

Small-interfering RNAs in the radar of the interferon system

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The expanding use of small RNA technology to silence specific genes shows no signs of slowing down. However, the discovery that it can induce components of the interferon system in animal cells should make us pause to consider potential non-specific effects of this technique.

Numerous reports over the past two years employing small-interfering RNAs (siRNAs) attest to the fact that it is a powerful tool for silencing gene expression in mammalian cells. There is a strong desire to apply siRNAs for therapeutic purposes against conditions ranging from viral infections to genetic diseases and cancer¹. Importantly, small RNAs were thought to overcome the problems associated with using long RNAs for silencing in mammalian cells, which activate the interferon system. However, two recent reports from Sledz *et al.*² and Bridge *et al.*³ demonstrate that under certain conditions, siRNAs may also activate components of the interferon system, thus potentially affecting gene expression more broadly. Thus these studies raise caution that such side effects might complicate the use of siRNAs both in the study of gene function and as potential therapeutic agents.

The interferon system is normally the first line of defence against viral infection⁴. Induction occurs when double-stranded RNA, which is a by-product of viral replication or viral gene expression, activates a multi-component signalling complex that includes PKR, a protein kinase and signal transducer. This results in activation of a signalling cascade that causes up-regulation of many interferon-induced genes. PKR also phosphorylates eIF2 α , the general translation factor, causing an arrest in protein synthesis. When the system is sufficiently activated, a cell essentially shuts down its operations and may eventually undergo apoptosis and die.

Although long RNAs of 500 base pairs or so are effective in achieving RNA interference in *Caenorhabditis elegans* and *Drosophila melanogaster*, they are not acceptable for this purpose in mammalian cells because they efficiently bind and activate PKR. Fortunately, the discovery that RNA interference is actually carried out by 21-base-pair

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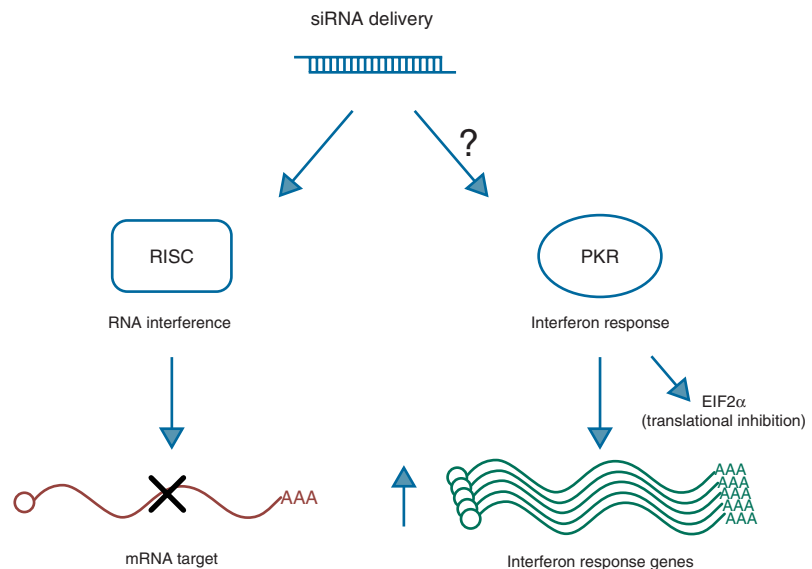


Figure 1 Specific and potential non-specific effects of small RNA technology. Small-interfering RNAs (double-stranded RNAs of approximately 21 nucleotides in length) are effective inhibitors of gene expression. They guide an RNA-induced silencing complex (RISC) to cleave specific mRNAs on the basis of sequence complementarity. They may also activate a double-stranded RNA activated protein kinase (PKR), causing upregulation of interferon-stimulated genes that may result in non-specific effects.

cleavage products of the long RNAs in *Drosophila* led to the development and successful use of synthetic siRNAs in mammalian cells^{5–8}. Even though siRNAs are double-stranded, because of their size they have been thought to fly below the radar of the interferon system, permitting effective targeting of specific mRNAs without perturbing other cellular activities. In fact, siRNA technology has rapidly become the preferred tool for knocking down gene expression of specific targets in a variety of mammalian cell lines. siRNAs can be synthesized *in vitro* and introduced into cells. In addition, several groups have developed DNA and viral vectors to deliver small genes encoding RNA hairpins that are processed by the ribonuclease Dicer into siRNAs *in vivo*, allowing stable and efficient RNA interference^{9–11}.

