

**Theoretical Methods of Introducing siRNA into Sheep & Goats affected with 'Scrapie'-
(Variation of BSE or 'Mad Cow Disease')**



By

Andrew Rich

(Wirral Grammar School for Boys-Sixth Form)

Jessica Hawe

(Wirral Grammar School for Girls-Sixth Form)

Michael Sullivan

(Wirral Grammar School for Boys-Sixth Form)

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PASS WITH DISTINCTION

Scrapie is a fatal, degenerative disease that affects the nervous systems of sheep and goats. It is caused by a TSE Prion Protein, and has been a well-known disease since the 18th Century, which does not appear infectious to humans. It is however highly infectious and transmissible among similar animals, and the only known solution to contain Scrapie (since it is untreatable) is to quarantine and destroy animals affected by it. Scrapie can continue in flocks and can also take place unpredictably in flocks that have not previously had the disease. RNA interference has the potential, using specific siRNA (short interfering Ribonucleic Acid), to 'silence' proteins that could cause disease. Due to these problems associated with Scrapie, this paper has been written to discuss how a specific siRNA could be introduced into nervous tissue to silence the process of a Prion modifying certain nervous tissue proteins, causing 'Scrapie'. The central idea for integrating the siRNA into cells is to use a preventative measure of producing the required siRNA within the body using 'Genetic Modification' to prevent the Scrapie Prion (PrP-Sc)'s target, PrP-C, from being produced in cells in the first place, thus starving the Scrapie Prion and preventing the disease from occurring. After discussion, our conclusion was; a genetically modified Bacterium (containing siRNA-which would be secreted) placed in the body would be able to prevent the Scrapie Prion's target protein from being made, therefore preventing Scrapie.

Scrapie

Scrapie is caused by a Prion. Prion diseases are very unusual because the pathogens are apparently neither viruses nor bacteria, being simply protein molecules, with no DNA or RNA of their own. Scrapie is caused by an abnormal Prion protein known as PrP-Scr, which affects the normal ('healthy') variant, PrP-C, which is naturally occurring in the brain. When the healthy proteins come into contact with the Prions, they change their shape by 'Allosteric regulation' and become diseased as well. The result is an uncontrollable chain reaction. In this case the PrP-Scr transforms all of the healthy PrP-C into more PrP-Scr, thus causing the disease to develop.

A special RNA type, siRNAs, can attach to a specific mRNA stretch, preventing these from being 'read', thus the protein encoded cannot be produced. This shuts down the production of proteins that go distorted in Prion diseases. Prion proteins are very similar to healthy proteins, but have a slightly different shape.

The discovery of RNAi

It all started when Professor Richard Jorgensen, a botanical scientist at the University of Arizona in Tucson in 1990, became interested in the esoteric mechanism that controls the regulation of genes in plants. Deciding to make purple petunia flowers even more purple, he injected them with the gene for that specific pigment coloration. However, the flowers flowered with white petals. Instead of the two sets of pigment-producing genes harmonising with each other, they seemed to interact by shutting each other off.



Diagram 2-Co-Suppression

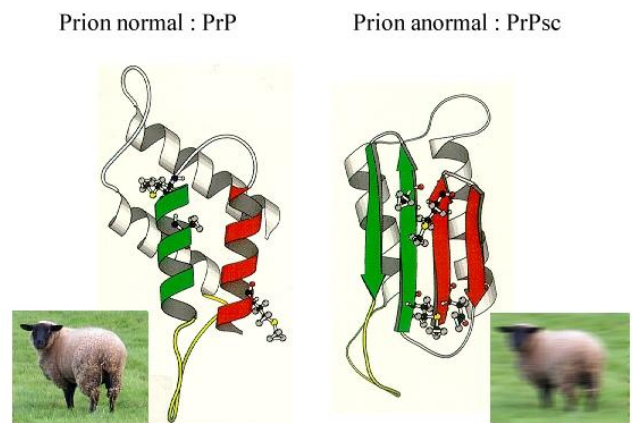


Diagram 1-Prions involved with Scrapie

They called the incident "co-suppression" because it seemed that both sets of pigment genes were preventing each other from working properly.

