
RNA Interference: a Novel and Physiologic Mechanism of Gene Silencing With Great Therapeutic Potential

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The post-genomics scientific era has evolved rapidly while achieving advanced understanding of the structure and function of the genes responsible for both the phenotypic characteristics of higher organisms and the pathophysiology of several genetic diseases. Researchers in the fields of oncology and infectious diseases have become more convinced of the great potential of molecular biology approaches to further develop highly specific diagnostic and less toxic therapeutic strategies. During the last two decades, approaches for the specific silencing of essential viral genes and cellular oncogenes were evaluated with optimism for developing directed therapies. However, there were drawbacks in the use of antisense oligonucleotides as a practical mechanism of achieving

gene silencing both *in vitro* and *in vivo*. Recently, a novel role for post-transcriptional gene silencing mediated by double-stranded RNA (dsRNA) was discovered in the experimental model of *C.elegans*. This mechanism, termed RNA interference (RNAi) has also been found in other eukaryotes, from plants to mammals, including humans. RNAi is presently being explored both *in vitro* and *in vivo* in functional genomics studies and possible therapeutic uses due to its highly specific and physiologic mode of gene silencing. This article focuses on the most current information available regarding the RNAi mechanism and its uses in models of cancer and infectious diseases.

Key words: RNA interference, Gene silencing, Therapeutic applications

Since the discovery of DNA half a century ago, the field of molecular biology has emerged with the challenge of deciphering the complexity of the genetic code from simple prokaryotes to highly complex eukaryotes, such as in the case of the human genome. The study and characterization of medically important genes in the fields of oncology and infectious diseases have evolved dramatically with the help of molecular biology techniques, in the post-genomics era. However, most of the techniques developed during the last decade to study gene function by transcription disruption *in vitro*, have proven to be of limited efficacy in human cells' models of disease and inappropriate for *in vivo* application in gene therapy.

Current technologies used to perform functional genomics studies are based on the central dogma of

genetics, which states that genomic DNA is transcribed to a gene-coding mRNA molecule and proceeds to be translated to a polypeptide with a determined function. Therefore, the disruption or mutation of a specific gene sequence should result in the shutdown of the desired protein synthesis or translation of a mutated form. Previous attempts to disrupt protein expression in human cells *in vitro* have been basically at the transcription level (mRNA), due to the difficulty of creating gene knockouts in these cells, as diploid organisms require both alleles to be disrupted. An early explored and promising technique for developing gene knockdown studies was antisense (1-3). The general mechanism of antisense involves basic Watson-Crick base pairing between single stranded antisense oligonucleotides (ssDNA, ssRNA, phosphorothioate, methylphosphonate or peptide nucleic acid) and the complementary mRNA sequence, which results in steric block to the ribosome during translation. Many approaches for enhancing the efficacy of antisense were developed, such as the incorporation of modified bases (phosphorothioates, methylphosphonates and peptide nucleic acids) to increase their stability and resistance to cellular nucleases both *in vitro* and *in vivo* (4-7). However, there were several drawbacks for their use, such as the limited cellular uptake, short-term effects

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(duration of downregulation for less than 6 hours), and high variability of results in several cell lines and primary cells (8-10). In addition, their uses *in vivo* for both functional or therapeutic studies have been limited due to their decreased stability in serum, poor availability to target tissues (11) and cellular uptake plus short-term silencing.

In the case of *in vivo* gene function studies involving animal models, the development of gene knockout mice revealed an excellent system for establishing particular roles of deleted genes. Even though this technology resulted in substantial in-depth knowledge of crucial routes at the functional genomics level from developmental biology up to immunology, it has some definite disadvantages. The gene knockout mice process is extremely laborious, intense, expensive, and in several cases may lead to lethal deletions which result in death of the embryos. In addition, gene knockouts are not suitable for studying multiple genes *in vivo*, as possible lethal deletions may occur. Furthermore, the use of gene knockouts could not be incorporated into genetic therapy towards blocking pathological genes, thus actual biomedical approaches must rely on the pharmacological targeting of specific proteins or enzymes produced by the specified genes.

