

THE USES OF RNA-INTERFERENCE IN THE DEVELOPMENT OF A CURE
FOR FOOT-AND-MOUTH DISEASE AND THE CONSEQUENT ETHICAL
IMPLICATIONS

PASS WITH MERIT

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In 2006 Professor Andrew Fire, of Stanford University, and Professor Craig Mello, of the University of Massachusetts Medical School, received the Nobel Prize in Physiology or Medicine for their discovery of RNA-interference, a process which ultimately halts protein synthesis, by degrading the mRNA post transcription and prior to translation. The discovery of RNAi could, in theory eventually, allow any gene to be suppressed, leading to a cure against RNA virus infections, such as Foot-and-Mouth Disease (FMD).

Research into the genetic make-up of organisms is an ongoing process. It is agreed, however, that the manipulation of information within a gene to produce a protein is an essential process in all organisms, in order to exist. Since the discovery of DNA and its characteristic double helix, by James Watson and Francis Crick, who won the Nobel Prize for their pioneering work in 1962, scientists have strived to overcome the mystery behind how protein synthesis is initiated by DNA in the nucleus of a cell, and yet carried out in the cytoplasm. Originally, it was thought that a single strand of RNA was converted from DNA, resulting in a complementary strand. Next, the RNA could be deciphered to allow the assembly of amino acids into a polypeptide chain in a specific sequence. In 1961, Francois Jacob and Jacques Monod proposed a model outlining that when the process of transcription occurs, using the DNA within a gene as a guide to produce a complementary strand, it produces a distinctive RNA with the specific role of acting as a template in the process of the assembly of proteins, this specific RNA was named messenger RNA or mRNA. Transfer RNA or tRNA was then identified as an RNA molecule that acted as an intermediary in the build up of a polypeptide chain from mRNA, this became the process of translation, a concept that had previously been predicted by Francis Crick.

After transcription, the length of mRNA that leaves the nucleus is notably shorter than the DNA to which it corresponds. This is due to modifications that take place prior to the mRNA leaving the nucleus. In eukaryotes there are regions of base sequences which do not code for amino acids, these are called introns, which are found in precursor-mRNA but are removed to make mature mRNA. In pre-mRNA there are also regions of base sequences called exons, these are then merged together by spliceosomes in a process called RNA splicing. This was proven by Phillip Sharp and Richard Roberts in 1977, who then went on to receive the Nobel Prize in 1993 for the split gene concept.

Post-transcriptional gene silencing (PTGS) in plants was a phenomenon that was widely accepted, though largely unexplained prior to the discovery of RNA interference. The concept of PTGS was based upon a considerable amount of ambiguity. After experiments conducted around 1990 it was proposed that a cloned gene combined into the genome could inhibit gene activity. This was later shown to be caused by a process that targeted RNA in the cytoplasm, post transcription, when the mRNA had left the nucleus via a nuclear pore. It was also noted the probability that PTGS served as a natural viral defence mechanism.

The term RNA-interference was at first used by Craig Mello for an unknown mechanism, the term was applied after he noticed inconsistencies in the effects that sense RNA and antisense RNA had in gene silencing. Fire and Mello first published their results from their experiments, exploring the phenotypic effects of worms after being injected with RNA, in '*Nature*' in 1998. The results from their research allowed them to draw a series of conclusions, which led Fire and Mello to comment on the probability that RNAi offered an explanation for the occurrence of post transcriptional gene silencing in plants. Fire and Mello's conclusions, as outlined in '*Nature*' were as follows: dsRNA triggered an efficient gene silencing response, as opposed to single stranded sense or anti-sense RNAs, which had little if any response. Also only dsRNA that was complementary to the mature mRNA sequence, rather than pre-mRNA, prompted a response. In addition Fire and Mello showed that the targeted mRNA was consequently degraded. Furthermore they also suggested that dsRNA acted catalytically to target homologous mRNAs, as very few numbers of dsRNA per cell were enough to achieve full silencing.