The Scientists repeated the experiment with other species of plants and got similar results, they then repeated the experiment with yeast, which also followed the trend. They called the mechanism 'Quelling'. At the same time as these experiments were taking place, molecular biologists had been working on something called "antisense" technology. This was a way of turning genes off in RNA.

Antisense worked by injecting into a cell a molecule of RNA that was complementary in its genetic sequence of chemical bases to the RNA of the cell involved in the synthesis of proteins from genes. It was hoped that an antisense strand of RNA would block the manufacture of a particular protein, thus shutting off the gene. After numerous experiments it was found that the antisense technique worked best when the RNA was injected in the form of a double-stranded molecule, instead of its usual single-stranded form.

Andrew Fire, a researcher at the Carnegie Institution at the Johns Hopkins University in Baltimore, Maryland, looked into the problem. He too had been interested in injecting antisense material into a nematode worm to study the switching on and off of genes. Dr Fire set up a "control" experiment which was not supposed to produce any results and was merely there to compare against the actual experiment. In fact it proved to be very influential.

"What we found was that our control experiments never worked properly," Dr Fire said, "Not only was the normal gene shut off but the gene we were putting in was shut off as well."

Dr Fire couldn't help but notice that the incidents were very similar to the original petunia experiment he had heard about. He called it gene "silencing" – the nematode version of co-suppression in petunias and quelling in yeast.

"My lab worked pretty hard to sort out what the actual structure of the RNA that was causing the silencing was. We were surprised because it turned out not to be the major component we were injecting, but a contaminant that is known to be present when you make RNA in a test tube," Dr Fire said. They figured out that the contaminant was double-stranded RNA – when a "sense" and an "antisense" strand wrap around each other to form a single molecule, rather like the double helix of DNA. The discovery revealed that, when RNA came in its double-stranded form, it was capable of silencing genes.

They called their discovery "RNA interference", or simply RNAi, and said how surprised they were about the power of double-stranded RNA to silence genes.

After these breakthroughs, other botanical scientists continued working on the 'double-stranded' problem. One of the scientists, David Baulcombe, was interested in how plants defended themselves against attack by viruses, using double-stranded RNA as their primary genetic material. Professor Baulcombe had reanalysed Dr Jorgensen's petunias and other plants and found very small strands of double-stranded RNA floating around in the cells. The teams of fruit fly scientists tried to find the same stretches of double-stranded fragments in their lab animals. They, too, eventually found small RNAs that were interfering with the action of the genes.

They found that the large double-stranded RNA molecules were being chopped down into smaller units of a precise length by an enzyme known as DICER. These units formed a dangerous complex with enzymes, which would identify and chop up the "messenger" RNA that transfers genetic information from the genes inside the cell nucleus to the protein synthetic areas of the cytoplasm.

Suddenly it was realised that it would be possible to make these short, double-stranded RNAs in a test tube and tailor them to target a specific messenger RNA from a particular gene. This would mean that scientists could turn off any gene at will.

A critical question asked was whether this would also work in the cells of mammals, including those of humans. If it did work, the medical potential could be enormous. It would mean that we could turn off genes involved in cancer, genes that allowed viruses to infect cells, genes that were involved in tissue rejection after transplant operations and, of course, genes of viruses that had already managed to infect a healthy cell. Scientists have managed, in the test tube, to make human cells resistant, to an attack by the polio virus as well as the Aids virus, HIV, which leads to many possible future developments for the future.

The Mechanism of RNA interference (see diagram 3)

RNA interference research has huge potential. It involves siRNA (short interfering Ribonucleic acid) preventing certain proteins from ever being made in the first place by destroying the mRNA that encodes the protein.

The siRNA, at first, is double stranded (ds) and consists of 20 – 25 nucleotides, with 2 nucleotides on each 3 overhanging. The dsRNA is broken apart by an enzyme (called DICER) into 2 strands (an antisense and a sense strand). The two siRNA strands, sense and anti-sense, are bound by the RISC (RNA induced silencing complex) related proteins, only the RISC complex containing the anti-sense strand is active, because this is the strand needed to pair with the mRNA.

The anti-sense RISC binds the mRNA sequence that it is matched perfectly with. (In this way, only specific proteins can be chosen for ‘silencing’.) If just one nucleotide is wrong, the siRNA may not bind. Likewise, as few as 11 neighbouring nucleotides matches with an unrelated mRNA can lead to ‘off-target silencing’, which in therapeutics could be disastrous.

