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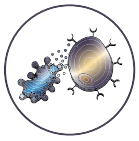

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Review Article

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RNA Interference: A Recent Approach for the Remedy of Various Diseases

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Abstract

RNA interference (RNAi) has been a recently discovered phenomenon for the analysis of regulation of gene expression in a variety of cells, by which target messenger RNA (mRNA) is cleaved by small interfering complementary RNA (siRNA). The technique is now established in *in-vitro* systems, and much work is focussed on adapting RNAi for *in vivo* application. This technology may also be used for therapeutic purposes, functional genomics and target validation. This review will describe the basic biological processes that drive RNAi, indicate current approaches for the treatment of various diseases, and forecast future areas of development. RNAi may provide new therapeutics for treating various diseases like thalassemia, cancer, neurodegenerative diseases, septic shock, macular degeneration, and also viral infection, although *in vivo* delivery of small interfering RNAs remains a significant barrier.

Keywords

Gene silencing; RNA interference; siRNA

Introduction

In the past few years, a novel mechanism for down-regulation of genes in mammalian cells has been discovered. Triggered by double-stranded RNA and acting by sequence-specific cleavage of the messenger RNA (mRNA) gene product, this phenomenon is known as "RNA interference," or RNAi. Already, having become the tool of choice for researchers to identify gene targets and elucidate function, RNAi has an exciting potential for therapeutic applications, that is just starting to be realized. RNA interference (RNAi), a process by which target messenger RNA (mRNA) is cleaved by small interfering complementary RNA (siRNA), is widely used for investigations of regulation of gene expression in various cells [1]. The delivery of siRNA (small interfering RNA) into cells *in vitro* has been shown to clearly inhibit gene expression. In the past few years, RNA interference (RNAi) has become the most widely used technology for gene knockdown. RNAi is a natural powerful mechanism, that is thought to have arisen for protection from viruses and transposons [2].

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History and Discovery of RNA Silencing

RNA silencing was first recognized in animals by Guo and Kemphues [3], who monitor that the introduction of sense or antisense RNA to *par-1* mRNA resulted in degradation of the *par-1* message in *Caenorhabditis elegans*. At that time, elimination of gene expression was frequently done by using antisense technology. Unpredictably, when Guo and colleagues carried out control experiments using only the sense *par-1* RNA, which would not hybridize with the endogenous *par-1* transcript, the *par-1* message was still targeted for degradation. This finding caused researcher to move around the current dogma. It was a big disclosure, when it was demonstrated that injection of double-stranded RNA had the most intense impact on gene silencing. This described phenomenon was discovered in *Caenorhabditis elegans* by Fire et al. [4], was called RNA interference (RNAi).

Knowing the degradation of mRNA through a process known as RNA interference (RNAi) directed by double-stranded RNA (dsRNA), in 2000 Zamore et al. [5] examined the molecular mechanism underlying RNAi. They discovered that RNAi has been ATP dependent, yet uncoupled from mRNA translation. During the RNAi reaction, both strands of the dsRNA are processed to RNA segments, 21-23 nucleotides in length. They also reported processing of long dsRNA by RNase III (Dicer) into shorter fragments of 21-23 nt intervals in *Drosophila* [5]. In 2001, Bernstein et al. [6] identified an enzyme, Dicer, which can produce putative guide RNAs. The enzyme has a distinctive structure, which includes a helicase domain and dual RNase III motifs. Dicer also contains a region of homology to the RDE1/QDE2/ARGONAUTE family, that has been genetically linked to RNAi [6].

In 2001, Elbashir et al. [7] showed that 21 nucleotide siRNA duplexes specifically suppress expression of endogenous and heterologous genes in different mammalian cell lines, including human embryonic kidney and HeLa cells. This promises a brilliant future of therapeutics [7]. And later in 2003 in mammalian cells, shRNAs was used to carry out the sequence-specific silencing [8-10]. In 2003, Song et al. [11] reported that siRNAs can be used therapeutically in whole animals.

