Towards clinical trials for DMD using exon-skipping obtained by combined gene and cell therapy

Final report of ICE 2010

The final meeting closing the second year of ICE (2010) was held in Monaco on 27 November 2010, gathering the leaders of the 12 research teams involved in the collaborative effort. The objective was to review the results obtained after two years, and to delineate goals for 2011. ICE-2010 centered on approaches aiming at rescuing mutated dystrophins by systemically administered device ensuring directed splicing manipulations at the primary transcript level to obtain a therapeutic exon skipping (TES). Efforts were concentrated upon the optimization of the strategy of autologous cell therapy, using patient’s or animal’s myogenic muscle stem cells equipped in vitro with the exon-skipping device that was previously elaborated (virally incorporated U7 opt antisens oligo). After systemic reinfusion the «therapeutic cells» are expected to proliferate in vivo in all muscle groups, including heart, thus promoting the synthesis of a truncated but functional dystrophin (quasi-dystrophin). Another exon-skipping strategy was explored consisting in the direct infusion of new antisens chemicals: tricyclo-nucleotides, invented by Christian Leumann (Bem) who has joined the ICE team.

The different strategies were tested in a variety of cellular models and in vivo animal models (mdx and mdx<sup>ck</sup> mice, GRMD dog, zebrafish normal and dystrophin-deficient). A special attention was also concentrated on the optimization and standardization of pre-clinical protocols paving the way for future clinical trials.

The budget assigned to ICE-2010 was 873,000 €, covering 4 main pivotal items: 1) tools and methods for systemic delivery of AAV vectors in large size animal models; 2) autologous stem cell therapy combined with exon skipping; 3) dystrophin rescue by tricyclo-DNA antisense oligonucleotides; 4) non-invasive evaluation of muscle rehabilitation applicable to clinical trials in DMD.

The most significant results obtained during year 2010 are organized below into 5 categories:
1. Validation in animal models of dystrophins and quasi-dystrophins obtained by TES (Therapeutic Exon Skipping)

- In mdx mice: the intramuscular electrotransfer of GFP labelled plasmidic human cDNA sequence missing exons 45 to 55 is followed by normal sarcolemmal localization of quasi-dystrophin (Keith Foster, London). This is a promising result since the 45-55 TES should apply to 75% of DMD cases with a gene deletion.

- In zebrafish: the intra-embryonic injection of GFP-labelled full-length and truncated human cDNA is followed by the appearance of human dystrophin (or quasi-dystrophin) at the end of muscle fibers, a normal site in this species. Experiments of FRAP bleaching (UV-induced) revealed two dystrophin compartments, mobile (40%) and fixed (60%). The protein produced by the full length human cDNA is functional since it rescues dysnul zebrafish mutants (Simon Hughes, London).

The experimental validation of 45-55 dystrophin has progressed favourably, but two points remain unaddressed: (i) the missing proteic domain contains the binding site of NOS (NO-synthase); (ii) among the rare reported subjects with a 45 to 55 deletion most are poorly- or non-symptomatic, however some patients develop a dilated cardiomyopathy. A decisive test would be to analyze the rescuing capacity of 45-55 quasi-dystrophin in animal models, such as mice resulting from cross-hybridization of mdx (or dKO dys and utr) with transgenic mice carrying a 45-55 del, or dysnul zebrafish.

2. Exon skipping by a vectorized su7opt device

This device is a kind of minigene that, once in the nuclei of muscle cells produces permanently transcripts with the appropriate antisense oligo promoting the desired TES. To enter the cell and target the nucleus the minigene must be included in a viral vector, either AAV (in vivo experiments) or a lentivirus (in cultured cells). This year the program focused mainly on the systemic administration of AAV vectorized device. The delivery efficiency of the vector strongly depends on the viral serotype, cardiac muscle being the most difficult target.

- Large scale production of viral particles was obtained for a variety of serotypes (Robert Kotin, Washington DC).

- Host-virus interactions: it has been previously found that the systemic administration to mouse and dog of su7opt vectorized in various AAV (serotypes 1, 5, 6 and 8) resulted in poor results, with rapid clearance of blood viral particles without delivery to muscle targets. This phenomenon is now explained. Thru immuno-affinity experiments followed by global proteomic analysis a circulating protein that binds specifically AAV1, 5, 6 and 8 has been discovered: G3BP (galectin3 binding protein) in dog, CRP (C Reactive Protein) in mouse (Fedor Svinartchouk, Evry).

