Hum Genet (2011) 130:583–605
DOI 10.1007/s00439-011-0995-8

REVIEW PAPER

RNAi: a potential new class of therapeutic for human genetic disease

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Received: 18 March 2011 / Accepted: 17 April 2011 / Published online: 3 May 2011

Abstract Dominant negative genetic disorders, in which a mutant allele of a gene causes disease in the presence of a second, normal copy, have been challenging since there is no cure and treatments are only to alleviate the symptoms. Current therapies involving pharmacological and biological drugs are not suitable to target mutant genes selectively due to structural indifference of the normal variant of their targets from the disease-causing mutant ones. In instances when the target contains single nucleotide polymorphism (SNP), whether it is an enzyme or structural or receptor protein are not ideal for treatment using conventional drugs due to their lack of selectivity. Therefore, there is a need to develop new approaches to accelerate targeting these previously inaccessible targets by classical therapeutics. Although there is a cooling trend by the pharmaceutical industry for the potential of RNA interference (RNAi), RNAi and other RNA targeting drugs (antisense, ribozyme, etc.) still hold their promise as the only drugs that provide an opportunity to target genes with SNP mutations found in dominant negative disorders, genes specific to pathogenic tumor cells, and genes that are critical for mediating the pathology of various other diseases. Because of its exquisite specificity and potency, RNAi has attracted a considerable interest as a new class of therapeutic for genetic diseases including amyotrophic lateral sclerosis, Huntington's disease (HD), Alzheimer's disease (AD), Parkinson's disease (PD), spinocerebellar ataxia, dominant muscular dystrophies, and cancer. In this review, progress and challenges in developing RNAi therapeutics for genetic diseases will be discussed.

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Introduction

Completion of the mapping of the reference copy of the human genome (I. H. G. S. Consortium 2001; Venter et al. 2001) has provided accessible and comprehensive annotation of the human genome and provided new opportunities for the treatment of genetic diseases that were once incurable. Despite some progress in understanding the causes of many genetic diseases and leading the development of new gene discovery technologies, the human genome project has yet to deliver on its promise to find the root causes of many common diseases and to develop therapies that target those genes implicated in genetic diseases. The project has revealed that the genome holds more complexity than had been previously thought, making it difficult to isolate the functions of the three billion DNA sequences the project has uncovered. The human genome database (HGDB) have implicated that out of 73,411 catalogued mutations responsible for human disease, more than 60% are caused by single-base substitutions (Fig. 1) and identified approximately 2,900 single-gene aberrations known to cause diseases that are rare in accordance with Mendelian genetics in addition to 100 commonly identified genetic diseases (Jon Cohen, http://www.technologyreview.com). For example, recent studies have linked more than 200 genes to cancer (Jon Cohen, http://www.technologyreview.com). It is now estimated that more than 10,000 human diseases have been defined as having genetic abnormalities, but little progress has been made with respect to the clinical application of this knowledge (Leachman et al. 2008).

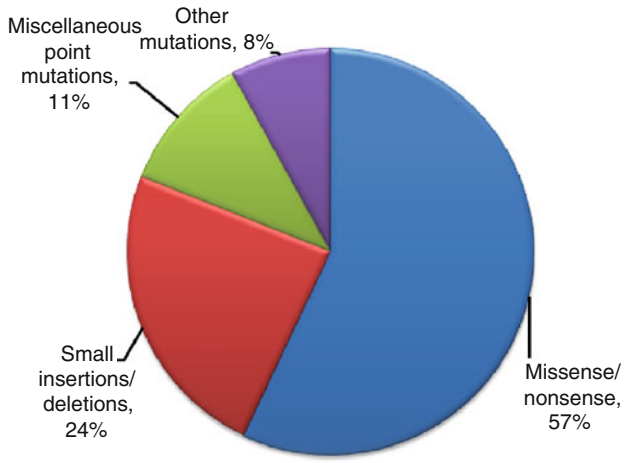


Fig. 1 Schematic representation of the prevalence of mutation types available from the human genome data base (HGDB, 2007-07-04). More than 60% of 73,411 catalogued mutations responsible for human disease are caused by SNPs. Adapted from HGDB (2007-12-04)

Further deepening of our understanding of genetics and genomics can potentially lead to the advent of individualized medicine. Although genomics has already contributed to improve diagnostics and a few treatments; the full utilization of this field cannot be realized unless new treatments are developed for all dominant negative disorders.

It is now widely accepted that small RNAs including microRNAs (miRNAs) play a central role as regulators that direct gene "expression" or the extent to which that protein is made. To add more to the complexity, the emerging field of epigenetics is now demonstrating how two individuals with an identical genetic background can have different characteristics.

Even if there are many well-characterized genes and their products with widely recognized association with number of diseases, many of these existing key targets still remain "undrugged". This is, in part, due to the polygenic nature of many genetic diseases, that is, there is more than one genetic factor contributing to the pathology of a disease or disorder, complicating the development of therapies since multi-therapeutic strategies to simultaneously modulate multiple targets are required.

Current therapies involving pharmacological and biological drugs are not suitable for these targets. This is because either the target is not accessible or structurally unsuitable for targeting or the disease-causing mutant variant cannot be differentiated from a normal variant of the target (Leachman et al. 2008). None of these therapeutic approaches can differentiate a target containing a SNP mutation. Although specific inhibitors of a particular protein could be developed, these are limited in scope and their specificity is questionable. Early gene therapy strategies which primarily focused on the introduction of a functional gene to compensate for a mutant allele has shown promise for conditions such as hemophilia (Petrus et al. 2010) and X-linked severe combined immunodeficiency (X-SCID) (Hacein-Bey-Abina et al. 2010), genetic diseases caused by the inappropriate upregulation of gene expression cannot be corrected by this approach. In addition, gene therapy relies on variety of viral vectors which has raised some safety concerns. Although, next generation viral vectors have demonstrated high safety levels there is a need to develop new approaches to accelerate targeting these previously inaccessible targets by classical therapeutics.

The emergence of RNA interference (RNAi) as a powerful gene silencing technology has provided new opportunities for interrogating clinically relevant questions by rapidly modulating gene expression and subsequent function of its product. Since its first description, there has been a notable increase in reports showing the utility of RNAi as a new generation of therapeutic intervention. The strong appeal of RNAi as a therapeutic is the potency, specificity, and ease of design and synthesis with which gene expression can be inhibited. This has led to a great deal of interest into this emerging field from academia to biotech and pharmaceutical companies to harness the potential of RNAi as a new class of drugs. This has led to a sudden growth of expectation that particularly RNAi or other antisense-based approaches can be applied to many diseases that were incurable before.

The implications were straightforward design where virtually any disease-related gene could, in theory, be targeted by synthesizing RNAi reagents such as double-stranded (ds) small interfering RNAs (siRNAs) in a short period of time with sequences that are complementary to their target sequences. It took only a couple of years for the pharmaceutical companies to take these drugs into clinical trials. Although many problems have been encountered during these trials, many lessons have also been learned. The biggest obstacle has been the delivery. It appears that the same problem that has inflicted the antisense drugs for over two decades has also been a main obstacle for RNAi, difficulty of delivery of such drugs to the tissues and cells where they are needed. Despite these recent developments, the renewed interest in other antisense-based and RNA-based drugs in recent years may provide some urgently needed breakthroughs for the entire field.

This review article provides background information on the RNAi phenomenon, its mechanism, and highlights the use of RNAi as a new class of therapeutics for human genetic diseases in tissue culture and in animal models as well as discusses the issues and technical challenges associated with this technology.

RNA interference

RNAi is a natural physiological sequence-specific gene silencing phenomenon mediated by double-stranded RNAs (dsRNAs) that can operate both at transcriptional and posttranscriptional levels (Dykxhoorn et al. 2003; Meister and Tuschl 2004; Zamore and Haley 2005; Chapman and Carrington 2007). It is a common phenomenon in nature, involving similar pathways in protozoa, plants, fungi, and animals and may have evolved as an innate defense mechanism to protect the host against potentially harmful foreign (virus) or endogenous (retrotransposon) dsRNAs (McManus and Sharp 2002). Although RNAi appears to have evolved as a natural defense against viruses or retrotransposons, various small non-coding RNAs are involved in RNAi, such as miRNA and Piwi-interacting RNA (piRNA) generated by various biogenesis pathways (Ghildiyal and Zamore 2009).

MiRNAs are endogenous, non-coding RNA molecules of ~22 nucleotides (nt) that play a major role in gene regulatory networks of a diverse range of biological pathways (Ambros 2004; Bartel 2004). They are transcribed by RNA polymerase II which can be located in independent non-coding transcripts or in introns of protein-coding genes; some are clustered in polycistronic transcripts that allow for coordinated expression. (Rodriguez et al. 2004) Many miRNAs are expressed in a tissue-specific and developmental-stage-specific manner (Lagos-Quintana et al. 2002). More than a decade after the discovery of the first miRNAs genes *lin-4* and *let-7* (Lee et al. 1993; Wightman et al. 1993; Reinhart et al. 2000) we now know that miRNAs are found across various eukaryota, and are often highly conserved throughout evolution (Zhang et al. 2007) implying a fundamental role for miRNAs. They play key roles in processes as diverse as early development, cell proliferation, differentiation, apoptosis, brain development, hematopoiesis, and maintenance of cell and tissue identity.

MiRNAs are predicted to regulate the expression of up to one-third of all human protein-coding genes but only a very small fraction of these interactions have been experimentally validated (Lewis et al. 2005). Based on computational predictions, miRNAs can have as many as 100 or more functional binding sites in the genome and consequently the potential regulatory circuitry regulated by miRNAs is large and complex (Lewis et al. 2005). Each miRNA regulates the expression of multiple genes, and most mRNA targets contain multiple miRNA binding sites within their 3′-UTR (Bartel 2009).

Altered expression of miRNAs has been linked to various developmental, physiological as well as pathophysiologic processes and have been linked to various disease states as well as different stages of the same disease including autoimmune, metabolic, neurologic, cardiovascular, cancer, and viral pathogenesis (He et al. 2005; Lu et al. 2005; Care et al. 2007; Chang et al. 2007; Cheng et al. 2007; Li et al. 2007; Chua et al. 2009; Alevizos and Illei 2010a, b). Because changes in miRNA expression levels has been described in variety of human diseases (Erson and Petty 2008), this provides opportunities for their use as novel drug targets or drugs as in miRNA mimics and biomarkers for diagnosis, prognosis, and monitoring disease activity (Alevizos and Illei 2010a, b).

