RNA Interference and its therapeutic applications

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Abstract

RNAi is a potent method, requiring only a few molecules of dsRNA per cell to silence the expression. Long molecules of double stranded RNA (dsRNA) trigger the process. The dsRNA comes from virus and transposon activity in natural RNAi process, while it can be injected in the cells in experimental processes. The strand of the dsRNA that is identical in sequence to a region in target mRNA molecule is called the sense strand, and the other strand which is complimentary is termed the antisense strand. An enzyme complex called DICER thought to be similar to RNAase III then recognizes dsRNA, and cuts it into roughly 22- nucleotide long fragments. These fragments termed siRNAs for "small interfering RNAs" remain in double stranded duplexes with very short 3' overhangs. However, only one of the two strands, known as the guide strand or antisense strand binds the argonaute protein of RNA-induced silencing complex (RISC) and target the complementary mRNA resulting gene silencing. The other anti-guide strand or passenger strand is degraded as a RISC substrate during the process of RISC activation. This form of RNAi is termed as post transcriptional gene silencing (PTGS); other forms are also thought to operate at the genomic or transcriptional level in some organisms. In mammals dsRNA longer than 30 base pairs induces a nonspecific antiviral response. This so-called interferon response results in a nonspecific arrest in translation and induction of apoptosis. This cascade induces a global non-specific suppression of translation, which in turn triggers apoptosis. Interestingly, dsRNAs less than 30 nt in length do not activate the antiviral response and specifically switched off genes in human cells without initiating the acute phase response. Thus these siRNAs are suitable for gene target validation and therapeutic applications in many species, including humans.

Keywords: RNAi, Delivery of siRNA, Therapeutic applications, Antivirus, Apoptosis, Cell.

Introduction

RNA interference (RNAi) is a molecular mechanism in which fragments of double-stranded ribonucleic acid (dsRNA) interfere with the expression of a particular gene that shares a homologous sequence with the dsRNA. In 2006, American scientists Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine for their work on RNA interference in the nematode worm *Caenorhabditis elegans*. RNA silencing is a sequence specific RNA degradation process that is triggered by the formation of double stranded RNA that can be introduced by virus or transgenes. Duplexes 21- nucleotide (nt) RNAs with symmetric 2-nt 3'overhangs are introduced into the cell mediating the degradation of mRNA. According to central dogma of molecular biology, proteins are made in two steps. The first step, transcription, copies genes from double stranded deoxyribonucleic acid (ds DNA) molecules to mobile, single- stranded ribo-nucleic acid (RNA) molecules called mRNA. In the second step, translation, the mRNA is converted to its functional protein form. Since there are two steps to making a protein, there are two ways of preventing one from being made. Scientists have made exciting progress in blocking the protein synthesis through the second step, translation.

One way they have accomplished this is by inserting synthetic molecules that triggers a cellular process called RNA interference (Thakur, 2003).

Before RNA interference was well charact-erized, the phenomenon was known by other names, including post transcriptional gene silencing, transgene silencing and quelling. Well before RNAi was discovered, RNA was used to reduce gene expression in plant genetics. Single-stranded antisense RNA was introduced into plant cells and hybridized to the homologous single-stranded "sense" messenger RNA. It is now clear that the resulting dsRNA was responsible for reducing gene expression.(Lee et al., 1993).

The revolutionary finding of RNAi resulted from the unexpected outcome of experiments performed by plant scientists in the Western World (Napoli et al., 1990; Jorgensen et al., 1994). While

trying to increase the purple colour in Petunias, scientists introduced a pigment producing gene under the control of a powerful promoter. Instead of expected deep purple colour, many of the flowers appeared variegated or even white. This observed phenomenon was named "cosuppression" since the expression of both the introduced gene and homologus endogenous gene was suppressed. There are two mechanisms through which silencing of both transgenes and endogenous loci could occur. In one mechanism transgene induced silencing was accompanied by heavy methylation of silenced loci, leading to transcriptional gene silencing. In the other mechanism post transcriptional gene silencing (PTGS) occurred.. Transgene cosuppression is not only limited to plants but has also been shown in fungi, Drosophila, C. elegans and rodent fibroblasts. This phenomenon has been well characterized in Neurospora crassa, where it is known as "quelling" and it occurs at the post-transcriptional level. It was clear that co-suppression in plants, quelling in fungi and RNAi in nematodes all shared a common mechanism.

