

### Exon skipping will change the fast Duchenne into the much slower Becker dystrophy

#### An interview with Professor Stephen D. Wilton

Professor Wilton is Head of the Experimental Molecular Medicine Group at the Centre for Neuromuscular and Neurological Disorders of the University of Western Australia in Nedlands near Perth. On 16 July 2006, after the Annual Conference of the American Parent Project Muscular Dystrophy in Cincinnati/Ohio, 13 - 16. July 2006, Professor Wilton answered questions of *Guenter Scheuerbrandt*, PhD (printed in italics) about exon skipping, the most advanced research approach towards a therapy of Duchenne dystrophy.

#### Exon skipping, clinical trials are beginning.

*Exon skipping is a technique that induces the protein synthesis in the muscles to ignore parts of the genetic message of the dystrophin gene so that a shorter than normal dystrophin is produced thus slowing down the fast progression of the symptoms of Duchenne dystrophy into those of the much slower Becker dystrophy. The potential drugs are antisense oligonucleotides, abbreviated AONs or oligos. During the important meeting here in Cincinnati, the details of this technique were discussed in great detail, we do not have to repeat them in this interview.*

*To begin, would you please explain to the families with Duchenne boys, what the very promising results of exon skipping research will mean to them who are waiting desperately for a cure of this terrible disease.*

I would not use the word cure, exon skipping would never cure Duchenne. At best we could reduce the severity, and in some cases, we may be able to reduce it a lot.

I would rather be cautiously optimistic and say that if we are going to make a difference with exon skipping, it would be a modest difference. And if it works better than we think, that will be great. It is very important, to keep the expectations very tempered.

*I am discussing exon skipping quite often with parents. I explain it to them as well as I can, and always add that it has only been tested in animals, and that nobody can say at the moment whether it will work in boys as well. Then I explain that clinical tests with Duchenne boys are being done now which will show whether exon skipping will work in children or not.*

This is why at this particular meeting I was saying that our lab was able to induce skipping of every exon in the dystrophin chain except exon 1 and exon 79. All the exons from 2 to 78, we can skip. We have a manuscript in preparation and it is taking longer than expected to tie so many ends together. Many exons are easily removed, and a few are more stubborn but we are developing ways to skip the difficult exons.

Our clinical trials have to be done slowly, steadily, and step by step, unfortunately for parents who want a treatment now. But what is important is that, as soon as one step is taken, we are ready to take the next one. Everyone is always very optimistic when a trial is started, hoping that the results will be positive. There are no great safety problems expected as just a single muscle is being treated and then later removed for analysis. There will be a greater risk when the trials progress to a whole-body treatment as much larger amounts of AONs will be administered and

there is a chance of some unanticipated side effect.

But one does not know how long the dystrophin lasts after two or four weeks of the initial treatment. In the proposed British trial, one to four weeks after the treatment, the entire muscle will be removed and assessed first by molecular testing to see if dystrophin is present. This will be a proof of principle to show that exon skipping is going to work in humans.

At this time, I think, one should not experiment with different doses or times, because the real proof will be a whole-body treatment, a systemic treatment to demonstrate that exon skipping really works in human muscles. And that is going to be very, very difficult, because we do not know how much AONs we will need, and how often they will have to be administered.

*Francesco Muntoni*, who will lead the British trial, once said "I have a nightmare: How do we work out the dose? In ten years time, we will still be discussing the same issues that concern the treatment with steroids now, one day on, one day off, 10 days on, 10 days off, or when the wind blows from the east!" And we have only two related steroids, and still don't know how they work.

Now for exon skipping, we will have to look at different compounds for different patients addressing different mutations. Each patient will respond differently. It is going to be very, very difficult research. But I may be overly pessimistic here.

*The Dutch researchers in Leiden are now trying to skip exons 51 and 46. And they will expand this to other exons. Will they have to go each time through the whole lengthy approval procedures?*

There will have to be approvals, because technically each new oligo is a new drug. But what we are hoping is that the oligos for the first trials are select examples, and that for the subsequent oligos in the pipeline the approval procedures could be shortened, if all oligos behave similarly and do not induce any side effects.

