May 20, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 17129-article.doc).

Title: Mucocele of the appendix: Dual gRNAs guided CRISPR/Cas9 system inhibits hepatitis B virus replication

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Thank you for the kind and thoughtful suggestions from the review of our manuscript. We have carefully considered 36 comments of 6 reviewers. According to their suggestions, the manuscript has been revised, and some additional experiments have been conducted.

Please find below our point-by-point responses to the reviewers' comments. In addition, the revised and added content in the revised manuscript is highlighted in yellow for easy identification.

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology.*

Sincerely yours,

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Point-by-point responses

Reviewer #1 (Comments to the Author):

Dr. Wang and colleagues have presented a highly interesting functional study in which they aimed to show that dual gRNA guided CRISPR/Cas9 system can suppress HBsAg and HBeAg as well as promote clearance of HBV cccDNA in cell culture. The authors conclude that the presented dual gRNA/CRISPR/Cas9 system might be a potential approach for eradication of HBV cccDNA and thus considered for new and additional antiviral treatment option of chronic hepatitis B. Overall, the manuscript is well written and concise in its content showing convincing data. The design of the study is well performed and the results are of high relevance. However, there are some comments which should be addressed.

1 Although the study design is well performed, I miss a control experiment showing that the used gRNA are specific and responsible for the significant reduction of HBsAg/HBeAg in the treated cell culture experiments. For that, does it make sense to analyse possible artificial action of a non-HBV sequences gRNA (nonsense gRNA)?

Response: Thanks for your suggestion. The control plasmid used in each experiment was pSpCas9 (BB)-2A-GFP (PX458) which expressed a nonsense gRNA (GGGTCTTCGAGAAGACCT). Thus, the effect of HBV-specific gRNA should be specific. The corresponding content has been added in the revised manuscript (Lines 116-118).

2 It is not clear to me from the figures 2 and 3 whether there is a significantly enhanced effect of dual gRNA versus single gRNA. If possible, could the authors show this by an extra diagram or comparison of their calculation?

Response: Thanks for your suggestion. The dual gRNAs indeed induced a significantly enhanced effect on the suppression of HBV replication comparing to that of single gRNA. We have shown the detailed data and the comparison of their calculation in the revised manuscript (Lines 227-241).

3 The presented results of reduction of HBsAg/HBeAg by gRNA and dual gRNA are convincing; however, what is the reason not to present quantitative measurement of newly synthesized HBV DNA in the supernatant by real time PCR? This could be done

easily by removing the transfected HBV-plasmid by DNAse digestion before lysing the HBV particles. The addition of HBV DNA data will enhance and verify the impact of the "serological" results.

Response: Thanks for your suggestion. To completely exclude the interference of HBV expression plasmid, we used the HepAD38 cells which stably express the HBV DNA and detected the effect of gRNA on the HBV DNA levels in the culture supernatant of this cell. The result revealed that HBV-specific gRNAs could also significantly reduce the HBV DNA level in the HepAD38 cell culture supernatant (Figure 5b), in line with that of HBsAg and HBeAg (Figure 5a). We have added this result in the Result section (lines 297-298, and Figure 5b)

4 Did the authors test the concentration effect of gRNAs in the cell culture experiments to show the specificity of the used gRNAs. In other words excess gRNA concentrations may possibly lead to incorrect results.

Response: The reviewer's concern is reasonable. We conducted an additional experiment to test the concentration effect of gRNAs on HBV replication. The result revealed that the suppression efficiency of gRNA on the HBsAg level was gRNA concentration dependent (Figure S2). The corresponding result was added in the revised manuscript (Lines 254-258).

5 Material and Methods, page 7, line 95. A reference or source of the HuH7 and HepAD38 cells is missing.

Response: Thanks for your suggestion. The references of HuH7 and HepAD38 cells have been added in the revised manuscript (Lines 123-125).

6 Results, page 10, line 149. I suggest to state that the sequences were blasted by Nucleotide Blast search (NCBI) instead of NCBI website.

