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Review Article

RNAi: A CHALLENGING PROSPECT IN THERAPEUTICS

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ABSTRACT

RNA interference (RNAi) represents a mechanism invented by nature to protect the genome. In the past few years the field has emerged at a surprisingly high pace. RNA interference (RNAi) has advanced from its basic discovery in lower organisms to becoming a powerful genetic tool and perhaps our single most promising bio therapeutic for a wide array of diseases. Numerous studies document RNAi efficacy in laboratory animals and the first clinical trials are underway and thus far suggest that RNAi is safe to use in humans. RNAi is a posttranscriptional process that can effect gene silencing through chromatin remodeling, blocking protein synthesis and cleaving specifically targeted mRNA. Since RNA interference has only been recently discovered, there are many possible future avenues for application. Its specificity makes it an ideal tool for knocking down single genes for studying gene function or for gene therapy. There appear to be numerous potential clinical and medical applications. The discovery that 21-23 nucleotide RNA duplexes (small-interfering RNAs, siRNAs) mediate RNAi in mammalian cells opened the door to the therapeutic use of siRNAs. Silencing of gene expression by siRNA is rapidly becoming a powerful tool for genetic analysis and represents a potential strategy for therapeutic product development. We highlight emerging solutions and concurrently discuss novel therapeutic RNAi-based concepts. The therapeutic advantages of siRNAs for treatment of viral infection, dominant disorders, cancer, and neurological disorders show great promise. The current rapid advances create realistic optimism that the establishment of RNAi as a new and potent clinical modality in humans is near.

Key Words: RNAi, siRNAs, therapeutic, gene silencing, disorders

INTRODUCTION

Functional genomics in recent years enormously increased our understanding of gene function. The post-sequence era now requires the simultaneous elucidation of the function of thousands of gene products. Despite its success, gene targeting by homologous recombination requires substantial time and money. Approaches using antisense oligonucleotides have proven useful, but are not generally successful. RNA interference (RNAi) opens a new door to efficiently silence gene expression in a variety of experimental settings. Any disease-causing gene as well as any cell type or tissue can potentially be targeted. This naturally occurring mechanism, which regulates gene expression, known as RNA interference (RNAi), has become a potent tool for modulating gene expression in several fields, such as functional genomics, drug validation, and transgenic design (Caplen 2004, Napoli et al., 1990) Post-transcriptional gene silencing was first described in Petunia flowers in 1990, wherein the introduction of a purple pigment-producing gene under the control of a promoter caused an unexpected white color. This phenomenon was termed co-suppression (Napoli et al., 1990). Work in Caenorhabditis elegans led to the discovery that doublestranded RNA (dsRNA) triggers RNAi(Fire et al, 1998). RNAi is a posttranscriptional process that can effect gene silencing through chromatin remodeling, blocking protein synthesis and cleaving specifically targeted mRNA. RNA interference (RNAi) is a regulatory mechanism of most eukaryotic cells that uses small double stranded RNA (dsRNA) molecules to direct homology-dependent control of gene activity (Almeida and Allshire 2005). Known as small interfering RNAs (siRNA) these ˜21–22 bp long dsRNA molecules have a characteristic 2 nucleotide 3′ overhang that allows them to be recognized by the enzymatic machinery of RNAi that eventually leads to homologydependent degradation of the target mRNA. In mammalian cells siRNAs are produced from cleavage of longer dsRNA precursors by the RNaseIII endonuclease Dicer (Zhang et al.,2004) .The silencing activity of RNAbased therapeutics is dependent on molecular interactions at specific intracellular sites. The combined requirement for

modulation of the pharmacokinetics and intracellular trafficking is a major challenge for improvement in the therapeutic efficiency of RNAi therapeutics (Fougerolles et al., 2007).

