REVIEW ARTICLE

Application of RNA interference in treating human diseases

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Abstract

Gene silencing can occur either through repression of transcription, termed transcriptional gene silencing (TGS), or through translation repression and mRNA degradation, termed posttranscriptional gene silencing (PTGS). PTGS results from sequence-specific mRNA degradation in the cytoplasm without dramatic changes in transcription of corresponding gene in nucleus. Both TGS and PTGS are used to regulate endogenous genes. Interestingly, mechanisms for gene silencing also protect the genome from transposons and viruses. In this paper, we first review RNAi mechanism and then focus on some of its applications in biomedical research such as treatment for HIV, viral hepatitis, cardiovascular and cerebrovascular diseases, metabolic disease, neurodegenerative disorders and cancer.

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Introduction

Epigenetic regulation of gene expression is a heritable change in gene expression that cannot be explained by changes in gene sequence. It can result in repression or activation of gene, referred to as gene silencing or gene activation, respectively (Vaucheret *et al*. 2001). During the 1990s, a number of gene-silencing phenomena that occurred at the posttranscriptional level were discovered in plants, fungi, animals and ciliates, introducing the concept of post-transcriptional gene silencing (PTGS) or RNA silencing. (Baulcombe 2000; Matzke *et al*. 2001) PTGS results in the specific degradation of a population of homologous RNAs. PTGS was first observed after introduction of an extra copy of an endogenous gene (or of the corresponding cDNA under the control of an exogenous promoter) into plants (Napoli *et al*. 1990; Smith *et al*. 1990; Van der Krol *et al*. 1990). Because RNAs encoded by both transgenes and homologous endogenous gene(s) were degraded, the phenomenon was originally called co-suppression. A similar phenomenon in the fungus *Neurospora crassa* was named quelling (Romano and Macino 1992; Cogoni *et al*. 1996). Fire *et al*. (1998) identified a related mechanism, RNA interference (RNAi) in animals. RNAi results in a specific degradation of endogenous RNA in the presence of homologous dsRNA either locally injected or transcribed from an inverted-repeat transgene (Tavernarakis *et al*. 2000; Vaucheret *et al*. 2001). They applied single-stranded antisense RNA and double-stranded RNA in their experiments. To their surprise, it was found that dsRNA was more effective at producing interference than either strand individually. After injection into an adult *Caenorhabditis elegans*, single-stranded antisense RNA had a modest effect in diminishing specific gene expression whereas double-stranded mixtures caused potent and specific interference (Fire *et al*. 1998; Zou and Yoder 2005). RNAi is a multistep process involves the generation of small interfering RNAs (siRNAs) *in vivo* through the action of the RNase III endonuclease '*Dicer*'. The resulting 21 to 23-nucleotide (nt) siRNAs mediate degradation of their complementary RNA (Shi 2003; Zou and Yoder 2005). Hamilton *et al*. (2002) have now discovered second category of siRNAs, long siRNAs (25 nt), distinguishable by size from 21–22-nt siRNAs class they had previously found (Hamilton and Baulcombe 1999; L. Timmons, H. Tabara, C. Mello and A. Fire 2002 Systemic RNAi. Mid-west Worm Meeting). Unlike the 21–22-nt siRNAs, long siRNAs do not participate in PTGS (Hamilton *et al*. 2002). ARGONAUTE4 and long siRNAs direct chromatin modifications, including his-

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tone methylation (Zilberman *et al*. 2003). In this review, we attempt to summarize the mechanism of RNAi and some of its applications in medicine.

Mechanism of RNAi

RNAi, which can cause the degradation of virtually any RNA, involves a simple mechanism. Long dsRNA is processed to short interfering RNAs (siRNAs) by the action of a dsRNA-specific endonuclease known as *Dicer* (Bernstein *et al*. 2001; Hammond *et al*. 2000). The resultant siRNAs are 21 to 24 nt in length, are double stranded, and have 3′ overhangs of 2 nt (Stevenson 2004).

