RNA interference: A new alternative for rheumatic diseases therapy

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ABSTRACT

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism preserved during evolution. This mechanism, recently described, is mediated by small double-stranded RNAs (dsRNAs) that can specifically recognize a target mRNA sequence and mediate its cleavage or translational repression. The use of RNAi as a tool for gene therapy has been extensively studied, especially in viral infections, cancer, inherited genetic disorders, cardiovascular and rheumatic diseases. Together with data from human genome, the knowledge of gene silencing mediated by RNAi could allow a functional determination of virtually any cell expressed gene and its involvement in cellular functioning and homeostasis. Several *in vitro* and *in vivo* therapeutic studies with autoimmune disease animal models have been carried out with promising results. The pathways of tolerance breakage and inflammation are potential targets for RNAi therapy in inflammatory autoimmune diseases. This review will present the basic principles of RNAi and discuss several aspects of RNAi-based therapeutic approaches, from *in vitro* tool design and target identification to *in vivo* pre-clinical drug delivery, and tests of autoimmune diseases in human cells and animal models. Finally, this review will present some recent clinical experience with RNAi-based therapy.

Keywords: small interfering RNA, gene expression, gene therapy, autoimmune diseases.

INTRODUCTION

RNA interference (RNAi) is a cellular mechanism responsible for post-transcriptional gene silencing (PTGS) acting on messenger RNA (mRNA). In the heart of this mechanism there is a double stranded RNA molecule (dsRNA) that, when incorporated in active form to an intracytoplasmic complex, binds to a complementary sequence of nucleotides located in mRNA target, thus, causing silencing by inhibiting translation and or mRNA degradation. There is evidence that dsRNAs are also involved with the maintenance of condensed chromatin regions and suppression of transcription in these regions vicinity. However, the association between transcription gene silencing (TGS) and RNAi is not yet completely understood. In fungi, the deletion of genes related to RNAi pathway causes loss of gene silencing and heterochromatin disruption. This phenomenon is not well understood at molecular level, but seems to have great importance to the proper functioning of genes and maintenance of genomic integrity.[1]

RNAi was first described in plants (petunias) in the early 1990s.[2] Transgenic plants, overexpressing genes for pigment production, presented white flowers due to pigment synthesis inhibition, caused by coordinate silencing of both transgene and endogenous gene. This phenomenon, known as "co-suppression" was also observed in other species of plants, fungi, and other organisms, but the mechanism leading to gene silencing was still unknown.[2-4]

In 2006, the American researchers Andrew Z. Fire and Craig C. Mello were awarded the Nobel Prize in Physiology and Medicine for participating in the elucidation of gene

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silencing by RNAi.[5] The mechanism occurs in many eukaryotic organisms,[6,7] and the dsRNAs involved can be classified according to their origin and function in at least three categories: miRNAs (microRNAs), siRNAs (short interfering RNAs), and shRNAs (short hairpin RNAs).[8]

MiRNAs represent endogenous small dsRNAs, approximately 22 nucleotides long, whose main function is to act as post-transcriptional silencer by inhibiting translation of target mRNA into protein. They were discovered just over a decade ago, in studies of *Caenorhabditis elegans*, and are now recognized as key regulators of gene expression in plants and animals. Genes that encode miRNAs are transcribed by RNA polymerase II in a long primary microRNA (pri-miRNA) that, inside the nucleus, is cleaved by a protein complex which comprises an RNase III (Drosha) and Pasha or DGCR8 protein (DiGeorge Syndrome Critical Region 8 protein) with a binding domain for dsRNA. The cleavage results in a precursor micro-RNA (pre-miRNA), with approximately 70 base pairs, containing a double stranded stretch and a single-stranded loop, forming a structure called hairpin. Pre-miRNA is exported to cytoplasm by exportin-5, and is cleaved by Dicer, generating a mature miRNA of about 22 nucleotides in length. Dicer is a RNase III essential in the process of RNAi. It presents a RNA binding domain, PAZ, which seems responsible for the size of generated dsRNA. Dicer is also involved in the incorporation of one miRNA strand into a complex called RNA-Induced Silencing Complex (RISC), to which it belongs together with Argonaut (Ago2) and other proteins.[9] RISC complex allows pairing between miRNA incorporated strand and target mRNA homologous region by complementary base pairing. Normally, when complementarity is total, mRNA degradation occurs, and when it is partial, translation repression and subsequent mRNA degradation occur (Figure 1).[10]