Mechanism of RNA Interference

RNA interference or RNAi (in some articles categorized in knockdown methods), is an exciting strategy for reverse genetics [12]. In RNAi, a double-stranded RNA (dsRNA), which operate as templates for a protein called "dicer ribonuclease III" (which produce short interfering RNAs or siRNAs), slices the long dsRNA molecules to fragments of ~ 20 nt [6]. The produced siRNAs by dicer become separated into two ssRNA, called the guide strand and the passenger strand (Figure 1). The guide strands integrate into a protein complex called RNA inducing silencing complex (RISC), which serve as sequence specific guides that target homologous mRNA molecules for destruction, and the passenger strand will be degraded [13,14]. Post-transcriptional gene silencing is the outcome of this event, which happened when the guide strand coupled with its complementary mRNA, and induces splicing by argonaute, which

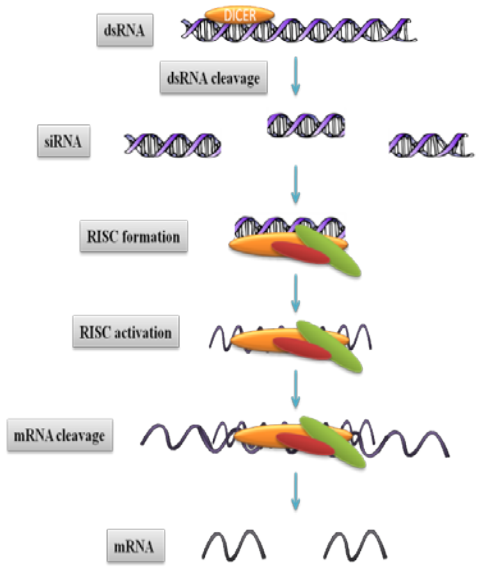

Figure 1: In RNAi, siRNA duplexes are produced naturally when an enzyme, Dicer, cleaves long double-stranded RNA (dsRNA), which becomes associated with an RNase-containing complex to form the RNA-induced silencing complex (RISC), which unwinds the siRNA duplex and releases the sense strand. The RISC-bound antisense strand then serves as a guide for targeting the activated complex to complementary mRNA sequences, resulting in subsequent mRNA cleavage and degradation.

is the catalytic component of the RISC complex. This process leaves the genomic DNA intact, but suppresses gene expression by RNA degradation [15].

Biological Functions of RNA Interference

Immunity

Not only the RNAi technology scores over many approaches with its ease, cost-effectiveness and rapidity, in order to be used in research [16], but also RNA interference is now regarded to be an evolutionarily conserved, existing in the entire eukaryotic kingdom, and its original function is serving as a defence mechanism against viruses and foreign nucleic acids [17]. Likewise to the silencing of genes by RNAi, viral functions can be silenced by the same mechanism, as well. Knowing this, virus expression vectors were developed to be used as vehicles with which to deliver siRNAs into cells.

The concept of "molecular immunity" results from a fusion of concepts from diverse fields, including plant biology, molecular genetics, immunology and biochemistry; they have contributed to describing a mechanism by which eukaryotes are able to regulate gene expression, fight double-stranded RNA (dsRNA) viral infections, and protect their genomes from genetic parasites, such as transposons (the DNA mobile elements that move from one location to another), retrotransposons (the DNA mobile element that use RNA intermediate), and retroviruses. In the last several years, numerous examples of RNA based intracellular molecular defense mechanisms have been described. All of these are epigenetic, and are based upon recognition of nucleic acid sequence homology at the mRNA level, the degradation of a homologous RNA by dsRNA, or triplex RNA [18,19]. RBGS has been shown to inhibit production of retroviruses (the Human Immunodeficiency Virus, HIV-1) [19,20], Rous sarcoma virus [20,21], RNA viruses [22], and a DNA virus (Human papillomavirus) [23].

Downregulation of genes

Recently, RNAi technology can be emerged as a powerful tool for studying the knockdown of genes *in vitro*. One study was done to inhibit the α-globin mRNA expression in erythroid precursor cells *in vitro* by RNAi, and may possibly be applied in reducing α-globin levels in severe forms of β-thalassemia, in a clinical setting [1]. For that purpose, siRNA mediated gene silencing can be used for the down regulation of a gene phenotype *in vitro,* with striking potency at relatively low compound costs. But the quantity of siRNAs must also be carefully regulated. Too many siRNAs can set off an undesirable immune response. In addition to these difficulties, studies have shown that RNAi can silence genes that have not been targeted. Although difficulties exist, RNAi does have some distinct advantages over traditional disease treatments. The main advantage is that RNAi is a natural cell pathway. The pathway's continued existence of the prokaryote eukaryote split to the present is a testament to its effectiveness. Another advantage of RNAi is that siRNAs can be easily mass produced. This speeds up experiments, and could mean that future treatments involving RNAi will be inexpensive. Current research was done on the downregulation of Akt1 expression in gastric cancer cells, in which Lentivirus-mediated RNA interference (RNAi) was used to silence the Akt1 gene [24]. The study confirmed that downregulation of Akt1 reduced chemotherapy tolerance of gastric cancer cells to cisplatin treatment.

