed in Levin and Henry<sup>[189]</sup>).

Many groups have found there are striking differences between toxicity profiles in rodents and non-human primates. Some of these differences are due to the biological differences between species. For example, the pattern of TLR expression is different in rodents and humans (TLR9 is not expressed in human myeloid cells, TLR8 is lacking in mice, etc.). Additionally, in many cases there are also important species-dependent sequence differences, such that an oligonucleotide-based drug perfectly complementary to a human target may not hybridize

**Table I.** RNA interference (RNAi) drugs in preclinical development

Company	Name	Disease	siRNA target	Stage
Alnylam Pharmaceuticals	ALN-PCS	Hypercholesterolemia	PCSK9	Preclinical
	ALN-TTR	TTR amyloidosis	TTR	Preclinical
Alnylam Pharmaceuticals/Medtronic	ALN-HTT	Huntington's disease	Huntingtin	IND application submitted 2009
Alnylam Pharmaceuticals/Novartis	—	Pandemic influenza	—	Preclinical
Alnylam Pharmaceuticals/Biogen	—	Progressive multifocal leukoencephalopathy	—	—
Benitec	—	AIDS	rHIV7-sh1-TAR-CCR5RZ	Preclinical
Benitec/Tacere Therapeutics	—	Hepatitis C	—	Preclinical
Calando Pharmaceuticals	CALAA-02	Solid tumors	HIF-1 $\alpha$	Preclinical
Cequent Pharmaceuticals	CEQ501	Familial adenomatous polyposis	$\beta$ -Catenin	Preclinical
Gradalis	—	Cancer	Stathmin-1	Preclinical
MDRNA	—	Hepatocellular carcinoma	—	Preclinical
OPKO Health	ACU-HHY-011	Wet age-related macular degeneration Diabetic macular edema	HIF-1 $\alpha$	Preclinical
	ACU-XSP-001	Allergy and inflammation	Syk kinase	Preclinical
	ACU-HTR-028	Wound healing antifibrotic	TGF- $\beta$ RII	Preclinical
Quark Pharmaceuticals	QPI-1007	Non-arteritic anterior ischemic optic neuropathy	—	Pre-IND application
	AHLi-11	Acute hearing loss	—	Preclinical
	—	Lung cancer/lung metastasis	—	Preclinical
	—	Acute lung injury/lung transplantation	—	Preclinical
	—	Acute and chronic neurodegenerative disease	—	Preclinical
Roche/Alnylam Pharmaceuticals	—	Liver-associated metabolic disorders	—	Preclinical
	—	Respiratory disease	—	Preclinical
	—	Cancer	—	Preclinical
RXi Pharmaceuticals	—	Rheumatoid arthritis	—	Preclinical
	—	Atherosclerosis	—	Preclinical
	—	Inflammatory bowel disease	—	Preclinical
	—	Hypercholesterolemia	—	Preclinical
	RIP140	Obesity Type 2 diabetes mellitus	—	Preclinical
Silence Therapeutics	Atu-111	Prostate cancer	—	Preclinical
	Atu-150	Liver cancer	—	Preclinical
Sirnaomics	STP-601	Ocular neovascularization diseases	—	Preclinical
	STP-702	Pandemic influenza	—	Preclinical
	STP-705	Wound healing	—	Preclinical
	STP-801	Organ transplant	—	Preclinical
	STP-503	Solid tumors	—	Preclinical
Sylentis	SYL040012	Ocular hypertension/glaucoma	$\beta$ 2 adrenergic receptor	Preclinical

Continued next page

Table I. Contd

Company	Name	Disease	siRNA target	Stage
Tacere Therapeutics/Oncolys Biopharma/Pfizer	TT-033/OBP-701	Hepatitis C infection	—	Preclinical
Tekmira/Alnylam	PLKSNALP	Cancer Solid tumors	PLK1	Preclinical
	ApoBSNALP	High-density lipoprotein	ApoB	Preclinical
ZaBeCor	Excellair	Asthma Allergy Lung inflammatory conditions Rheumatoid arthritis Systemic lupus erythematosus Cystitis Conjunctivitis Parkinson's disease Atherosclerosis	Syk kinase	IND application approved