RNA interference (RNAi) has been called “one of the most exciting discoveries in biology in last couple of decades” (Fire et al., 1998) while Science called it “Breakthrough of the Year” in 2002 (Couzin, 2002). RNAi is an ancient sequence specific gene silencing mechanism in eukaryotes, originally discovered as a natural inherent property of plants and invertebrates as a defense mechanism against viral pathogens and transposons (Fire et al., 1998). Besides the antiviral function of RNAi, there is evidence that it plays an important role in regulating cellular gene expression (Szweykowska-kulinska et al., 2003). Since its discovery small interfering RNA (siRNA), less than 30 base pair (bp), has become the most powerful and indispensable tool in gene function studies in mammalian cells and development of RNAi based drugs for the prevention and treatment of human diseases such as viral infections, tumors and metabolic disorders as a viable alternative for various diseases in the near future because of its high knockdown efficacy and specificity (Mittal, 2004). A number of groups are evaluating the antiviral potency of RNAi by using HCV-specific siRNA, exposing the possibilities and problems of siRNA-mediated antiviral agents. While RNAi has been a tremendous resource for studying mammalian gene function without the laborious use of gene knockout techniques, the next frontier is to harness this powerful technology for therapeutic purposes. Since gene silencing by RNA interference (RNAi) became possible by using short interfering RNA (siRNA) of less than 30 base pair (bp) in mammalian cells, siRNA has become the tool for gene function studies. Because of the high knockdown efficiency and specificity of siRNAs, it is hoped they will become a useful therapeutic agent preventing pathogenic gene products associated with diseases, including cancer, viral infections and autoimmune disorders (Mittal, 2004, Sioud 2005, Ryther et al., 2005, Hannon and Rossi 2004, Dorsett and Tuschl 2004, Caplen and Mousses 2003).A number of groups have evaluated the antiviral potency of RNAi by using HCV-specific siRNA, exposing the possibilities and problems of siRNA-mediated antiviral agents.

RNA interference (RNAi) methods are also based on nucleic acid technology, however unlike antisense and triplex approaches, the dsRNA activates a normal cellular process leading to a highly specific RNA degradation, and perhaps more importantly, a cell-to-cell spreading of this gene silencing effect in several RNAi models. As yet this 'systemic' spreading has not been observed in mammals; however, several recent RNAi studies coupled with extensive mouse and human cDNA homology to a newly discovered molecular component of cell-to-cell RNAi spreading in worms are truly encouraging (Winston et al., 2002).

RNAi has been extensively used invitro for identifying and validating gene function, especially when specific inhibitors were unavailable (Whitehead et al., 2009). Moreover, RNAi mediated functional gene silencing has been employed invivo in experimental animal studies (de Fougerolles 2007, Song et al., 2003), as well as in ongoing clinical investigations (Castanotto and Rossi 2009, Whitehead et al., 2009). Although some approaches did not directly target endogenous, but pathogen genes (Bitko et al., 2005) , or have been questioned to be of reduced specificity (Kleinman et al., 2008) , others showed successful endogenous gene suppression in rodents and non-human primates invivo (Aleku et al., 2008, Bhandari 2006, Lomas-Neira et al., 2005, Perl et al., 2005).

THE DISCOVERY OF RNAi

The discovery of RNAi has been heralded as a major scientific breakthrough that happens only once every decade or so, and represents one of the most promising and rapidly advancing frontiers in biology and drug discovery today. RNAi is a natural process of gene silencing that occurs in organisms ranging from plants to mammals. RNAi was originally discovered in Caenorhabditis elegans as a biological response to exogenous double-stranded RNA molecules (dsRNA) which induce sequence-specific gene silencing (Fire et al., 1998) .It was discovered as an endogenous property of plants (Napoli et al., 1990, Jorgensen et al., 1996), and perhaps exists as a defense mechanism against viral pathogens or uncontrolled transposon mobilization (Ahlquist 2002). Subsequently this phenomenon was observed and experimentally demonstrated in worms (Fire et al., 1998, Guo

and Kemphues 1995), flies (Kennerdell and Carthew 1998 and 2000), and vertebrates (Li et al., 2000 and Svoboda et al., 2001).