RNAi can also be induced by exogenous delivery of dsRNAs that have been introduced to the cell in the form of synthetically made or vector-encoded (plasmid or virus) shRNA or siRNAs (Seyhan et al. 2005, 2007; Quon and Kassner 2009) (Fig. 2). Mechanistically, when an siRNA or its precursors, such as shRNA or shRNAmirs (shRNA imbedded in a miRNA scaffold) are introduced to target cells, siRNA duplex is taken up by specific proteins involved in natural miRNA biogenesis. Primary-miRNAs (pri-miRNAs) or shRNAs containing miRNA sequences are cleaved in the nucleus to hairpin pre-miRNAs by Drosha (Gregory et al. 2004). Pre-miRNAs or shRNAs are then exported to the cytoplasm by exportin-5 (Yi et al. 2003). shRNAs are then processed by the cytoplasmic endonuclease Dicer into a double-stranded RNA (19–25 nucleotides in length) generating 2 nucleotide overhangs at the 3′ termini. These duplexes now called siRNAs, are recognized and bound by a multiprotein complex called the RNA-induced silencing complex (RISC) (Robb and Rana 2007). The RISC unwinds the siRNA duplex and one strand enters the RISC (Chua et al. 2009), hybridizes to complementary messenger RNA (mRNA) while the passenger strand is cut by an enzyme within the RISC, argonaute 2 (Ago2), and removed from the RISC complex. The "guide" strand of the siRNA directs RISC-mediated endonucleolytic cleavage of the mRNA at a single phosphate across from nucleotides 10 and 11 from the 5′end of the siRNA ''guide'' strand, triggering mRNA degradation thereby preventing the translation of the mRNA (Hamilton and Baulcombe 1999; Hammond et al. 2000; Zamore et al. 2000; Elbashir et al. 2001a). However, if a guide strand does not have perfect complementariness with the mRNA, gene silencing can occur at the translational level. This is the usual case for micro RNA (miRNA) induced RNAi. The guide strand remains within the RISC and can turnover, repeatedly binding and continuing the silencing of the target mRNA (Aagaard and Rossi 2007; DeVincenzo 2009), leading to long term sustainable silencing of the target gene. Even though, both strands of an siRNA can induce RNAi (Elbashir et al. 2001a; Harborth et al. 2001; Nykanen et al. 2001; Schwarz et al. 2003), the strand with lowest thermodynamic stability at its 5′ end forms a more stable complex with RISC leading to cleavage of the target mRNA. The siRNA strand that serves as the guide reflects

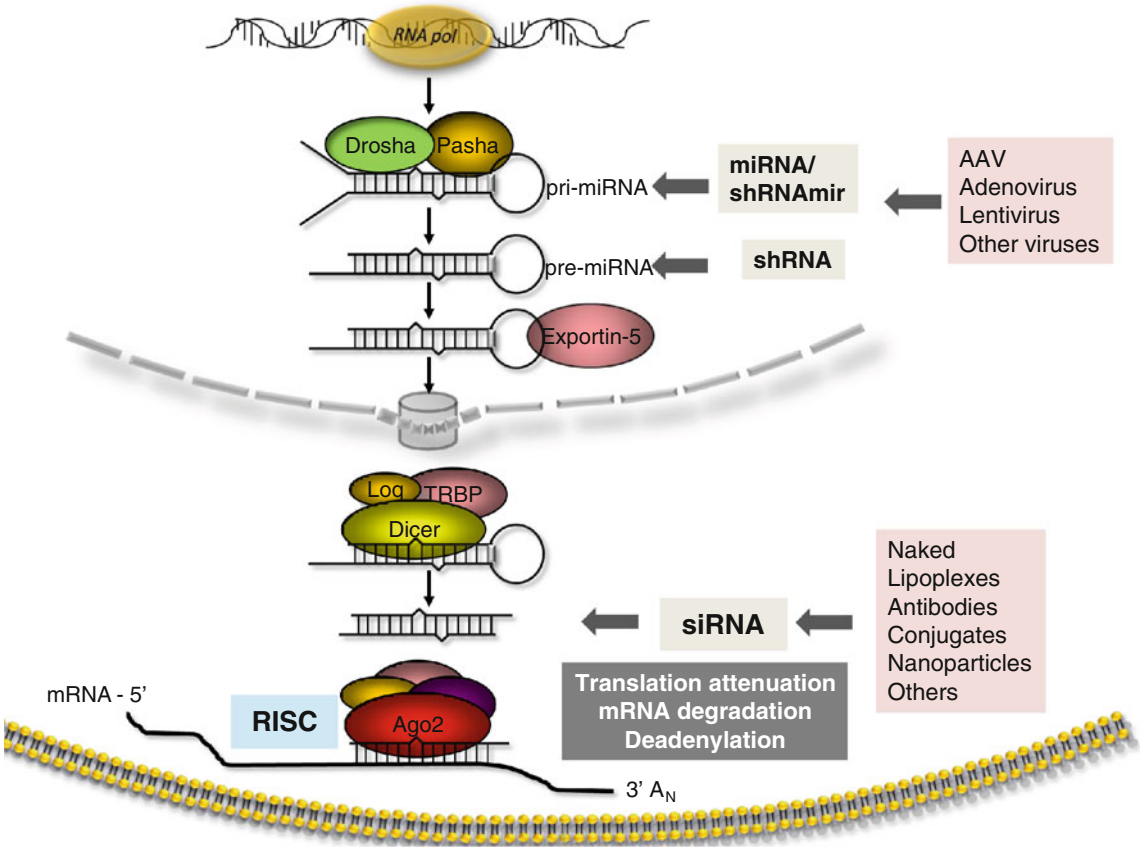


Fig. 2 Mammalian RNAi biogenesis and therapeutic opportunities. A pri-miRNA transcript is first transcribed by RNA polymerase II as hairpins with large single-stranded flanking sequences in the nucleus (primary miRNA, pri-miRNA). These hairpins are initially processed by the enzyme Drosha/Pasha into precursor miRNA (pre-miRNA) and exported to the cytoplasm by Exportin-5 and cleaved by Dicer in a complex with Loq/Trbp. After strand separation, one strand of the miRNA, once loaded into the RISC complex, guides translation repression, degradation of the targeted mRNA or deadenylation. To reprogram the RNAi pathway for therapeutic intervention, viral vector-mediated nuclear expression of miRNAs, shRNAs or shRNAmirs (shRNAs imbedded in a miRNA scaffold) or alternatively, cytoplasmic delivery of siRNAs (mimicking mature miRNAs) are used. *Top right* Endogenously transcribed shRNAs are encoded by plasmid or viral vectors are either exported to cytoplasm directly if they are transcribed by RNA polymerase III promoters (U6 or H1) or processed by Drosha–DGCR8 to generate precursor shRNAs if they contain miRNA-derived flanking sequences and transcribed by RNA pol II promoters (e.g., CMV). These precursors are exported to the cytoplasm by exportin 5 and subsequently bind to the Dicer–TRBP–PACT complex, which processes the shRNA into siRNA for loading into RNA-induced silencing complex (RISC). *Bottom right* Exogenously delivered siRNAs directly bind the RISC ribonucleoprotein complex, which contains a helicase that unwinds the duplex siRNA in an ATP-dependent reaction and argonaute 2 (Ago2) and TRBP. The activated RISC with siRNA guide strand direct mRNA cleavage catalyzed by Ago2. An RNA with a perfect match to a target mRNA behaves like an siRNA and results in mRNA degradation, whereas an RNA with a partial match functions as an miRNA and causes translational repression. *Pri-miRNA* primary miRNA, *pre-miRNA* precursor miRNA, *RISC* RNA-induced silencing complex, *TRBP* transactivation-response RNA-binding protein. Adapted from Grimm (2007)

the relative thermodynamic stability of the 5′ ends of the two siRNA strands (Khvorova et al. 2003; Schwarz et al. 2003). siRNAs that exhibit near absolute asymmetry, with only one strand of the siRNA capable of entry into the RISC, are said to be functionally asymmetric (Schwarz et al. 2003). Since then several design strategies, backbone and terminal nucleotide modifications, such as 2′-O-Me or LNA have been used to artificially destabilize the 5′-end of siRNA duplex favoring only the guide strand's entry into RISC and concomitantly destroy the sense, or ''passenger,'' strand (Khvorova et al. 2003; Schwarz et al. 2003). This has several repercussions; (1) reduced or minimal sequence-dependent off-targeting effects mediated by passenger strand of siRNA, (2) improved silencing by guide strand of siRNA, and (3) potential avoidance of Toll-like receptors due to these modifications (Behlke 2008).

RNAi as a new class of therapeutics

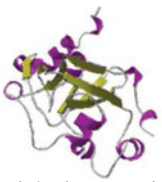
RNAi is well suited to probe the biological function of individual genes or genes in a pathway or genes known to

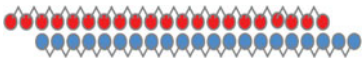
be associated with diseases, including inherited genetic diseases, viral pathogens and discover novel genes critical to pathogenic processes (Seyhan 2010). Therefore, RNAi has major implications for basic and biomedical research that may lead to a number of clinical applications (Davidson and Boudreau 2007; Gonzalez-Alegre et al. 2007; Lingor and Bahr 2007). RNAi, in theory, is capable of suppressing gene function in a large set of diseases. For example, RNAi has been used to silence exogenous disease-causing genes encoded by pathogens (Martin and Caplen 2007; Arbuthnot 2010), such as human immunodeficiency virus (HIV) (Li et al. 2006; Kumar et al. 2008; Castanotto and Rossi 2009), hepatitis C virus (HCV) (McCaffrey et al. 2003a; Ilves et al. 2006; Khaliq et al. 2010), hepatitis B virus (HBV) (McCaffrey et al. 2003b), and Semliki Forest virus (Seyhan et al. 2007) as well as endogenous genes that play essential roles in the disease process (Dillon et al. 2005). Since mismatches in the duplex formed by siRNA and its target RNA have been shown to abolish RISC-mediated target cleavage (Harborth et al. 2001; Holen et al. 2002; Amarzguioui et al. 2003), this has enabled the design of siRNAs to distinguish between wild-type RNA sequences and mutant transcripts for targeting autosomal dominant genes. Because of its potency and exquisite specificity to its targets, RNAi provides an opportunity to suppress alleles of genes exhibiting spontaneous or inherited polymorphisms and alternative splicing with single point mutations found in inherited disorders, genes specific to pathogenic tumor cells, and genes that are critical for mediating the pathology of various other diseases. The siRNA-mediated selective inhibition of mutant genes in negative dominant human genetic diseases has been demonstrated, and has resulted in a considerable interest in RNAi technology as potential therapeutic agents for human disease.

Comparison of RNAi with traditional pharmaceutical drugs

Although RNAi mimics the pharmacological inhibition of target protein by active compounds, RNAi provides several advantages over conventional pharmaceutical or biologic (i.e., antibodies, therapeutic proteins and peptides, and vaccines) drugs (Table 1). The principal advantage of RNAi is that all targets, including 'undruggable' targets become druggable with RNAi since any transcript that encodes a protein that causes or contributes to a disease, in theory, can be targeted by RNAi (Perrimon et al. 2010). Often, it has been challenging to find active compounds or biologics that effectively inhibit disease-associated proteins. Most drugs work by blocking the action of a protein/enzyme, but some proteins, because of their structure or location cannot be readily targeted; hence, are "undruggable". Therefore, reducing the levels of the mRNA from which they are translated, instead of suppressing protein activity alone increases the number of druggable targets (DeVincenzo 2009).

