The new great promise in drug innovation: RNA interference from the laboratory to the clinic

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Introduction

THE RNA molecule has been recently identified as a possible drug capable of revolutionizing the way we provide treatment for a large number of human diseases. The biological activities of these molecules, which are generally used in the form of double-stranded RNA, were discovered in the 1990s through the identification of mechanisms of RNA interference and are based on the ability of these molecules to reduce (interfere with) the expression of certain target genes. The therapeutic potential of double-stranded RNA is enormous, particularly in genetic processes that require inhibition of gene products. Among the target diseases are therapies for tumors and infectious agents, especially viruses, but several other human diseases will also be controlled by the action of these molecules. The relatively large size of RNA molecules, especially compared to some chemical drugs, can be one of the problems to be faced by research on the topic, but what draws attention is the ease with which these molecules can be designed, depending solely on the sequence of the target gene basis. Several clinical protocols have been tested in humans in recent years, with promising results. It is possible that soon this type of drug will be found in pharmacies: it pays to know why!

RNA is not just a mere DNA messenger

In the 1980s the scientific community was stunned by the revelation that RNA molecules have catalytic properties, like enzymes. Thus, these molecules were named ribozymes and demonstrated that RNA can perform functions that far exceed those of simple messenger for protein synthesis. But that was only the beginning. In the 1990s, results with transgenic plants began to generate completely unexpected data: plants that over-expressed pigment production genes (and therefore should produce more pigmented, i.e., dark purple flowers) produced white flowers, due to the absence of pigment synthesis! In this case, what occurred was the unexpected silencing of the transgene and endogenous gene of these flowers' cells.

The molecular mechanism leading to gene silencing remained unknown for a few years until the groups of American scientists Andrew Z. Fire and Craig C. Mello, working with the nematode worm *Caenorhabditis elegans*, found that double-stranded RNA molecules (dsRNA) could silence gene expression (Fire et al. 1998). The experiments that led to this finding are extremely simple: when analyzing the effect of gene silencing of antisense as well as sense single-stranded RNA molecules (in relation to the direction of the target gene's mRNA molecule), the researchers found that the effect of the use of double-stranded molecule increased by 100-fold. In view of the ability of dsRNA to interfere with gene expression, the mechanism began to be called RNA interference or simply RNAi.

The mechanism of RNA interference works in human cells

This silencing mechanism was initially demonstrated in worms, plants and insects, but it took three years for its existence to be demonstrated in human cells (Elbashir et al., 2001). These experiments indicated the evolutionary importance of RNAi mechanisms in eukaryotes in general and sparked ideas for the potential technological use of dsRNA molecules as the basis for gene silencing for therapeutic purposes in humans. Furthermore, researchers also identified small dsRNA molecules (in the form of hairpins) synthesized by the cells themselves from their genome, whose function is to control the expression of cellular genes. These endogenous RNA, which became known as microRNA (miRNA), demonstrate that the RNA functions in the cell far exceed those of a simple message. Doctors Fire and Mello were awarded the Nobel Prize for Medicine in 2006 for their discovery.

How RNA interference works in the cell

RNA interference mechanisms are well known. Basically, endogenous genes are transcribed as a long mRNA containing multiple grouped miRNA. These primary miRNA are known as pri-miRNA and contain sequences of palindromic bases, so that their alignment generates dsRNA structures in the form of a hairpin. These molecules are further processed in the cell nucleus and cleaved by RNAse (Drosha), forming structures with about 70 bases, known as pre-microRNA (pre-miRNA). These molecules are exported to the cytoplasm (by the Exportin-5 enzyme) and are again processed by an RNase Dicer, which generate the mature miRNA, i.e., duplexes of 19 to 23 base pairs. The Risc complex (RNA induced silencing complex) is the protein platform that associates with the miRNA and promotes the interaction of one of the dsRNA strands (RNA-guide) with mRNA molecules. This interaction occurs by complementarity in the RNA guide and mRNA sequence, usually in the 3' untranslated region (3'-UTR), resulting in target mRNA silencing, either by mRNA degradation or repression of translation into proteins. In the case of translational repression, complementarity does not need to be complete, which indicates that a single

miRNA can regulate hundreds of genes and each target gene can be regulated by multiple miRNA, thus demonstrating the complexity of this gene regulatory network. Figure 1 shows a scheme depicting in simplified form how the RNA interference mechanism works (for a review, see Liu, 2008).

