RNA interference therapies – a RISCy business?

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Disruptive technologies that never were

The fundamental principle of molecular biology now is widely known outside the scientific community. Cells contain DNA which is organised into genes, genes are transcribed into messenger RNAs (mRNAs), and the mRNA messages are translated into proteins. Thus genes encode proteins, which are largely responsible for the processes of life, and indeed of disease.

This paradigm has suggested a number of therapeutic approaches. For example, gene therapies seek to treat genetic disorders through augmenting the patient's own complement of genes by the introduction of an exogenous gene, and antisense therapies seek to treat disease by specifically obstructing the expression of a disease-associated gene. However, neither of these approaches has been particularly successful, in that (so far) both have failed to generate a stream of approvable therapeutic products. Yet each was at one time heralded as a new, disruptive therapeutic modality, and each was the basis for several biotech companies, some of which still are extant.

Now we see another novel 'disruptive' technology, which is attracting eerily familiar headlines and media attention. Like antisense, RNA interference (RNAi) has been widely 'hyped' as a technique for specifically down-regulating the expression of any gene. The basic principle is that administration of appropriate double-stranded (ds) RNA sequences to a cell will cause specific degradation of mRNA sequences complementary to the dsRNA. In theory, then, one can treat a disease caused by over-expression of gene X by administration of a carefully designed dsRNA sequence that specifically recognises the mRNA transcribed from gene X.

Why interfere?

The normal purposes of the RNAi machinery in cell biology remain unclear. It has been postulated that RNAi is a primitive antiviral response (some viruses have RNA genomes), or a mechanism to limit potentially deleterious retrotransposon movements (retrotransposons are sequences of DNA with similarities to some viral genomes). Evidence for the latter includes the observation that nematodes with dysfunctional RNAi machinery also have increased levels of transposon activity.

It also has been suggested that endogenous siRNAs may affect developmental processes (eg in embryogenesis) by influencing the association of proteins with genomic DNA, leading to gene silencing by prevention of transcription. For example, there are reports that developmental defects sometimes are associated with poor expression of RNAi-associated genes. Furthermore, another group of interfering RNAs, called small temporal RNAs (stRNAs; also known as microRNAs or miRNAs), are thought to have a role in gene regulation. These miRNAs may influence a number of processes relevant to development eg apoptosis, proliferation and differentiation. They are said to have their effect by binding to

the untranslated ends of mRNAs (3' UTRs), although it may be that some can mediate RNA destruction in a manner similar to the siRNAs.

Thus, in normal circumstances, RNAi appears to be a system for defence against viral pathogens and also for direction of cell fate. There is no doubt that the discovery of RNAi is a significant scientific advance which has exposed the existence of a hitherto unsuspected mechanism of gene regulation. The question of whether the advance will lead directly to a therapy for a human disease still is open.

A game of Dicer

Before examining the potential of RNAi therapies, it may be useful to summarise the molecular mechanism of RNAi. In many organisms, it seems that dsRNAs in a cell are recognised by an RNase III-like enzyme called Dicer and cut into short lengths (about 20-25 nucleotides). The products of this 'dicing' are known as short interfering RNAs (siRNAs). The siRNAs in turn are recognised and bound by a complex called RISC (RNA-induced silencing complex), which contains another RNase enzyme (not Dicer). The siRNAs in the complex unwind in an ATP-dependent step, so that they are available for binding.

Thus the siRNA in RISC is able to interact with other RNA sequences, and hence can recognise and associate with complementary mRNAs. In this way, the siRNAs can 'guide' the RISC enzyme to specific mRNA sequences, allowing knock-down of gene expression through RISC-mediated destruction of mRNA. The RISC enzyme cuts the mRNA around the middle of the region bound by the siRNA, thereby preventing translation of the mRNA into protein. The specificity of the siRNA-mRNA interaction often appears to be very high, and in many cases a single nucleotide mismatch may significantly decrease the extent of gene expression knockdown.

In mammalian cells, the situation is less straightforward, in that administration of dsRNA often results in an antiviral response characterised by non-specific gene shutdown and cell death. This generalised response may be mediated by activation of RNases (resulting in degradation of a broad range of mRNA targets) or by activation of a protein kinase, which then inactivates a translation initiation factor (leading to repression of translation of a broad range of mRNAs). However, administration of 'pre-diced' siRNAs does not induce mammalian cell death. Instead, the exogenous siRNAs are recognised by RISC and thus mediate down-regulation of gene expression in the manner described above.

The demonstration of this mechanism in mammalian cells in vitro has led to much speculation about the possibility of developing RNAi therapeutics to address those human diseases which involve deleterious over-expression of particular genes. Interest in this area has been further stimulated by reports suggesting that the RNAi mechanism can work in vivo (for example to down-regulate expression of hepatitis C virus protein in the mouse). However, success in a specific mouse model is not necessarily indicative of potential success in human patients (numerous mice have been cured of various diseases, for example cancer, in various ways, very few of which have been developed into effective treatments in people).

