Author's response to reviews

Title: The oncofetal gene survivin is reexpressed in osteoarthritis and is required for chondrocyte proliferation in vitro

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Version: 3 Date: 23 June 2011

Author's response to reviews: see over
Dear Sirs,

we thank the reviewers and the editor for their helpful comments and we feel that the resulting manuscript has improved substantially.

In the following we have responded to the reviewer’s comments by inserting our responses accordingly.

Reviewer's report

Title: The oncofetal gene survivin is reexpressed in osteoarthritis and promotes proliferation and cell survival in chondrocytes in vitro

Version: 2 Date: 12 May 2011

Reviewer: 1

Reviewer's report:
Major Compulsory Revisions:
1. It is not accurate to say in the concluding paragraphs of the Abstract, Results, and Discussion that this is the first time that survivin has been studied in human chondrocytes (see Gagarina V, Carlberg AL, Pereira-Mouries L, Hall DJ: and a quick search finds that it has been studied in other forms of arthritis (PubMed search yields 18 papers), as well as in chondrosarcoma. Also, a quick search of either survivin or Birc5 with bone yields over 100 papers. Thus, it is incorrect to say that “no report exists analyzing survivin’s role in the musculoskeletal apparatus beyond an “oncofoetal” condition”.

Response: We thank the reviewer for her most constructive comment on the revised manuscript. A role for survivin in the musculoskeletal system has been discussed in various preceding publications. While the attention of most of these manuscripts has been focused on cancer and the inflamed synovial membrane in rheumatoid arthritis, no publication has examined a potential role of survivin in arthritic human cartilage. The reviewer mentioned the study of Gagarina et al. (J Biol Chem. 2008 Jan 4;283(1):648-59). Here, the authors report on the induction of IAP family (XIAP, survivin, cIAP1, cIAP2) protein expression by cartilage oligomeric matrix protein in 293 and HeLa cell lines and in primary human chondrocytes. No data concerning survivin expression in untreated (i.e. untransfected) human chondrocytes is shown or reported. Either empty vector or control siRNA were transfected when survivin mRNA or protein was detected. Furthermore, no controls for the determination of the specificity of the detection of survivin has been shown or described. Nevertheless, we included the important study of Gagarina et al. in the introduction and discussion. Moreover, we revised the manuscript concerning the significance of the study.

2. Methods (RNA extraction and real-time PCR) and Figure 1G: Please indicate whether the RNA was extracted directly from cartilage (and if so, how the samples were handled to give good yield and quantity) or from cell isolates.
Response: In Figure 1 G relative gene expression rates of human cartilage specimens were detected by real time PCR. For RNA isolation, a protocol primarily described by the group of Thomas Aigner was used [McKenna LA, Gehrsitz A, Söder S, Eger W, Kirchner T, Aigner T Effective isolation of high-quality total RNA from human adult articular cartilage. Anal Biochem. 2000 Nov 1;286(1):80-5]. The high average yield of RNA reported, 8.4 µg/g for normal and 6.7 µg/g for osteoarthritic cartilage from the original publication were not reached in the present study. The methods section and figure legend have been revised and extended.

3. Figure 4: Please write in the figure legend what the in vitro ischemic conditions are.

Response: Figure legend has been revised accordingly.

4. Overall, the Discussion is poorly referenced.

Response: Discussion has been revised accordingly.

5. The in vitro findings do not link up directly with the in vivo descriptive findings of the increased survivin expression in chondrocyte clusters in OA cartilage. An obvious in vivo model would be the growth plate during embryonic development, where hypoxia and ER stress can occur. Such an analysis would round out the paper nicely. At the very least, a more complete study on the molecules upstream or downstream of survivin involved in survival and stress would be warranted, including colocalization studies in the human cartilage samples.

Response: We agree with the reviewer, that the data gained from in vitro experiments in primary human chondrocytes cannot replace in vivo models. Yet, the present study describes the expression of survivin mRNA and protein in human osteoarthritic cartilage. Furthermore, in vitro experiments confirmed the expression of survivin in primary human chondrocytes and indicated towards a role for survivin in chondrocyte proliferation and antiapoptosis. We thank the reviewer for the most stimulating suggestion to include supplementary in vivo experiments in the current manuscript. Understandably, these questions need to be clarified in future studies.

Minor Essential Revisions:

i. There are several errors of English expression that need to be corrected.

Response: We thank the reviewer for carefully examining the manuscript.

ii. endochondral", not “enchondral”.

Response: Text has been revised accordingly.

ii. Write “chondroitinsulphate” as two words. Etc.

Response: Text has been revised accordingly.
Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests.

