Silence of the Genes

2006 Nobel Prize in Physiology or Medicine

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The 2006 Nobel Prize in Physiology or Medicine was awarded to Andrew Fire and Craig Mello for discovering "RNA interference – gene silencing by double-stranded RNA". The Nobel Committee at the Karolinska Institute in Sweden selected them for the award for unraveling "a fundamental mechanism for controlling the flow of genetic information" that is "already being widely used in basic science as a method to study the function of genes and may lead to novel therapies in the future". This has been one of the fastest Nobel Prizes conferred in physiology or medicine, considering that Fire and Mello published their path-breaking article in the journal *Nature* in 1998, less than ten years ago.

What is RNA Interference?

Genetic information is stored as sequences of nucleotides of double-stranded DNA in a specialized organelle within the cell called the nucleus. This information is first copied into messenger RNA (mRNA) by a process called *transcription*. Upon synthesis, mRNA moves out into the cytoplasm where the information is finally decoded into protein by a process called *translation*. The protein molecules are the workhorses that carry out all the cellular functions. This two-step process of decoding genetic information 'from DNA through RNA to protein' has been fondly termed the *Central Dogma* of molecular biology by Francis Crick (see *Resonance*, Vol. 9, November 2004).

The first step of making mRNA from DNA is also known as 'gene expression'. The human genome codes for ~35,000 genes and all these genes are not expressed in every cell. The time and site of gene expression is very precisely regulated. In any cell, only

some genes are 'activated', whereas others are 'repressed'. An error in gene regulation may lead to fatal consequences for the organism. Cells have specialized proteins called 'transcription factors' that activate/repress genes depending on the requirement. The process of transcription has been an active field of research for several decades (See *Resonance*, Vol. 12, pp.47–53, March 2007).

RNA interference (RNAi) is a novel mechanism for controlling gene expression. In this mechanism, tiny double-stranded RNA molecules called 'small interfering RNA' (siRNA) degrade cellular mRNA that has sequence similarity with them. As a result, even though a gene has been expressed and mRNA has been made, proteins are not formed. In this context, it should be remembered that RNAi always represses the target gene function; it can never activate it. And it does so in a very "degrading manner".

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The siRNA molecules that initiate the RNAi process are really tiny, ~21 nucleotides long, and can trigger degradation of mRNA molecules that can be >100 times larger than them. And if these mRNA molecules are destined to code for proteins with important cellular functions, such degradation can result in devastating consequences for the cell. Thus, these siRNA molecules remind us of little matchsticks that have the potential to cause large-scale destruction with a single flick.

Origin of the Concept

RNA-induced gene silencing was first demonstrated in plants. Back in 1986, two biologists from Stanford University expressed a gene in the opposite orientation in a cultured plant cell line and observed that the gene product was reduced. Expression from the opposite direction makes an *antisense* mRNA that is complementary to the endogenous *sense* mRNA produced by the normal gene. The *antisense* strand binds to the *sense* strand and blocks protein synthesis. This method of gene inhibition was termed *antisense technology*. The antisense technology soon became a

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popular method in plant genetic engineering and agriculture. The technique yielded only partial success and the extent of gene inhibition depended on the strength of the promoter used (promoter is a DNA element that initiates gene expression). Since the interaction between the sense and the antisense is stoichiometric and there is no catalytic amplification of the destruction message, the phenomenon did not suggest involvement of any cellular machinery.

In the early 1990s, plant biologists in USA and the Netherlands observed a very strange phenomenon in the horticultural plant *Petunia hybrida*. Normal petunia produces purple coloured flowers. In order to increase the intensity of the colour, they expressed an extra copy of a gene involved in the synthesis of the pigment in a transgenic petunia. To their surprise, they noticed that this resulted in reduced flower colour instead of an increase. The transgenic flowers started losing colour in patches. Introduction of an extra copy of the gene had 'silenced' the endogenous gene! This phenomenon was termed 'co-suppression'. A similar phenomenon was observed in the fungus *Neurospora crassa* and was termed 'quelling'. Although the mechanism of such processes remained largely unclear, it was shown that the nature of gene suppression was post-transcriptional and was mediated by RNA degradation. Moreover, it was proposed that degradation of the RNA could be mediated by double-stranded RNA. A nascent *RNA interference* theory was already in the making!