We are working with modified oligos of a different chemistry called *morpholinos*. This type of compound has already been systemically administered to humans as potential antibiotics. They have been shown to be safe, because they are not broken down in the body, they seem to be perfectly stable. Already extensive trials have been done with them, and thus may not need such extensive clinical tests as, for instance, the other modified AONs, the Dutch researchers are using, the 2O-methyl-thioate-protected ones, which have not been administered to humans.

The morpholinos, on the other hand, have a completely different backbone. They are uncharged and because they

are so weird, there is no way they can be integrated into the genome. So, what we are doing is really not a gene therapy, but rather a modification of gene expression.

If the first few morpholinos are shown to be safe as muscular dystrophy drugs, I would hope that the people in charge of drug regulation would take previous experiences on the morpholino safety profiles into consideration, and it may be possible to slightly relax the guidelines on what has to be done for new oligos. If we had to do extensive testing for every oligo, we might as well stop now, it would just be completely unsustainable and we would not be able to treat many of the different mutations causing Duchenne muscular dystrophy.

One of the most important aspects to consider is, that the oligos are not simple conventional drugs, but that we would be offering a personalized treatment, a customized molecular therapy for a boy with Duchenne.

Now, the company AVI Biopharma in Portland, Oregon is developing morpholino oligos as antiviral agents, which could potentially go to hundred of thousands of people. And therefore, with something going to the general population, you must have extensive safety testing to make sure that 0.1% of them do not have any unexpected or unforeseen adverse reactions. In contrast, our Duchenne oligos would not be administered to thousands of people but rather to very specific cases of Duchenne dystrophy. So if there is an adverse event, and we hope that won't happen, it would concern only one or two boys, and since these patients will be very closely monitored during the treatment, any adverse effects should be seen quickly and appropriate steps taken.

There could still be effects dependent on the different nucleotide sequences of the oligos. We have to be aware of that risk. But this would be a case of weighing the risks against the benefits. Even if there were some adverse side effects with these morpholinos in addressing Duchenne mutations, the benefits of restoring some dystrophin expression could outweigh the risks. Steroids have numerous side effects but these are currently accepted as the best treatment option.

At this meeting, *Dominic Wells* showed that when he was just injecting AONs directly into the muscles of mice, he was getting very good exon skipping at the RNA level after 14 weeks. So these morpholinos are inducing exon skipping for 14 weeks. And the protein is going to be more stable than the RNA. So after 14 weeks, you should get a lot of dystrophin, and that may possibly last for up to 26 weeks, that is 6 months! I never expected that this would last as long. It is working better than anticipated. And if there were an adverse reaction after a boy is given a morpholino, perhaps there are ways of controlling that. And then, the boy would perhaps have an uncomfortable week, and then it is going to be 6 months before the next treatment. Again, we have to work out whether a treatment twice a year would be adequate. We could compare this with chemotherapy and radiotherapy in cancer treatment. There are terrible side effects. And they are accepted there because there is nothing else.

## Two types of antisense-oligonucleotides.

*The Dutch researchers are trying their oligos, the 2O-methyl-phosphothioates, to skip first exon 51, and you and the*

*British will test morpholinos. But you will work together, will you? And what is the reason why the Dutch work with this other type of oligos?*

The Dutch are looking at a variety of different exons, and the English have done a comparison and found that compounds which we developed, in particular the morpholinos, were working very well, and decided to use them for their clinical trial which is still in the planning stage. They will try to skip exon 51, too, to have essentially a parallel study to the Dutch trial. So, they will be able to compare different chemistries, different sequences, and compare the efficiency of these different treatments. That really could not be done if they were trying to skip another exon. So, if both types of AONs are being shown to be safe, work on both of them is going to be pursued. Hopefully, both will give similar results.

No one really knows what is going to happen when there is a long term exposure to any of these different oligos. It is even possible that one treatment will need a combination of oligos. The idea is, that competition is healthy, and that we don't keep all the eggs in one basket. If both systems are promising, both have to be pursued.

