Duchenne Muscular Dystrophy: Exon Skipping with U7 gene transfer.

On 3 December 2004, the journal "Science" published an article with the title "Rescue of Dystrophic Muscle Through U7 snRNA-Mediated Exon Skipping" by the authors *Aurélie Goyenvalle, Adeline Vulin, Françoise Fougerousse, France Leturcq, Jean-Claude Kaplan, Luis Garcia*, and *Olivier Danos* of the Généthon Institute in Evry near Paris (Science 306, 1796-1799, 2004).

Dr. Luis García presented the results of this research approach to effectively treat Duchenne muscular dystrophy at the meeting of the Spanish muscular dystrophy association ASEM in Barcelona on 11 June 2005 and at the Parent Project conference in Cincinnati on 8 July 2005. The following report is an attempt to explain the scientific facts to the families with Duchenne boys in a way they might understand.

First, a few words concerning the abbreviation "**RNA**", which means "ribonucleic acid": Its molecular structure is a chain with alternating sugar and phosphoric acid units as in the strands of the genetic material, the double helix DNA. The sugar units in RNA are ribose, in DNA, they are desoxyribose. Each ribose of RNA carries one of the four bases adenine, guanine, cytosine, and uracil, the "genetic letters", abbreviated A, G, C, and U. (In DNA, the base thymine, T, is used instead of U). RNA is much more labile than DNA. DNA may last for thousands of years, RNA, however, does not last long, nature, therefore, uses it for the rapid transport of genetic information within the cells.

Next, the way **dystrophin** is made should be explained. Dystrophin is the protein that is needed for the stability of the muscle cell membranes. The instructions for the biosynthesis of dystrophin are written in its gene with 2,220,223 genetic letters in a linear sequence. (The genetic letters are scientifically called *nucleotides* or *base pairs.* The dystrophin gene, which is located on the X-chromosome, is by far the largest human gene. It consists of 79 active regions, the *exons*, and of the much longer *introns* between them. Only the exons contain the information for the construction of the protein.

In the nuclei of the muscle cells, the entire instructions of the exon and intron regions of the dystrophin gene are copied into the pre-messenger RNA, pre-mRNA. The introns of this copy or transcript are then eliminated and the exons joined - spliced together - one after the other, to the messenger RNA. This mRNA travels to the ribosomes, the protein "factories", in the cytoplasm of the cells. In the ribosomes, the genetic message stored in the 79 exons of the mRNA is used for the construction of the protein dystrophin which consists of 3,685 amino acids, the building blocks of proteins. The completed dystrophin then moves to the inside of the muscle cell membrane where it is part of a complex with many other proteins that is necessary for the transmission of the muscle force and the stability of the cell membrane under mechanical stress.

(A note: Scientists like to talk about the things they are working with as if they have only one of each. They say: *one* protein, *the* dystrophin, *the* gene, *the* muscle fiber, etc. In reality, they are producing and are working with millions or billions of them. For instance, there are 114 billion dystrophin molecules in one gram of muscle tissue.)

Now, the cause of **Duchenne muscular dystrophy** is explained: The instructions for dystrophin on its gene begins with the genetic word for the amino acid methionine, that is, with the three letters, the triplet or codon ATG. Then, 3,684 further three-letter words follow without any spaces between them. Each of these codons means one of the 20 different amino acids which nature uses to construct proteins. Sometimes, in the muscles of a boy, the genetic instructions for making dystrophin contain a mistake, a *mutation*. If that has happened, the borders of the three-letter words behind the mistake may have moved by one or two letters, so that the reading frame is shifted *out-of-frame.* The words may now mean different amino acids or even a premature stop sign like the codon TGA. Then, the dystrophin protein cannot be completed. The protective structures on the membranes of the muscle cells desintegrate, the muscles deteriorate and the result is Duchenne muscular dystrophy with all its devastating consequences.

To understand what the researchers at Généthon have done, the technique of **exon skipping** is now explained: The aim of exon skipping, or "jumping over exons", is to normalize the disturbed reading frame, to make the out-of-frame situation *in-frame* again. This can be done by preventing the use of one or several selected exons directly before or after the site of the mutation, so that they are no longer included in the mRNA. The exon or the exons to be skipped have to have the correct structure of their borders which would normalize the shifted reading frame. The genetic information of these left-out exons is then not used for the protein synthesis. When the correct reading frame is restored after the skipping, dystrophin can be made again, but it is shorter than

normal. In many cases, this somewhat shorter dystrophin can still protect the muscle cells to a certain extent. The result then is the change of the fast Duchenne dystrophy into the much slower progressing Becker dystrophy.

Splicing of exons is a complicated procedure which needs at least five different splicing factors and two secondary factors, all of them small nuclear RNAs, snRNAs, at least nine different SR proteins and, in addition, about 250 other proteins. Special genetic sequences within an exon and on its borders are necessary for the splicing reaction to proceed correctly. If some of these sequences are blocked, such a targeted exon is not spliced to its neighbors any more, it is *skipped*. The blocking can be caused by artificially made short RNAs consisting of about 20 genetic letters or nucleotides. Their sequence must be constructed in such a way that they can attach themselves specifically to the splice-promoting sequences and desactivate them so that the particular exon, targeted in this way, is not spliced any more.

This blockade works because the artificial RNA has a sequence which is the reverse of the splice-promoting sequences, it is an **antisense oligoribonucleotide**, abbreviated AON. The prefix "oligo" means that it contains only a few genetic letters. Such an AON attaches itself exclusively to the exon sequences to be blocked because its antisense sequence has been made complementary to the these sequences so that a completely paired Watson-Crick structure is possible, that is, that a U is always opposite of an A and a G opposite of a C and vice versa.