Reviewer's report

Title: The oncofetal gene survivin is reexpressed in osteoarthritis and promotes proliferation and cell survival in chondrocytes in vitro

Version: 2 Date: 9 May 2011

Reviewer: 2

Reviewer's report:
The current manuscript describes the production of survivin by OA chondrocytes and its role in in vitro proliferation. As such the finding is new and worth publishing. However, quite some adaptations need to be made.

Question 1: Is the question posed by the authors well defined?
The question if the oncofetal gene survivin is reexpressed in OA is well defined in the introduction. Also the clinical relevance is nicely addressed.

Question 2: Are the methods appropriate and well defined?
In general the methods lack some detail
- A description of negative controls for immunohistochemistry for survivin is missing in the M&M section.

Response: We thank the reviewer for the helpful annotation. We revised the material and methods section accordingly.

- The methods section describes immunofluorescence for survivin on paraffin sections of cartilage tissue. However, there is no mentioning of the corresponding result (probably because paraffin embedding often results in autofluorescence).

Response: Figure 1 A and B show immunofluorescence for survivin on paraffin embedded sections. We clarified this in the figure legend and in the results section. Of note, after the omission of either primary or secondary antibody no significant autofluorescence was detected.
How long was the BrdU exposure of the cells?

Response: Cells were exposed for BrdU incorporation over 4 hours. Material and methods section has been revised accordingly.

When more than two groups are included in an experiment, ANOVA with appropriate post hoc testing should be performed.

Response: We thank the reviewer for this helpful comment. ANOVA and post hoc test according to Bonferroni have been applied. See revised manuscript.

Question 3: Are the data sound?

In the results section the authors present data on the difference between healthy and OA cartilage. The distinction is made microscopically. Could the authors describe how they distinguish between healthy and OA. Did they used a H&E staining or safranin-O staining and a scoring method? Is the healthy part of the osteoarthritic cartilage used from the OA patients? Describe in M&M section.

Response: We revised the materials and methods section to clarify the histologic distinction between arthritic and non-arthritic cartilage.

Irrelevant differences in the experiments with inhibition of survivin by RNAi have been described. Groups treated with survivin siRNA should be compared to those transfected with irrelevant siRNA, in this case GFP siRNA and not to untransfected cells. Possibly performing the right statistical analyses (including the comparison between GFP vs survivin) may help. Additional experiments with a different stimulus (e.g. IL-1/TNF) may shed more light.

Response: The reviewer is right. In the revised manuscript GFP- and survivin siRNA treated cultures are compared in Figure 4. Additionally, analysis of variance followed by Bonferroni’s post hoc test were performed to reveal statistical significance.

An decrease in BrdU staining should also be reflected in a decrease in cells in S phase. Authors should comment on this discrepancy.

Response: The reviewer emphasizes correctly the discrepancy between the unaltered S phase fraction 48 hours after the knock down of survivin and the reduction of BrdU uptake 48 hours after the transfection of survivin specific siRNA. The differences seen in both markers could result from the heterogeneity of both experimental methods. First, the S phase fraction is not a reliable measure for the detection of cellular proliferation because the DNA distribution gives no kinetic information about cycling or noncycling cells. Whereas, the used BrdU assay yields a sensitivity in detecting cellular proliferation comparable to a radioactive thymidine assay [Maghni K, Nicolescu OM, Martin JG. Suitability of cell metabolic colorimetric assays for assessment of CD4+ T cell proliferation: comparison to 5-bromo-2-deoxyuridine (BrdU) ELISA. J Immunol Methods. 1999 Mar 4;223(2):185-94.]
Furthermore, for FACS PI staining cells were cultured over 48 hours under exposure to siRNA before being harvested and stained with propidium iodide. This differs considerably from the BrdU assay. Here, 48 hours after the knock down of survivin living cells were exposed over 4 hours to BrdU. Nevertheless, we stressed the discrepancy in the main text and thank the reviewer for providing the opportunity to improve the revised manuscript substantially.

Question 4: Does the manuscript adhere to the relevant standards for reporting and data deposition?
Yes, except for the issues mentioned elsewhere.

Question 5: Are the discussion and conclusions well balanced and adequately supported by the data?
In OA the problem is apoptosis of the chondrocytes, so reexpression may be beneficial for OA. This could be better addressed. Do OA chondrocytes better withstand apoptotic cues then healthy chondrocytes?

Response: Manuscript was revised accordingly.

Question 6: Are the limitations of the work clearly stated?
- It cannot be stated with certainty that macroscopically healthy OA carilage is equivalent to healthy cartilage from non-affected joints. If the authors think so, they have to show some evidence from literature that this is the case.

