Towards clinical trials for DMD using gene and cell therapies combined with exon skipping

Two interim workshops of the ICE consortium have been held in Monaco on January 17 and June 6, 2009. They gathered 25 participants belonging to the 15 partner teams of the ICE network. The objective of these meetings was to review the current state of progress half-way after the launching of the first year of the ICE program.

The objective of the first year of the ICE project was to build tools and resources necessary to overcome the different bottlenecks that currently prevent us from starting clinical trials with combined exon-skipping/cytokera. Six months after the launch of the project, the amount of “deliverable” material effectively delivered, or about to be delivered, and the multiplicity of issues explored are encouraging. It bodes well for the completion of the project in due time. The usefulness of some strategic reorientation and the reasons to extend the program over a second year will be considered at the next Monaco meeting (23 January 2010).

Following is a synthesis of the material that was delivered in each “word-package”.

«Work-Package 1»: Validation of quasi-dystrophins

- Validation of potentially therapeutic quasi-dystrophins by in silico data mining of the UMD-DMD Cochin database: Association of minimally symptomatic or even asymptomatic phenotypes with in-frame deletions terminating in exon 51 validates this exon as a target for mono-skipping in 6 of the 7 patterns of local deletion correctable by this measure. For the proposed master-key corrective del 45-55 pattern, phenotypes are of the expected BMD type, but of varying severity. It appears that mining the database has its limits, because the accuracy of every piece of molecular and clinical information is not assured in each individual instance. It is thus a necessary but not sufficient preliminary step that must be followed by a return to the paper files and to the patient. The availability of a CGH-array exploring the entire DMD gene (exons + introns) will make it possible to correlate the breakpoint boundaries with the clinical presentation.

- Custom-made preparation of truncated cDNA plasmids coding for a variety of supposedly functional quasi-dystrophins: the methodology is mastered and already 10 different constructs (each in two forms: with or without GFP coding sequence) have been prepared and delivered to other teams for in vivo validation.

- In vivo validation of the above-mentioned quasi-dystrophin cDNA: this is achieved in the two following animal models:
  (i) In mdx mice (intra-muscular local delivery by electro-transfer).
(ii) in *dys"null* Zebrafish by transgenesis. This novel promising model has been validated by showing that it is rescued by human full-length dystrophin cDNA and various truncated quasi-dystrophin cDNAs. GFP constructs proved very useful to show that the human material is attached to the same cellular structures as the resident dystrophin (curiously at the fiber ends exclusively), and is not expressed in other tissues than muscle. GFP-human dystrophin was successfully used to investigate protein turnover (or relocalization) by a method of photo-bleaching (FRAP method). The relationship between the function of dystrophin at the fibre ends and that at the lateral muscle fibre surface in mammals remains to be elucidated.

« Work-Package 2 »: Exon-skipping devices

- Setting up a resource for ex-vivo testing of the efficacy of current U7-based exon-skipping devices on DMD patients cell material. A robust protocol is now operational, in which patients cultured myotubes are derived either from primary myoblast cultures or from myo-induced fibroblasts (when muscle biopsy is not possible), then transduced with LV vectorized exon-skipping devices. Successful exon-skipping (expected mRNA and quasi-dystrophin) has been obtained for exons 51, 46, 45 and 16. Cell material from DMD patients is now routinely being obtained (over 50 samples already)

- Optimization of U7-based exon-skipping cassettes. The mouse U7 sequence is now replaced by the U7 human version (coined «SU7»). A «sticker» (3-4 nt) to add at the 5’ end of antisense sequence has been delineated (by modeling and mutagenesis). It ensures optimal binding («kissing») of the loop and appropriate folding of the resulting snRNP. Concatenated cassettes aiming at multiple exon-skipping have been obtained, and successfully validated in GRMD dogs where double targeting is necessary to obtain frame correction.