RISC vs. Reward

Indeed, there are theoretical grounds to suppose that the translation of RNAi into effective therapeutics is unlikely to be straightforward. Some potential problems in the application of RNAi in a clinical context are briefly overviewed below.

In order to escape degradation and to penetrate the mammalian cell membrane in vivo, the siRNA usually needs to be complexed in some way, eg to liposomes. If the delivery vehicle (be it a liposome, or a modified virus, or some other vector) can enter any cell, then huge amounts of vector would have to be applied to the body to allow for non-specific uptake of the vector by non-target cells. One way around this is so-called anatomical targeting, where physical means are used to confine the vector to the tissue of interest, but this is not possible in all circumstances. A better solution would be to target the vector so that it is taken up only by the desired cells. But this approach raises its own problems – finding a molecule that is expressed only on the target cells, and which also allows the complex to be taken across the cell membrane and directed to appropriate (non-degradative) cellular pathways, is not easy and indeed may not be possible for all cell types. This is particularly the case for malignant disease, in which tumour heterogeneity and genetic lability suggest that not all target cells will express any given tumour-associated molecule.

Importantly, regardless of the exact type of vehicle used to protect the RNA and promote its transport into the cell, there will be issues concerning the efficiency of delivery, ie how many of the target cells will actually take up the delivery vehicle, and then how much of the siRNA will reach appropriate cellular compartments. Where the target tissue consists of very large numbers of cells spread over many anatomical compartments, such as in disseminated malignant disease, the delivery problem is non-trivial.

Even if the therapeutic sequence were applied in sufficient quantity for it to be taken up not only by non-target cells but also by every target cell in quantities sufficient for effective down-regulation of the target gene, there may still be problems related to (i) side-effects and (ii) sustainability of effect.

The issue of side-effects is raised by the probability of the RNAi vehicle being taken up by non-target cells. Side effects then could come about due to the target mRNA being expressed in non-target cells. For example, a gene which is harmful when over-expressed in cancers may be essential when expressed at low levels in normal cells, and thus elimination of expression in normal cells could have very undesirable effects. In addition, side-effects also could be produced by the siRNA mediating degradation of non-target mRNAs, although this is thought to be unlikely (but not impossible) due to the specificity of the RNAi mechanism.

In terms of sustainability of effect, it is clear that application of siRNA sequences will not confer permanent protection. In tissue culture, for example, the effect of a transient transfection is said to wear off after a few days. Hence there has been interest in the development of vectors to allow permanent production of siRNAs, eg by means of viral vectors directing the expression of small hairpin RNAs (shRNAs) that get processed into siRNAs in vivo. Continuous expression of shRNAs from a recombinant virus might in theory allow sustained gene expression knockdown, but then we have to consider all the issues and problems (delivery, efficiency, safety, expression shut-down, etc) that are associated with gene therapy approaches and viral vectors.

A further problem relates to restrictions on the numbers of genetic targets that are suitable for RNAi therapies. One aspect of this is the relatively low number of

patients suffering from diseases which are single-gene disorders, since monogenic disorders often represent quite rare genetic diseases. However, it is these monogenic disorders that will be most suited to RNAi approaches, as therapy of multigenic disorders might require simultaneous delivery of several different RNAi sequences to target cells, which would multiply the delivery problems referred to above.

Another aspect relates to the (probably small) number of diseases which are of a type that would relax the delivery constraints outlined above. For example, it may not be necessary for the siRNA to be delivered to every single target cell if a therapeutic effect could be produced by down-regulation of gene expression in only a proportion of target cells, eg such that expression of an unwanted protein is depressed to below a threshold level. However, these diseases may not represent significant patient populations.

Finally, it should be noted that not all siRNA sequences have the desired effect in mammalian cells; only about one sequence in three appears to work. For those sequences that work, the effectiveness varies but appears never to be 100%, ie the technique constitutes a knock-down approach rather than a knockout approach, which may also affect the theoretical extent of its therapeutic applicability. The reasons for the variability in effectiveness have not been established.

The little siRNAs that might

As laboratory tools, siRNAs have the potential to rapidly advance the state of knowledge of human biology and of molecular pathogenesis. Therefore, the excitement over RNAi as a target validation technique, to make sense of the diversity of new genes discovered by genomics research, may well be justified. However, there are significant problems to overcome before we see the routine application of RNAi-based therapies. In summary, potential issues include the necessity to stabilise and efficiently deliver siRNAs to a significant proportion of target cells in vivo, and the possibility of side-effects in non-target cells. Side effects would be less likely where the target mRNA is not expressed in normal cells, for example viral RNA, or where the target mRNA is significantly different from related mRNAs in normal cells, eg due to mutation (for example in malignant disease). However, effective delivery remains a significant challenge, and will be critical to widespread use of RNAi therapies.

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