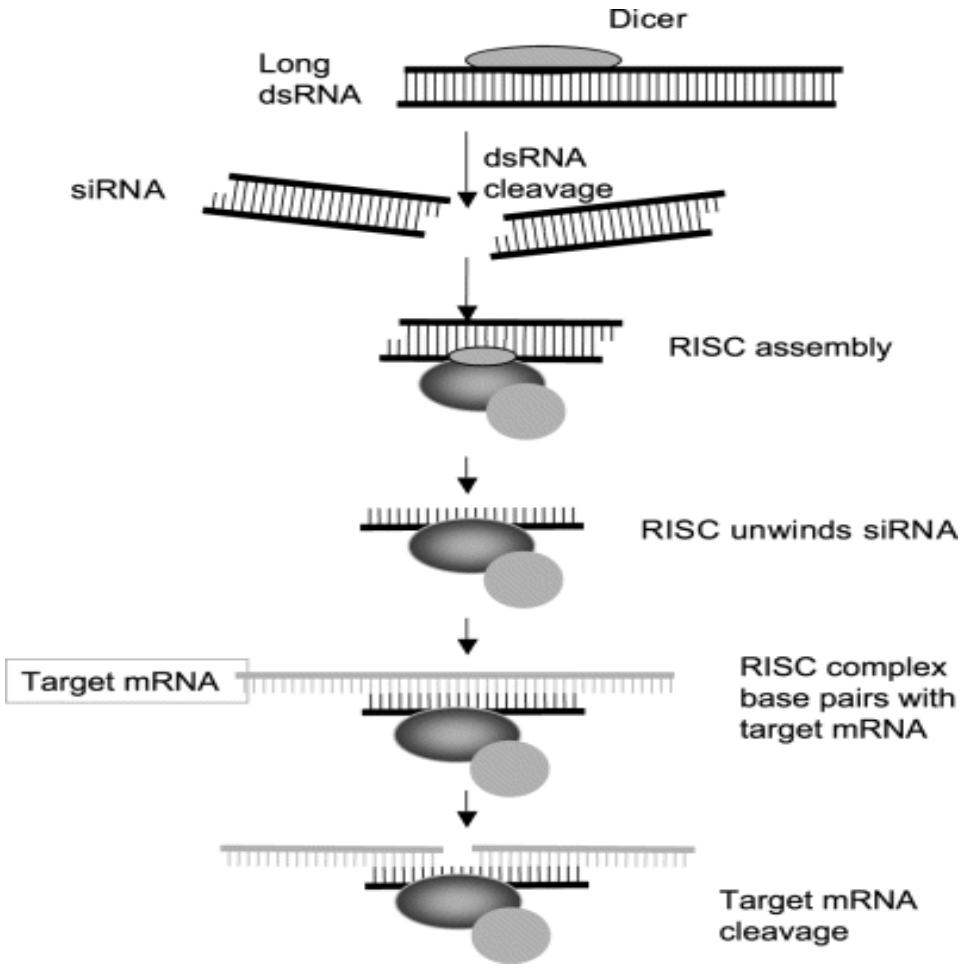
The genetic code in mRNA is described as being the 'sense' sequence, and injecting 'antisense' RNA, which can pair with the mRNA, also had no effect. But when Fire and Mello injected sense and antisense RNA together, they observed that the worms displayed peculiar, twitching movements. Similar movements were seen in worms that completely lacked a functioning gene for the muscle protein. When sense and antisense RNA molecules meet, they bind to each other and form double-stranded RNA. Could it be that such a double-stranded RNA molecule silences the gene carrying the same code as this particular RNA? Fire and Mello tested this hypothesis by injecting double-stranded RNA molecules containing the genetic codes for several other worm proteins. In every experiment, injection of double-stranded RNA carrying a genetic code led to silencing of the gene containing that particular code. The protein encoded by that gene was no longer formed.

After a series of simple but elegant experiments, Fire and Mello deduced that double-stranded RNA can silence genes, that this RNA interference is specific for the gene whose code matches that of the injected RNA molecule, and that RNA interference can spread between cells and even be inherited. It was enough to inject tiny amounts of double-stranded RNA to achieve an effect, and Fire and Mello therefore proposed that RNA interference (now commonly abbreviated to RNAi) is a catalytic process.Indeed varieties of endogenous small, regulatory RNAs have been identified in several organisms, and are assumed to elicit their roles via an RNAi degradative pathway (Lagos-Quintana et al., 2001 and Lee and Ambros2001).

