RNAi: a discovery that revolutionized biomedical researches

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REPORT

*I*ntroduction

One of the most important breakthroughs in modern biology was discovering that double chain small or large RNA molecules are able to regulate gene expression. Formerly it was thought RNAs have just two broad functions in cells: Single-stranded messenger RNAs (mRNAs) are vital intermediaries in gene expression, transmitting information between DNA and protein. Ribosomal and transfer RNAs have structural, catalytic and information-decoding roles in the process of protein synthesis [1, 2].

In 1998 Fire and Mello observed in *Caenorthabditis elegans* that double-stranded RNA (dsRNA) was the source of sequence-specific inhibition of protein expression, which they called RNA interference (RNAi) [3]. This phenomenon was observed at first time in plants. In early 90´s plant biologists working with petunias found that introducing numerous copies of a gene that codes for deep purple flowers led to an even darker purple hue, but rather to plants with white or patchy flowers [4]. That indicated that both transgenes and plants genes have been suppressed. However, this phenomenon was not well understood until Fire and Mello published their results.

A great advance in this field was produced when similar process of gene silencing observed in plants and *C. elegans* were reported in many others eukaryotes resulting in an evolutionarily ancient mechanism in numerous organisms including insects, fungi, mammalian and others.

Determining the presence of this phenomenon in mammalian was a hard work due to the interferon response against dsRNA larges which induce non specific degradation of mRNA. Interferon triggers the degradation of mRNA by inducing 2-5 oligoadenylate synthase which actives RNAse L. In addition interferon activates PKR leading to a global inhibition of mRNA translation [5]. Nevertheless, in 2001 Tuschl and colleagues introduced chemically synthesized small interfering RNA (siRNA) into mammalian cells and showed sequencing specific silencing of gene expression in several mammalian cell lines without non-specific effects [6, 7].

Although the gene silencing presents differences in animals, fungi and plants, the process and its molecular participants are basically the same. It is considered that this is an ancestral process to which a function of maintenance of the integrity of the genome is attributed since it suppresses the transposon mobilization, the accumulation of repeated DNA in the germ line; it destroys aberrant, incomplete or unstable messenger RNA and it also works naturally against the viral infections constituting a defense mechanism in many organisms [1, 8].

It is known that the inhibition of gene expression triggered by small interference RNA can take place at three levels; they are: the transcriptional, the post transcriptional and the translational level.

*M*echanism of post transcriptional gene silencing

Silencing begins in the cell by formation of the dsRNA, which could be originated by different ways depending on the specie. The dsRNA is processed by the enzyme called Dicer which is a type III RNAase [6]. This enzyme produces small double strand RNA fragments of 21 to 25 nt [9].

Once the enzyme Dicer cleaves, the dsRNA are used as a guide for the multiprotein complex called RNA-Induced Silencing Complex (RISC). RISC includes several components, among them endo and exo RNA nucleases, a helicase and others [10]. RISC unwinds the double-strand siRNA and the activated complex with the associated antisense siRNA strand targets the homologous mRNA transcript for cleavage and subsequent degradation. In this way occurs the exact matching and the RISC cleaves the mRNA at the middle of the complementary region, 10 nt upstream of the oligonucleotide matched with the 5' end of the *siRNA guide*. The degradation occurred in a specific mRNA sequence manner and the specific gene is silenced. Then the RISC-*siRNA* complex finds another target mRNA and the process is repeated.

It is known that few dsRNA are only required in order to trigger the mechanism for degradation of approximately 5000 mRNA transcripts [11].

*T*ranscriptional gene silencing

One of the functions of the RNA silencing is the control of the activity of the transposable elements through the formation of heterochromatin. The transposable elements are DNA sequences, highly abundant in eukaryotic organisms, having the capacity of moving around to different positions within the genome of a single cell [12]. The activity of these elements means the possibility of causing mutations and therefore the lost of gene functionality. The induction of silencing by these transposable elements is facilitated by the fact that they are repeated in the genome and the contiguous insertion of several elements may cause the production of mRNA with complementary sequences forming double stranded RNA [13]. In the cases of birds and mammals the mechanism is triggered by the

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symmetric transcription of opposed promoters when there are many repeated sequences and/or many copies of a transgene originating dsRNA. In plants and nematodes the mechanism is triggered by the action of an RNA depending RNA polymerase (RpRd). Once the Dicer generated the *siRNA*, the RNA-induced initiation of transcriptional gene silencing (RIST) complex (complex homologous to RISC) recognizes the non-sense strand, while the complementary strand is degraded. The RIST-siRNA complex recruits the proteins that methylate the histones as well as the DNA sharing sequence homology, transforming the chromatin into the silenced form (heterochromatin). The methylated histones can also recruit proteins that extend the silencing state [14].

