Author's response to reviews

Title: A short-term in vivo model for giant cell tumor of bone

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

Thank you for reviewing our manuscript “A short-term in vivo model for giant cell tumor of bone”.

The name of the ethical committee that approved this study is “Ethik-Kommission der Ärztekammer Westfalen-Lippe und der Medizinischen Fakultät der Westfälischen Wilhelms-Universität Münster”, file number 2008-279-f-S.

The commentaries of the reviewers were very helpful to us and will increase the quality of the manuscript. Here are our point-by-point responses to the concerns of the reviewers. We submitted a revised version as well as a manuscript with highlighted changes.

We hope that the paper is now suitable for publication in your journal.

Sincerely,

Maurice Balke, MD
(corresponding author)
Response to Referee 1:

1. Page 5, giant cell suspension. After the GCT tissue was homogenized, was there any giant cells (multinucleated cells) in the cell pellet? This information is important for the hypothesis of GCT solidification.

   ➤ This is an interesting point, unfortunately we did not evaluate the cell pellet with regard to this specific question. However, given the fact that a relatively large portion of the tumor tissue was homogenized, it seems very unlikely that no multinucleated cells were present.

2. Page 5, Thawing giant cell suspension. What the number of the cells were contained in the 20µl suspension?

   ➤ We were not able to count the exact number of cells. The solution consisted of tumor cells, blood cells, debris as well as components of the spongy bone. Therefore it was not possible to reliably count the cell numbers. However, based on experiences with grafting of cell lines, usually one to 3 million tumor cells are necessary to form a solid tumor on the CAM.

3. Page 8, second paragraph. “vascularized without significantly increasing in size” means that tumor cells didn’t increase? The growth ratio of tumors should be calculated by some methods during culture.

   ➤ The tumor volume didn’t increase over time once the tumor was established. A calculation of the growth rate of the tumors during culture would be interesting, but since there was no relevant growth we did not perform this analysis. One has to keep in mind that the model is a short-term model which recapitulates the first tumor cell-host interactions such as invasion and angiogenesis, but not tumor expansion.

4. Page 8, FISH. In figure 5, it’s difficult to identify vascular endothelium. HE or another endothelial immunohistochemical stain with the same section should be shown.

   ➤ You are right that it is somehow difficult to identify the endothelium. Because we were limited in the number of figures and added another figure requested by another reviewer we decided not to show another HE staining. From figure 3 it is clear that the tumors are vascularised. In figure 5 B some erythrocytes (within the blood vessels / vascular endothelium) are indicated by white arrows.

5. Page 10, Discussion third paragraph. The author hypothesized that high embryonic mortality rate in GCT culture was a result of GCT tumor aggressiveness. But commonly osteosarcoma is thought to be more aggressive than GCT. And this study seems to show that GCT cells didn’t increase, while osteosarcoma cells grafted grown up in CAM [Author’s paper published in 2010]. What factor was defined as the more aggressive one in GCT?

   ➤ We mentioned in the discussion that the reasons remain speculative, but as you said the term “aggressiveness” is confusing when comparing GCT to osteosarcoma. We changed the respective paragraph to “…but it is possible that the mortality rate may correlate with tumor (graft) and host interactions.” Even though speculative, it might well be that secreted factors from the grafted tumor cells perturb embryonic development.

6. Page 11, first paragraph. This theory is interesting in the point of tumor solidification, but there is one question with this theory. What do the authors think about the origin of multinucleated cells in solid tumor cultured in CAM?