In mammalian cells, it had been known that introduction of dsRNA activated the IFN pathway and RNA binding protein kinase and induced non-specific degradation of RNA, translation inhibition and cell death (Gil and Esteban 2000 and Takaoka et al., 2003). However, Elbashir et al., succeeded in inducing RNAi machinery in mammalian cells by using 21 nt of small dsRNA (called small interfering RNA; siRNA) without a non-specific response against the dsRNA (Elbashir et al., 2001). This finding was a major breakthrough for the application of RNAi. Thereafter, siRNA became a widespread tool for specific gene silencing.

MECHANISM OF GENE SILENCING

It is generally accepted that the RNAi cascade is initiated when the host cell encounters a long dsRNAfroma virus or endogenous source (Hannon and Rossi 2004). These long dsRNAs are cleaved into shorter dsRNA segments (siRNA) by the Dicer protein, ribonuclease III type protein (Bernstein et al., 2001). The RNA segments are 19–22 nt containing 5′ phosphate and 3′ overhangs with 3′ hydroxyl termini in both the strands. These siRNAfragments are then incorporated into a large protein assembly, called the RNA-induced silencing complex, (RISC) (Hammond et al.,2000), wherein the sense strand of siRNA is removed by a helicase associated with the RISC. The RISC with the antisense strand specifically cleaves mRNA which has a complementary sequence to the antisense strand Elbashir et al., 2001and Hannon 2002. The cleaved mRNA is then degraded immediately in the processing body (Sheth and 2003).



MECHANISM OF RNA INTERFERENCE

The entire mechanism can be divided into two phases – the initiation phase, and the effector phase, (Coller and Parker 2005).

Initiation phase: The double stranded RNA (siRNA or miRNA) which are expressed in, or introduced into the cell, is processed by a Ribonuclease III i.e., Dicer enzyme into 21–23 bp RNA (Sen and Blau 2006, Siomi and Siomi 2009, Bernstein et al., 2001). These result into dsRNA with two to three nucleotide long overhangs on 30 ends. These overhangs with a 50 phosphate group (Elbashir et al.,2005) are necessary for siRNA induced silencing complex. Dicer has different homologs in different species. Animals usually encode a single type of Dicer to generate various class of small RNAs with the exception of C. elegans and Drosophila each encoding two Dicers. Dicer requires a RNA helicase domain for its functioning (Sledz and Williams 2005). It also requires ATP in certain species which is however, not the case for humans (Provost et al., 2002, Zhang et al., 2002). Dicer is also assisted by a DNA binding protein i.e., TRBP (Chendrimada et al., 2005 Nykanen et al., 2001).

Effector phase: The processed dsRNA along with the Dicer gets incorporated into the RNA induced silencing complex (RISC). However, it is only one strand i.e., the guide strand (Antisense) which gets incorporated and causes gene silencing. The other strand i.e., the passenger strand (Sense) gets cleaved by certain Argonaute proteins(Matranga et al., 2005). The 50 antisense region of functional siRNA are found to be thermodynamically less stable than 50 sense strand providing the basis for strand selection entering the RISC. The guide strand now guides the entire RISC to target mRNA. There are four domains in RISC viz. Helicase, Exonuclease, Endonuclease and Homology searching domains. These together bring about the gene silencing (Sledz and Williams 2005, Nykanen et al., 2001).

In mammalian cells, dsRNA molecules, including siRNA and short hairpin RNA (shRNA) transcribed from a polymerase III promoter of a DNA fragment can efficiently induce RNAi activity. shRNA exported to

the cytoplasm from the nucleus via Exportin-5 and the GTP-bound form of its cofactor Ran, (Lund et al., 2004 and Yi et al., 2003) utilizes the RNAi cascade in the same fashion as siRNA.

siRNA

The field of small interfering RNAs (siRNAs) as potent sequence-selective inhibitors of transcription is rapidly developing. When small double-stranded RNAs, called siRNA, are introduced into cells, they mediate posttranscriptional gene silencing of a specific target protein by disrupting messenger RNAs (mRNAs) containing complementary sequences (Elbashir et al., 2001, Fougerolles et al., 2005). Any disease-causing gene as well as any cell type or tissue can potentially be targeted.

