5th International Round Table in Monaco, 14 January 2006

Translation of exon skipping to the clinic: how to make it a cure for Duchenne Muscular Dystrophy?

Several pre-clinical research projects using exon skipping are emerging, with the objective of treating Duchenne Muscular Dystrophy.

Two years after the Round Table of January 2004 which concluded with the possibility of utilizing exon skipping as a therapeutic strategy, the clinical potential of this strategy has been confirmed and has been translated into different research approaches (molecular, pharmaco-chemical, gene therapy, autologous cell therapy) which have all attained pre-clinical status today. Several clinical trials are already in preparation, but in parallel, basic research remains to be done in order to answer some unresolved questions (such as the immune response, systemic injection, production of batches of drugs). The international community which is concerned by Duchenne Muscular Dystrophy is mobilizing to cross a crucial frontier: at the initiative of the Parent Project USA an international consortium has been created and was presented for the first time in Monaco on January 15, 2006, with the goal of raising funds to finance clinical trials in Duchenne Muscular Dystrophy utilizing the promising technique of exon skipping.

A chain of events

Monaco, 17 January 2004, the second Round Table raised the question about the possibility of restoring the reading frame of dystrophin in patients with Duchenne muscular dystrophy (DMD) by utilizing molecules which interfere with the process of maturation of mRNA (the technique known as exon skipping). The positive in vitro and in vivo results presented at this time by specialists (T. Partridge-GB, J. Van Deutekom-Holland, S. Wilton-Australia) have provided a promising roadmap for proceeding to clinical trials. However, the scientists have underlined the transitory nature of the treatment and to circumvent this problem, Swiss (D. Schümperli), Italian (I. Bozzoni) and French (L. Garcia) groups have shown that a combination of this approach with a gene therapy strategy with the aim of producing therapeutic antisense molecules in a stable and continuous manner in dystrophic fibers could be envisaged. This strategy rapidly proved to be positive in 2004 with the publication of the work of L. Garcia’s team in Science, which described the success of exon skipping in the mouse using a small U7 nuclear RNA as a “shuttle” for the antisense sequences, introduced in an AAV viral vector, leading to a massive and stable restoration of dystrophin associated with a significant amelioration of the phenotype in the mdx mouse.

Monaco, 14 January 2006, two years later, the Duchenne Parent Project France and the Association Monégasque contre les Myopathies invited scientists and physicians implicated in the creation of projects with the goal of restoring the reading frame in Duchenne Muscular Dystrophy in order to review the progress achieved in using either synthetic oligos, gene therapy, gene therapy combined with cell therapy, or pharmacologic approaches. In two years, the application of exon skipping to Duchenne Muscular Dystrophy has given rise to at least 4 therapeutic approaches for targeting a treatment: synthetic antisense oligonucleotides (AON, morpholinos), gene therapy (AAV-U7), integration of U7 in myogenic stem cells and a pharmacology of splicing. The pertinence of these different approaches and the therapeutic
perspectives that they offer was discussed by the 25 scientists and physicians, international specialists, in the presence of about 10 representatives of Duchenne Muscular Dystrophy associations.

Imminent beginning of the Dutch AON clinical trial, while the British MDEX Consortium is using morpholinos

The Dutch team of J. Van Deutekom has a collection of AONs (using 2’-O-methyl phosphorothioate chemistry for synthesis) today which targets the majority of human dystrophin exons. The validation of these AONs was performed ex vivo on patients’ cells in culture and in vivo using a humanized transgenic mouse with an integral copy of the human dystrophin gene (Hdys). This is the most advanced group clinically, because it is ready to begin a phase I clinical trial targeting exon 51 in the coming weeks in partnership with Prosensa. The DMD patients will receive an intramuscular injection, with no therapeutic benefit expected at this stage.

The results obtained in mice indicate that administration by the systemic route is conceivable. The doses and the frequency of injections remain to be determined. Long-term toxicity studies also need to be undertaken. The British MDEX consortium (F. Muntoni) in collaboration with the laboratories of S. Wilton (A) and Q. Lu (USA) is performing similar studies, but using a different chemistry for the synthesis of oligonucleotides, the morpholinos. These groups are concentrating particularly on intra-venous administration. The preclinical results on the mdx mouse are very promising; numerous muscles are attained after six injections of morpholinos but the heart seems refractive to systemic treatment. Furthermore, long-term accumulation/toxicity studies of this product are lacking. The MDEX consortium envisages the launching of a clinical trial in 2007, similar to that of the Dutch.