In spite of the drawbacks that DNA based therapeutics may have encountered in the past, here we describe a potential emerging molecular technology for use in the study of gene expression (functional genomics), and most interestingly, to specifically and effectively silence disease-causing genes both *in vitro* and *in vivo*. Recently, a novel gene silencing function for RNA was discovered in eukaryotic cells by Fire and colleagues in the nematode *Caenorhabditis elegans* (12-15) and was named RNA interference (RNAi). This RNA-induced silencing pathway was previously observed in plants (16,17). However, the actual mechanism for gene downregulation after homologous RNA introduction into plant cells was not well understood. The mechanism of RNAi has also been described in several eukaryotic organisms such as *Neurospora* (18), *Drosophila spp.* (19-21), and mammals (22-23), including human cell lines and primary cells. The most interesting characteristic of RNAi, is that this mechanism has evolved naturally in these organisms, probably as the result of a developed intrinsic defense towards RNA viruses and transposons, making it an ideal physiologic approach for both *in vitro* and *in vivo* gene silencing (24,25).

Mechanism of RNAi. RNA interference (RNAi) occurs after a long double-stranded RNA molecule (dsRNA) is introduced to a cell and is processed in the cytoplasm by a two-subunit RNase III-family enzyme named Dicer, which catalyses the digestion of the dsRNA molecule into smaller,

21-23 nucleotide-long dsRNA fragments termed small interfering RNA (siRNA) molecules. Each of these siRNA molecules consists of both a sense and antisense strand to the homologous RNA target (mRNA) sequence (22). These siRNA molecules serve as a trigger for the RNA Induced Silencing Complex (RISC) cellular machinery, composed of associated proteins and RNA nucleases, which selectively degrade the target homologous mRNA (Fig. 1). Although this process of post-transcriptional gene silencing seems to be highly specific to the target homologous RNA sequence, in mammalian cells there is another non-specific outcome of introducing dsRNA molecules longer than 30 base pairs. This response is mediated through activation of the protein kinase R (PKR) pathway, which induces interferon-alpha (IFN- α) production, the so called interferon response (26-28). By this process, the interferon response results in general protein synthesis shutdown and leads to cellular death through apoptosis. Thus, the interferon response seems to be another intrinsic cellular defense against invading RNA viruses in mammalian cells. Therefore, for RNAi in mammalian cells to be effective and specific in inhibiting gene expression, the effector dsRNA duplexes (siRNA) must not exceed 30 base pairs in length.