And again, if something happens after three years of morpholino treatment, then we will fall back onto the 2O-methyls. But if we have just nothing than the morpholinos at that moment, we would be in trouble. But in addition to the morpholinos and the 2O-methyls, there are other types of AONs available with other chemistries we are working with.

## The first exon skipping experiments.

*Who actually had the first idea about exon skipping?*

I think it was developed simultaneously at several different places. I had been doing work on revertant fibers with dystrophin that appear spontaneously in Duchenne muscles, and was trying to work what the mechanism was. And to me it was logical that it was some exon skipping mechanism. That was about in 1994, and we found some gene transcripts, some functioning mRNA for shortened dystrophin. And then at the end of 1996, at a meeting in Lake Tahoe, I met *Ryszard Kole* from the University of North Carolina. He was talking about suppressing the abnormal splicing in the  $\beta$ -globin gene as a therapy for thalassemia. As far as I know, he was the first person to modify the expression of a gene by modified splicing. Afterwards, Ryszard and I were talking quite some time, and then one of those moments happened where something hits you almost literally like a brick! It was just so obvious, that this was the way of going. If you could use AONs to suppress abnormal splicing, why not apply the same approach for normal splicing, too? Within a few weeks of getting back to Perth, I received some oligos from Ryszard. We had some cultures with muscle fibers going that were not very good, but we did some experiments anyway right away, and we got exon skipping in our very first experiment with one of Ryszard's oligos.

*Then you were the one who applied for the first time this technique on muscular dystrophy?*

In a way, yes, at least in an attempt to remove a disease causing mutation from the dystrophin mRNA. But I think, *Masafumi Matsuo* in Kobe in Japan also applied AONs to the dystrophin gene transcript. He did it earlier, but I was

not aware of this at the time. But he was actually inducing a mutation, the "Kobe" deletion of exon 19 in a normal dystrophin gene transcript. He was probably the first person who was doing exon skipping in the dystrophin gene.

He has now treated a Duchenne boy with a deletion of exon 20 by injecting into his blood stream a special DNA oligo against exon 19. According to a recent publication, 6% of the normal amount of dystrophin mRNA was found and some dystrophin protein, too. But from the data I saw, it was not convincing. However, he could show that systemic exon skipping in Duchenne boys is possible without dangerous side effects.

I think the main problem there was the choice of this DNA oligo, that, as I understand it, reacts with the RNA in such a way that the mRNA is destroyed by an enzyme called RNaseH. It would have been a race between the exon skipping mechanism and the mRNA degradation by RNaseH. We repeated Matsuo's work in mice and found that this type of DNA oligo works on some targets but not others. Furthermore, when we used an ordinary 2O-methyl oligo of the same sequence to exon 19, this compound was 30 to 40 times more efficient. The reason was that it is a stable oligo, which gets into the cells to induce exon skipping and does not induce the RNaseH mechanism.

### When will there be an exon skipping therapy?

*The gene was found 20 years ago. Everybody was very excited then that next year, we would have a cure. What can one actually say to the parents who feel that the time for their boys is running out? Two years ago, Gertjan van Ommen of Leiden University told me in an interview, that it will take about 10 years until exon skipping will work for the boys. Two years are gone, so eight years are left. I have asked our families whether they really wish to know that, and their answer was: "Yes, we wish to know it, and we understand that estimates like this cannot be precise, and that this estimate does not mean that in January 2014 exactly there will be something for our son." What is the situation now? How long do you think it would take?*

Yes, I know these are terrible things. This is the sixth year that I am coming to the Parent Project meetings. Unfortunately, I meet new people each year, and each year, some people are not here any more. But we have to proceed slowly and carefully and one step after the other to avoid mistakes that would make the time to wait even longer.

However, I am optimistic that, if the first clinical trials are done carefully and safely, then very quickly we plan to follow up with new trials. And the new trials would include new targets, that would skip other exons to address different mutations. So there will be no waiting for years to see what happens and analyzing the results. If we get a positive result in one trial, we will always start the next stage as soon as possible.