Once bound by the RISC complex, the mRNA is cut at the binding site, and because of this cleavage, the mRNA is further cleaved by nucleases and broken down by the cell’s ‘maintenance’ proteins. The active RISC complex is recycled and moves on to destroy more mRNA strands. With proper siRNA introduction, one protein can be singled out in a cell or organism, and ‘silenced’.

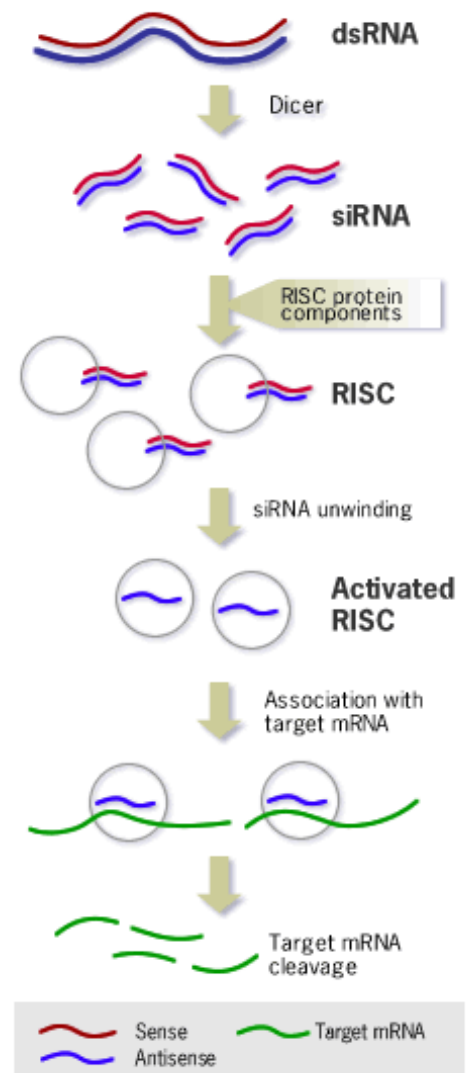


Figure 3-The Mechanism of RNAi

As a result, certain proteins produced by cells that cause certain diseases (e.g. cancers) can be prevented due to the necessary mRNA being destroyed before it reaches a ribosome and produces a polypeptide/protein (Protein synthesis). This method can also be used when dealing with viruses, which insert their genetic material into the host cell's DNA. If the virus contains RNA, it must first turn its RNA into DNA using the host cell's mechanism, before inserting it into the host DNA. Once it has taken over the cell, viral genes are then copied thousands of times, using the mechanism the host cell would usually use to reproduce its own DNA. Then the host cell is forced to convert this viral DNA into new protein shells; the new viruses created are then released, destroying the cell.

Possible Methods of Introducing siRNA into specific cells of Sheep & Goats affected with 'Scrapie'

Researchers reasoned that shutting down healthy protein production using siRNAs would break the process by depriving diseased Prions of their ability to spread. Professor Kretschmar, director of the Prion Centre of Munich's Ludwig Maximilian University, said "If brain cells are to produce siRNAs, you have to smuggle in the corresponding gene, but presumably we'll never manage to equip all the cells in the brain with this gene".

Therefore, part of the task for the Scientists included finding out how many cells they have to genetically block to treat Scrapie or other Prion diseases successfully, so to do this they performed an experiment involving mice. Professor Alexander Pfeifer, director of the Institute of Pharmacology of the University of Bonn said, "We modified the brain cells of mice in such a way that they were able to produce siRNAs in place of the 'healthy' PrP-C protein", this along with them using RNA interference cut back the production of PrP-C in the mice brains.

It was discovered that even if only a few cells in the brain could produce the siRNAs, the mice died at the same time as the control mice (on average after 170 days), whereas the mice treated with siRNA in 97% of their brain lived much longer, for example 230 days. Professor Pfeifer concluded that "SiRNAs seem to be a promising therapeutic option for Scrapie, CJD or BSE. However, it will take years before the method can be used on human beings".