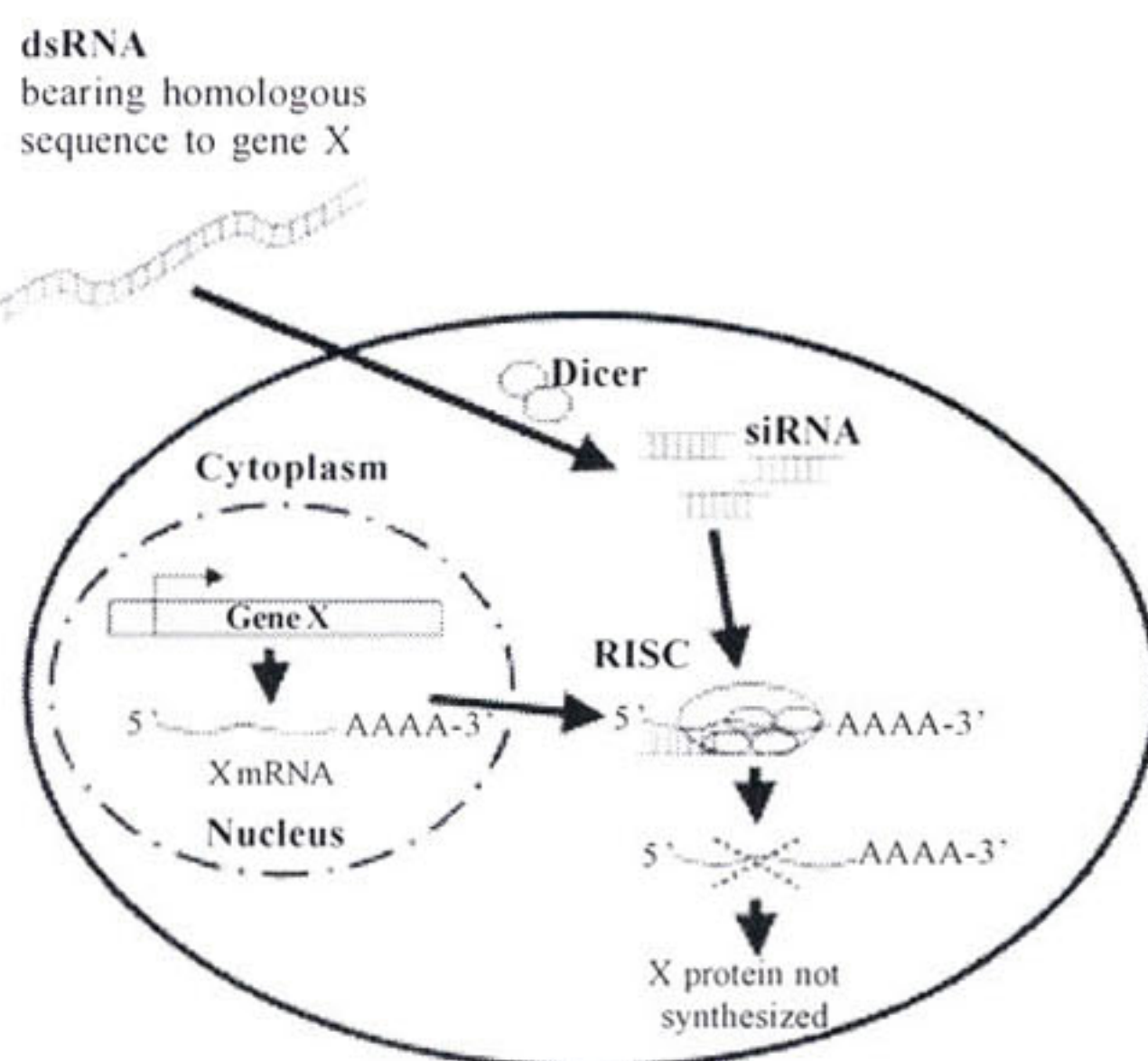


Figure 1. Mechanism of RNA-mediated interference in eukaryotic cells. A dsRNA molecule is introduced to the cell and digested by Dicer to produce active siRNA molecules. The siRNAs produced (21-23 nucleotide long) must be homologous to the target mRNA for the RISC proteins to induce its silencing.

Advantages of RNAi induction over the antisense approach. Prior to the discovery of RNAi, there were some widely used post-transcriptional gene silencing techniques, mostly utilizing modified single-stranded

antisense oligonucleotides. The most commonly studied oligonucleotide modifications include phosphorothioate, methylphosphonate, and 2-O-methyl linkages. These chemical modifications result in an increased resistance to serum and cellular nucleases, thus resulting in an increased stability when compared to unmodified nucleic acids (29). Some major disadvantages affecting the clinical use of these chemically-modified oligos are that they must be artificially synthesized, are non-physiological molecules, have poor cellular uptake and cannot be synthesized intracellularly. Therefore, these modified oligonucleotides should be administered at high doses and multiple regimens for them to be successful for gene silencing. However, the problem of effectively delivering oligonucleotides *in vivo* for targeting particular tissues or cells remains a difficult task.

Another oligonucleotide modification that results in higher resistance to cellular nucleases is the utilization of peptide nucleic acids (PNAs). PNAs contain peptidic linkages in their structural backbone, which provide increased stability to nuclease degradation (30,31). These molecules have been used successfully for RNA and DNA hybridization assays, and were initially thought to have good potential for mRNA targeting due to their high stability in serum (32). However, a major drawback of PNAs for selective targeting of mRNA is their poor cellular uptake (3). Also, due to their amino acidic nature, PNAs may non-specifically interact with cellular proteins, hampering their interaction with the target mRNA.

On the other hand, RNAi offers a more suitable tool for effective gene silencing in terms of their high sequence specificity, simplicity of synthesis (double-stranded RNA), viral and non-viral mediated cellular delivery, and the capacity of intracellular synthesis by siRNA expression vectors or cassettes (24,33). All of these characteristics make RNAi a powerful tool for potential siRNA-expressing DNA vaccines against specific viral and tumorigenic genes. In addition, the physiologic nature of the RNAi mechanism in human cells combined with its high specificity and its null cellular toxicity, promises a safer application of this approach to successful gene therapy (34).

