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

Authors:

Hailan Yao (helen919@163.com)
Yangde Zhang (zyd99@189.cn)
Feng He (hefengfeng2001@163.com)
Caihong Wang (caihong@bjmu.edu.cn)
Zonghui Xiao (xiaozunghui@vip.sina.com)
Jizhen Zou (zheweiliu@163.com)
Fang Wang (cipwangfang@gmail.com)
Zhewei Liu (zheweiliu@163.com)

Version: 2 Date: 12 May 2012

Author's response to reviews: see over
Dear Ms. Pangilinan and Reviewers:

On behalf of my co-authors, we appreciate very much for giving us an opportunity to revise our manuscript. We also appreciate reviewers for your thoughtful comments and constructive suggestions on our 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 helpful for revising and 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:

[Reviewers’ comments in italic, authors’ response in roman]

**Reviewer #1 Henry Fechner:**

*Yao et al. have investigated the antiviral efficiency of shRNAs directed against the 2B gene of CVB3 for treatment of CVB3 myocarditis and pancreatitis. The shRNA was delivered to mice via tail vein injection using plasmid and lentiviral vectors in a prophylactically model. Anti-CVB3 shRNA treatment resulted in increased survival rates, reduced viral titers and attenuated tissue damage in the CVB3 infected animals. Although several studies have been published in the field of RNAi based therapy of CVB3 infections the study presents some new data and complete our knowledge.***

**Major Compulsory Revisions**

1. The vectors that were used seem to have an GFP expression cassette but I wonder that no data were shown confirming vector mediated GFP expression in the heart and
the pancreas, where the strong antiviral effects were seen. To confirm vector transfection/transduction efficiency the distribution of GFP expression in the target organs should be determined (immunohistochemistry for GFP or direct detection of GFP in tissue slides).

We have now added data showing the distribution of GFP expression in the heart and pancreas by peroxidase immunohistochemistry using a primary antibody against GFP. It shows that lentiviral vectors Lenti-2B and Lenti-NC express GFP efficiently in heart and pancreas of mice (new Figure 6), as well as pGCL-2B and pGCL-NC (Fig1).

![Image of IHC-GFP and Untreated Control for heart, pancreas, and liver](image)

Fig1. The distribution of pGCL-2B in mice. BALB/c mice were injected with pGCL-2B (40 µg/mouse) through tail vein, 24 h post injection, 8×10³ pfu/mouse of CVB3 was used as the challenge, and then euthanized at day 7 post CVB3 infection. Immunohistochemical analysis of GFP expression was performed on the sections prepared from the hearts, pancreas and livers.

2. To achieve high transfection rates in vivo plasmids will be injected by
hydrodynamic tail vein injection. Did the authors employ the hydrodynamic injection?

We did not use the hydrodynamic injection. Instead, we used Entranster\textsuperscript{TM}-in vivo transfection reagent to delivery the plasmids. Entranster\textsuperscript{TM}-in vivo is a nano-polymer transfection reagent synthesized by Engreen Biosystem Co, Ltd. To confirm the plasmids transfection efficiency, we determined the GFP expression in the hearts, pancreas and livers by Immunohistochemical analysis (Fig1).

3. A statistical analysis was carried out but in the figures and figure legends no p-values were shown. Please add. I am also in doubt about the application of the statistical test which was described in the material and methods. For in vivo experiments with a small number of animals in a group the Mann–Whitney U test is well suited.

We have reanalyzed the data from in vivo experiments using Mann–Whitney U test, and added the p-values into the figures and figure legends. The virus titers in the hearts and pancreases of mice infected with Lenti-2B were approximately $4\log_{10}$ lower than those in the control group ($P<0.01$ versus Lenti-NC group for both organs, n=5 in each group) (new Figure 4). Virus titers were $1\log_{10}$ lower in the heart and $2\log_{10}$ lower in the pancreas of mice that received pGCL-2B than that in the respective control mice (new Figure 5).