   ➤ The multinucleated giant cells originate from the human tissue, as they are positive for the red human chromosome staining in the FISH. Weather they are still present from the human tissue or newly developed during solidification of the tumors on the CAM remains speculative.
Response to referee 2:

1. Since a newly developed model is presented, the methods should be explained and discussed more in detail:
   a) A schematic drawing or a photography of the CAM assay should be provided to get a better imagination of how the whole preparation looks like.
   b) What are the techniques and the equipment used to be able to remove albumen from the egg, to detach the embryo, to cut a small window in the eggshell, to seal the window with a tape, to lacerate the CAM surface, and to deposit the tumor suspension?
   ➔ Since we were limited in the number of figures for this article we decided not to add any instructional drawings. But inspired by your comments we recorded 6 instructional videos showing all relevant steps for the CAM assay and added these videos as additional files.
   ➔ In the Material and methods section we added “For further information of the technique of the CAM assay see instructional videos in the ‘additional material’ section (Additional files 2 to 7: Movie 01 to Movie 06).” to the CAM paragraph.

   c) What happened with the embryos after removal of the tumors? Are there any hints for metastases in the embryos?
   ➔ The embryos were disposed after tumor removal. No further evaluation of the embryos was performed, thus we are not able to give information on metastases. Further studies with respect to this question are planned.

2. A detailed experimental protocol should be provided. Why is the number of Controls and grafted CAM-assays different?
   ➔ We hope that the videos provide missing information. There are no specific reasons for the differences in the numbers of the controls and grafted eggs. For each experiment / different tissue specimen a different charge of eggs was used, thus the number of available eggs differed. The available amount of tumor grafts varied due to the different sizes of the human tumor material.

3. Scale bars should be provided for the Figures 1, 2, 4, and 5.
   ➔ Done as recommended for Figures 1, 2 and 4 and changed the respective figure legends

4. In the Figure legends, there is no description of image “D” in Figure 4.
   ➔ We added “in D” as recommended

5. Would it be possible to present more parameters in the Result section?
   a) The growth rates (V mm³) over time of the 10 GCTs should be presented.
   ➔ Because there was no increase in size over time, the growth rates over time were not calculated

   b) What were the standard deviations (or maximum vs. minimum) per group of the tumor volumes presented in Table 1?
   ➔ We added the SD as recommended.

   c) Would it be possible to demonstrate when first perfusion of newly formed vessels in the tumors took place?
   ➔ We did not record this parameter. However, since tumors appeared red after 48h, it is estimated that tumor angiogenesis is initiated quite early, starting around the second day.
d) Was there an increase of the vascular density over time?

*We did not perform any analysis regarding this question. The most important fact is that tumors attracted chick blood vessels, thus proving that there was an interaction between the human tumor material and the chick host.*

6. The advantages and limitations of the model should be discussed more in detail

a) Since an in vivo model is used, in the Discussion there should be described which parameters can be measured in principal with the CAM assay in vivo by using intravital microscopy or other technical devices.

b) In which way drugs could be administered to the tumors – e.g. i.v. or locally by superfusion?

c) Would it be possible to use the CAM assay for a longer time than 6 days? If not, what are the reasons for this?

» More detailed discussion of the CAM assay itself would be out of scope of the present paper. We referred to the former paper of the author on osteosarcoma ([http://www.ncbi.nlm.nih.gov/pubmed/20202196](http://www.ncbi.nlm.nih.gov/pubmed/20202196)) which is more of “instructional” character. Longer than 6 days is not possible because the CAM starts to degenerate around day 18 and the chick hatches at day 21.
Response to referee 3:

- Results. “Histological and Immunohistochemical findings” paragraph. “The giant cells reacted for CD68 and CD51, indicating that these cells were osteoclasts; there were also CD68+ macrophages in the mononuclear component...”. CD68 and CD51 can both be expressed by macrophages that derive from the same precursor of osteoclasts. Therefore, it is hard to establish if the staining is really associated to an osteoclast-like or to a macrophage-like phenotype. Moreover, the figure showing results on CD68 and CD51 is not included in the paper, although it is commented in the result section. According to the aim of the manuscript, the demonstration of the formation of osteoclasts rather than reactive macrophages in the GCT model is quite striking. In fact, the authors state that osteoclast cultures form GCT are quite difficult to obtained, and therefore an alternative model is needed. On the contrary, both monocytes and stromal-like cells can be easily isolated and characterized, as documented by several papers. The figure showing the CD68 and CD51 staining should be added, and additional demonstrations that osteoclasts rather then macrophages were obtained are needed.