Translational gene silencing

The first evidence of translational repression was observed in *C. elegans*, where it was demonstrated that the specific miRNA, a class of short single-stranded RNAs that are encoded in the genomes of most multicellular organisms, *can reduce the protein synthesis without modifying the mRNA levels* [15].

In humans the mechanism begins gradually with the generation of large primary transcripts (pri-*RNA*) synthesized by the RNA polymerase II in the nucleus. The pri-RNA has an internal structure with sequences of imperfect complementarities bind by a loop which is called hairpin. The processing is started by the enzyme Drosha together with the protein DGCR8 that has a dsRNA binding domain [16]. Drosha, as well as Dicer, is an RNAase III having catalytic and binding domains specific to dsRNA. Drosha/DGCR8 cleaves the recently transcripted pri-RNA in hairpins of approximately 70-100 nt called pre-miRNA. Afterwards the pre-miRNA are exported into the cytoplasm by means of the nuclear export factor Exportin-5 and once there, they are processed by Dicer, generating a single strand RNA molecule of approximately 21-25 nt [17]. Afterwards the mature miRNA is assembled in a ribonucleoprotein miRNP complex that guide the search of target mRNA complementary or almost complementary to it. The mRNA has binding sites to miRNP in its untranslated 3' region, and if sequence of the target messenger and the microRNA are not exactly complementary, the translation is affected. In this way the protein synthesis is interrupted by avoiding the elongation or termination of the translation, but not its initiation. In few occasions the sequences of the miRNA and the mRNA are exactly complementary, and only in this case the degradation of the messenger is induced [1, 12].

The genes which expressions are regulated by miRNA are frequently transcription factors and genes involved in the development. The miRNA can be involved in the regulation of the development as well as in cellular processes such as homeostasis. It is considered that in humans the regulation by miRNA could affect up to one third of the total genes, which indicate the great relevance of this mechanism in the cell function [18].

RNA interference as research tool

The studies about RNA interference have reached great importance in the last years due to its potential in clinical and biomedical research. At present, there are libraries of siRNA expression vectors against human genes in order to determine their functions. The large dsRNA facilitate the effective silencing of genes because they have several sequences compatible with the target mRNA. Several strategies have been designed for introducing the desired sequences into the cells. For example, the introduction of short chemically or enzymatically synthesized dsRNA or through the expression of molecules such as short hairpin (shRNA), expressed in vectors within the cells [19]. These findings permitted the wide use of this technology for studying the functions of genes and the potential therapeutic applications.

Clinical potentialities of the RNA interference

RNA interference is an important tool for studying the function of genes in mammalian cells and it is considered as a novel and powerful alternative for the treatment of several human diseases.

Several researchers have used the siRNA as a tool for elucidating the mechanisms causing the uncontrolled cell proliferation and cancer. RNAi became a potential candidate for cancer treatment. One example of the principle of RNAi as anticancer drug is the oncoprotein Bcr-Abl, which is characteristic of the chronic myeloid leukemia that has been selectively regulated in cell cultures using synthetic siRNA or by transduction with lentiviral vectors containing shRNA [20]. There are several genes selected as target for studying diseases, where the gene silencing have been used effectively, such as hepatitis C virus, human papiloma virus, human immunodeficiency virus, oncogenes as Ras, surface receptors such as CD4, CD25, CXCR4, Fas receptor, etc. [7, 21].

The efficacy of RNAi in the treatment of diseases was reported in the case of the age related macular degeneration. This was the first study where the RNAi was used as a drug to depress the expression of the gene coding for the vascular endothelial growth factor (VEGF), which causes the wet form of the macular degeneration. This drug is injected in the vitreous cavity reaching the cytoplasm of the pigment epithelium cells inhibiting the VEGF. According to the preliminary results, its administration is safe and well tolerated [22]. The challenge in the development of therapies based on RNAi is the stable release of the siRNA into the cells.

RNAi Europe 2007

Workshops, events and other meetings are carried out every year around the world to discuss the novelties related with this field. A congress denominated RNAi Europe is organized since 2004 and the last one was held in Barcelona, Spain in September of 2007. Three topics were considered in this congress: RNA interference technologies, Emerging Themes in RNA interference and Therapies based on RNA interference.