Argonauts are proteins present in RISC complex, characterized by the presence of conserved domains (PAZ and Piwi). They bind to siRNA and miRNA, and have endonuclease activity directed against the mRNA strand complementary to siRNA or miRNA. Argonaut proteins are also responsible for selecting the siRNA strand, which will be incorporated into RISC.[11,12]

The shRNAs are double-stranded RNAs built to present a similar structure to miRNAs. They can be exogenously synthesized and introduced into the cell or transcribed inside the cell from vectors encoding the shRNA linked to a RNA polymerase III promoter. In this case, the transcript is processed by Dicer in the same way as miRNAs.[13]

The siRNA molecules are synthetic double-stranded RNA of 19 to 30 pb, acting through the pairing of complementary sequences to mRNA target messenger, causing its degradation and therefore specific gene silencing. Target gene silencing

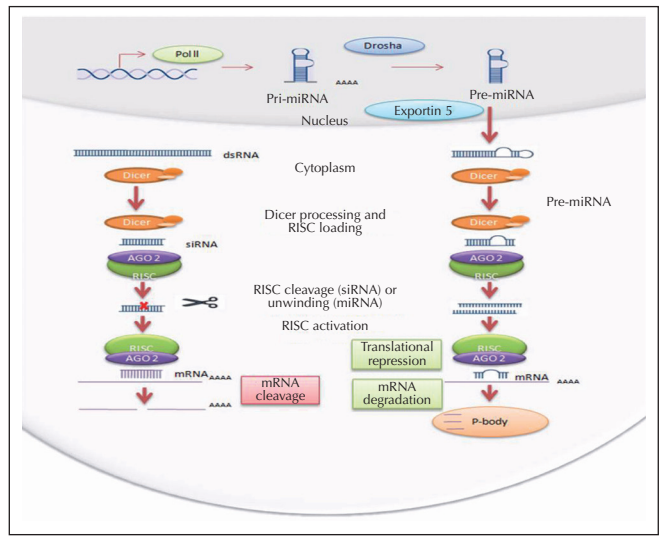

Figure 1. Chart of the pathways leading to interference by microRNAs and siRNAs.

provides information about which function the protein coded by this gene performs in the cell.[14]

Recently, the existence of a link between RNAi and GW bodies (GWB) has been established,[15,16,17] GWBs seem to be the sites involved in mRNA translation repression by miRNAs. The GWB (or P-bodies) are recently described cytoplasmic structures involved in post-transcriptional control of gene expression.[18] This control regulates turnover of mRNA, removal of aberrant RNAs, and nonsense mutations. GWB formation seems to depend on specific proteins and RNA, particularly miRNAs.[15]

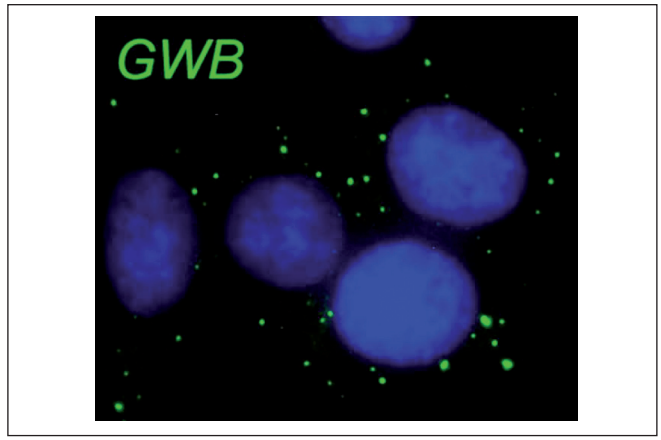

Figure 2. Immunofluorescence on HEp-2, showing presence of GW bodies (visualized in green) in cytoplasm. The nuclei were stained with DAPI (blue). Photomicrograph courtesy of Dr. EK Chan, Department of Oral Biology, University of Florida, Gainesville, USA.

