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LETTER

Therapeutic exon 'switching' for dysferlinopathies?

We read with interest but also some surprise, the recent 'Therapeutic exon skipping for Dysferlinopathies?' article by Aartsma-Rus et al,[1] published in the Eur J Hum Genet (advance online publication, 10 February 2010; doi:10.1038/ejhg.2010.4).

This report contains some inaccuracies and mistakes, and we do not agree with some of its main contents. Therefore, for the sake of the scientific and patients communities, we consider it essential to point out and discuss some of the results and subsequent conclusions appearing in the 'results and discussion' section, as well as in figures and tables.

Antisense-mediated exon skipping constitutes a relevant therapy for several genetic diseases associated with premature termination codons (PTCs) or frameshift mutations lying in dispensable in frame exons. However, knowledge of the specific targeted gene, mRNA and resulting protein, is, among others, an essential pre-requisite toward designing pertinent exon-skipping strategies. In the report by Aartsma-Rus et al,[1] it appears that several of these pre-requisites have been overlooked. Here we intend to discuss and contradict some important assertions and results that appear in both the text and the figures of the article by Aartsma-Rus et al,[1] and place our comments in the perspective of a recently published report from our group on the same matter.[2]

DYSFERLIN EXON PHASING

Exon phasing is the main key toward designing exon-skipping strategies. Surprisingly, a discrepancy regarding DYSF exon 32 phasing appears in the article by Aartsma-Rus et al (Figure 3)[1] when compared with the previously published report by Wein et al (Figure 1).[2] We first thought that this discrepancy resulted from the use of different symbols for phasing in these two reports. However, in Figure 3,[1] a frame shift in the use of symbols corresponding to exonic ends (0, +1, or +2) starting from exon 15 appeared. According to the Leiden muscular dystrophy pages database (http://www.dmd.nl/dysf_home. html) and our own report,[2] exon 15 is predicted to splice after +2 nucleotides within codon 466, whereas Aartsma-Rus et al represent this exon with a symbol corresponding to a splice after +1 nucleotide in the same codon. Upstream from exon 15, phasing was represented (eg, for exon 3) with the correct symbol.[1] Although this phasing error does not modify the frame per se from exons 15 to 55, it would, however, have severe consequences in case exon skipping would be used as a therapy. As examples, exons 13–15 are skippable according to Aartsma-Rus et al,[1] whereas, in reality, such a skipping combination does not preserve the reading frame (http://www.dmd.nl/dysf_home. html and Wein et al (Figure 1)[2]) and would introduce a PTC in the following exon 16 [r.1181_1397del; (p.Met394_Trp466 > ArgfsX4)]. Exons 15 and 16 are also considered as a possible skipping combination according to both Table 2 and Figure 3,[1] while such a skipping

strategy would introduce a PTC in exon 20 [r.1354_1480del; (p.Phe452_Glu494 > ArgfsX133)]. In contrast, multiple exon skipping for exons 14–18 or 15–18 is considered as pathogenic by the authors,[1] and patients carrying mutations in these exons would thus be excluded from a potential exon-skipping therapy according to Aartsma-Rus et al.[1] Actually, this region can be considered as a strong candidate for exon skipping as it encodes part of the C2C calcium sensor domain, a region which is potentially dispensable, as its complete absence leads to a mild phenotype, as previously reported by Krahn et al.[4] In the region encompassing exons 14–18, 11 different mutations have been reported to date as disease causing in the Leiden muscular dystrophy pages database (http://www.dmd.nl/dysf_home. html) (respectively four different nonsense mutations, three different frame-shifting mutations and four different missense mutations), corresponding to a total of 17 patients who would be potential candidates for exon skipping. We feel that these erroneous informations need to be corrected as they would have some important negative consequences for patients and their families who might be misled with respect to their potential participation to clinical trials.