The success of exon skipping in the dog with AAV-U7 reinforces the chances of success of a therapeutic trial despite unresolved problems.

The French group of L. Garcia is continuing its work on gene therapy with AAV and the U7 micro-gene coding the antisense sequence which is necessary for exon skipping, using this technology on the GRMD dog model, in which it is necessary to skip not one but three exons to restore a functional dystrophin. The intramuscular injections of AAV-U7 in the GRMD have led to very encouraging results, comparable to those in the mouse, which is very promising for human treatment, all the more so because this large animal permits simulation of future human trials and determination of limits. For the moment the vector is administered by injection under pressure at the level of one limb, because of the lack of a vector which can spontaneously cross the endothelial barrier of the blood capillaries. The major efficacy of the U7 option has thus been confirmed, but the use of the viral vector raises the problem of the immune response against the envelope of the vector after the first injection, which would prevent the re-injection which would be required for sequential treatment with therapeutic doses. Current efforts are now concentrating on the development of a protocol for immunomodulation which would permit re-injection of AAV.

A phase I clinical trial, expected in 2007, is in the process of elaboration at Genethon and the Institut de Myologie. In order to evaluate tolerance and the success of exon skipping without disqualifying DMD patients, intramuscular injections, with no benefit or major risk except that of being vaccinated against the therapeutic vector will be carried out on asymptomatic female heterozygote carriers. Lee Sweeney’s American group is also preparing to launch a clinical trial with the same technology, probably targeting exon 53.

The vector + U7 system is opening new perspectives for cell therapy which should materialize in the form of clinical trials in the medium-term.

The U7 system has been transposed to a retroviral vector (lentivirus) in order to implant the micro-gene in myogenic stem cells with the perspective of autologous cell therapy (using the patient’s own cells). J. Tremblay (Canada) reviewed his experience in heterologous myoblast transfer (the donor is related to the patient) in DMD patients (phase 1 trials). The intramuscular injection procedure is laborious (the injection sites are 1mm apart) but practicable in small muscles. The main limit of this approach is immunologic and linked to graft rejection. The utilization of lenti-U7 vectors, however, opens perspectives for the continuation of this research. In fact, preliminary experiments on myoblasts in culture from patients treated by
lenti-U7 have revealed significant levels of dystrophin.

**Y. Torrente** (Italy) has performed a phase 1 trial of cell therapy by injecting AC133+ autologous stem cells into patients. The advantage of these cells is that they can be extracted from muscle and/or blood and that they are compatible with systemic administration (at least in mice). The research presented demonstrated the feasibility of generating muscle fibers expressing repaired dystrophin from AC133+ cells from Duchenne patients previously infected by a lenti-U7 (exon 51), *in vivo* in an immuno-deficient mdx mouse (SCID).

**J. Morgan** (Great Britain) presented a similar project, but using another variety of stem cells harvested from the synovial space.

**M. Sampaolesi** (Italy) presented his research on the utilization of “mesangioblasts”, a variety of stem cell with high myogenic capacity isolated from muscular blood vessels. The optimism raised by these cells is due to the fact that they seem to have the capacity to re-colonize dystrophic muscle when injected by the intra-arterial route. The results are all the more promising because they seem to be confirmed in the GRMD dog model. In this case also, access to lenti-U7 vectors should make it possible to avoid immunological problems inherent in the use of heterologous cells.

**And why not a pill one day? A step has been taken in the direction of research on drugs acting on splicing.**

**J. Tazi** (France) presented the research of his group which converges towards the elucidation of the splicing mechanism and the identification of new targets among the factors implicated in this mechanism, to correct splicing anomalies in genetic diseases. He has undertaken a search for drugs which could interfere with the molecular partners implicated in this reaction, and Duchenne muscular dystrophy seems to be an ideal candidate for validating the drugs which have been identified. Screening of hundreds of drugs has led to the identification of 2 molecules which are selectively active in skipping exon 23 in the mdx mouse. These promising results have made it possible for this group to continue screening for molecules which could be useful for human exons. This may represent a fundamental turning point in the quest for future drugs to treat Duchenne Muscular Dystrophy.
Therapeutic approaches of exon skipping for DMD: where we are, what remains to be done

Patrick Dreyfus, MD, PhD

What is exon skipping
Exon skipping is a new method, based on natural process, allowing to cut out one or several exons from a nuclear pre-messenger RNA. The altered transcript will be translated by the cellular machinery into a shorten protein. This is obtained by forcing the nuclear pre mRNA processing to skip the region containing the mutation which will be viewed as an intron and cut out. Exon skipping implies to know precisely the mutation and the sequences of the splicing sites near the mutation. Different sites in the pre mRNA and different chemicals can be targeted to achieve the exon skipping. In this Round Table conference, these aspects were discussed including molecular characteristics, delivery, efficacy and side effects.

