

dsRNA-Mediated Interference of Gene Expression: Past, Present, and Future

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Introduction

Double-stranded RNA-mediated interference (RNAi) is a powerful genetic phenomenon that was first observed in higher plants and later discovered in *Caenorhabditis elegans*, *Drosophila melanogaster*, insects, fungi, and vertebrates (1). The direct injection of sequence-specific double-stranded RNA into a variety of organisms will cause selective post-transcriptional gene silencing (PTGS), and the inability to produce the protein corresponding to the injected gene (2). While organisms such as *C. elegans* and *D. melanogaster* have shown successful use of dsRNA to achieve gene silencing, mammals are unable to exhibit RNAi using dsRNA (3). In mammals, dsRNAs longer than 30-nt will activate an antiviral response, which will lead to the nonspecific degradation of RNA transcripts, the production of interferon, and the overall shutdown of host cell protein syntheses (4). As a result, the long dsRNA will not produce RNAi activity in mammalian cells (3). Nevertheless, gene specific suppression in mammalian cells can be achieved by using a new approach involving *in vitro* synthesized ~ 21-nt short interfering RNAs (siRNAs) (4). These synthesized siRNAs are long enough to stimulate gene-specific suppression but short enough to avoid the host interferon response (3). These new RNAi approaches for gene-specific silencing are being used to gain insight into gene function as a tool for screening whole genomes, to easily produce the loss-of-function phenotypes, to produce RNAi function *in vivo*, and to help researchers investigate the genome for candidate drug interventions, therapeutic techniques, and vaccine targets (5).

The History Behind RNAi

While the discovery of the RNAi phenomenon is usually associated with the nematode *C. elegans*, the knockout of expression of a particular gene was actually first observed in plants (6). Through the introduction of multiple transgenic copies of a gene to the *Petunia* plant, gene silencing of not only the transgenic, but also the endogenous gene copy was observed (6). This event was termed cosuppression. Cosuppression notably involves a silencing effect that can “move within the plant,” or that will spread to a transgene that shows no homology to the suppressed transgene (6). Fungi are observed to exhibit a pathway, termed quelling, that is similar to cosuppression in plants. This quelling pathway led to the identification of a gene whose wild-type function is needed for the silencing effect (6). In the fungi *Phytophthora*, an internuclear transfer of the silencing signal, which demonstrated the use of an RNA intermediate, was observed. This movement of the silencing effect in both plants and fungi produce evidence that the silencing information can be replicated. These studies with plants and fungi have aided in unraveling the mysteries of RNA interference (6).

Andrew Fire and Craig Mello originally discovered RNA interference in 1998 (7, 2). Fire was working with *C. elegans* at the Carnegie Institute of Embryology in Baltimore when he discovered that the injection of a dsRNA molecule complementary to

a particular gene would result in the lack of production of that gene product (2). Fire examined the silencing effects of dsRNA at the cellular level in a transgenic line of *C. elegans*. These worms expressed two different green fluorescent protein (GFP)-derived fluorescent-reporter proteins in body muscle cells (8). When these transgenic animals were injected with dsRNA directed to the *gfp* gene, there was a visible decrease in the fraction of fluorescent cells (8). The affected cells did not contain either of the reporter proteins. The unaffected cells were found to contain the GFP proteins and were fluorescent (8). Fire also used a target transcript, *mex-3*, that is abundant in the gonad region and in early embryos of *C. elegans* to show that the injection of dsRNA can produce either a decrease or an elimination of mRNA. The animals that were injected with a dsRNA segment that was derived from *mex-3* contained no *mex-3* mRNA (8). However, the animals that were injected with *mex-3* antisense RNA retained the *mex-3* mRNA (8).

There are several methods by which dsRNA can be introduced into a worm, including the following: injection into the gonad or other body parts, soaking of the animal in dsRNA, transcription from a transgene, or more surprisingly by the ingestion of *Escherichia coli* that has been overexpressed with a particular dsRNA (6). Fire found that the injection of a small amount of both sense and antisense dsRNA strands per cell was sufficient to silence the homologous gene's expression throughout the worm and in the first generation progeny (9). Since the discovery of RNAi in *C. elegans*, many different organisms, including *D. melanogaster*, *Trypanosoma*, plants, and vertebrates such as the mouse and zebrafish, have demonstrated RNAi susceptibility. It was through the study of RNA activity in these organisms that the mechanism for RNAi was uncovered (6).