The major bottleneck in the development of siRNA therapies is the delivery of these macromolecules to the desired cell type, tissue or organ. siRNAs do not readily cross the cellular membrane because of their negative charge and size. Cellular delivery of chemically synthesized or in vitro transcribed siRNAs is usually achieved by cationic liposome based strategies. The disadvantage of lipid based delivery schemes in vivo is the rapid liver clearance and lack of target tissue specificity. Cationic polymer and lipid-based siRNA complexes have been used for systemic delivery in mice (Sorensen et al.,2003 and Sioud et al., 2003) . Liposomes and duplexed siRNAs are complexed in vitro and the resulting siRNA containing vesicles are taken up by cells via the endosomal pathway. siRNAs are released into the cytoplasm where they associate with RISC. Typically, transiently transfected cells in cultures show gene silencing for three to five days. However, non-dividing cells may show sustained silencing for several weeks Song et al., 2003 and Bartlett and Davis 2006). Optimization of the in vivo stability of siRNAs has been accomplished by chemically modifying the RNA backbone, and several strategies are now available for improved in vivo stability that may eventually reduce the requirement for high dosage. These include 2′F, 2′O–Me and 2′H substitutions in the RNA backbone, all of which increase serum stability. Importantly, selective modifications of the backbone do not seem to reduce RNAi efficiency (Pederson 2004). Until recently most trials have relied on duplexed 21-mer RNA species, but current research indicate that longer (25–27 mer) RNA species that undergoe intracellular processing by Dicer may increase the potency by

channelling the duplexes through Dicer where the siRNAs are handed off to RISC (Kim et al., 2005 and Siolas et al., 2005) thereby further reducing the required concentrations of siRNAs for achieving a therapeutic effect. Other in vivo delivery approaches for siRNAs include conjugation of cholesterol to the siRNA sense strand (Soutschek et al., 2005), antibody-protamine fusions that bind siRNAs (Song et al., 2005), cyclodextrin nanoparticles (Hu-Lieskovan et al., 2005) and aptamer-siRNA conjugates (McNamara et al., 2006). Each of these approaches resulted in tissue or cell type specific targeting, thereby expanding the therapeutic potential for in vivo siRNA delivery.

shRNA

A small hairpin RNA or short hairpin RNA (shRNA) is a sequence of RNA that makes a tight hairpin turn that can be used to silence gene expression via RNA interference. shRNA uses a vector introduced into cells and utilizes the U6 or H1 promoter to ensure that the shRNA is always expressed. Soon after the realization that synthetic siRNA could be introduced into mammalian cells and trigger RNAi without activating type 1 interferon responses, researchers discovered that DNA-based expression cassettes could be generated that express short hairpins (shRNA) or separate sense and antisense 21 mers from Pol III promoters. These promoters have well defined transcriptional start sites and simple terminators. Consequently the have become the most popular choice for DNA-based gene silencing in molecular biology. The minimal shRNA expression system includes a PolIII-promoter, directly followed by at least 19 nucleotides of sense (or antisense) target sequence, a 4–10 base loop, the complementary antisense (or sense) target sequence and finally a stretch of at least four to six U's as a terminator. Alternatively, a dual promoter system with individual expression of the two RNA strands can be used. The separately expressed strands hybridize and produce functional siRNAs Lee et al., 2002, Miyagishi and Taira 2002). The duplex RNAs produced by either system are substrates for nuclear export by the exportin-5 pathway. The hairpins are further processed by Dicer to yield functional siRNA duplexes whereas the siRNAs enter RISC directly. shRNA is transcribed by RNA polymerase III. shRNAs can also be made for use in plants and other systems, and are not necessarily driven by a U6 promoter. In plants the

traditional promoter for strong constitutive expression (in most plant species) is the cauliflower mosaic virus 35S promoter (CaMV35S), in which case RNA Polymerase II is used to express the transcript destined to initiate RNAi.