Response: Thanks for your nice suggestion. The sentence " the gRNA sequence was blasted in the NCBI website " has been revised as "the gRNA sequences were blasted by Nucleotide Blast search (NCBI)" in the revised manuscript (Lines 190-191).

7 Discussion section, page 16, line 256. I know that most authors used the elderly data of 350 million chronically HBV infected; however, there are new data from the

WHO that there are now 240 million (Hepatitis B Fact sheet N°204; Updated March 2015; http://www.who.int/mediacentre/factsheets/fs204/en/). Please correct.

Response: Thanks for your helpful suggestion. The new data from WHO has been added in the revised manuscript (Lines 313-314).

Reviewer #2 (Comments to the Author):

The study of Jie Wang et al, is very interesting because it addresses a topic of maximum current interest: the possible gene therapy of HBV infection given the limitations of current treatments by a promising strategy the CRISPR/Cas9 system. The studio is well posed and the experimental development is excellent for what it is considered important its distribution among medical community. However, some point must be solved: Major point:

1 The discussion should include information about the real possibilities of the application of this treatment strategy to human patients, both ethical level of the introduction of plasmid human and material potential risks based on current knowledge.

Response: Thanks for your suggestion. There is still far away for the treatment of HBV infected patients by CRISPR/Cas9 system. However, instead of PX458 plasmid, CRISPR/Cas9 system could be developed by recombinant adeno-associated virus system which is a potential vector for gene therapy. Therefore, there is the possibility that CRISPR/Cas9 system is used to treat HBV infected patients. The corresponding content has been added in the revised manuscript (Lines 370-377).

Minor points: 1-page 8: include the nucleotide positions of the primers (page 8) as well as those of the gRNA (Table 1).

Response: Thanks for your suggestion. The nucleotide positions of the primers and gRNAs have been added in the revised manuscript (Lines 147-153, and Table 1).

2 Explain the search criteria for the possible gRNA (page 10). On this same page is used twice the word "striking", we recommend reviewing the style of these phrases.

Response: Thanks for your suggestion. We have explained the detailed searching criteria for possible gRNA in the Result section (Lines 185-188). Also, the word "strikingly" has been deleted in the revised manuscript.

3 Explain the methodology of gRNAs toxicity study, indicating the significance of MTT.

Response: Thanks for your advice. Since MTT assay is a colorimetric assay for assessing cell viability. To exclude the possibility that the HBsAg or HBeAg suppression observed in our study was the result of cytotoxicity, we conducted MTT assay to detect the cell viability when gRNAs over-expressed. We found gRNAs did not induce the cytotoxicity, suggesting that gRNAs could specifically suppress HBV replication. The methodology of gRNAs toxicity study and the significance of MTT have been explained in the revised manuscript. (Lines 210-214)

4 The surprising event described on page 12 line 188-189 ("the antagonist effect for HBsAg production") should be explained in the discussion.

Response: Thanks for your suggestion. We have explained "the antagonist effect for HBsAg production" in the discussion part of the revised manuscript (Lines 337-342).

5 In order to facilitate the paper reading to non initiated readers , please include a brief description of the CRISPR / Cas9 system mechanism in the methodological section , mainly explaining the cutting and ligation of affected sequences.

Response: Thanks for your nice advice. The cutting and ligation mechanism of CRISPR/Cas9 system was added in the methodological section of revised manuscript (Lines 113-115).

Reviewer #3 (Comments to the Author):

The study aims to develop a CRISPR/Cas system for cleaving the covalently closed circular HBV DNA (cccDNA) of different genotypes. The authors utilise the CRISPR/Cas system to cleave HBV DNA of the genotypes A, B, C and D. Guide

RNAs (gRNAs) were developed to target different HBV sequences, and their functionality assessed using HBV plasmid constructs (for genotypes A, B and C). The cell line HepAD38 was used for demonstrating the functionality of the gRNAs in the context of a HBV genotype D sequence. Studies similar to the submitted work have been already published; the authors extend the studies to demonstrate the feasibility against different HBV genotypes. The authors demonstrate the functionality of the gRNAs using pBB4.5-HBV plasmid constructs as target molecules. Circumstantial evidence via PCR is provided to show that cccDNA can be cleaved using the HepAD38 cell line.