A few years later plant virologists made a similar observation. In their research they aimed towards improvement of resistance of plants against plant viruses. At that time it was known that plants expressing virus-specific proteins show enhanced tolerance or even resistance against virus infection. However, they also made the surprising observation that plants carrying only short regions of viral RNA sequences not coding for any viral protein showed the same effect. They concluded that viral RNA produced by transgenes can also attack incoming viruses and stop them from multiplying and spreading throughout the plant. They did the reverse experiment and put short pieces of plant gene sequences into plant viruses. Indeed, after infection of plants with these modified viruses the expression of the targeted plant gene was suppressed. They called this phenomenon "virus-induced gene silencing" (VIGS").

Mechanisma of RNAi

Long molecules of double stranded RNA (dsRNA) when introduced into the cell cytoplasm trigger the process. The dsRNA comes from virus and transposon activity in natural RNAi process, while it can be injected in the cells in experimental processes (Elbashir et al., 2001). In the initiation step the "trigger" dsRNA molecule, usually several hundred base pair long, is cleaved to form 21-23 bp double stranded fragments known as short interfering RNAs (siRNAs). In the effector step the duplex siRNA are then unwound by a helicase activity associated with a distinct multiprotein complex known as the RNA-induced silencing complex (RISC). The strand of the dsRNA that is identical in sequence to a region in target mRNA molecule is called the sense strand, and the other strand which is complimentary is termed the antisense strand. An enzyme complex called DICER that is similar to RNAase III then recognizes ds RNA, and cuts it into roughly 22- nucleotide long fragments. These fragments termed siRNAs for "small interfering RNAs" which remain in double stranded duplexes with very short 3' overhangs, then act as templates for the RNAi inducing silencing complex to destroy the homologous message, thus specifically suppressing its expression. This form of RNAi is termed as post transcriptional gene silencing (PTGS), other forms are also thought to operate at the genomic or transcriptional level in some organisms.

Systemic Silencing in Animals

Animals have a system for amplification and spread of silencing. This is quite evident in C. elegans. If these animals are injected with dsRNA, or if they are allowed to feed on bacteria that produce dsRNA there is systemic silencing of a corresponding endogenous RNA. It is presumed that the signal molecule is produced in the *C. elegans* cells that receive dsRNA. This signal then moves and causes RNA silencing in other cells that become sources of secondary signal (Weinberg et al., 2006).

RNAi in Mammalian Cells

After its discovery, RNAi was rapidly employed as a powerful tool for large-scale reverse genetic screens in C elegans, where it can be easily induced by direct injection of dsRNAs, by feeding worms with bacteria engineered to express si- RNAs, or simply by soaking the animals in medium containing siRNAs. Furthermore, in nematodes, RNAi acts systemically and is a long-lasting heritable event due to the presence of RNA-dependent RNA polymerases (RdRPs) which allow the amplification of the trigger and the perpetuation of the silencing process.

In mammals, however, initial efforts to use RNAi for specific gene silencing encountered more difficulties, especially due to the induction of nonspecific inhibition of gene expression resulting from the activation of the interferon (IFN) response pathway by dsRNAs longer than 30 bp. Hence, the RNAi pathway was thought to be nonfunctional in mammalian cells, where dsRNA longer than 30 base pairs induces a nonspecific antiviral response. This so-

called interferon response is characterized by the activation of the RNA-dependent protein kinase, leading to phosphorylation and there by inactivation of the translation initiation factor eIF-2a which results in a nonspecific arrest in translation and induction of apoptosis. Moreover, the synthesis of 2'–5' polyadenylic acid results in the activation of the sequence nonspecific RNaseL. This cascade induces a global non-specific suppression of translation, which in turn triggers apoptosis.