Methods for Inducing RNAi

Chemical synthesis of siRNA duplexes. Currently, there are several commercial suppliers who offer tailor-made synthesis of specific 21 nucleotide-long siRNA molecules designed accordingly to the desired mRNA target sequence. There are also increasing numbers of validated siRNA libraries available towards many cellular genes, which may be an affordable means of selecting

effective sequences. The general structure composition of an siRNA molecule is 5'-AA(N19)UU-3', where N stands for the bases adenine (A), guanine (G), cytosine (C), or uracil (U). The siRNA molecules are designed based on the following guidelines (35,36): first, selection of a 19 nucleotide-long target sequence (N19) preferably upstream from the desired mRNA start codon (AUG), leaving a UU overhang at the 3' end (this results in a RNA duplex of 21 nucleotides); second, a G/C content of 20-50% in the duplex siRNA; and third, assessment of lack of homology to other genes by performing a gene sequence comparison (BLAST) for the desired target mRNA sequence from the organisms' genome database (<http://www.ncbi.nlm.nih.gov/BLAST/>). This is followed by the evaluation of multiple (3 or 4) possible regions on the target mRNA to determine the most efficient siRNA molecule against the desired target. There are several online softwares (<http://design.rnai.jp/sidirect/index.php>) with designed algorithms which allow the user to search for multiple siRNA sequences with the minimum requirements for an effective gene silencing experiment (37).

Long dsRNA molecules. As mentioned previously, the cellular mechanisms of RNAi are primed by the recognition of long dsRNAs (~200bp) by the Dicer enzyme. Apparently, these long dsRNAs mimic some naturally occurring viral genomes, explaining at least an evolutionary appearance for RNAi in eukaryotes. Introduction of dsRNA longer than 30 base pairs in mammalian cells triggers a robust interferon response, which causes non-specific shutdown in protein synthesis (26-28). However, there are commercially available kits for *in vitro* production of siRNA cocktails using long dsRNA (100-200 base pairs) and recombinant Dicer. This approach may lead to increased chance of gene silencing due to the fact that hundreds of diverse effective siRNAs towards the same mRNA sequence can be produced. Therefore, using siRNA cocktails can bypass the need for designing and assaying individual siRNA molecules to select the most efficient sequences.

Intracellular siRNA expression: DNA based siRNA expression cassettes (SECs) and viral vectors. One of the most versatile RNAi approaches is the construction of siRNA expression DNA cassettes or expression vectors (Fig. 2). Basically, SECs consists of an RNA pol III promoter region, followed by the sense and antisense sequences (19 nucleotides long) of the homologous target mRNA, joined together by a non-hybridizing nucleotide sequence (loop region). The transcribed RNA self-hybridizes its sense and antisense sequences forming a secondary structure called short hairpin RNA (shRNA). This intermediate dsRNA structure is further processed intracellularly by Dicer to generate the active siRNA duplex

(38-42). The fact that active siRNA molecules can be synthesized intracellularly by the use of SECs, suggests the potential for stable specific gene silencing, and thus the possible application for DNA based therapeutics (33,34). These SECs can be transfected directly into cells *in vitro* for high throughput screening or short term silencing, or can be cloned into selectable expression plasmids (bearing antibiotic resistance genes) or integrated viral vectors for stable long term studies or *in vivo* delivery. In this way, long term gene silencing studies can be performed by selecting stable transfected clones of cells expressing the desired siRNA.