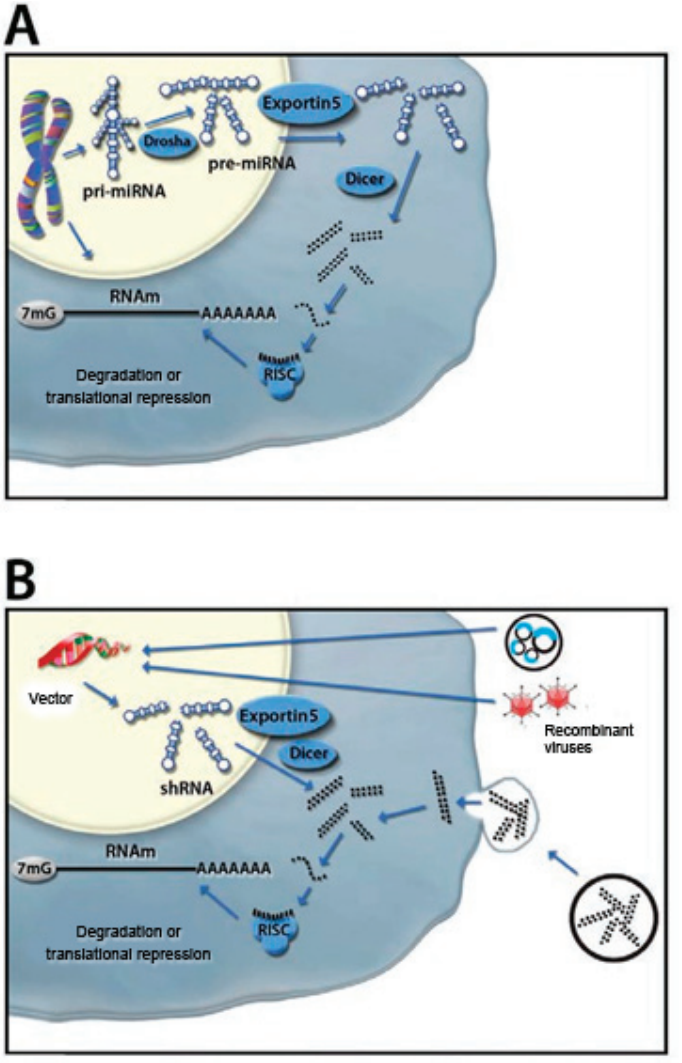

Figure 1 – RNA interference activity in animal cells. A) Schematic of the RNA interference mechanism indicating the main proteins/protein complexes involved; B) schematic showing how we can use these mechanisms to guide gene silencing, with RNA duplexes (siRNA), or viral or non-viral vectors for shRNA expression.

The discovery of gene regulation through RNA interference has also revolutionized the way the role of specific genes is studied in biology. The use of exogenous dsRNA molecules as a tool can reduce the expression of specific genes (an effect known as knock-down), so that changes induced by this strategy enable identifying the functions this gene plays in the cell. In these experi-

ments the sequence of dsRNA molecule is defined by the researcher to interfere in the target gene of choice. This strategy can also be used directly in vivo (in animals or in humans), and hence the possibility of drug development (Tiemann & Rossi, 2009).

Basically, two different approaches may be used. In the first, gene vectors usually derived from viruses (such as adenoviruses, retroviruses or adeno-associated viruses) are transduced into cells to express genes that are transcribed in dsRNA molecules in the form of a hairpin (similar to miRNA). These molecules are known as shRNA (short hairpin RNA) and target the gene to be silenced. The shRNA is cleaved by Drosha and processed by the cellular RNA interference machinery. Although this approach requires the production of viral vectors, which is not a simple process from the drug production perspective, as we shall see there are some therapeutic strategies being tested, such as clinical protocols in humans using vectors expressing shRNA.

