

RNA interference

Viruses, Overcoming Problems and Future possibilities

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ABSTRACT

RNA interference (RNAi) is a relatively new method in gene technology. It is the inactivation of gene expression by double-stranded RNA. Since the first example of gene silencing was spotted in petunias in 1986 the technology has expanded into mammalian cells and amongst others entered the fight against HIV, cancer and foot and mouth .RNAi for silencing genes can be achieved by 21-to 23- nucleotides of small interfering RNA (siRNA). Advantages include specificity, lack of side effects, and a fast onset of action that can overcome some of the problems associated with vaccination. Difficulties encountered include the need to inject/deliver siRNA into cell, the short lived effect, and viral mutation rendering the current siRNA ineffective. siRNA are also ineffective in bacteria. Antibody fusion and si-hybrids can overcome some of these difficulties and are discussed in relation to HIV/foot and mouth. This is a new area of disease therapy which given appropriate resources promises to deliver useful therapies in fight against disease.

INTRODUCTION

RNA interference is a relatively new method in gene technology. Many diseases could be completely eliminated by allowing scientists to selectively turn off genes.

The first example of gene silencing was spotted in petunias in 1986. A scientist named Richard Jorgenson was attempting to change the colour of petunias to a deep purple colour. To do this, he added an extra gene (extra RNA) that coded for the colour, to an already purple petunia. The result after adding this extra gene for deep purple was unexpected, the flower turned bright white. It was not clear to scientists what had triggered this effect at the time. In 1998, two scientists, Dr. Craig Mello of The University Massachusetts and Dr. Andrew Fire of the Carnegie Institute in Washington conducted an experiment to help solve this (1). Their experiment involved injecting worms with a short piece of RNA that was the genetic mirror image of a muscle gene. The results were worms that twitched, just like another family of worms from which the muscle gene had been removed altogether. This told Mello and Fire that the RNA they had injected must have shut off or silenced the muscle gene in the injected worms.

This suggested that when Jorgenson inserted the extra purple-producing RNA into the petunia, he had activated Dicer. This is an enzyme that chops up dsRNA (double stranded RNA) causing disease. It works on both bacterial dsRNA and viral dsRNA. The dicer enzyme was activated when the extra purple gene was added to the cell. Thinking it was a virus, the dicer enzyme shut down all colour producing genes to protect the flower from the foreign substance. Therefore Jorgenson got a flower with no colour.

But how can we turn it to our advantage? The basic idea is to repeat what Mello and Fire did with their worms and switch off trouble-making genes linked to certain diseases. The RNAi genes could be delivered to vulnerable cells using modified viruses, or could be injected directly into specific cells (2).

The process

1. In mammalian cells, RNAi can be achieved by 21-to 23- nucleotides of small interfering RNA, called siRNA. Small interfering RNAs siRNA or RNAi can be generated by chemical synthesis; these are in vitro transcription or intra-cellular transcription with a vector.

2. Upon cell entry, the dsRNA is cleaved by the nuclease Dicer into double-stranded small interfering RNAs (siRNAs) of 21 nucleotides in length.
3. These siRNAs are recognized by the RNA-induced silencing complex (RISC), and assembly of one siRNA strand into RISC is used to identify complementary mRNAs, thereby targeting them for destruction.
4. RNAi allows for the sequence-specific destruction of mRNAs (see figure1)

We could use it to produce therapies for illnesses in any organism. Currently, a large amount of research is dedicated to cancers. The possibility exists to effectively ‘kill off’ the cancer cells if the target mRNA cleavage was the cancer gene (3).

Viral infections are also important potential targets for RNAi-based therapies. Reducing the activity of key viral genes would cripple the virus, and numerous studies have already hinted at the promise of RNAi for treating viral infections. In laboratory-grown human cells, investigators have stopped the growth of HIV, polio, hepatitis C and other viruses.

But there are some problems with the technique. Injecting and transfecting the dsRNA seems to be the only way to distribute siRNA to the cells. Another problem is that the silencing effect only lasts for a short while, before the results start to deteriorate. So if we take a viral disease for example HIV, and inject the affected being, the silencing process would not be able to reach all of the cells as the silencing outcome would gradually lessen. How would we overcome this? We need to find a way to release siRNA constantly into the organism.