Exogenous synthetic siRNAs or endogenous expressed siRNAs can also be incorporated into the RNA-induced silencing complex (RISC), thereby bypassing the requirement for dsRNA processing by *Dicer*. siRNAs are incorporated into the multiprotein RISC. A helicase in RISC unwinds the duplex siRNA, which then pairs by means of its unwound antisense strand to messenger RNAs (mRNAs) that bear a high degree of sequence complementarity to the siRNA (Stevenson 2004). Cleavage of the target mRNA begins at a single site 10 nt upstream of the 5′-most residue of the siRNA—target mRNA duplex (Elbashir *et al*. 2001). Although the composition of RISC is not completely known, it includes members of the Argonaute family (Hammond *et al*. 2001) that have been implicated in processes directing post-transcriptional silencing (Stevenson 2004). Argonaute proteins were first implicated in RNAi when the RNAi-deficient 1 (rde-1) gene was identified in a large-scale genetic screen for proteins required for RNAi in *C. elegans* (Tabara *et al*. 1999; Zamore 2006). Argonaute proteins are essential components of the RNAi machinery that associate with distinct classes of small RNAs to exert their effector functions. One branch of the Argonaute family, the PIWI subfamily of proteins, form complexes with Piwi-interacting RNAs (piRNAs) and are essential for restricting the activity of transposons in the germ line. Argonaute proteins are associated with small interfering RNAs (siRNAs) or microRNAs (miRNAs), and silence gene expression by either siRNA guided cleavage of the target mRNA transcript, or by miRNA-mediated post-transcriptional repression involving both translational inhibition and/or mRNA degradation. In *Drosophila* there are three PIWI proteins and two proteins of the argonaute family, AGO1 and AGO2. Genetic and biochemical evidence has demonstrated functional specialization in fly AGO proteins, with AGO1 binding to miRNAs and AGO2 being associated with siRNA-mediated-gene silencing. Functional specialization extends to the biogenesis pathways associated with these small RNAs; miRNAs are processed from endogenous hairpin precursors by cleavage events involving the RNaseIII enzymes *Drosha* and *Dicer1* (Dcr-1) with its partner loquacious (Loqs). siRNAs loaded into AGO2 are processed from long dsRNAs by *Dicer2* (Dcr-2) and its partner R2D2, but until recently only siRNAs from exogenous long dsRNAs had been reported in flies and mammals (Rivas 2008). There are two small RNAs in the RNAi pathway: small interfering RNAs (siRNAs) and microRNAs (miRNAs) that are generated via processing of longer dsRNA and stem loop precursors (Novina *et al*. 2002; Yin and Wan 2002; Tijsterman and Plasterk 2004). *Dicer* enzymes play a critical role in the formation of these two effectors of RNAi (Tijsterman and Plasterk 2004). They can cleave long dsRNAs and stem-loop precursors into siRNAs and miRNAs in an ATP-dependent manner, respectively (Tan and Yin 2005).

The biogenesis of miRNAs is a multistep process (Kim 2005). A primary miRNA transcript (pri-miRNA) (Lee *et al*. 2002), which is frequently synthesized from intronic regions of protein-coding RNA polymerase II transcripts (Cai *et al*. 2004; Lee *et al*. 2004), is first processed by a protein complex containing the double-strand specific ribonuclease *Drosha* in the nucleus to produce a hairpin intermediate of 70nt (Lee *et al*. 2003). This precursor miRNA (pre-miRNA) is subsequently transported by exportin-5/RanGTP (Lund *et al*. 2004; Yi *et al*. 2003) to the cytoplasm where it is cleaved by another dsRNA specific ribonuclease, *Dicer*, (Bernstein *et al*. 2001; Hutvagner *et al*. 2001) into miRNA duplexes. After strand separation of the duplexes, the mature single-stranded miRNA is incorporated into an RNA-induced silencing complex (RISC)-like ribonucleoprotein particle (miRNP) (Hutvagner *et al*. 2001; Martinez *et al*. 2002a; Tang 2005; Yekta *et al*. 2004; Weiler *et al*. 2006) (figure 1).

RNAi has several applications in biomedical research, immune system and health care such as treatment for HIV, viral hepatitis, cardiovascular and cerebrovascular diseases, metabolic disease, neurodegenerative disorders and cancer. Some of these applications are reviewed in the following sections (table 1).

Effect of dsRNAs on the immune system

There is significant debate over the ability of siRNAs and longer dsRNAs to induce innate immune response (Manche *et al*. 1992; Sledz *et al*. 2003; Kim *et al*. 2004). Though mammalian cells are exquisitely sensitive to the introduction of dsRNA, molecules less than 30 bp in length are generally believed to avoid induction of interferon (IFN) pathways (Manche *et al*. 1992; Kariko *et al*. 2004; Kim *et al*. 2004; Siolas *et al*. 2004). Recent studies have also suggested that long (27–29 bp) dsRNAs and shRNAs that enter the RNA interference (RNAi) pathway in a *Dicer*-dependent fashion provide more efficient gene silencing than shorter, *Dicer*-independent substrates (Kim *et al*. 2004; Siolas *et al*. 2004; Reynolds *et al*. 2006).

Introduction of dsRNA molecules into most mammalian cells causes global, nonspecific suppression of gene expression. Although Toll-like receptor 3 has been identified as a dsRNA-response protein (Alexopoulou *et al*. 2001), the two best characterized dsRNA pathways found in most cell types signal through the dsRNA recognition proteins PKR (dsRNA-dependent protein kinase) and 2-,5-oligoadenylate synthetase, and have been recognized for over 20 years.