- Construction of a bifunctional U7snRNA skipping cassette to improve efficacy for «difficult» exons. It carries an antisense sequence targeting an appropriate exon-specific sequence involved in splicing + a universal free hanging tail with canonical binding sites to powerful splicing repressors (ESS) to obstruct the spliceosomal machinery. It has been validated ex vivo on DMD patients myoblasts (del 49-50) (LV vector), in vivo in mdx mouse (AAV1 vector), in hDMD and in double KO mice (dys/utr) which recovered normal ability to move. These results are now published (Goyenvalle et al, Mol Therapy, online 19 may 2009).

- Master-key device to skip 11 exons from 45 to 55 (that would rescue a maximum of deletion patterns): Difficult problem not yet resolved (multiple strategies are tried: targeting the two terminal exons, targeting every exon individually). The fact that this region is subjected to spontaneous alternative splicing prevents testing of the efficacy by sizing the transcripts, forcing reliance on western-blotting.

- Optimization of U1-based exon-skipping cassettes by exploring the world of miRNA. Muscle specific promoters of some miRNA (skeletal, smooth, heart) have been identified and used to drive the skipping cassette in U1 constructs. Eleven chimeric miRNA promoters have been constructed, vectorized in LV and validated for exon 51 skipping.

« Work-Package 3 »: Autologous cytotherapy strategies

- Using CD 133 cells: Progress has been achieved in the biological characterization, in the purification and the amplification of these cells obtained from muscle and from blood. CD 133 transduced by the exon skipping device behave as expected after intra-muscular engrafting, with indication of a ‘by-stander’ effect (i.e. evidence that exon-skipping is initiated in myonuclei neighbouring those bearing the corrected nuclei).

- Using mesoangioblasts: Progress in the understanding of the embryonic origin of these cells and the biological factors orienting them towards myogenic determination.
- **Dissemination of the CD 133 methodology from Milan to other labs of the ICE consortium:** This transfer of « savoir-faire » is in process (UCL, Cochin, Genethon, Institut de Myologie). In several of these sites the purification from control muscle is already operational with a satisfactory yield, as well as the amplification (minipreps - not yet preparative). The very special medium necessary to cultivate these cells is provided by Ivan Torrente. A company has been approached to manufacture the medium (very costly: 30 000 €/liter, but there will be a negotiation to obtain better price) and of amounts to produce (the labs must announce their needs).

- **Improving culture methodology for myogenic precursors:** A preconditioning method to ensure myodifferentiation of potentially myogenic precursor cells (human MSC) has been devised. It consists of a polyacrylamide/collagen matrix of optimal elasticity (11 kPa) producing excellent viability and spectacular yields of dystrophin after intramuscular injections (nearing 100 %). These cells are astonishingly well tolerated in immunocompetent mdx (not explained for the moment).

« Work-Package 4 »: The systemic delivery issues

- **Large scale production of AAVs:** an industrial process of production in Baculovirus and quality control protocols have been established. Pharmaceutical grade batches of rAAV6 U7smOPT for local and systemic administration in sufficient amounts to be applied to dog (in which ca 10^15 vg per dog of 10kg is necessary) are obtained. The same methodology is now being applied to AAV8. Mass production is now routinely accomplished and delivered to the ICE community.

- **Immunogenicity of AAV6 and 8 in dogs:** a failure of systemic administration of AAV6/optU7 by intracardiac (left ventricle) route at dose of 10^14 particles in 1.5 kg GRMD has been observed. It is explained by pre-existing circulating neutralizing antibodies against AAV6 (role of systematic prophylactic antiparvoviral vaccination at birth?). In fact the precise mechanism of neutralization is still not elucidated. AAV8 which was believed to be a good substitute because it seemed not to be neutralized in vitro by GRMD serum, is in fact also neutralized in vivo, but to a lesser degree.

- **Systemic delivery of rAAV6 in GRMD dog by extra-corporeal circulation:** in order to by-pass the pulmonary filter a protocol of ECC has been devised and optimized in normal dogs with infusion of AAV in the arterial incoming blood. Rapid clearance of virus from blood, between 7 and 12 min, was observed. The distribution and skipping efficiency are under current investigation. Biocoating of circuitry is expected to avoid loss by an adhesion of viral particles. 2 GRMD dogs were injected by ECC in February. The tolerance was very good, but the delivery very bad, indicating that the neutralization bottleneck is still a problem.

