RNA interference and double-stranded-RNA-activated pathways

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Abstract
RNAi (RNA interference) has become a powerful tool to determine gene function. Different methods of expressing the short ds (double-stranded) RNA intermediates required for interference in mammalian systems have been developed, including the introduction of si (short interfering) RNAs by direct transfection or driven from transfected plasmids or lentiviral vectors encoding sh (short hairpin) RNAs. Although RNAi relies upon a high degree of specificity, recent findings suggest that off-target non-specific effects can be encountered. We found that transfection of siRNAs can result in an interferon-mediated activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway and global up-regulation of interferon-stimulated genes. This effect is mediated in part by the dsRNA-dependent protein kinase PKR, as this kinase is activated by the 21 bp siRNA, and is required in response to the siRNAs. However, the transcription factor IRF3 (interferon-regulatory factor 3) is also activated by siRNA as a primary response, resulting in the stimulation of genes independent of an interferon response. In cells deficient in IRF3, this response is blunted, but can be restored by re-introduction of IRF3. Thus siRNAs induce complex signalling responses in target cells, leading to effects beyond the selective silencing of specific genes.

Intense interest in the field of RNAi (RNA interference) has facilitated its rapid movement from a biological phenomenon to a valuable research tool used to silence target gene expression. This application is now widely used to determine gene function and has potential as a therapeutic agent. Although the mechanism has only recently been uncovered, the concept of expression interference has been a recurring theme in biology for over 20 years. Schmidt and colleagues identified a non-toxic strain of Aspergillus flavus and found ds (double-stranded) RNA to be the cause of this attenuation (described in [1]). More than a decade ago, a post-transcriptional silencing event in petunias that was attributed to dsRNA was reported [2]. In yet another experimental system, Romano and Maciano [3] described a similar silencing mechanism that they termed quelling, in the fungus Neurospora crassa. These studies hinted at the existence of a well-conserved mechanism of dsRNA-mediated specific gene silencing. However, it was the elucidation of the mechanism responsible for this response that led to the realization of its potential as a powerful research tool for manipulating gene expression.

Drawing upon these previous studies, Fire and Mello determined that dsRNA molecules, like those produced during viral replication, induce a potent and specific gene-silencing response in the nematode, Caenorhabditis elegans [4]. Subsequent work by multiple groups showed that the potentially harmful viral dsRNA is recognized and cleaved into 21–23 nucleotide siRNAs (short interfering RNAs) [5]. The siRNA is then incorporated into the RISC (RNA-induced silencing complex), which targets the mRNA of an homologous sequence for degradation much more efficiently than antisense-mediated silencing (reviewed in [6]). Since organisms such as C. elegans and Drosophila use the specific RNAi pathway to respond to dsRNA-containing challenges, it was relatively easy to apply this concept to artificial assays set up to determine gene function.

It was initially thought that this process would not work in mammalian systems due to the presence of an innate immune defence mechanism directed against dsRNA-containing challenges, such as virus infection. Introduction of dsRNA molecules into most mammalian cells causes global, non-specific suppression of gene expression.

Although Toll-like receptor 3 has been identified as a dsRNA-response protein [7], the two best characterized dsRNA pathways found in most cell types signal through the dsRNA recognition proteins PKR (dsRNA-dependent protein kinase) and 2′,5′-oligoadenylate synthetase and have been recognized for over 20 years. Activation of both of these pathways by dsRNA results in the general inhibition of protein synthesis. PKR autophosphorylates in response to dsRNA and subsequently phosphorylates its substrates, one of which is eIF2 (eukaryotic initiation factor 2), leading to translation inhibition [8]. The activation of 2′,5′-oligoadenylate synthetase results in the formation of an oligoadenylate, which serves as a substrate for the endoribonuclease oligoadenylate endoribonuclease (OAS) and activates the RNaseL pathway.

Key words: double-stranded RNA (dsRNA), interferon, RNA interference (RNAi), RNA polymerase, short interfering RNA (siRNA), transcription.

Abbreviations used: ds, double-stranded; IgG, interferon-stimulated gene; mRNA, microRNA; PKR, dsRNA-dependent protein kinase; RNAi, RNA interference; shRNA, short-hairpin RNA; siRNA, short interfering RNA; UTR, untranslated region.