**ApoB**=apolipoprotein B; **HIF-1 $\alpha$** =hypoxia-inducible factor-1 $\alpha$ ; **IND**=investigational new drug; **PCSK9**=proprotein convertase subtilisin/kexin type 9; **PLK1**=polo-like kinase 1; **rHIV7-sh1-TAR-CCR5RZ**=recombinant HIV-sh1-trans-acting response element-chemokine (C-C) motif receptor 5 ribozyme; **siRNAs**=small interfering RNAs; **TGF- $\beta$ RII**=transforming growth factor- $\beta$  receptor type II; **TTR**=transthyretin. '—' indicates that the information is not available.

with the corresponding mRNA in the animal species used in toxicity studies. In such cases, it might be appropriate to design and synthesize a surrogate oligonucleotide species-specific drug that is complementary to the mRNA for the target gene in the relevant animal species; however, ultimately, it will be critical to evaluate the immunological properties of candidate nucleic acid drugs in human primary immune cells. From what is known about the synthetic process and what is understood about synthesis-related impurities, we can predict that species-specific oligonucleotides will have similar chemistry manufacturing and controls as the sequence used in clinical trials. These animal sequence surrogates could be tested alongside the human sequence in toxicity studies to establish if there are biologic effects due to excessive silencing of the target gene and whether possible toxicities in rodents, such as immunostimulation, would be clinically relevant in humans. This concept has been part of the toxicity profiling of antisense drugs for the last decade.<sup>[190,191]</sup> There are cases when surrogate drugs are not needed simply because the target does not exist or has a completely different function in the species in which the toxicity studies will be performed. Such is also the case when the target mRNA is derived from an infectious agent not present in the host.

Another important consideration is what to do when the drug's oligonucleotide sequence has only a few mismatches between the human mRNA target and the corresponding animal species that will be used in the toxicology studies. If there are one or two mismatches near the termini of the siRNA or if

these mismatches are not within the seed sequence, it is possible that there would still be a silencing effect, i.e. the degree of activity will largely depend on the placement of the mismatches and might largely be target specific. The general recommendation in such cases is to test whether slight differences in sequences are reflected in the activity of the compound. If this is not the case, we believe there would be no need to use surrogate sequences to characterize unwanted effects due to excessive pharmacologic effects. On the other hand, what if these few mismatches do have a great impact on the activity of the compound? Would it be preferable to change those few mismatches into the nucleotides that will confer full sequence complementarity and run the toxicology studies with such a surrogate only? Or should a completely new species-specific sequence be designed and analysed for toxicity effects in parallel with the human-specific one? The answer to these questions will have a broad impact on the candidate sequence choice because development process costs will increase dramatically if full toxicology studies are needed with the human-specific and surrogate sequences in parallel. The scope of the toxicity studies when surrogate molecules have been used includes pharmacology studies, subchronic and chronic studies, reproductive toxicity studies, immunotoxicity studies, and even carcinogenicity studies.<sup>[189]</sup> Short-term assays such as safety pharmacology studies are too short for there to be an antisense effect, so have not been widely performed in the case of surrogates, except when safety pharmacology endpoints are included in chronic and subchronic studies.

