

A Study of Equine Sarcoids and how
Development in RNA Interference
Could eliminate the cancerous cells

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ABSTRACT

RNA interference (RNAi) could potentially hold the key to cures for many viral diseases, as well as a cure for cancer. Research into RNAi started in the early 1990's on plants. But the recent research on RNAi is in developing technologies in which RNAi can be achieved in mammalian cells by activation of the RNAi pathway by use of siRNA. In this paper I am going to discuss how RNAi could be used to eliminate the cancerous cells of equine sarcoids. I will also discuss the problems in the RNAi process in mammalian cells and the ethical issues involved in RNAi.

INTRODUCTION

(i) DNA

DNA is the hereditary material in humans and almost all other organisms. Nearly every cell in a person's body has the same DNA. Most DNA is located in the cell nucleus. DNA is a polymer and is composed of many monomers called nucleotides. Nucleotides consist of a phosphate group, pentose sugar and a nitrogenous base. DNA is double stranded, there are two polynucleotide strands running anti-parallel. The two strands twist to form a double helix, joined by hydrogen bonds between the base pairs.

A gene is a length of DNA that codes for the production of a specific polypeptide (or protein). Proteins allow and control thousands of complex chemical reactions in the cell. To get make proteins from genes, the process is as follows:

1. Transcription of DNA – DNA spirals separate (RNA polymerase) and the base instructions are copied to mRNA (messenger RNA).
2. The mRNA leaves the nucleus and travels to the ribosomes.
3. Translation takes place at the ribosome when the mRNA arrives, producing new proteins. (Translation could be stopped if the mRNA never arrives, meaning any gene can be switched off at any time).

(i) RNAi is responsible for turning of genes. But how does it work?

Prior to the development of RNAi, a phenomenon called gene (or RNA) silencing was described in plants, firstly by the observations and reports of unexpected outcomes in experiments performed by plant scientists in the US and Netherlands. Around 1990, by means of experiments, it was noted that a coloured gene incorporated into the genome, could inhibit the expression of a homologous sequence, as well as stimulate gene activity. In an attempt to alter the flower colour of Petunias, to a deeper colour of purple, the gene for the colour purple was artificially over expressed. The over expressed gene was expected to result in a darker coloured flowers but instead produced less pigmented, fully or partially white flowers, shown in *Figure 1*. This phenomenon was named homology-dependant gene silencing. It was evident RNA played a key role in gene silencing; however the phenomenon remained ambiguous until the discovery of RNA provided an explanation.



Figure 1
The colour of the petunias as a result of an over expressed gene.

Following this observation, plant virologists observed a similar unexpected phenomenon when working on improving plant resistance to viral diseases. Viral forms contain RNA, a virus has a characteristic genetic fingerprint and some viral forms are double stranded. They observed an experiment, in which short sequences of plant genes were introduced into viruses. The results coincided with the research's predictions, and showed that the target gene was suppressed in the infected plant.

(iii) Earlier Research

After the initial observations in plants, many scientists around the world researched this phenomenon, in laboratories, on other organisms. Andrew fire and Craig C. Mello's discovery was particularly notable as it represented the first identification of the causative agent for the phenomenon. Fire and Mello were awarded the Nobel Prize in physiology or medicine in 2006 for their pioneering work.

In 1998 Fire and Mello published their break through study on the mechanisms of RNAi, in their research paper, 'Nature'. Fire and Mello tested the effect of RNA injected into the worm, 'C. elegans'. The scientists established that only the injection of double stranded RNA (dsRNA) lead to an effective loss of the target mRNA. They presented a series of conclusions from their research and commented that RNAi could provide an explanation for the phenomenon discovered in plants for several years. Fire and Mello ended their paper contemplating about the possibility that dsRNA could be used by the organism for physiological gene silencing.

In the follow up study, published in PNAS, published in the same year, Fire provided agreeable evidence that mRNA is the target for dsRNA. Also that dsRNA exerts its effects prior to translation. It was also indicated that the RNA mechanism could potentially be a specific 'tactical' approach to viral defence in lower organisms.