From Fire and Mello's break-through results, it was able to conclude that in gene silencing the targeted mRNA is degraded, as a result of the presence of dsRNA, at its post-transcriptional level. After the revelation of RNAi a correlation between the occurrence of PTGS in plants and the presence of small chain RNAs was discovered. It was also shown that dsRNA initiates PTGS in plants. The specific mechanisms of RNAi were further investigated, and it was proposed that dsRNA is first processed and cut into smaller fragments, by the enzyme DICER, that are 21-23 nucleotides long to become small interfering RNA (siRNA). It was demonstrated that then a short antisense strand, from a double stranded small interfering RNA molecule, attached itself to a multi-component complex known as the RNA- induced silencing complex or RISC. This short anti-sense RNA targets the RISC to the complementary mRNA strand and links by base pairing. The RISC complex splits the mRNA which is subsequently degraded. RNAi ensures that the mRNA never reaches the ribosome, and consequently translation cannot take place. This mechanism could be essential in the prevention of foot-and-mouth disease if its positive sense genome could be prevented for encoding for a viral polypeptide.

The difficulty of employing the RNAi mechanism in mammals is due to the presence of long strands of RNA, which instigate an interferon response in cells. The production of interferon is triggered when foreign material, such as a virus, invades a cell. Interferon acts as a potent chemical messenger that signals for the immune system to destroy an invading pathogen; this feature makes interferon an essential part of immunity. Nonetheless the interferon response is not always enough, as proven by the sheer number of viruses that cause infection. Though interferon is essential, in this context it can almost be considered a nuisance, as it is blocking the way for a potentially greater defence mechanism. However, it has been observed that, introducing siRNA of no longer than 23 nucleotides long does not provoke the interferon response, and therefore allows RNAi to take place.

Foot-and-Mouth Disease (FMD) is an acute infectious disease, caused by a RNA virus from the picornaviridae family of the aphtovirus genus. There are seven distinctive serotypes, and within each serotype further subtypes. FMD was the first animal virus in which such dissimilar serotypes were discovered. This creates a dilemma in vaccine production as immunity to one serotype does not confer immunity against another. Also between subtypes there appears to be great antigenic variability, resulting in poor protection against other subtypes of the same serotype. The FMDV (Foot-and Mouth Disease Virus) is made up of a protein icosahedral capsid which contains infectious, single stranded positive sense RNA made up of approximately 8400 nucleotides.

FMD is one of the most contagious animal diseases and clinical signs are the same for each serotype affecting all cloven-hoofed animals. Characteristic clinical signs are pyrexia and anorexia, followed by the development of vesicles primarily in and around the mouth and muzzle and on the feet, and in the female on the skin around the udder and teat. FMD spreads rapidly and is very resistant, and it can survive in an appropriate environment for up to a month. Transmission can occur by direct or indirect contact, animate vectors such as humans can act as carriers, as well as inanimate vectors such as vehicles. The virus is also airborne so can travel up to 60km over land, and even more impressive distances over sea.

An outbreak of FMD has significant economic consequences, especially in less developed countries, and in the UK is a notifiable disease due to rapid recognition of the disease being of the utmost importance. Economic losses arise from the seriously affected productivity of farm animals, and where the disease is not controlled and becomes an endemic, a total ban on the movement of live animals is implemented, which has further economic implications due to heavily reduced trade. The 2001 outbreak in the UK was reported to have cost up to £4.1 billion, which would have included loss of productivity, the cost of diagnostic services, slaughter of infected or at-risk animals, disposal of carcasses and compensation to farmers, amongst other expenses.

All viruses must use a host cell in order to replicate and in doing so they take full advantage of the host cell's own metabolic facilities. Outside of a host cell a virion is chemically inert and therefore is unable to carry out metabolic processes or biosynthetic functions. All viruses follow the central dogma of molecular biology, despite the range of diversity shown in different viruses' genome structure, which is that all genetic information flows from nucleic acid to protein. In the FMDV, after penetration of the host cell by endocytosis, the RNA genome is uncoated and delivered into the cytoplasm. Initially, the RNA must be translated to produce viral proteins required for RNA replication and for the packaging of new virions from the RNA. As the FMDV is a positive, single stranded RNA genome it functions similar to mRNA, acting as a template for RNA replication and encoding for a viral polypeptide, therefore no mRNA is required. In the FMDV RNA replication happens in two distinct stages; first the synthesis of the negative strand takes place, using the complementary positive strand. Then using the negative strand, the synthesis of many infectious positive strands occurs. Many of these infectious FMDV RNAs are packaged by the structural proteins to produce new virions. Infection is spread as the viral genome is introduced into new host cells which repeat the process.

