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| Department of Health and Human Services LogoDEPARTMENT OF HEALTH AND HUMAN SERVICES | National Institutes of Health  National Institute of  Environmental Health Sciences  P. O. Box 12233  Research Triangle Park, NC 27709  Website: http://www.niehs.nih.gov |

To: *Journal of Visualized Experiments (JoVE)*

From: Negin P. Martin, Ph.D.

Subject: Manuscript JoVE58327 - Editorial and Peer Review Rebuttal

June 21th, 2018

Dear Editor Dr. Wu and Reviewers,

Many thanks for taking the time to review our manuscript. Please find our revised manuscript entitled " Laser-assisted Lentiviral Gene Delivery to Mouse Fertilized Eggs" attached. We hope that the new revisions address all comments and concerns. We have reviewers’ comments and our improvements to the paper listed below.

I appreciate your time and should you have any questions or concerns please feel free to contact me.

Sincerely yours,

Negin P. Martin, Ph.D.

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**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. Done.

2. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file. We have 3 figures uploaded as .tiff files.

3. Please remove the titles and Figure Legends from the uploaded figures. The information provided in the Figure Legends after the Representative Results is sufficient. Done.

4. Figure 1: Please label in Figure 1 the days when the images were taken. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. The figures were marked with Day 0-4 and a scale bar was added to the image.

5. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available. Done – no antibodies used.

6. Please provide an email address for each author. Done – on the first page of manuscript.

7. Introduction: Please rephrase the Introduction to include a clear statement of the overall goal of this method. The introduction was modified to the best of our abilities and we hope that the purpose is clearly stated in our new version. Please feel free to contact me if you need further modifications.

8. Please define all abbreviations before use. Done.

9. Please use SI abbreviations for all units: L, mL, µL, h, min, s, °C, etc. Done.

10. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc. Done.

11. Please use subscripts in chemical formulae to indicate the number of atoms, e.g., H2O, CO2, etc. Done.

12. Please place the ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution’s human research ethics committee. Done.

13. 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. All company names and catalog numbers were removed.

For example: Hamilton Thorne Biosciences, System Biosciences, Charles River, Millipore, Sigma, Molecular Biotechnology, etc.

14. 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. Please see manuscript for the added protocol details.

15. 2.1: Please specify the euthanasia method and describe how to isolate ovaries. Done.

16. 2.4: What is the incubation temperature? Done.

17. 5.2: Please remove the weblink which contains commercial information. Done.

18. 5.5: Please add more details to this step. This step does not have enough detail to replicate as currently written. Alternatively, add references to published material specifying how to perform the protocol action. More details and references were added.

19. References: Please do not abbreviate journal titles. We used Endnote JoVE style in the latest version. But, it still abbreviates the journal titles. Is there another style that we should use?

**Reviewers' comments:**

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

**Reviewer #1:**

Manuscript Summary:

In this manuscript, the authors described a novel method to generate transgenic mice using lentiviruses. They used laser to punch a hole on zona pellucida, and then cultured these punched embryos with lentiviruses. Based on their result, the authors could obtain ~70% transgene rate, and declared this method would be more friendly for researchers without embryo micromanipulation.

Major Concerns:

None

Minor Concerns:

1. In line 128, the authors should make sure whether they prepare 1x solution of hyaluronidase "in water". Great point. We prepared 100x stock in water and then dilute to 1x in M2. This was added to the protocol steps.

2. In line 138, the authors should take ovaries and oviducts. Corrected in the manuscript to reflect Ovaries and Oviducts.

3. In line 177, the authors should describe the size of "holes" the laser punctured, since this is the key point of this technique. The size of the hole, diameter 10 um, was added to the manuscript. Also, Figure 2 was modified to show an actual picture of the holes with scale bar.

4. In line 187, the authors should describe the volume of KSOM drop since it is highly related to the concentration of lentiviruses. The authors should also describe the optimal concentration they suggest here. All requested information was added.

5. In line 230, the authors may not need to describe "Number of integrated lentiviral genome copies was validated by genotyping the pups tail samples" since they did not should this result. We removed the statement and also included the result of our genotyping for readers.

6. In line 229, the embryo transfer rate is relatively low (12/58). The PI may provide opinions for this aspect. We hypothesize that the lentiviral treatment may have played a role in embryo development and contributed to the low embryo transfer rate. The viral treatment has an adverse effect on blastocyst formation and the transduction may also impede development. We also included references from published literature about the expected rate of embryo transfer with NSET and our results.

7. In line 249, the authors should label in different images and describe the different developmental stages of fertilized oocytes in the figure legend. All requested information was added to Figure 1 and the representative result section in the manuscript.

8. In figure 2, it would be better to provide the real images. This was a great improvement. The new version of Figure 2 has an actual picture of the perforated embryos with a scale bar.