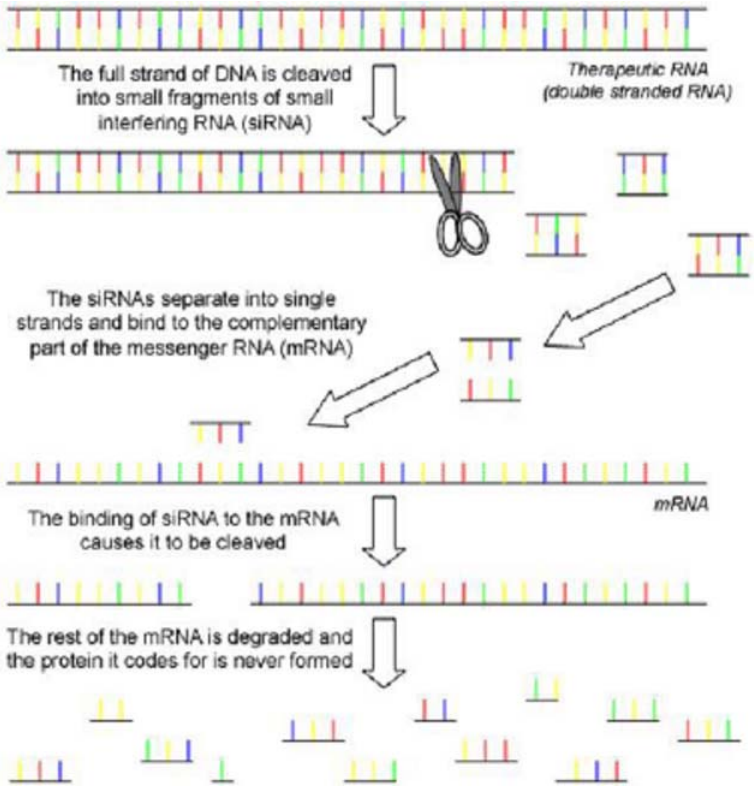

Figure 1. Schematic picture of RNAi mechanism.

Table 1. Applications of RNAi in treating human disease.

Disease	Case study	Reference
Cancer		
Lymphoblastic leukemia	Using siRNAs specific for the BCR–ABL transcript to silence the oncogenes	Scherr *et al*. (2003), Wohlbold *et al*. (2003)
Pancreatic and colon carcinomas	The use of retroviral vectors to introduce interfering RNAs specific for an oncogenic variant of K-RAS	Brummelkamp *et al*. (2002), Wilda *et al*. (2002)
Colonic adenocarcinoma	Downregulation by miRNAs miR-143 and miR-145	Michael *et al*. (2003)
Bladder cancer	Treatment by miRNAs as biomarkers	Saito *et al*. (2006)
HIV	Downregulation of the cellular cofactors required for HIV infection by RNAi	Jacque *et al*. (2002), Coburn and Cullen (2002), Novina *et al*. (2002), Martinez *et al*. (2002b), Capodici *et al*. (2002), Banerjea *et al*. (2003), Li *et al*. (2003), Hannon and Rossi (2004), Surabhi and Gaynor (2002)
Viral hepatitis	Inhibition of *Fas* expression by siRNA	Song *et al*. (2003)
Cardiovascular and cerebral vascular diseases	Using RNAi to intervene in the process of atherosclerosis or to reduce the damage to heart tissue and brain cells	Reddy (2007)

Table 1 (*contd.*)

Disease	Case study	Reference
Ocular diseases	Shutting down production of VEGF by siRNA	Campochiaro (2006)
Malaria	RNAi can identify the genetic factors shape the vector parasite relationship may be crucial to identifying new genetic means of controlling mosquito-borne diseases	Brown *et al*. (2003), Vlachou and Kafatos (2005)
Metabolic disease and neurodegenerative disorders	Treatment of these diseases with miRNAs as potential therapeutic targets	Colussi *et al*. (2000), Weiler *et al*. (2006)

Activation of both of these pathways by dsRNA results in the general inhibition of protein synthesis (Williams 1999). PKR initiates a signalling cascade that results in the production of interferons. Interferon, in turn, activates cellular signalling pathways that culminate in the nucleus with the upregulation of interferon-stimulated genes, mediators of antiviral and antiproliferative and pro-apoptotic activity (Kumar *et al*. 1994). Cells exposed to interferon are sensitive to very low levels of dsRNA, eventually leading to cell death. These properties initially precluded the use of RNAi based on long (> 100 bp) dsRNA targets of *Dicer* as an effective research tool in these systems (Sledz and Williams 2004).