- Promising results obtained with scAAV9-su7opt: systemic administration of this serotype to mdx (Luis Garcia, Paris) and to dKO (dystrophin⁻/⁻/utrophin⁻/⁻ mdx) mice promotes efficient skipping of exon 23 with production of dystrophin (Aurélie Goyenvalle, Oxford). AAV9 thus appears to be an optimal choice for systemic administration of U7 antisense oligonucleotides. Yet mass production of this serotype is not possible, as long as specific antibodies are not available (Robert Kotin, Washington DC).

- Design of a novel non-immunogenic AAV retaining an adequate delivery capacity after systemic administration: all natural AAV particles are immunogenic, whatever their serotype. Capsid-less AAV would theoretically be non-immunogenic. We found that arterially injected «naked» viral genomes do retain the capacity to enter cells and reach muscle nuclei with acceptable yields. Such a vector, coined AAV0 (zero) is expected to accommodate
transgenes of unlimited length and should lend itself to mass production. This is one of the most significant results obtained this year (Luis Garcia, Paris).

- Direct targeting of myocardium: this has been obtained by intra-myocardial multiple injections thru an arterial catheter of AAV-vectorized U7 constructs in dog. This elicited a local production of dystrophin. The efficacy depends on the serotype (AAV6 >> AAV2, 8 et 9) (Lee Sweeney, Philadelphia).

- Multiple skipping of exons 45 to 55 restablishes the reading frame in 75% of DMD cases with a gene deletion yielding a functional quasi-dystrophin (vide supra). Several attempts have been tried ex vivo using various U7-AON vectorized in a lentivirus. The step-by-step approach faced some hurdles: (i) there is a background of spontaneous alternative splicing in this region of the transcript; (ii) the different spliced forms are difficult to quantify by qRT-PCR; (iii) some exons are more resistant to AON (antisense-oligonucleotide) induced skipping (47, 52, 53) (Aurélie Goyenvalle, Oxford). In contrast an en-bloc skipping of exons 45 to 55 was obtained by a single Su7 AON device targeting one ESS (Exon Skipping Suppressor) motif located in exon 55. This was demonstrated firstly in ex vivo experiments using myoiduced fibroblasts taken form DMD subjects with a 45-50 and 48-50 deletion. These ex vivo results were then implemented in vivo in mdx4cv (stop in exon 53) in which the expected 45-55 salvage quasi-dystrophin was obtained. (Luis Garcia, Paris). This important step forward is one of the highlights of ICE 2010 data.

We are about to overcome the hurdles that prevented successful systemic administration of AAV vectorized su7opt: the best AAV vector is scAAV9 because it reaches heart muscle, and is not neutralized in dog by G3BP (vide supra). This is why setting up a mass production of this AAV serotype is a priority on the agenda. On the other hand AAV0, a naked antigen-free vector retaining a good ability to deliver, may be the ultimate alternative. It is urgent to further enforce experimentally the great expectations regarding non-immunogenicity and unlimited size capacity. This decisive step will require ample stocks of AAV0 genomes. Another burning priority is to validate the « one-shot » 45-55 exon skipping by a single AON.

3. Autologous stem cells harnessed with the su7opt exon skipping device

The selected cells are muscle-derived “AC-133” precursors (Yvan Torrente, Milan). This material is difficult to isolate and to amplify ex vivo without losing its myogenic properties. The most significant advances are:

- The preparation of AC-133 cells retaining their in vivo myo-regenerating capacity was reproduced for the first time in another lab, thru the utilization of another less expensive culture medium (EGM2 Lonza) (Jenny Morgan, London).

- Quality control: the experimental laboratory processes of isolation and amplification of AC-133 cells derived from dog and human muscle have been optimized. A battery of tests have been standardized to control the quality of the cells at each step of the procedure (Genosafe, Evry).

- Heterogeneity of AC-1133 cells: this important feature has been evidenced, even after starting from one single cell clone. It has an impact on cell viability, cell proliferation, and in vivo myoregenerating capacity. At the present time it is a major hurdle on the way to clinical trials (Yvan Torrente, Milan).