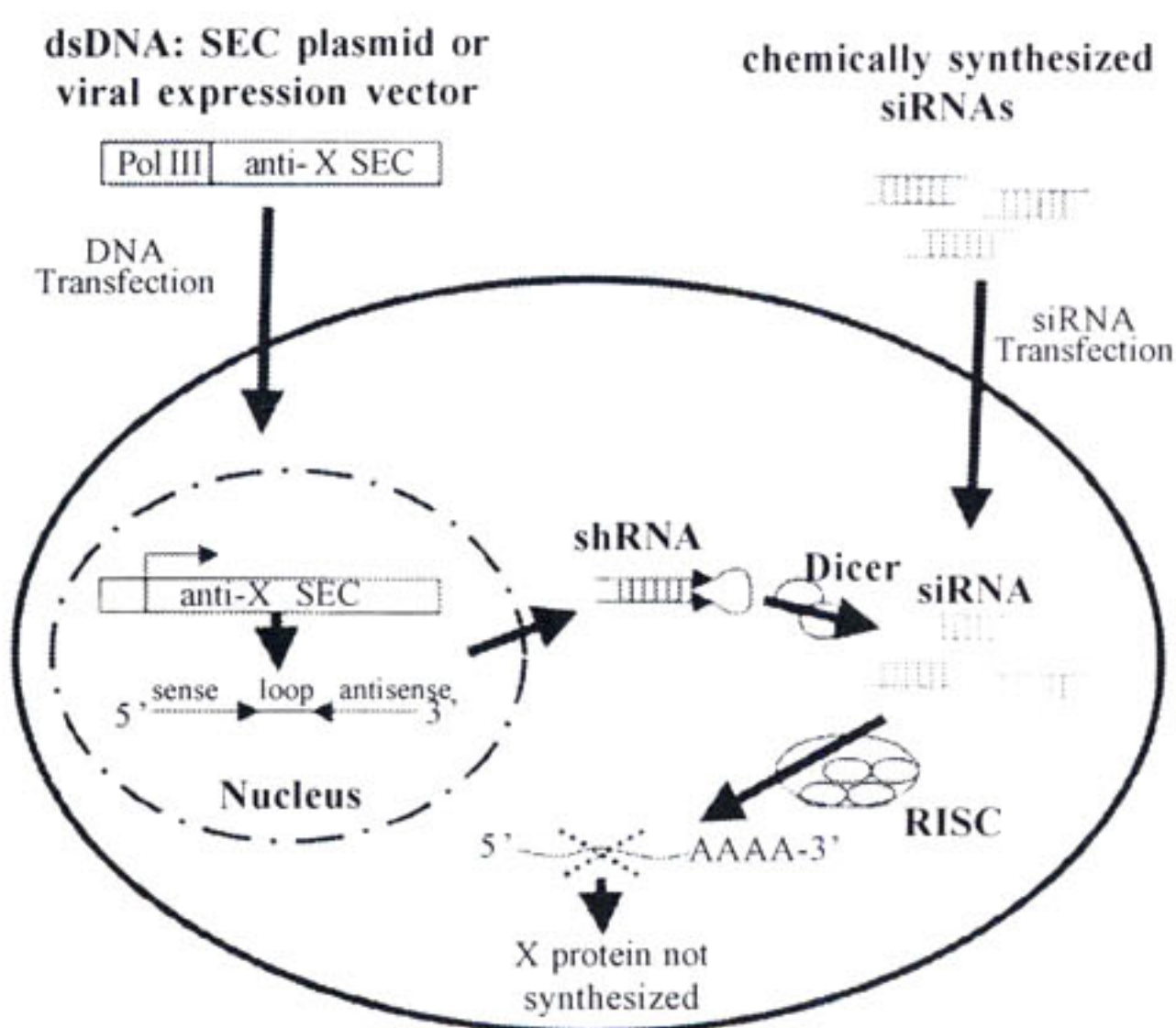


Figure 2. Methods for inducing RNAi. Small interfering RNA (siRNA) can be chemically synthesized and transfected into cells for short-term gene silencing studies. On the other hand, DNA vectors can be designed to express sequence specific siRNA intracellularly (siRNA expression cassettes = SEC). Incorporating a selection marker or gene can be used to select stable clones of gene-knockdown cells for long-term silencing studies.

RNAi as a potential tool for gene therapy. As the RNAi mechanism has been extensively studied and exploited as a powerful strategy for post-transcriptional gene silencing studies in human cells, its therapeutic potential has also been demonstrated in several *in vitro* and *in vivo* models of disease (24,33,34). The effective inhibition of tumor growth and stimulation of apoptosis *in vitro* in immortalized cells has been achieved by blocking several associated oncogenes such as the signal transduction protein Stat 3 and the anti-apoptotic *livin* gene (ML-IAP/KIAP) through RNAi (43-45). Moreover, inhibition of tumor

growth *in vivo* has also been observed after silencing genes for the epidermal growth factor receptor (EGFR) and the Polo-like kinase 1 (PLK1) in mice (46,47).