General aspects related to therapy using RNAi

The great potential of RNAi has resulted in successful strategies for silencing a wide variety of protein-coding genes, providing important information about gene function and coded proteins. The high specificity of this method allows interfering with disease-related alleles, which differ from normal allele by only in a few nucleotides. RNAi is much more potent than other antisense strategies that use short antisense strands of DNA and ribozymes.[19]

RNAi has been the method of choice for gene silencing in mammalian cells due to its selectivity and potency. Use of RNAi as a therapeutic approach has been considered highly promising in fighting diseases in which abnormal expression of certain genes can be identified as the cause or contributing factor. Among these diseases, we can mention cancer, dominant genetic disorders, autoimmune diseases, and viral infections.[20]

There are currently over a dozen biotechnology companies dedicated to developing clinical applications of siRNA in several human diseases.[21] RNAi has been used successfully in experimental models for silencing genes critical for tumor cells viability, proliferation, and spread.[22,26] So far, however, the greatest contribution of RNA interference has been the technological revolution introduced in the process of new drug discovery. The use of RNAi reduces the number of potential targets, allowing us to centralize efforts on most promising genes.[27,28] RNAi has also become a powerful tool for validation of drug therapy targets.

siRNAs and shRNAs

There are basically two strategies to induce RNAi. In the first, pre-synthesized siRNAs are introduced into target cells. Although many strategies apply siRNAs of 21-23 base pairs, the longer siRNAs with 27 nucleotides (nt), which are processed by Dicer, are more effective in promoting silencing. These longer siRNAs have a 3' end with 2nt unpaired and a blunt end allowing that Dicer cleavage results in a single siRNA. Eventually, the blunt end may activate signaling proteins and induce unwanted production of interferon by stress response pathways, however, being more powerful, the 27nt siRNAs can be used in smaller doses, avoiding this effect.[29] Because the silencing attained with siRNAs is transient, new strategies were developed to obtain a more permanent silencing. The second strategy is based on the introduction of vectors encoding-shRNA, resulting in stable, long-term cell silencing. shRNA transcription is controlled by RNA polymerase II or III promoter sequences, depending on the desired expression. High levels of shRNAs are obtained with RNA pol III promoters. The shRNAs, controlled by RNA pol II promoters and transcribed as long precursors that mimic pri-miRNAs, can be specifically expressed in a particular tissue and, because they are effective at low levels, they avoid saturation of RNAi pathway components.[20,29]

Selection

There are three important attributes that should be taken into consideration when selecting a siRNA: potency, specificity, and nuclease stability. The initial step to identify good siRNAs candidates start with a bioinformatics project. Several algorithms have been developed to select siRNAs with the specificity and stability characteristics desired, and currently there are *in vitro* methods to quickly identify candidates capable of silencing a particular gene of interest.[30] Still, siRNAs must be evaluated experimentally to determine the efficacy of gene silencing and verify the absence of unwanted effects, named off-target effects.[31]

Potency

siRNAs can be designed to silence any desired gene, often with *in vitro* activity in nanomolar or lower concentrations. Although the use of algorithms increases the chance of identifying effective siRNAs, sometimes, it fails to provide potent siRNAs.[30] A bit longer siRNAs, which need to be processed by Dicer before being incorporated into RISC complex, seems to result in greater efficiency. However, these longer molecules are more difficult to synthesize and more likely to activate an unwanted immune response. [31]

Specificity

One of the critical factors for successful RNAi use is the siRNA ability to silence specific mRNA target. The siRNA-mediated gene silencing can be highly specific, as evidenced by selective silencing of alleles that differ in a single nucleotide.[32] However, siRNAs can also recognize and interfere with the expression of mRNAs with partial homology to target mRNA. In fact, the assessment of transcriptional activity *in vitro* has shown that siRNAs can alter the mRNA levels of genes that are not an intended target (off-target effect), though with less intensity.[33,34] A careful design of siRNA molecule and the introduction of chemical modifications on guide strand ribose residue can reduce or prevent most of the so-called off-target effects without affecting siRNA performance on desired gene.[35]

Furthermore, siRNAs may eventually induce undesired effects by activating the innate immune response mechanisms used in viral defense. Activation of Toll-like receptors (TLR),

particularly TLR7 which recognizes dsRNA, is a major concern. The activation of these receptors on plasmacytoid dendritic cells results in production of type I interferons and proinflammatory cytokines.[36]

Stability

Unprotected siRNA molecules are degraded in human plasma, with a half-live of minutes.[3738] To convert siRNAs into optimized drug, chemical modifications with the ability to extend molecule half-life without affecting its biological activity have been thoroughly investigated.