4. Despite authors have cited several publications employing anti-CVB3 shRNAs for treatment of CVB3 infections in vitro and in vivo the list is incomplete. Please insert
Fechner et al. 2008, JMM, which was one of the first studies demonstrating efficiency of anti-CVB3 shRNAs in vivo.

This reference has been added and commented upon (new reference #25).
Reviewer #2 Andreas Henke:

To the authors:

In the present manuscript the authors demonstrate a set of experiments to analyze the antiviral activity of short hairpin RNA (shRNA) against the 2B sequence of CVB3, which is expressed either by plasmid DNA or recombinant lentiviruses. Experiments were performed under in vitro and in vivo condition and demonstrate the inhibitory effect of this shRNA on virus replication and inflammatory reaction. So far, the results are convincing but prior publication several comments should be addressed and further experiments should be performed as it is stated here:

Minor essential revisions:

1. Next time, please provide a manuscript with numbered lines. This will help to direct comments.

We have added the numbered lines into the revised manuscript.

2. Please look carefully at your text, especially in view of duplications (page 7, chapter “Viral plaque assay” first line) or missing punctuations (page 11, first chapter, last line).

We have corrected those accordingly (line 16, page 7 and line 12, page 12).

3. M+M section, “Cell cultures, virus…..”: Please explain why a MOI of 0.01 was used. And please describe the origin of the CVB3 strain.

We used CVB3 at MOI of 0.01 to evaluate the impact of shRNA-2B on CVB3
replication in HeLa cells because the cytopathic effect appeared distinct difference between shRNA-2B group and shRNA-NC control group at 48 h post CVB3 infection. Firstly, to determine the dosage of plasmid we used in the experiment, HeLa cells in a 12-well plate were transfected with pGCL-2B at a series of doses and GFP-expressing cells were visualized by fluorescence microscope after 24 h. Our data showed that 1.25µg/well was the lowest dose of pGCL-2B to reach about 60% of cells expressing GFP (new figure 1A) and was less toxic to the cells. Then, after transfected with pGCL-2B (1.25µg) for 24 h, HeLa cells were infected with CVB3 at MOI of 0.1, 0.01 or 0.001 and CPE was observed 48 h later. Our data showed at 48 h post infection with CVB3 at MOI of 0.01, the rate of CPE formation in HeLa cells treated with pGCL-2B was about 20%, while the rate is about 80% in cells treated with pGCL-NC. This protective effect was absent at the high CVB3 dose of 0.1 MOI, and the low CVB3 dose of 0.001 MOI led to only 40% of cells showing CPE even in pGCL-NC-treated cells. Thus we used CVB3 at MOI of 0.01 to observe obvious differences between the two groups. Coxsackievirus B3 used in our study is the M strain, a kind gift from Dr S.A. Huber, University of Vermont, U.S.A. We have indicated this on line5, page5.

4. M+M section: “Recombinant plasmid......”: Please provide a much better description how the recombinant lentiviruses were generated (e.g. origin of the virus etc.).

We have added the description about the recombinant lentiviruses generation into
method part (line15, page5). pHelper 1.0, pHelper 2.0 and pGCSIL-GFP plasmids were purchased from Shanghai GeneChem Co. Ltd (Shanghai, China).

5. M+M section, “Organ virus titers....”: A much better analysis of the vial load in infected tissue is based on the weight of the analyzed organ. The viral titer should be described as pfu/gram. Please provide these data.

We have changed the viral titers in the infected tissues according to the weight of the heart or pancreas recorded initially, and expressed the titer as pfu/g (line7, page7; new figure4 and new figure5).

6. M+M section, “Viral plaque assay”: Why did you use cell monolayer with only 90 % confluence?

We used cell monolayer with 90% because this density could ensure to form the plaques and left some spaces for cell reproduction.