Why Exon Skipping?
The nuclear transcript of a DNA carrying a mutation, the pre mRNA, carries also the mutation. The pre mRNA is then processed in the nuclei of the cells, the introns are cut out and the exons are spliced to give the mature mRNA ready to be exported in the cytoplasm and translated. This highly complex process should be very accurate to achieve a readable RNA. According to the mutations numerous events are possible; the most severe for the cell and for the organism will be a complete lack of the product of the gene or an altered non functional protein. Sometime the result will be a functional shorten protein. Taking advantage of the knowledge of RNA processing, it is possible to restore a functional protein after having skip the site of a mutation, which is eliminated from the RNA.

Application of exon-skipping to DMD.
In a large majority the mutations carried by DMD patients lead to an out of frame mRNA, which is translated into a truncated protein that cannot support the functions of the genuine dystrophin.
It is possible to skip some exons in order to synthesized an "in frame" RNA. This imply the knowledge of the sequence of the DNA, the site of the mutation carried by the patient and to be able to target the right site. In this condition, the mRNA will be shorter than the canonical mRNA encoding the full length dystrophin. But several lines of evidences demonstrate that a shorten dystrophin can be totally functional. In the muscle of some DMD patients and in the animal models, it is easy to find muscle fibers expressing dystrophin. These fibers, called revertant, are spontaneously able to skip the mutation by an unknown process. Some patients carrying a Becker muscular dystrophy (BMD) often express a shorten dystrophin. Many experiments on animal models of DMD demonstrate that a shorten dystrophin can be located at the right place, interact with the other proteins of the dystrophin complex and restore almost normal contraction properties to the muscular fibers.
It appears from these data that the lack of certain regions of the dystrophin protein do affect its properties.
To get exon skipping it is necessary to target specific sequences of the pre messenger RNA with molecules specially and specifically. Three types of molecules are already known for their pharmacological efficiency: The antisense oligonucleotides (AON). The antisense oligonucleotides (AON) are developed by the group of Judith van Deutekon, in Netherlands, and the group of Steve Wilton in Perth Australia. The chemical nature of the AON are different and imply different behaviour of the active molecule. J van Deutekom utilizes 2'O methyl oligonucleotides that are close to the natural chemical form of RNA. The AONs produced by specialized fine chemical companies at a clinical grade were designed and injected into the muscle or intravenously. Their half-life is seven days, which implies frequent and iterative injections to get a permanent effect. Beside the 2'O methyl oligonucleotides, the morpholinos have a different chemical structure that is very similar to the natural oligonucleotide...
but have a long half-life, likely because they are not recognized by nucleotidases.

U7-AAV for direct delivery, utilizes a different way; the DNA coding for the pharmacological small RNA is vectorized by an adeno-associated virus (AAV). The U7 RNA is a natural small RNA molecule, which is devoted to the splicing of some nuclear proteins. Its sequence has been modified to target the desired sequence in the dystrophin pre messenger RNA. Indeed the natural U7 RNA is still expressed by the cells. The AAV carrying the U7 RNA is either intramuscularly or intravenously injected. AAV, is preferentially internalized by muscular fibers and cardiomyocytes cells which will express the small RNA, called U7. The main advantage of U7-AAV is the very long lasting effect after one injection.

These molecules interact with different sequences of the RNA, but so far, no rule have been found to predict the efficiency of a sequence, it is necessary to test the pharmacological effect on cells in vitro. But it appears that some sequences are efficient in vitro but not in vivo.

U7 integration into myogenic stem cells. To manipulate myogenic stem cells, the U7 DNA is carried by a another virus, the lentivirus, that is capable to transfret the stem cells in vitro. The modified cells are the injected intramuscularly. This method derived from the numerous research on the cell therapy for muscular dystrophies. The main limitation is the very low integration of the grafted cells in the muscles. This is likely a consequence of the competition of the grafted cells with the natural myogenic cells in the muscle and their poor diffusion.

**Attractions of AON**

They work. The results presented demonstrate a good expression of a slightly shorten dystrophin, clearly detected with specific antibodies. The dystrophin is localized at the muscular membrane and associates the other proteins characterizing the dystrophin complex.

