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Standard Review

RNAi in medicine: current and future perspectives

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The discovery of 21 – 23 nucleotide RNA duplexes, called small interference RNA (siRNA) may well be one of the transforming events in biology in the past decade. RNAi can result in gene silencing or even in the expulsion of sequences from the genome. Efforts to understand its mode of action have revealed a central role in gene regulation and host defense. The specificity, efficiency and potency of RNAi makes it an attractive tool for analyzing the function of genes. RNA interference can be exploited artificially to inhibit the expression of any gene of interest. RNA interference systems could be used clinically to suppress gene expression as a therapeutic strategy in many diseases characterized by elevated gene function. Finally, as a therapeutic tool, it has shown enormous promise in the control of a large array of diseases. This review focuses on the potential therapeutic use of RNAi for various diseases, the current understanding of RNAi biology, and how RNAi has been utilized to study the role of different genes in the pathogenesis of cancer, HIV, infectious diseases, HBV, cardiovascular diseases, cerebral diseases, neurogenerative diseases, malaria, among others.

Key words: RNA interference – cancer – HIV – hepatitis B – neurogenerative diseases – malaria – infectious diseases.

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1. INTRODUCTION

In the early days of molecular biology, genes were first defined through the description of their mutant phenotype. Forward genetics has the advantage that the phenotype of the mutant gives a clue to the function of the gene. But with the advent of large-scale genome sequencing, thousands of genes have been identified without of their function. Reverse genetics is now the most effective way to assess the function of a gene, but so far there has been no general method for reverse genetics other than gene targeting by homologous recombination, which is slow and costly. Antisense approaches, such as antisense oligonucleotide and ribozyme technologies, have been useful in reverse genetics, but only to a limited degree. By contrast, the promise of small interfering RNA (siRNA) technology to 'knock down' the expression of any gene in vertebrate cells is set to revolutionize reverse genetic approaches (Michael et al., 2002). In 1998, and at the time of the completion of the *Caenorhabditis elegans* genome project, Andrew Fire and Craig Mello described this new technology based on the silencing of specific genes by double stranded RNA (dsRNA); a technology they called RNA interference (RNAi).

2. RNA interference - Biology

The discovery of 21–23 nucleotide RNA duplexes (Elbashir et al., 2001; Dykxhoorn et al., 2003; Eugene Berezikov et al., 2006), called small interference RNA (siRNA) may well be one of the transforming events in biology in the past decade. Production of small interfering RNAs (siRNAs) that bind to and induce the degradation of specific endogenous mRNAs is now recognized as a mechanism that is widely employed by eukaryotic cells to inhibit protein production at a posttranscriptional level (Olivier Milhavet et al., 2003; Meister et al.,2004). In animals, micro RNAs are transcribed as long primary transcripts (pri-iRNAs) by RNA polymerase II enzyme Esquela-Kerscher and Slack, 2006). They are cropped into hairpin-shaped pre-iRNAs by the nuclear RNase III Drosha-DGCR8 complex. The processing intermediates, pre-iRNAs are exported out of the nucleus by exportin-5 (Exp-5) and subsequently cleaved by the cytoplasmic RNase III, Dicer, into ~22 nucleotides duplexes (Lee et al., 2002; Basyuk et al., 2003; Bryan R Cullen 2006). One strand of the duplex (the mature micro RNA) is incorporated into the RISC (RNA –induced Silencing Complex) or iRNP complex, a protein complex that, when bound to the micro RNA is responsible for the post-transcriptional regulation of the corresponding protein (Denli et al., 2003; Gregory et al., 2005; Matranga et al., 2005; Rand et al., 2005). This complex down regulates the gene expression in one of two ways. (i) Translation inhibition, in case where micro RNA only partially complements to its corresponding mRNA and (ii) Target mRNA cleavage, in case where there is a near perfect complementary between the iRNA and the mRNA.

Salient features of RNA interference are **-** double stranded RNA, high degree of specific gene silencing with less effort, highly potent and effective, silencing can be introduced in different developmental stages, systemic silencing, avoids problems with abnormalities caused by a knocked out gene in early stages and silencing effects passed through generations.