Some changes involve phosphate group alterations of linkage between nucleotides, which confer exonuclease stability and are well tolerated.[37-40] Changes in sugar residues, such as methylation, which confer resistance to endonuclease, although generally tolerated, depend on location within molecule. In general, changes in sugar sense strand are better accepted than in guide strand, or antisense.[21,4]

The minimum changes necessary for stability of a given siRNA can be assessed by the study of siRNA degradation products in plasma. Some groups had stability and efficiency improvements of *in vivo* deliver complexing siRNA with cholesterol,[42] atelocolgen,[43] and polietilenimina.[43]

Introduction of siRNAs *in vivo*

The main obstacle for using siRNAs as therapeutic drugs is to achieve its penetration into cell via cytoplasmic membrane to incorporated it into the RNAi pathway and cause degradation of target mRNA in selected tissue. In the absence of transfection agents or high pressure, most cells do not incorporate siRNA.[45,46]

Due to its negative charge, siRNA does not penetrate easily through hydrophobic cell membranes.[20,47] However, silencing *in vivo* has been reported after direct administration of naked siRNAs to anatomically isolated sites (intravitreal, intranasal, and intrathecal), showing the possibility of delivering siRNAs into eyes, lungs, and central nervous system.[48,51] However, the therapeutic use of RNAi in a broader sense depends on whether siRNA molecules can be associated with other pharmacological properties such as bioavailability and selectivity for target cells.[31,42]

Several approaches use intravenous injection of chemically modified siRNAs, through cholesterol conjugation, or protected within cationic liposomes. Cholesterol conjugation has shown good results, because it prolongs siRNA half-life in circulation by binding to lipoproteins, which are resistant to kidney filtration and also offer protection to action of plasma nucleases. Although effective, cholesterol conjugation is not a selective method, because cholesterol-siRNA-complex can be endocytosed by cholesterol receptors found in all cell types.[20] Cationic liposome binding to siRNAs, which has also been widely studied, improved pharmacokinetics and reduced siRNAs toxicity, although it will also result in broad spectrum of cellular action.[31]

Selective mechanisms for siRNA delivery to cells and tissues are being intensively studied. One possibility is the binding of siRNAs to aptamers that bind to specific cell receptors. Aptamers are RNA or DNA molecules capable of binding with high affinity to ion, oligosaccharides, and a large variety of proteins and glycoproteins, such as thrombin, L-selectin, P-selectin, vasopressin. Besides having high affinity and specificity for their ligands, aptamers can be chemically synthesized, and is therefore very attractive for therapeutic use, where quality control is critical.[52]

The introduction of cell-specific siRNAs using siRNA bound to a fusion protein of immunoglobulin-protamine Fab was recently reported. The binding of siRNA is mediated by interaction with the protamine portion, and cellular specificity is dependent on the Fab portion of antibody molecule.[45] The delivery to specific cells facilitates siRNA incorporation by endocytosis. Several groups have studied the use of viral vectors to introduce shRNAs into organisms.[53,54] However, the use of viral vectors is still questioned regarding therapeutic use safety.[55]

Preclinical studies with RNAi in autoimmune rheumatic diseases

The last three decades were marked by rapid advances in molecular biology techniques, which are now widely available for routine research groups worldwide. Clear examples include microarray and methods of gene silencing as the small interfering RNAs (siRNA) and cell transfection with antisense constructs. For research groups interested in studying disease pathogenesis, these new techniques enable rapid screening of genes that are expressed in different ways both in health and disease and are probably involved in disease onset and progression.[56] In this context, much progress has been made in understanding the pathophysiology of connective tissue diseases. These diseases comprise a broad spectrum of disorders in which the pathophysiology and clinical presentation are quite different. However, discovery of key mechanisms and molecules involved in connective tissue disease pathogenesis have significantly increased treatment options for its systemic and organ-specific complications. While conventional non-selective therapies do not, yet,

have importance daily in clinical setting, the majority of new agents developed focus on specific molecules or mechanisms that have a functional role in systemic disease pathogenesis or organ-specific manifestations. Several cytokines, chemokines, transcription factors, and molecules expressed on immune cell surface (CDs, adhesion molecules) have been investigated for molecular therapy with RNAi.