Table 1 Comparison of RNAi with traditional pharmaceutical drugs





Small molecules	Biologics (proteins and antibodies)	RNAi
Antagonism or agonism of target	Antagonism or agonism of target	Antagonism only
Extracellular and intracellular targets	Extracellular targets	All targets, including nondruggable targets
Not all targets can be modulated selectively and potently (conformation driven)	High selectivity and potency	High selectivity and potency
No allelic specificity	Low allelic specificity	High allelic specificity
Lead ID and optimization slow	Lead ID and optimization slow	Lead ID and optimization rapid
Low cross species reactivity	Low cross species reactivity	High cross species reactivity
Easy to manufacture	Difficult to manufacture	Easy to synthesize and manufacture
Easy to deliver	Difficult to deliver	Difficult to deliver

The principal advantages of RNAi over small-molecule and protein therapeutics are that all targets, including "druggable" and "nondruggable" targets, extracellular and intracellular targets and mutant alleles can be targeted with RNAi. One of the major advantages of sequence-based targeting technologies is the ability to design precisely targeted therapeutics for almost any target sequence both coding and non-coding sequences, regardless of the function of the gene product, whether that function is known, and in the absence of any target protein structure information. Adapted from Bumcrot et al. (2006)

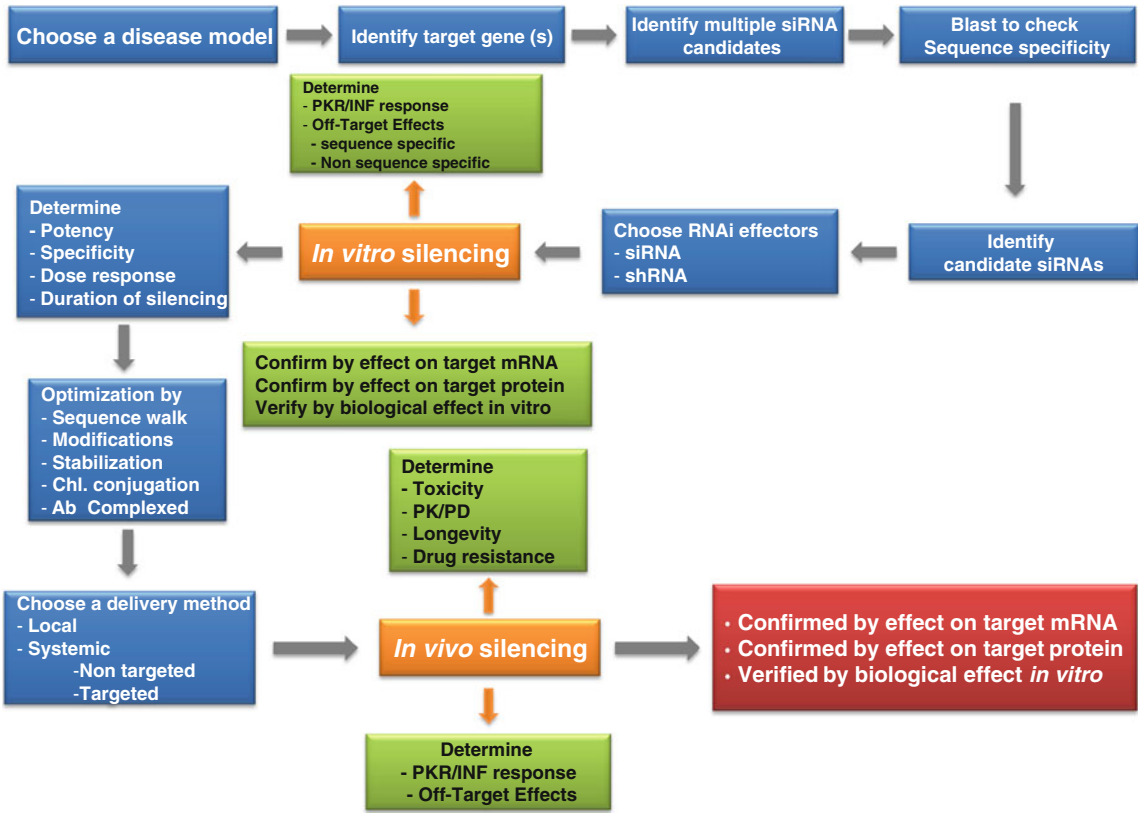


Fig. 3 Schematic representation of siRNA drug candidate design workflow. Adapted from Yang and Mattes (2008)

RNAi offers more specificity and flexibility than traditional drugs since the only requirement for its design is a unique ~20 nucleotide sequence on the targeted RNA sequence for Watson–Crick base-pairing. The identification of potent and specific siRNA sequences is rapid (Fig. 3). It usually involves a combination of siRNA selection algorithms (Seyhan et al. 2007; Seyhan 2010) or a gene-walk strategy to identify functional and specific siRNAs from a panel of siRNA (Ilves et al. 2006; Vlassov et al. 2007) or gene-specific siRNA libraries (Seyhan et al. 2005).

It is also possible to design cross-species specific siRNAs in which key target sequences are conserved across all the relevant species used in safety and efficacy studies, thus enabling the development of a single siRNA drug candidate from the research stage through to clinical studies (de Fougerolles et al. 2007).

As for biologic drugs, the main challenges are limited target space as in small molecules and production. For example, biologics are applicable only to certain targets that are expressed on cell surfaces or circulating systemically. Additionally, acceptable cellular production levels of protein or peptide therapeutics are often difficult to achieve. For biologics as a therapeutic class, production and aggregation are the major issues, whereas, siRNA molecules are synthetic and easy to produce. Because of these, RNAi has become the focus of the bio-pharmaceutical industry to develop novel RNAi therapeutics, based mainly on siRNAs, to target viral infection, cancer, hypercholesterolemia, cardiovascular disease, macular degeneration, and neurodegenerative diseases (Sah 2006).

Altogether, RNAi presents an innovative therapeutic approach providing a major new class of drugs that could meet some unmet medical needs.

RNAi for dominant human genetic diseases

Individuals with negative dominant genetic diseases are heterozygous, that is, they carry both a normal and a mutant allele of the same gene which may lead to a disease-causing phenotype if the normal allele cannot compensate for the lost function of the mutant allele. Furthermore, the mutated gene could also cause the disease by gain of function, which could be a novel function or enhancement of an existing function. The solution to gain-of-function diseases is challenging and historically, gene therapy has been used to deliver a normal copy of the gene to the patient to treat only the loss-of-function mutations. In addition, the safety

concerns involving viral vectors used in gene therapy have slowed its adoption.

The challenging task to differentiate an SNP in the mutant allele transcript from a normal allele by other therapeutics underlined the potential of RNAi for the treatment of many genetic diseases and disorders caused by dominant, gain-of-function mutations. Mutant allele transcripts of dominant genetic disorders can be suppressed at the mRNA level with allele-specific siRNA which exclusively destroys the mutant, disease-causing transcript, while leaving the normal transcript intact thereby reducing mutant protein synthesis and any toxicity associated with it. Indeed, many recent reports have indicated the utility of this strategy in vitro or in animal models demonstrating how SNPs in mutant allele transcripts can be used as selective targets for RNAi-mediated inhibition of mutant gene expression (Miller et al. 2003, 2004).

These developments have resulted in the recognition of RNAi as a potential therapy for many genetic diseases. Although RNAi therapy is well suited for dominant genetic disorders where disease-causing mutations are in some instances in a single gene, targeting a single mediator may have limited success for treatment other than monogenic diseases. Because the mechanisms that underlie the polygenic and multifactorial etiology of complex diseases including autoimmunity, metabolic diseases and cancer are not clearly defined; an understanding of diseases at the molecular level and possibly modulating multiple targets simultaneously may be necessary to develop novel therapeutics for these diseases (Yang and Mattes 2008). In diseases where pathogenesis is aligned with many possible targets, such as in polygenic diseases, RNAi drugs can be designed to target multiple targets and may be used in combination to suppress function of those genes simultaneously.

Design of allele-specific siRNAs

Two main strategies are used to design siRNAs: (1) a gene-walk strategy on a target mRNA sequence in which potent and selective siRNA can be identified from a panel of siRNAs (Fig. 4); (Ilves et al. 2006; Vlassov et al. 2007; Vaishnaw et al. 2010) or (2) siRNA selection algorithms (Seyhan et al. 2007) for screening and selection of lead siRNAs. Once candidate siRNAs are identified from either approach, various steps are followed to select lead siRNAs which include a bioinformatics screen to identify siRNAs

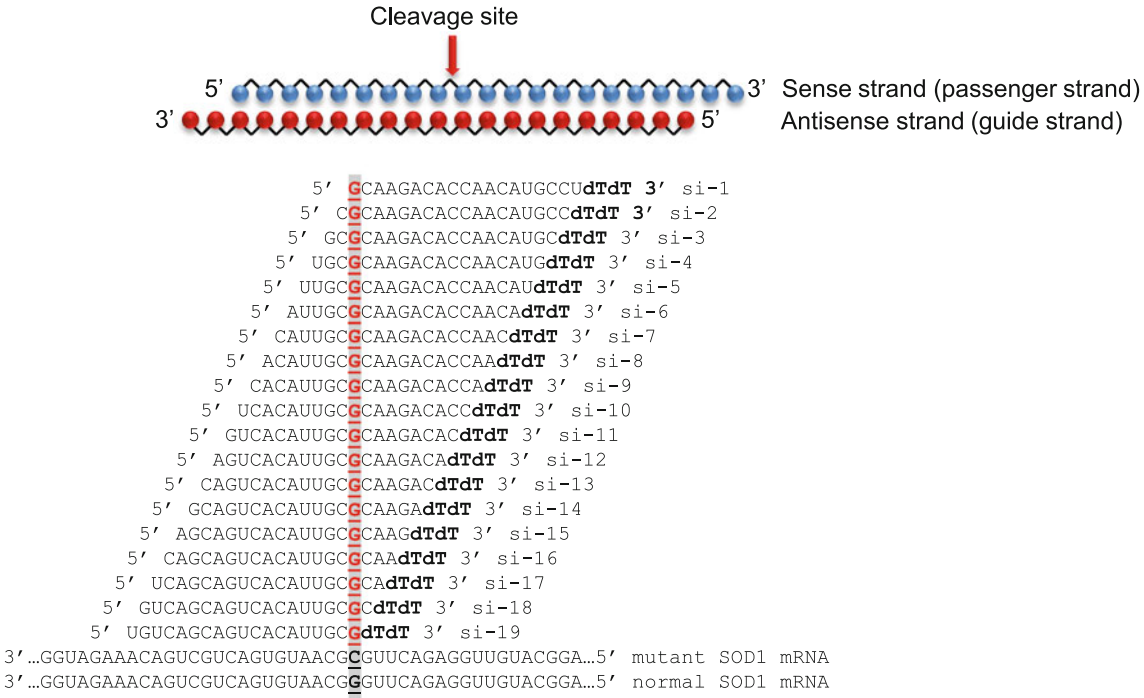


Fig. 4 Design of an allele and SNP specific siRNA targeting human SOD1 sequence. *Top panel* Structure of siRNA. siRNA structure is composed of two strands, a 19 base pair region of duplex, with two unpaired overlapping nucleotides on the 3′ end of each strand. The site at which the mRNA target is cleaved (leaving the antisense strand intact) is indicated by an *arrow* (adapted from DeVincenzo 2009). *Bottom panel* Schematic depiction of a tiled siRNA gene-walk strategy to identify potent and SNP-selective siRNA targeting human SOD1 sequence. siRNA guide strands having the G;G mismatch with the normal SOD1 mRNA is indicated. To target mutant SOD1. siRNAs designed such that they contain a G nucleotide complementary to the mutant C nucleotide in the mutant SOD1 transcript. Adapted from Schwarz et al. (2006)