Like siRNA delivery DNA-based shRNAs face the problem that negatively charged nucleic acids do not readily pass the cellular membrane without facilitating carriers. Direct plasmid delivery can be achieved using high-pressure tail vein injections in mice. However, this strategy is limited to the liver and due to transient heart congestions it is not suitable for clinical use. Vector-based shRNA systems usually rely on delivery via viral vectors. A number of platforms exist including the popular adenovirus- and adeno-associated virus-(AAV) derived vectors that provide an efficient delivery vehicle for transient shRNA expression. Retroviruses on the other hand allow the researcher to stably silence target genes since the virus-encoded proteins mediate integration into the host cell chromosomal DNA. Retroviral vectors that have found clinical interest are either based murine leukemia virus (MLV) or lentivirus (e.g. HIV, FIV or EIAV).

Therapeutic Applications of RNA Interference

Recent progress in the potential therapeutic applications of siRNAs is owing largely to major breakthroughs in delivery. Systemic delivery of therapeutic amounts of anti-ApoB siRNAs in Chimpanzees was recently accomplished by the use of bilayer liposomes (Zimmermann et al.,2006). These important proofs of principle studies demonstrated that it is safe to systemically delivery therapeutically effective doses of siRNAs to primates, paving the way for other future systemic applications of RNAi. Listed below are some potential diseases that may be therapeutic targets for RNAi in future years.

RNAi in Cancer

The use of RNAi for cancer treatment has mostly relied on the concept of targeting over expressed or mutated oncogenes to directly kill the cells and, while in vitro results have been promising, in vivo experiments have been less impressive. This is in part due to a major impediment to the clinical use of RNAi therapy, namely, the need to deliver these macromolecules to the desired cells, tissues or organs to elicit gene silencing.

Furthermore, to work effectively RNAi therapy needs to be delivered to each and every cancer cell to elicit direct and specific killing. Several studies have demonstrated some in vivo efficacy in the delivery of siRNAs using various strategies such as complexing siRNAs with cationic lipids, nanoparticles, polyethyleneimine, cyclodextrin, chitosan, and collagen .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 whereas 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 there by 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).

The ability of RNAi to silence disease-associated genes in cell culture and animal models has spurred development of RNAi-based reagents for clinical applications to treat diseases including cancer (Howard, 2003; Wall and Shi, 2003). siRNAs are readily synthesized with low production costs compared to protein or antibody therapies. In addition, siRNAs have favorable pharmacokinetic properties and can be delivered to a wide range of organs (Braasch et al, 2004). However, their stability in blood and delivery methods are challenges that must be solved for developing effective RNAi reagents for cancer therapy. Several groups have been investigating the use of alternative backbone and nucleotide modifications to improve the clinical properties of these reagents. For example, by conjugating the 3’-end of the sense strand of siRNA with cholesterol through a pyrrolidine linker has markedly improved the pharmacological properties of siRNA molecules (Soutschek et al, 2004). The Cholesterol- conjugated siRNA are more resistant to nuclease degradation, exhibit increased stability in blood by increasing binding to human serum albumin and show increased uptake into liver. Another

study has shown that boranophosphate modified siRNAs were 10 times more nuclease resistant than unmodified siRNAs (Hall et al, 2004). In addition, boranophosphate siRNAs were more potent than unmodified siRNAs and appeared to act through the standard RNAi pathway.

Interestingly, RNAi may also be exploited to silence pathways that facilitate the effects of traditional cancer drugs. This includes targeting of the multidrug resistance gene (MDR1) for re-sensitization to chemotherapy (Nieth et al., 2003) and silencing of double-strand break repair enzymes for enhanced effects of radio- and chemotherapy (Collis et al., 2003) .