- **Delivering AAV to heart muscle:** a protocol of transendocardial delivery of rAAV6 (thru vascular catheterization) has been established in GRMD dogs. The choice of AAV serotype depends on the animal model: AAV9 is better in rodents; AAV6 is better in dogs and non-human primates.

« Work-Package 5 »: Preparing clinical trials

- **The proceedings of the « Treat-NMD/ICE meeting on WP5 » held in Paris on 25th November 2008 are provided by Francesco Muntoni.** This chart of guidelines is of great importance, and the full document is attached as an Appendix.

- **In silico prescreening of candidate patients:** for any given mono- or multiskipping rescuing pattern, a list can be instantly obtained from the French UMD-DMD database thru its built-in specifically designed tools. In addition the Cochin UMD-DMD database (ca 1500 probands ie 60 % of patients in the national database) is backed by paper files, with necessary information for further scrutiny of eligibility.
- **Developing tools for functional assessment of treated individuals**: several existing devices to measure local residual muscle strength have been evaluated. Another promising line of evaluation is accelerometry to give a global indication of motility. It appears that for still ambulant boys the 6 minute-walk test (employed by the PTC company) is simple and reliable. For non-ambulant patients a new device called « Piano » is useful since it explores the endurance of hand flexors and extensors.

- **Checking the tolerance of autologous CD 133 cells**: intra-muscular infusion in five DMD recipients of their own (non-corrected) cells was not followed by any clinical or histological adverse event.

- **Preclinical study to investigate fate and tolerance of mesoangioblasts**: this has been done (i) in humans where variable proliferation and myogenicity was obtained, indicating that the methodology is not yet operational; (ii) in normal and GRMD dogs in which homing and persistence has been assessed by indium 111 labelled cells.

« **Work-Package 6**: Management  

Counts and statistics: a list of the 15 teams with their respective funding allocation (Total = 1,693,191 €) has been prepared by Inserm-Transfer. Seven teams have already received their share (2/3 of total funding).

**Supplementary data provided at the June meeting by guest speakers:**

*Philippe Moullier (Nantes)* presented his data obtained in non-human primates (macaques) and long-term AAV survival after 5 years, with no evidence of integration.

*Muriel Audit (Genosafe, Evry)* exposed her expertise in terms of biosafety and compliance to regulation of Biosafety agencies.

See attached:

**Appendix with the Clinical Trials Guidelines elaborated at the WP5 « TreatNMD/ICE » joint meeting (Institut de Myologie, Paris 25 nov 2008) and recorded by Francesco Muntoni.**

Report written by Jean-Claude Kaplan, (ICE Advisory Board) and reviewed by Terry Partridge (ICE Advisory Board), and Luis Garcia (ICE scientific coordinator).

12 June 2009
WP5 « TreatNMD/ICE » joint meeting (Institut de Myologie, Paris 25 nov 2008)

Towards clinical trials for DMD using gene and cell therapies combined with exon skipping strategies

report established by Francesco Muntoni

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Jamel Chelly (ICE Partner, Geneticist, French DMD database curator, Institut Cochin, Paris)

Apologies:
Adrian Thrasher (Immunologist, Institute of Child Health, UK)

Aims

This meeting has been planned to discuss the details of how to design and perform the proposed DMD multinational clinical trial using the combination of autologous cell transplantation and gene therapy.

Specific aims are to agree on the topics mentioned below, in order to facilitate each of the centres into an application for ethical and local institute approval:

a. patient inclusion and exclusion criteria,
b. clinical trial design,
c. toxicology consideration,
d. manpower and timelines for the trials.