Application of RNAi in biomedical research and health care

RNAi is being used for a variety of purposes including biomedical research and health care (Gupta 2006) and has begun to produce a paradigm shift in the process of drug discovery (Hannon and Rossi 2004). In order to meet this objective, dsRNA molecules have been designed for silencing of specific genes in humans and animals. Such silencing RNA molecules are introduced into the cell to facilitate activation of the RNAi machinery. This method has already become an important research tool in biomedicine. Several recent publications show successful gene silencing in human cells and experimental animals. For instance, a gene causing high blood cholesterol levels was shown to be silenced by treating animals with silencing RNA. Plans are also underway to develop silencing RNA as a treatment for cardiovascular diseases, cancer, endocrine disorders, and virus infections (Gupta 2006), such as those caused by the hepatitis C virus (HCV) and the human immunodeficiency virus (HIV) (Hannon and Rossi 2004).

RNAi and cancer

Cancer is a genetic disease in which mutational and/or epigenetic changes in a genome lead to stepwise deregulation of cell proliferation and cell death mechanisms (Weiler *et al*. 2006). There are two classes of genes in which mutations cause cancers. Oncogenes were initially identified as genes carried by viruses that cause transformation of their target cells. A major class of the viral oncogenes has cellular counterparts that are involved in normal cell functions. The cellular genes are called proto-oncogenes, and in certain cases their mutation or aberrant activation in the cell to form an oncogene is associated with tumour formation. About 100 oncogenes have been identified. The oncogenes fall into several groups, representing different types of activities ranging from transmembrane proteins to transcription factors, and the definition of these functions may therefore lead to an understanding of the types of changes that are involved in tumour formation (Miller and Therman 2001).

Tumour suppressors are detected by deletions (or other inactivating mutations) that are tumourigenic. The mutations represent loss-of-function in genes that usually impose some constraint on the cell cycle or cell growth; the release of the constraint is tumourigenic. It is necessary for both copies of genes to be inactivated (Miller and Therman 2001; Lewin 2003).

RNAi is being explored as a way to inhibit the expression of genes involved in oncogenesis. The translocation of the Philadelphia chromosome (Ph) generates a fusion gene called *BCR–ABL*. The translation product of this gene creates a constitutively active protein tyrosine kinase that induces and maintains leukaemic transformation in chronic myelogenous leukaemia and Ph-positive acute lymphoblastic leukaemia (Stevenson 2004). The siRNAs specific for the BCR–ABL transcript have been shown to silence the oncogenic fusion transcripts without affecting expression levels of normal c-ABL and c-BCR transcripts (Scherr *et al*. 2003; Wohlbold *et al*. 2003).

Pancreatic and colon carcinomas, in which *RAS* genes are often mutated, provide another example of the use of RNA silencing in treating cancers. In many cases, the *RAS* oncogenes contain point mutations that differ by a single-base mutation from their normal counterparts. The use of retroviral vectors to introduce interfering RNAs specific for an oncogenic variant of K-*RAS* (called K-RAS^{V12}) reduces the level of K-RAS^{V12} transcripts and effects a loss of anchorage-independent growth and tumourigenicity (Brummelkamp *et al*. 2002; Wilda *et al*. 2002). Studies of these kind provide

proof-of-concept for RNAi-based strategies aimed at reversing tumourigenesis. A major factor confounding cancer treatment is resistance to chemotherapeutic agents. The siRNAs have been used to decrease the drug resistance of cells *in vitro* by inhibiting the expression of *MDR1*, a multidrug transporter with a major role in multidrug resistance (Nieth *et al*. 2003).

Evidence is emerging that particular miRNAs may play a role in human cancer pathogenesis (Weiler *et al*. 2006). For example, deletions or mutations in genes that code for miRNA tumour suppressors might lead to loss of a miRNA or miRNA cluster, and thereby contribute to inappropriate stabilization of oncogenes (McManus 2003; Gong *et al*. 2005). The results of a recent large-scale miRNA study suggest that 50% of miRNA genes are frequently located in cancer-associated genomic regions or fragile sites (Calin *et al*. 2004). The genes encoding mir-15 and mir-16 are located at chromosome 13q14, a region that is deleted in the majority of B-cell chronic lymphocytic leukaemias (B- CELL) (Calin *et al*. 2002), and in other cancers such as mantle cell lymphoma and prostate cancer (Stilgenbauer *et al*. 1998). Interestingly, none of the protein-coding genes in this region were found to cause B-CLL (Migliazza *et al*. 2000), suggesting that mir-15 and mir-16 may possibly function as tumour suppressors. MiRNAs, miR-143 and miR-145, display significant downregulation in colonic adenocarcinoma samples compared to matched normal mucosa tissues (Michael *et al*. 2003). Putative mRNA targets of these miRNAs include several genes that have been implicated in oncogenesis such as RAF1 kinase, G-protein 7 and tumour-suppressing subfragment candidate 1, although molecular interaction of these genes with their putative miRNA counterparts *in vivo* remains to be proven (Weiler *et al*. 2006).