RNAi in HIV treatment

Several groups have shown that siRNAs can inhibit HIV replication effectively in culture. HIV infection can also be blocked by targeting either viral genes (for example, *gag*, *rev*, *tat* and *env*) or human genes (for example, *CD4*, the principal receptor for HIV) that are involved in the HIV life cycle. This is promising, as antiviral therapies that can attack multiple viral and cellular targets could circumvent genetic resistance of HIV. But these results have been achieved by transfecting the siRNAs into cells, and getting the siRNAs to function *in vivo* is likely to be a more difficult task. To get around the delivery problem, many groups have designed promoter systems that can express functional siRNAs when transfected into human cells. Early results have shown that this can decrease replication of HIV considerably and the group is currently working on inserting the system into a lentiviral vector to test its effectiveness in acute HIV-1 infections.

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 individualsare 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.

RNAi in Cardiovascular and Cerebrovascular 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 moleculesin 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).

HBV (Hepatitis-B Virus) as an RNAi target

Hepatitis B virus (HBV) infection increases the risk of liver disease and hepatocellular carcinoma. 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 a 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 compact genome lacks redundancy, resulting in very limited sequence

plasticity thus preventing the virus from evading RNAi through mutation. Thus ideally, a single RNAi 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 sh-RNAs in different *in vivo* models and found high efficiencies of their constructs, albeit for only relatively short periods (up to 26 days).

The registered agents currently available for the treatment of HBV infections, such as interferon and nucleoside analogues, can dramatically decrease HBV DNA levels and induce particular HBeAg loss, but will rarely cause HBsAg loss in chronic hepatitis B patients (Janssen et al., 2005, Papatheodoridis and Manolakopoulos 2009, Stroffolini et al., 2007). RNA interference, on the other hand, can theoretically be directed to cleave any target RNA, providing a novel methodology for anti-HBV therapy (Arbuthnot et al., 2007). Using RNAi as an inhibitor for HBV effectively reduces viral antigen levels, including HBsAg. It can be speculated that RNAi-treatments may offer complementary effects for current anti-HBV therapy. However, the final application of RNAi-based anti-HBV drugs depends on the development of effective and safe RNAi delivery systems.

RNAi in Neurodegenerative Disorders

RNA interference (RNAi) mediates gene silencing in a sequence-specific manner and has proven to be an exceptionally valuable discovery for bench scientists. In the laboratory, RNAi technologies provide efficient means for validating drug targets and for performing reverse genetics to study gene function. Patients may also benefit from RNAi as applications extend to potential human therapies. RNAi-based treatments are being investigated and may provide hope for patients suffering from cancer, viral infections, or genetic diseases for which effective therapies are currently lacking. Notably, several independent studies have demonstrated that RNAi therapy can improve disease phenotypes in various mouse models of human 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). 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 incertain 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 neurodegenerative 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. 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.

Conclusion

The discovery of RNA interference less than a decade ago was a turning point for molecular biology. RNAi has become a powerful tool for studies of gene function in mammals. It provides the researcher with the ability to silence virtually any gene with artificial triggers of RNAi and utilizing the cellular machinery for efficient targeting of complementary transcripts. However, before we reach that stage additional studies need to be performed, mostly focused on the development of efficient delivery methods to the human nervous system and on the assessment of potential toxic effects. Hopefully, significant deleterious consequences will not occur in humans, as they could carry negative consequences for this field as occured in the recent past with more traditional modalities of gene therapy. Despite the limitations therapeutic targets needs to be carefully designed.

Viral escape and off-target effects from RNA silencing is a major problem for developing effective RNAi based antiviral therapy but that can be overcome by finding highly effective target sites, targeting more than one gene at a time such as combinatorial treatment with several siRNAs and modification in siRNA design. Effective delivery vehicles such as lipidosomal, cationic and biodegradable polymers with positive charges should be chosen as good means to deliver siRNA in vivo for long lasting RNAi activity. Given the pace of new findings and discoveries of applications, we anticipate that RNAi will be a major therapeutic modality within the next several years. Hopefully RNAi will revolutionize the treatment of human disease in the same way that it has revolutionized basic research.

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