Major compulsory revisions:

1. Results section, “Inhibition of CVB3 replication in HeLa cells”: In this set of data several experiments are missing. Based on the fact that the original publication to demonstrate the antiviral shRNA against CVB3 is published in Chinese, it would be appropriate to add additional samples (only shRNA-2B and control shRNA) to this experiment. Furthermore, please indicate why you used 1.25 µg DNA of pGCL-2B and a MOI of 10 of Lenti-2B in your experiments. Dose-response analyses and
time-course experiments would be appreciated. Please provide pictures to show transfection and transduction rates (based on EGFP expression). Why did you analyze only the supernatants? What was the intracellular virus concentration? Did you see any cytopathic effect in your protected cells? Why was the lentiviral expression less effective in comparison to the plasmid transfection (especially in view of your in vivo experiments)?

These experiments have been performed. We used 1.25 µg DNA of pGCL-2B in our experiments because that 1.25 µg/well was the lowest dosage of pGCL-2B to reach about 60% of cells expressing GFP and was less toxic to the cells (new figure 1A). RNAi vector pGCL-2B reduced the virus titers in HeLa cells in a dose- and time-dependent manner (new figure 1D and C). We have corrected the expression of lentivirus infection unit to TU/ml instead of MOI because of the titers determined from the GFP expression of infected cells by fluorescence microscopy. As new figure 2A shows that Lenti-2B at a dose of 10 TU/cell or 100 TU/cell could reduce CVB3 titer efficiently compared with that in control cells treated with Lenti-NC. The antiviral effect was almost absent at the dose of 1 TU/cell because of the low transduction efficacy of the lentivirus. Thus, we used a dose of 10 TU/cell of Lenti-2B in our experiments. The pictures to show transfection and transduction rates based on GFP expression have been shown in new figure 1E and new figure 2B. New figure 1E group pGCL-2B shows the GFP expression at 72 h post transfected with pGCL-2B. New figure 2B group Lenti-2B shows the GFP expression at 96 h post transfected with Lenti-2B. We analyzed the virus titers only in the supernatants because the
supernatants were easy to collected continuously at several time points, and about 80% of cells occurred CPE to lead to cell lysis at 48 h post CVB3 infection in two control groups, in which most virus particles were released into the supernatants at that time. We have detected the intracellular virus titers at early time points after infection, the differences of CVB3 titers between shRNA-2B group and control group were consistent with those in supernatants. Thus we showed the data collected from supernatants. We saw the CPE in our protected cells, and the CPE was limited in small area. At 48 h post infected with CVB3 at MOI of 0.01, rate of CPE formation in HeLa cells treated with pGCL-2B was about 20%, while the rate was about 80% in cells treated with pGCL-NC. In in vitro experiments, the lentiviral expression exhibited less effectiveness in comparison to the plasmid transfection because lentivirus expressed later than plasmid. When infected with CVB3 at 24 h post transfection with pGCL-2B, the cells expressing GFP was about 60%. While, when infected with CVB3 at 48 h post transduction with Lenti-2B, the cells expressing GFP was less than 50%. Thus when infected with CVB3, lentivirus expressed shRNA less than plasmid and caused less protected effect against CVB3. However, in in vivo experiments, the expression of shRNA-2B with lentiviral vector exhibited higher inhibitory efficiency on viral replication than with plasmid vector (New figure 3, 4, 5).

2. Results section, “Inhibition of CVB3 replication in coxsackievirus-induced myocarditis model”: Again, please indicate the reasons to use the indicated dosages
of plasmid DNA and lentiviruses. Dose-response analyses would be much better. Why did you infect treated animals with CVB3 at 24 h? In view of a therapeutic approach, treatments should be performed prior or at least at the same time of virus challenge. Furthermore, please indicate how often you repeated the in vivo experiments.