In a second approach, small dsRNA molecules can be introduced directly into cells in culture, aiming at the specific degradation of the target gene. These molecules are known as siRNA (small interfering RNA) and can be introduced into the cell using different strategies. Currently, several biotech companies offer siRNA molecules for any human (or mouse) gene the researcher wishes to silence. An important feature is the size of the duplex, which should be between 19 and 30 base pairs, since larger sizes can induce nonspecific interferon responses in animal cells.

In cell culture, the introduction of siRNA molecules is generally made by the formation of complexes of these duplexes with chemical or lipid polymers, usually cationic. The complex is endocytosed by cells, and RNA duplexes are released into the cytoplasm and processed by the RNA interference machinery, promoting the target gene silencing. siRNA has been widely used in functional genomics studies and in different clinical trials because of the robust and specific gene silencing induced by these molecules. Easy manipulation and the existence of siRNA sequences for any human genome gene have made the use of this tool accessible to different groups of basic and applied research.

Interfering with gene expression in vivo

The discovery of RNA interference mechanisms in human cells is relatively recent. However, it was promptly verified that the use of dsRNA as a tool can also be done in vivo, directly in animals. Obviously, most of these pre-clinical trials have been conducted in mice, but larger animals such as monkeys have also been used with promising results, thus encouraging the development of studies directly in humans, and several clinical protocols are already being tested. The therapeutic potential of silencing via RNA has been investigated for various diseases such as cancer, respiratory disorders, inflammation, neurological and autoimmune disorders, and infectious processes (Figure 2). Some phase I clinical protocols testing the safety of the use of therapies in humans have been

completed and it was found that siRNA molecules have been introduced in individuals with no regard for any possible toxic effects. More than that, in several cases it was possible to identify the functioning of these duplexes in silencing the target gene, as desired.

The development of RNAi-based therapy has become an attractive and promising research area, particularly in cases where inhibition of certain proteins was unsuccessful by traditional pharmacological methods. Furthermore, as the development of RNA duplexes depends only on knowledge of the target gene sequence, the development of these drugs is extremely simple compared to traditional drug design, which requires knowledge of the crystallographic structure of the target protein, which is no guarantee of success in determining the active ligands in these structures. Although there are cases in which duplexes may act on targets other than the expected ones (effect known as off-target), several experimental protocols have demonstrated the specificity provided by the sequence of these molecules, thus encouraging further research in the area.

In general, the studies use well investigated methods for the introduction of DNA into organisms, using traditional gene therapy procedures. However, some differences in the use of these two approaches are clear and should be taken into account when choosing the methodology. The siRNA molecule is much smaller (between 19 and 30 base pairs), which facilitates its delivery in relation to RNA- or DNA-dependent genetic vectors (typically several thousand base pairs). Nevertheless, RNA is a much more unstable molecule and can be degraded in vivo by RNase. This characteristic required several studies on chemical modifications that increase its stability in the organism, without harming the action of these molecules with drugs.