EXON 32 SKIPPING

One of the most relevant natural proof-of-principle to suggest dysferlin as a pertinent target for at least a subset of patients, arose from a report by Sinnreich et al who reported a very mild and late onset phenotype in one patient carrying an in frame deletion of exon 32.[3] Recently, by using two different antisense oligonucleotides (AON) either alone or in combination, we have succeeded in efficiently skipping DYSF exon 32 from both control and patient's cells.[2] Surprisingly, the single AON toward targeting and skipping exon 32 (H32DYS1) (Table 1) designed by Aartsma-Rus et al was shown to be inefficient (Figure 4).[1] DYSF exon 32 is a small exon (78 bps) and we thus checked whether the H32DYS1 AON was overlapping our previously reported ESE1- and/or ESE2-AONs.[2] To our surprise, H32DYS1 was not matching any sequence in DYSF exon 32. A thorough investigation throughout the dysferlin gene revealed that the AON sequence H32DYS1 targets a sequence included in exon 34 of dysferlin and that a switch between AONs H34DYSF2 targeting exon 32 and H32DYS1 targeting exon 34, has been introduced by Aartsma-Rus et al,[1] potentially explaining the absence of efficiency of these two AONs. Indeed, the AON H34DYSF2 (positions 7–26 in exon 32) is overlapping the ESE1-AON (positions 3–23 in exon 32) described in Wein et al.[2] We are aware that such inversions may occur at several stages during experimental protocols design and processes, but we feel that this is precisely why this type of information needs to be carefully checked before publication, even more so when another article is published on the same matter as was the case in this instance, with our article[2] published online 2 months before, and appropriately referenced in the article by Aartsma-Rus et al.[1]

To further confirm that the sequence included in the AON H34DYSF2 (in fact, AON targeting exon 32) could indeed efficiently skip DYSF exon 32, we performed an exon-skipping experiment using AON H34DYSF2 on the same patient's cells used in our previous study and carrying a PTC in exon 32, using the same forward and reverse primers in exons 30 and 33, respectively.[2] As demonstrated in Figure 1, H34DYSF2 was quite efficient to skip exon 32 in our hands. We did not check the AON H32DYSF1, but we are convinced that a skipping of exon 34 would have been observed. However, as mentioned by Aartsma-Rus et al (Table 2),[1] DYSF exon 34 skipping could be pathogenic and, although another AON targeting exon 34 seems to
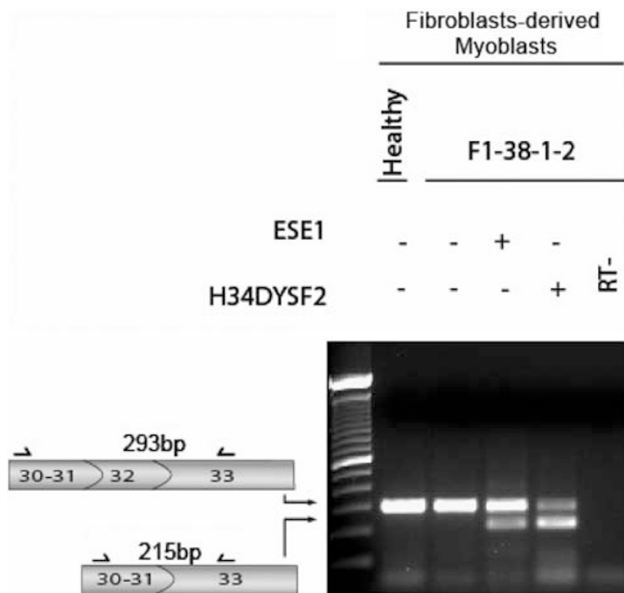
Figure 1 Dysferlin exon 32 skipping analysis. RT-PCR analysis of the transcript region flanking exon 32 (exons 30–33) in control fibroblasts-derived myoblasts and fibroblasts-derived myoblasts from patient F1-38-1-2. After treatment with H34DYSF2, a shorter transcript fragment, not present in the negative control, was observed at the expected size (215 bp) and indicated the efficient skipping of exon 32. A similar result was observed after treatment with ESE1-AON used as a positive control.

be functional (H34DYSF1), exon 34 does not, in our opinion, represent a first choice therapeutic target.