To confirm the protective effect of pGCL-2B on CVB3 infected mice, we have used three doses of pGCL-2B at 20 µg/mouse, 30 µg/mouse or 40 µg/mouse respectively and observed the survival of mice. pGCL-2B at 40 µg/mouse could improve the lifespan of mice, while pGCL-2B at low doses were not able to improve the survival rate. Thus, we used the pGCL-2B at 40 µg/mouse to perform our experiments. Similarly, in Lenti-2B experiments in vivo, we used three doses of Lenti-2B at $1 \times 10^7$ TU/mouse, $5 \times 10^7$ TU/mouse or $1 \times 10^8$ TU/mouse respectively, the highest dose could significantly improve the survival rate. Thus, we adopted Lenti-2B at $1 \times 10^8$ TU/mouse in subsequent experiments. We infected the treated animals with CVB3 at 24 h post transduction, in consideration of the later expression of lentivirus and the different ways to infect with lentivirus and CVB3. We also infected animals with CVB3 at the same time or prior to transduction with lentivirus, but the results could not reach the one as expected. We repeated the in vivo experiments three times.

3. Results section: Figure 5: Please provide much better histology pictures, especially to show infiltration in myocardial tissue of control animals. By looking at your pictures inflammation in the myocardium is hard to see at all. Please mark necrotic cells and calcifications. IHC staining: Please provide controls missing the first
antibody. To show long time effects of tissue protection it would be very helpful to sacrifice mice later during the course of infection. Then, please use Sirius-Red staining to show fibrotic tissue. In case you are planning those experiments you have to reduce the CVB3 dose to obtain surviving animals in control groups. By the way, what was the reason to use $8 \times 10^3$ pfu?

We have picked out other histology pictures especially to show infiltration, necrosis and calcification in myocardial and pancreatic tissue of control animals and marked these with arrows respectively. We have also added the IHC staining controls missing the primary antibody (new figure 6). As you pointed out we must reduce the CVB3 dose to observe the long time effects of tissue protection. We used the CVB3 with $8 \times 10^3$ pfu/mouse because this is lowest lethal dose and all mice die within ten days after challenge, which was a LD100 of the virus.

4. Results section: As you stated, both systems express EGFP as well. Please show pictures of EGFP expression in heart and pancreas samples.

We have added the pictures showing GFP expression in heart and pancreas samples (new figure 6).

5. Discussion section: page 11, second paragraph last lines: Here you speculate that “2B protein might have other important functions”. I agree, but a very obvious explanation could be that due to shRNA-2B activity the whole translation process of the viral polyprotein is hampered which consequently leads to reduced viral progeny
production.

We now discuss this more fully in the Discussion. Our study indicated that shRNA-2B can reduce CVB3 progeny production by cleavage of viral genomic RNA, which results in the suppression of viral particle assembly with intact CVB3 RNAs and hampering of the whole translation process of the viral polyprotein. On the other hand, it was reported that 2B protein plays a major role in suppressing apoptotic host cell response by manipulating intracellular Ca\(^{2+}\) homeostasis and, thereby, in extending the life span of the host cell. However, this RNAi by shRNA-2B may enhance host cell death and subsequently limit CVB3 replication. Therefore, shRNA-2B inhibits CVB3 replication not only directly through RNAi but also indirectly through enhancing host cell death.
Reviewer #3 Jae Hwan Nam:

The authors investigate the potential of a shRNA targeted at 2B gene of coxsackievirus B3 to suppress of viral replication in vitro and in vivo, and improvement of survival rate and histological pattern after CVB3 challenge by using recombinant lentivirus system expressing 2B targeted shRNA. Their data are clear to show their final decision which is that shRNA-2B is a potentially therapeutic agent for the treatment of enterovirus-induced diseases. In that data is OK. However, I concerned its novelty. As the authors already mentioned about previously published papers (Virus Genes 36:141, J Virol 79:8620 and so on…), previous papers already showed that recombinant lentivirus expressing shRNA against CVB3 is usefulness to treat enterovirus-mediated diseases. The authors mentioned the ability of 2B gene as target for RNAi technique. However, they already published about it. Only difference between this manuscript and previous papers is target region for shRNA.