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Scientists working on plant viruses noticed that plants resist the spread of viral infection by degrading the viral RNA. In a reverse experiment, when a small fragment of a plant gene was introduced into a virus, infection by such virus resulted in the silencing of the endogenous copy of the same plant gene and the silencing spread systemically. This was termed *virus-induced gene silencing* (VIGS). These experiments indicated that a general cellular mechanism is involved in both plant defense against virus and gene silencing in plants. It also demonstrated that a small fragment of the RNA is sufficient to initiate RNA degradation.

These results were published in *Nature* in 1997, only a year before Fire and Mello published their paper on the nematode *C. elegans*. However, these experiments failed to explain the phenomenon and a unified mechanism remained elusive.

Unlike plant biologists, Fire and Mello introduced RNA by microinjecting it into the body cavity of the worm.

The Observations of Fire and Mello

What did Fire and Mello do that others had missed out? They were studying the mechanism of RNA interference in a small worm called *Caenorhabditis elegans*. Unlike plant biologists, Fire and Mello introduced RNA by microinjecting it into the body cavity of the worm. They first injected single stranded RNA molecules of a gene called *UNC-22* that is normally expressed in the muscle cells. Loss of *UNC-22* function does not affect the survival of the worm, but introduces twitching in its movement. Fire and Mello observed, as the plant biologists did, that introduction of many molecules of the antisense RNA into the worm resulted in a modest change in the levels of the *UNC-22* RNA, and consequently produced a little twitching. To their surprise, they observed that simultaneous introduction of sense and antisense RNA resulted in severe twitching in the worm! Could it be that the double stranded RNA that resulted from base-pairing of the

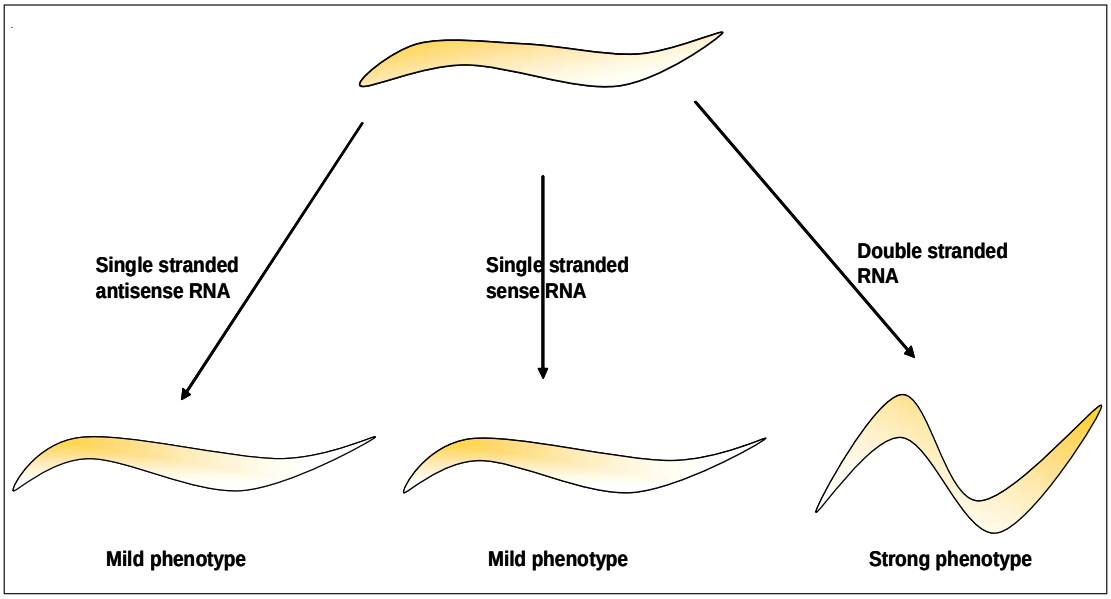

Figure 1. Microinjecting the worm with either sense or antisense strand of* UNC-22 *RNA resulted in little phenotypic changes. However, when double stranded RNA was injected, strong twitching phenotype type was observed.