These findings give promise to future developments of using siRNA to breed cattle which cannot produce any PrP-C and would therefore be resistant to BSE. Due to Scrapie being a similar disease, Scientists have recently attempted to selectively breed for Scrapie resistance in sheep, by breeding sheep that naturally produce the siRNA instead of the PrP-C protein, see The National Scrapie Plan's PDF file for details at

<http://www.sac.ac.uk/mainrep/pdfs/scrapieresistance52104.pdf>

However, the method in which they cut back the production of PrP-C has not been released or specified, and so this is where our project discusses our theories of how we could implement the specific siRNA into the required cells to cut back PrP-C production, to prevent or treat Scrapie.

Possible Methods

Our initial possible methods include:

1. Genetic modification of cells to produce siRNA specifically for BSE in Sheep.
2. Organ cloning-with cells that naturally produce siRNA
3. Genetic Modification of a Bacterium to produce an exponential amount of siRNA
4. Using Liposomes to package pre-made strands of the specific siRNA and injection into the bloodstream.

Methods 1 and 2

Our first idea was to genetically alter cells to produce siRNA, similarly to how plants defend themselves against attack by viruses, using double-stranded RNA as their primary genetic material. This would involve genetically altering the cells of Blastocyst lambs, to set up the mechanism of the preventing the naturally occurring PrP-C from being made in the first place; creating a perfect immunity to the Scrapie Prion PrP-SC. However, stem cell research at the moment is very limited, due to controversial issues and the difficulties of recognising the specific DNA strand to prevent the PrP-C from being created.

Even though prenatal genetic alteration does not, in theory, kill the early developing organism, it does essentially change them in a way that cannot be reversed. Thus, it is disputed that 'prenatal genetic alteration' indeed does disturb that individual: resulting that they will not develop either physically or mentally in their mature state.

Furthermore, when discussing the idea of genetically altering cells of Sheep, there were mainly thoughts about possible side-effects to the treatment, as potentially cancers could be formed or there could be deformities of cells and tissues due to the siRNA cancelling out certain proteins from being produced. This issue would also be apparent with organ cloning as it the possibility of replicating cells once the cloned organ is implemented could possibly grow out of control or die. Therefore, for this project, we decided not to follow this line of research to find a way of implementing the siRNA into Sheep.

Method 3

Starting at the basics; to implement the siRNA into body the molecule would have to be protected and carried to specific cells/tissues using a vector. Now, this vector has to be able to get to its target without being destroyed, therefore our first ideas were that it could be possible to use genetic engineering to replace part of a bacterium's plasmid with chains of siRNA. See Figure 4 for an example of Genetic Modification of Bacteria to produce a particular substance.

Now for this to be possible, and safe, the bacterium would have to be pre-existing within Sheep, meaning that it would already have some kind of immunity to the Sheep's immune system, so that there would be not immune reaction to the bacteria, meaning it would not be destroyed before the essential siRNA is produced on a large scale. In addition, due to the fact that the selected bacteria would naturally be living within the Sheep, would give the indication that it flourishes naturally such conditions, and so it happily reproduces within the body; a perfect environment for manufacturing the siRNA, reproducing many times and so supplying a sufficient quantity of siRNA to be useful. Also using bacteria would prevent any chance of deformities of cells that usually develop normally without the siRNA.

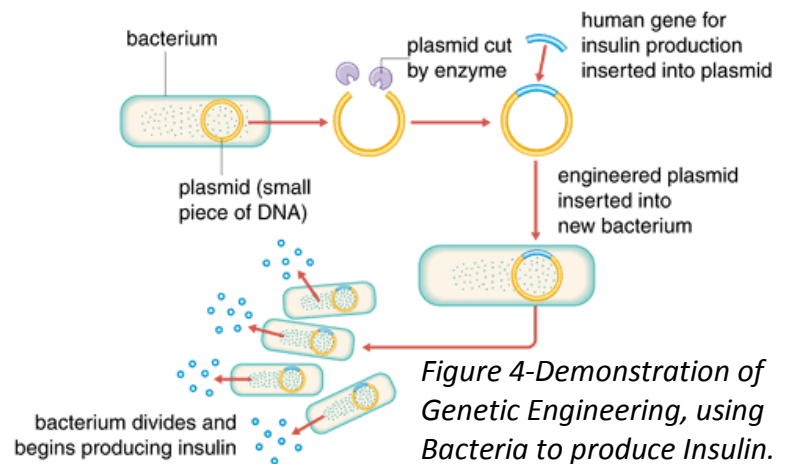


Figure 4-Demonstration of Genetic Engineering, using Bacteria to produce Insulin.