1 The quality of the manuscript could be substantially improved by directly demonstrating that the cccDNA level in HepAD38 cells are indeed reduced after transfections with gRNA/Cas9 expression vectors versus mock gRNA/Cas9 expression vectors. Do you have any direct evidence by Southern blot that cccDNA can be cleaved? This should be feasible, cccDNA can be detected in the HepAD38 cells. Zhou et al (2006; Antiviral Res 72: 116-124) have shown that cccDNA can be detected 10 hours after tetracycline removal. For the studies, how was the cell line treated? When were the experiments done, how many hours after tetracycline removal?

Response: The reviewer's concern is reasonable. Indeed, the cleavage of cccDNA should be confirmed by southern blot, and cccDNA can be formed in the HepAD38 cells under the control of tet-off system. However, It is difficult to confirm the gRNA-induced destruction of cccDNA by southern blot for the low transfection efficiency of the PX458 palsmid. We will demonstrate the gRNA-induced destruction of cccDNA by southern blot when the CRISPR/Cas9 system was combined with the recombinant adeno-associated virus system. In addition, to get enough cccDNA for detection, we transfected the gRNA expression vector into HepAD38 cells at least 7 days after tetracycline removal. The reviewer's concern has been reflected in the revised manuscript (Lines 361-365 and 165-166).

2 Please provide more details in the Materials and Methods section, how many "mol" of the expression vectors (HBV containing plasmids versus gRNA/Cas9 vectors) were used?

Response: Thanks for your suggestion. Since the ratio of HBV containing plasmids versus gRNA/Cas9 vectors was different in the different experiments, we added the detailed amount of the expression vectors transfected to cells in each figure legend of the revised manuscript.

3 Lines 84-86: please clarify or provide more details regarding the pBB4.5-HBV1.2C construct. The 1.2 HBV genotype C construct is based on a genotype 1.3 genotype D construct - a 1.3 construct should comprise more sequences than a 1.2 construct, so it is unclear what "based on" means.

Response: Thanks for your suggestion. Although a 1.3× construct comprise more sequences than 1.2× construct, both 1.2× and 1.3× HBV construct can efficiently transcript into 3.5kb pregenome RNA and produce HBV particle. To construct pBB4.5-HBV (genotype C) expression plasmid, the pBB4.5-HBV1.3 (genotype D, G1896A mutation) plasmid was digested with PstI and NheI enzymes to remove the 1.3×HBV (genotype D), and then inserted the 1.2×HBV (genotype C) into the digested backbone of pBB4.5 vector. So, the construction of pBB4.5-HBV(genotype C) expression plasmid is based on the pBB4.5-HBV1.3 (genotype D, G1896A mutation). The above information has been reflected in the revised manuscript (Lines 193-109).

4 Line 95: Please indicate reference for HepAD38 cells: reference 24 in manuscript; for HuH-7 cells: Nakabayashi, H., et al. 1982. Growth of human hepatoma cell lines with differentiated functions in chemically defined medium. Cancer Res. 42:3858–3863.

Response: The references of HuH7 and HepAD38 cells have been added in the revised manuscript (Lines 123-125).

5 Line 202: The functionality of the gRNAs were tested using the gRNA/Cas9 expression vectors in the context of the pBB4.5-HBV1.2 C, and pGEM-HBV1.3A and B plasmids. Why was the plasmid pBB4.5-HBV1.3 (genotype D) not included? This would have completed the set of experiments using plasmids with HBV sequences.