3. Therapeutic Applications of RNA Interference

RNAi was described by Jennifer Couzin in the journal *Science* as the "Break through of the Year" in 2002 having the potential to become a powerful therapeutic drug toward targeted and personal medicine. RNAi-based therapeutics has potentially significant advantages over traditional approaches to treating diseases, including broad applicability, therapeutic precision, and selectivity avoiding side effects associated with currently marketed pharmaceutical products. This widespread applicability, coupled with relative ease of synthesis and low cost of production make siRNAs an attractive new class of small-molecule drugs. RNAi-based drugs are designed to destroy the target RNA and therefore stop the associated undesirable protein production required for disease progression (Julian Downward, 2004; Peter Sandy, 2005; Ryther, 2005; Cristina Rondinone, 2006; Morris, 2006). Finally, as a therapeutic tool, it has shown enormous promise in the control of a large array of diseases. This review focuses on the potential therapeutic use of RNAi for various diseases, the current understanding of RNAi biology, and how RNAi has been utilized to study the role of different genes in the pathogenesis of cancer, HIV, infectious diseases, HBV, cardiovascular and cerebral vascular diseases, neurogenerative diseases, malaria, diabetes and obesity.

3.1 RNAi in Cancer: Advances, Challenges and opportunities

There are two general abnormalities in cancer cells- they exhibit dysregulation of the cell cycle resulting in uncontrolled growth and they are resistant to death as a result of abnormalities in one or more proteins that mediate apoptosis. In cancer cells, proto-oncogenes have frequently been activated by various mechanisms, producing oncogenes that act in a dominant fashion (Nam and Parang, 2003; Hwang and Mendell, 2006). In epithelial tumors, point mutations are predominant where-

as hematological malignancies often show gene fusions that result from chromosomal translocations (Arndt Bork-Borkhardt, 2002). The goals for RNAi approaches for cancer therapy are therefore to knock out the expression of a cell cycle gene and/or an anti-apoptotic gene in cancer cells thereby stopping tumor growth and killing the cancer cells. To selectively eliminate cancer cells without damaging normal cells, RNAi would target a gene specifically involved in the growth or survival of the cancer cell, or the siRNAs would be selectively delivered into the cancer cells by transfection (Olivier Milhavet et al., 2003). Because interference RNA can function either as tumor suppressor or as oncogenes, they have been referred as "Oncomirs" (Auror Esquela-Koscher and Frank Slack, 2006). Recent evidence has shown that miRNA mutations or mis-expression correlate with various human cancers (Auror Esquela-Koscher and Frank Slack, 2006), further asserting their oncogenic or tumor suppressor function. miRNAs have been shown to repress the expression of important cancer-related genes and might prove useful in the diagnosis and treatment of cancer (Calin et al., 2004 ; Lu et al., 2005; Auror Esquela-Koscher and Frank Slack, 2006). A recent study (Lu et al., 2005) showed that about 50% of annotated human miRNAs are located in areas of the genome, known as fragile sites that are associated with cancer and indicates a crucial role in cancer progression (Lin et al., 2005).

The differential expression of certain miRNAs in various tumours might become a powerful tool to aid in the diagnosis and treatment of cancer. Northern-blot analyses and miRNA microarrays have been useful in determining tissue-specific 'signatures' of miRNA genes in humans (Liu et al., 2004; Nelson et al., 2004; Miska et al., 2004; Monticelli et al., 2005). Researchers are now using miRNA-expression signatures to classify cancers and to define miRNA markers that might predict favorable prog-nosis (Iorio et al., 2005; Cimmino et al., 2005; Chan et al., 2005; He et al., 2005; O'Donnell et al., 2005). Gene therapies that use miRNAs might be an effective approach to blocking tumour progression. miRNAs such as *let-7*, which has been shown to negatively regulate the Ras oncogenes, and miR-15 and miR-16, which negatively regulate *BCL2*, are promising candidates for cancer treatment. miRNAs have been shown to repress the expression of important cancer-related genes and might prove useful in the diagnosis and treatment of cancer (Cimmino et al., 2005; Calin et al., 2005).

Recent evidence has shown that microRNAs play a significant role in the development of different forms of cancer. Based on these important findings, Scientists can pursue a range of product development opportunities that will harness the power of microRNAs in the diagnosis and treatment of many different forms of cancer in the years ahead. The key to understanding the roles of iRNA in cancer will be identifying and validating the critical targets responsible for the phenotypic effects of miRNA loss- or gain-of-function. Another potential challenge for future studies relates to the probable tissue-specific functions of some iRNAs.

