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12th February 2020

Dear Dr. Nguyen,

We are grateful to you and the reviewers for taking the time to assess our manuscript and provide thoughtful critique. We have addressed the editorial and referee's requests, which have improved the manuscript. In addition, we provide a detailed point-by-point response below. If you require any further information, please do not hesitate to get in touch.

Yours sincerely,

Professor David C. Hay

Chair of Tissue Engineering

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

--We have thoroughly proofread the manuscript.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

--We have updated the Materials Table to address these requests.

3. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

--We have removed the embedded figures from the manuscript and uploaded each figure separately.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Nucleofector, Amaxa, etc.

--We have removed commercial language in the manuscript.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

--We have modified our protocol steps and referenced original papers wherever necessary.

6. 3.1: Growth conditions?

--Now included in the manuscript text.

7. 3.11: What is the tube size?

-- Now included in the manuscript text.

8. Please specify all experimental parameters used throughout.

--Complete.

9. 3.13: What volume is used to resuspend the cells?

-- Now included in the manuscript text.

10. 3.14: What are the electroporation settings used? Please provide the parameters to decouple the protocol from the device.

-- Unfortunately this is not possible. We have provided as much detail as we can.

11. 3.15: The medium is warmed to what temperature?

-- Now included in the manuscript text.

12. 3.16: Incubator is at what temperature?

-- Now included in the manuscript text.

13. 3.21: Temperature?

-- Now included in the manuscript text.

14. 3.26: How is lysis done? Please note that if this is to be filmed, we need the details here.

--The procedures are from the link provided and this will not be filmed.

15. 3.28: Sequence how?

--Sanger sequencing is provided by the sequencing company.

16. 4.3: How is this done? If this is to be filmed, we need the details here.

--The step-by-step procedures are provided in the referenced paper. This will not be filmed.

17. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
18. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

-- Highlighted.

19. Please note that steps 1 and 2 are not appropriate for filming as it is too abstract (Decision making).

-- Note taken.

20. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

-- We have revised the Discussion accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The article by Wang et al. describes the method of introducing point mutations into human pluripotent stem cells using Cas9 nickase system. They targeted HNF4α gene which has important roles in endoderm differentiation and inserted two point mutations in the exon 8. This paper uses HNF4α gene as an example to describe the genome editing technique and is not related to endoderm differentiation or the role of HNF4α in endoderm differentiation from pluripotent stem cells. However, the abstract creates wrong impression about the context of the current methods and is not appropriate for this method article.

--We agree with the referee and have removed the sentence that might cause wrong impression in the abstract.

The method can be used by many researchers who would like to insert a mutation or correct a mutation in human stem cells.

Major Concerns:

A majority of the manuscript in the protocol refers to previous papers and the experimental steps are not described in detail. For instance, the sections explaining designing and constructing Cas9n/sgRNA and piggyBac-based target vector and removing transposan are very brief compared to the other sections and the important steps were explained as "follow the published protocol". It is important that the authors explain each step.

-- We agree and have modified the text to provide more experimental details, in combination with the appropriate references, to better equip the reader.

A second example: what is meant by "appropriate" space and orientation in single-guide RNA design ?;

--We have provided more details in the revised manuscript, explaining this in the NOTE text.

Details of cloning reactions, PCR reactions, sequencing, and primer sequences are not included and explained in a step by step manner. The list of required materials and equipment is not available. If all this information is already in the published literature then what is the purpose of this report?

--Our article's focus is on human pluripotent stem cell genome editing using an up-to-date and reliable CRISPR/Cas9 system. Therefore, we concentrated on the novel aspects of our study. The molecular details requested are sufficiently documented in the literature to enable our scientific audience and we were keen not to duplicate this.

The authors describe nucleofecting stem cells in detail but critical pieces of information is missing such as "volume" of DPBS and "medium", "percentage of O2".

--We agree and the relevant Information has been added in the revised manuscript.

Poorly defined and subjective terms should be avoided such as "maintain the cells in good condition" or "maintain the colonies well".

--We agree and the subjective terms have been removed.

Total number of colonies screened by PCR, and the number positive colonies are not provided. Percentage of heterozygous, homozygous, and non-targeted clones are not available to consider whether the technique is efficient or not.

--We agree and the numbers have been added in the discussion for editing HNF4a gene.

There are additional concerns in the Figures; 1) Why is the WT image missing in Figure 4A? 2) What does blue color represent in IgG control in Figure 4B?

--We apologize for the missing image, caused by a technical failure. The blue color represents DAPI staining and it has been explained in the updated figure legend.

Reviewer #2:

Manuscript Summary:
The manuscript is of high inpact for the stem cell community in academia and industry interested in efficient and robust CRISPR/Cas9-based geneome editing tools. The protocol is descirbed in sufficient details and summarizes the current state-of-the-art. Some minor aspects should be implemented into a revised version of the manuscript.

Minor Concerns:

1. It wold be reasonable to refer to the respective Addgene stock numbers, wherever plasmids are deposited in that repository.

--We agree, the plasmids used in the manuscript from Addgene are listed in the Materials Table.

2. Similarly, for the detailed "Name of Materials-Table" catalogue numbers of the given products need to be provided.

--We agree, the relevant catalogue numbers have been added in the updated Materials Table.

3. The PGK promoter is prone to rapid silencing in human pluripotent stem cells, as the authors shortly discussed in the manuscript. Other promoter systems as used by other groups might be considered and discussed as alternative strategies (e.g. the CAG promoter, used by Eggenschwiler et al; see: https://www.addgene.org/browse/article/25051/)

--We agree that this is a good suggestion. We have not explored this in our lab, but have discussed this briefly in the manuscript.

4. the "hypoxic" and "normoxic" conditions should be described more precisely. Most probably, depletion of oxygen to reach 5%O2 in the incubator is meant by "hypoxia" and the ambient 20% O2 (actually 'hyperoxia') is refered as "normoxia".

--We thank the reviewer for the clarification and have modified the manuscript text accordingly.