MicroRNAs as robust diagnostic and prognostic biomarkers

MiRNAs are excellent biomarkers for the diagnosis and prognosis of cancer. Due to their gene regulation activities, the potential for using miRNA in cancer therapy is evident. So-called anti-miRNA oligonucleotides (AMOs), which are designed to be complementary to oncogenic miRNAs, are able to specifically inhibit miRNA activity in tumours. On the other hand, overexpression of miRNAs that act as tumour suppressors might also be beneficial for anticancer therapy. MicroRNAs provide not only promising therapy approaches for cancer, but also for many other diseases like virus infections or cardiovascular diseases, in which they are also involved as gene regulators. While the understanding for the gene regulation driven by miRNAs is under extensive research focus, the knowledge about the mechanisms regulating the gene expression of the miRNAs themselves still needs to be broadened. Amongst others, miRNAs are thought to be controlled by epigenetic mechanisms not only due to their tissue and tumour specific expression patterns. As a matter of fact, several miRNAs have shown to be regulated by DNA methylation. Treating human bladder cancer cells with demethylating agents, Saito *et al*. (2006) have shown that ~5% of the human miRNAs became upregulated more than three-fold. The strongest effect was seen in miR-127, whose corresponding gene was found to be embedded in a CpG island. After epigenetic reactivation of miR-127, one of its target genes, the proto-oncogene BCL6, became downregulated, leading to the assumption that miR-127 acts as a tumour suppressor gene. In cases like these, an epigenetic anticancer therapy becomes feasible (Lange and Stahler 2009).

RNAi as a treatment for HIV

HIV was the first infectious agent targeted by RNAi, perhaps because the life cycle and pattern of gene expression of HIV is well understood (Hannon and Rossi 2004). Synthetic siRNAs and expressed shRNAs have been used to target several early and late HIV-encoded RNAs in cell lines and in primary haematopoietic cells including the TAR element (Jacque *et al*. 2002), tat (Lee *et al*. 2002), rev (Lee *et al*. 2002; Coburn and Cullen 2002), gag (Novina *et al*. 2002; Park *et al*. 2002), env (Park *et al*. 2002), vif (Jacque *et al*. 2002), nef (Jacque *et al*. 2002), and reverse transcriptase (Surabhi and Gaynor 2002; Hannon and Rossi 2004).

Despite the success of RNAi-mediated inhibition of HIV-encoded RNAs in cell culture, targeting the virus directly represents a substantial challenge for clinical applications because the high viral mutation rate will lead to mutants that can escape being targeted (Boden *et al*. 2003). Therefore, RNAi-mediated downregulation of the cellular cofactors required for HIV infection is an attractive alternative or complementary approach. Cellular cofactors such as NF-κB (Surabhi and Gaynor 2002), the HIV receptor CD4 (Novina *et al*. 2002), and the co-receptors CXCR4 and CCR5 (Martinez *et al*. 2002b) have been successfully downregulated by RNAi, resulting in the inhibition of HIV replication in numerous human cell lines and in primary cells including T lymphocytes and haematopoietic stem-cell-derived macrophages (Jacque *et al*. 2002; Capodici *et al*. 2002; Coburn and Cullen 2002; Martinez *et al*. 2002b; Novina *et al*. 2002; Surabhi and Gaynor 2002; Banerjea *et al*. 2003; Li *et al*. 2003; Hannon and Rossi 2004).

RNAi to treat viral hepatitis

Acute liver failure and subacute liver failure, induced by viral hepatitis, are associated with high mortality rates. In animal models of liver failure, liver toxins and viral hepatitis lead to apoptosis of hepatocytes through cell-death receptors such as *Fas* (Stevenson 2004). The siRNAs targeted to *Fas* RNA and delivered by high-pressure injection into the tails of mice were able to reach the liver, inhibit *Fas* expression, and protect mice from hepatitis (Song *et al*. 2003). Similarly, siRNAs that target CASP8 RNA (encoding caspase 8) pre-

vent acute liver failure induced by *Fas* activation (Zender *et al*. 2003; Stevenson 2004).