3.2 Infectious diseases

Diseases caused by viruses and bacteria continue to be major causes of death worldwide and are an increasing concern because of the emergence of resistant strains and the potential use of infectious pathogens by terrorists (Tan et al., 2000; Franz and Zajtchuk, 2002; Olivier Milhavet et al., 2003). The prominent viral infectious diseases include HIV, Influenza, Hepatitis, and West Nile virus and from bacterial infectious diseases pneumonia and sepsis are the prominent examples. The ability of RNAi to inhibit the replication or cellular uptake of viruses and other infectious agents has been clearly demonstrated in cell culture studies (Leonard and Schaffer, 2005). Transfection of human cells with siRNAs directed against different genes in the poliovirus genome resulted in resistance of the cells to infection with poliovirus (Gitlin et al., 2002). The ability of siRNAs targeting the gene encoding the death receptor Fas to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis was tested by Song and colleagues (Song et al., 2003). Intravenous injection of Fas siRNA specifically reduced Fas protein levels in the livers of mice during a 10-day period. Fas siRNA treatment abrogated hepatocyte necrosis and inflammatory infiltration and markedly reduced serum concentrations of transaminases demonstrating a clear heaptopro-tective effect of the siRNA therapy. The observations made in reference (Jopling, 2005; Krützfeldt et al., 2005) was the specific cellular miRNAs can facilitate virus replication, together with the finding that several pathogenic human viruses encode miRNAs that undoubtedly are important in the virus life cycle *in vivo*, suggests that technologies that allow the specific inhibition of miRNA function, such as the recently described antagomir molecules, could have a future role in the treatment of virally induced diseases.

3.3 RNAi in HIV treatment: Advances, challenges and opportunities

HIV was the first infectious agent targeted by RNAi, perhaps because the lifecycle and pattern of gene expression of HIV is well understood. 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, tat, rev, gag, env, vif, nef, and reverse transcriptase (Jacque et al., 2002; Novina et al., 2002; Leonard and Schaffer, 2005; John J Rossi, 2006). 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 leads to mutants that can escape being targeted (Martinez et al., 2002). Therefore RNAi-mediated downregulation of the cellular cofactors required for HIV infection is an attractive alternative or complementary approach. Cellular cofactors such as NFk-B, the HIV receptor CD4, and the co-receptors CXCR4 and CCR5 (Capodici et al., 2003) have been successfully down regulated 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 (Gitlin et al., 2002; Martinez et al., 2002; Boden et al., 2003; Capodici et al., 2003; John J. Rossi, 2006). There are nonetheless, drawbacks in targeting cellular HIV cofactors because non-infected cells will inevitably be targeted and as well, leading to toxicities that are similar to those observed with the current anti-retroviral drugs. Viral targets will need to be included in any successful strategy using RNAi and these targets should be sequences that are highly conserved throughout the various clades to ensure efficacy against all viral strains (Jacque et al., 2002).

An alternative approach to relying solely upon RNAi as an anti-HIV approach is mixing a single shRNA with other antiviral genes to provide a potent combinatorial approach. This has been successfully accomplished by co-expressing an anti-*tat/rev* shRNA, a nucleolar- localizing TAR decoy, and an anti-CCR5 ribozyme in a single vector backbone (Cordelier et al., 2003). A somewhat different combination used a shRNA with a dominant negative Rev M10 protein in a co-expression system (Li et al., 2005). Future investigations should focus on developing more potent combinations of iRNAs with mixtures of noniRNA antivirals for testing in preclinical settings.

The delivery of iRNAs to HIV-infected cells is also a challenge. The target cells are primarily T lymphocytes, monocytes and macrophages (Olivier Milhavet et al., 2003; Cordelier et al., 2003; Anderson and Akkina, 2005; Dmitriy Ovcharenko et al., 2006). Since synthetic siRNAs do not persist for long periods in cells (Julian Downward, 2004), they would have to be delivered repeatedly for years to effectively treat the infection. Systemic delivery of siRNAs to T lymphocytes is probably not feasible owing to the immense number of these cells. Using viral vectors to deliver anti-HIV-encoding short hairpin RNA (shRNA) genes is also problematic, and systemic delivery is not yet practicable because the immunogenicity of these vectors themselves precludes performing multiple injections (Olivier Milhavet et al., 2003; Gitlin et al., 2002; Michienzi et al., 2003). Therefore the preferred method is to isolate T cells from patients, which are then transduced, expanded and re-infused into the same patients. In clinical trial, T lymphocytes from HIV-infected individuals are transduced *ex vivo* with a lentiviral vector that encodes an anti-HIV antisense RNA (Morris and Rossi, 2004; Morris and Looney, 2005). The transduced cells are subsequently expanded and reinfused into patients. This type of therapeutic approach would also be applicable to vectors harboring genes that encode siRNAs. A different approach is to transduce isolated haematopoietic progenitor or stem cells with vectors harbouring the therapeutic genes. These cells give rise to all the haematopoietic cells capable of being infected by the virus. Haematopoietic stem cells are mobilized from the patient and transduced *ex vivo* before reinfusion (Esquela-Kerscher and Slack, 2006) and demonstrated it is more feasible approach (Amado et al., 2004; Banerjea et al., 2003).