If the principle of RNA interference could be applied to the FMDV then it is possible that treatments could be synthesised that could help to prevent the spread of FMD in identified carriers. Though Fire and Mello based their conclusions on the superiority of dsRNA in achieving gene silencing, in preference to single stranded RNA, I am suggesting that through the manipulation of the RNAi mechanism, it could be just as successful in inhibiting the spread of FMD by the degradation of the FMDV RNA.

As aforementioned, the problematic obstacle with the RNAi mechanism is the provoked interferon response due to long strands of RNA; however the introduction of much shorter strands of RNA, known as small interfering RNA (siRNA) does not provoke the interferon response, and allows RNAi to take place. Certain scientific reports, by Martinez and colleagues, have expressed their surprise after experiments suggested that single stranded small interfering RNAs provoke just as much, if not more, of a specific immune reaction as their double stranded counterparts. This would imply that single stranded siRNA could be just as effective in place of dsRNA in the RNAi pathway. If scientists could synthesis artificially or otherwise single stranded siRNA of the antisense strand of FMDV RNA, then introduce it into an infected organism by transfection methods, I believe, it would allow the RNAi mechanism to occur.

Once siRNA has been successfully delivered to an infected cell, the siRNA orchestrate the degradation process of the mRNA, in the case of the FMDV it will lead to the degradation of the positive sense RNA, which acts as its own mRNA. The antisense single stranded siRNA will then become associated with the multi-component RNA induced silencing complex (RISC). The single stranded RNA of the FMDV does not present a dilemma here, as in double stranded siRNA the duplex must uncoil prior to associating with the RISC, so the RISC only contains a single strand of siRNA. Once the antisense strand and RISC are assembled, the antisense RNA guides the RISC to target the positive sense RNA of the FMDV by complementary base pairing and the positive sense strand is linked into the complex. The positive sense strand is cleaved by RISC and consequently degraded. This prevents the further synthesising of viral polypeptides due to translation being unable to take place as the positive sense genome of the FMDV cannot any longer act as mRNA, halting the spread of infection.

Of course I have simply laid out a theory and before anything can be presented as fact, in depth scientific experiments have to be conducted. What I have suggested above is only a theory based on the current scientific understanding of the RNAi pathway and my ideas for future developments. However with any scientific research come certain ethical implications that are arguably unavoidable, because until a theory or a drug is tested, on subject organisms expanding from a few cells 'in vitro' to large clinical 'in vivo' tests, the implications, side effects and real discoveries cannot be made clear.

The use of animals in laboratory testing has been, and will be, for some time a controversial issue, and for the foreseeable future animal testing will remain an essential aspect to scientific research. Scientific researchers that use animals in their work are under pressure to balance their loyalties to the progression of medicine and science with the responsibility and the duty of care to their animal subjects. To some, animal testing is a demonstration of one's ethical conscientiousness and obligation to society to seek cures and preventative measures, such as research and experiments into the RNAi pathway as a way of halting the spread of an infectious disease, such as FMD. However there is also the completely opposing, almost violent, opinion that animal testing is unnecessary and hugely wasteful, putting animals, who are unable to give their consent, lives at risk. They also argue that procedures are carried out in a synthetic and uncompassionate environment, where the individual animal's needs are simply overlooked. Neither extreme, in my opinion, is correct. It is specifically the combination of fulfilling the responsibility to society and the duty of care to each individual animal subject, under the best circumstances as possible, that holds the ethical higher ground. The Animal Scientific Procedures Act, 1986, regulates the supply and breeding programme of animals intended for scientific purposes, and all laboratories must adhere to a strict code of conduct which stipulates the minimum standards expected, which includes the criteria for the environment in which animals are enclosed and the expectation for humane killing. Nonetheless there are still many measures that can be taken to ensure an animal's welfare during their involvement in laboratory testing.

In July 2009 the Home Office released its annual animal experimentation statistics. In which it was seen that 2008 revealed the highest figure in the past 20 years for the number of scientific procedures involving animals, a startling figure of 3.7 million animals. The basis of good laboratory animal practice has been derived from a publication by Russell and Burch, named 'The Principles of Humane Experimental Technique', which was first printed in 1959. It proposed the three 'Rs' of laboratory animal welfare: replacement, reduction and refinement. Replacement with non-animal models does appear to be the most appealing answer to concerns over animal testing, and although non-animal research is being carried out, and some scientists are dedicated to developing satisfactory non-animal models, animals will still be involved in scientific procedures for a time yet.

