Author's response to reviews

Title: Short hairpin RNA targeting 2B gene of coxsackievirus B3 exhibits potential antiviral effects both in vitro and in vivo

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Author's response to reviews: see over
Dear Dr. Marshall and Prof. Henke:

Thank you for your letter and the comments on our revised manuscript entitled “Short hairpin RNA targeting 2B gene of Coxsackievirus B3 exhibits potential antiviral effects both in vitro and in vivo” (ID:1176956252655028). These comments are very valuable and constructive for improving our paper. We have studied the comments carefully and made corrections accordingly, which we hope will meet your requirements for acceptance. The main corrections in the revised version of the manuscript and the responses to the reviewer’s comments are as follows:

[Reviewer’s comments in italic, authors’ response in roman]

**Reviewer: Andreas Henke:**

*Minor essential revisions:*

1. **Page 3, line 8:** Do you really think that CVB3 “is among the most common and significant agents of infectious illness in humans”? What about HIV, HCV, HEV, influenza virus, Mycobacterium tuberculosis, denguevirus etc. etc.

   Thank you for your reminding. This sentence on page 3, line 8 described about CVB3 was not suitable. We have corrected that Coxsackievirus B3 (CVB3) is among the most common and significant causative agents of heart muscle disease in human.

2. **Please look carefully at your text again, especially in view of missing blanks (e.g. page 5 line 9) or unnecessary words (e.g. page 21, line 11 “CVB3”).**

   We have corrected these accordingly.

3. **Please provide a reference for the virus strain (CVB3M), which you received from**
Dr. Huber.

The reference for the virus strain has been added (new reference #18).

4. Page 6, line 14: The headline of this paragraph does not really fit the text. Here, you describe how the mice were treated and when the samples were taken. You are not describing the myocarditis model.

The headline of this paragraph has been changed to “Short hairpin RNAs treatment in vivo”.

5. Page 7 line 6: Here it is better to write: “In the supernatants, infectious virus were analyzed by…..”

We have changed this accordingly.

6. Page 7, line 11: From where did you receive you VP1 antibody?

We have corrected “VP1 antibody” to “anti-coxsackievirus B3 monoclonal antibody” (page 7, line 12). We purchased anti-coxsackievirus B3 monoclonal antibody from a company of Chemicon International.

7. In general, please state in the text how often each experiment was repeated and what is demonstrated at each figure (mean +/- SD of all experiments or a representative result?).

We have added the statement that experiments were carried out 3 times (page 5, line
13 and page 7, line 1). We have also added the statement about the mean ± standard deviation in legends of figure 1 A, 1 B, 1 C, 1 D, 2 A, 4, and 5 respectively.

8. Figure 3 A and B: Here a better labeling of the y-axis would be “Surviving animals [%]”, right now you are using “percent” twice.

We have deleted “(%)” in labeling of the y-axis of figure 3 A and B.

9. Page 20, line 18 and page 21, 3: Here, I guess, you mean “weighted” instead of “weighed”.

We have changed this accordingly (page 7, line 6, page 21, line 18 and page 22, line5).

**Major compulsory revisions:**

1. Figure 1 A and B: Please provide mean +/- SD concerning these experiments.

Figure 1B: Here, the distance on the x-axis between 18-24 h (6 h) and 24-36 h (12 h) is identical. Please adjust the diagram. Figure 1 A and B: Please provide a precise description how the transfection rate was determined. Figure 1E pGCL-NC: Please state that the identical picture is demonstrated via LM and FM. It seems that not the same section is demonstrated (also Figure 2B Lenti-NC). Figure 1 and 2: How was the transfection/transduction rate between your 2B expressing systems and the controls? Any differences?

We have added “mean ± SD” in both new figure 1 A, 1 B and their figure legends. We
have adjusted the distance on the x-axis of new figure 1 B. New figure 1 A and B: The transfection rate of pGCL-2B was determined by the percent of the area of GFP-expressing cells versus that of whole HeLa cells in one field calculated using Anymicro DSS™ digital shoot system (Beijing Yutianshijiweiye Technology Development Co. Ltd., China) (page 9, line 4). The transfection rate of control plasmid was similar to that of pGCL-2B (page 9, line 13). Figure 1 E pGCL-NC and 2 B Lenti-NC: the pictures demonstrated via LM and FM are from the identical section. Initially, to show the result more clearly, we cut the original picture to meet the space limitation. However, in this process, due to the technical issue, it did not show the exactly same field. Thus, it seems that the pictures demonstrated via LM and FM were not from the same section. Now we have showed the original pictures demonstrated via LM and FM from the same section (New figure 1 E and 2 B).

2. Results section, page 10, line 9 starting “Inhibition of CVB3 replication in coxsackievirus-induced myocarditis model”. Dose-response analyses would be much better. In your response to my comment during the first review process you mentioned that you tried other dosages as well. Why did you omit this in the revised manuscript? If you don’t get any protection if CVB3 is applied prior or at the same time as the treatment started what is the meaning of the experiments at all? Nobody is going to inject people with lentiviruses prior any infection.

We had tried threes dosages of plasmid or lentivirus in survival experiments. The results showed that the highest dosage could improve significantly the life span of
mice; however, transfection of pGCL-2B or Lenti-2B with lower doses had no difference on survival between treated group and control group (page 11, line 5). We did not get the expected protection when CVB3 was applied prior to or at the same time as the treatment started. This may be due to the slow expression of lentivirus/plasmid and the different ways of infection with lentivirus/plasmid and CVB3. Another reason is that the titer of CVB3 used was a LD100 of the virus, the high titer of CVB3 needs a high dose of shRNA and certain length of time to express. We agree to the reviewer’s comment that the treatment should be done after or at the same time of the CVB3 infection. Thus, ideally low CVB3 titer should be used to infect the animals before treatment. However, considering the main purpose of our experiments is to identify the effective antiviral target on CVB3 gene and demonstrated the sequence is also effective in an animal model. We think it may not be really necessary to repeat the experiments to test the CVB3 titer and the time of treatment for this model in this study. We believe that because of the limited application of lentivirus vector in human gene therapy, the effective method of shRNA delivery in human needs further studies.

3. Figure 6: Here, please give an exact description what “untreated control” means (which sample, why only IHC-CVB3).

We had added the exact description about “untreated control”. It means the control was treated without the first antibody in IHC staining (page 22, line 16). We showed one picture of one tissue in IHC staining experiments, because the difference between
IHC-CVB3 and IHC-GFP is the first antibody.

Yours sincerely,

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