Fire and Mello injected the RNA into the worm and observed the phenotype in the offspring, demonstrating that RNA interference was heritable.

sense and antisense strands was responsible for the phenomenon? When they purified such double stranded RNA from the mixture of single strands and injected it into the worm, they observed similar results; severe twitching. So it was the double stranded RNA after all!

Fire and Mello repeated the experiment with three more *C. elegans* genes, and observed a similar effect every time; phenotypes similar to the null mutants of the respective genes were observed. Thus, the phenomenon is 'specific' and works for all genes. Fire and Mello injected the RNA into the worm and observed the phenotype in the offspring, demonstrating that RNA interference was heritable. Even when the amount of injected RNA was reduced to such an extent that each cell in the progeny worm had only a few molecules of RNA, absolute silencing of the gene was observed! This indicated that an amplifying mechanism is in operation. So there must be a "catalytic machinery" in the cell.

The Elegance of *C. elegans*

Why was it that the RNAi studies carried out in *C. elegans* became so successful and informative compared to studies in other organisms? It is mainly due to the simplicity of the method by which one can deliver foreign RNA in this organism. *C. elegans* is grown in laboratories by feeding them with the common laboratory bacterium *E. coli*. If the bacterial cells contain double-stranded RNA (dsRNA) that has complementarity with the *C. elegans* genes, then RNAi can be induced in the worm simply by feeding them with this bacterium. This "delivery by feeding" is perhaps the simplest method to induce RNAi in any organism. Soaking the worm in dsRNA-containing solution also induces interference. If more precise delivery is required, the RNA is usually microinjected into the body of the worm. This is the method that was used by Fire and Mello in their study. And since they microinjected the RNA into the worm, they knew precisely what they were putting in. It was neither the antisense RNA nor the sense strand; it was the double stranded RNA that

held the key. The other advantage was that gene silencing by RNAi is heritable in *C. elegans*; dsRNA can be injected in one worm and the effect can be observed in all offspring as they grow to maturity.

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The simplicity and elegance of *C. elegans* as a model system have led to two important discoveries that earned Nobel Prizes in Physiology and Medicine. First, the phenomenon of *programmed cell death* or *apoptosis* was discovered that led to the 2002 Nobel Prize to Sydney Brenner, John Sulston and Robert Horvitz (see *Resonance*, Vol.8, pp.89–94, March 2003). Studies on cell death were possible in *C. elegans* because this worm is made of a small number of cells (~1000) whose division and growth can be monitored by light microscopes without cutting open the organism. The second discovery is RNAi. *C. elegans* is perhaps the only model organism that has earned two Nobel Prizes!

The Mechanism of RNA Interference

What actually happened when Fire and Mello injected double stranded RNA into the worm? Why did it degrade the cellular mRNA? Why did only double stranded RNA initiate mRNA degradation whereas either of the single strands had little effect? Their work did not answer these questions, but it surely initiated a spurt of research activities in this field. Many laboratories throughout the world became interested in RNAi research using various model systems. Genetic and biochemical studies carried out in *Arabidopsis*, *C. elegans*, *Drosophila* and yeast in the past 7-8 years have identified a cellular mechanism involved in RNA interference that is conserved in all eukaryotes. Findings from these studies are quite astonishing. It is now clear that cells have a fully equipped 'destruction squad' to degrade the RNA that needs to be degraded. In efficiency and specificity, this destruction machinery is comparable to the protein destruction machinery in the cell. The discovery of protein degradation machinery also led to the Nobel Prize in Chemistry in 2004, and the Prize went to Aaron Ciechanover, Avram Hershko and Irwin Rose. (See *Resonance*, Vol.10, pp.41–49, January 2005.)