And one way how we might accelerate the work is, that instead of trying to skip only one exon at a time, we might target two exons or even more simultaneously, thus make multi-exon skipplings. Why not using a cocktail of oligos to skip several exons at once? We have actually done this in cell culture with a mixture of oligos to remove exons 50, 51, 52 and 53 simultaneously, and it works reasonably well. And another mix we have worked with, addressed

exons 6, 7, and 8, which are the exons the dystrophic dog needs to have skipped. So we could try it on the dog before working with humans. Many different mutations could be addressed by one of these cocktails. And one advantage of using a cocktail is that you could do safety testing for three or more oligos at one time. And we could even combine different oligos. That would accelerate research considerably.

So, as it looks now, Gertjan's estimate is quite realistic, maybe the time will be shorter, 5 to 6 years from now on until we can treat the first boys with good results.

*When talking to families, I often hear them saying: "Why did Duchenne hit us? What is the reason?" "The reason are the mutations, and they occur at random, they cannot be predicted", I answer. But mutations are just tools of evolution. If there were no mutations, we would not be here, there would not be real life on earth, perhaps just slime. But evolution also made scientists - like you - who are trying to right this, to repair the gene, to solve that problem by finding a therapy.*

It is an interesting way of looking at it. It is true, without evolution, we wouldn't have even evolved. To look at it, everyone is different, everyone has probably slightly different dystrophin genes. In some cases there are single base changes in the protein coding exons, but these change one amino acid and are generally not significant, unless that was one very important amino acid. Every dystrophin gene is subtly different, and the genetic variation extends to other genes and the genes that control gene expression. We are a very complicated genetic package and that is likely to be the reason for the different clinical symptoms we find in different Duchenne patients.

### Manufacturing the oligos.

*Who actually makes the oligos? They are probably made automatically.*

I personally made the 2O-methyl oligos we used with a machine we have in the lab. I pressed buttons to key in the AON sequences, I filled the reagents and kept them topped up, and I lost lots of sleep, when I had to watch the synthesis. The chemicals are very expensive and I hate wastage so the synthesiser is kept running around the clock when loaded with the chemicals. Now, there are many companies who make oligos but I prefer some control over the process. Also I get to learn more about the chemistries. Once we optimized our 2O-methyls in cultured cells, we got in contact with AVI Biopharma in Oregon, the company that now makes our morpholinos. They do this in a collaborate arrangement and have been very supportive and good to work with. More importantly, they can make morpholinos to clinical grade for the trials.

*Will these oligos be expensive when everything is ready for the boys?*

They will be expensive, but not as expensive as the production of viruses for gene replacement. Exon skipping will be many times cheaper. The cost of making these oligos is substantial, and we still need lots of morpholinos with different sequences. But if we can design oligos that work very efficiently in small quantities, which you can administer at a low dose and still are therapeutic, then these drugs might not be so expensive.

## Early preclinical diagnosis will be important.

*If exon skipping or another technique works, should it not be applied early before the muscles disappear? I still have my screening laboratory working, but it is dying. I wish I could find some investor who could wait five years until he would get his money back and more of it by promoting and doing newborn screening for muscular dystrophy. In the United States, there are now pilot programs in Atlanta and Columbus/Ohio.*

Early detection will be important. And possibly, if exon skipping was to work and shown to be safe, then, after an early diagnosis, you might start treating before any symptoms at all. And that could make a big difference. So I think newborn screening for muscular dystrophy is a good idea and should be made available everywhere.