Interestingly, dsRNAs less than 30 nt in length do not activate the PKR kinase pathway. This observation, as well as knowledge that long dsRNAs are cleaved to form siRNAs in worms and flies and that siRNAs can induce RNAi in Drosophila embryo lysates, prompted researchers to test whether introduction of siRNAs could induce gene-specific silencing in mammalian cells. Indeed, siRNAs introduced by transient transfection were found to effectively induce RNAi in mammalian cultured cells in a sequence-specific manner. The breakthrough for the use of RNAi in mammalian cells came when Elbashir et al (2001) and Caplen et al (2001) showed that siRNA, when directly introduced into mammalian cells, does not trigger the RNA-dependent protein kinase response but effectively elicits RNAi, presumably by directly associating with RISC. Targeted gene silencing in mammalian cells by the application of siRNA is well established. The high degree of sequence specificity inherent to the technology is emphasized by several reports showing that even a 1–2 nt mismatch in the siRNA sequence hampers targeted gene silencing. The effectiveness of siRNAs varies - the most potent siRNAs result in >90% reduction in target RNA and protein levels. The most effective siRNAs turn out to be 21 nt dsRNAs with 2 nt 3'overhangs. Sequence specificity of siRNA is very stringent, as single base pair mismatches between the siRNA and its target mRNA dramatically reduce silencing. Unfortunately, not all siRNAs with these characteristics are effective. The reasons for this are unclear but may be a result of positional effects.

Selection and Designing of Small Interfering RNA

Elbashir et a.l, (2002) reported a number of guidelines for the design of siRNA molecules. Several design tools are also available from the internet. Although one can follow these guidelines it is still necessary to test several siRNAs, targeting distinct regions within the gene of interest, because there is great variability in the capacity of an individual siRNA to induce silencing. One may have to test three or four siRNAs in order to find one that results in more than 90% reduction in target gene expression.

This reduction in target gene expression may be related to one or more of the following factors: incorporation of siRNA into RISC and stability of RISC; base pairing with mRNA; cleavage of mRNA and turnover after mRNA cleavage; secondary and tertiary structures of mRNA; and binding of mRNA-associated proteins. Accordingly, Vickers et al., (2003) found a significant correlation between mRNA sites that are RNase H sensitive (i.e. accessible) and sites that promote efficient siRNA-mediated mRNA degradation. Moreover, placing the recognition site of an efficient siRNA into a highly structured RNA region abrogated silencing.

Schwarz et al., (2003) and Khvorova et al., (2003) found that the decision regarding which of the two strands of a siRNA molecule is incorporated into RISC was crucial in determining the efficiency of gene silencing. In order to target specifically a given mRNA for degradation, the antisense strand of the siRNA duplex, which is complementary to the mRNA, must be incorporated into the activated RISC. They found that the absolute and relative stabilities of the base pairs at the 5'ends of the two siRNA strands determine the degree to which each strand participates in the RNAi pathway. The strand with lower 5' end stability is preferred. As a consequence, a highly functional siRNA is characterized by lower internal stability at the 5'end of the antisense strand as compared with less effective duplexes (Chiu and Rana (2003).

To comply with the rules promoting asymmetric incorporation into RISC, the base pair at the 5' end of the siRNA antisense (guide) strand should have a lower thermodynamic stability compared with the 3'-end. In addition, is is recommended that each strand must have 2-nt 3' overhangs; A/U base pairing at the 5' end of the antisense strand and G/C base pairing at the 5' end of the sense strand; AU richness in the 5' terminal third of the antisense strand; avoidance of introns , 5' and 3' untranslated regions, regions within 75 bases of the start codon and sequences with >50% Guanine and Cytosine content; maximizing sequence divergence from related mRNA. Despite applying these criteria, differences in silencing efficiencies between siRNAs occur, and occurrence of offtarget siRNA activity has been recognized as an important factor in siRNA experimental design (Reynolds et al., 2004).