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The RNA degradation machinery is present in the cytoplasm of the cell. Cytoplasm is the site of three types of natural single stranded RNAs: mRNA, rRNA and tRNA. All these three species of single stranded RNA are recognized and used by the protein synthesis machinery for making new proteins. However, when a double stranded RNA appears in the cytoplasm, it is first recognized by a RNA-splitting enzyme called DICER. The DICER protein, along with other proteins, binds to the incoming double stranded RNA and cleaves it into small, ~21 nucleotide long siRNA. The siRNA is then transferred to a large RNA-protein complex called RNA-Induced Silencing Complex (RISC). RISC, among other protein components, contains a *helicase* enzyme that unwinds the double stranded siRNA and removes one strand from

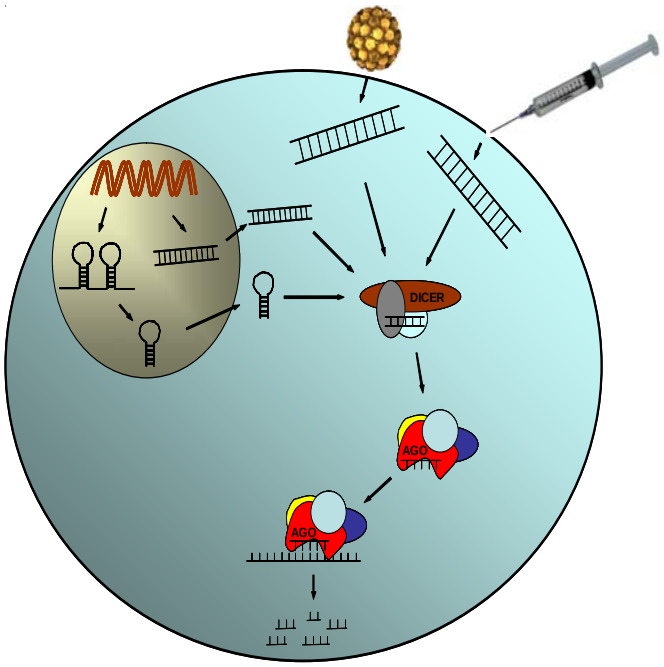

Figure 2. Schematic representation of RNA interference machinery in the cell. Endogenous precursors of siRNA are produced in the nucleus (golden circle) by transcription of miRNA genes or transposon-induced repeats. The miRNA precursor is cleaved into small stem-loop structure by a nuclear RNase III type endonuclease called DROSHA. Double stranded RNA molecules of both these origins are transported into the cytoplasm where they are recognized by another RNase III type endonuclease named DICER. DICER also recognizes exogenous double stranded RNA molecules that are introduced either by viral infection or by microinjection. DICER, in complex with other proteins and RNA, cleaves the precursor siRNA into small, ~21-nucleotide long, double stranded siRNA. This siRNA is then transferred to a RNA-protein complex called RISC, which unwinds the siRNA and removes one strand from the complex. The RISC complex, thus armed with a single stranded small RNA, recognizes and degrades cellular mRNA with sequence homology, possibly by a component protein called ARGONAUTE (AGO).

the complex. The resulting RISC, armed with a tiny single-stranded RNA *probe*, identifies the mRNA that has sequence similarity with the *probe*, by complementary base pairing. Once base pairing takes place, the resulting double stranded region is nicked by the RISC, triggering complete degradation. The mechanism of mRNA nicking is not yet clear, but most likely the ARGONAUTE (AGO), a RISC component protein with ribonuclease like domain, performs the cleavage. The RISC acts like an enzyme complex that *catalyzes* the cleavage reaction; hence a few molecules of siRNA are sufficient to exert a strong silencing effect.