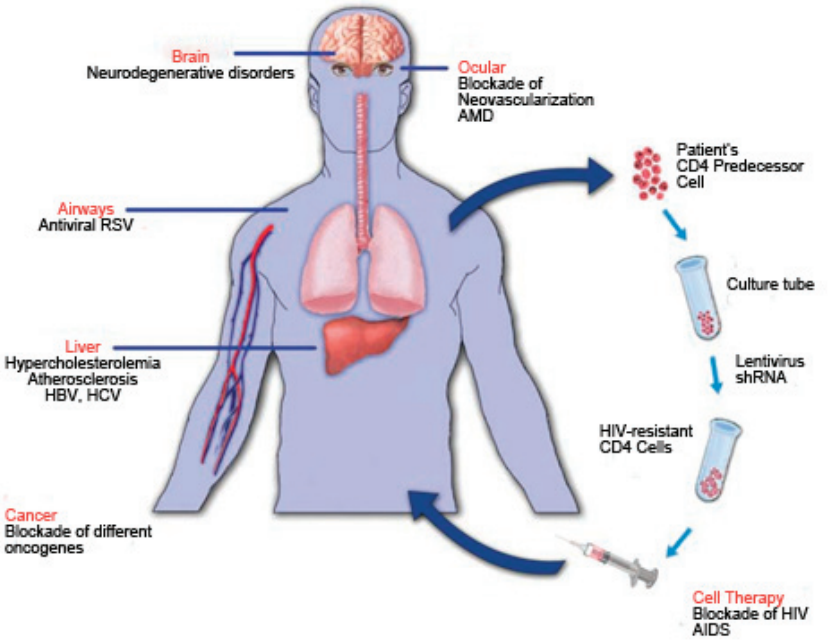

Figure 2 – Main pathways used for gene silencing through direct action in human protocols with RNA interference.

Tests in animals use siRNA or shRNA through various routes of administration, depending on the gene and the target tissue. For example, intravenous administrations allow the duplex to be directed specifically to the liver, where it is internalized into cells. In turn, the introduction of siRNA by intraocular injection ensures a direct effect in that organ, with the advantage that the eye is a compartment of limited size, thus enabling the use of few molecules, which are also effective in gene silencing. Also very successful has been the introduction of RNA duplexes by inhalation, when the target is the airways. Furthermore, siRNA molecules have been introduced directly into the skin, enabling reaching various points of the body, although preferably more superficial tissues. In general, these in vivo applications use, as in cell culture, polymers or lipid complexes (polyplexes or liposomes) that make up the nanoparticles, which are endocytosed for introduction of duplexes into the cells. Curiously, however, in many applications siRNA saline solutions are used directly and these are still effective, promoting the silencing of the target gene in the organism. However, it is quite possible that in many of these cases the use of nanoparticles improves the efficiency of the drug. In the case of shRNA, usually virus-derived gene vectors are used for gene expression in the cells. As in other gene therapy procedures, some cases are directly applied in vivo (intravenously, for example), but there are also cases of ex vivo application, which enables modifying the stem cells of the organism with the vector expressing shRNA (and promotes the target gene silencing). Subsequently, the modified cell is reintroduced into the patient for the purpose of obtaining therapeutic benefits.

Interfering in the human genome for therapeutic purposes

Although the existence of the RNA interference mechanism in human cells has been known for a relatively short time (since 2001), it is surprising to see how quickly clinical protocols using RNAi were initiated in humans. This speed and relative success in some of these protocols enable predicting that in a few years these molecules will be sold in our pharmacies and/or applied in our hospitals (Tiemann & Rossi, 2009).

Among the main indications for the use of RNA interference are those involved in ophthalmic therapies. This is basically due to the compartmentalization of the eye, which allows direct administration of intravitreal injections. The eye compartment is freed of nucleases, and siRNA molecules are introduced into target cells. These advantages have been exploited by drug therapies using oligonucleotides, already on the market (Vitravene®, Novartis and Macugen®, Pfizer). With RNA duplexes (siRNA), clinical trials have focused on the treatment of age-related macular degeneration (AMD). The development of vascularization at the back of the eye, especially in persons over sixty years of age, impairs vision in AMD patients, and treatment with siRNA aims to silence the expression of genes related to vessel development, such as VEGF-I (vascular endothelial growth factor). Phase II/III clinical trials in 217 patients showed an improvement in visual acuity in two-thirds of the patients, demonstrating that this technology may be close to being approved for marketing. However, there are some problems related to the administration system (intraocular injection) and interestingly, despite positive results, there is controversy as to the fact that part of the effects is due to non-specific immune responses, and not to specific silencing by siRNA (Hubschman et al. 2009). Similar studies have been conducted using siRNA against VEGF-A in diabetic patients with macular edema.