The skipping of one or more exons happens in the cell nucleus on the level of RNA which has copied the genetic instructions of the gene. That means that the AONs do not change the mutated gene itself. So it could, because of its damaged information, continue to create further harm unless the AONs are present. However, the AONs are still not sufficiemntly stable, they disappear with time, therefore, they probably must be introduced into the muscles repeatedly.

The exon-skipping technique described so far is being further developed by the research teams of *Judith van Deutekom* in Leiden, *Terry Partridge* in London, and *Steve Wilton* in Perth, above all with the aim to make the AONs stable for a sufficiently long time after their injection into the blood circulation. This is being done by adding to them small protective molecular structures. Exon skipping with artificially made AONs is already being clinically tested on Duchenne boys in the Netherlands.

The researchers at the Généthon institute have now extended this technique by attempting to instruct the muscle cells to make continuously the AONs themselves so that they do not have to be applied repeatedly. This can be achieved by transporting into the muscles the genetic information for the construction of the AONs. Preliminary research towards this approach was performed by the teams of *Irene Bozzoni* in Rome and *David Schümperli* in Berne. Their original idea was to use **U7-snRNAs**, small RNAs which are necessary for the maintenance of chromosomes, but which have a structure similar to the splicing factors. These U7-snRNAs can be modified so that they are able to cause exon skipping. The researchers in France have developed this technique further and applied it to repair the point mutation in exon 23 of dystrophin of the dystrophic mdx-mouse. By skipping this exon, the premature stop sign caused by this mutation, can be avoided.

Olivier Danos, *Luis García* and their colleagues at the Généthon institute have added to the rather short DNA sequence of the **U7-snRNA gene** the information for two antisense sequences. (It is important to know at this point, that the snRNAs, like all other RNAs too, are also "made" by genes.) These additional DNA-sequences in the U7-snRNA gene are 24 and 20 letters long and are designed in such a way that, after they are copied into RNA, can attach themselves specifically to two sequences of the mouse dystrophin pre-mRNA. One is located at the end of intron 22 and the other at the border of exon 23 with the following intron 23. And if these two sequences are blocked, exon 23 with its premature stop sign is being skipped.

In order to transfer this modified U7-gene - U7 SD23/BP22 - into the muscles, it was, together with additional control sequences, inserted into the genetic material of **adeno-associated viruses** (AAV) of a type-2 genetic structure with a type-1 protein shell. Up to 20 trillion of these modified and harmless viruses were injected in one portion directly into two different muscles of 37 mdx-mice. After six weeks, up to 80% of the cells of the treated muscles had new shortened dystrophin which did not contain any more the 72 amino acids determined by the normal sequence of exon 23. And this new dystrophin was still present one year after this single injection of the vectors, as the modified viruses are called. The new dystrophin had also migrated to its normal position underneath the muscle cell membranes, and the "rescued" muscle cells looked quite normal under the microscope. The dystrophic processes in the mdx muscles, that is, their accelerated degeneration and regeneration, were completely halted. And there was also no immune reaction against the new dystrophin.

Then, five other mdx-mice were treated similarily, but the virus vectors were not injected into their muscles directly but into the **blood circulation** of their legs. After one month, more than 80% of the fibers of

all checked leg muscles had new dystrophin, and also other proteins of the dystrophin complex, which were analyzed, had reappeared. This means, that the binding sites of the new dystrophin to these proteins had the normal structure.

And finally, the **muscular function** was studied by measuring the spontaneous contraction of the treated muscles after they were forcefully lengthened. This normally very reduced function of the mdx-mice had returned to normal, if the muscles contained more than 70% of new dystrophin. And treated mdx-mice, which were physically stressed by downhill running on a treadmill, did not develop the usual muscle damage found in non-treated mdx-mice.

The results of earlier experiments with the transfer of other genes into the muscles of mice, rats, dogs and monkeys suggest that the U7 transfer with AAV viral vectors could lead to a **permanent rescue** of the dystrophic muscle fibers. In monkeys, genes transferred in this way were found to be active up to six years. The adenoviruses do not insert the gene sequences they carry into the chromosomes of the muscle cells, therefore, the risk that other genes could be disturbed by the insertion of the new U7 gene seems to be low.

In preliminary experiments performed after the work published in the Science article, the U7-gene transfer technique was applied to treat the **dystrophic GRMD-dog**. In contrast to the mdx-mouse, which is not significantly handicapped by the absence of its dystrophin, the dystrophic dog has severe clinical symptoms similar to boys with Duchenne muscular dystrophy. Its dystrophin gene has a point mutation in the splice receptor region of exon 7 so that this exon is deleted and the reading frame after the deletion is shifted leading to a premature stop sign with the result that the dog has no dystrophin in its muscles. By skipping the three exons 6, 7, and 8 simultaneously, the reading frame can be repaired. This multi-exon-skipping was possible by the transfer of two different modified U7 genes - U7 C6ESE2 and U7 C8ESE1 - by the AAV vector at the same time. Two months after the injection of the two vector preparations directly into the muscles of one leg of the dog, almost normal amounts of dystrophin could be found around the injection site. It has to be shown now whether the new dystrophin, that is shorter than the normal one, does indeed lead to milder symptoms in the dog that are comparable to those of human Becker patients.

In a first human **clinical study**, this very promising technique will soon be tested on adult female Duchenne carriers to see whether exon 51 can be skipped in this way. Like all new therapeutic procedures, this technique will have to go through the usual phases of clinical studies. As this combination of exon skipping and gene transfer - two completely new approaches -, interferes with the information of a human gene, special care must be taken to reduce all possible risks. Even if all necessary future research steps proceed without any difficulties, it will take still several years until this technique will be available as a safe and effective therapy for boys with Duchenne muscular dystrophy.

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