Response: Sorry for the confusion. Because the pBB4.5-HBV1.3 (genotype D) was a G1896A mutated expression plasmid which does not expresses HBeAg, we just use it to construct the pBB4.5-HBV1.2 C plasmid and did not use it to detect the CRISPR/Cas9-mediated suppression of HBV replication. Instead, we used the HepAD38 cells stably expressing genotype D HBV to assess the effect of HBV-specific gRNA on the replication of genotype D HBV.

6 Lane 231/232: " .... destroy HBV genome of genotype A-D". This relates possibly to Fig 4f, but only genotypes A, B, and C are shown. Please clarify.

Response: Thanks for your helpful reminding. As shown in Figure 4e, the target genome sequence of gRNA-13 in genotype B HBV harbors a one base difference as compared to genotypes A/C/D. In Figure 4f, we only detected genotypes A, B, and C and did not detect the genotypes D because the wildtype genotypes D plasmids are not available. Therefore, we revised this reslut as "As shown in Figure 4e, the target genome sequence of gRNA-13 in genotype B HBV harbors a one base difference as compared to genotypes A/C/D. However, dual gRNAs composed by gRNA-1and gRNA-13 could also destroy HBV genome of genotype B (Figure 4f). " (Lines 284-287).

7 Line 252: "... HBV cccDNA (Figure 4a, 4c and 5c)." Figures 4a and 4c do not relate to cccDNA.

Response: Thanks for your suggestion. The sentence has been changed as "PCR amplification confirmed that the fragment between two cleavage sites of dual gRNAs in HBV expressing template was removed (Figure 4a and 4c). Similarly, HBV-specific gRNA could also guide Cas9 to destroy HBV cccDNA (Figure 5d)" (Lines 307-310).

8 Line 302: "... in HepAD38 cells was significantly down regulated by HBV-specific gRNA, ..." Fig. 5c does not show a significant down-regulation; the PCR based result shows clearly that a substantial amount of cccDNA is not cleaved.

Response: Thanks for your suggestion. The sense primer used to detect the cccDNA level in Figure 5c (revised version) covers the cleavage site of gRNA-12. Since the

indel mutation induced by gRNA-12 may inhibit the PCR amplification of cccDNA even if the fragment between gRNA-5 and gRNA-12 was not removed, so the wildtype cccDNA level in HepAD38 cells was significantly suppressed by gRNA-5 and gRNA-12 in Figure 5c. However, in Figure 5d (revised version), although a substantial amount of cccDNA is not cleaved, it still contains the indel mutation induced by gRNA-12. So the downregulation of the wildtype cccDNA level in in HepAD38 cells was more obvious than the amount of the cleaved cccDNA.

9 Line 308: Comment. The results show that in the context of targeting cccDNA and not plasmid DNA, only a fraction of cccDNA is cleaved; the authors acknowledge this by proposing that the CRISPR/Cas9 system could be useful in combination with other treatment options.

Response: Thanks for your comment. Although only a fraction of cccDNA is cleaved, the indel mutation induced by HBV-specific gRNA could also inhibit the transcription of cccDNA, according to the effect of single gRNA on the inhibition of HBV replication (Figure 2a and 2b). These results suggested that the CRISPR/Cas9 system could significantly suppress the replication of HBV and the production of HBsAg and HBeAg by inducing the cccDNA cleavage and the indel mutation. Therefore, we proposed it may get the synergistic effect in inhibiting HBV replication when the CRISPR/Cas9 system combined with other treatment options, such as NA or IFN-α.

Reviewer #4 (Comments to the Author):

In this article, the authors designed 15 gRNAs and evaluated the effects of these gRNAs on HBV replication in vitro. The author firstly demonstrated that both single gRNA and dual gRNAs could suppress the secetion of HBsAg and HBeAg in cell lines of Huh7 and HepAD38. They also reported that dual gRNAs exerted stronger inhibitory role in HBV replication compared with single gRNA. Finally, the possible role on cccDNDA was also determined. The authors then concluded that dual gRNAs guided CRISPR/Cas9 system might be a potential approach for eradication of persistent HBV cccDNA. Overall, the study is well designed. However, there are still some concerns in the current manuscript. Minor comments:

1 The dual gRNAs guided CRISPR/Cas9 system has been demonstrated to inhibit the replication of HBV cccDNA, rather than destroy or clearance. However, it is not accurate for authors to use "clearance" or "destroy" in the title page. Therefore, the authors should polish these kinds of writings throughout the full manuscript.