## Then there is a message of hope?

*At the end of this interview, would you please say something to the parents to keep hope after an important meeting like this one in Cincinnati?*

First let me give you a surprise: The progress with the morpholinos, that has been made in the last year, was astounding, we were getting results beyond our expectations. We are optimistic that they will work far, far better than we anticipated. At the beginning of our work, we found that the morpholinos work very poorly in cell culture. And when we started the work in mice with in-vivo injections, we were doing all these fancy tricks to get the morpholinos into the muscles. It was working in principle but then we just tried a type of negative control, the morpholinos in saline, just in a 0.9 % common salt solution, and that worked beautifully, without any carrier, without any lipofectin or any other agent to enhance delivery. The pure morpholinos in saline, the simplest possible way of

administering it, worked very, very efficiently! And then the people at AVI Biopharma attached some little peptide tags on them to enhance delivery even more, and these things work very, very nicely in the mouse. Now, we have to try this in humans. The people of AVI, who make the morpholinos, are very reactive and innovative. They are developing new chemistries, making new modifications, and it is a wonderful collaboration with them. So, morpholinos are the best for the moment. I am hoping, these guys will come up with something even better in the future.

But to finish, I want to say that this Parent Project meeting has been very positive. There are so many different approaches of research for muscular dystrophy at the moment. There are pretty good trials on gene replacement, on read-through, there are two trials on exon skipping, there is the myostatin work, there are so many different things that are going on, and the steroids are being studied in great detail. There is reason for a lot of hope.

But it is never going fast enough. I wish we had a cure yesterday. Someone said: to me: "Cure muscular dystrophy and then retire". I am 50 years old now, and I think I will continue working in this field for a long time. But if we can make a difference soon, it will be by exon skipping. That will buy some time until something better or more permanent comes along. It is not a perfect treatment. But it is the best we can do at the moment with lots of oligos and with no new quantum leaps in technology.

*Thank you very much, certainly also on behalf of many of the people all over the world who will read this interview: the boys, their parents and their relatives, their doctors and the others who care for them, the Duchenne researchers and perhaps even influential people who could change things so that you and your colleagues get the funds and the opportunity to reach our goal soon, a therapy for Duchenne muscular dystrophy.*

## Some scientific facts explained.

**Genes** are functional units of the genetic material **desoxy-ribonucleic acid, DNA**. Its structure looks like an intertwined ladder, the *double helix*. The rungs of this ladder consist of four different small molecules, the **bases**: *adenine, guanine, thymine, and cytosine* (abbreviated A, G, T, C). For spatial reasons, the rungs can only contain two types of base combinations, the **base pairs** A-T and G-C. Therefore, the **sequence** of the **bases** on one strand is *complementary* to the sequence on the other.

Most of the genes carry the instructions for the construction of **proteins**. In the cell nucleus, the genetic instruction of active genes is **expressed**, it is copied, **transcribed**, to another genetic substance, the **pre-messenger ribonucleic acid** or **pre-mRNA**, also called the **transcript**. Most genes consist of active regions, the **exons**, which contain the information for the proteins, and of "inactive" ones, the often much longer **introns**, which, however, may be important for the control of gene expression. After transcription, the introns are removed from the pre-messenger RNA, and the transcribed exons **spliced** together to the **messenger RNA, mRNA**, which then

moves to the **ribosomes**, the protein synthesizing structures outside the nucleus. The ribonucleic acids, **RNAs**, use the base U, *uracil*, instead of the similar base T of the DNA.

In the messenger RNA, three consecutive bases, a **codon** or *triplet*, specifies one amino acid according to the **genetic code**. Three of the 64 different codons, UAA, UAG, and UGA, are **stop codons**, where protein synthesis is terminated. There are no spaces between the codons. In the ribosomes, the genetic instruction of the messenger RNA is used for the construction of proteins from 20 different kinds of their building blocks, the **amino acids**.

Duchenne and Becker muscular dystrophies are caused by a **mutation** or damage of the **dystrophin gene** which carries the information for the protein **dystrophin**. The gene is located on the X chromosome. With a sequence of 2,220,223 **nucleotides**, "genetic letters", it is by far the longest human gene. Only 11,058 nucleotides, 0.5%, in the 79 exons of the dystrophin specify the sequence of its 3,685 amino acids.

Dystrophin is needed for the mechanical stability of the

muscle cells. It is located on the inside of the muscle cell membranes to which it is anchored by many other proteins, the **dystrophin-glycoprotein complex**.