Hepatitis induced by the hepatitis B virus (HBV) and by HCV is a major health problem. At present hundreds of millions of individuals are infected worldwide. There is an effective vaccine against HBV, but this treatment is only useful for the prevention of viral infection and there is no vaccine for HCV (Hannon and Rossi 2004). Therefore, hepatitis caused by these two viruses has been an important target for potential RNAi therapy. The first demonstration of RNAi efficacy against a virus *in vivo* involved hydrodynamic co-delivery of an HBV replicon and an expression unit encoding an anti-HBV shRNA in mice (McCaffrey *et al*. 2003). This study demonstrated that a significant knockdown (99%) of the HBV core antigen in liver hepatocytes could be achieved by the shRNA, providing an important proof of principle for future antiviral applications of RNAi in the liver. Subgenomic and full-length HCV replicons that replicate and express HCV proteins in stably transfected human hepatoma-derived Huh-7 cells have been used to study the effects of various antiviral drugs (Lohmann *et al*. 1999; Blight *et al*. 2000; Pietschmann *et al*. 2001; Ikeda *et al*. 2002; Hannon and Rossi 2004).

In another *in vivo* study, siRNAs were used to treat fulminant hepatitis induced by an agonistic *Fas*-specific antibody in mice. Anti-*Fas* siRNAs were hydrodynamically injected into the antibody-treated mice: 82% of the mice survived for 10 days of observation whereas all control mice died within three days (Song *et al*. 2003). Importantly, mice already suffering from auto-immune hepatitis also improved after the *Fas* siRNA treatment. So it may be feasible to use siRNAs to ameliorate the severity of certain diseases by targeting the inflammatory response pathways rather than the infectious agent (Hannon and Rossi 2004).

As with HIV therapeutics, delivery of the siRNAs or shRNA vectors is the main challenge for successful treatment of HCV. The method of delivery used in several *in vivo* studies—hydrodynamic intravenous injection—is not feasible for the treatment of human hepatitis (Hannon and Rossi 2004).

In mice, genetic material can be introduced into hepatocytes using catheters or even localized hydrodynamic procedures (Eastman *et al*. 2002), but it is yet to be determined whether such procedures can be used to deliver siRNAs in larger mammals (Hannon and Rossi 2004).

RNAi and cardiovascular and cerebrovascular diseases

Cardiovascular disease most commonly results from the progressive occlusion of arteries in a process called atherosclerosis, which can ultimately culminate in a myocardial infarction or stroke, resulting in the death of cardiac muscle cells or neurons. Although some of the cells die rapidly by necrosis, many other cells die more slowly by apoptosis; data from animal studies suggest that such cardiac myocytes and brain neurons that die by apoptosis can be saved (Mattson 2000; Zhao and Vinten-Johansen 2002). It may be possible to use RNAi technology to intervene in the process of atherosclerosis or to reduce the damage to heart tissue and brain cells that patients suffer following a myocardial infarction or stroke (Reddy 2007).

siRNA and ocular diseases

The eye is a relatively isolated compartment, which is easily accessed, and its enclosed nature makes ocular diseases ideal targets for siRNA-based therapies (Wallach 2004). siRNA injected into the vitreous cavity readily diffuses throughout the eye and is detectable for at least five days, the amounts used for intraocular injections are small compared to those used for systemic application and so as siRNA gets out of the eye it is diluted and is difficult to detect. The sequence specificity of siRNA resulting in targeting of a single gene combined with local administration in the relatively isolated confines of the eye provides an ideal way to study eye-specific effects of gene disruption (Campochiaro 2006).

The first ever clinical trial in man administering an siRNA-based therapeutic was initiated in autumn 2004 by Acuity Pharmaceuticals in patients with AMD (age-related macular degeneration). An siRNA targeting the growth factor VEGF (vascular endothelial growth factor), which is believed to be a primary cause of overgrowth of blood vessels in the 'wet' form of AMD, was administered by intravitreal injection. The primary goal of this phase 1 study was to evaluate the safety of the siRNA. Independently, Sirna Therapeutics developed a siRNA targeting VEGF receptor 1 for AMD (Sirna-027) and started a clinical trial a few months later. A single intravitreal dose of Sirna-027, ranging from 100–800 μg, appears to be safe and well tolerated with no systemic or local adverse events related to the drug. So far, according to the clinical investigators, all patients enrolled in this trial have experienced visual acuity stabilization during their trial participation. Since no dose-limiting toxicity has been observed, dose escalation will continue with the objective of defining a maximum tolerated dose (Cejka *et al*. 2006).

Further clinical trials for siRNA-based compounds are also planned, and the diseases targeted cover a broad range spanning from ocular diseases, viral infections and cancer to metabolic disorders. In a recent study, Bitko *et al*. (2005) evaluated a siRNA strategy targeting RSV (respiratory syncytial virus) and PIV (parainfluenza virus), two major pathogens causing croup, pneumonia and bronchiolitis (Easton *et al*. 2004). Alnylam, an siRNA company probably holding the biggest share of intellectual property regarding RNAi technology, is about to initiate clinical trials for targeting RSV. To date, no effective therapies for established RSV-induced disease or prophylactic vaccines preventing full-blown infection are available, despite intensive research efforts (Maggon and Barik 2004; Cejka *et al*. 2006).