- Discussion, Third paragraph. “Histologically the GCTs in our model closely resembled the pulmonary metastases of GCT......the latter were generally less numerous and less multinucleated than in the original tumor, a common morphological finding in GCT lung nodules”. The association between GCT model in CAM and GCT lung metastases is rather speculative. Moreover, the cited reference reports that in lung metastases, there are few multinucleated cells in respect to the primitive tumor, but the number of nuclei are not mentioned.

- Discussion, Third paragraph. “Thus the model would appear to simulate the early phase of tumor seeding, one of the initial steps in the development of a metastasis or local recurrence”. This statement is speculative. Moreover, results obtained about the similarities with lung nodules are contradictory in respect to the aim of the study. In fact, the authors state that their GCT model is more similar to the lung nodules rather that to the primitive tumor. GCT metastases are very rare in GCT patients, and are not the best model to study GCT biological

The reviewer has questioned the nature of the giant cells in the lesion which developed on the CAM. It should first be appreciated that the lesions are very small and that all the usual cytochemical (eg TRAP) and functional (eg lacunar resorption) markers to confirm the osteoclastic nature of the giant cells in these lesions could not be employed. As requested, we have now added a figure showing CD51 expression by giant cells (now Figure 5). We have supplemented this with a figure showing absence of CD14 expression. CD14-, CD51+ expression is characteristic of osteoclasts (see below). We were fully aware that macrophages and osteoclasts, as well as macrophage polykaryons express CD68 – indeed one of the authors was the first to describe this. We have now clarified our findings with regard to the antigenic phenotype of the giant cells identified in the lesions on the CAM and correlated them with findings in both osteoclasts and osteoclast-like giant cells in GCTB with appropriate references (Page 6, para 4; page 8 para 3).


- Results. “Histological and Immunohistochemical findings” paragraph. “Tumor giant cells frequently contained more than five nuclei in the original tumors but were smaller and contained fewer nuclei in the cultured samples...” also macrophages can have 4-5 nuclei. See above for comments.

The respective paragraph has been changed.
and molecular mechanisms that develops in the bone microenvironment.

- **We agree that our assertion that the association between the GCT model in the CAM and GCT lung metastases is speculative. However, as has been suggested in several investigations (including the one we have referenced and the reviewer has quoted), these metastases most likely form by seeding from primary tumours and implanting in the lung. We noted that the tumours appeared to grow on the CAM rather than invade it, producing an implant-like rather than infiltrative growth pattern. This is the reason why we have drawn attention to the potential of the model we describe to study the development of GCT lung metastases. The reviewer, however, is correct in pointing out that our previous referenced study noted only that lung metastases contain fewer multinucleated cells and not giant cells with fewer nuclei. (This was an impression of one of the authors and has not been formally verified). Reference to the number of nuclei has been deleted (Page 11, para 1 – rewritten).**

- References. Add the year to the reference n. 49.

  - **Done as recommended**

- Figures. In figure 4a and B, the authors show indicate with arrows the multinucleated cells because they are poorly evident.

  - **Done as recommended, indicated by arrowheads**

- It is hard to be convinced that multinucleated cells can resist to a cycle of freezing and defrosting. It could be interesting to investigate if the multinucleated cells in the GCT specimens are newly formed and derived from implanted human monocytes or if they are rather derived directly from the cell suspension obtained from the tumor biopsies. Suggested experiment: the TRAP staining associated to nuclear staining of defrosted cells cultured in vitro at 3-4 days after seeding.

- To demonstrate that among the obtained cells in the GCT in the CAM there are osteoclasts, a cell suspension isolated from the tumor mass, as previously done for tumor biopsies, could be seeded for a bone resorption assay.

  - **GCT multinucleated cells did resist the treatment described – they are remarkably hardy – as evidenced by the morphological and immunophenotypic findings. It is not possible to carry out TRAP staining on the fixed specimens we employed or investigate if the giant cells are newly formed from monocyte/macrophages within the tumour due to the small size of the sample available for study. Studies, however, are planned to confirm that the giant cells are capable of lacunar resorption.**