The reduction of the number of animals involved in scientific procedures may well seem the easiest to rectify, however it is important to minimize the numbers of animals involved without compromising the statistical value of the research. Inadequate sample sizes could jeopardise the conclusions drawn from the experiment, and mean that it would have to be repeated, wasting resources and involving yet more animals in laboratory tests. It has been suggested that insufficient animal researcher statistical training is resulting in poor experimental designs, which could be contributing to the increasing number of animals involved in laboratory experiments. Reduction should include the development of methods to obtain comparable levels of results whilst insisting on reduced animal involvement.

The original idea of refinement was simply to minimise animal suffering and limit the amount of stress put upon animals during clinical trials. More recently however the concept of refinement was broadened by Buchanan-Smith, to include considerations concerning individual animal welfare in relation to minimising potential or actual pain, suffering, distress or lasting harm and the effect on each individual's overall well being. This can be achieved by providing environmental enrichment to create comfortable and secure surroundings, and also by the appropriate use of analgesic and anaesthetic. A study by Hudson and Bhogal in 2006 suggested that 60 per cent of scientific procedures on animals carried out in the UK are done so without the aid of any anaesthesia. By not using any form of anaesthetic or analgesic researchers may be compromising the validity of their own results, as pain suffered by an animal subject will undoubtedly affect the animal's normal physiology.

If greater awareness and application could be shown towards a relatively simple strategy; replacement, reduction and refinement, then not only could lives be saved, but also the experiences of all laboratory animals could be enhanced. This in turn could then also greatly improve the quality and validity of experimental results.

To conclude, scientific knowledge has its limits set firmly in the understanding of today, however the potential of scientific knowledge knows no limits, and will continue to surprise mankind for some time yet. As demonstrated by Fire and Mello's discovery of the RNA interference mechanism. Just over fifty years ago surprisingly little was known about the genetic make up of organisms at all, but today we are able to look specifically at gene control. With the revelation of RNAi Fire and Mello offered an explanation behind the previously described phenomenon in plants, PTGS. The direct action observed in RNAi suggested that this phenomenon could be employed to suppress specific genes. It has also more recently been suggested that an organism depends on the RNAi mechanism to allow the correct function of its cells and for complete development. The spread of infection by RNA viruses could be blocked by RNAi, if only the interferon response in mammals could be averted, by the introduction of small interfering RNA. If the RNAi pathway could be manipulated to incorporate single stranded RNA, as opposed to dsRNA, then infection from the FMDV could be prevented. Of course different siRNA would have to be synthesised according to the different serotypes, and then the subtypes within each of the seven serotypes of the FMDV. The prevention of infection of FMD would have an enormous economic and social impact in the long term, and bring to an end the devastating consequences that follow an outbreak of FMD. It would also avert huge suffering in infected livestock, leading to the slaughter of entire herds at a time. This would save the individual farmer, as well as the government, from huge economic implications, which is even more essential in less developed countries, where an outbreak of FMD causes even greater terror and chaos amongst people.

As outlined earlier, such extensive scientific research requires clinical tests involving animals, though perhaps not the most ideal situation, at the moment, mankind has come up with no better alternative. However, through the better application of a clear strategy about the minimum expectations for the standard of research laboratories, animal distress and pain, whether intentional or accidental, can be minimised for potentially more accurate results. Replacement, reduction and refinement underline the thesis to all good code of conducts concerning animals involved in laboratory testing.

My personal opinions, theories and conclusions as expressed in this paper, are based on the scientific findings and information that scientists have achieved to date. However RNAi continues to appear to have much more depth to it, than we can currently understand, and therefore will remain to be a huge area for research for many more years to come. For that reason, some of my theories as already presented, are still largely based on speculation about what could be achieved in the future, and based on the information available to me at the time of writing. It is impossible to speculate how much more there is still to learn about RNAi or the prevention of gene expression, and it will be greatly interesting to see what RNAi could achieve in the future, as the nature of science and newer times brings new scientists with alternative ideas. However the discovery of RNAi has undoubtedly raised the bar about the expectations of possible applications of RNAi in medicine in the future.

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