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2′,5′-oligoadenylates that bind to and activate RNaseL. This enzyme then non-specifically cleaves both cellular and viral RNAs, also causing translation inhibition [9].

In addition to its role as a translational inhibitor, PKR also acts as a signalling transducer. Following activation, PKR initiates a signalling cascade that results in the production of interferons. Interferon, in turn, activates cellular signalling pathways that culminate in the nucleus with the up-regulation of interferon-stimulated genes, mediators of antiviral and antiproliferative and pro-apoptotic activity [10]. Cells exposed to interferon are sensitive to very low levels of dsRNA, eventually leading to cell death. These properties initially precluded the use of RNAi based on long (>100 bp) dsRNA targets of Dicer as an effective research tool in these systems.

In 2001, Tuschl and colleagues found that they could initiate RNAi in mammalian systems by the intracellular introduction of artificially synthesized mimics of Dicer products of 21–23 bp [11]. By excluding long dsRNAs from the process, researchers hoped to also eliminate non-specific side effects, resulting in activation of the interferon system.

The enthusiasm that greeted this successful application in the mammalian system led to its widespread application and an underlying assumption of specificity. However, there was an accompanying failure to take into account the multiple roles that short dsRNAs can have in regulating signal transduction and gene expression. In particular, while a large number of dsRNA-binding proteins had been identified and the structural requirements for activation of enzymes such as PKR were known, these were overlooked [12,13]. The alternative pathways turn out to be a source of non-specific off-target effects. Some of these are not apparent unless global gene expression studies are performed that look at the effects of siRNAs beyond the expected suppression of target gene expression. A siRNA-specific, as opposed to a target-specific, trend emerged from these studies and up-regulation of interferon-stimulated genes, as well as non-specific effects on non-targeted genes are frequently observed.

Studies into a distinct type of short dsRNAs, miRNAs (micro-RNAs), have added to our understanding of how cells deal with short dsRNA molecules. miRNAs make up a group of small non-translated RNAs that regulate the timing of developmental events in an organism. Similar to siRNAs, miRNAs are 21–25 nucleotides long, mediate the down-regulation of target genes, and are produced as a result of Dicer activity. Despite these similarities, miRNAs down-regulate target gene expression after translation initiation by binding to a region of partial complementarity in the 3′ UTR (untranslated region) of the target mRNAs and appear to be the primary products of Dicer action during unstressed conditions [14].

Since such similar formation pathways exist for the two types of short RNAs, it is feasible that a subset of siRNAs will have miRNA activity. Gene expression studies aimed at exploring this possibility have found that this is the case. A subset of siRNAs have been shown to induce post-translational suppression of target gene expression, as seen with miRNA activity. In addition, the non-specific inhibitory effects are seen at low siRNA concentrations and only partial complementarity to the suppressed gene is required. While a single mismatch between an siRNA and its target will reduce specific silencing efficiency, that same siRNA may still be able to down-regulate the expression of non-targeted genes that contain regions of partial complementarity [13,14].

When computational analyses were performed on the siRNAs, multiple potential 3′ UTR targets were identified that contained partial sequence similarity to each strand of a given siRNA [14]. Even a relatively stringent algorithm, allowing for target matches with a minimum of eight complementary nucleotides at either end of the siRNA and G:U wobble base pairing between the siRNA and the 3′ UTR target, produced multiple hits in nine out of 26 siRNA strands tested [14]. If siRNAs can indeed act as miRNAs and control the expression of multiple genes, global expression profiles must be analysed to ensure siRNA specificity, especially when these studies are used to determine gene function.

Although siRNAs were initially thought to be too short to induce a dsRNA-initiated response initiated by PKR or 2′,5′-oligoadenylate synthetase, early assays (using what would now be considered non-optimized siRNA conditions) resulted in up-regulation of certain classic ISGs (interferon-stimulated genes), including GBP1, CCL2, FGF2, CXCL11 and ISG20. The observed up-regulation was not seen in the control or mock-transfected samples, proving that the effect was due to the intracellular presence of the ds siRNA. These non-specific effects were concentration-dependent and appeared to be consistent for all chemically synthesized siRNAs tested. Microarray experiments revealed that chemically synthesized siRNAs trigger the activation of signalling components that overlap, but are not identical with, those regulated by interferon [15]. Similarly, we reported up-regulation of the ISG Stat 1, in addition to activation of the dsRNA-response protein PKR, in response to multiple chemically synthesized siRNAs [16]. While recent optimization studies of siRNAs through efficacy-determining algorithms (as discussed later in this review) can alleviate these effects, significant ISG up-regulation by siRNAs has been noted. However, not all chemically synthesized siRNAs cause ISG up-regulation, although in most cases this has been determined by measuring the level of only OAS2, a highly inducible ISG [14,17]. There are a number of possibilities to explain this result, including siRNA sequence specificity and/or cell-type specificity.