DISPENSABLE DOMAINS OF DYSFERLIN AND EXON SKIPPING
We have also some disagreements regarding the strategy presented by Aartsma-Rus et al.[1] In Table 2 of their report, it is stated that the skippable exons exclude those encoding the C2 domains. It should be pointed out that the first and only natural proof-of-principle of non-deleterious single exon skipping of dysferlin corresponds to the in frame deletion of one exon encoding part of the C2D domain.[2,3] In addition, we have also reported a large homozygous deletion in a mildly affected patient, removing five out of the seven predicted C2 domains in dysferlin.[4] This represents a solid basis for multiple exon skipping targeting C2 domains in dysferlin, or for mini-gene transfer strategies in dysferlinopathies.

CONCLUDING REMARKS
As we showed in the first report on exon skipping in dysferlinopathies,[2] Aartsma-Rus et al showed that, when properly designed, AONs may be efficient to skip exons in the dysferlin mRNA. However, they could not demonstrate any bypass of mutated exons in *DYSF* as they only used normal control cells in their experiments. Over the last few years, numerous articles, several of them being outstanding reports by the researchers from the center for human and clinical genetics in Leiden (for review, see Aartsma-Rus and van Ommen[5]), have reported exon skipping as a promising therapy particularly for Duchenne muscular dystrophy. Additional proof of technical feasibility of exon skipping is thus probably no longer required as far as it does not add to the general knowledge.

This is precisely why we think essential to emphasize, for patients and clinicians, the real possibilities opened by exon skipping in dysferlinopathies. We went into these comments and considerations in depth, as articles reporting potential therapies have a major impact on the clinical and genetic community, as well as for patients, and their content must be of as much help as possible and should only deliver real and accurate information. While demonstrated as a technically feasible approach in dysferlinopathies, exon skipping should only be applied when its scientific relevance (pertinent exons *vs* patient's phenotypes, domains' function, converging arguments for modularity, …) determined by experts in the field is based on solid data obtained from the largest possible cohorts of patients.

WEB RESOURCES
The URL for data presented here are as follows: Leiden muscular dystrophies mutation database, http://www.dmd.nl/dysf_home.html.

NOMENCLATURE
Theoretical deletions of respectively exons 13–15, and exons 15–16, inducible by exon skipping, are described using the human *DYSF* sequence (NM_003494.2) and the nomenclature of the Human Genome Variation Society (www.hgvs.org/mutnomen) in its 12 October 2004 version, as an uncertainty for a precise description would have been introduced using the 12 May 2007 version.

Nicolas Lévy[1,2], Nicolas Wein[1], Florian Barthelemy[1], Vincent Mouly[3], Luis Garcia[3], Martin Krahn[1,2] and Marc Bartoli[1]
[1]Faculté de Médecine de Marseille, Université de la Méditerranée, Inserm UMR_S 910 'Génétique Médicale et Génomique Fonctionnelle', Marseille, France
[2]AP-HM, Département de Génétique Médicale, Hôpital d'enfants de la Timone, Marseille, France
[3]Institut de Myologie, Groupe Hospitalier Pitié-Salpêtrière, Université Pierre et Marie Curie/Paris 6/Inserm UMR_S 974, CNRS UMR 7215, Paris, France
E-mail: Nicolas.Levy@univmed.fr

1 Aartsma-Rus A, Singh KH, Fokkema IF *et al*: Therapeutic exon skipping for dysferlinopathies? *Eur J Hum Genet* 2010, e-pub ahead of print 10 February 2010.
2 Wein N, Avril A, Bartoli M *et al*: Efficient bypass of mutations in dysferlin deficient patient cells by antisense-induced exon skipping. *Hum Mutat* 2010; **31**: 136–142.
3 Sinnreich M, Therrien C, Karpati G: Lariat branch point mutation in the dysferlin gene with mild limb-girdle muscular dystrophy. *Neurology* 2006; **66**: 1114–1116.
4 Krahn M, Wein N, Lostal W *et al*: Partial functionality of a mini-dysferlin molecule-identified in a patient affected with moderately severe primary dysferlinopathy. *Neuromuscul Disord* 2008; **18**: 781.
5 Aartsma-Rus A, van Ommen GJ: Progress in therapeutic antisense applications for neuromuscular disorders. *Eur J Hum Genet* 2010; **18**: 146–153.