with minimal off-target effects and cross-species specificity, in vitro assays for potency and nonspecific cytotoxicity, and assessment of in vivo pharmacology (de Fougerolles et al. 2007; Vaishnaw et al. 2010) leading to the identification of highly selective and potent allele-specific siRNAs. Examination of the mechanism of action of siRNA target recognition and subsequent target cleavage have suggested that siRNAs are most selective for a particular SNP in the target when the polymorphic nucleotide is complementary to the mid-region of the siRNA, leading to selective cleavage of only the mutant mRNAs while leaving the normal mRNAs intact (Miller et al. 2003, 2004; Ohnishi et al. 2008). Although siRNAs with incomplete homology to their target mRNA sequence can also silence gene expression; the central region of the siRNA:mRNA interaction site (between positions 10 and 11 from the 5′ end of the guide strand) is thought to be critical for cleavage of mRNA by the RISC complex leading to target mRNA degradation (Elbashir et al. 2001b) but may not be required for the less efficient silencing by inhibition of translation (Ding et al. 2003; Doench et al. 2003; Saxena et al. 2003). Since some mismatches at this site have been shown to block RISC-induced target mRNA cleavage (Harborth et al. 2001; Holen et al. 2002; Amarzguioui et al. 2003) (Schwarz et al. 2006), this property was used to design highly selective and SNP-specific siRNAs where the targeted mRNA can differ at a single base-pair from that of the normal allele. These studies suggest that that the discriminating nucleotide must be positioned centrally within the inhibitory RNA duplex. Because this single SNP is the only determinant for target selectivity of mutant genes in dominant human diseases with SNPs, the siRNA design is limited to the region surrounding the SNP mutation (Fig. 4). In the case where disease allele-specific silencing is not sufficiently produced, the specificity can be improved by incorporating additional peripheral mismatches in the siRNA guide strand (Miller et al. 2003, 2004). For example, by manipulating peripheral pairing by introducing mismatches in those areas, Miller et al. (2003, 2004) showed that the specificity of these siRNAs can be further improved. This suggests that more than one factor contributes to siRNA specificity for differentiating against a SNP: (1) the overall base-pairing efficiency between the siRNA guide strand and mRNA within RISC and (2) base-pairing between siRNA and mRNA at the central position across the RISC-mediated cleavage site. Nonetheless, each allele-specific siRNA must be custom designed and experimentally verified.

Once a potent and selective siRNA is identified, it can be incorporated into shRNA hairpins that can be expressed from plasmids or viral vectors that retain the efficacy and allele specificity of the original duplex. Allele-specific inhibition of mutant gene expression can be achieved with vector expressed shRNAs as well (Miller et al. 2003, 2004).

RNAi therapy of monogenic diseases

Because many neurodegenerative diseases are, at least in part, associated with toxic gain-of-function mutations where conventional active compounds are not suitable for precision targeting of these types of diseases, RNAi has become an attractive strategy for selective inhibition of the mutant gene expression as a potential therapy for neurodegenerative diseases and others that require surgical precision for targeting mutant alleles (Schwarz et al. 2006).

Utility of this strategy has been demonstrated in several dominant genetic diseases, such as amyotrophic lateral sclerosis (ALS) (Ding et al. 2003; Maxwell et al. 2004; Harper et al. 2005; Ralph et al. 2005; Raoul et al. 2005), Huntington's disease (HD) (Harper et al. 2005), Alzheimer's disease (AD) (Miller et al. 2004), slow channel congenital myasthenic syndrome (SCCMS) (Abdelgany et al. 2003), spinocerebellar ataxia type 3 (Miller et al. 2003, 2004), sickle cell anemia (Dykxhoorn et al. 2006; Samakoglu et al. 2006) and cancer (Martinez et al. 2002). Although none of these studies have yet progressed into human trials, they demonstrate the utility of RNAi technology as new class of therapy for treating these conditions in preclinical models.

RNAi therapy for dominant genetic diseases caused by point mutations

Amyotrophic lateral sclerosis

ALS is characterized as a progressive motor neuron degenerative disease of the brain and spinal cord that leads to mortality. Mutations in the Cu, Zn superoxide dismutase 1 (SOD1) gene cause a toxic gain-of-function phenotype that is thought to be the main cause of the disease (Gurney et al. 1994). Several recent studies have shown the benefit of selective inhibition of the mutant allele of SOD1 gene (Ding et al. 2003). Because wild-type SOD1 performs important functions, it is important to selectively eliminate expression of only the mutant allelic transcript. Since mutations in SOD1 are SNP mutations, researchers have successfully shown selective suppression of a mutant SOD1 allele, demonstrating potential use of RNAi for the treatment of ALS. For example, Schwarz et al. (2006) identified siRNAs that discriminate between the wild-type and mutant alleles of the human Cu, Zn superoxide dismutase (SOD1) gene in Drosophila embryo lysate using a reporter assay.

Furthermore, a viral RNAi strategy has also been used to suppress mutant SOD1 in a transgenic mouse model of ALS showing therapeutic benefit via improved motor neuron survival, delayed disease onset and increased lifespan of the animals (Ralph et al. 2005; Raoul et al. 2005).

RNAi therapy for dominantly inherited nucleotide repeat diseases

There are many incurable autosomal dominant neurogenetic diseases caused by nucleotide repeat expansion with available treatments limited to symptomatic intervention (Denovan-Wright and Davidson 2006). For example, HD and spinocerebellar ataxia (SCA) are caused by the expansion of CAG repeats encoding glutamine in the coding region; whereas, in myotonic dystrophy, the CUG or CCTG expansions are in non-coding regions. It is this mutant RNA which forms the etiology of the diseases.

Spinocerebellar ataxia type 3

Spinocerebellar ataxia 3 (SCA3) is a dominantly inherited neurodegenerative disease, that is, the mutant ataxin-3 gene is present in cells alongside the normal gene, and despite the presence of the normal gene, and the mutant gene causes the disease. SCA3 is a progressive, untreatable, neurodegenerative disorder and is caused by a dominant expansion of CAG repeats in the ataxin-3 gene (Paulson 2000; Paulson et al. 2000). Because RNAi (and other antisense-based approaches) is currently the only strategy that has potential as a therapy for targeting mutant alleles of dominant genetic disease genes, it may be possible to target SNPs within the mutant allele with tight linkage to the repeat mutation, rather than the mutation itself (Miller et al. 2003, 2004). For example, researchers used an associated SNP to selectively target the mutant Machado-Joseph disease/SCA-3 allele, a polyglutamine neurodegenerative disorder, in which the selective targeting of the disease-causing CAG repeat was not possible (Miller et al. 2003, 2004).

Because of its exquisite specificity, RNAi has been used to target the mutant ataxin gene in mice. For example, SCA is mimicked in a transgenic mouse model that expresses high levels of a pathogenic form of a human ataxin-1 with 82 CAG repeats in cerebellar cells (Burright et al. 1995). Mutant mice that received intracerebellar injections of an AAV1 expressing shRNA directed against human ataxin-1 shortly before disease onset have demonstrated improved motor coordination, cerebellar morphology, and characteristic ataxin-1 inclusions in Purkinje cells (Xia et al. 2004). In another study, researchers designed shRNA hairpins selective for silencing the mutant ataxin-7 transcript in a heterozygous ataxin-7 disease model (Scholefield et al. 2009). They showed significant reduction of levels of toxic mutant ataxin-7 protein with decreased mutant protein aggregation and retention of normal wild-type protein in a non-aggregated diffuse cellular distribution. Allele-specific mutant ataxin-7 silencing was also obtained with the use of primary miRNA mimics (Scholefield et al. 2009).

Huntington's disease

The disease-causing polyglutamine (polyQ) proteins encoded by CAG-repeat containing transcripts have been identified in Huntington's disease (HD). HD is a progressive, untreatable, neurodegenerative disorder that is caused by a dominant expansion of CAG repeats within the huntingtin gene (HTT) (Paulson 2000; Paulson et al. 2000). It is an autosomal dominant disorder, that is, patients are heterozygous, possessing both a normal and a mutant HTT allele. Currently, there is no cure for HD and treatments can only alleviate disease symptoms (Harper 2009); therefore, HD patients may benefit from therapies capable of suppressing the expression of mutant HTT. Such CAG-repeat expansions encoding polyQ have also been characterized in at least nine other neurodegenerative disorders. PolyQ expansion confers a dominant toxic property on the mutant protein that is associated with aberrant accumulation of the disease protein in neurons (Zoghbi and Orr 2000). Because these CAG repeats also present in many normal transcripts in surrounding healthy tissues, disease-causing CAG repeats cannot be exclusively targeted by siRNA. However, targeting the diseased regions of the brain with siRNAs or viral vector-encoded shRNAs appears to remain as a viable approach (Davidson and Paulson 2004).

RNAi as a potential therapy for CNS disease has since been evidenced by experiments performed in various HD mouse models, which were developed to manifest striatal pathologies and motor abnormalities similar to those observed in human with HD (Harper et al. 2005; Rodriguez-Lebron et al. 2005; Rodriguez-Lebron and Paulson 2006; Wang et al. 2005; Machida et al. 2006; DiFiglia et al. 2007; Huang et al. 2007; Franich et al. 2008; McBride et al. 2008; Boudreau et al. 2009; Drouet et al. 2009) For example, Harper et al. showed that intrastriatal treatment of transgenic mice containing mutant human huntingtin with 82 or 144 CAG repeats with AAV expressing shRNA against human huntingtin resulted in improvement of disease pathology and lifespan extension (Harper et al. 2005). Although, none of these studies used comparable methods including HD animal models, RNAi reagents, delivery methods, or methods to measure phenotypic changes, each study supports RNAi as a potential therapy for HD.

