Author's response to reviews

Title: Suppression of Local Invasion of Ameloblastoma by Inhibition of Matrix Metalloproteinase-2

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Suppression of Local Invasion of Ameloblastoma by Inhibition of Matrix Metalloproteinase-2

Anxun Wang, Bin Zhang, Hongzhang Huang, Leitao Zhang, Donglin Zeng, Qian Tao, Jianguang Wang, Chaobin Pan

Dear Dr. Edwards,

We wish to thank you and the reviewers for their thoughtful comments on our manuscript. We have addressed all the reviewers’ comments in the revised manuscript and also give a point-by-point response to their concerns below. We also solicited the help of an English-speaking editor to correct language problems.

Sincerely yours,

Anxun Wang
Reviewer 1

Major

1) The authors have characterized the culture using cytokeratins 14, 16 and 18. There are evidences showing that cytokeratins 13 and 19 are consistently expressed in both ameloblastoma and tooth germ. I believe that the culture should be labeled with antibodies against these CKs.

**Answer:** In order to characterize ameloblastomas, which are of epithelial origin, the expression patterns of cytokeratin were examined which has been performed in many studies (1–9). Harada *et al* [6] found that ameloblastoma epithelial cells express cytokeratin 8, 14, 18, 19, but not CK10, 16. Although CK13 or CK19 were expressed in most of ameloblastoma, they are not the special markers of ameloblastoma. In different histological type of ameloblastoma the expression of CK13 or CK19 was different [10, 11]. In our study, CK used to confirm the epithelial origin of the ameloblastoma. We chose CK 14, 16, 18 and found that CK14 and 16, but not CK18 were expressed in ameloblastoma cells indicating that the cultures were primarily composed of ameloblastoma cells of epithelial origin.


3. Thesleff I, Eknblom P. Distribution of keratin and laminin in ameloblastoma.


2) Results in Figure 1 are not well described. What kind of microscopy is presented in Figs A and B? Furthermore, to better illustrate and to strengthen the manuscript, cytokeratin staining should be carry out through immunofluorescence instead of peroxidase.

**Answer:** Phase-contrast microscopy is presented in Figures 1A and B and the title of the figure has been modified to include this information. While both immunofluorescence and peroxidase can be used to detect cytokeratins, we found that more studies appeared to use peroxidase (1-6) why is why we used peroxidase to detect cytokeratin in this study.


3) Fig. 1D shows non specific nuclear staining for MMP2. Why? What kind of controls were used for immunofluorescence?

**Answer:** In Figure 1D nuclei were counterstained with propidium iodine (PI) and we have modified the figure title to reflect this. In this study, controls were indeed included in which the first and/or second antibodies were withheld. In the control samples, the green fluorescence can not be detected in the cytoplasm, only the red fluorescence was obvious in the cell nucleus (data not shown).

4) Table (invasion assay) show results mock control. Why? Were these results not different from scrambled RNAi groups?

**Answer:** There was statistically significant difference between pcDNA-TIMP-2 and pRNA-MMP-2 transfection with the mock, lipifectamine, or vector controls. Our manuscript has been revised to reflect this difference (table 1).

5) The zymogram of figure 2 shows only one gelatinolytic band corresponding to MMP2. Is this the proenzyme or the active form? It would be interesting to run in the same gel a MMP standard, to confirm the nature of the band.

**Answer:** In Figure 2, the gelatinolytic band corresponded to the active form of MMP-2. In our raw data, we detected two gelatinolytic bands--one for the
proenzyme form of MMP-2 and the other for the active form of MMP-2. In the manuscript, we analyzed the activity of MMP-2, and therefore only included information pertaining to the active form of MMP-2. Further, we utilized a protein marker to confirm the nature of the bands in the each gel. Two bands at molecular weights of 72 KD and 64 KD (corresponding to the proenzyme form or active form of MMP-2) were identified.

6) Gelatin zymograms usually detect MMPs 2 and 9. The authors should include in the results the expression of MMP9, in order to demonstrate the specificity of MMP2 knockdown.

**Answer:** In this study, both MMP-2 and MMP-9 were detected in the gelatin zymogram. The MMP-9 was not different after MMP-2 knockdown compared to pre-knockdown values. According to the design of MMP-2 siRNA, the sequences were checked using nucleotide BLAST to ensure that it had no homology to any other known human gene, including MMP-9 (as described in the manuscript). Therefore, it is specific to knockdown MMP-2 and then we subsequently elected to only show the active form of MMP-2 in our study.

7) Figure 2 shows an immunoblot with expression of MMP2. Why was only one band detected? The same blot showed a band corresponding to GAPDH. I assume this is a loading control, but there is no mention of loading controls in material and methods.
**Answer:** In the raw data, two bands of MMP-2 were detected—one for the proenzyme form and the other for the active form. In our study, the objective was to compare the activity of MMP-2 so we only included data for the active form of MMP-2 (the 64KD band).

GAPDH served as a loading control. It has been revised in material and methods.

**Minor**

The manuscript will benefit from professional language editing.

**Answer:** We solicited the help of an English-speaking editor to correct language problems.
Reviewer 2

Major Revisions

1. MMP-2 aka gelatinase A is a collagenase type-4. It has been implicated in digestion of basement membrane containing collagen type 4. There has not been much evidence for its role in ECM degradation mainly constituted by collagen type 1. Authors have investigated the role of MMP-2 in local invasion that involves ECM degradation. On the other hand, MMP-1 is a collagenase type-1 and has been implicated in ECM degradation. There has been no investigation for the expression or activity of MMP-1 and how it may have been affected by MMP-2 down regulation. This is a major limiting factor of the study, and has not been commented upon by authors.

Answer: Extracellular matrix (ECM) is composed of interstitial matrix and basement membrane. MMP-2, which degrades basement membrane containing collagen type 4, is important in the invasiveness of tumor cells [1]. So the degradation of the ECM must be changed as the degradation of basement membrane. The manuscript has been revised as suggested.


2. Majority of MMP production in cancers of epithelial origin has been attributed to activated stromal cells of mesenchymal origin in previous investigations involving intra-ductal epithelial carcinoma of breast. As shown by authors,
ameloblastoma cells have epithelial markers. In this investigation involving ameloblastoma cell culture there has been no consideration given to the MMP production by the matrix cells in vivo. Another limitation of the study that has not been commented upon.

**Answer:** While the majority of MMP production in cancers of epithelial origin has been attributed to activated stromal cells, many studies have revealed that ameloblastoma cells express and secrete MMP (1,2). In our study, ameloblastoma cells expressed MMP-2 and the purpose of our study was to investigate the relationship between MMP-2 activity and the invasion of ameloblastoma cells. As such, we did not study the effect of the MMP production by matrix cells. Nonetheless, this is a good question and we will continue to study the effect of MMP production by the matrix cells.


Minor Revisions

1. Authors need to revise the title for the manuscript.
**Answer:** The title has been revised as ‘Suppression of Local Invasion of Ameloblastoma by Inhibition of Matrix Metalloproteinase-2’

2. Authors may like to comment upon the decrease in invasiveness of cells from 53.3% with 2 microgram to 50.5% with 3 microgram of pcDNA-TIMP-2.

**Answer:** For the decrease in invasiveness of the ameloblastoma cells, there was a significant difference between 1µg and 2 µg or 3 µg of pRNA-MMP-2 or pcDNA-TIMP-2, but no significant difference between 2 µg with 3 µg of pRNA-MMP-2 or pcDNA-TIMP-2. The manuscript has been revised to reflect this data.