The phenomenon of silencing pathologic genes through RNAi has been utilized with effectiveness for viral infection and replication inhibition in Hepatitis B and C viruses, HIV-1, respiratory syncytial virus (RSV) and severe acute respiratory syndrome virus (SARS). There are recent *in vitro* studies involving replication inhibition of Hepatitis C virus by targeting the NS3 and NS5B proteins required for viral replication (48-50), as well as *in vivo* inhibition of Hepatitis B virus replication in mice after introduction of siRNA against structural viral proteins mRNAs (51). The same effectiveness of gene knockdown has been observed for inhibiting *in vitro* the synthesis of the Spike (S) protein from the recently emerged SARS-associated coronavirus (SARS-CoV), the etiologic agent of the severe acute respiratory syndrome (52,53). SARS-CoV shares similar proteins and replicative cycle of other coronaviruses, as the S protein. This protein is required for viral envelope interaction with the host cell membrane during the infection process. Effective knockdown of S protein by RNAi demonstrates a putative approach to interfere with the virus replication in host cells.

Certainly, the Human Immunodeficiency Virus (HIV) has been one of the most challenging infectious agents because of its worldwide human threat, lack of an effective prophylactic vaccine, and ineffective means for its cure despite the development of highly active anti-retroviral therapy (HAART). Recently, RNAi has been tested effectively *in vitro* for inhibiting CD4⁺ cell infection by HIV-1 through silencing of the CCR5 (54) and CXCR4 (55) coreceptors. The CCR5 and CXCR4 coreceptors are required for proper viral entrance to CD4-bearing monocytes and T lymphocytes, by macrophagetropic and lymphotropic viruses, respectively. Primary infection with HIV-1 is associated to macrophagetropic virus strains which infect mostly monocytes and macrophages bearing both the CD4 receptor and CCR5 coreceptor. Although the CCR5 coreceptor is required for chemokine signaling, individuals lacking the CCR5 alleles appear to have normal immune responses (56,57). Therefore, targeting the CCR5 coreceptor with RNAi appears to be an effective means to inhibit primary infection by macrophagetropic strains of HIV-1. Another study demonstrated the inhibition of viral replication in HIV-infected cells by siRNAs directed against Tat protein (58). HIV Tat is required for proper replication and transcription at the TAR region located in the provirus LTR. Herein, the Tat protein associates in conjunction with the NFκ-B host cellular transcription factor to initiate transcription of viral genes and replication of the viral RNA genome within the infected host cells.

There is no doubt that the regulation executed by expressed HIV-genes in chronically-infected human cells could be better understood through the use of RNA interference towards specific targets (59,60). Furthermore, the potential applications of this technology towards molecular therapy (gene therapy approaches) in both infectious diseases and cancer is rapidly attracting the optimistic attention of clinical researchers and biopharmaceutical sectors.

Resumen

La era científica post-genómica ha evolucionado a paso acelerado, alcanzando un entendimiento más profundo en cuanto a la estructura y función de genes asociados al fenotipo y patofisiología de un diverso repertorio de enfermedades genéticas en organismos superiores. Investigadores biomédicos en las disciplinas de la oncología y las enfermedades infecciosas, han descubierto el enorme potencial de usar técnicas de biología molecular para desarrollar e implementar estrategias de diagnóstico más específicas y efectivas, al igual que tratamientos más efectivos y menos tóxicos. Durante las últimas dos décadas, se han estudiado con optimismo estrategias para bloquear la expresión de oncogenes y genes virales con el fin de desarrollar terapias específicamente dirigidas. Sin embargo, la estrategia más prometedor y estudiada envolviendo el uso de oligonucleótidos antisentido, resultó ser la menos apropiada para fines del bloqueo en la expresión genética tanto *in vitro* como *in vivo*. Recientemente, un novedoso rol para el ARN de doble cadena (dsRNA) en cuanto al bloqueo de la expresión genética post-transcripcional, fué demostrado en el modelo experimental del nemátodo *C. elegans*. Este mecanismo, se nombró ARN de interferencia (RNAi) y ha sido demostrado ser funcional en otros organismos eucarióticos, desde plantas hasta mamíferos, incluyendo a humanos. Actualmente se exploran las posibilidades de éxito en cuanto al uso y aplicaciones del RNAi, tanto *in vitro* como *in vivo*, en estudios de genética funcional y posibles usos terapéuticos, debido a su mecanismo fisiológico de bloqueo en la expresión genética. Éste artículo discute la información más reciente disponible en cuanto al mecanismo de RNAi y sus usos en modelos de estudio de cáncer y enfermedades infecciosas.

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