Viral vector mediated delivery to hematopoietic cells, including stem cells, is a feasible approach for shRNA gene delivery. Clearly, the barriers that initially confronted therapeutic applications of RNAi for HIV infection are rapidly being broken down, and one can expect to see this powerful cellular process applied clinically to HIV-1 infected patients within the year.

3.4 HBV (Hepatitis-B Virus) as an RNAi target

Over 500 million people worldwide are infected with one or more different and unrelated types of human hepatitis virus. Such individuals are at a high risk of developing acute or chronic hepatic disease, and ultimately dying from sequelae. Although a vaccine is available for hepatitis A and B virus, treatment options for chronically infected patients are limited, and particularly ineffective in case of hepatitis C virus (HCV) infection (Mast et al., 1999; Bowen and Walker, 2005). A promising new avenue currently being explored is to harness the power of RNA interference for development of an antiviral therapy (Grimm and Kay, 2006). The timing to pursue this particular approach is excellent, with the first *in vivo* animal models for HCV infection becoming available (McCaffrey et al., 2003; Persengiev et al., 2004; Taylor and Naoumov, 2005; Locarnini, 2005), and the technology for liver-specific expression of short hairpin RNAs advancing at a rapid pace. Very recently Kusov *et al* (2006) achieved an efficient and sustained suppression of the viral infectivity after consecutive applications of an siRNA targeting a computer-predicted hairpin structure. This siRNA holds promise as a therapeutic tool for severe courses of HAV infection and in addition, these results provide new insight into the structural bases for sequence specific RNAi. HBV makes extensive use of overlapping reading frames within its DNA genome (Cheng et al., 2005), suggesting that while the viral DNA itself cannot be targeted; the multiple HBV RNAs will make the virus highly susceptible for RNAi. HBV is in fact an excellent candidate for therapeutic RNAi because its unusually co-

mpact genome lacks redundancy, resulting in very limited sequence plasticity thus preventing the virus from evading RNAi through mutation. Thus ideally, a single iRNA can potentially target multiple viral transcripts simultaneously, efficiently inhibiting not only viral gene expression, but also DNA replication (Wieland and Chisari, 2005).

Most noteworthy findings were obtained by McCaffrey et al. (2003), Shlomai and Shaul (2004), and Uprichard et al. (2005) who independently assessed anti-HBV shRNAs in different *in vivo* models and found high Efficiencies of their constructs, albeit for only relatively short periods (up to 26 days). An important consistent finding in these studies was that inhibition of viral gene expression did not require active viral replication (Cheng et al., 2005; Ren et al., 2005; Grimm and Kay, 2006), suggesting that RNAi strategies are excellent options as adjutants to conventional anti-HBV therapies, for example with inhibitors of the viral reverse transcriptase. The current understanding of RNAi-related mechanisms raises considerable hope that we will see the clinical evaluation of efficient, safe and specific antiviral RNAi therapeutics for treatment of virally induced human liver diseases in the not too-distant future.

3.5 Cardiovascular and Cerebral vascular Diseases

Cardiovascular diseases are the leading cause of death in the United States and many other industrialized countries. These diseases are commonly results from the progressive occlusion of arteries in a process called Atherosclerosis, which can ultimately culminate in a myocardial infarction or stroke (Forbes et al., 2004). Atherosclerosis involves damage to vascular endothelial cells, local production of inflammatory cytokines, and the recruitment of macrophages to the site of foam cell formation; in addition to these, apoptosis of foam cells and vascular smooth muscle cells occurs (Geng and Libby, 2002). The severe irritability that occurs in heart or brain cells during a myocardial infarction or stroke results in the death of cardiac muscle cells and neurons. Although some of the cells die rapidly by necrosis, many other cells die more slowly by apoptosis; data from animal studies by Zhao and Vinten-Johansen (2002) and Mattson *et al* (2000) shown that such cardiac myocytes and brain neurons are saved from die by apoptosis.

RNA interference (RNAi) utilizing small interfering RNAs (siRNAs) is a recent advance that provides the possibility of reducing gene expression at the post-transcriptional level in cultured mammalian cells (Elbashir et al., 2001). This technology exploited 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. A key step in the process of atherosclerosis is the up-regulation of cell adhesion molecules in vascular endothelial cells, which play an essential role in the recruitment of macrophages to the site of endothelial damage. The production of cell adhesion molecules can be selectively suppressed in cultured cells (Jarad et al., 2002).