The work from Sledz *et al.* on page 834 of this issue of *Nature Cell Biology* and Bridge

et al. in *Nature Genetics* suggest that siRNA technology may induce components of the interferon system, posing a complication that had been widely dismissed (Fig. 1)^{2,3}. Both studies used sensitive assays to follow some of the many components of the cell's innate response to double-stranded RNA, and indeed, some siRNAs were causing activation of PKR and the induction of multiple interferon response genes. This effect was detected with synthetic siRNAs that were electroporated into cells and with siRNAs produced within cells by the expression of short hairpin sequences. While activation of the interferon system in these experiments was modest, the important point is that a non-specific response is being triggered by the use of siRNAs that is unrelated to the specific mRNA targeted for downregulation.

Both papers document significant non-specific changes in gene expression as a

consequence of the delivery of siRNAs. Using synthetic siRNAs, Sledz *et al.* observed at least a twofold induction of 52 out of 850 putative interferon-stimulated genes examined. Importantly, they made use of knockout cell lines to show that this non-specific effect depends on PKR and other components of the interferon system, suggesting that double-stranded RNA is indeed the culprit. Using DNA vectors that encode small RNA hairpins, Bridge *et al.* observed that among the 100 most strongly induced genes, 27 are known to function downstream of the interferon pathway. In particular, they observed a 50-fold induction of the interferon stimulated gene *OAS1* with one siRNA vector alone, and a 500-fold induction when two vectors were used simultaneously. However, not all of their silencing vectors, nor all of the synthetic siRNAs tested, caused the effect, suggesting that the ability to induce the interferon system depends on some aspect of both the siRNA sequence and their method of delivery.

Both studies agree that increasing the quantity of siRNA delivered enhances the effect. However, it is difficult to reconcile some differences in the details. Sledge *et al.* report a non-specific induction of the interferon system with six different siRNAs tested, all of which were synthesized chemically or by *in vitro* transcription. Bridge *et al.* observed an effect on *OAS1* induction only with the viral vectors that they tested, but they cannot rule out that synthetic siRNAs might have an effect on other genes in the pathway because this was not directly tested. They do hypothesize that the phenomenon might be triggered when the small-hairpin RNAs synthesized from the vectors saturate the endogenous siRNA processing machinery, allowing

longer, unprocessed double-stranded RNAs to accumulate. Still, how likely one is to encounter induction of the interferon system when performing such experiments has not yet been established.

To some extent, these results are not altogether surprising. Short RNAs were shown some time ago to bind and activate PKR *in vitro*¹². In fact, PKR possesses two copies of a double-stranded RNA-binding domain, and in this respect it is like Dicer, the enzyme that generates siRNAs¹³. Therefore, one could expect that an active dimer of PKR could also interact with a double-stranded RNA as short as approximately 22 base pairs¹⁴. Although such short RNAs may not efficiently activate a full-blown antiviral response, which would result in apoptosis, under certain circumstances high levels of short RNAs do induce enough PKR kinase activity to generate the effects observed in these studies.

An important question now is whether detection of siRNAs by the cell's anti-viral radar system is a problem for investigators. So far, no cytopathology associated with a strong antiviral response have been described as a non-specific effect of siRNA treatment. These two recent papers do not test for or report such an effect either; rather, through the use of very sensitive assays, they reveal induction of a subset of the system's components. There will be some settings where the induction is germane to the system under study, as in the investigation of the innate immune response itself. In addition, as pointed out by Bridge *et al.* this phenomenon will not be problematic in cell lines in which the interferon response is already partially or fully disabled¹⁵. However, in light of their findings, both groups point out

that care should be taken in interpreting results of specific siRNA experiments.

Importantly, many worry that an induction of an interferon response will thwart the objective of extending siRNA technology to uses in patients. Therefore we will have to determine what is an acceptable level of interferon system activation. In addition, alternative strategies are needed to reduce the induction of non-specific effects. One way is to use the lowest effective dose, as Bridge *et al.* advocate. Furthermore, perhaps strategies could be borrowed from viruses that have evolved means of resisting the cell's responses against them. Once the features of the siRNA delivery methods that are causing the problem have been defined, they can hopefully be changed to avoid them. One can expect that many different strategies will be considered to do what is necessary to move siRNA technology from the laboratory to the bedside, and to ensure that it is a reliable tool for basic research. □

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