(iv) The Defence *Figure 2*

1. dsRNA (double stranded RNA) invades the cell
2. dsRNA is identified
3. Enzyme dicer chops dsRNA
4. This activates RISC (RNA-Induced Silencing Complex)
5. RISC opens the strands and copies the fingerprint
6. The matching RNA is destroyed

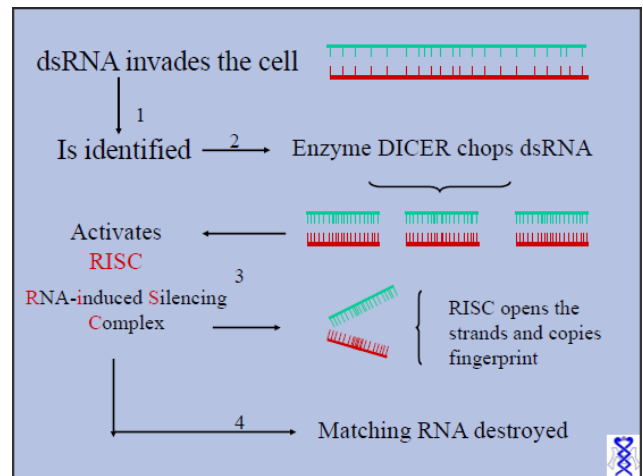


Figure 2 the defence mechanism

(v) Problem

However mammals including humans do not employ this defence mechanism. The enzyme dicer and RISC are both present in higher animals, but the presence of long chains of dsRNA provides a complex interferon based inflammatory response that shuts down all protein production.

RNAi pathway does exist in higher mammals, specifically cells, via RNAi pathway can 'learn' to destroy specific genetic fingerprints carried by foreign RNA, and very importantly their own mRNA. Nether the less, the RNAi pathway does not come into play because the presence of long strands of dsRNA triggers the inferon response shutting everything down.

(vi) the Solution

the solution would be to introduce short double strands of RNA (siRNA) into the cell, smaller than 23 nucleotide pairs in length. The siRNA does not trigger the inflammatory response but does trigger the interference response. RNAi is a revolution in medicine, if the siRNA can be introduced into the cell with a specific fingerprint, the RNA, including the mRNA, can be destroyed i.e. can turn off any gene.

DISCUSSION

The future of RNAi research is exciting and there are many different medical opportunities to be considered within this field. My proposal for future RNAi research will be the treatment for equine sarcoids. This should be done to improve the quality of life for the animal.

It is not fully known what causes equine sarcoids, it is thought to be caused by either exposure to the bovine papilloma virus, or mutations in the gene that cause the tumours to grow but is classed as cancerous. Some speculate face flies may spread the virus, so good fly control may eliminate new cases. Equine sarcoids are common and usually benign but occasionally can be very invasive, invading deeper tissues beneath the skin. They are basically a form of skin cancer in horses. Sarcoids come in various amounts and sizes, some horses may have single sarcoids whereas others may have many. They can also occur on all types/breeds of horses all over the world. There are six types of sarcoids; Occult; verrucous; Nodular; Fibroblastic; Mixed; Malignant. The most common site for sarcoids are areas with thin skin, limited or no hair and have a tendency to sweat. Sarcoids are not appealing to the eye, but more importantly they can cause other problems to the horse. As they more commonly grow in sweaty areas, sarcoids may be in the path of the horses tack, therefore becoming sore or putting the horse out of work. This would be very uncomfortable for the horse. Examples in figure 3.



*Figure 3
Images of
sarcoids on
horses*

Existing treatment

Sarcoids have proven to be quite difficult to treat. However there are a number of procedures, because there are several treatments indicates there isn't one good way in which to treat a sarcoid. Some of the treatment include: surgical excision, banding with rubber rings, cryosurgery (freezing), BCG injection, chemotherapeutic injection, topical cytotoxic therapy, and radiation. Furthermore these treatments are not proven to cure the sarcoid, but to simply control the lesions and it can also be quite expensive. It is proven that the majority, over 50%, of sarcoids grow back after being surgically removed. Sometimes it is impossible for the sarcoid to be removed in such a manner because of the awkward positioning.

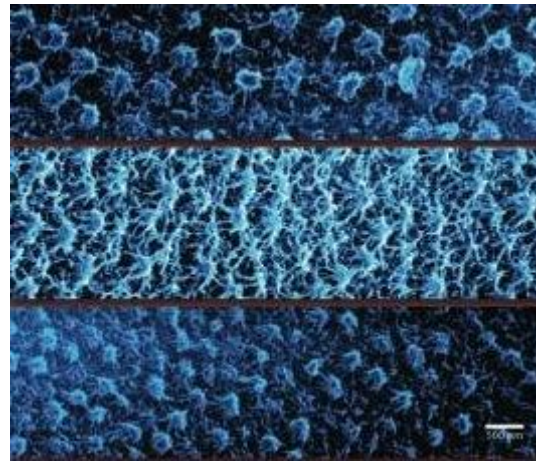
Now let us consider how proteins are formed from DNA in order to grow and produce new cells. Transcription of DNA will occur, the DNA spirals will separate and the base instructions will be copied to mRNA, including the base code for the sarcoid. The mRNA will then leave the nucleus and begin to travel to the ribosomes. Once the mRNA has reached the ribosome, translation will occur, producing new proteins.

