Dear Dr. Alaghemandi,

We would like to thank you for considering our manuscript for publication. We have gone through the editor and reviewer comments and addressed them to the best of our ability, and have submitted a rebuttal as seen below. We hope that you will view our manuscript favorably as we believe that our novel method will be of interest to those in the stem cell and liver regeneration fields and hope to hear from you soon,

Sincerely,

Yuval Rinkevich

Jonathan Tsai

Irving L Weissman

**Editorial comments:**

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.

2. Please provide at least 6 keywords or phrases.

3. Please define all abbreviations before use.

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.

5. 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.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

7. Protocol: Please remove the Regents and Equipment from the beginning of the protocol. Please list them in the Material Table.

8. 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.

9. Protocol: 1.4 What is the appropriate environment? Please provide a quantitative measure.

10. Protocol: 2.3 What is the standard dissecting scope? How much light is adequate?

11. Protocol: 2.4 What is the standard isoflurane anesthesia chamber?

12. Protocol: 2.5 What are the temperature settings used?

13. After revising the protocol, 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.

14. Please include a title of each figure legend.

15. Figure 3: Please make sure that the vertical axis is in percent not a fraction of 1. Please provide more information in the figure legend.

16. Figure 4: Please label all the panels and use SI units (i.e. m, mm). Also, please describe all the panels in the figure legend. In the bottom right, is that part of panel B or C or panel D? Please use the Greek symbol mu in the micron abbreviation.

17. Figure S1 Should arrows for “day 7” and “day 56” point to a white space?

18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

We thank the editor for these comments and have made all the corresponding changes in the text.

**Reviewers' comments:**

Reviewer #1: Manuscript Summary:

This is a well written protocol on an important model of liver regeneration in neonatal mice. This protocol adds to the recent body of work on the important regenerative window in neonatal mice. The protocol is clear, and should be a very useful method for investigators.

Major Concerns: None

Minor Concerns: The current figures do not show crucial steps of the surgery, which would make it easier for investigators to perform the surgery (in addition to the video). It would be important for the authors to discuss the reproducibility of the protocol, as well as potential pitfalls and percentages of survival.

Due to the microscope nature of this surgery, we have found it difficult to obtain high quality images of the intermediate steps of the surgery, and hope that the video will be able to adequately show these steps. We have added text regarding the reproducibility, pitfalls, and survival rates in the manuscript.

Thus, I recommend it for publication in JOVE after addressing these minor points.

Reviewer #2: Manuscript Summary:

The authors present a surgical method to study liver regeneration in neonates. This is done to assess real liver regeneration when compared to compensatory hyperplasia of the remaining lobe which is the result of partial hepatectomy in adult mice. The authors present their protocol of resecting the apex of the liver in neonate mice and show by HE staining and EDU as a regeneration marker that there is specific regeneration at the site of resection.

The specified method woulb be of particular interest as regeneration is studied in resected lobes but not in remaining lobes as after standard two third partial hepatectomy. Therefore the physiologic environment stays intact.

Major Concerns:

- It remains unclear if the model can show specific biological processes that add information in addition to standard hepatectomy.

We acknowledge this reviewers’ point and invite the reviewer to read the publication of which this method is based on for biological relevance and our pertinent findings.

- To standardize the extent of resection will be extremely difficult. First, because of the lack of anatomic landmarks and second because of the small size of livers in neonates. How did the authors ensure standardization of experiments.

- It remains unclear if the lack of liver tissue or just surgical injury leads to the differences desbried in figure 3 and 4. The authors should add other controls and also show that the effect is dose dependent: They should assess Proliferation with different extent of liver resection in neonates.

- The authors show only differences in liver weight in figure 3 but not other regeneration markers. To measure liver weight is typically not specific and precise enough because of inherent high variability and blood contents of the liver.

- Morphology: The authors need to show by the use of specific markers that that parenchymal and non-parenchymal cells (stellate cells, vasculature, infiltrating and resident inflammatory cells and others) are similar in the regenerated lobe compared to remaining tissue.

We acknowledge that all the points that the reviewer has raised are highly important but we believe are ultimately out of the scope of our methods manuscript and have been already covered in our recent publication. Please see Tsai et al, 2017 for the all the information requested.

- The authors need to state that approval for this type of experiments has been obtained.