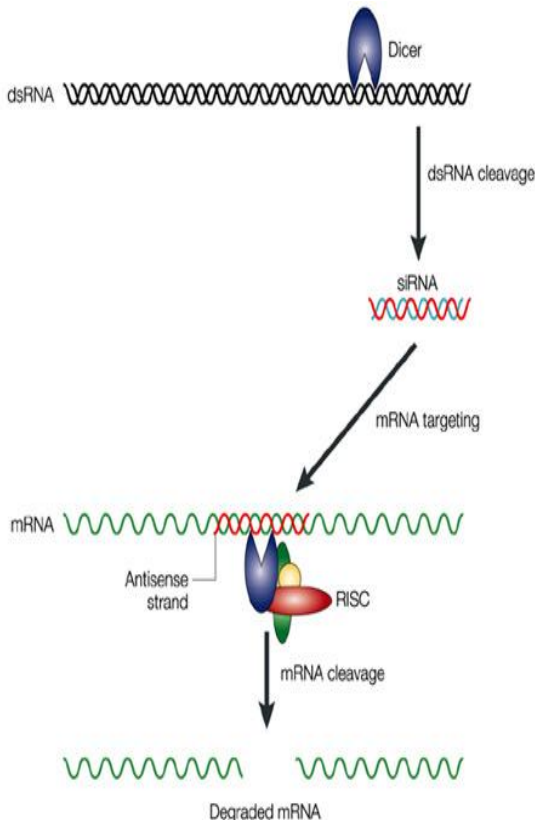


Figure 1

DISCUSSION

Viral disease

Foot and mouth disease

This is one of the most dangerous infections of cloven-hoofed animals. It is a highly contagious vesicular disease affecting more than 33 species of cloven-hoofed animals, including domestic animals such as cattle and swine, and wild animals such as buffalo and goats (4). It is a constant threat to the dairy and beef industries in the Middle East and other regions of the world, despite intensive vaccination programmes. It is also a good place to start as curing or beginning to alleviate the symptoms will benefit both the worlds of Medicine and Veterinary Medicine. Foot and mouth disease is an economically important because of the speed of its transmission. Routine vaccination may not be effective for early protection in an outbreak situation and this is where RNAi can change the outlook in the disease.

Vaccination of healthy animals is possible but immunity is virus type-specific, there is no cross-protection, and it is short-lived. There is also the danger that vaccinated animals, although protected against developing the disease, may become carriers if exposed to new infections of the virus (5). Current measures for the control of FMD outbreak include routine vaccination, control of animal movement, and slaughter. In the developed world this disease is a serious matter for the farmer, but causes little concern to the consumer. But in developing countries foot-and-mouth can have a much wider and far more serious impact (6).

The disease is caused by a very small RNA virus, which is 28nm in diameter. There are seven distinct serotypes of the virus (see figure 2). Whilst they all look alike, each serotype stimulates a different immune response. Immunity to one serotype does not protect against another, and this is a big problem when it comes to making vaccines.

Serotypes foot-and-mouth disease virus
O
A
C
SAT* 1, 2, 3
Asia 1

* Southern African Territories

Figure 2

The most common method of transmission is contact between infected and susceptible animals. Infected pigs can release up to 400 million infectious units each day in their breath. Under humid conditions, the virus survives well in the atmosphere. This makes the wind an important mechanism for FMD transmission.

Foot and mouth is a virus which constantly evolves and mutates. Viruses can also vary widely in their basic biology and reproductive mechanisms, which suggests that unique strategies are needed to inhibit each particular virus. The virus can be inhibited by targeting essential viral mRNAs. Small interfering RNA (siRNA) can be used in a rapid and effective antiviral approach. (See figure 1.)

To silence the Foot and mouth gene, we would need to produce siRNA that would have the complementary base pairs to the target mRNA which contained the gene for Foot and Mouth. This would mean that the dsRNA would have to be fragmented by the enzyme Dicer into siRNA that would contain these complementary base pairs. Therefore when RISC (RNA-induced Silencing Complex) was activated the siRNA would associate with the gene that coded for Foot and mouth disease. When this target mRNA was cleaved and hence taken out of the mRNA, translation would not be able to occur on that particular base sequence that coded for foot and mouth. The amino acids and therefore proteins made would not be produced from the Foot and mouth gene as it has been removed from every piece of mRNA in the body – the code for the

production of proteins containing the Foot and mouth disease never gets to the stage of translation.