**Table II.** RNA interference (RNAi) drugs in clinical development

Company	Name	Disease	Target	Stage	Comments	Status
Alnylam Pharmaceuticals/Kyowa Hakko Kirin/Cubist Pharmaceuticals	ALN-RSV1	RSV infection	Nucleocapsid (N) gene of RSV genome	Expanded phase II	Safety, tolerability, antiviral activity evaluation	Ongoing
Alnylam Pharmaceuticals	ALN-VSP	Liver cancers and solid tumors	Kinesin spindle protein and VEGF	Phase I	Safety, tolerability, pharmacokinetics and pharmacodynamics evaluation	Initiated 2009
Benitec	—	AIDS, lymphoma	rHIV7-shI-TAR-CCR5RZ	Phase I	—	Ongoing
Calando Pharmaceuticals	CALAA-01	Cancer and solid tumors	M2 subunit of ribonucleotide reductase	Phase I	Tolerability, safety profile, maximum tolerated dose evaluation	Ongoing
OPKO Health	Bevasiranib	Wet age-related macular degeneration	VEGF	Expanded phase III	Dose ranging, safety, efficacy evaluation	Under review
	Bevasiranib	Diabetic retinopathy, diabetic macular edema	VEGF	Phase II	Dose ranging, safety, efficacy evaluation	Completed
Quark Pharmaceuticals/Pfizer	PF4523655/RTP-801i-14	Wet age-related macular degeneration	RTP801	Phase II	Dose ranging, safety, efficacy evaluation	Ongoing
	PF4523655/RTP-801i-14	Diabetic retinopathy, diabetic macular edema	RTP801	Phase II	Dose ranging, safety, efficacy evaluation	Ongoing
Quark Pharmaceuticals	QPI-1002/Akli5/I5NP	Acute kidney injury	TP53	Phase I/IIa	Dose escalation, safety, pharmacokinetics evaluation	Ongoing
	QPI-1002/DGFi	Delayed graft function in kidney transplantation	TP53	Phase I/II	Dose escalation, safety, pharmacokinetics evaluation	Ongoing
Sirna Therapeutics (Merck)/Allergan	Sirna-027/AGN-745	Wet age-related macular degeneration	VEGFR-1	Phase II	Dose escalation, safety, pharmacokinetics evaluation	Halted
Silence Therapeutics	Atu027/Atu093	Lung cancers	—	Phase I	—	Initiated 2009
Senetek	—	Brain tumors, glioblastomas	Tenascin-C	Phase I	—	Ongoing
TransDerm	TD101	Pachyonychia congenita	Keratin 6a N171K	Phase I	Safety and toxicity evaluation	Completed

**rHIV7-shI-TAR-CCR5RZ** = recombinant HIV-shI-trans-acting response element-chemokine (C-C) motif receptor 5 ribozyme; **RSV** = respiratory syncytial virus; **RTP801** = hypoxia-inducible factor 1-responsive gene; **TP53** = tumor protein p53; **VEGF** = vascular endothelial growth factor; **VEGFR-1** = vascular endothelial growth factor receptor-1. '—' indicates that the information is not available.

When designing a toxicity study with a surrogate drug it becomes necessary to decide whether to run a full dose-response curve for the surrogate or for the human sequence. For practical purposes and humane reasons, some have argued for the use of a single active dose of the surrogate unless there is marked toxicity associated with the reduction in target protein.<sup>[189]</sup>

All RNAi-based therapeutic agents currently in development are chemically synthesized and none are produced from biological reactions. As a result, these compounds have been regulated not as biologics but as traditional drugs and have been tested in non-clinical assays like small molecules, with complete toxicity characterization in genotoxicity, safety pharmacology, subchronic

and, when appropriate for the indication, chronic toxicity and carcinogenicity assays. Nevertheless, some aspects of oligonucleotide-based therapeutics are much more akin to biologics and could be covered by the International Conference on Harmonisation S6 guidance. For example, the chemical characterization of these compounds often shows that they have complex profiles more similar to biologics than to traditional drugs.<sup>[192]</sup> Additionally, their metabolism is similar to the catabolism of biologics that are reduced to amino acids, in that oligonucleotide-based drugs are cut down to nucleotides by nucleases.

In order to maximize returns from their RNAi-based drug development programs, companies within the field have invested considerable resources to retain a strong intellectual property portfolio and protect their franchises. The use of RNAi as claimed in many patents has led to a significant number of litigations as companies compete for ownership of this emerging technology in a fiercely competitive field. As the market evolves, companies are seeking to file patents based on structural and chemical modifications as well as specific therapeutic targets as delivery of the RNA structures is still a major hurdle in this field. In recent years, a significant number of new patent applications have been filed, yet the number of issued patents remains small.

There are several key patents or patent families in the field of RNAi therapeutics: Fire and Mello, Tuschl I/II, Kreutzer-Limmer I/II, Benitec, Crooke, Glover, and Hannon. The strongest patent portfolios have been developed by Alnylam Pharmaceuticals and Sirna Therapeutics (now Merck) and many small RNAi players have chosen to negotiate licensing deals with one or other of these. Others have developed proprietary intellectual property and are awaiting the outcome of these so-called fundamental patent applications.