In vivo Systemic Delivery of siRNAs

Direct injection of siRNA into the blood would

be ineffective because of rapid degradation of the RNA by serum ribonucleases. However, it was recently demonstrated that chemical modification can protect the siRNA molecule from degradation and might even prolong the silencing effect due to slower depletion within the cell. Several groups reported different approaches for systemic in vivo delivery of siRNAs. Soutschek et al (2004) described intravenous injection in mice of chemically modified naked siRNAs coupled to a cholesterol group chemically linked to the terminal hydroxyl group of the sense strand to promote entry into the cells. In vivo delivery of chemically modified siRNAs encapsulated into liposome particles has been reported by Morrisey et al (2005). Song et al (2005) described an antibody-based delivery system which could offer a possibility for systemic, cell-type-specific siRNA delivery. Thus far, synthetic siRNAs have been applied in animals via hydrodynamic transfection (i.e. the intravenous injection of a substantial dose of siRNA within a large volume of liquid), resulting in a knockdown efficiency up to 70–80%, at least in some organs, including liver, kidney, spleen, lung and pancreas. Using this method, the silencing of either Fas receptor or caspase-8 resulted in a clearly measurable protection from severe Fas-induced liver damage. In vivo application of siRNA against genes of the hepatitis B virus also led to an effective inhibition of virus replication. This method is of course not applicable to humans. It is also limited by the fact that siRNA can only be delivered to a certain set of organs and it is not possible to target specific organs or cells. Development of cell-specific or organ-specific delivery systems for siRNA, as is required for broad in vivo application of this technique, is indeed a demanding task. One promising avenue is the complexation of siRNAs with the polyethyleneimine (PEI) which efficiently stabilizes siRNAs and upon systemic administration leads to the delivery of the intact siRNAs into different organs (Aigner, 2006).

Therapeutic applications of Small RNAi

The most obvious clinical uses of RNAi are for diseases in which selective depletion of one or a few specific proteins would be expected to slow or halt the disease process in the affected cells. Ideally this would be accomplished with no or tolerable side effects. RNAi may provide new therapeutics for treating viral infections, neurodegenerative diseases, septic shock, macular degeneration, cancer and other diseases (Dallas and Vlassov,2006).

Intially, the prevention of liver disease was attempted by targeting genes linked to apoptosis control in the liver in two mice models of autoimmune hepatitis. siRNA was directed against caspase (Zender et al., 2003 and Fas (Song et a.l, 2003) and was delivered hydrodynamically via the tail and portal vein respectively. In both the cases there was successful reducing hepatocyte necrosis and inflammation, and protected the mice from future chronic fibrosis. Since then, several experiments using RNA interference to target respiratory viruses have been attempted. Intially influenza virus was chosen due to its significant public health issues and lack of wholly effective vaccine (Tompkins et al., 2004). Proteins were targeted that are highly conserved across several sub types of influenza and which are essential for viral replication. It was found that combination of intranasal delivery and intravenous injection was most effective in inhibiting the virus replication at the site of infection. Kumar et al (2006) described that promising results for the therapeutic potential of RNAi in treating viral encephalitis, both virus specific and across species. They induced RNAi in mice with either a lentivirally expressed short hair pin RNA or a synthetic siRNA.

Kim et al, (2008) applied adenoviruses expressing siRNA both before and after foot and mouth disease virus (FMDV) infection in vitro and in vivo. Treatment after FMDV infection gave effective viral inhibition, but a combination of treatment before and after FMDV infection gave the best results in IBRS-2 cells. They obtained high survival rates in suckling mice by the use of therapeutic injections following challenge. The results of their study suggest that treatment with siRNA could enhance antiviral effects and may be helpful in the control of FMDV in an outbreak.

Limitations of RNAi

Despite the proliferation of promising cell culture studies for RNAi-based drugs, some concern has been raised regarding the safety of RNA interference, especially the potential for "off-target" effects in which a gene with a coincidentally similar sequence to the targeted gene is also repressed. A computational genomics study estimated that the error rate of off-target interactions is about 10%. One major study of liver disease in mice led to high death rates in the experimental animals, suggested by researchers to be the result of "oversaturation" of the dsRNA pathway.

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