Most recently, RNAi has been implicated in epigenetics, or the changes in gene expression not caused by the DNA code and that occur across at least one generation (7). One type of epigenetic regulation is a result of changes in the level of compaction of chromatin, which can alter the genes that are expressed. Through the study of different organisms, the siRNAs that are responsible for RNAi have been identified to have great control over the form of chromatin (7). Because of the control that siRNAs have, they can shut down or delete portions of DNA, instead of only silencing them temporarily. However, the mechanism for the chromatin control exhibited by siRNAs is not yet known (7).

Gene Suppression in Mammals

The antiviral defense mechanism in mammals is activated when dsRNAs longer than 30 nt are present; this causes nonspecific suppression of gene expression (4, 9). Consequently, until recently RNAi has been limited to plants and animals in which long dsRNAs could induce gene-specific silencing (4). By using chemically synthesized ~21-nt siRNAs, the obstacles involving the achievement of RNAi in mammals have been overcome (4). Because siRNAs do not activate the antiviral pathway, RNAi can be effectively induced in mammals (9). The efficiency of siRNAs varies. A 90% reduction in target RNA and protein levels can be produced using the most potent form of siRNA, a 21-nt dsRNA with a 2-nt 3' overhang (9). The effective use of siRNA also requires strict sequence specificity with the target mRNA in mammalian cell cultures. A single base pair mismatch between siRNA and target messenger RNA (mRNA) will dramatically reduce the silencing effect (9). There are different DNA vector-based strategies that have

been developed for the syntheses and introduction of siRNAs into mammalian cells. These strategies are cost effective, and could be major developments of RNAi technology *in vivo* (4).

With these new methods of silencing using siRNA understood, the best form of delivery of siRNA must be determined. An initial experiment involving siRNA gene silencing in mice failed to produce either a positive delivery method or a long-lived silencing effect (11). The siRNA probes were injected into the mouse's tail vein by way of a high-pressure hydrodynamic method. While the silencing effect was present in the mouse, it lasted only two to three days (11). Because humans cannot withstand such high pressure and volume delivery methods, new developments for siRNA technology in mammals will have to be developed in order to observe the silencing effect in humans (11).

The 3' overhangs of *in vitro* siRNA are important in gene silencing because their structural features allow a more effective gene inhibition in mammalian cells. The overhangs that involve two uridines have been found to exhibit a more efficient inhibition than overhangs of AA, CC, or GG (3,4). It was recently reported that, using these optimal structural features, siRNA could be synthesized *in vivo*, with the aid of RNA polymerase III promoters (3, 4). RNA polymerase III (Pol III) is an optimal promoter because it normally transcribes small noncoding transcripts that are not capped at the 5' and 3' ends, it initiates transcription at defined nucleotides, and it terminates transcription at specific bases (4, 10). There are currently two methods that involve Pol III to produce strong gene-specific silencing. The first of these two methods is modeled after naturally occurring microRNAs (miRNAs). These miRNAs are ~22-nt hairpins (4). Expression constructs of inverted repeats form short hairpin loops (shRNA) that get processed by the Dicer protein and regulate gene expression *in vivo* (4, 12). The second method involving miRNAs uses two Pol III promoters placed in tandem, or on two separate vectors, to guide transcription of a sense and antisense strands that are 19 nt in length, and match the targeted gene sequence (4). These sense and antisense strands are believed to form siRNA *in vivo*. The hairpin method of gene silencing appears to be more efficient than the siRNA production from two separate vectors (4).

Another way to obtain small RNAs for gene silencing *in vivo* is by using RNAi plasmid vectors that allow modified miRNAs to act as siRNAs (4). Naturally occurring miRNAs are noncoding, single-stranded, ~22-nt RNAs that are processed from ~70-nt hairpin RNA precursors by the Dicer protein (3, 4). Unlike siRNAs that target RNA substrates for degradation, natural miRNAs can inhibit gene expression at the transcriptional level (4). RNA transcript levels have also been reduced by the siRNA-like function of artificially engineered single-stranded miRNAs whose sequence is complementary to the target RNAs. These artificial miRNAs act as siRNAs in that they are able to induce target RNA degradation (4). These DNA vector-based approaches to gene inhibition appear to function in transfected, as well as endogenous, genes (3). Many different types of cells including the following: primary neurons, fibroblasts, T cells, pluripotent embryonic carcinoma cells, cells derived from cervical carcinoma, osteosarcoma cells, breast cancer cells, and kidney cells have also demonstrated gene inhibition using the DNA vector-based approach (4). While RNAi can inhibit expression of genes in many cells, siRNA-mediated gene silencing in mammals does not completely eliminate the gene product. Because of the incomplete inhibition of gene function, RNAi

in mammals is a knockdown, rather than a knockout, approach to gene silencing. However, there is the possibility that RNAi could generate the knockout phenotype associated with a gene (4).