Alzheimer's disease

Similar to SCA1 and HD, Alzheimer's disease (AD) is believed to be caused by toxic gain-of-function mutations which result in the accumulation of amyloid beta-containing plaques or tau-containing neurofibrillary tangles (Hardy and Selkoe 2002). Because of this, amyloidal precursor protein (APP) and its processing enzymes are potential therapeutic targets for RNAi (Singer et al. 2005). The therapeutic potential of RNAi has been demonstrated for AD by demonstrating allele-specific gene silencing by shRNA to selectively suppress mutant APP (Rodriguez-Lebron and Paulson 2006). To deliver shRNA, researchers used an AAV5 vector and injected this virus into the hippocampus of a transgenic APP-mutant mouse model of AD. This resulted in a significant reduction of mutant APP, while the expression of normal murine APP was not affected (Rodriguez-Lebron et al. 2009). Consequently, mice treated with shRNA displayed improved psychometric performance (e.g., spatial learning and object recognition tasks) (Rodriguez-Lebron et al. 2009), demonstrating the utility and high specificity of RNAi for this debilitating neurodegenerative disease.

Frontotemporal dementia

Frontotemporal dementia (FTD) is characterized by the progressive degeneration of the frontal and anterior temporal cortex of the brain. Frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17), Tau mutations are the cause of the disease which leads to the formation of neurofibrillary tangles subsequent to neuronal dysfunction and degeneration. Although the precise mechanisms by which these mutant proteins cause neuronal injury are unclear, compelling data suggests that these mutant proteins themselves initiate the disease pathogenesis. Researchers have also used siRNA in HeLa cells to specifically target a missense Tau mutation, V337M, that causes frontotemporal dementia with parkinsonism linked to chromosome 17 (FTDP-17) (Miller et al. 2003, 2004). Moreover, the study demonstrates that suppressing the expression of the mutant protein by RNAi should ameliorate or prevent the disease (Yamamoto et al. 2000). This study demonstrates that RNAi is capable of selectively targeting disease alleles differing from normal alleles by as little as a single nucleotide, as in frontotemporal dementia, and highlight a key role for SNPs in extending the utility of siRNA in dominantly inherited disorders. However, these experiments must be validated in animal models. Currently, there are murine models available for many dominant disorders such as MJD_SCA3 (Cemal et al. 2002), HD (Lin et al. 2001), and FTDP-17 (Tanemura et al. 2002), and should accelerate the in vivo testing of siRNA-based therapy for these and other human diseases.

Parkinson's disease

Similar to other neurodegenerative diseases, PD which affects nearly 2% of adults and is the second most common neurologic condition is a consequence of the accumulation of cellular proteins and abnormal protein clearance. This leads to the formation of cytoplasmic Lewy bodies (Eriksen et al. 2005) which consists mainly of α-synuclein (Spillantini et al. 1997). α-Synuclein is aggregation-prone and is toxic when over-expressed or mutated (A53T and A30P mutations have been identified in familial PD) (Polymeropoulos et al. 1997; Singleton et al. 2003); therefore, it is a rational target for RNAi therapy. The function of α-synuclein is not clear and α-synuclein knockout mice do not show any adverse effect, indicating that suppression of α-synuclein in vivo by RNAi strategy may have no adverse effect in human (Abeliovich et al. 2000).

To demonstrate the utility of RNAi strategy, researchers used a lentiviral expressed shRNA approach to successfully suppress only the expression of the A53T mutant allele in a rat model where mutant human α-synuclein transgene was expressed in rat brain. Although RNAi silencing of mutant α-synuclein was successful in the rat model, the therapeutic efficacy of suppressing mutant α-synuclein has yet to be determined (Sapru et al. 2006). Future studies are expected to determine whether silencing mutant α-synuclein provides therapeutic benefit in mouse models for PD. Since these studies utilize a gene therapy approach, applications in human are not expected any time soon.

Slow channel congenital myasthenic syndrome

In another study, researchers (Abdelgany et al. 2003) demonstrated allele-specific inhibition of a pathogenic mutant acetylcholine receptor subunit by RNAi for the treatment of the SCCMS as a model for an excitotoxic autosomal dominant neurological disorder. SCCMS is a disorder of the neuromuscular synapse caused by dominantly inherited missense mutations in genes encoding the muscle acetylcholine receptor (AChR) subunits (Croxen et al. 2002) and provides a model for allele-specific gene silencing by RNAi. The authors selectively inhibited mutant AChR containing the a-subunit SCCMS mutation aS226F using siRNA and shRNA in mammalian cells expressing wild-type or mutant AChR subunits and successfully discriminated the mutant allele from the wild-type transcripts when the nucleotide mismatch is at position 9 in the guide strand of siRNA and shRNA.

There are several other CNS disorders where RNAi therapy may prove useful in treatment. For example, RNAi

has been used to reduce neuropathic pain, prevent neuronal death induced by brain ischemia, minimize the damage caused by prion proteins, and induce apoptosis or increase chemotherapy susceptibility in brain tumors (Gondi et al. 2004; Zhang et al. 2004; Pfeifer et al. 2006; Dore-Savard et al. 2008; White et al. 2008; Sun et al. 2009).

Pachyonychia congenita

Pachyonychia congenita (PC) is a rare, dominant negative keratin disorder with no known cure. Utilizing RNAi technology, Kaspar et al. (Leachman et al. 2008) have designed an siRNA that selectively inhibited a mutant allele of *KRT6A*, the most commonly affected PC keratin. Preclinical in vitro studies have demonstrated the potent and selective inhibition of the mutant allele by RNAi and reversal of the cellular aggregation phenotype. These early studies finally paved the way for a phase Ib clinical trial (Leachman et al. 2008, 2010). For this (Leachman et al. 2008) phase Ib study, a single patient with pachyonychia congenital was registered and the safety and efficacy of siRNA (TD101) was tested in a 17-week, prospective, double blind, split-body, vehicle-controlled, dose-escalation trial on a single patient. SiRNAs or vehicle controls were delivered via injection into symmetric plantar calluses on the opposite feet. The results showed no adverse effects during the study or in the 3-month washout period. Patient assessment and clinical efficacy measures demonstrated regression of callus on the siRNA-treated, but not on the vehicle-treated foot enabling RNAi a potent and only available treatment of PC. RNAi therapy for PC is currently in phase IIb clinical trial with a promising outcome. If phase II efficacy study is demonstrated, this will be the "first-in-skin" RNAi therapy in the treatment of dominant negative genetic disorders.

RNAi therapy for human diseases caused by aberrant splicing

Many genetic diseases are caused by the aberrant exon splicing resulting in the production of alternatively spliced variant gene expression (Gaur 2006). Alternatively spliced mRNAs not only lead to protein diversity in vertebrates, but are also the cause of the 35–70% of human genes that generate alternatively spliced transcripts that are associated with numerous human genetic diseases and various forms of cancer.

The disease-associated alternative splicing is often caused by mutations of the *cis* and *trans* acting elements leading to the expression of variant forms of mRNA, the normal and the abnormal. The unbalanced expression of both isoforms of genes involved in processes ranging from cell cycle regulation to angiogenesis have been implicated in certain forms of cancer (Gaur 2006) and other diseases. Since aberrant splicing is associated with many diseases, the potential of RNAi and antisense-based technologies for modulating mRNA splicing is well recognized. Two strategies are used to do this: (1) splice junction modulation by antisense-based approaches where splice junction is blocked or shifted such that the aberrant splicing is blocked (Kole et al. 2004; Graziewicz et al. 2008) or (2) tools that specifically target and degrade a disease-linked mRNA isoform (Zhu et al. 2005).

Sickle cell anemia

An SNP mutation in the sickle β-globin gene (β^S), a single nucleotide substitution in codon 6 (GAG → GTG) of the Hb β-chain gene is the cause of sickle cell anemia (SCA) (Dykxhoorn et al. 2006). The Glu to Val substitution produces an Hb variant (Hb^S) that polymerizes upon deoxygenation to produce long rigid fibers that distort red blood cell (RBC) reducing flexibility. The sickled RBCs are less able to transit through the microvasculature leading to vasoocclusion, localized hypoxia, painful crises, and organ damage and are susceptible to hemolysis leading to anemia. Sickling increases sharply with deoxy sickle Hb concentration and decreases with increasing fetal hemoglobin (HbF) concentration. At present, there is no cure. Hydroxyurea (HU) treatment, which increases fetal γ-globin subunit production; thus, decreasing sickle cell crises, is the only treatment available (Charache et al. 1987). Disease severity has been reduced in mouse models of SCA when gene therapy was used to increase the intra erythrocytic concentration of a modified βA mutated in position 87Thr3Gln, a key residue for the antisickling properties of HbF (Pawliuk et al. 2001).

More recently, RNAi has been used (Dykxhoorn et al. 2006) for SNP-selective targeting of disease-causing alleles of human β-globin. In this study, researchers used allele-specific siRNAs designed such that the SNP of the β-globin mRNA was aligned at nucleotide position 10 of the guide strand of siRNA and their efficacy were tested on an allele-specific luciferase reporter in tissue culture. In vitro results demonstrated that β^S siRNA exclusively suppressed the β^S gene, without affecting the expression of the normal βA or β-globin genes. Interestingly, despite the high specificity of the β^S siRNA, the corresponding βA siRNA not only silenced βA but also silenced the β^S reporter and cDNA construct. Researchers identified that central mismatches consisting of pyrimidine:pyrimidine or pyrimidine:purine residues did not completely block RNAi activity while bulky mismatched purines at position 10 completely blocked RNAi activity suggesting that mismatches at the mRNA cleavage site involving pyrimidines are tolerated.

Moreover, suppression of β^S expression by RNAi would result in unbalanced α chain expression, leading to α chain precipitation, and hemolysis. This imbalance might be mitigated if silencing β-globin resulted in compensatory enhanced expression of fetal γ-globin. Because of this, siRNA-mediated silencing could be done in conjunction with the therapeutic expression of *βA87Thr3Gln*-globin (Pawliuk et al. 2001) or β-globin (Samakoglu et al. 2006) to provide the dual advantages of silencing and replacing the mutant allele with an antisickling form of globin.

To reduce the expression of Hb^S by silencing the *βS* gene by RNAi without diminishing the expression of the β or the normal *βA* gene, researchers used a strategy by coexpression of a shRNA against $\beta^S$and a γ-globin transgene and showed the therapeutic benefit in human erythrocytes derived from lentivirally transduced hematopoietic stem cells (Samakoglu et al. 2006). Because sickling increases sharply with deoxy sickle Hb concentration and decreases with increasing fetal γ-globin concentration, a large clinical benefit could be obtained with relatively modest pan erythroid suppression of β-globin.

Cancer

In general, there are three potential targets for cancer therapies: (1) genes that are part of cancer-associated pathways, (2) those that are involved in tumor-host interactions, or (3) those that are part of chemo- or radiotherapy resistance (Gartel and Kandel 2006; Pai et al. 2006; Takeshita and Ochiya 2006; Grimm 2007).