3.6 Neurodegenerative Disorders

Recent rapid progress in the applications of RNAi to mammalian cells, offers new approaches to drug target identification and validation for treatment of disease. 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 (Steven et al., 2004). RNA interference (RNAi) is a powerful new gene knockdown technique that permits tissue-specific, temporally controlled suppression of gene expression (Rodriguez-Lebron, and Paulson, 2006). 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 (Raoul et al., 2006). 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 (Link, 1995; Miller et al., 2004), dopamine-producing neurons in the substantia nigra that control body movements in Parkinson's disease (Feany and Bender, 2000), and spinal cord motor neurons in amyotrophic lateral sclerosis (Elia et al., 1999). Specific genetic mutations are responsible for a small percentage of cases of Alzheimer disease, Parkinson's disease and Amyotrophic lateral sclerosis (Hardy, 2001), whereas all cases of Huntington's disease result from mutations (polyglutamine expansions) in the Huntington protein (Rubinsztein, 2002). Studies of patients, and of animal and cell culture models of each disease, have revealed shared biochemical cascades that result in neuronal death. Those cascades include increased oxidative stress, dysregulation of cellular calcium homeostasis and apoptosis (Zhao and Vinten-Johansen, 2002). As a result, different strategies preventative and therapeutic intervention strategies have been employed in neurodegenerative disorders. One strategy is to block the disease-specific events that are believed to initiate the neurodegenerative process, whereas the second strategy targets downstream events in the neurodegenerative cascade (Olivier Milhavet et al., 2003). Recent studies have shown that cultured neurons can be efficiently transfected with siRNAs, leading to effective silencing of target genes (Rubinsztein, 2002). In one study it was shown that cultured neurons can be depleted of the p75 neurotrophin receptor, a protein in the TNF receptor family that has been implicated in neuronal apoptosis in

certain settings (Higuchi et al., 2003). Pro-apoptotic members of the Bcl-2 family (Colussi et al., 2000) and caspases (Quinn et al., 2000) have been effectively targeted and neuronal death prevented using RNAi methods. Caplen and colleagues (2002) performed studies aimed at determining whether RNAi could be used to target the pathogenic process in inherited neuro-degenerative disorders caused by polyglu-tamine expansions and showed loss of ARGFP aggre-gates by 80% in cotransfected S2 cells. Therefore, RNA interference could have considerable therapeutic potential in poly (Q) neurodegenerative disorders. Thus, now it is possible, at least in cell culture, to selectively silence a transcript associated with an important group of genetic diseases by RNAi.

Despite the clear therapeutic potential of RNAi-based gene therapy (Harper et al., 2005; Raoul et al., 2005; Miller et al., 2005) several issues are challenging translation towards the clinic. The first concerns the RNAi-mediated depletion of the wild-type allele. Indeed, the dominant trait of inheritance of neurodegenerative disorders often implies that only one allele harbours the causative mutation. Therefore, silencing of the wild-type gene could have a deleterious effect, owing to the loss of normal physiological functions (Zeitlin, 1995). To circumvent this limitation, allele-specific siRNAs discriminating between wild type and mutant allele have been designed (Link, 1995; Miller et al., 2003). Nevertheless, for neurodegenerative diseases associated with a large number of different point mutations in the same gene, such as ALS-linked mutations in SOD, a selective silencing may not be clinically appropriate. Indeed, design of allele-specific siRNA is not so straightforward. Moreover, the silencing efficiency of the allele-specific siRNA may vary with the nature and the position of the point mutation. Finally, all the safety concerns of each newly designed siRNA will have to be addressed before its translation into the clinical situation. An interesting alternative consists of a gene replacement technology that potentially allows for the knockdown of the vast majority of mutated genes and the synthesis of a wild-type protein refractory to RNAi-based silencing (Raoul et al., 2005; Rodriguez-Lebron and Paulson 2006). By retaining normal gene function and switching off a potential broad range of mutations, this system opens a therapeutic avenue to a wide variety of dominantly inherited neurodegenerative disorders.