Research using RNAi

Because RNAi can function *in vivo*, there are developing methods for its use in genetic research in mammals (13). Using RNAi, researchers will now be able to combine multiple knockdown constructs in one animal to generate multiple loss-of-function phenotypes in one generation with ease. By designing unique oligonucleotides targeting specific splice variants, foreign or mutant genes may be possible to produce because of highly selective RNAi (13). The vector-based RNAi methods are also able to achieve loss of function phenotypes that do not result in loss of genomic information of the gene of interest (13). Because the genomic information is still present, there is the possibility to restore the expression of the gene once the RNAi vector is either removed or silenced (13). Advances in research techniques would also be possible if the ability to turn the expression of a gene on and off were possible. Because the RNAi system used in most mammals requires the aid of the polymerase III promoter, which can be manipulated to be susceptible to repression, this on and off switch may be possible (13).

Therapy using RNAi

Much like the body's immune system, the natural function of RNAi is to protect the genome from invasion by mobile genetic elements, like viruses and transposons (1). RNAi protection can be accomplished by inhibiting cellular proteins that allow viral entry and replication, or by silencing the viral gene (13). One of the most promising uses of RNAi involves the creation of disease-resistant cells that can repopulate a diseased organ. Possible RNAi vectors for this therapy include those for cancer and viral infections. Cancer target genes that are being considered for RNAi therapy that may produce tumor regression include dominant mutant oncogenes, amplified oncogenes, translocation products, and viral oncogenes. Possible viral infection target genes for viral clearance include viral genes, host genes required for virus propagation, and resistant cell population (13). RNAi pathways that are conserved within mammalian cells may be used for antiviral therapy (12). There is promising research being done that examines the use of RNAi that limits the infection produced by viruses in cultured cells. These culture cell studies focus mainly on HIV and the poliovirus (12). An experiment, using Magi cells as a model system, directed siRNAs against different regions of the HIV-1 genome and demonstrated a reduction of more than 95% in viral infection after the cotransfection of the siRNAs with an HIV-1 proviral DNA (12). In another experiment, cotransfection of HIV-1 proviral DNA with an *in vivo* transcribed siRNA targeting the HIV-1 protein produced a reduced level of the viral antigen in viral supernatants, along with a corresponding loss of viral integration in 293 cells (12). Experiments in mice involving other pathogenic viruses, such as the poliovirus, Rous Sarcoma Virus, and Hepatitis-C virus, have been weakened after a transfection with siRNAs (10). While the results of RNAi's involvement in the life cycle of a virus are good indicators of the potential that RNAi has for antiviral therapy, there are still many obstacles that must be overcome before RNAi can be used in humans (12). RNAi has also been shown to have a potential for facilitating drug screening and development (5). In the future, identifying genes that

can confer drug resistance, or genes that have mutant phenotypes that are improved by drug treatment may be a task that is regulated by RNAi (5).

Conclusions

In 2002, *Science* magazine awarded siRNAs with the title of “Breakthrough of the Year” (7). Because RNAi has the ability to easily create loss-of-function phenotypes, laboratories are now using the RNAi methods to study gene function, instead of gene “knockouts” (7). The traditional knockout method involves the use of embryonic stem cells (ES cells) and homologous recombination to achieve gene targeting (15). Through the modification of a stretch of DNA, a targeted gene is knocked out and exchanged for the endogenous DNA sequence of the stem cell. These modified stem cells are implanted into the womb of a “foster mother” (15). This “chimeric” mother can then pass the knockout mutation to its progeny (15). This knockout approach to deleting a gene is a laborious process that can now be discarded in some cases, and replaced by RNAi that can simply turn down the expression of a particular gene (7). From its origin in plants, RNAi has demonstrated silencing of genes in organisms such as fungi, *D. melanogaster*, *C. elegans* and vertebrates (1). Because the long dsRNAs that are used to inhibit gene expression in lower animals and plants cannot be used in mammals, new developments for the delivery of siRNAs into mammalian cells, through various vector methods, are enhancing the use of RNAi (4). In most species, RNAi produces a post-transcriptional gene silencing however, in some species RNAi can also lead to translational repression, DNA methylation, heterochromatin formation, or DNA elimination (14). These findings suggest that RNAi can control both transcriptional and post-transcriptional gene expression, and that they can function in the nucleus and cytoplasm of a cell (14). Because of the knowledge that RNAi may hold the promise for new drug and therapeutic techniques, the RNAi approach to gene silencing has taken the research world by storm (9). With new information about RNAi becoming available at rapid rates, RNAi is revolutionizing the world of functional genomics (9).

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