The oncogenes that are the most promising targets are associated with malignant transformation, and are either amplified, mutated, result from chromosome or gene rearrangement, or are exogenously introduced by transforming viruses in human tumors. Although multiple genetic mutations have been identified in most of human tumors, including dominant mutant oncogenes; it is often not clear which of these mutant oncogenes are continuously required for tumorigenesis (Brummelkamp et al. 2002). Many other cancer targets for RNAi therapies can be found in recent reviews (Gartel and Kandel 2006; Pai et al. 2006; Takeshita and Ochiya 2006; Grimm 2007; Kim 2009). Hence, RNAi has become a logical strategy to selectively target each of these mutant alleles that define the oncogenesis, including novel fusion proteins that define many forms of cancer (Damm-Welk et al. 2003; Barik 2004; Ameyar-Zazoua et al. 2005). For example, researchers used a lentiviral vector to target *bcl-2*, whose overexpression specifically in human gliomas mediates protection from apoptosis (Kock et al. 2007). The researchers also used another vector to express a secreted form of TRAIL, an extraneous apoptosis-inducing agent. Delivery of both vectors into glioma cells and subsequent transplantation into nude mice resulted in a significant reduction of tumors, exemplifying the utility of both RNAi and gene therapy technologies. Similarly, others used a lentiviral RNAi approach to inhibit *BRAF* and *Skp-2* genes that are frequently up-regulated and mutated in melanoma cells and showed significant anti-tumor efficacy from this combinatorial strategy (Sumimoto et al. 2006).

Fusion oncogenes which are especially prevalent in lymphoproliferative cancers are attractive targets for RNAi, as chimeric mRNA is unique to tumor cells. The *bcr-abl* fusion oncogene in chronic myelogenous leukemias (CML) resulting from t(9;22) translocations is an example. Researchers (Sengupta et al. 2006) targeted the *bcr-abl* junction in primary $CD34^+$ CML cells using an Epstein–Barr virus-encoded shRNA in primary $CD34^+$ CML cells which led to apoptosis (Sengupta et al. 2006). They also demonstrated the utility of gene therapy strategy by co-expressing a dominant negative form of p27 (a cdk inhibitor) which potentiated the effect.

RNAi in combination with anticancer drugs have also been investigated in mice to test whether a combination therapy can promote apoptosis and reduce tumor burden. For example, Chen et al. (2004) targeted the TEL-PDGFßR oncogene (leads to leukemia in human) using retroviral RNAi in mice. Inhibition of this gene extended disease latency and survival of mice that received transplants, but also mediated synergism with imatinib, an inhibitor of tyrosine kinases. Similarly, Landen et al. (2005) demonstrated that inhibition of tyrosine kinase receptor EphA2 by siRNA resulted in a 50% reduction of tumor size and the combination of siRNAs and paclitaxel resulted in a 50% reduction of tumor size in ovarian cancer cells (Landen et al. 2005).

Many other fusion oncogenes in solid tumors (e.g., mucoepidermic salivary gland tumors or rhabdomyosarcomas) are other potential targets for RNAi therapy (Larsen and Rasko 2005; Komiya et al. 2006; Taulli et al. 2006; Venturini et al. 2006) and additional cancer targets for RNAi therapies can be found in recent reviews (Gartel and Kandel 2006; Pai et al. 2006; Takeshita and Ochiya 2006; Grimm 2007; Kim 2009).

RNAi strategy has also been described to effectively silence other cancer related genes, including mutant forms of p53, KRas and Bcr-Abl (Dillon et al. 2005). The tumor suppressor which most frequently mutates in human cancers is p53 (Berns 2010). Reactivation of p53 in tumors, which induces programmed cell death and cell-cycle arrest is an attractive therapeutic strategy. Almost half of all human cancers contain point mutations of p53. Because of this, selective suppression of mutant gene would be the logical strategy for suppression of cancer causing mutant allele by RNAi. However, two recent studies suggested that

restoring p53 activity in mouse models of cancer affects only advanced tumors, leaving early lesions untouched (Feldser et al. 2010; Juntilla et al. 2010).

In pancreatic and colon carcinomas, Ras genes are often mutated providing an opportunity for targeting this mutant allele with RNAi technology. In many cases, the Ras oncogenes contain SNP mutations that differ by a single-base mutation from their normal alleles. Therefore, RNAi has been a logical strategy for selective suppression of mutant KRas. Bernards et al. (Brummelkamp et al. 2002) used a retroviral RNAi strategy for SNP-selective suppression of only the oncogenic Ras (KRas V12) allele in human tumor cells resulting from a G3T transition in codon 12 but not normal KRas (Brummelkamp et al. 2002). The results showed effective inhibition of the level of K-RasV12 transcripts in with virally transduced cells resulted in loss of anchorage-independent growth and tumorigenicity (Brummelkamp et al. 2002; Wilda et al. 2002). The KRasV12 specific siRNA and normal KRas mRNA contained a purine:purine (A:G) mismatch at position 9 relative to the 5′ end of the guide strand of the siRNA, supporting the hypothesis that that the juxtaposition of these bulky residues would not target the normal allele; thus making this siRNA highly selective.

In another example RNAi has been used for the selective targeting of fusion gene called Bcr-Abl. Bcr-Abl is the result of the translocation of the Philadelphia chromosome (Ph) which leads to expression of a constitutively active protein tyrosine kinase that induces and maintains leukemic transformation in chronic myelogenous leukemia and Ph-positive acute lymphoblastic leukemia. The exclusive specificity of the RNAi approach was employed to target exclusively the oncogenic Bcr-Abl transcript. Results from this study have demonstrated the selective suppression of the oncogenic fusion transcript Bcr-Abl without affecting normal c-ABL and c-BCR transcripts (Scherr et al. 2003; Wohlbold et al. 2003).

From all the cancer RNAi programs to date, only Bcr-Abl safety and efficacy clinical trials are complete (Koldehoff et al. 2007). However, this trial treated a single patient with recurrent CML by systemic delivery of a formulated siRNA and Bcr-Abl knockdown in circulating leukemic cells was difficult to assess due to prior chemotherapy (Vaishnaw et al. 2010). Nevertheless, these results suggest that RNAi can be exploited for allele-specific inhibition of tumor-specific genes or oncogenic mutant genes to reverse the oncogenic phenotype of cancer cells.

Genetic skeletal diseases

The utility of RNAi to target mutant alleles in dominantly inherited skeletal genetic disease has also been recognized (Wallace 2010). As in other studies, siRNAs can be designed to specifically distinguish mutant from normal alleles of a genetic skeletal disease and block only mutant allele expression. This could convert a dominant negative disorder, i.e., a disorder in which the product of the mutant allele interferes with the function of the normal allele product, to a disorder that results from haploinsufficiency or functional loss of one allele. For families in which both forms occur, manifestations are usually milder in the form resulting from haploinsufficiency, such as osteogenesis imperfecta type I—haploinsufficiency and osteogenesis type II—dominant negative. (Horton 2005) In summary, there is a potential clinical benefit to be exploited from RNAi-based therapeutic strategy.

RNAi for dominant muscular dystrophies and other myopathies

X-linked recessive Duchenne muscular dystrophy (DMD) is the most common muscular dystrophy (Flanigan et al. 2001), followed by the dominant disorders, myotonic dystrophy type 1 (Harper 1989) and facioscapulohumeral muscular dystrophy (FSHD) (Flanigan et al. 2001; Tawil and Van Der Maarel 2006; Tawil et al. 2010). Mutations in at least 29 known genes lead to various dominant muscular dystrophies and other related myopathies.

In recessive genetic muscular dystrophy disorders such as DMD, gene therapy strategies have been used to replace defective or missing genes. However, gene replacement strategies are not indicated for treating dominant genetic diseases where reduction or elimination of the abnormal allele would prevent the onset of disease in individuals with these mutations. Until the emergence of RNAi strategy, there was no practical strategy to reduce or eliminate mutant genes for dominant muscular dystrophies.

Since RNAi approach could potentially be applied to suppress dominant disease genes, including those involved in muscular dystrophy and related myopathies, several reports have shown the utility of this technology in vitro and in vivo in animal models for these diseases. Consequently, demonstration of RNAi therapeutic efficacy in one myopathy could act as a proof-of-concept role for an entire class of disorders.

Myotonic dystrophy type 1

DM1 is the most common dominantly inherited muscular dystrophies. Development of targeted treatments to these diseases would potentially have the broadest benefit for patients with dominant muscle disease, making them logical candidates for RNAi therapy.

DM1 is caused by CTG trinucleotide repeat expansion in the DMPK 3′ UTR, which causes nuclear retention of

this toxic mRNA (Cho and Tapscott 2007). Patients with this genetic abnormality develop myotonia, resulting in skeletal muscle weakness and cardiac conduction abnormalities that are often fatal. Heterozygous and homozygous knockout animal models for DMPK both manifest skeletal and cardiac muscle sodium channel gating abnormalities that parallels conduct ion defects in human DM1 (Berul et al. 1999; Mounsey et al. 2000; Lee et al. 2003). Older DMPK± knockout mice also demonstrate mild, variable sarcomeric disorganization, myofiber regeneration, and decreased force production (Reddy et al. 1996). While these phenotypes support that DMPK haploinsufficiency may contribute to some DM1 pathologies, modulation of this genetic disease by RNAi is complicated because normal and mutant DMPK alleles are identical except for the 3′ UTR trinucleotide repeat expansion.

Because inhibition of normal DMPK could reduce therapeutic benefit of RNAi by inhibiting the expanded mutant allele, an alternative approach must be developed to target the mutant allele with disease-linked polymorphisms, located outside the CTG repeat area. For example, using a miRNA based approach one can target specifically the mutant DMPK 3′ UTR CTG repeat expansion. This should result in a therapeutically sufficient inhibition of mutant DMPK, potentially resulting in haploinsufficiency-related DM1 phenotypes.

Alternatively, an siRNA specific to both alleles can be used to target both the mutant and normal DMPK while expressing a normal DMPK modified with base changes that prevent its regulation by the therapeutic siRNA. However, this approach requires a gene therapy approach to express both the shRNA and the DMPK gene from an expression cassette. Another problem in the field is compartmentalization of RNAi reagents with the targeted mRNAs. It has been shown that mutant DMPK RNA is localized in the nucleus, whereas siRNA is considered to be cytoplasmic, questioning if an RNAi strategy could be effective for targeting. Interestingly, a number of studies have shown that RNAi also functions in the nucleus (Ting et al. 2005; Kim et al. 2006; Kim and Rossi 2008). It was shown that RNAi can suppress the expression of localized nuclear RNA, such as 7SK and DMPK (Langlois et al. 2005; Robb et al. 2005; Ohrt et al. 2008; Weinmann et al. 2009), implying that RNAi strategy for DM1 is possible, provided that issues discussed above as well as issues with effective delivery strategies are addressed before human use.