However, siRNA has limitations when used in disease prevention, such as a short duration of action. (See problems section below).

HIV

The Human Immunodeficiency Virus (HIV) is a virus that attacks the body's immune system. A healthy immune system provides a natural defence against disease and infection. It is a worldwide issue, leading to AIDS. AIDS is a huge menace for public health, but in Africa it also has a deep economical impact (7). Some animals can carry viruses that are similar to HIV, such as FIV (Feline Immunodeficiency Virus) found in cats or SIV (Simian Immunodeficiency Virus) found in apes. Simian Immunodeficiency Virus (SIV) is very similar to Human Immunodeficiency Virus (HIV) and it is used to study the condition in animal models. In both HIV and SIV, the level of virus in the blood, or 'viral load', is important because when the viral load is high, the disease progresses and it depletes the patient's immune system. This eventually leads to the onset of Acquired Immune Deficiency Syndrome (AIDS), where the patient cannot fight infections which would be innocuous in healthy individuals.

There is currently no publicly available vaccine or cure for HIV or AIDS. HIV antiretroviral drug treatment is the main type of treatment for HIV. The treatment consists of drugs that have to be taken every day for the rest of a person's life.

The aim of antiretroviral treatment is to keep the amount of HIV in the body at a low level. This stops any weakening of the immune system and allows it to recover from any damage that HIV might have caused already. Taking two or more antiretrovirals at the same time vastly reduces the rate at which resistance would develop, making treatment more effective in the long term, so the antiretrovirals are taken combined (8).

The problem is that there are currently only around 20 anti-HIV drugs. The virus eventually finds ways of resisting the combinations of the drugs, meaning over time, the patient can run out of combinations.

Studies have shown that siRNAs can inhibit HIV replication effectively in culture. HIV infection can also be blocked by targeting either viral genes or human genes that are involved in the HIV life cycle. This is promising, as antiviral therapies that can attack multiple viral and cellular targets could avoid genetic resistance of HIV. These results have been achieved by transfixing the siRNAs into cells, and getting the siRNAs to function in the body is likely to be a more difficult task.

Here is the method that could be carried out: the short piece of double-stranded RNA (HIV) enters into a cell, carrying the genetic code, which can be chopped into pieces by the enzyme Dicer. These little pieces, called small interfering RNA (siRNA), can in certain conditions associate with cellular proteins to create a complex with a built-in template (see diagram figure 3), and which uses this template to know which mRNA to destroy.

Since mRNA is the cell's messenger for carrying the genetic instructions from the DNA to the place where proteins are made, then the destruction of certain bits of mRNA means that certain genetic products will never get made, and the spreading of HIV will be inhibited.

Overall, we need to silence the main structural protein in the virus, and the human protein which the virus needs to enter the cell. This would impair the virus in infected cells and would limit its spread into healthy cells.

RNAi discovery is having a substantial impact; it is exciting we have the potential to knock out a protein without harming the cell.

But HIV mutates and evolves resistance so rapidly that any single target for an RNAi therapy won't be sufficient. This is where we look at the problems of RNAi.

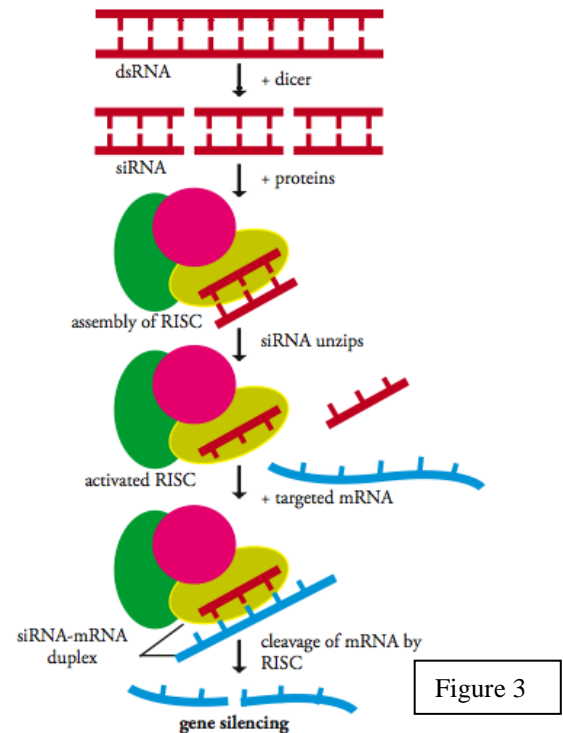


Figure 3

PROBLEMS

Getting RNAi therapies into specific parts of the body and across the cell membrane is the main challenge. This is currently hindering selective gene silencing. The major barrier to using RNAi as a therapy is to move it from blood to inside the cell.