9. In figure 3, the authors need to label these mice in the image. The transgenic pups were labeled with an arrow in Figure 3 and the genotyping result was added to the figure legend.

**Reviewer #2:**

Manuscript Summary:

The report describes the use of a laser equipment (XYclone Laser System) for delivery of a transgene construction into the mice zygote by perforation of the zona pellucida. The idea is into the scope of the journal. However, this information was reported by the same authors this year in Transgenic Research, showing different technical parameters with different laser treatments performed in different experiments. In addition, graphic material (i.e. photos and diagrams) were also reported in that paper. So, the question is which is the new information about the method or technique reported previously? In my opinion, the contribution of the current MS is not too different than the method available in the previous paper.

After the publication of our paper in *Transgenic Research*, we were approached by *JoVE* to prepare a video demonstrating the procedure for perforating the zona pellucida of the mouse fertilized eggs. We hope to provide more details and demonstrate how the zona is perforated for lentiviral gene delivery in this publication.

As called to attention by the editor, novelty is not a requirement for publication in *JoVE*. To give an example, the video about transformation of bacteria in *JoVE* is regularly watched by our students during first week of laboratory orientation. It is not novel but provides a wealth of information, a detailed protocol, and a visual demonstration of the method. *JoVE* videos are an amazing educational tool for researchers.

**Reviewer #3:**

Manuscript Summary:

The MS presents an interesting method using laser to thin the zona pellucida of early embryos and perform lentiviral transduction without the need of laborious embryo micromanipulation. The method is already used in several laboratories and species, with good results. The MS is well written, english is clear and the methods as well described. In order to help the authors improve the MS, I wrote below some recommendations:

Major Concerns:

- XY Clone is a commercial name, I recommend you to use a generic name throughout the MS since this protocol should be carried out using any brand of laser. We agree with the reviewer that any laser used for *in vitro* fertilization can be substituted in place of XYClone laser. We replaced all “XYclone laser” with “laser” in manuscript except for material for section 3 of the protocol where we demonstrate the perforation. In section 3, we had to give detailed information about how to setup and perforate the zona. Each laser is setup differently, so we left the XYClone laser in that section but added the statement: “Other lasers, typically used for *in vitro* fertilization, can be substituted for XYClone laser to perforate fertilized eggs.” at the end of the protocol step 3.1.

- I would not say either that the laser is economical (abstract, line 49), since several laboratories cannot afford this cost. Our laboratory is very small with limited resources. Since the laser can be used for gene delivery (Viral Vector Core), *in vitro* fertilization (Comparative Medicine Branch), and embryo dissection (Stem Cell Biology), two of our departments combined resources to buy the laser. The laser is very small and similar in size to an objective lens. It is setup within a few minutes and can be transported and shared among several labs easily. In contrast, microinjection stations at our institute are not shared among biologists in different labs. The economical reference is because the laser is half the price of a microinjection station (based on pricing in our institute) and the cost can be shared among several laboratories/departments. However, we respect reviewer’s wishes and removed the comment about the laser being economical. Please note that we have no relationship with the XYClone manufacturers.

- Regarding representative results, please include how many embryos were incubated with lentivirus and how many were GFP+ at morula/blastocyst stage (in vitro efficiency). We included the requested result in the representative data section.

- It seems that very few pups were born after embryo transfer, could this be due to some problems in the embryos linked to the use of lentivirus? or rather due to the use of NSET? Please clarify. We hypothesize that the lentiviral treatment may have played a role in embryo development and contributed to the low embryo transfer rate. The viral treatment has an adverse effect on blastocyst formation and therefore, transduction may also impede development. We also included references from published literature about the expected rate of embryo transfer with NSET and our results.

- Please include the number of integrated lentiviral genome copies in the representative results to evaluate overall efficiency. This data was added to the manuscript.

Minor Concerns:

Introduction

Line 63: include "among other species" after pig, since other transgenic species have been produced also Done

Line 75-76: include other references for this statement, since removal of zona pellucida is a standard procedure in several techniques without affecting embryo development or causing any harm. Our apologies. We only use murine embryos for research at our institute and we meant to discuss mouse fertilized eggs without zona in that statement. Fertilized eggs of larger mammals, for example humans, develop well without the zona. We corrected the text to reflect the above and included a reference for the mouse zona in the manuscript.

Results

Since the used lentiviral vectors had GFP, it would be good to include in vitro efficiency before embryo transfer (how many GFP embryos/total infected embryos) This result was included in the representative data section.

Protocol

1.2 : The mice need to be superovulated first, please include that if necessary. We did not super ovulate the mice in these experiments. This was added to the protocol.

2.1 : change "isolate ovaries" for "isolate ovaries and oviduct" Done

4.1 : lentiviral vectors remain in the KSOM drop until blastocyst development? Please clarify if the media needs to be changed. We added to the protocol that the media is not changed during the entire experiment.

Materials table

Hyaluronidase is duplicated, please delete one Done