So how could RNAi be used to stop the translation of the mRNA? One possibility is to use siRNA. If we could find the gene for sarcoids in a horses DNA then siRNA could be put into the cells of the sarcoid, complementary to the sarcoid gene. RNAi could then take place, as we have used siRNA the inflammatory response will not take place but the interference response will. The siRNA will be detected in the cytoplasm and the enzyme dicer will chop up the siRNA, in turn, activating the RISC which will open up the strands and copy the genetic fingerprint. The matching RNA, the mRNA for the sarcoid gene, will then be destroyed. The mRNA will be destroyed prior to it reaching the ribosomes, therefore translation will not occur and the gene will not multiply, resulting in a silenced gene.

One problem with the above method is getting the siRNA into the cells and how it will be used once inside the cell. The research into getting siRNA into cells is fairly recent and not yet fully proved exactly how to do it effectively. However, by doing the research, there has been several theories on how to do this. However, siRNA's are difficult to deliver into cells and current delivery approaches result in cytotoxicity, poor percentage of cells, changes in the overall transcription and biology of the cells, and even DNA damage.

One such theory is a process in which 'hydrogels', shown in *Figure 4* are used to sneak a particular type of siRNA into the wanted cells. This research has shown by using hydrogels less than 100 nanometres in size, the siRNA can be sneaked in and once in siRNA can turn on the programmed cell death the body uses to kill mutated cells. Once inside the cell siRNA has a limited life span but by keeping it protected inside the hydrogel nanoparticles, it gives more time to allow only small amounts of siRNA to be released at any one time. This allows the siRNA cells to get into the cell safely and acts as a protective barrier around them. This process is good as once the hydrogels are inside, the particles have a slow release profile that leaks out the siRNA over a timescale of days, allowing it to have a therapeutic effect, therefore spending time to destroy each cell. This theory gives an indication on how siRNA is released once inside the cell but still gives no indication on how it is put into the cell initially.

Figure 4
Hydrogen Nanoparticles



Another theory is called the 'siRNA method'. This is a process in which synthetic double stranded siRNA, created *in vitro* (referring to processes and experiments occurring outside an organism in an artificial environment), is transfected (the introduction of DNA into a recipient eukaryote cell and its subsequent integration into the recipient cells chromosomal DNA. Usually accomplished using DNA precipitated with calcium ions) into the cells. Shown in Figure 5. Once present, the double stranded siRNA is denatured, and bound by the RISC complex, where it goes on to perform its duty. Shown in Figure 6. This siRNA is still small double stranded siRNA. This is a good way in which to get siRNA into cells as it is as the strand sequence has been heavily researched and compared to a huge genomic library to assure specific and targeted silencing with guaranteed accuracy. Therefore targeting and silencing the cells wanted.

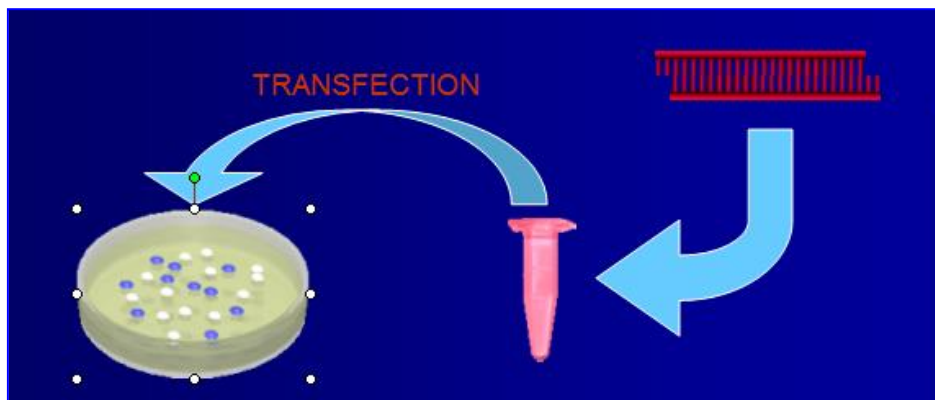
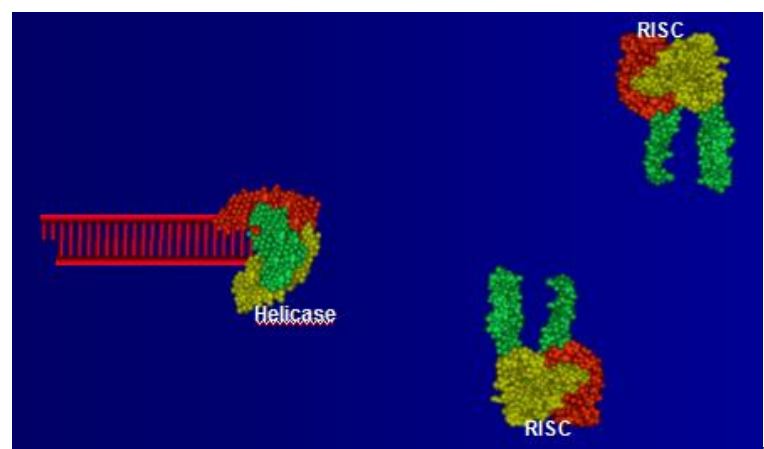


