Author’s response to reviews

Title: Oct4 upregulates osteopontin via Egr1 and is associated with poor outcome in human lung cancer

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Response to comments from reviewers and editors

We truly appreciate all constructive comments and suggestions from both reviewers. We have adopted all the suggestions in the revised manuscript. The following are our point-to-point response to reviewers’ comments. (The comments are shown with italic and bold font).

Editor Comments:

1. The Keywords have been moved to below the Abstract (page 5 line 6).
2. All the anesthetic and euthanasia methods used on the animals were described in the section of animal study (page 15 line 15).
Reviewer 1:

1. To support the conclusion of the Oct4-upregulating Egr1 and OPN, site-directed mutagenesis was suggested to confirm the putative binding site of Oct4 at Egr1 promoter.

   Response: In figure 2, we have performed a serial deletion of Egr1 promoter to detect the putative binding site of Oct4. Then ChIP assay confirmed the binding of Oct4 to the DNA fragment encompassing the region from -413 to -309 bp within Egr1 promoter.

2. The data of patients should be calculated to explore whether Oct4 expression in tumor tissues could be associated with Egr1 and OPN.

   Response: We have shown the correlation of Oct4 and Egr1 expression (figure 1B) and Egr1 and OPN (figure 1C) in human tissues. These correlations revealed significance statistically.

3. The data of animal model should be separated from the cell model and showed tumor nodules directly.

   Response: The data of cell model has been moved to new figure 5.

4. The immunostaining results of animal models should add immunostaining results of OPN and positive control.

   Response: We added the Oct4 stain in new figure 4.

5. How many mice were used in the animal experiments?

   Response: Four mice were used for each group (page 15 line 13).

6. Figure 1: the immunostaining results are not very clear.

   Response: The picture quality of figure 1 has been modified.
Reviewer 2:

1. It is not clear what statistical analysis were done in detail. As it is described that it was just Student’s t-test, but more detailed information is necessary. Otherwise, there is a question on the reasonable value of the results obtained. The calculations using different groups are performed and examination of the assumptions of variance such as e.g., checking the normality of the variables as well as their homogeneity in variance (the variance of the data should be the same) are should be proved as done. All experimental variables have to be characterized by normal distribution and homogeneity of variances, which can be confirmed using the parametric Shapiro-Wilk test, and the Kolmogorov-Smirnov test for normality. Homogeneity of variance is, on the other hand, calculated by the Levene’a test and the Brown-Forsyth test, at the significance level (p) of 0.05. Since the criteria of normality of the tested variables are proved, it is possible to use parametric tests to search for differences between the groups of subjects described by the analyzed variables with e.g., t-test or ANOVA. What was done and in what extent within the shown study it not defined correctly in the text?

Response: For the section of statistical, we added wording to make it more clear, as shown here. After checking with Shapiro-Wilk test or the Kolmogorov-Smirnov test, all variables do not present as normality (all p<0.05). Thus, the non-parametric approach, including Wilcoxon rank-sum test or Kruskal-Wallis Test, is used to compare the difference between the targeted groups. The section of statistical analysis is reformatted. (page 16 line 2)

2. It is stated on page 17 and in Figure 1D that "tumors with higher expression of Oct4, Egr1, and OPN were not associated with higher recurrence rates compared with those with lower expression of Oct4, Egr1, and OPN, respectively (Figure 1D)". Is this contrary to the whole concept? Why such a phenomenon could be observed?

Response: Indeed, all these three biomarkers, Oct4, Egr1 and OPN all tended to associate with higher recurrence rates of lung cancer, but none of them reached significance statistically. The p values will be shown in the legend of figure 1D. By Kaplan-Myer analysis, high OPN expression dose associated with inferior disease relapse survival in human lung cancer (page 30 line 8).

3. Figure caption for Figure 1C should have rather "Egr1" instead of "Oct4".

Response: We changed it (page 30 line 7).
4. It is written that levels of OPN were quantified by ELISA. This is not a very accurate and precise technique in my opinion. Was the quantitative method applied in the study appropriately validated?

Response: The protein levels of OPN were measured in the medium after secretion from cells. ELISA assay would have been much more reliable to assess the soluble protein amount in my opinion.

5. No basic statistics were performed for data in Supplementary Figure S2.

Response: The experiment was performed only once, as a result we did not analyze the data statistically.