3.7 Antiproliferative and Proapoptotic miRNAs

miRNAs play essential roles in many basic physiologic processes including proliferation, differentiation, and apoptosis. The function of a given miRNA is dictated by the milieu of targets that are expressed in a given cell type. Thus, an miRNA that regulates both proproliferative and antiproliferative targets may act as a tumour suppressor in some cancers and an oncogene in others, depending on which targets are driving tumorigenesis (Hwang and Mendell, 2006). MicroRNAs with proproliferative and antiapoptotic activity would likely promote oncogenesis and may be over expressed in cancer cells. The most comprehensively studied example of miRNA locus with these properties is the mir-17 cluster, consisting of six miRNAs: miRs-17-5p, -18, -19a, -19b, -20, and -92. This miRNA cluster is located on human chromosome 13q31, a region that is frequently amplified in several types of lymphoma and solid tumors (Ota et al., 2004; Hayashita et al., 2005; He et al., 2005). The other miRNA with antiproliferative and proapoptotic activity are likely to function as tumour suppressor genes and thus may be under-expressed in cancer cells (Hwang and Mendell, 2006). The family of let-7 miRNAs represents a clear example of this. The let-7 family consists of a group of highly conserved miRNAs in multiple species including *C. elegans*, Drosophila, and vertebrates. let-60/RAS was recently identified as a target of let-7 in *C. elegans* (Johnson et al., 2005). The regulation appears to be conserved in humans where three RAS genes, known to be potent oncogenes, have also been demonstrated to be directly regulated by human let-7. Since RAS dysregulation is a key oncogenic event in lung cancer, loss of let-7 may contribute to pathogenesis in this disorder. Indeed, let-7 is generally expressed at low levels in cancerous lung tissue compared to normal tissue and low expression of this miRNA correlates with shorter postoperative lung cancer survival (Takamizawa et al., 2004; Johnson et al., 2005). miR-15a and miR-16-1 represent additional miRNAs with likely tumour suppressing activity. These miRNAs are located on human chromosome 13 in a region frequently deleted in B-CLL. Cimmino *et al* (2005) identified a conserved target site for miR-15a and miR-16-1 in the 3' UTR of BCL2, a potent inhibitor of cell death. Consistent with a regulatory interaction between these miRNAs and this gene, the levels of miR-15a and miR-16-1 are inversely correlated with Bcl2 protein levels in samples from CLL patients. Furthermore, over expression of miR-15a and miR-16-1 in a leukemia cell line results in decreased Bcl2 protein expression, activation of the intrinsic apoptosis pathway, and ultimately cell death. Thus, loss of these miRNAs may contribute to elevated Bcl2 expression and pathologic cell survival in B-CLL (Hwang and Mendell, 2006).

Potential challenge for future studies relates to the probable tissue-specific functions of some miRNAs because in some cancer cells act as oncogenes and in others tumor suppressors. Although we are at an early stage in our understanding of the roles of miRNAs in cancer, the importance of these molecules in this disease process are clear. Undoubtedly, continued efforts to delineate miRNA function in physiologic and patho-physiologic states will reveal novel insights into normal cellular and developmental biology and the normal cell-

ular and developmental biology and the mechanisms that are disrupted when these processes go awry.

3.8 Malaria

Despite intense efforts to eradicate, malaria remains a leading cause of morbidity and mortality worldwide. The World Health Organization (2004) estimates that nearly half a billion clinical cases of malaria occur each year, with over one million deaths. Recent evidence strongly suggests that RNAi can play a key role in identifying which genetic factors shape the vector parasite relationship may be crucial to identifying new genetic means of controlling mosquito-borne diseases (Vlachou et al., 2005). RNAi can, and indeed has been made inheritable in *Anopheles* 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; Brown and Catteruccia, 2006). 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- and time-specific promoter, dsRNA expression can be tailored to coincide spatially and temporally with the journey of the parasite through the mosquito. The cell autonomous nature of transgenic RNAi in *Anopheles* (Ito et al., 2002), implies that the loss-of-function phenotype would only be observed in those cells where target gene and dsRNA are co-expressed, avoiding the pleiotropic effects of the loss of that gene in other non-target tissues. Conceptually, 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. Only few publications to date have described characteristic RNAi-like silencing phenomena [loss-of-function-like phenotypes, reduction in cognate messenger RNA (mRNA) levels, and the presence of miRNA species] (Kumar et al., 2002; Malhotra et al., 2002; Mohammad et al., 2003; Li et al., 2004; Gissot et al., 2005). In one such in vivo study so far published, injection of *P. berghei*-infected mice with siRNAs targeting the *P. berghei* cysteine protease, berghepain, resulted in its specific down-regulation (Blandin et al., 2002; Mohammad et al., 2003; Tina et al., 2006; Volz et al., 2006). Injections resulted in only approximately 0.01% of the siRNA being internalized into the parasite, and the observed 40–50% reduction in berghepain mRNA levels did not alter the parasitemia of the siRNA-treated mice. More studies will be needed to elucidate the mechanisms of gene silencing observed in *Plasmodium* and to assess the therapeutic potential of RNAi. The urgent need to develop new therapeutics for malaria control warrants a thorough assessment of the possible application of this novel technology.

3.9 RNAi and Metabolic diseases

Over the past years RNA interference (RNAi) has exploded as a new approach to manipulate gene expression in mammalian systems. More recently, RNAi has acquired interest as a potential therapeutic strategy. 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 and other oligo-nucleotide approaches (Soutschek et al., 2004; Rondinone, 2006).