Off-target effects

Off-target activity is defined as the nonspecific silencing of genes, other than those for which the sequence of siRNA has been manufactured [25]. Offtarget effects can be reduced by avoiding the incorporation of sense strand (siRNA), and promoting the incorporation of antisense strand (siRNA) of siRNA duplex into the RISC complex. 5′-phosphate group is essential for the siRNA strand to act as a guide strand. So, modification of the 5′-phosphate group of the sense strand to a 5′-O-methyl, can effectively avoid sense strand RISC (siRNA–RISC) formation [26]. In earlier studies, it was found that 11 contiguous matches between siRNA and the off-target mRNA can result in silencing of protein production [27]. In one experiment, 16 siRNAs were designed to target the same specific coding region and the expression profiles of each siRNA, then compared. As well as silencing the target gene, it was found that each siRNA showed specific, repeatable, off-target gene silencing with only a small number of gene regulations in common, *i.e.* the off-target effects were specific to the siRNA, not the target [27].

Applications and Current Prospects of RNAi

In circumstance with the present work of RNAi, we can think of its revolution in the field of genetic engineering for its potential to control the gene expression, which helps in the therapeutic approach for the treatment of various diseases, as well as for the use of a tool in functional genomics. The capability to operate RNA silencing has become a broad diversity of practical applications of biotechnology, ranging from molecular biology to gene therapy in animals. This process can be induced experimentally with high efficiency and targeted to a single specific gene, or a multigene family. The most commercially RNAi is used as a therapeutic agent. While there are many technical hurdles to be overcome before the technology would

be of application to man, there has been much research in animals into the potential of RNAi as a therapeutic [28]. Many approaches have been explored, and many more can be envisioned to modify the splicing pattern of a mutant pre-Mrna, or eliminate a mRNA that bears a disease causing mutation to achieve therapy. Increasing knowledge of RNA biology and chemistry is stimulating. There are many powerful approaches that qualifying siRNA compounds, for developing as therapeutic drugs. Due to its mRNA targeting strategies, it has many advantages over traditional therapeutic drugs. As RNAi interferes with translation and not with DNA transcription, siRNA may not interact with chromosomal DNA. This lack of DNA interaction greatly reduces concerns about possible adverse gene alteration that might result from DNA-based gene therapy [29]. The interaction of siRNA with mRNA, not protein, also makes it possible to reduce the production of harmful proteins before synthesis. Another merit of siRNA as a therapeutic drug is that a wide range of target proteins can be utilized for gene silencing to treat diseases [30].

siRNA design and possible delivery strategies

In recent year, the antisense strategies for mRNA silencing have been growing rapidly. siRNAs are small dsRNAs, generally 20-24 nt in length, that are processed from longer dsRNAs. One strand is the 'guide' strand and directs silencing, with the other strand the 'passenger', being degraded [31,32]. siRNAs usually show full complementary to their target mRNA, and cleavage occurs 10-12 bases from the 5′ guide strand binding site [5,7]. The most effective siRNA have a medium GC content at about 35-55% [25,33]. According to some researchers, too low GC content may destabilize the structure of siRNAs, while too high content may delay RISC assembly. Several strategies for inducing siRNA-mediated gene silencing have been developed, such as chemical or enzymatic synthesis, DNA plasmid vector, or cassette and viral vector. Synthesis, purification and annealing of siRNAs by industrial chemical processes is becoming increasingly popular. This method is rapid and purity is generally high. This may be the best approach for initial "proof of principle" experiments. Effective design of synthetic siRNAs relied on a detailed investigation of the characteristics of naturally occurring siRNA molecules, to ensure effective uptake by RISC and specific silencing of the targeted gene. The sequence selection process has no other constraints. It is important to note that the structure within the targeted mRNA appears to have minimal effect on the availability of the mRNA target and efficacy of the siRNA silencing approach. To-date, successful silencing has been achieved using the above method to select the target sequence, although the method is essentially random, with respect to accounting for mRNA structure.