Response: We agree to the reviewer's concern. Although we found that CRISPR/Cas9 system could destroy the cccDNA (Figure 5c), but the CRISPR/Cas9 system mainly inhibit the replication of HBV cccDNA. We have changed the title of the article "The dual gRNAs guided CRISPR/Cas9 system promotes the clearance of hepatitis B virus cccDNA" to "The dual gRNAs guided CRISPR/Cas9 system inhibits hepatitis B virus replication" and polished the writings of "clearance" and "destroy" throughout the revised manuscript.

2 It is a common problem for gRNA for the specificity of targeting. How did the authors identify the success of gRNA containing vector in the experiment? Therefore, the authors should show the details of gRNAs design, transfection method, and verification for the success of construction in the section of methods.

Response: According to the reviewer's suggestion, we have showed the details of gRNAs design (Lines 185-188) and transfection method (Lines 125-130). As for the verification for the success of gRNA containing vector construction, we used direct sequencing method. This has been added in the revised manuscript (Lines 192-193).

3 Is there any time-dependent manner for the effects of gRNAs on the secretory levels of markers of HBV replication, including HBeAg, HBsAg and HBV DNA? Here, the authors only set the time point as 72 hours after transfection into cell lines.

Response: The reviewer's concern is reasonable. We did not detect the time-dependent manner for the effects of gRNAs on HBV replication. In general, the expression level of Cas9 protein in the gRNA containing vector was highest at 48-72 hours post transfection. So in this study, we detected the effects of gRNAs on HBV replication at 3 days post transfection.

4 In Fig 4(f), why the genotype D was missed?

Response: Thanks for your comment. It is the same to the answer for comment 5 of reviewer 3.

5 Both single gRNA and dual gRNAs could significantly reduce the production of HBsAg and HBeAg in cell culture supernatant. How about the quantitative changes of HBV DNA in cell culture supernatant? The authors should provide a column table of quantitative changes of HBVDNA in figure 4.

Response: Thanks for your comment. It is the same to the answer for comment 3 of reviewer 1.

6 There is still far away for the treatment of HBV infected patients. At least, the results of this experiment should be validated in HBV animal models. Therefore, the authors should discuss such kind of limitation in the discussion.

Response: We agree to the reviewer's concern. We will validate the effect of HBV-specific gRNA on HBV replication in HBV trangenic mice by hydrodynamic injection method or use the adenovirus or adeno-associated virus system in future. The reviewer's concern has been reflected in the revised manuscript (Lines 371-377).

7 The mechanism for the possible inhibitory role of gRNAs in cccDNA should be well discussed. Is there any direct action of gRNAs on cccDNA replication? Is it the reason why the possible indirect role of gNRAs in the decreased cccDNA replication due to the inhibition of whole HBV genome?

Response: According to the reviewer's concern, we have discussed the possible inhibitory role of gRNAs in cccDNA. We found that the single gRNA could significantly inhibit HBV replication (Figure 2a and 2b), indicated the indel mutation of cccDNA induced by HBV-specific gRNA could inhibit the cccDNA replication through the direct change of cccDNA sequence. Besides, dual-gRNAs could also destroy the cccDNA by removing the fragment between two cleavage sites (Figure 5c). Taken together, the indel mutation and destruction of cccDNA should be the main reason of gRNA-induced suppression of cccDNA replication. The corresponding comment has been reflected in the manuscript.