- Divide the task of developing an effective systemic therapy in smaller achievable aims
- An intramuscular experimental part likely to be required by regulatory authorities
- This will be a proof of principle, safety, non-efficacy driven proof of concept study
Choice of patients

- Ideally patients from age 10+ selected
- As exon 51 skippable very saturated by other studies, suggestions is to move into another target (Exon 53 or 46)
- Small number of patients to be studied in each centre, say 3-5 patients per centre
  - Preselection using skin biopsy and MyoD transfection
  - Preceding muscle biopsy sufficient for entry (repeated biopsy not a necessary entry at study)

Choice of cells

- Ac133+ cells freshly isolated from donors. Discussion on whether these should come from muscle or blood.
  - Advantage of cells from muscle is that they are more efficient in making skeletal muscle;
    disadvantage is that a muscle biopsy is required and especially if one targets DMD patients
    who are not very young.

Choice of endpoints

- Very important to be able to recognise the nature of positive fibres and differentiate this from revertant fibres.
- As it will be difficult to select patients based on deletions of epitope recognised by ant dystrophin antibodies, other means should be devised.
  - A possibility is to develop an in situ system to detect lentiviral vector; if this is not feasible, the
    procedure should take into consideration the possibility to do a sham injected site + a cell
    treated site.

Work to do in the interim

- Compare and harmonise methodologies to isolate and culture AC133 + cells in the laboratories involved in the study
- Verify myogenicity of human muscle derived AC133 following intramuscular injection in immunodeficient mice
  (obtain muscle via biobank from patients; from orthopaedic surgical procedures from controls, following the biobank ethics protocols);
- Verify myogenicity of blood derived stem cells. This can be done using normal donors
  - make contact with blood bank.
- Set up techniques to allow the detection of lentiviral U7 virus in situ
- Start the evaluation and validation of outcome measures for future regional delivery studies. Lower limb better than arm because of accessibility of femoral artery vs subclavian artery.
  - Evaluate therefore quantitative methods for recording strength in the age range of the patient population which will be subjected to future trials. For example foot dorsi/plantarflexion with the device used at IdM.
- Organise a meeting of interested relevant parties (therapists and physical evaluators) to
  decide the range of outcome measures which ought to be assessed.
- Assess efficacy of different extracellular matrix environment in determining the fate of blood
  derived ac133 cells.
- Start to consider the questions in terms of protocol design and toxicology package that the
  regulatory authorities will likely ask us.
- Start planning for meetings with the regulatory authorities, ideally held together with the
  various national PIs, in order to get early feedback regarding the development of the protocol.
Towards clinical trials for DMD using gene and cell therapies combined with exon skipping

One goal
Overcome enduring hurdles in order to initiate clinical trials using exon skipping strategies

ICE at present

Year 1 - 2008/2009
Budget: 1.7 million €

2 promoters
Association Monégasque contre les Myopathies
Duchenne Parent Project France

4 countries
France, Italy, United Kingdom, U.S.A

12 institutions
INSERM
University of Milan
University College London
King’s College London
Royal Holloway University London
University of Rome
University of Oxford
University of Pennsylvania
National Institute of Health Bethesda
Institut de Myologie Paris
Imperial College London
INSERM-Transfer
15 partners

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Olivier Danos, Necker Hospital, Paris, & University College, London
Francesco Muntoni, Institute of Child Health & Great Hormon Street Hospital, London
Kay Davies, Oxford University
George Dickson, Royal Holloway University, London
Dominic Wells, Imperial College, London
Simon Hughes, King’s College, London
Irene Bozzi, Sapienza University of Rome
Yvan Torrente, University of Milan, Maggiore Policlinico Hospital, Milan
Lee Sweeney, University of Pennsylvania, Philadelphia
Robert Kotin, NHBL, National Institutes of Health, Bestheda
Anton Ottavi, Inserm-Transfert, Lyon, France

- 90 researchers and clinicians -

6 Work Packages

1 Evaluation of the therapeutic value of truncated dystrophins completed by exon skipping
2 Design and upgrading optimized-snRNAs for dystrophin rescue
3 Autologous stem cell therapy using LV vectors encoding opt-snRNAs
4 Widespread delivery of AAV vectors encoding opt-snRNAs: Clinical relevance in GRMD
5 Design of the future clinical trials
6 Management

- 42 deliverables -

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