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ESPS PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology
ESPS manuscript NO: 17129
Title: The dual gRNAs guided CRISPR/Cas9 system promotes clearance of hepatitis B virus cccDNA
Reviewer's code: 00053556
Reviewer's country: Egypt
Science editor: Ya-Juan Ma
Date sent for review: 2015-02-22 20:16
Date reviewed: 2015-04-08 14:17

CLASSIFICATION	LANGUAGE EVALUATION	SCIENTIFIC MISCONDUCT	CONCLUSION
[Y] Grade A: Excellent	[Y] Grade A: Priority publishing	Google Search:	[ ] Accept
[ ] Grade B: Very good	[ ] Grade B: Minor language polishing	[ ] The same title	[ ] High priority for publication
[ ] Grade C: Good	[ ] Grade C: A great deal of language polishing	[ ] Duplicate publication	[ ] Rejection
[ ] Grade D: Fair	[ ] Grade D: Rejected	[ ] Plagiarism	[Y] Minor revision
[ ] Grade E: Poor		[Y] No	[ ] Major revision
		BPG Search:	
		[ ] The same title	
		[ ] Duplicate publication	
		[ ] Plagiarism	
		[Y] No	

COMMENTS TO AUTHORS

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. o Transfection of cells: source of cell lines as well as the incubation condition has to be mentioned. o Detection of HBsAg and HBeAg: It is not a qualitative assay, quantitative detection needs more clarification. o 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. o DetectionofHBVcccDNA: 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. o Statistical methods are missing and have to be mentioned. 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...." ? 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. ? Line175: combination of two HBV-specific gRNAs (dual gRNAs): The ratio in use is better to be mentioned. ? 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. 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.

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ESPS PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology
ESPS manuscript NO: 17129
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Reviewer's code: 00225318
Reviewer's country: Spain
Science editor: Ya-Juan Ma
Date sent for review: 2015-02-22 20:16
Date reviewed: 2015-03-27 15:10

CLASSIFICATION	LANGUAGE EVALUATION	SCIENTIFIC MISCONDUCT	CONCLUSION
[ ] Grade A: Excellent	[ ] Grade A: Priority publishing	Google Search:	[ ] Accept
[Y] Grade B: Very good	[Y] Grade B: Minor language polishing	[ ] The same title	[ ] High priority for publication
[ ] Grade C: Good	[ ] Grade C: A great deal of language polishing	[ ] Duplicate publication	[ ] Rejection
[ ] Grade D: Fair	[ ] Grade D: Rejected	[ ] Plagiarism	[Y] Minor revision
[ ] Grade E: Poor		[Y] No	[ ] Major revision
		BPG Search:	
		[ ] The same title	
		[ ] Duplicate publication	
		[ ] Plagiarism	
		[Y] No	

COMMENTS TO AUTHORS

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 CRIDP/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. Minor points: 1-page 8: include the nucleotide positions of the primers (page 8) as well as those of the gRNA (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. 3-Explain the methodology of rgRNAs toxicity study, indicating the significance of MTT. 4- The surprising event escribed on page 12 line 188-189 ("the antagonist effect for HBsAg production") should be explained in the discussion. 5-In order to facilitate the paper reading to non initiated

readers , please iInclude a brief description of the CRISPR / Cas9 system mechanism in the methodological section , mainly explaining the cutting and ligation of affected sequences.

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ESPS PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology
ESPS manuscript NO: 17129
Title: The dual gRNAs guided CRISPR/Cas9 system promotes clearance of hepatitis B virus cccDNA
Reviewer's code: 00013065
Reviewer's country: Germany
Science editor: Ya-Juan Ma
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CLASSIFICATION	LANGUAGE EVALUATION	SCIENTIFIC MISCONDUCT	CONCLUSION
[Y] Grade A: Excellent	[Y] Grade A: Priority publishing	Google Search:	[ ] Accept
[ ] Grade B: Very good	[ ] Grade B: Minor language polishing	[ ] The same title	[ ] High priority for publication
[ ] Grade C: Good	[ ] Grade C: A great deal of language polishing	[ ] Duplicate publication	[ ] Rejection
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[ ] Grade E: Poor		[Y] No	[ ] Major revision
		BPG Search:	
		[ ] The same title	
		[ ] Duplicate publication	
		[ ] Plagiarism	
		[Y] No	

COMMENTS TO AUTHORS

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)? 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?

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. 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. Minor comments: 1) Material and Methods, page 7, line 95. A reference or source of the HuH7 and HepAD38 cells is missing. 2) Results, page 10, line 149. I suggest to state that the sequences were blasted by Nucleotide Blast search (NCBI) instead of NCBI website. 3) 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.

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ESPS PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology
ESPS manuscript NO: 17129
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Reviewer's country: Australia
Science editor: Ya-Juan Ma
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CLASSIFICATION	LANGUAGE EVALUATION	SCIENTIFIC MISCONDUCT	CONCLUSION
[ ] Grade A: Excellent	[ ] Grade A: Priority publishing	Google Search:	[ ] Accept
[ ] Grade B: Very good	[Y] Grade B: Minor language polishing	[ ] The same title	[ ] High priority for publication
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[ ] Grade D: Fair	[ ] Grade D: Rejected	[ ] Plagiarism	[Y] Minor revision
[ ] Grade E: Poor		[Y] No	[ ] Major revision
		BPG Search:	
		[ ] The same title	
		[ ] Duplicate publication	
		[ ] Plagiarism	
		[Y] No	

COMMENTS TO AUTHORS

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

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ESPS PEER-REVIEW REPORT

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Reviewer's country: China
Science editor: Ya-Juan Ma
Date sent for review: 2015-02-22 20:16
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CLASSIFICATION	LANGUAGE EVALUATION	SCIENTIFIC MISCONDUCT	CONCLUSION
[ ] Grade A: Excellent	[ ] Grade A: Priority publishing	Google Search:	[ ] Accept
[ ] Grade B: Very good	[Y] Grade B: Minor language polishing	[ ] The same title	[ ] High priority for publication
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		BPG Search:	
		[ ] The same title	
		[ ] Duplicate publication	
		[ ] Plagiarism	
		[Y] No	

COMMENTS TO AUTHORS

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

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ESPS PEER-REVIEW REPORT

Name of journal: World Journal of Gastroenterology
ESPS manuscript NO: 17129
Title: The dual gRNAs guided CRISPR/Cas9 system promotes clearance of hepatitis B virus cccDNA
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Science editor: Ya-Juan Ma
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CLASSIFICATION	LANGUAGE EVALUATION	SCIENTIFIC MISCONDUCT	CONCLUSION
[ ] Grade A: Excellent	[Y] Grade A: Priority publishing	Google Search:	[ ] Accept
[Y] Grade B: Very good	[ ] Grade B: Minor language polishing	[ ] The same title	[Y] High priority for publication
[ ] Grade C: Good	[ ] Grade C: A great deal of language polishing	[ ] Duplicate publication	[ ] Rejection
[ ] Grade D: Fair	[ ] Grade D: Rejected	[ ] Plagiarism	[ ] Minor revision
[ ] Grade E: Poor		[Y] No	[ ] Major revision
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		[ ] The same title	
		[ ] Duplicate publication	
		[ ] Plagiarism	
		[Y] No	

COMMENTS TO AUTHORS

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