It is easy to forget that RNAi is a natural process that operates in mammals as well as in lower organisms and plants. The mechanism probably evolved as a way to fight off pathogenic viruses. Many viruses have their genetic material made from RNA. So when they infect a cell, the RNAi pathway strikes back, shutting off key viral genes and aborting the infection.

1. The major task is how to deliver the siRNA complexes to all cells requiring them. While siRNAs work well if delivered into tissues that are relatively easy to access, the delivery remains a problem.

In one study using a guided missile strategy using antibody fusion works. We can deliver the drug specifically to the cells we want to target, while using less of the drug and it is less toxic. A Harvard researcher has fused an antibody fragment (for instance an antibody to LFA1, a protein manufactured by active T cells) to a fragment of protamine. While the protamine binds to siRNAs, the antibody discriminates between disease activated T cells and resting T cells (10.)

2. Another problem with the technique is that the siRNA are very short and specific. Both Foot and mouth and HIV are viral diseases, so how would we cope with the changes in viral RNA?

Let's take HIV for an example. We already know that in the lab, a gene silencing mechanism prevents the virus's replication in T cells, by cleaving its RNA and turning off its main proteins. But the virus mutates and evolves resistance so rapidly that fighting it will take more than a single RNA target.

It should work well at first but eventually the HIV virus mutates to become RNAi resistant. How could we fix this? If the virus mutates too much, we might be able to switch RNA sequences, which would be faster than developing another small molecule. Finding good drug candidates is costly and time consuming, and we have to take these factors into account.

3. Also, it isn't always a cure- it may be necessary to keep a level of siRNA present. How to do this is still a question that needs resolving.

Cancer is a good example to use here. The idea of a simple switch-off is especially appealing in cancer, where tumours generally arise from anomalous genes. But unless the RNA reaches every tumour cell and wipes it out, the cancer will return.

Furthermore, siRNA molecules cannot function in bacteria, which are widely used in biological research experiments. The siRNA molecules also cannot be delivered into cells without disrupting the cells, and the genes do not remain suppressed for long. I was unable to come up with a method to solve this; however I have found that scientists in Livermore's Biology and Biotechnology Research Program Directorate have overcome the problems of delivery and the duration of effectiveness by changing the molecular composition of the conventional double-stranded RNA molecules used in siRNA. What they did was to combine a single strand of RNA with a complementary single strand of DNA to create short hybrid RNA-DNA molecules, called siHybrids.

The siHybrids are more stable than siRNAs. They passively enter cells and remain stable in the presence of the enzymes in a cell. As a result, the hybrid molecules have more efficiency than siRNA, and their effects last up to 10 times longer than those of the conventional molecules. Additionally, siHybrids cost half as much to produce, and unlike siRNA, they are effective in bacteria.

Ethical issues

Safety and ethical issues have to be stressed surrounding the use of RNAi. As it is a relatively new process, caution has to be taken with experimentation. This also relates to any experimentation with gene therapy, but scientists are desperate to find solutions to RNAi -in particular the problem of finding the specific gene for HIV or Foot and mouth disease. We cannot silence the particular gene without it being identified first. Therefore animal experimentation is taking place; the ethical considerations involve the number of animals left harmed through the process of finding the particular gene.

Also, what if we find RNAi to be a cure for every disease we apply it to? Would this result in overpopulation in both developed and lower economically countries? When we apply this to

animals –especially animals in the wild we could be destroying the process of natural selection. Different populations of animals could increase, which could affect the outcomes of the food chain.

CONCLUSION

RNAi is a discovery that could change disease as we know it in future generations. The possibilities are endless. In 2006 Williams et al. published a study in which they explored the use of RNAi to stop sperm from fertilising eggs. They used the technique to silence a gene encoding a sperm-binding protein which is normally present on the surfaces of human or mouse ova. The resulting eggs, which lacked the protein, were unable to interact with sperm and so remained unfertilised. Current contraceptives involve the manipulation of female hormones or placing copper devices in the uterus, both of which can cause problems for some individuals. This alternative method of temporarily switching off a gene that's essential for fertilisation is a big development as a future birth-control strategy.