Although siRNA silencing appears to be extremely effective by selecting a single target in the mRNA, it may be desirable to design and employ two independent siRNA duplexes to control for the specificity of the silencing effect [34]. This recommendation is only for specificity, for it is yet unknown if the targeting of a gene by two different siRNA duplexes would be more effective than using a single siRNA duplex. It is believed that the rate-limiting component of the siRNA effect is the availability of cellular nuclease components, and not mRNA target availability. Therefore, doubling the number of siRNA duplexes is not expected to double the rate or efficiency of silencing [35].

An efficient siRNA delivery system includes a cationic group for ionic interaction with siRNA, poly ethylene glycol (PEG) for steric hindrance, an endosomolytic group for endosomal disruption, and a targeting ligand for site-specific delivery [34]. In 2001, Elbashir et al. [35] reported that chemically synthesized 21 base-pair double-stranded RNA molecules suppressed the target gene with high specificity. Since then, chemically synthesized siRNA has been widely used for biological researches to identify gene functions. Chemically synthesized siRNA can be modified with functional group by chemical reaction, for improving the stability *in vivo,* and reducing non-specific gene suppression. In term of high-throughput applications, vector based strategies are favoured because such strategies enjoy the advantages of much lower cost, and the ability to regenerate. The advantage of the vector-based siRNA is the capability of removing those cells that are not transfected with the plasmids, by selecting the transfected cells with antibiotic resistant genes. Virus vectors also enable the delivery of siRNA expression cassettes into cells with higher transfection efficiency, and in case of lentivirus and retrovirus, it is easy to make stable knockdown cells by integration into the genome [36].

Applications in clinical research and diagnosis

RNAi was recently used to inhibit lethal infection by the filovirus Ebola in a primate model [37]. This strategy protected animals from death, including those that received siRNAs, only after the onset of the infection. RNAi-based therapies are also under development for hepatitis B virus (HBV) [38,39], and hepatitis C virus (HCV) [40]. The liver was also one of the first organs targeted in the development of RNAi-based therapies for cancer [41]. One study used SNALPs targeting PLK1, a cell cycle protein that is crucial for the activating phosphorylation of many cell cycle proteins; inhibition of PLK1 induces cell cycle arrest and tumour cell apoptosis [42].

siRNA delivery to specific cell types is a key challenge for turning siRNA into therapeutics. A number of cell specific ligands, including polysaccharide, antibody, antibody fragment and peptide have been employed to achieve targeted delivery of siRNA. Its present success and future development will clearly benefit from the scientific experiences acquired from related gene-silencing technologies, such as antisense oligonucleotides, ribozymes, and DNAzymes [25]. For the future development of RNAi based therapies, it is important to perform a risk-benefit analysis, and to respect the autonomy of the human subject or patient, by considering the risks of generating infection competent viruses or introducing genetic changes in germ line cells [43]. However, future research needs to address the important challenges relating to more effectively design, enhanced biological stability and efficient targeted delivery *in vivo.* RNAi approaches offer the promise of a certain degree of control over the Huntington's disease [44]. Recent studies reveal that pharmacological manipulation of Tmprss6 with RNAi therapeutics is a useful approach to remedy iron overload diseases linked with diminished hepcidin expression, and might have effectiveness in modifying disease-associated morbidities of β-thalassemia intermedia [45]. However, there are several challenges in the potential use of RNAi in the treatment of these diseases [41].

Conclusion

The area of RNAi is however rapidly expanding, and new innovation is being made on a daily basis. RNA silencing approaches have been quickly developed and employed in plants, animals and many fungus species, as a tool for exploring gene function. The therapeutic potential of RNAi is theoretically unrestricted to any

disease-related gene, that can be amenable to RNAi-mediated silencing. The mechanism of RNAi shows that it is a powerful tool for biochemical studies and developing technologies, and a ray of hope for various challenging diseases. This review summarizes the discovery and translation of this approach, all the way from concept to the clinic. The future advances in RNAi technology are expected to expand our capability to design and use RNAi libraries for genetic screening in mammals. Currently, the RNAi-based medicines are running their way into the clinic. If we can overcome the questions about the safety and specificity of the RNAi experiments, we may possibly clear out the way of introducing this method into a real cure. This area is expected to perceive novel development in the near future.

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Conflicts of Interest

The authors declare no conflicts of interest.

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