Author's response to reviews

Title: The Immunosuppressant FTY720 (Fingolimod) enhances Glycosaminoglycan Depletion in Articular Cartilage.

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Author's response to reviews: see over
Dear Dr Changhai Ding,

Thank you for reviewing of our work entitled “The Immunosuppressant FTY720 (Fingolimod) Enhances Glycosaminoglycan Depletion in Articular Cartilage”. We very much appreciate the comments of the reviewers. The manuscript has been improved to address their suggestions:

Reviewer: Stefan Toegel

Major Compulsory Revisions
*) The Methods section needs revision regarding the description of experiments and statistical aspects. Please address the following issues:

We apologize for the inadequate Material & Methods section. It has been completely revised to provide sufficient information.

+) How were the cartilage explants prepared (size, amount/weight for each assay,...)? Please see p.7, second paragraph.

+) How many animals were included in the study (n=?)) Please see p.5, second paragraph.

+) Viability assay: Information on technical replicates and time periods for incubations are missing. How many times were the experiments repeated? The results of the viability assay are not given or discussed in the manuscript. Please see p.5, second paragraph and p.8, fourth paragraph.

+) Information on cell culture conditions (including treatment, incubation periods,...) preceding RT-qPCR assays must be given in the methods section. It remains unclear (also from the figure legend) how many technical replicates were used within each experiment. Were the 3 individual experiments conducted with cells from different animals? Did the authors validate the use of GAPDH as reference gene for the experimental setup of the study? For guidelines on the documentation of RT-qPCR methods and results see: http://www.ncbi.nlm.nih.gov/pubmed/20858237

Information on cell culture conditions, number of replicates and animals, validation of GAPDH and minimum information for publication of qRT-PCR according MIQE précis is now provided on p.5 and 6 and supplementary tables 1 and 2.

+) Again, cell culture details (including replicates) for WB assays are unclear. Please see p.6 second paragraph.

+) GAG release: Again: n=?, replicates, how many individuals? Furthermore, description on IL1ß and TNFa treatments is missing. Please see p.7 second paragraph.

+) Histological examination: Again: n=?, replicates, how many individuals? Please see p.8 second paragraph.
Statistics: Consider ANOVA (plus post-hoc) test as the appropriate statistical test for comparing the means of three or more unmatched groups.

We have recalculated statistics using ANOVA followed by the appropriate post-hoc analysis. P-values that have changed are highlighted in the text and have been corrected in the figures.

*) The authors should discuss their findings with respect to recent studies on the impact of FTY720 in animal models:

Thank you for your input. We have amended the Discussion section to include these important recent studies. Please see p.13 first paragraph.

Minor Essential Revisions

*) From my understanding, Angyal et al in their study (reference 14) do not conclude that FTY720 enhances damage severity as stated by the authors in their discussion (page 10, first paragraph). Could the authors justify this point? Indeed Angyal et al. did not find a statistically significant difference between FTY720 treated and untreated mice. However, a clear trend towards more arthritis severity was observed when animals were treated after arthritis had evolved (Angyal et al., Fig.4a). In the light of our results, one could speculate that the difference is due to additional damage of FTY720 in the presence of inflammatory mediators.

*) The standard deviation of one bar (TNFa treated, 1μM FTY720-P treated) is missing. We excuse for this error. It has been corrected.

*) An interesting point of the study is the “dichotomous” effect of FTY720 in bovine chondrocytes, which might warrant a more detailed discussion. We have extended the discussion, please see p.13, second paragraph.

Reviewer: FRANCESCO GRASSI

Major compulsory revisions.
1. Statistical analysis needs some attention. Given the experimental design and the size of the sampling the use of Student T test for data analysis requires a more accurate justification. The authors should clarify what is the rationale for this choice over other tests (i.e. ANOVA analysis of data) and provide supporting information regarding their claim that data are normally distributed.

Thank you for your comments. We have recalculated statistics using ANOVA followed by the appropriate post-hoc analysis. Normal distribution of data was assured using Kolmogorov–Smirnov test. A detailed description is provided on p.8, third paragraph. P-values that have changed are highlighted in the text and have been corrected in the figures.

Minor essential revisions.
2. Methods, page 5: the method section for western blot analysis lacks several important details that should be provided in a revised version.

A detailed description is provided on p.6, second paragraph.
3. Methods, Results and Figure legends. Duration and range of concentrations of treatment with FTY720-P are incoherently stated (5 or 8 days of duration of treatment; 0.1-3 #M or 0.5-3#M concentration range). Please adjust accordingly.

Indeed the treatments were of different duration. DMB is more sensible than safranin O staining. Therefore, explants assessed by histochemistry were treated longer. For DMB analysis explants were treated with 0.5 to 3μM FTY720-P, as we did not observe changes in GAG depletion with lower concentrations.

4. The biological significance of iNOS downregulation by FTY720-P could be further discussed in the context of cartilage homeostasis. Did FTY720-P have any affect on unstimulated (basal) expression levels of iNOS? FTY720-P also reduces the basal expression of iNOS. This finding and its impact on cartilage homeostasis are now discussed on p.13, second paragraph.
5. In figure legend 1, the authors could restate that the data refers to chondrocytes grown in monolayer. We have amended figure legend 1 accordingly.

6. In figure legend 3, the label 'A' is missing in the text. We excuse for this error. It has now been corrected.

Reviewer: Klaus Bobacz
Remarks:
1. The authors state that the phosphorylated form of the compound FTY720 was used. Did the authors test the non-phosphorylated form on articular chondrocytes?

We thank you for this important remark. We tested non-phosphorylated FTY720 and observed a reduction in iNOS and MMP-13 expression. The maximal effect was observed after 6h and was less pronounced than with FTY720-P. This indicates that chondrocytes are able to phosphorylate FTY720 to its active form. We have added this information on p.9, second paragraph.

2. How many different animals were used and how many independent experiments were performed for each readout system. This should be clearly stated in the Materials & Methods section.

We apologize for the lacking information. A detailed description is now provided in the Material & Methods section.

3. Cartilage samples were acquired from adult animals. Did the authors also test cartilage derived from juvenile animals? A comparison would have been interesting since degenerative changes that occur in the elderly could influence the effects of FTY720.

We did not test cartilage from juvenile animals in the current study. We chose to work with adult cartilage to simulate the situation of human RA and OA. Both are diseases of the adult and elderly. We agree however that a comparison between juvenile and adult cartilage would be very interesting. It is possible that S1P (and FTY720-P) signaling in juvenile cartilage is different from adult. Such a situation was found in endothelial cells (http://www.ncbi.nlm.nih.gov/pubmed/18765664). Despite the interest in this topic, we felt that such a comparison would lead beyond the scope for the current study.

We thank you and the reviewers for their efforts and hope to have addressed all concerns. Thank you for consideration of our work for publication in *BMC Musculoskeletal Disorders*.

Sincerely,
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