Regarding osteoarthritis (OA), a number of cytokines and enzymes have been associated with joint damage and, in recent years, gene therapies aimed at cytokines and/or signaling pathways, activated in inflammatory processes, have brought new hope for treatment. IL-1 and TNF-α are considered the main inflammatory cytokines taking part in this disease. [57] Therefore, they are potential targets for OA therapy. NF-κB is one of the key transcription factors associated with inflammatory pathway, and various methods have been used to inhibit the induction of NF-κB, such as the use of antisense oligonucleorides[58] and adenoviral constructs of mutated IkB.[59] Recently, a study of OA in animal model has shown the efficacy of siRNA specific for NF-κB p65 subunit.[60] RNA interference was able to inhibit the expression of several genes related to NF-κB signaling pathway, such as genes of cyclooxygenase-2 (COX-2), nitric oxide synthase-2 (NOS-2), and matrix metalloproteinase-9 (MMP-9). These mediators are associated with initial progression of joint damage in osteoarthritis model and are induced in chondrocytes exposed to IL-1 and TNF-α. These results demonstrate that siRNA specific for NF-κB p65 is a potential candidate in preventive gene therapy for early stage OA.

The ability to inhibit NF-κB activity becomes even more interesting when its power of action on immune cell activation is broadly examined, as in some protocols that seek to inhibit maturation of dendritic cells and thus restore the immune tolerance. The Canadian biotechnology company Tolerothech Inc. has explored the use of siRNA in preclinical studies to silence inflammatory Th1 pathway. This company developed the drug ToleroVaxT, which is a siRNA specific for IL-12 p35 subunit. ToleroVaxT use induces immune deviation from Th1 response to Th2 response with high levels of IL-4 and IL-10 and low FNI-γ production. Inhibition of dendritic cells (DCs) allostimulatory activity in mixed cultures of leukocytes became possible with this tool.[61]

Because age is considered another risk factor for OA development, chondrocytes senescence has also been investigated with siRNA, for its correlation with decreased extracellular matrix synthesis by chondrocytes that also show less responsiveness to growth factors. The $p16^{INK4a}$ protein has an important role in cell cycle control and senescence by acting as a cyclin competitor that control cell output of G1 phase and leading to senescence. OA patients show high expression of $p16^{INK4a}$, and *in vitro* silencing of this protein in chondrocytes from patients with OA led to a change in their characteristics senesced, increased expression of some chondrocyte-specific genes, and overall improvement in their repair ability. Inhibition of $p16^{INK4a}$ by RNAi can be exploited in a therapeutic strategy for blocking senescence of articular chondrocytes relevant to OA treatment and prevention.[62]

SiRNA therapeutic strategies have also been investigated *in vitro* in synovial cells of rheumatoid arthritis (RA). One of the mechanisms causing joint degradation in RA is the presence of synovial cells abnormally resistant to apoptosis, which produce high levels of proinflammatory cytokines and metalloproteinases. Induction of apoptosis is a common property of death receptors, most of them belonging to the family of tumor necrosis factor (TNF). However, some kinds of TRAIL receptors, as TRAIL 4, have antiapoptotic activity. In a study by Terzioglu *et al*. (2007), it was observed that gene therapy using siRNA specific for TRAIL 4 combined with pro-apoptotic TRAILS transfection cleared synoviocytes resistant to apoptosis. These receptors expression balance may be a new gene therapy strategy to sensitize synoviocytes from RA patients to apoptosis.[63] In collagen-induced arthritis animal models, some studies with siRNA targeting TNF-α silencing are being conducted. Some forms of RNAi delivery are being tested, such as siRNA direct delivery by electroporation in joint tissue, or siRNA systemic delivery using liposome as carrier. Both studies are showing good results, significantly decreasing joint inflammation.[64,65]