Figure 5 siRNA Method
Transfection

Figure 6
siRNA denatured and bound to RISC complex



My theory for one possible use of RNAi is to use both of the theories above and combine them as they both have some good point about them. My theory would take the same pathway as in the 'siRNA method', targeting the sarcoid gene, as that process assures specific and targeted silencing, meaning only the sarcoid gene would be destroyed. But I would cover the siRNA used in this process with hydrogels as the slow release of the siRNA allows it to keep up a sustained attack so that it can continue to interrupt the production of proteins, preventing the gene for sarcoids recovering. Meaning that once treatment has been done there would be a great chance of the sarcoid never returning, unlike the previous treatment, where it was more likely to come back than not. Also this treatment could be combined with another, for example radiation, therefore improving the horses' chances even further.

Expense is also an issue that is needed to be covered. This process of using RNAi to treat sarcoids maybe an expensive process but this will drastically reduce the risk of the regrowth of cancerous cells. It will also cause no more irritation to the horse. Earlier treatments were also costly but they also carried a great risk that they would re-grow again, meaning more procedures. Therefore meaning earlier procedures were pointless. Overall, I think that the RNAi procedure would be the more sensible option.

If RNA interference is to be used therapeutically, we should perform a risk-benefit analysis. It is ethically relevant to perform a risk-benefit analysis since ethical obligations about not inflicting harm and promoting good are generally accepted. RNA interference is considered a new and promising therapeutic approach, but the ethical issues of this method have not been greatly discussed, however I will go through some ethical issues that may arise from the use of RNAi.

Clinical trials with RNAi have now begun, but major obstacles, such as off-target effects, toxicity and unsafe delivery methods, have to be overcome before RNAi can be considered as a conventional drug. In order to do this a risk-benefit analysis needs to be completed in order to weigh the pros and cons on use of RNAi. The terms harms and benefits are ethically relevant concepts since ethical obligations or principles about not inflicting harm (nonmaleficence) and promoting good (beneficence) are generally accepted. There are several different ethical theories of the principles of nonmaleficence and beneficence, including; the foundation of the utilitarian theory, which says that ethically right actions are those that favour the greatest good for the greatest number; the Hippocratic Oath, which expresses an obligation of beneficence and an obligation of nonmaleficence: "I will use treatment to help the sick according to my ability and judgment, but I will never use it to injure or wrong them". Therefore risk-benefit analysis clearly is an ethical issue.

Generally, participants in a clinical trial benefit from having access to promising new approaches that are often not available outside the clinical trial setting, and they receive regular and careful medical attention from a professional research team. Furthermore, the participants may be the first to benefit from the new method under study. Lastly, the results from the study may help others in the future. However, when participating in a clinical trial you are open to some possible risks.

For example, new drugs or procedures under study are not always better than the standard care to which they are being compared. The new treatments may have side effects or risks that physicians do not expect or that are worse than those resulting from standard care.

Nether the less, my research paper is based on sarcoids on horses and I would like to mention the ethical issues about the animals. I think that animal testing should be extremely limited as the animals have rights too. We as humans should look after and do our best to care for the animals, not use them for our own selfish reason. Moreover in this case it is necessary to do a small amount of animal testing as the therapy is being used on animals, for their own benefits, to remove the sarcoid gene.

CONCLUSION

I have mentioned that the future of RNAi research is vast and at the moment we are only in the early stages of using RNAi in mammary cell. I hope in the near future RNAi can be used effectively and efficiently to cure all kinds of cancers and viruses, not only in the medical profession but widely in the veterinary profession too. My proposal for using RNAi to eliminate cancerous sarcoid cell may be among the great advances in RNAi later on in life. I have looked at the problems faced when using RNAi in mammary cells, and how procedures can be combined to overcome problems and give a greater success rate. During this project I have enjoyed researching RNAi and learning how it can be used to benefit both humans and animals in many different ways.

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