The liver’s central role in the control of glucose homeostasis is subject to complex regulation by substrates, insulin, and other hormones. Insulin resistance is a major hallmark in the development of type II diabetes, which is characterized by an impaired ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in muscle. A few studies utilized RNAi technology to target key genes involved in the regulation of gluconeogenesis and provided in vivo proof-of-principle for the development of RNAi-based therapeutics for diabetes (Koo et al., 2004; Taniguchi et al., 2005). One group (Taniguchi et al., 2005) developed an adenovirus-mediated RNAi technique that utilizes shRNAs to substantially and stably knock down insulin receptor substrates; IRS-1 and IRS-2 expression, specifically in the livers of mice to better understand the roles of these proteins in hepatic insulin action. By knocking down IRS-1 and IRS-2 separately and together in liver, they showed that IRS-1 signaling may be more closely linked to the regulation of genes involved in glucose homeostasis, whereas IRS-2 signaling may have specific roles in the regulation of hepatic lipid metabolism. Vector-based RNAi approach (Hanson and Reshef 1997; Makimura et al., 2002) was used to induce posttranscriptional gene silencing 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. Treatment of diabetic mice with PEPCK siRNA caused a 50% decrease in hepatic PEPCK protein content and was sufficient to lower blood glucose and to improve glucose tolerance (Makimura et al., 2002). These data reinforce the significance of PEPCK in sustaining diabetes-induced hyperglycemia and validate liver-specific intervention at the level of PEPCK for diabetes gene therapy.

Type I diabetes originates from autoimmune/inflamematory destruction of insulin-producing β cells in the pancreatic islet (Castano and Eisenbarth, 1990). Islet transplantation offers a potential cure for type I diabetes. However, its success has been limited, due to loss of cells by apoptosis. Therefore, any process that would inhibit apoptosis or related insults should increase the yield of viable islets available for transplantation from one donor pancreas. Two different studies have described the successful introduction of siRNA directly into pancreatic islet cells, both during in situ perfusion and from intravenous tail vein injection (Bradley et al., 2005a,b; Brown and Catteruccia, 2006). In one of the studies, the insulin 2 (Ins2) gene was targeted with siRNA, and mice received siRNA via hydrodynamic tail vein injection. In vivo delivery of siRNA to pancreatic islets revealed a 33% reduction in Ins2 mRNA levels. These studies provided the first steps for the use of RNAi technology in pancreatic islets and may provide a solution to maintain viable cells for transplantation.

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 other diseases such as obesity, neuropathic pain, and depression (Rondinone, 2006). After the *in vitro* success in down-regulating gene expression in neurons, chemically synthesized siRNAs were tested for their *in vivo* gene knockdown ability in the brain (Makimura et al., 2002; Shishkina et al., 2004) and observed the physiological functions of mammalian genes especially those expressed in brain. The companies like Sirna Therapeutics Inc., CytRx Corporation, Alnylan Pharmaceuticals developmed chemically modified siRNAs to deliver systematically, opening the possibility of RNAi therapeuptics for a range of different diseases, such as metabolic and cardiovascular diseases (Zinker et al., 2002; Powelka et al., 2006).

Several studies have demonstrated efficient *in vivo* delivery of siRNAs and therapeutic benefit in mice in earlier studies. The ongoing and future preclinical studies in animal models will hopefully help optimize RNAi therapeutics for applications in humans. Although development of RNAi-based therapeutics for diabetes is in its infancy, early clinical studies are soon to begin assessing the use of this new class of therapeutics to tackle metabolic diseases, including diabetes and obesity.

4. The Future of RNA Interference in Medicine

Inhibiting the expression of genetic information has long been the domain of classical genetics. The discovery of an endogenous mechanism for regulation of gene expression, RNA interference (RNAi), has created a small revolution in functional genomics, which is rapidly spreading into therapeutics. SiRNA-mediated gene silencing is generally believed to be highly sequence-specific. The demonstration of efficient and sequence-specific gene silencing by synthetic siRNAs and expressed shRNAs has led to the beginning of a revolution in mammalian functional genetics (Mittal, 2004). Advances in the design of targeting molecules and in their delivery methods have allowed the interrogation of several biochemical and signaling pathways relevant for basic physiological and complex disease processes (Peter Sandy et al., 2005) and shRNAs will likely establish RNAi as a standard novel therapeutic tool in the treatment of human diseases.