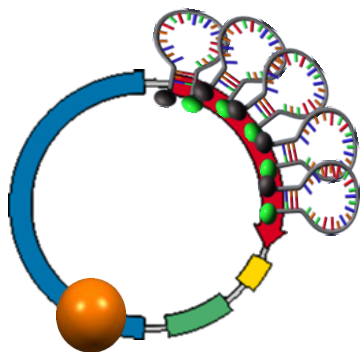


Figure 5-Modified Bacterium Plasmid with siRNA, ready for replication via Binary Fusion, after which, the siRNA is secreted, (The secretion of the siRNA should be an area of research for the future)

This method has many advantages (including ones already discussed):

- Theoretically very simple to place the siRNA strands in the plasmid.
- Also due to bacteria being able to copy everything within themselves, the siRNA can be copied and be produced on a mass scale, as the bacteria divide by Binary fission a plentiful supply of siRNA is produced so as much of the PrP-C is prevented from being produced as possible.
- The amount of Bacteria is exponential so more and more siRNA can be produced.
- Bacteria are not connected to actual Sheep's body so can be moved to specific areas of the body, via the blood to the nervous system.
- Plus, if the bacteria become harmful, you can potentially destroy it using antibiotics, so there is a 'safety net' with the method. If implemented, after the first couple of days, run basic health checks on the animal: check lymph nodes and temperature. After checking if complications have occurred prescribe strong antibiotics to the animal.
- Because the bacteria used would originate from the sheep, there would be not immune reaction to the bacteria so would not be destroyed before siRNA is produced on a large scale.
- In addition, a constant supply of siRNA would be present, if the bacteria survives and is not destroyed by the Sheep's immune system.

In addition, as Scrapie Prions first enter through the intestines, and first appear in the lymph nodes, especially in Peyer's patches at the small intestine, it could be possible for the siRNA to reach the same nervous system cells in the same fashion via the Lymphatic system. As a result, this would require the necessary Bacteria to be present in the intestines at all times. However, for this to be effective and for Scrapie not to develop, this treatment must be implemented in the very early stages of the Sheep's life, so that enough siRNA can be produced to prevent PrP-C from being developed, or at least limit the PrP-Sc from converting PrP-C, so that eventually, if the Sheep was infected, then the disease would be starved and so would dissipate.

However, like most new developments in Science, Genetic Engineering has its 'concerns':

Complications and Ethics

These concerns range from ethical issues to a lack of knowledge on the effects genetic engineering may have. One major concern is that once an altered gene is placed in an organism, the process cannot be reversed.

Ethically, due to 'Genetic Engineering' only being a recently development branch of research, there is great uncertainty as to whether there is the possibility that the bacteria chosen for modification would develop into a new disease-causing micro-organism, perhaps one that would be more detrimental to the Sheep than Scrapie; however that risk would only be dismissed with experimental tests that would prove otherwise.

In addition, an efficiency problem with 'Genetic Engineering' is that, approximately, only 1-2% of genetically engineered bacteria take to the new inserted section of plasmid, the others either not accepting it, destroying it or not replicating it, so it is not an efficient process at the moment, but that could be an aim for the future of this method; improving the engineering efficiency.

Another further difficulty with it is that, the bacterium used has to be specifically harmless or already useful to the Sheep's body. To do this would require many samples and trials to ensure that a harmful bacterium is not used, and therefore potentially preventing a more harmful contagion.

In addition, this method would be very expensive as the bacteria used would be an example of a transgenic organism, which is an organism altered by genetic engineering. GEO (genetically enhanced organism). Basically, genetic engineering involves genetic material being changed by other than random natural breeding, transferring genes from one organism to another. These require skill and knowledge to be carried out properly, and so require specialists for this development to work. (An example of Transgenics that are used nowadays is; bST (Bovin Somatotropin) which is a genetically engineered enzyme produced by GE bacteria which is used to stimulate milk production in cows).