A systemic delivery is possible, which is an easy way to deliver the molecule to a large number of muscles.

There is probably no immune problems. The lack of dystrophin in the DMD patients does mean that dystrophin is a non self protein, because generally other dystrophin related protein are still expressed in other organs. Moreover, some revertant fibers exist and are producing a shorten dystrophin.

The AON are easily withdrawn and the target can be modified. This is important to be able to fit the best treatment to a specific patient.

**Problems for AON**

Their effect is temporary. The half life of the AON is 7 days, but is not known for the morpholinos. The problem is indeed the necessity to reinject the AON.

Does not work on heart. For unknown reasons, the heart does not express dystrophin after several intravenous injections.

Toxicity effects at effective doses is unknown. It is likely that AON, with their short temporary effect would be poorly or non toxic. The morpholinos, that are very stable could have a toxic effect due to their accumulation in the nuclei. This will be carefully study.

**Attractions of AAV-U7**

They work. Results on mice and dogs demonstrate the highy efficient effect of U7, more than 95% of the muscular fibers are positive for dystrophin and the dystrophin complex is present. The muscular force is recovered.

Localized systemic delivery is possible, in dystrophic dog injected into a forelimb vein, a large number of muscles express a rescued dystrophin.

No immune problems with transgene or dystrophin. The transgène is a small RNA which does not elicit any antibody reaction. For the dystrophin the problem is discussed above.

Long-term effects of single treatment is due to permanent expression of U7. Further investigation are needed to be determined.

Could target the heart; The AAV can be internalized by cardiomyocytes.

**Problems for AAV-U7**

Immune response to vector coat proteins. The organism receiving one AAV injection produces antibodies against proteins of the vector. This could limit the use of the AAV vector. Several ways are studied to overcome this difficulty.

Systemic delivery is not straightforward. The amount of virus to be injected and the volume of injection are directly linked. Dogs carrying a muscular dystrophy were injected intravenously, in a full limb. The results presented are very convincing.

There is commitment to the target sequence; this is a consequence a the long last effect. The sequence of the modified U7 should be carefully
designed and tested. The AON could give the right sequence to use.
Possibility of integration of U7 into the DNA has been shown in human, but this is now discussed. The effect of this integration is not known unforeseeable.

**Attractions of U7 integration into myogenic stem cells.**
Since several years, the use of stem cell to repair damaged muscle is studied. Many cells have been proposed: myoblasts, bone marrow-derived cells, blood-borne stem cells (AC133), synovial cells, mesoangioblast stem cells. AC 133 and myoblasts were transfected in vitro by a lentivirus carrying U7 DNA and IM* injected.

The cell therapy has some attractions:
- Avoids immune problems of vector, the transfection of the cells takes place in vitro, thus the virus is "seen" by the receiver.
- Could use autologous stem cells, another way to avoid immune response.
- Could provide benefits of a cell-therapy.

**Problems for U7 integration into myogenic stem cells.**
- It puts inefficient processes in series.
- Delivery is dependent on stem cells.
- Commitment to the target sequence.
- Requires integration of transgene.
- There may be better genetic modifications.

**How to translate pre-clinical results into the clinic**
- Proof of principle
- Netherlands IM injection (exon 51 2OMethyl chemistry).
- MDEX IM injection (exon 51 morpholino)
- Planned to be completed by the end of the year.
- Both groups planning early move into IV* trials once results from IM studies available.

**NOTE**- costs for such trials are huge
- Toxicology, GMP production comparable trial £20,000 per subject hospital costs alone.
- AAV U7 preliminary IM trial in carriers (exon 51).
- Move into patients/systemic move less clear.

**Questions that require further work for the planned studies**
- Dose- what is the optimum dose? How will this relate to side effects?
- Regime- how often is dosing going to be needed to maintain dystrophin expression?
- Is long term administration low risk?
- Other exons- how quickly can they be brought on line and what are the regulatory issues involved?
- Other chemistries- further modifications are likely with time?
- Who will fund the ongoing initiative?
- Who is going to supply the product long term?

Exon skipping is probably the most important progress in the cure of muscular dystrophy since this disease is well characterized. For the first time more than 90% of the muscular fibers are expressing a fully functional rescued dystrophin which also restore the muscular force. Different small RNA molecules have the property to target the site of skipping; they are more complementary than in competition. Other very small non RNA molecules have the same interaction with the pre mRNA. It is too early to evaluate the future of these molecules; but promising preliminary results were shown.

(*IM: intra-muscular, IV: intra-vascular)
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