Response: The reviewer is right. We have revised the corresponding sections of the manuscript and used a more balanced nomenclature.

- The effects of inhibition of survivin found are relatively small. The authors should mention this in their discussion.

Response: Both, the effect seen on cell cycle distribution and on BrdU uptake after 48 hours are significant. The conclusion drawn from the apoptosis assays have been discussed more carefully in the revised manuscript.

Question 7: Do the authors clearly acknowledge any work upon which they are building, both published as unpublished?
Not always. In the discussion references are lacking. For example, in the description of survivin’s participation in the passenger complex or in the statement on “prominent functions of survivin”. On the whole, 19 references are somewhat limited.

Response: The discussion and reference list has been extended accordingly.

Question 8: Do the title and abstract accurately convey what has been found?
- In the title the authors state that survivin promotes proliferation and cell survival. However, what they showed is that inhibition of survivin inhibits proliferation, which is not the same. Authors should change the title to “is required for proliferation”, otherwise they would falsely suggest that adding or overexpressing survivin would enhance proliferation. Moreover, the mere trend in the effect of inhibition of survivin on apoptosis does not support the conclusion “promotes...
survival”.

**Response:** We thank the reviewer for the comment on the title of the manuscript. It has been considered in the revised manuscript.

-Again, the suppression of survivin in stressed chondrocytes did not lead to a significant difference in apoptosis, as P<0.0678. As mentioned in the Discussion, there is a trend, and authors should rather use this term in their abstract or leave it out altogether.

**Response:** The conclusion drawn from the apoptosis assays have been discussed more carefully in the revised manuscript.

**Question 9:** Is the writing acceptable:
Overall the writing is acceptable. Slides are not “cooked” but “boiled” (M&M). “Brood capsules” should be “chondroid nests” or “chondrocyte clusters”.

**Response:** We thank for the help revising the manuscript. Writing has been improved, specific remarks have been considered.

Overall conclusion and recommendation for the authors:
In the current study the authors address an important difference between ‘healthy’ and OA chondrocytes, which leads to better understanding of the role of chondrocytes in a pathologic condition as osteoarthritis. However, to be accepted some minor essential revisions should be made. The authors should describe how they distinguished between healthy and osteoarthritic chondrocytes and cartilage and which staining they used (M&M). The authors should use the GFP-siRNA-transfected chondrocytes as the control for their statistics and clearly show significant differences between groups before speaking of increased or decreased activity. Also, the authors should clearly acknowledge any work upon which they are building by adding the right references. Statistics should be redone and the appropriate conditions compared.

**Level of interest:** An article of importance in its field

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:**
I declare that I have no competing interests
Reviewer's report

**Title:** The oncofetal gene survivin is reexpressed in osteoarthritis and promotes proliferation and cell survival in chondrocytes in vitro

**Version:** 2 **Date:** 2 May 2011

**Reviewer:** 3

**Reviewer's report:**
Lechler and co-authors studied the expression of oncogene survivin in cartilage of patients with osteoarthritis (OA).

1) A quick search for survivin and joints using PubMed retrieved 18 publications, 14 of them are original. Thus, the claim of priority is overestimated. Previous publications in the field need to be included in the introduction and the novelty of the findings need to be discussed in relation to previous publications in the field.

*Response:* The reviewer is right. In the revised manuscript additional key publications have been cited, and we balanced the interpretation of our findings.

2) All samples of the articular cartilage were obtained from patients with osteoarthritis undergoing prothesis surgery. Thus, all cartilage tissues were severely affected and no normal healthy tissue was actually studied. Statement about “normal” tissue should be changed in the abstract and in the results.

*Response:* The manuscript has been changed accordingly.

3) Description of RT-PCR does not meet recent recommendations for gene expression analysis. This may be improved by adding the amount of mRNA used for RT-PCR and indication of a second reference gene used.

*Response:* The description of the RT-PCR has been revised accordingly. We used β-actin a second reference gene. No significant discrepancies were seen when values were normalized against both house keeping genes. This has been added in the revised manuscript.

4) Which number of cells was used for transfection analysis? What was cell survival at 24 and 48h after transfection in specific siRNA culture and in GFP siRNA culture?

*Response:* For the transfection analysis cells were seeded in 6 well dishes at 1.5 × 10^5 per 3.5 cm well, 24 hours before knockdown was performed. This information has been included in the materials and methods section. Cell proliferation, caspase 3/7 activity and cell cycle distribution were assessed. From these assays no definite conclusion can be drawn about cellular survival.

5) The aim and main findings need to be carefully re-evaluated.
Response: Key conclusions have been carefully re-evaluated and the manuscript has been changed accordingly.

Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests: I declare that I have no competing interests.