There are three types of mutations of the dystrophin gene: **deletions**, if one or more entire exons of the gene are missing, **duplications**, if parts of the gene are repeated, and **point mutations**, if single base pairs are exchanged, eliminated or added. As the three-letter codons of the messenger RNA is read in the ribosomes one after the other without interruption, the **reading frame** is not disturbed, if the mutation deletes or adds entire codons of three base pairs each. In this case, the reading frame remains **in-frame** and the dystrophin made is longer or shorter than normal. If this change affects non-essential structures of the dystrophin, it can still be partly functional. Then the benign form of dystrophy, **Becker muscular dystrophy**, develops.

If, however, the mutation shifts the reading frame by one or two base pairs, the reading frame becomes **out-of-frame**. Then, a number of incorrect amino acids is incorporated into the protein starting at the mutation site until finally a new and **premature stop codon** is reached. The incomplete dystrophin cannot fulfill its normal function, it disappears and **Duchenne muscular dystrophy** develops.

**Splice sites** are specific sequences inside the exons and at the borders of exons and introns which are essential for the correct removal of the non-coding intron sequences from the pre-mRNA. The splicing itself is accomplished by **spliceosomes**, a complex of many small RNAs and proteins.

The **exon skipping** technique tries to change a Duchenne mutation into a Becker mutation. If a mutation disturbs the reading frame and thus causes Duchenne dystrophy, the reading frame can be **restored** by artificially removing from the messenger RNA one or more exons directly before or after the deletion, duplication or the exon containing a point mutation.

Exons can be eliminated from the mRNA with **antisense oligoribonucleotides**, AONs. They are short RNA structures consisting of about 20 nucleotides whose sequences are constructed in such a way that they attach themselves only at the complementary sequence inside the exon to be removed or at its borders *and nowhere else*. These AONs thus interfere with the splicing machinery so that the **targeted exons** are no longer included in the mRNA, they are *skipped*.

*The gene itself with its mutation is not altered by exon skipping*, but its mRNA no longer contains the information of the skipped exon or exons. As this mRNA is shorter than normal, the dystrophin protein is also shorter, it contains fewer amino acids. If the missing amino acids are part of non-essential regions of dystrophin, like the central rod domains, the resulting shorter protein can often still perform its stabilizing role of the muscle cell membrane. The result would be the change of the severe Duchenne symptoms into the much milder symptoms of Becker muscular dystrophy.

A note for those who learnt some chemistry in school: **Oligonucleotides** are short pieces of the two kinds of nucleic acids, DNA and RNA, (oligo means few). The two strands of **DNA**, desoxyribonucleic acid, consist each of a chain of alternating phosphate and desoxyribose units, their backbone. Desoxyribose is a sugar molecule with five carbon atoms, and the second carbon atom has its usual oxygen atom missing. Each sugar unit carries one of the four "genetic" bases on its first carbon atom. **RNA**, ribonucleic acid, has normal ribose units in its backbone with an oxygen on its second carbon atom. **Nucleotides** are the building blocks of both kinds of nucleic acids. Each nucleotide consists of one ribose, one base and one phosphate. So there are four different **ribonucleotides** and four different **desoxyribonucleotides**. The oligos discussed in this interview are **antisense oligoribonucleotides**, AONs. *Antisense* means that their base sequence is in reverse order to a specific target sequence of a splice site in the pre-mRNA.

The two kinds of oligos or AONs used for exon skipping are protected oligoribonucleotides so that they are not destroyed in the muscle cells by enzymes. The Dutch are using the **2O-methyl-phosphothioates**, also called *methyl thioates* or *2O-methyls*. They have a methyl group, a carbon with three hydrogen atoms, on the oxygen of the second carbon of the ribose units and a sulfur atom instead of one of the oxygen atoms of the phosphate groups. The **morpholinos** the British-Australian researchers are using, have one of the phosphate oxygens replaced by a dimethyl amide group, a nitrogen carrying two methyl groups, and the entire ribose units replaced by morpholino rings, six-membered rings, each consisting of four carbon atoms, one oxygen and one nitrogen atom with hydrogen atoms attached to the carbons.

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