Among the oral presentations in the session related to therapies based on RNA interference, the work presented by Dr. Takahiro Ochiya, Director of the National Cancer Centre Research Institute, of Tokyo, Japan was outstanding. He used a drug delivery system (DDS) for the siRNA mediated by nanoparticles

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of atelocollagen that showed great potential for practical application of gene suppression using siRNAs for cancer therapeutics. He showed results of the systemic application of siRNA *in vivo* against human prostate cancer and metastasis. The formation of the complex between siRNA and atelocollagen avoid the degradation of the siRNA without inducing unspecific effects [23].

Dr. Judy Lieberman, Senior Investigator of the Harvard Medical School, USA showed the results of a method for cell-specific systemic delivery of siRNAs by mixing siRNAs with an antibody fragment fused to protamine that was designed with the objective to silence gene expression *in vivo* with exquisite specificity in cells bearing the receptor recognized by the antibody.

Dr. Marta Izquierdo, Professor of Biochemistry and Molecular Biology of the Autonomous University of Madrid used RNA interference in melanoma-derived B16-F10 cancer cells showing that the combination of JunB knockdown and c-Jun/JNK inactivation leads to cell cycle arrest and apoptosis. Inactivation of both, c-Jun and JunB, provides a valuable strategy for antitumor intervention.

The possibility to silence the HIV using RNAi was discussed by Karin von Eije from Amsterdam University who showed results of the HIV *in vitro* inhibition using siRNA or shRNA and comparing both methods. There was a strong inhibition of the HIV in transduced cells expressing siRNA simple and it was observed that shRNAs against conserved sequences restricted the possibility of viral escape and that the combined expression of four shRNA avoided the viral escape. She discussed that the HIV chronic infection could be treated by *ex vivo* gene therapy [24].

Paul McCormac, Director of Process and Analytical Development of Avecia Biotechnology Inc. showed the results in the production of siRNA for therapeutic purpose. They are able to produce several siRNA and they are also scaling up the RNA production.

Interesting results were also presented in the sessions related to RNA interference technologies and Emerging Themes in RNA interference.

Susan Magdaleno, RNAi Manager of Ambion, talked about that one of the most critical aspects of siRNA experimental design is the selection of effective, potent and specific siRNAs for knocking down expression of the target(s) of interest. She presented a new algorithm that permits the design of siRNAs, predicting those that could be toxic and eliminating these sequences.

Teresa Rubio, Group Leader of Bio-Rad showed new methods to silence genes for a longer length of time when compared to standard siRNAs, allowing efficient transfection of siRNA into a wide variety of cultured mammalian cells with extremely low cytotoxicity.

Another conference was presented by Mark Behlke, Vice President of Molecular Genetics of the Integrated DNA Technologies. He talked about the *in vivo* use of Dicer-substrate siRNA which are specially designed to be processed optimally by Dicer, increasing their potency because they are better assembled in the natural processing route. He stated that the siRNA of more than 21 mer are up to 10 times more potent than the 21 mer siRNA. They used an asymmetry 25/27 mer that showed higher potency for the siRNA.

Jost Seibler, RNAi Group Leader of, Artemis Pharmaceuticals showed the results of a doxycycline inducible system developed by Artemis Pharmaceuticals for the temporal control of shRNA expression in mice. A mouse model of reversible insulin resistance was generated. The progression of the disease correlates with the concentration of doxycycline, and the phenotype is reversible shortly after withdrawal of the inductor.

Jesper Wengel, Professor at the University of Southern Denmark worked with LNA (Locked Nucleic Acid)-modified siRNA and sisiRNA (short internally segmented interfering RNA). The LNA modification improved the stability of the siRNA and reduced the off targets effects.

Beate Neumann, Scientist of the European Molecular Biology Laboratory, EMBL, Heidelberg, Germany used the Time-Lapse Microscopy for identifying the genes that play a role in the mitosis of HeLa cells and also studied the function of tubulin [25].

Volker Patzel, Group Leader of the Max Planck Institute for Infection Biology, Berlin, Germany developed a technique of siRNA-mediated gene silencing in prokaryotes. This method was used to specifically silence chromosomal and episomal genes in Gram+ and Gram- bacteria as well as in mycobacteria opening promising perspectives regarding validation of prokaryotic gene functions [26].

RNAi is gaining popularity as a tool in biomedical and molecular biology investigation as well as in its therapeutic potentialities. In this case, the RNAi has the advantage of being a natural mechanism that exists in the organism, it is highly specific and potent and its effectors are small molecules that have few possibilities to be toxic or to elicit an immune response. The main challenge today is to find methods that deliver siRNA efficiently *in vivo* and therefore most researchers in this field are working with this objective. Studies that explore administration routes such as the inhalation are under evaluation for safety and efficiency in current clinical trials.

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