Phase II clinical protocols are also being conducted to combat infections by respiratory syncytial virus (RSV). In this case, siRNA molecules against viral genes are administered to the patients by inhalation. Apparently, RSV viruses replicate superficial epithelial cells from the lung which, in turn, are able to endocystose RNA duplexes, so that viral silencing is efficient. Clinical trials with 88 healthy adult individuals demonstrated that siRNA molecules were safe, with infection levels significantly lower compared to individuals who received placebo (De Vincenzo et al., 2010).

RNA interference as antiviral therapy is also being clinically tested in AIDS therapy. Interestingly, in these studies HIV-derived viral vectors (the etiological agent of AIDS) have been used for shRNA expression in stem cells of patients. These vectors, known as lentivirus, are capable of permanently integrating into the genome of the cells, which are then reimplanted in the patient. This process produces virus-resistant autologous cells (mainly T4 lymphocytes, the target of the HIV virus), thus allowing the patient to recover. Data have been promising, indicating that even eighteen months after initiation of therapy, the implanted cells continue to express shRNA (Singh & Gaur, 2009).

In addition to the clinical protocols mentioned here, the RNA interference technology is also being used in clinical trials for many other diseases, such as hepatitis B, different types of tumors (including melanoma), hypercholesterolemia, acute kidney injury, etc. Many target genes are being tested against cancer: oncogenes, cell-cycle and apoptosis mediators, genes involved in protein degradation and stability, angiogenesis, molecules related to metastatic invasion and cell adhesion. Moreover, our proposal is the use of siRNA as co-adjuvants in therapeutic treatments with ionizing radiation and chemotherapeutic agents. To that end, we are currently testing the silencing of target genes such as those encoding anti-apoptotic, multidrug resistant proteins and even proteins involved in DNA repair. Certainly, given the number of preclinical tests under way, the list of target diseases to be tested directly in humans through different strategies using RNA interference should be greatly expanded.

The gold rush in the development of new RNAi-based therapies

The discovery of RNA interference mechanisms, although recent, has sparked a true revolution in the way the functioning of genes in the cells is studied, and also opened up prospects for direct in vivo intervention in gene action. In many cases of human diseases there is an increase in the expression of some genes, and controlling these genes through silencing sparks hope for novel forms of therapy. Chemical inhibitors remain an extremely interesting alternative for many of these diseases, but the RNA interference mechanism provides a simple approach (search for sequence complementarity) for obtaining drugs, thus expanding its potential use and reach. Efforts to use this new biotechnology in the clinic have been in tune with one of the most important recent examples of translational medicine, in which trials move quickly form the laboratory to the patient's bedside. The challenges are still great, with the need for new strategies to maximize the action of effector molecules within cells, as well as of mechanisms of molecule administration and delivery to target organs. Many strategies are proposed, in theory, for targeting these molecules to with a view to ensuring greater specificity in the target tissue, but tests are necessary to demonstrate effectiveness in practice.

The challenges, however, do not seem to scare big pharmacological companies worldwide. Small businesses set up by researchers are being acquired by multinational companies, which are investing billions in research and development, believing that the drugs and strategies developed using the RNA interference approach are capable not only of being successful for specific diseases, but also create models for other diseases. These investments have been so egregious to the point of resembling a new gold rush for the use of knowledge gained from decades of studying the human genome, in the design of new drugs. However, the application of RNA interference-based therapy should be carefully considered, since it still requires intensive research throughout the process of

developing therapeutic protocols. In Brazil these studies are still in their infancy, so that only a few research groups in universities have mastered the technology for the use of RNA interference. Even fewer groups are directly involved in the development of new technologies based on this approach. If this true intellectual silencing persists in the area, we will have to pay dearly for the patents and the use of novel drugs that will most likely be sold in the coming decades.

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Abstract – The discovery of gene silencing mechanisms in our own cells using Rna interference is very recent. However, in less than a decade scientific investigation has progressed enough to make us see that, very soon, we will use this knowledge for therapeutic purposes. RNA duplexes are potential pharmaceutical drugs and there are high investments in this new strategy. The promising gene therapy seems to finally reach maturity with these new tools.

Keywords: RNA interference, Gene silencing, Cancer, Gene therapy, Viral infection.

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