Author's response to reviews

Title: Dual exon skipping in myostatin and dystrophin for Duchenne muscular dystrophy

Authors:

Dwi U Kemaladewi (d.u.kemaladewi@lumc.nl)
Willem MH Hoogaars (w.m.h.hoogaars@lumc.nl)
Sandra H van Heiningen (s.h.van_heiningen@lumc.nl)
Samuel Terlouw (samuelterlouw@gmail.com)
David JJ de Gorter (d.de_gorter@lumc.nl)
Johan T den Dunnen (ddunnen@humgen.nl)
Gert Jan B van Ommen (g.j.b.van_ommen@lumc.nl)
Annemieke Aartsma-Rus (a.m.aartsma-rus@lumc.nl)
Peter ten Dijke (p.ten_dijke@lumc.nl)
Peter AC 't Hoen (p.a.c._t_hoen@lumc.nl)

Version: 5 Date: 7 January 2011

Author's response to reviews: see over
Leiden, December 23, 2010

Subject: Second resubmission

Dear BMC Medical Genomics editors,

On behalf of my co-authors I would like to resubmit the manuscript titled ‘Dual exon skipping in myostatin and dystrophin for Duchenne muscular dystrophy’ by Kemaladewi et al. for publication in BMC medical genomics.

We appreciate the chance given to revise our manuscript. The comments and details pointed by the reviewer were very valuable to our study. A point by point response to the reviewer is addressed below.

The reviewer has referred to a study by Kang et al. published on Oct 5, 2010. Since our initial submission was on May 28, we would like to suggest a comparison of our results with the results of the above paper in separate “note added in proof” section.

We believe that our study is valuable for a broad group of researchers, not only those interested in myostatin exon skipping. We look forward to your decision.

Yours sincerely,

Dwi Utami Kemaladewi
Center for Human and Clinical Genetics
Leiden University Medical Center
Einthovenweg 20, 2333 ZC Leiden, the Netherlands.
Tel: +31-71-5269423
Fax: +31-71-5268285
E-mail: d.u.kemaladewi@lumc.nl
Reviewer’s report
Even though the authors have answered some of the major points, I am still not convinced by the experiments presented here. My main point concerns the in vivo experiments. I still do not agree with author’s conclusion “Myostatin AON induced exon 2 skipping in cell cultures and to a lower extent in the mdx mice”.
Moreover, the authors did not show any functional data related to these in vivo injection as hypertrophy for instance. Furthermore, a recent paper has shown a beautiful skipping of myostatin exon 2 in normal mice as well as in C2C12 cells (Kang et al, Mol Ther, 2010).

Response: Our initial submission was long before the paper by Kang et al. was published. Therefore, we would like to compare our results obtained with the 2'-O-methyl-PS AONs to the results obtained with the morpholino AONs described in mentioned paper in a separate “note added in proof” section.

- Major Compulsory Revisions
1. According to supplementary figure 1A, the efficiency of the skipping is not dependent on the AON concentration. But in fig 2B, the skipping efficiency is AON concentration dependent. Can the authors give an explanation for that?
Response: We thank the reviewer for the comment. We would like to point out that the human primary myoblasts were used in the experiment described in supplementary figure 1A. As we explained in the previous response, we observed experimental variability due to the nature of the cells.

2. The results presented in figure 2D are not consistent with the observed non-skipped band in figure 2B for 7304.1 and DL589.2 cells. Moreover it would have been much more relevant to analyze the skipped RNA instead of the non-skipped products.
Response: We thank the reviewer for pointing out the mistake. The legends of figure 2D were switched during image processing. The concentrations were inverted and thus did not match with the corresponding figures 2B. This has been corrected in the resubmitted version. We would like to emphasize that the quantification is based on 3-4 experimental replicates, whereas the gel corresponds to one representative experiment.
We have chosen to quantify the non-skipped product with QPCR because it provides information about the level of myostatin knockdown. Moreover, the level
of knockdown as determined with QPCR shows a good correlation with the level of exon skipping shown on gel, so we feel this justifies the use of this method.

3. The figure supp1B is not convincing at all (I cannot see any skipping) and may be removed of the manuscript.
Response: Removed.