Furthermore, another ethical issue, from a more radical view is that certain religious people oppose genetic alteration due to the method 'going against nature', and thus we do not have 'the Right to play God'. This may not specifically apply to Bacterial modification; however, it is still an ethical issue that must be considered, as with all other scientific breakthroughs that are similar to Stem Cell research and cloning.

Regardless of all of these issues, the potential for genetic engineering is incredible. However, further testing and research will be required to educate people on the advantages and disadvantages to genetic engineering. With the recent breakthroughs in cloning, the capabilities of changing organism characteristics are unpredictable.

On a more practical view, there could be an issue with the treatment itself, particularly if Farmers object to giving their sheep and goats the new treatment, i.e. nervous about the risk of losing their flock to complications. Therefore Veterinary surgeons and clinicians would need to be convinced, this can be done by obtaining results from clinical trials. With these, you could express your confidence of the accuracy of your results by publishing them in a veterinary medical magazine to promote the treatment.

Other issues:

Despite the original task asked for the siRNA to be delivered to specific cells, for this method it may not be possible due to the conditions required for the bacteria to flourish. Whereas if we were concentrating on a cancer for the siRNA to attach to and breakdown, in which we would probably choose Liposomes to transport the material to the cancers because of their natural ability to target cancer, however due to Scrapie directly affecting the Nervous system after entering the Sheep's Lymphatic system and thus entering the blood before going to the Nervous system, the siRNA required to prevent the PrP-Sc's target Prion PrP-C may have to follow the same route, however the only other possibility would be use a bacterium found naturally in the Sheep's Nervous System, providing a shorter route to travel (theoretically).

4) Other possibilities- Liposome Use

Liposomes are tiny vesicles made out of Phospholipids. Phospholipids are found in stable membranes composed of two layers (a bi-layer). In the presence of water, the heads are attracted to water and line up to form a surface facing the water. The tails are repelled by water, and line up to form a surface away from the water. In a cell, one layer of heads faces outside of the cell, attracted to the water in the environment. Another layer of heads faces inside the cell, attracted by the water inside the cell. The hydrocarbon tails of one layer face the hydrocarbon tails of the other layer, and the combined structure forms a bi-layer.

The use of Liposomes for the siRNA would involve packing them to contain the necessary siRNA, for the siRNA to reach the cells and then undergo a version of Endocytosis, releasing the siRNA into the specific cells, where

the Liposome becomes fused into the cell's plasma membrane, and thus secreting the siRNA into the cell, (instead of the siRNA being packaged into a vesicle. This way the siRNA is directly passed into the necessary cells, however there needs to be some kind of mechanism that cause the Liposomes to release the siRNA into the Nervous tissue, instead of other cells. That would need to be an area of research for the future, if this method were to be used. However, we feel that the method using a Bacterium would be more effective, sustainable and easier to implement, in theory.

Conclusion

In conclusion, we feel that an effective method of preventing Scrapie would involve starving the Prion (PrP-Sc) of the Prion that it converts, (PrP-C). To do this, we feel that Genetic Engineering of a benign bacterium to cause it to contain, produce and release siRNA into the sheep's body, would be an effective way of preventing or limiting the amount of PrP-C present, therefore providing an immunity to PrP-Sc or reducing the effects of the disease to the point that it breaks down in the body. However, there are complications with this method at present, specifically the fact that 'Genetic Engineering' is quite inefficient, at the moment, and to able to give sheep the required Bacteria would require a huge production line. With only about 1-2% of Bacteria taking to Genetic modification, not all of the sheep population would have the immunity. On the other hand, as the bacteria would flourish in the body, a sufficient quantity would be maintained in the body, therefore only one implementation of the bacterium would be necessary for a lifelong immunity (in theory). Once Genetic Engineering becomes more efficient, this problem would be resolved.

Genetic Engineering plays a fundamental part in the practicality of this method, and so this idea can only develop with its advances, therefore unless there are major breakthroughs to improve its efficiency, making this method widely available, it would require a lot of research however, we feel that if this method, despite its present inefficiency, proves to be effective in preventing Scrapie, then this method would be a fundamental success, as this could potentially result in a cure/preventative for Mad Cow Disease or Creutzfeldt-Jakob Disease, because they are similar Transmissible Spongiform Encephalopathy (TSE) diseases. We feel confident with the theory of our method, however for it to be safe and to implemented, obviously, trials and experiments would need to take place.



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