One of the seminal patents enabling the development of siRNA therapeutics, filed by Nobel Laureates Andrew Fire and Craig Mello and their associates in 1998, is owned by the Carnegie Institution of Washington and the University of Massachusetts. It was issued in 2003 and has been widely licensed on a non-exclusive basis. Other seminal patents include the first and second Tuschl patent families (Tuschl I and II). The first Tuschl patent (Tuschl I) was jointly filed by Thomas Tuschl, Philip Zamore, Philip Sharp, and David Bartel. This patent covers the therapeutic application of RNAi technology, in particular the use of siRNA for sequence-specific inhibition of gene expression. Further work carried out by Tuschl, a scientific advisor to Alnylam Pharmaceuticals, led to a new patent (Tuschl II), which describes the ability of synthetic siRNA to induce RNAi, and adds the requirement for overhangs at the 3'-positions. Other key patents include the Kreutzer-Limmer

series, which covers various oligonucleotide structures and siRNAs directed towards over 125 disease targets. However, the first granted European patent belonging to this family was recently revoked and others are undergoing complex opposition and prosecution procedures.

In 2003, Alnylam Pharmaceuticals launched a program, InterfeRx, through which it grants licenses for the development of RNAi therapeutics under the company's broad portfolio of issued and pending patent rights relating to siRNA molecules and their use as therapeutics. Alnylam Pharmaceuticals also offers non-therapeutic license agreements to life science reagent and service providers.

When interest in RNAi first began to develop, a patent surfaced from the Australian company Benitec that describes the use of transcribed RNA to induce RNAi, with a priority dating back before Fire and Mello, for use in animals and humans. A dsRNA, with the sequence of one strand matching the targeted mRNA, is delivered to the cell and initiates the silencing process (which Benitec calls DNAdirected RNAi, ddRNAi). However, examples were initially only given for work in plants. In a subsequent patent application, the work was expanded to include mammalian cells. The company has encountered a number of legal difficulties, primarily as a result of disputes in various jurisdictions by Nucleonics Inc. Benitec has a number of licensees, including a strategic cross-licensing with Alnylam Pharmaceuticals (CombiMatrix Corporation).

Companies are also starting to apply discoveries in miRNA to diagnostics and therapeutics. Leading companies in each field are now beginning to define their positions and are seeking to form strong alliances with academic institutions as early as possible. Key patents in miRNA have been applied for by the Max Planck Institute (including Tuschl III that protects the use of a specific miRNA, miRNA-122, in viral hepatitis), the Rockefeller University, University of Massachusetts Medical School, and Johns Hopkins University. Alnylam Pharmaceuticals and Isis are exclusive co-licensees of the Tuschl III series. However, no patents have yet been granted in relation to miRNA based therapeutics or diagnostics. Licenses for key patents are owned by Alnylam Pharmaceuticals, Isis and Sirna for therapeutic purposes and by Rosetta Genomics, Exiqon, Asuragen and Stratagene for diagnostics. Of interest, Regulus Therapeutics is a joint venture between Isis Pharmaceuticals and Alnylam Pharmaceuticals, created to discover, develop, and commercialize miRNA therapeutics.

In addition to RNAi technology, leading companies have also been submitting applications covering specific disease targets. Nevertheless, some segments of the industry view claims to gene targets as uncompetitive and such patents may be

challenged vigorously. To date, most therapeutic-based companies hold patents regarding target-specific RNAi, with delivery of RNAi compounds remaining the unsolved problem.

## 9. Conclusions

Shortly after its discovery in 1998, RNAi rapidly emerged as a novel therapeutic approach to treat human disease. RNAi-based therapeutics hold the promise of significantly expanding the number of 'druggable' targets by overcoming the major limitations of existing drugs, and companies are moving to invest in this growing field based on an endogenous mechanism that is highly specific and potent.

Overcoming the obstacles described above remains one of the most crucial challenges on the road to bringing RNAi-based drugs to the market, but given the immense resources that are being invested in improving their safety, efficacy and delivery, the coming years are likely to present exciting advances towards the use of RNAi-based therapeutics for the treatment of an increasing number of pathologies otherwise 'undruggable' by existing therapies.

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