4. Fusion index: to justify their incapacity to analyze the fusion index of the transfected cells, the authors say they have observed a severe toxicity after 3 days. They suppose that this toxicity is caused by the transfection reagent. However according to figure 2A, where nice myotubes after 500nM 5’-FAM-Ctrl AON transfection are shown, the toxicity does not seem to be severe. On the other hand, they have shown that the best exon skipping is obtained with 100 nM AON which is the lowest concentration tested. But, this does not mean that 100 nM is the best concentration. The authors may try some lower concentrations, which may result in an improvement of the skipping and reduce the toxicity of the transfection.
Response: We still agree with the reviewer that this functional assay is a key experiment. Nevertheless, toxicity issues due to prolonged effects of the transfection and low serum medium hampered the differentiation assay in all human cells models we tested. Regarding figure 2A, we mentioned in the legend that the image was taken 3 hours post transfection. We indeed have tried 50 nM AON but the skipping efficiency was lower, thus we considered 100 nM as a threshold.

5. In figure 6A, the presentation of the figure is not clear. The “+” and “-” must be added in each column. Moreover, the data presented in mdx 1 are not convincing. For the matter, an in vivo experiments realized on 2 mice is not enough. Moreover, as for the in vitro experiments, it would be useful to quantify the skipping efficacy for MSTN and DMD AONs.
Response: The “+” and “-” signs have been added based on the reviewer’s suggestion. As the AON showed low skipping efficacy in vivo, we think that it is not essential to expand the group. As proposed in the discussion, further research should focus on optimizing the delivery to fibroblast/satellite cells.
6. Figure 6B: a low level of exon skipping is still observed in the data presented here. Moreover, there is an important variation between 2 mice.

**Response:** The reason behind performing this multiple injection experiment was to improve the skipping efficiency. Even though limited number of animals was used, we feel that the message we want to give is clear. As stated in our discussion, multiple injections could improve the skipping efficiency but not enough to achieve decent skips and to further assess the hypertrophic phenotypes.

7. In their answer, the authors have declared they have removed all the data corresponding the UTRN-/- mouse as requested. But, they still mention all these data as “data not shown”. These data are not convincing at all and must be totally removed from this manuscript (methods, results and discussion)

**Response:** Removed

Minor Essential Revisions

1. The article by Kang et al (molther, 2010) must be added at least in the introduction.

**Response:** As this paper came out during the resubmission process, we propose to add it into a separate “note added in proof” section.

2. In the methods, the 7304-1 cells are cultivated with 75% FBS. I suppose the authors mean 25%.

**Response:** We thank the reviewer for the comment. The medium was supplemented with 15% FBS. The method has been changed.

3. In figure 2B, since the best skipping is observed with the concentration of 100 nM, the control AON should also have been used at 100 nM. Could also the authors include the DNA ladder like for the KM109 cells.

**Response:** Based on what we have observed, no significant difference was shown between samples transfected with 500 nM or 100 nM control AON. With this manuscript we include a supporting figure for reviewer, where we provide examples of independent experiments depicting this observation.
However, we did not include the 100 nM concentration in every experiment since we did not see a difference with the 500nM or NT condition. Therefore, for a consistency in the figure presentation, we have left out the 100 nM control AONs data.

4. The authors must change the sentence “myostatin skipping results in premature codon stop in exon2 “ by “myostatin skipping results in premature codon stop in exon3”

Response: We thank the reviewer for the comment. This has been changed.

5. In figure 4 A, the total amount of skipped and unskipped RNA is not the same in all the wells. Can the authors give an explanation for that?

Response: Based on our understanding, the reviewer’s question is about the dystrophin PCR. Dystrophin expression was low and variable in these cells, most likely because the (heterogenous) culture consisted not only of differentiated myotubes but also of dystrophin negative fibroblasts. Myostatin expression in the same samples is comparable because fibroblasts and myoblasts are source of myostatin and therefore the expression level is higher.

6. The authors should provide the sequence of the primers used for the different PCR and quantitative PCR.

Response: The sequences of the primers are now included in the manuscript.