An ethical issue I am also interested in is animal experimentation.

Non-animal tests cannot yet replace animal tests completely, some animal testing is still essential. Scientific questions exist that can only be answered by performing animal experiments. And some animal-based tests are required by law, for example for the toxicological testing of chemicals. To try and replace animal experimentation, I have investigated the use of RNAi to replace it.

The research can work by targeting and silencing human genes in human cells and tissue. RNAi can also use any cell from the human body and so doesn't rely on the use of controversial embryonic stem cells. A dramatic impact on research will take place, as we would have the ability to inactivate selected genes in human cells in the lab. Also we would be studying the correct genes in humans, instead of scientists taking a risk by studying on the wrong genes in different species altogether, mice for example. For development of treatments in the veterinary field, we could use cells from any animal that suffers from the illness – with no ethical issues on unnecessary suffering of animals. However more research needs to take place for this to come into action (11).

To fully evaluate RNA interference, we should also be thinking of the disadvantages it brings to society.

- High investment costs, RNAi is one of the largest research industries
- Economical view -Significant time and money is spent
- 12-15 yrs and an average of £500 million to develop
- Years of scientific education
- 12-15 years worth of research and development

As well as costs, some people argue that as much as researchers know about RNAi, there are still too many gaps. A lot of money is being invested, a lot of time spent, and no real clinical evidence or successful trials have yet been carried out. But I don't think it is something to give up on-many mysteries of RNAi have yet to unfold, but one thing that is certain is that these small RNAs have changed the way scientists think about how DNA and RNA work.

Overall RNAi needs more development but the idea of it is truly fascinating. It can make major changes in both Medicine and Veterinary Medicine. After identifying the gene responsible for each particular condition, we can silence it and possibly cure the disease in humans or animals. Already, RNAi is being investigated in clinical trials. If enough resource is put into its development, we can change the future for good.

The future's bright, the future's RNAi.

References

The discovery of the RNAi mechanism by Andrew Fire and Craig Mello

1. www.the-scientist.com/news/display/24964/

The introduction of RNAi to science

2. http://www.vet.uga.edu/ID/tripp/siRNA_VMES2005.pdf

RNAi and cancer biology and treatment

3. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WWY-494C5B7-1&_user=10&_coverDate=08%2F31%2F2003&_rdoc=1&_fmt=high&_orig=search&_sort=d&_docanchor=&_view=c&_searchStrId=1248168595&_rerunOrigin=google&_acct=C000050221&_version=1&_urlVersion=0&_userid=10&md5=53fa70e449db3e039a0c1cfec392389e

Foot and mouth disease

4. J. D. McLauchlan, W. M. Henderson 'The Occurrence of Foot-and-Mouth Disease in the Hedgehog under Natural Conditions', *The Journal of Hygiene*, Vol. 45, No. 4 (Dec., 1947), pp. 474-479
5. **Pereira, H. G.** 1981. Foot-and-mouth disease, p. 333-363. *In* E. P. J. Gibbs (ed.), *Virus diseases of food animals*.
6. http://en.wikipedia.org/wiki/Foot-and-mouth_disease

HIV

7. <http://www.tht.org.uk/informationresources/hivandaids/>
8. Blankson, J. N., Persaud, D., Siliciano, R. F. (2002). "The challenge of viral reservoirs in HIV-1 infection". *Annu. Rev. Med.* **53**: 557–593. [doi:10.1146/annurev.med.53.082901.104024](https://doi.org/10.1146/annurev.med.53.082901.104024). [PMID 11818490](https://pubmed.ncbi.nlm.nih.gov/11818490/).

microRNA - A revolution in gene expression

9. www.jyi.org

Delivery of siRNA research

10. <http://labs.idi.harvard.edu/lieberman/NewsItems/Commentaries/Mol%20mMed%20Commentary-%20antibody-directed%20cell-type-specific%20delivery%20of%20siRNA.pdf>

RNAi and the charity which focuses on overcoming animal experimentation

11. <http://www.drhadwentrust.org>

The Resource Centre for RNAi Technology

12. www.rnaiweb.com

RNA interference and Gene Silencing - History and Overview

13. www.ambion.com