Regarding systemic lupus erythematosus (SLE) diseases, studies in animal models have demonstrated the potential of RNAi therapy. Mice homozygous for mutation in the gene encoding Roquin protein show a great increase in receptor expression of inducible co-stimulating T cells (ICOS), with consequent lymphocytes accumulation and development of a syndrome similar to SLE.[66] Roquin protein contains RNA binding domains and may be observed in association with stress granules and GW-bodies. This close association leads to questions on the possibility of Roquin being able to direct some mRNAs to a decay route by miRNA in GW-bodies. The miR-101 is a key component in suppressing the expression of ICOS, and the expression of miR-101 was able to reduce the levels of co-stimulatory receptor. It is believed that, due to a mutation in mice Roquin protein, the mRNA of ICOS is not properly addressed to GW-bodied, which prevents their expression control by miR-101.[66] There are many studies showing the importance of proteins able to regulate miRNA-mediated

repression, directing target mRNA location to GW-bodies. Interestingly, a large number of autoantibodies against key components of RNA interference machinery have been described, both in mice and in humans,[67] including autoantibodies against Argonaut proteins and Dicer.[68]

In autoimmune diseases with fibrosis clinical manifestations, such as systemic sclerosis, TGF-β1 is a potential target for treatment and prevention. Because this cytokine has an important role in fibrosis pathophysiology of various diseases, the use of RNAi targeting this molecule may prevent fibrosis development or even improvement of this condition, as noted in the study by Takabatake *et al*. (2005) of glomerulonephritis animal model. In this study, the use of siRNA against TGF-β1 led to a significant improvement in fibrotic matrix progression and expansion.[69]

The profile analysis of miRNA expression has also proved to be a useful tool in investigating the genetic mechanisms involved in the predisposition and pathogenesis of human diseases, since they have an essential role in embryogenesis and cell differentiation. Koralov *et al.* (2008) showed a role of miRNAs in the early stages of B cell development in experimental model of conditional Dicer knockout.[70] Dai *et al*. (2008) studied the expression profile of miRNA in SLE patients, and identified 16 miRNAs differentially expressed in SLE group compared with normal controls,[71] indicating that miRNAs assessment is a potentially useful biomarker tool of probable factors involving autoimmune rheumatic diseases pathogenesis.

Perspectives

Although therapy with RNAi treatment is associated with a high degree of specificity, recent findings suggest that off-target effects and other potential complications can be noted. Some siRNA may induce post-translational suppression of unwanted gene products, as observed by miRNAs activity, which may have the ability to act on different gene products. This inhibitory effect occurs when non-specific siRNA is used in low concentrations and presents partial complementarity with target gene.

The development of siRNA with a simple change that will lead to a partial matching can not only reduce the efficiency of silencing, but increase the chance of pairing with transcripts whose silencing is not wanted and have regions of partial complementarity. Other nonspecific effects of siRNAs have also been observed. For example, alternative pathways of cellular response against dsRNA can be activated; resulting in activation and increased expression of genes typically associated with innate immune response, including genes for type I interferon (INF) gene expression.[36] In addition, toll-like receptor (TLRs), as TLR7 and TLR8, may recognize certain sequences of siRNA and induce immune response with INF production.[36] Further studies will be needed to investigate the frequency of nonspecific recognition and adverse effects of using RNAi and shRNA, as well as to identify the structural sequences responsible for causing such processes. Potential strategies to reduce induction of IFN effects or TLRs activation include developing algorithms for siRNAs design and chemical modifications capable of reducing their immunogenicity and extend their lifetime, preventing degradation by RNases. Another possibility that should be closely investigated relates to safety with regard to possible mutagenesis insertion and malignant transformation, or the possibility of immune responses development against proteins of viral vectors used for transfection in some therapy models.[34] In general, the use of RNAi as a therapeutic strategy is an attractive alternative and potentially promising. The results of experimental models of autoimmune disease and *in vitro* studies with human cells have been encouraging, but this therapeutic model future clinical application still requires vigorous investigations of efficacy, selectivity; development of strategies to avoid off-target effects; and system optimization for siRNAs delivery.

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