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The double stranded RNA can appear in the cytoplasm from various sources. When it comes from the transcription of a nuclear regulatory gene that does not code for proteins, it is called micro RNA or miRNA (see below). The pre-miRNA transcripts are usually very large and are processed in the nucleus to a small stem-loop structure by a DICER-like enzyme called DROSHA. When the double stranded RNA arise from the transcription activities of transposon-induced repeats[1], it is called repeat-induced short interfering RNA (rasiRNA). Both miRNA and rasiRNA, after processing, are transported into the cytoplasm, where they are recognized by DICER. Among the external sources of double stranded RNA in the cytoplasm, infection by double stranded RNA viruses is the most common. Both viral RNA and the RNA that was injected by Fire and Mello are recognized and processed by DICER.

[1] When 'jumping genes' hop from one location to another, they create a duplication of the target sequence.

The basic RNAi machinery described above is conserved in almost all the metazoans. Variations of the mechanism have evolved in certain organisms during the course of evolution. For example, rasiRNA does not lead to mRNA degradation, but induces chromatin modification and gene silencing in yeast, *Arabidopsis* and *Drosophila*. Further, apart from mRNA degradation, miRNA of *Arabidopsis*, *Drosophila*, *C. elegans* and mammals also inhibits protein synthesis by binding to the target mRNA and posing as a stearic hindrance.

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Biological Function of RNAi

RNA interference is found in almost all eukaryotes and, in a rudimentary form, in bacteria as well. What is the evolutionary origin and selective advantage of such a destructive mechanism in the cell? It is believed that RNAi acts as 'innate immunity' in lower organisms to fight against viral attack.

RNAi is also used to keep the *jumping genes* or *transposons* in check. Transposons are DNA elements that are capable of jumping around in the genome. If a transposon gets inserted into an essential gene and disrupts its function, the survival of the organism can be threatened. There are certain transposons called retro-transposons that replicate via an RNA intermediate. Since the original DNA copy of this transposon remains intact, the number of retro-transposons progressively increases in an organism, leading to extra load on the genome. Thus, keeping the transposons in check is in the best interest of the organisms. RNAi mechanism may have been evolved to perform such a job.

The RNAi machinery also comes handy in normal gene expression, especially during the development of an organism. Many chromosomal genes code for small RNA molecules that do not make any protein, but control other gene products. Such small RNA is called miRNA. Upon transcription and processing in the nucleus into the stem-loop structure, miRNA molecules move into the cytoplasm where they are recognized by the RISC complex and processed into smaller products that degrade the target mRNA. Majority of the developmental genes are spatially regulated by miRNA through RNA interference.

Discontent among Plant biologists

Everyone in RNA interference research was happy to see that the topic got recognition through the award of a Nobel Prize. Some, however, expressed disappointment that plant biologists were not included in the Nobel share. The concept of RNA interference started emerging in early 1990s from the work carried out by the

plant biologists. Some argue that, non-inclusion of the plant biologists in the prize was justified because they made several discrete discoveries that could not be united into a single phenomenon, nor could the findings lead to a mechanism that explained the phenomenon. Others, however, point out that the plant scientists had already emphasized the major findings by Fire and Mello that have been noted by the Nobel Committee. For example, they already knew that small RNA molecules could induce degradation of endogenous RNA in a sequence-specific manner, that the phenomenon is post-transcriptional in nature and non-cell autonomous. They even proposed that double-stranded RNA is involved in the interference and that the process can be amplified! Fire and Mello, however, demonstrated these points beyond doubt through simple and elegant experiments. While leaving the plant biologists out of this Nobel share is justified or not can be debated, the person who made fundamental contributions that lead to the RNAi discovery and possibly deserves to share the prize is David Baulcombe of John Innes Center, Norwich, UK.

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Application of RNAi technology

Discovery of RNAi technology and its mechanism has opened up new possibilities in the field of basic science, biotechnology, medicine and agriculture. There has been a tremendous surge in research in this field since the publication of Fire and Mellow paper. A search in the *Pubmed* (public database of published papers in the field of biology) with the key word "RNA interference" now yields ~9000 articles, and the number is fast increasing. The potential of application of the technology in various fields of science is enormous and the use of siRNA in the field of biomedical field is expected to revolutionize medicine and health care.