In addition, it was determined that not all siRNAs targeting a particular gene are effective to the same degree at silencing its expression. Because no obvious trend could be determined that ruled an siRNA’s level of efficiency, such as secondary structure at the target sequence site or location of the target region within the gene, a systematic analysis was needed to define specific determinants of siRNA efficiency.

Researchers from different reagent supplier companies, including Dharmacon and Ambion, have expended considerable effort determining the siRNA characteristics that increase functionality while minimizing the non-specific
effects. These two ideals may not be mutually exclusive; a highly efficient siRNA can be used at low concentrations, reducing the potential for siRNA concentration-dependent, non-specific side effects. Dharmacon’s study in particular identified eight characteristics associated with siRNA functionality and the application of an algorithm that incorporates these criteria to siRNA design greatly improves the success rate of siRNA selection [18]. Applying these constraints to siRNA design not only increases the silencing efficiency of the target, but also decreases the number of test siRNAs that must be designed before finding one of desired efficiency.

Under these optimized conditions, RNAi assays using chemically synthesized siRNAs have been optimized and non-specific side effects are much reduced. However, there are drawbacks. Assays using this method are obviously transient in nature, and the suppressed phenotype is lost within approx. 1 week. While this approach is reliable for short-term studies of gene expression, it cannot substitute for the utility of knockout mouse models. This is not a concern in organisms such as *C. elegans*, because they express an RNA-dependent RNA polymerase that facilitates the propagation of siRNA expression. In these systems, the suppressed phenotype is not only maintained, but is also passed on to future generations [19]. Another consideration is that siRNA synthesis is very costly, and this limits the number and scale of experiments that can benefit from this technique.

To address these issues, a system for the stable expression of siRNAs has been developed. Mammalian expression vectors were designed to direct the synthesis of siRNAs from integrated vectors [20,21]. In most cases, the target-specific insert is made up of a 19-nucleotide sequence complementary to the target, followed by a short spacer and the reverse complement of the same target sequence. Once transcribed, a 19 bp stem-loop structure, termed shRNA (short-hairpin RNA), forms that is able to induce the down-regulation of target gene expression via elements of RNAi machinery. A polymerase III promoter was initially used in these constructs, as they produced RNAs that mimic the requirements for an efficient siRNA. These requirements include, but are not limited to, the absence of a polyadenylate tail and a termination signal that yields transcripts with a 3′ overhang.

This idea has been applied to kits that are commercially available which enzymically synthesize large amounts of siRNAs in vitro using a T7 RNA polymerase-based system. Clearly, the siRNAs produced by this method will also produce a transient phenotype, but it allows for the rapid, and relatively inexpensive, production of large amounts of siRNAs. While very effective at silencing target gene expression, the siRNAs synthesized from T7 RNA polymerases have moved farther away from the natural biological process of RNAi and non-specific side effects have emerged.

It was initially noted that siRNAs produced from both the expression of the endogenous vectors [17] and from the transfection of *in vitro* transcribed siRNAs [16] induce a robust interferon response. Microarray studies illustrate the concentration-dependent, global up-regulation of ISGs in response to T7-synthesized siRNAs (Figures 1 and 2).

These non-specific effects represent a robust interferon response, unlike that seen in earlier studies using chemically synthesized siRNAs, where only a subset of ISGs were up-regulated. While the direct cause of these side effects
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Figure 2 | Primary and secondary signalling by siRNA

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substantial, the focus is not to discredit siRNA experiments, but to develop strategies that maintain a high level of siRNA efficiency while minimizing the non-specific off-target effects that have been associated with siRNA expression.

References

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