RNAi and malaria

Despite intense efforts, malaria remains a leading cause of morbidity and mortality worldwide. Recent evidence strongly suggests that RNAi can play a key role in identifying the genetic factors shape the vector parasite relationship may be crucial to identifying new genetic means of controlling mosquito-borne diseases (Brown *et al*. 2003; Vlachou and Kafatos 2005). RNAi can be made inheritable in *Anophele* mosquitoes by stably transforming the mosquito with a transgene that contains two copies of the target gene arranged in an inverted repeat configuration (Brown *et al*. 2003). Hairpin RNA is expressed *in vivo* whenever the inverted repeat is transcribed from an upstream promoter. By placing dsRNA expression under the control of a tissue-specific and time-specific promoter, dsRNA expression can be tailored to coincide spatially and temporally with the journey of the parasite through the mosquito. Both parasite receptors and immune components protective of the parasite are putative targets for engineering parasite resistance through RNAi and, in principle, mosquito strains that have been rendered refractory to malaria transmission could be released in the field to replace wild type, permissive populations and achieve malaria eradication (Reddy 2007).

miRNA in metabolic disease

RNAi can be used to silence endogenous genes involved in the cause or pathway of metabolic diseases and holds considerable promise as a therapeutic approach to silence disease-causing genes, particularly those that encode so-called 'non-drugable' targets. In addition, the high potency, specificity and chemical structure of siRNAs may eliminate the toxicity and adverse events commonly seen with small molecule drugs. Several studies have demonstrated efficient *in vivo* delivery of siRNAs and therapeutic benefit in mice. Taniguchi *et al*. (2005) developed an adenovirus-mediated RNAi technique that utilizes shRNAs to substantially and stably reduce the expression of insulin receptor substrates IRS-1 and IRS-2 specifically in the liver of mice to better understand the roles of these proteins in hepatic insulin action. By knocking down IRS-1 and IRS-2 in the liver separately and together, they showed that IRS-1 signalling may be more closely linked to the regulation of genes involved in glucose homeostasis, whereas IRS-2 signalling may have specific roles in the regulation of hepatic lipid metabolism (Taniguchi *et al*. 2005). Vector-based RNAi approach was also used to induce PTGS of hepatic PEPCK using nonviral gene delivery. PEPCK is the rate-controlling enzyme in gluconeogenesis and altered rates of gluconeogenesis are responsible for increased hepatic glucose output and sustained hyperglycemia (Hanson and Reshef 1997). RNAi holds promise for the development of novel therapeutic strategies for disorders that are yet difficult to treat and might be beneficial for the treatment of diseases, such as obesity, neuropathic pain and depression (Reddy 2007).

In addition, two recent publications have described miRNAs as potential therapeutic targets for the treatment of diabetes and obesity. In both studies, the respective mRNA targets were predicted and subsequently experimentally validated in cellular systems (Weiler *et al*. 2006). The pancreatic islet-specific mir-375 was found to modulate glucose-stimulated insulin secretion and exocytosis, by blocking the expression of myotrophin, a protein associated with neuronal secretion (Poy *et al*. 2004). In a similar manner, Esau *et al*. (2004) suggested a potential implication of miRNAs in the maturation of human adipocytes. Modified oligonucleotide complementary to miR-143 effectively suppressed adipocyte differentiation by modulation of its putative target ERK5, a protein previously known to be implicated in MAP kinase signalling pathways, but which had not yet directly been linked to adipocyte differentiation (Weiler *et al*. 2006; Esau *et al*. 2004).

RNAi and neurodegenerative disorders

Advances in targeted delivery of RNAi-inducing molecules has raised the possibility of using RNAi directly as a therapy for a variety of human genetic and other neural and neuromuscular disorders. Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis (ALS) are examples of relatively common age-related neurodegenerative disorders that are increasing as average life expectancy increases. Each disorder is characterized by the dysfunction and death of specific populations of neurons: hippocampal and cortical neurons involved in learning and memory processes in Alzheimer's disease, dopamine-producing neurons in the substantia nigra that control body movements in Parkinson's disease, and spinal cord motor neurons in amyotrophic lateral sclerosis. Specific genetic mutations are responsible for a small percentage of cases of Alzheimer's and Parkinson's diseases and amyotrophic lateral sclerosis (Hardy 2001), whereas all cases of Huntington's disease result from mutations (polyglutamine expansions) in the huntington protein (Rubinsztein 2002). Recent studies have shown that cultured neurons can be efficiently transfected with siRNAs and that the targeted genes are effectively silenced. Pro-apoptotic members of the Bcl-2 family (Colussi *et al*. 2000) and neuronal death prevented, using RNAi methods. Also, DiGeorge syndrome is a rare congenital disease whose symptoms vary but include heart defects and characteristic facial features. It is caused by a large deletion from chromosome 22 and two recent studies revealed that *dgcr8*, a gene located within the deleted region, encodes a protein which interacts with *Drosha* during the processing of primary miRNA transcripts (Gregory *et al*. 2004; Landthaler *et al*. 2004).