Gene Expression Studies

Studies on gene function have traditionally been restricted to a few model organisms where mutants could be generated. RNAi

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offers a new and simple alternative to knock-down gene expression and study gene function in many plants and animals that could not be studied earlier. We can now study the function of human genes in cultured cells simply by introducing siRNA homologous to the gene we are studying. Several important crop species are polyploid (more than two sets of genome), making it difficult to analyze gene function by Mendelian genetics. RNAi has now been successfully used in wheat (that has 6 sets of the genome, and hence 6 copies of each gene) to repress all the copies of a gene and study its function. Similarly, siRNA can also be designed so that it will lead to degradation of a family of gene products that share sequence similarity. Functional studies of such genes are difficult using genetics since the effect of mutation in one member of the family is often compensated by the other members.

Medicine and Health

The discovery of RNAi technology has perhaps the greatest impact in the biomedical field. In principle, this technology can be used to knock-down any gene that is involved in causing a disease. No other single technology has offered such a promise in the field of gene therapy.

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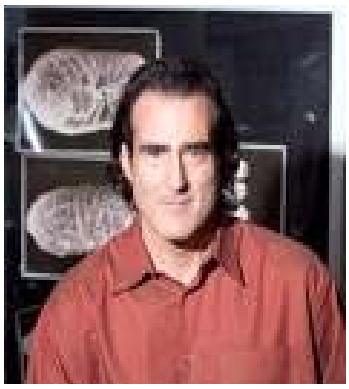
Using computer modeling, scientists have been successful in designing siRNA that silence a gene product with high efficiency and absolute specificity. The technique has already been used to suppress viral genes and silence replication of the viruses that cause human diseases such as cervical cancer, hepatitis, measles and influenza. Success has also been obtained in case of genetic diseases such as Huntington's disease and cancer (both virus as well as mutations in the cellular genes can cause cancer). Most studies, however, are in the experimental phase in cell culture. Clinical trials of the RNAi-based treatment have only begun. A major area of research in RNAi-based gene therapy has been on the efficient, safe and specific delivery of siRNA to the infected tissue.

Like any new technology, RNAi-based treatment has its own share of safety concerns. Safety becomes even more important in this case, since the siRNA designed to silence a specific gene has the potential to silence other homologous genes. Careful and meticulous computational methods will be required to design siRNA molecules that would silence only the target gene. A recent study in mouse has shown that RNAi-based drug delivery against a liver disease resulted in high death rate of individuals, even though the liver failure was cured.

Crop Improvement

Manipulation of crop plants for improving agricultural traits and increasing disease resistance is possible through RNAi technology. The success again is restricted to the laboratory experiments and no RNAi-based transgenic plant products have yet appeared in the market. Nevertheless, the possibilities are promising.

A major area of focus has been in reducing toxic products in plants by suppressing the toxin-producing genes. Such techniques have already been used successfully to reduce harmful chemicals in cotton, tapioca, tobacco and tomato. If these successes survive the field trials, market acceptability of these products will increase. The other area where RNAi has been used in agriculture is generation of disease-resistant crops. Success has been obtained in many crop species including tomato and papaya.



Andrew Fire and Craig Mello, recipients of 2006 Nobel Prize in Physiology or Medicine

Conclusion

Nobel Prizes are usually given to discoveries that have changed the face of science and opened up new possibilities. And in this context, the 2006 Nobel Prize for the discovery of RNA interference is well deserved. It has completely changed our understanding of gene regulation. Proteins are no more the sole regulators of transcription – tiny RNA molecules have taken up large responsibilities in this process. Genome-wide studies on small RNA molecules and their targets predict that almost one-third of human genes are regulated by micro RNA! We are going to witness more surprises in this field in the coming few years.

Suggested Reading

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