Reviewer #5 (Comments to the Author):

Comments to the Editor: Thanks for inviting me to review the article entitled " The dual gRNAs guided CRISPR/Cas9 system promotes clearance of hepatitis 2 B virus

cccDNA". The authors are advised to put in consideration the suggested remarks provided in the area of comments to author. Comments to the authors: Minor Comment: o Minor editing polishing is needed o Language evaluation: "A". Comments to Authors:

1 TITLE Reflect the major content of the article.

2 ABSTRACT It gives a clear delineation of the research background, including important data and conclusions.

3 INTRODUCTION Provides sufficient background regarding the studied topic and the aim of the study is clearly defined.

4 MATERIALS AND METHODS: Full description is provided for this section; however and in order to satisfy the reader, some important issues are better to be elaborated.

4.1 Transfection of cells: source of cell lines as well as the incubation condition has to be mentioned.

Response: Thanks for your suggestion. The source of cell lines as well as the incubation condition has been mentioned in the revised manuscript (Lines 123-125).

4.2 Detection of HBsAg and HBeAg: It is not a qualitative assay, quantitative detection needs more clarification.

Response: Thanks for your suggestion. The reviewer's concern has been reflected in the manuscript (Lines 140-143).

4.3 Detection of HBV DNA fragments cleaved by dual gRNAs: Reference is needed for the primers in use, as well as the source of Taq mix. Also the concentration of each primer has to be mentioned. The DNA marker in agarose gel is also missing.

Response: Thanks for your suggestion. The primers used for the detection of HBV DNA fragments cleaved by dual gRNA were designed in this study. The PCR Taq mix, the concentration of each primer and the DNA marker have been added in the revised manuscript (Lines 157, 158 and 162).

4.4 Detection of HBV cccDNA: The quantity of the two gRNA/Cas9 dual expression vectors has to be mentioned as well as the incubation condition. More details regarding rolling Circle Amplification (RCA) to selectively amplify cccDNA are better to be included.

Response: Thanks for your suggestion. The quantity of the two gRNA/Cas9 dual expression vectors as well as the incubation condition have been mentioned in each figure legend of the revised manuscript. The detailed regarding rolling amplification to selectively amplify cccDNA has been added in the revised manuscript (Lines 302-303).

4.5 Statistical methods are missing and have to be mentioned.

Response: Thanks for your suggestion. Statistical methods have been mentioned in each figure legend of the revised manuscript.

5 RESULTS: An overall theoretical analysis of the study results is well covered. Provide sufficient experimental data, however, some important data were missing and has to be covered: Line 161-162, 164, 166: P value is missing and has to be mentioned, e.g:"HBV genome exhibited higher HBsAg suppressing...."

5.1 Mitochondrial Tetrazolium Assay: MTT assay was mentioned in this section, without being described in the materials and methods section. It has to be fully written and mentioned in its proper place.

Response: Thanks for your suggestion. Mitochondrial Tetrazolium Assay has been mentioned in the materials and methods section of the revised manuscript (Lines 175-182).

5.2 Line175: combination of two HBV-specific gRNAs (dual gRNAs): The ratio in use is better to be mentioned.

Response: Thanks for your suggestion. The ratio of two HBV-specific gRNAs (dual gRNAs) has been added in the revised manuscript (Lines 242-243 and 516-517).

5.3 Figures & tables are well presented, however, unit of measurements of HbsAg/ HBeAg in Y axis of figures 2 (a/b) and 3(a-e) are lacking and better to be added.

Response: Thanks for your suggestion. Since the Y axis of figures 2 (a/b) or 3 (a-e) reflects the relative level of HBsAg/HBeAg, so there was no unit.

6 Discussion: The section is almost well organized; an overall theoretical analysis concerning the provided data is well covered.

7 REFERENCES: Relevant and sufficient references were adequately cited; however, the authors have to follow the journal style in writing this section. Also, PMID/DOI is not well maintained for all the cited references.

Response: Thanks for your suggestion. According to the journal style, PMID/DOI has been added in the revised.

Reviewer #6 (Comments to the Author):

The paper is well written and informative with perspective for clinical application, even if the study is a pre-clinical one and limited to demonstration of efficacy without signinficant cytotoxicity, always in vitro.

Response: Thanks for your comments.