RNAi and stem cells

In the six years since the initial report, RNAi has now been demonstrated to function in mammalian cells to alter gene

expression, and has been used as a means for genetic discovery as well as a possible strategy for genetic correction. An equally popular topic over the past six years has been the proposal to utilize embryonic stem cells or adult stem cells as cell-based therapies for human diseases (Zou and Yoder 2005).

Recent studies have begun to investigate the role of miRNA during normal stem cell development in mammalian and nonmammalian systems. Houbaviy *et al*. (2003) identified miRNAs in undifferentiated and differentiated mouse embryonic stem (ES) cells. Some of the miRNAs seemed to be ES-specific, and these ES-specific miRNAs were repressed as ES cells differentiated into embryoid bodies and were undetectable in adult mouse organs and tissues (Zou and Yoder 2005). Finally, miRNAs isolated from adult hippocampal neural stem cells played a critical role in mediating neuronal differentiation (Kuwabara *et al*. 2004). miRNAs are also involved in the regulation of stem cell development in nonmammalian organisms. Martinho *et al*. (2004) recently identified that maintenance of primordial germ cell (PGC) self-renewal and inhibition of PGC differentiation towards somatic cell fate in *Drosophila* requires the non-coding pgc miRNA. RNA-polymerase-typeII-dependent transcription is normally repressed in PGCs of many animals during early development and this phenomenon might be important for the maintenance of germ line fate by preventing somatic cell differentiation. Germ cells lacking pgc miRNA, expressed a number of genes important for differentiation of nearby somatic cells (Zou and Yoder 2005).

In *C. elegans*, sequential stage-specific expression of the lin-4 and let-7 miRNAs triggers transition in the complement of heterochronic regulatory proteins to coordinate developmental timing (Reinhart *et al*. 2000). Besides lin-4 and let-7, 15 new miRNAs have been cloned and some of them vary in abundance during *C. elegans* larval development (Leen and Ambros 2001). Thus constitutive miRNAs expressed in the *C. elegans* may play important roles in the regulation of development (Zou and Yoder 2005).

RNAi applications to research

Stem-cell function

ES cells are pluripotent stem cells derived from the inner cell mass of the 3.5-day-old mouse blastocyst (Evans and Kaufman 1981; Zou *et al*. 2000). These cells are an attractive model to study the molecular regulation of cell lineage commitment and cellular differentiation because ES cells can give rise to cells derived from all three primary germ layers: endoderm, mesoderm and ectoderm. Therefore, the ability to selectively knockdown specific target genes would aid in the understanding of multiple aspects of early development (Zou *et al*. 2003). Yang *et al*. (2001) demonstrated the ability to diminish expression of a target gene in undifferentiated ES cells by *in situ* production of long dsRNA from a transient transfection of a plasmid harbouring a 547 bp inverted repeat or by direct transfection of a 740-bp dsRNA made by *in vitro* transcription. However, these long dsRNAs could only mediate RNAi in undifferentiated ES cells but not differentiated ES cells. As an alternative approach, Zou *et al*. (2003) transfected well-differentiated ES cells with siRNA, and found that these oligonucleotides were effective in diminishing the expression of such genes as PU1 and c-EBPα (Zou *et al*. 2003).

Oct4 is a transcription factor which has been characterized as a key regulator of ES cell pluripotency. Genetic studies have indicated that the expression level of Oct4 is important in early lineage commitment of ES cells. Oct4-deficient embryos fail to form an inner cell mass (ICM) but remaining cells commit to the trophoblast lineage (Velkey and O'Shea 2003). Velkey and O'Shea examined whether suppression of Oct4 expression via RNAi would alter ES cell lineage commitment decision. In their study, ES cells were transfected with plasmids containing an independently expressed reporter gene and an RNA polymerase type III promoter to constitutively express small stem-loop RNA transcripts corresponding to Oct4 mRNA. Cells transfected with Oct4 shRNA demonstrated reduced levels of Oct4 mRNA and exhibited characteristics of trophectodermal differentiation. More recently, Oct4 siRNAs delivered by transfection were effective in both human and mouse ES cells in diminishing Oct4 expression (Hay *et al*. 2004).

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