Current clinical trials

A recent search on a database (http://www.clinicaltrials.gov) revealed 2,269 studies when ‘‘gene therapy’’ was searched, while only four when “RNA interference OR RNAi” were used. When searched for siRNA, it resulted in 19, OR shRNA showed only one OR miRNA showed 20 studies. However, most of the miRNA studies in these studies examined endogenous miRNAs as biomarkers for disease and for pharmacogenomics studies. In addition, few safety and feasibility studies were identified, which were based on the delivery of siRNA therapeutics for cancer (5 studies), macular degeneration (5 studies), kidney injury (2 studies), autosomal dominant inherited diseases (1 study), hypercholesterolemia (1 study), and hepatitis (1 study).

The following conclusions are drawn from this analysis: (1) while gene therapy-based studies dominate the list and are considered for a wide range of conditions, RNAi-based therapeutics are in its infancy and (2) clinical trials to date focus largely on siRNAs.

In a recent report, the authors discussed RNAi clinical trials in detail (Vaishnaw et al. 2010). Based on this report, a total of 14 RNAi therapeutic programs have entered clinical trials in recent years. One half of these involve local/topical delivery to the eye (four), respiratory tract (two) and skin (one) while the rest are systemic delivery targeting liver (two), hepatic and extrahepatic cancer (three), kidney (one), and leukocytes (one).

To date, only Bcr-Abl (Koldehoff et al. 2007) and TD101 (Leachman et al. 2008) (Transderm Inc., Santa Cruz, CA, USA) have completed safety and efficacy studies that have been published (Vaishnaw et al. 2010). The Bcr-Abl study treated only one patient with recurrent CML by systemic administration of a formulated siRNA against Bcr-Abl (Koldehoff et al. 2007). The TD101 study (Leachman et al. 2008) also treated a single patient over a 17-week period with pachyonychia congenita and no adverse events were reported during the trial or in the 3-month washout period.

Challenges and future perspectives to RNAi therapeutics

There has been increasing interest in harnessing the naturally occurring RNAi mechanism, which enables degradation of a specific mRNA, as a novel pharmacological approach to human disease.

As discussed earlier, from a drug discovery perspective, RNAi strategy has some unique advantages over conventional drugs such as small molecules or antibodies.

However, there are many issues that have to be addressed before RNAi therapeutics can enter clinical trials, including steps required for selection of potent siRNAs, the use of chemical modifications to confer appropriate biopharmaceutical properties (RNAi reagents should remain stable in biological fluids and avoid rapid clearance;

the design of formulations should enable delivery to a target tissue and activate the RNA-RISC for RNAi in the target cells), and screening of these products for safety and efficacy, including assessments for potential off-target effects. Major problems with RNAi and any other antisense-based technologies are delivery, specificity and stability of the RNAi reagents. There are two main issues in developing RNAi as a therapy: (1) avoiding off-target effects and (2) efficient delivery to targeted tissues. Another potential complication is the competition with the endogenous miRNA machinery that can be very detrimental in vivo (Grimm et al. 2006). Moreover, with RNAi, only the antagonism of the specific molecular target is possible, whereas small molecules, proteins and antibodies can act as agonists of a molecular target.

Off-targeting effects and siRNA design considerations

Cells are controlled by the dynamic actions of many gene networks interacting with one another. Because of the existence of these dynamic complex gene networks, the loss-of-function of any gene by RNAi may affect other genes, pathways or the entire network (Zhao et al. 2005). Because of this, off-target effects are a major problem of RNAi-based therapeutics.

Off-targeting effects can occur when: (1) the sequence of the RNAi reagent is identical or nearly identical to other mRNA sequence causing a phenotype (Jackson et al. 2003). Careful choice of the siRNA duplex sequence can eliminate such sequence-dependent effects by simply removing these sequences removed from siRNA designs on a continuing basis; (2) sequences that are identical to "seed" regions (nucleotides 2–8 from 5′-end of the guide strand) of the endogenous miRNAs which can cause miRNA-like unrelated gene silencing by pairing with a complementary sequence in the 3′ UTR region of an unrelated mRNA (Jackson et al. 2003; Lin et al. 2005; Birmingham et al. 2006; Jackson et al. 2006a, b). This happens when the siRNA sequence partly pairs with a weakly complementary sequence in the 3′ UTR region of an unrelated mRNA. In this case, siRNA may function similar to a miRNA leading to suppression of an irrelevant gene expression through mRNA degradation or translational block. This problem can be eliminated by screening out siRNAs for the presence of any known human and cross-species specific miRNA seed sequences by BLAST search. Unintended off-target effects in mRNA can be further assessed by mRNA microarrays, which generally show that few off-target mRNAs are reduced by more than twofold, although for some genes changes of twofold or less may be clinically significant. However, it is more challenging to examine the impact of unintended changes in protein expression. Additionally, to minimize such off-targeting effects, the second residue in the guide strand of the siRNA (a key residue in the seed region for endogenous miRNA activity) has been chemically modified to block unintended off-target effects without interfering with silencing of the target gene (Jackson et al. 2006a, b); (3) double-stranded RNA (dsRNA) can activate the antiviral type I interferon response in a sequence-independent fashion [e.g., activation of protein kinase (PKR) signaling] (Sledz et al. 2003); however, this problem is usually exacerbated when saturating concentrations of siRNAs are introduced to cells (Sledz and Williams 2004); (4) siRNAs can activate innate immune responses by interacting with toll-like receptors (TLRs) (Agrawal and Kandimalla 2004) on the surface of cells or in endosomes (Hornung et al. 2005; Judge et al. 2005) or other signaling pathways are triggered. The presence of GU-rich motifs in the exogenously delivered siRNA or shRNA 33 (Judge et al. 2005) (e.g., 5′-UGUGU-3′ or 5′-GUCCUUCAA-3′) have been identified as the major contributors to this type of response and have only been observed with primary peripheral blood leukocytes and plasmacytoid dendritic cells. In addition to avoiding TLR-activating sequences, chemical modifications of the siRNAs that do not abrogate silencing can be used to block TLR activation. Several strategies are available to bypass the unintended immunostimulatory effects of siRNA drugs, and this potential toxicity is not likely to impede siRNA drug development. Candidate siRNAs for clinical use must be screened to verify that they do not activate inflammatory pathways. Since the number of effective siRNAs for any gene can be numerous, excluding siRNAs that bind to TLRs is not likely to affect silencing of any target gene. This will help to identify potential nonspecific effects that may be due to general cellular responses to siRNA delivery methods or to RNAi itself; and (5) siRNAs or shRNAs may saturate the cell's RNAi machinery; thereby, inhibiting the function of endogenous miRNAs although this is less of a concern for the exogenously delivered siRNAs than the vector expressed shRNAs. This type of nonspecific effect was seen when siRNAs interfere and/or compete with the processing and function of endogenous miRNAs. High level expression of miRNA-like shRNAs, using viral vectors (Grimm et al. 2006), has been shown to interfere with endogenous miRNA nuclear export by exportin 5 (Grimm et al. 2006). Since they are directly incorporated into RISC, exportin 5 is not involved in the function of artificial siRNAs. Nonetheless, it is essential to use the lowest level of siRNA or shRNA that achieves an efficient knockdown. Given that the level of stable shRNA expression achieved by lentiviral or retroviral vectors is comparatively modest, nonspecific activation of the innate immune response by these vectors appears less likely.

Although siRNAs will not compete with miRNAs at the upstream miRNA biogenesis pathway, high intracellular quantities of siRNAs can compete for limiting amounts of Dicer (for longer siRNA precursor drugs) or RISC. Longer siRNAs that need to be processed by Dicer but are still below the 30 nucleotide cutoff may be more effective at silencing than 21 nucleotide siRNAs, possibly because Dicer processing helps to incorporate siRNAs into the RISC (Kim et al. 2005). In animal studies induction of interferon-responsive genes has not been detected with these siRNAs. Changing the method of siRNA delivery and 'titrating down' the amount of siRNA delivered may decrease unwanted effects. This can be accomplished by the use of multiple siRNA species for each target gene in pools of three to five to maximize knockdown and minimize off-targeting effects caused by high concentrations of individual siRNAs.

It has been suggested that one siRNA can cleave as many as ten cognate mRNAs. This catalytic nature of mRNA targeting by siRNAs, where the same RISC can be turned over to silence mRNAs suggest that a potent siRNA will effectively function at much lower concentrations without saturating the endogenous miRNA machinery. It has been estimated that, it may take only about 1,000 siRNA molecules/cell to silence gene expression efficiently (an estimate derived from the frequencies of individual endogenous miRNAs in cells; Cullen 2006). Quantitative information about the numbers of siRNAs required for efficient gene silencing would be important for establishing safe dosing regimen for RNAi drugs and to avoid potential toxicity. Because most siRNAs have some effect, any RNAi reagent that will be used as a therapeutic must be carefully assessed for any potential off-targeting effects. Careful choice of the siRNA duplex sequence and designing siRNA sequences devoid of any of the above mentioned sequences can minimize or eliminate this type of off-targeting effects. One strategy to prevent the nonspecific activation of the cellular interferon response due to long stretches of RNA duplex may be to design the siRNAs or shRNAs such that they do not contain any stretches of perfect RNA duplex of $\geq$11 bp (Cullen 2006). For example, natural miRNA precursors contain such imperfect duplexes with varying degrees of mismatched or bulged bases that disrupt the perfectly matched duplex structure of the miRNA stem (Lagos-Quintana et al. 2001). Although, some of the naturally occurring endogenous miRNAs are expressed at high levels, yet presumably they do not trigger the interferon response, thereby, one strategy would be to mimic the structure of native miRNA precursors in the designs of siRNA to avoid inducing nonspecific effects. Several reports have shown the utility of this strategy by introducing miRNA-like structural features into design of siRNA or shRNAs including symmetrical bulge near the center of the predicted 19–20 bp stem of the duplex (Cullen 2006) such that no stretch of perfect dsRNA of $\geq$11 bp will be present in the duplex (Manche et al. 1992).

Several reports have demonstrated design features which can be incorporated into the design of siRNA or shRNAs to circumvent sequence-dependent (specific) or sequence-independent (nonspecific) off-targeting effects by the RNAi reagents during the in vitro or in vivo experiments (Pei and Tuschl 2006). In addition, interferon responses and cell activation and/or differentiation that might be induced by the delivery methods or reagents used for the RNAi reagent delivery to target cells must also be examined to identify the source of any off-targeting effect during the optimization and pilot screen phase of screens.

Delivery

For any RNA targeting drugs (siRNA, miRNA mimetic or antimir, antisense, LNA, ribozyme, etc.,) the delivery of these reagents to target cells at therapeutically effective concentrations appears to be the most significant challenge as it has been for the antisense-based drugs. This issue has been a major concern for the further advancement of RNAi and similar antisense-based technologies and a major breakthrough is needed to overcome this problem. Since delivery to cells has been the major obstacle, as a consequence this restricts the therapeutic efficacy and provide only partial inhibition of target. The partial inhibition of target gene expression may be sufficient to provide therapeutic benefits in diseases including neurodegenerative diseases such as PD and AD but diseases such as cancer suffers significantly from limited targeted delivery.