Although there are weak points as mentioned above, their data are confirmed and supported previous reports, and work strategy, writing and discussion with data are reasonable. So I suggested the authors re-make this manuscript as ‘short communication or note’ format.

We thank the reviewer for this comment. After carefully considering this comment and reading our manuscript again, we would like to make the following response. In this study, we evaluated the antiviral activity of shRNA against the 2B sequence of CVB3, which was expressed either by a plasmid or a recombinant lentivirus. We performed a set of experiments both in vitro and in vivo. Our previous work on the
selection of effective siRNA target sequences and the antiviral evaluation of 2B siRNA in vitro provided solid foundation for the current investigation. Although several studies of siRNAs that target other sites have been published, this study showed our completed data about shRNA-2B and compared the different efficiency between plasmid vector and lentiviral vector at the same time. Importantly, this study focused on in vivo evaluation using an established viral myocarditis mouse model, we analyzed the effects of treatment on the viral damage of the organ and disease severity not only in the heart but also in pancreas. These data are more valuable than our previous results obtained from using HeLa cells only. This is a major advance on this specific study. Thus, we hope to present more useful data to the readers.
Reviewer #4 Decheng Yang:

Manuscript by Yao H et al. designed and evaluated the anti-CVB3 activity of shRNA-2B in cell culture system and in a viral myocarditis mouse model. The authors constructed plasmid and lentiviral vectors expressing shRNA-2B targeting CVB3 2B region. They found that siRNA-2B significantly reduced CVB3 titer in HeLa cells and in mouse organs. They also found that shRNA-2B treatment reduced the tissue damage in mouse heart and pancreas, thus increased mice survival rate and prolonged life span of the animals. This study is interesting and the finding is important. Particularly, the work has a significant amount of studies using the recombinant lentiviral vector and an established mouse myocarditis model, which enhances the significance of the studies. Therefore, this report concludes that shRNA-2B is a promising candidate for anti-CVB3 drug development. Based on these investigations, this reviewer suggests that this manuscript can be considered for publication in the journal after minor modifications.

1. In the Result (page 8). Line 5 from top to line 8: “In our previous study, ...anti-CVB3 in HeLa cells” can be deleted because the same information has been mentioned in Background on page 4. Instead, the authors can describe more on the cloning of shRNA into the vectors in the Method or Result section.

We have deleted these sentences and added description for the shRNA vector in the Method (page 5).

2. In Discussion (page 10). The first sentence needs references on CVB3-caused direct...
damage of the tissues and the diseases in the organs.

We have added the references (page 11).

3. In Discussion (page 11), line 6 from bottom: “It was reported that 2B protein...[26]. In this study, surprisingly, CVB3 replication was inhibited by shRNA targeting 2B region, implying that 2B protein might have other important functions we need to further explore”. This discussion may be modified. The inhibition of CVB3 replication by shRNA-2B is not surprising data. It is an expected result. The authors may discuss the connection between their data and the reference [26] in another way: On one hand, reference 26 reported that CVB3 2B gene can suppress apoptotic host cell response..... and extend the life span of host cells. On the other hand, this study found that shRNA-2B targeting of CVB RNA can inhibit expression of CVB3 genes by cleavage of CVB3 genomic RNA. Thus, this RNAi by shRNA-2B will enhance host cell death and subsequently limit CVB3 replication. Therefore, the authors should indicate in their discussion that shRNA-2B inhibits CVB3 replication not only directly through RNAi but also indirectly through enhancing host cell death.

We have rewritten this part according to the reviewer’s comments (line 2-11, page 11).

4. Some minor English grammar problems need to be corrected.

We have corrected some grammar problems.
We tried our best to improve the manuscript and made some changes in the manuscript. We appreciate the comments and suggestions from the editors and reviewers earnestly, and hope that the revisions will meet with the editorial requirements for acceptance of publication in the journal.

Yours sincerely,

Hailan Yao

Corresponding author:

Name: Zhewei Liu E-mail: zheweiliu@163.com