Understanding the role of micro RNAs in cell biology and human health represents a major advance in the growing trend towards the development of new diagnostic and therapeutic products which are fore front of persona--lized medicine and biotechnology, this focus on the earliest stages of disease progression, by controlling and diagnosing diseases at the genetic level. This approach makes it possible to target disease while avoiding many of the abundant side effects associated with pharmaceutical products. microRNAs play a significant role in the development of many serious diseases, including different forms of cancer. Specific microRNA-based agents have been shown to increase apoptosis (prog-rammed cell death) in certain cell lines, while other sets of microRNA based agents have been found to provoke cell growth. Based on these important findings, companies can pursue a range of product development opportunities that will harness the power of microRNAs in the diagnosis and treatment of many different forms of cancer in the years ahead. RNAi-mediated down-regulation of cellular targets that encode receptors required for viral entry proved to be effective in the HIV treatment. The power of RNAi as an anti-HIV agent has propelled development of RNAi-based gene therapy approaches for the treatment of HIV infection in humans. Viral vector mediated delivery to hematopoietic cells, including stem cells, is a feasible approach for shRNA gene delivery for treatment of HIV.

RNAi-based functional analysis of genes associated with human recessively inherited neurodegenerative diseases can be addressed through an impressive development of various viral platforms (Deglon and Hantraye, 2005; Rodriguez-Lebron, 2006). Through these methods, understanding of neuronal death mechanisms could allow the identification of potential targets for RNAi-based gene silencing and open therapeutic perspectives for sporadic cases of neurodegenerative disorders. RNAi can also play a key role in identifying the genetic factors which shape the vector parasite relationship and this may be crucial to identifying new genetic means of controlling mosquito-borne diseases like malaria. Injections resulted in only approximately 0.01% of the siRNA being internalized into the parasite, and the observed 40–50% reduction in berghepain mRNA levels did not alter the parasitemia of the siRNA-treated mice. More studies will be needed to elucidate the mechanisms of gene silencing

observed in *Plasmodium* and to assess the therapeutic potential of RNAi. The urgent need to develop new therapeutics for malaria control warrants a thorough assessment of the possible application of this novel technology.

Ongoing and future preclinical studies in animal models will hopefully help optimize RNAi therapeutics for applications in humans. Although development of RNAi-based therapeutics for diabetes is in its infancy, initial clinical studies will assess the use of this new class of therapeutics to tackle metabolic and other diseases. Because of the potential of RNAi for therapeutic intervention, major efforts should now be placed on preclinical studies using this technology.

5. Challenges for RNAi as a therapy

Two key challenges in developing RNAi as a therapy are avoiding off-target effects and ensuring efficient delivery. One potential risk for side effects emerges from the feature that distinguishes RNAi from other antisense technologies - the use of cellular machinery for directing sequence-specific silencing. Using iRNAs to target specific cellular or viral transcripts in essence hijacks the endogenous RNAi machinery, and we know little about the potential for saturating the RNAi pathway in primary cells, although saturation of RISC is demonstrable in cultured cells (Hutvágner et al., 2004). Consequently, endogenous RNAi pathways could potentially be affected by siRNAs. It will be important to pay close attention to basic research studies on off-target effects of siRNAs and on the design of effective iRNAs (Saxena et al., 2003; Scacheri et al., 2004). A better understanding of the mechanisms that lead to nonspecific effects of short dsRNAs is essential before the use of siRNAs or taking shRNAs to human-based patient trials.

The issue of delivery has restricted the antisense field for almost two decades. Targeted delivery to specific cell or tissue types is still not a practical reality for oligonucleotide-based therapeutics. The alternative approach is viral-vector-mediated delivery of therapeutic shRNA genes. Because this type of delivery results in gene therapy, there are several associated safety concerns, and systemic delivery of viral vectors is still a major hurdle. Nevertheless, the potency and potential for general therapeutic utility of RNAi is prompting renewed vigour into delivery-related research. It remains to be determined whether backbone-modified, nuclease-resistant siRNAs will move to the clinic more quickly than synthetic deoxyoligonucleotides.

6. Conclusions

The ability to target a specific gene or genes using siRNAs or vector-mediated RNA expression methods, suggests the potential of RNAi to block the disease process or relieve symptoms of the disease. Depletion of proteins critical for the cell cycle, such as cyclins, cyclin dependent kinases, or telomerase, might be effective in treating cancers and some neurodegenerative disorders. Blocking the production of anti-apoptotic proteins such as Bcl-2, inhibitor of apoptosis proteins and antioxidant enzymes may be used to kill cancer cells. Conversely, RNAi-mediated suppression of expression of apoptotic proteins (Bax, Par-4, p53, AIF, and caspases, for example) may slow or stop the degenerative processes such as myocardial, neurological, and autoimmune disorders. Ligands, receptors, and downstream signal transduction proteins critical for a specific disease process may offer suitable targets. Similarly, genes that encode proteins involved in oxidative stress and inflamemation are potential targets in autoimmune and infectious or inflammatory diseases. The more obvious and widely studied viral and bacterial genes targets should offer biomedical scientists a critical springboard in moving RNAi-based therapeutic intervention in infectious diseases a notch higher.

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