The main reason why nucleic acid-based drugs as well as other biologics (e.g., mAbs and other protein-based drugs) have this problem is that: (1) nucleic acids-based drugs are highly negatively charged requiring approaches to shield the negative charge and (2) the molecular weight of a typical siRNA is greater than 13 kDa while a typical antisense weighs 6.5 kDa, much larger than the conventional small molecule pharmaceutics which are usually $<$0.5 kDa.

Since delivery appears to be the main bottleneck as well as the stability, off-target effects, and immune detection and subsequent reactions (see for example (Zimmermann et al. 2006), many researchers have focused their efforts for circumventing these issues by modifying siRNA or attaching them to agents that will chemically stabilize them against serum nucleases until they reach their therapeutic targets and retain them in circulation long enough to improve pharmacokinetics.

Systemic delivery of chemically stabilized siRNAs

Systemically delivered siRNAs face degradation by nucleases, and the use of viral vectors are currently not feasible for human trials because of safety concerns, although new generation viral vectors have demonstrated high safety levels. Chemically stabilized siRNAs have been used successfully to inhibit endogenous genes in living mice providing opportunities for improvement (Soutschek et al. 2004). Chemical modifications such as 2′O-Me, and 2′F of bases and phosphorothioate modifications of the RNA backbone that stabilizes RNA duplex against serum nucleases are the most frequently used modifications that improve stability of siRNAs whereas addition of polyethylene glycol (PEG) moiety improves bioavailability by slowing down the excretion from the kidneys.

Macromolecules such as cholesterol has also been used successfully to improve bioavailability and tissue penetration of siRNAs leading to effective inhibition of the expression of the gene encoding apoprotein B (*apoB*) in the mouse liver and jejunum (Soutschek et al. 2004). Researchers demonstrated that the cholesterol-conjugated siRNAs were more stable in serum and highly potent at lowering *apoB* expression resulting in lower total cholesterol and LDL cholesterol levels. The utility of this approach was demonstrated later in non-human primates with similar efficacy (Zimmermann et al. 2006). Additionally, antibody-protamine (Song et al. 2005; Zhu et al. 2005) cyclodextrin nanoparticles (Hu-Lieskovan et al. 2005) and aptamers (McNamara et al. 2006) were used as conjugates that resulted in tissue or cell type-specific targeting.

These studies demonstrate that backbone modification and attachment of conjugates to improve bioavailability of siRNAs are safe to systemically deliver therapeutically effective doses of siRNAs, paving the way for other future systemic applications of RNAi.

Delivery of siRNAs via various modalities

Full implementation of RNAi as a therapeutic requires the delivery issue to be resolved. Toward this goal several delivery modalities involving nonspecific delivery systems, such as liposomes and cholesterol, have been successful in enhancing siRNA uptake and reducing degradation. Complexing siRNAs to liposomes or attaching siRNAs to peptide-based polymers are the most promising technologies. Additionally, some unique chemical modifications, nanoparticels, virosomes and unique design strategies have been developed to improve serum stability and potency of RNAi reagents.

Systemic delivery of siRNAs has been shown to be most effective in highly vascularized tissues, such as the liver and spleen. While the hydrodynamic delivery, although successful in small lab animals (Ilves et al. 2006), is inappropriate for humans.

Targeted or focal delivery

Systemic delivery of siRNAs to specific cell or tissue type through cell surface receptors is the most ideal scenario to provide the maximal therapeutic benefit with least side-effects.

Toward this end, peptides, aptamers or monoclonal antibodies that recognize specific cell surface receptors have been conjugated to siRNAs, allowing entry into target cells that carry the required receptor (Muratovska and Eccles 2004; Juliano 2005; Chu et al. 2006; Kumar et al. 2008).

The utility of targeted delivery of siRNA was demonstrated for the first time in human phase I clinical trial involving the systemic administration of siRNA to patients with solid tumors by employing a tissue-targeted nanoparticle (Davis et al. 2010). The study reported a reduction in both the specific M2 subunit of ribonucleotide reductase (RRM2) mRNA and the protein levels when compared to pre-dosing tissue indicating that siRNA administered systemically to a human can lead to suppression of a target gene by an RNAi mechanism of action. However, the delivery of the siRNA to the central nervous system (CNS) (Begley 2004) is the most challenging, since large molecules including siRNAs cannot cross the blood–brain barrier (BBB). Although several recent studies have shown the feasibility of intracerebral and intrathecal administration of siRNA in animal models of CNS disease, this strategy is not optimal for humans. Since most of the human genetic disease of CNS appears to be good targets for RNAi, any improvement for the delivery across the BBB or round the BBB will make a significant impact in the treatment of diseases involving CNS. Several strategies to deliver siRNAs across the BBB are in development, including “trojan horse” liposomes which have been developed to overcome many of the problems with siRNA delivery to the CNS (Boado et al. 2007). In this approach, siRNAs are encapsulated in liposome that contain PEG that both stabilize the siRNA and improves their bioavailability by retaining them in the circulation longer by slowing renal excretion. Monoclonal antibodies specific for cell receptors on tissues are attached to the PEG to permit transcytosis across the tissue. Liposomes can ferry siRNAs or shRNA expression vectors in which tissue-specific promoters can be incorporated for tissue-specific expression. Furthermore, multi-targeting ligands can be incorporated into the liposomes for targeting multiple targets simultaneously. For example, two different monoclonal antibodies or even aptamers (McNamara et al. 2006) can be coupled to the liposomes,

one is specific to BBB to allow transport across the BBB and a second is specific to a cell type-specific receptor to facilitate the delivery of nucleic acid cargo to the appropriate cell (Lovett-Racke 2009).

Aptamers have also shown their potential for siRNA delivery. A prostate specific membrane antigen (PSMA) aptamer selected from a library was successfully used to selectively target a prostate cancer cell line LNCaP expressing PSMA inducing cell death (McNamara et al. 2006). In another study, researchers used an siRNA-aptamer chimera which the aptamer was selected to target an anti-HIV envelope (gp120) and siRNA was designed to target HIV tat/rev common exon and showed selective inhibition of HIV replication only in cells infected with HIV (Zhou et al. 2008).

Various site-specific (focal) delivery strategies including direct injection of siRNAs to specific tissue or organ have been used to demonstrate the utility of RNAi-mediated gene inhibition; however, mode of delivery of siRNAs and duration of their effect are limited to specific tissues or organs and by the half-life of the siRNAs, respectively (Boison 2010).

Focal delivery of RNAi reagents often involves direct injection of synthetic and chemically stabilized siRNAs ("naked" or complexed with liposomes) into the target tissue or organ (Wolff and Budker 2005; Leachman et al. 2008) or inhalation (e.g., lungs) directly into a target organ or tissue. For example, intraocular injection of siRNA for the treatment of age-related macular degeneration (AMD) is analogous to intraocular injection of other pharmaceutical drugs. However, distribution of siRNAs throughout the tissue and into the targeted cells is challenging requiring repeated injections to achieve long-term effect.

As for the treatment of diseases of CNS, because of poor penetration of the BBB and direct injections into the brain is not a feasible option for humans, RNAi treatment of diseases of CNS remains a major challenge. Although pumps and other delivery devices have been proposed, they are not practical and can cause infection.

Duration of silencing

Although synthetic siRNAs are easy to produce, their effects are transient and dictated by the half-lives of siRNAs requiring repeated application similar to conventional drugs. Improvements in the siRNA design via backbone modifications (e.g., 2′-O-Methyl) and attachment of bulky conjugates (cholesterol or PEG) have improved their stability and bioavailability of siRNAs by stabilizing them against serum nucleases and reducing their excretion from the kidneys.

However, this transient effect also makes them safer as compared to gene therapy approaches which tend to be permanent depending on the vector used (AAV although is considered gene therapy approach, its effect is transient).

Repeated administration of siRNAs via local injection or inhalation into lungs might be useful for early clinical studies or in select cases (Leachman et al. 2008), long-term silencing by RNAi can only be achieved using viral vectors engineered to express shRNAs (Raoul et al. 2006).

Lentiviruses and adeno-associated viruses (AAVs) are the most frequently used viral vectors for this purpose. Because lentiviruses integrate into the genome, they are advantageous when targeting dividing cells, so that therapeutic gene expression can be maintained through cell division, whereas, AAVs may be advantageous for targeting nondividing or quiescent cells, such as neurons in the CNS. Viral vectors can permit some cell specificity based on the type of virus, promoters that express shRNAs, and cell surface molecules that mediate viral entry. Because AAVs replicate episomally and do not integrate into genome of the host, they are perceived safer because they do not cause insertional mutagenesis.

Albeit focal injection have been used as a way to deliver the virus to specific tissue or organ, the ability of virus to diffuse within tissue and transduce target cells limits their use (Davidson and Breakefield 2003). Recently, researchers have reported that an AAV serotype 8 and 9 can cross the BBB and infects a variety of neuronal cells and astrocytes throughout the spinal cord and brain, when delivered into the vasculature of neonatal mice (Harding et al. 2006; Foust et al. 2009).

Despite the safety concerns associated with viral vectors, the long term and possible regulatable expression of RNAi reagents from tissue-specific promoters and relative ease of delivery of genetic cargo within these vectors makes them promising technology. Because of this, AAV-based vectors might be most suitable for those applications as they combine safety, tropism for brain cells, and long-term efficacy (McCown 2005; Noe et al. 2009; Riban et al. 2009; Gray et al. 2010).

Conclusions

In recent years, from an academic curiosity, RNAi has become a robust technology for studies of gene function in mammals and a potential new class of therapy against many human diseases. Early proof-of-principle studies in animal models have supported the utility of RNAi as selective and potent gene expression inhibitors without apparent toxicity.

Although a number of preclinical studies are in progress, many challenges must be addressed before RNAi can become a marketable drug. Because the delivery has been the major problem for RNAi and other antisense-based

approaches, further progress in this area is required. Towards this goal, strategies encompassing multiple delivery technologies including novel conjugation and formulation strategies or even a gene therapy approach using safer vectors must be explored.

While large scale phase III clinical trials and regulatory approvals still remain distant, translation of animal studies using basic RNAi research has progressed into early human trials which few of them are in progress. Nonetheless, the field must progress further to enable the necessary advancement of this therapeutic class to patients with genetic diseases where there is otherwise no cure and any potential failure resulting from a number of factors such as delivery and clinical trial design should not hamper this progress. Given the pace of new findings and discovery of applications, RNAi will remain as a major new class of therapy in the foreseeable future.

Review criteria

PubMed and internet were used to identify articles published on the subject discussed in this review. Following search terms used to identify relevant articles: "RNA interference" AND "genetic disease", "inherited disease" AND "dominant negative genetic disease", or "SNP" AND "RNAi" were used. The search was restricted to the recent studies involving RNAi and all searches were limited to human inherited genetic diseases published in English.

Acknowledgments I would like to thank Jenifer Weissert and Jocelyn Jakubik for helpful comments and critical reading of the manuscript.

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