- Therapeutic trials in GRMD dog: 6 animals received arterial infusion of autologous AC-133 cells harnessed with the su7opt exon skipping device vectorized in a lentivirus (10⁸ cells via subclavian route + 10⁸ cells via femoral route). Encouraging results were obtained both at biological and clinical levels. No side effects were observed. The degree of histological and...
functional recovery varied significantly from one dog to the other, suggesting that there are “good” and “bad” responders. Unexpectedly there was no correlation with the degree of dystrophin rescue. Functional improvement was evaluated by the “6 min walk test”, ability to climb stairs, and attested by video recording. It is already possible to say that this strategy combining splicing and cell therapy is able to elicit a sustained muscle regeneration with progressive appearance of dystrophin (7% of dys positive fibers after 6 months). These animals are still alive, allowing one to pursue long term surveys. These results belong to the most important advances obtained in 2010 (Yvan Torrente, Milan).

The results obtained in dogs are promising. They represent a first proof of concept of the therapeutic relevance of the strategy combining exon-skipping and cell therapy. A major hurdle is the heterogeneity of AC-133 cells, a material remaining difficult to calibrate. One of the priorities for 2011 is to optimize the experimental steps for cell purification and amplification, to find reliable biological markers of myo-regenerative capacity and to elucidate the relationship with muscle satellite cells.

4. A new generation of antisense oligonucleotides for exon skipping: tri-cyclonucleotides

- The chemistry of these compounds has been improved by introducing a phosphorothioate bridge, with large scale production (Christian Leumann, Bern).
- In vivo efficacy was demonstrated by systemic administration (IM, SC or IV) to mdx mice of tricyclo directed against exon 23. After 8 weeks dystrophin was seen in several skeletal muscles and in diaphragm (Luis Garcia, Paris). These results are encouraging and offer an interesting alternative to the other exon-skipping strategies in use in the ICE consortium.

This line of investigation is worth pursuing in 2011.

5. Getting ready for clinical therapeutic trials

At this point it is essential to standardize and secure the experimental protocols to be used in the ensuing clinical trials. This is meant to comply with scientific, ethical and regulatory requirements. Action was taken in the following themes:

- Specifications concerning good practice and quality control have been formally established. The main parameters are: quantification of viral vectors; quality and quantity assessment of transcripts corrected by exon-skipping; vector toxicity and possible impact upon differentiation of transduced cells (Séverine Pouillot, Genosafe).
- A pre-clinical protocol using the GRMD dog model has been elaborated and experimented as a prelude for future clinical trials. It consists in the intravenous limb loco-regional administration of AAV8-U7. Various doses (2.5 $10^{13}$ vg; 5.10$^{12}$ vg; 2.5.10$^{12}$ vg) were injected in an isolated posterior limb, each dose in 3 dogs. Neutralization by the G3BP protein mentioned earlier was overridden by the large amounts of viral particles. Biological and functional results confirmed previous pilot experiments conducted at ENVA (veterinary school in Paris) since 2005 by Luis Garcia and Stephane Blot (Laurent Servais, Paris).
- Methods for objective and reproducible assessment of muscular function recovery must be available for future clinical trials. Efforts have been focused on non-invasive methods: (i) sensitive ergometric mechanical tests have been elaborated and validated after being applied to survey the progression of functional deterioration in a cohort of 100 non-ambulatory patients suffering from progressive muscle dystrophy; concurrently actimetric and accelerometric tests are being designed to achieve continuous control of motricity at home.
Lessons from other clinical trials conducted elsewhere have been drawn, essentially the AVI-BIO (type 1b/2) trial using the AVI-4658 product (phosphorodiamidate morpholino oligonucleotide targeting exon 51) coordinated by Francesco Muntoni. Various doses were administered intravenously in 19 still ambulant patients (age 5 to 15 y) resulting in some restoration of dystrophin. The degree of restoration was variable among the patients, with “good” and “bad” responders, as observed in dogs (see §3). The best results were obtained with the higher doses (20 mg/kg). The best level of dystrophin restoration reached 55% of positive fibers (Francesco Muntoni, London).

The results obtained during the preclinical stage are meant to prepare a file to obtain the agreement from the regulatory authorities (AFFSAPS) for a first loco-regional clinical trial in DMD patients.

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