We have added this line under an ethical statement preceding our Procedures section.

- What should be the primary read out after partial lobular hepatectomy. Proliferation marker or percentage of whole in comparison to an unresected lobe? Please specify. After defining a primary read out, the authors should suggest a power calculation in order to detect differences in regeneration.

There are multiple read outs depending on the question being tested experimentally. We have shown both proliferation in terms of EdU positivity and regeneration as a percentage of the whole.

Minor Concerns:

- What are the reasons that the authors did not use analgetic drugs?

We apologize for this omission and have added in the analgesic regimen.

- The results of individual mice should be shown with dots rather than mean and error bars.

As we have done n > 30 surgeries, we believe this data would be better represented as mean with error bars.

- The authors described that they used various genetic modified animals. The results with these mice are not described.

The results for these mice are ultimately very similar to the C57BL6 mice and were used for functional studies and were thus not included in the methods manuscript. Please see Tsai et al 2017 for these results.

Reviewer #3: Manuscript Summary:

Tsai et al have presented a methods paper on a new surgical approach for studying liver regeneration in neonatal and juvenile mice. The paper is interesting and the method presented could advance studies on liver regeneration relevant to clinical pediatric adverse events. This is in contrast to existing rodent liver regeneration methodologies, which are performed on predominantly adult rodents and are used not to model a clinical situation, but rather to study more fundamental regenerative processes. Overall, the submission is judged favorably. However, in my evaluation, some major corrections, clarifications, and additions are required before this paper can be accepted for publication, as specified below.

Major Concerns:

1) In the abstract, introduction, and elsewhere, the mechanistic presentation of liver regeneration is misleading. Thus, it is stated in the abstract (lines 33-36) and in various related ways elsewhere that "Adult liver regeneration after 70% partial hepatectomy results in hepatocyte hypertrophy in remaining lobes with restoration of metabolic activity but with permanent loss of the injured lobe's morphology and architecture." Whereas this is not untrue, it is misleading, in particular in the focus on hypertrophy and omission of cell cycle events. During regeneration there is, indeed, some hepatocyte hypertrophy, but the regenerative growth is matched by DNA replication, such that there is no change in hepatic nuclear:cytoplasmic ratios. The hypertrophy is associated with increased cell ploidy that has been shown, at least in some situations, to result from complex dysregulation of cell cycle checkpoints (Duncan, 2009; Gentric, 2015 - both citations missing from manuscript). Among adult organs, the cells of the liver (hepatocytes and others) are eccentric in their ability to reenter the cell cycle, proliferate, and occasionally escape checkpoints to allow some degree endoreduplication and thus some degree of hypertrophy. A more complete and less misleading presentation of the liver regenerative process should be used.

We thank the reviewer for this point and have changed our wording in this line to reflect this.

2) Lines 75-77. The highly speculative conjecture about non-liver organs is not supported by data in this paper nor by cited literature. This should be omitted.

We have omitted this statement as this reviewer has requested.

3) Lines 68-99 require careful exhaustive citation of the original literature to support each of the many statements.

4) Lines 111-115. All mouse strains used need citation and accession numbers (e.g., JAX stock #s or equialent). All strains reported must be available for unrestricted use to the research community, and this should be explicitly stated.

We have included these citations as this reviewer has requested.

5) Lines 120-122. Complete scientific details of the relative juvenile hepatotoxicity of corn oil, DMSO, and alcohol this should be presented. DMSO and ethanol are generally considered far more hepatotoxic than corn oil, so this is a potentially important observation in juveniles that needs to be supported by complete detailed statistically validated data.

We believe this is out of the scope of the manuscript as we are not testing the use of chemical damage in the liver, but proposing a new injury model. We are consistent in the use of DMSO in our neonatal mice and do not use corn oil in these procedures, therefore the use of alternate chemicals should have no impact on our method.

6) Lines 128-129. The specialized animal care needs to be specified in enough detail to allow other researchers to replicate the procedure and survival rates.

We have included this as the reviewer has requested.

7) Use of analgesic, or rationale for not using analgesic, should be addressed.

We have included our analgesic regimen as the reviewer has requested.

8) Statement of institutional ethical approval of procedures is not provided.

We have included this as the reviewer has requested.

Minor Concerns:

1) Lines 59-62. Confusing sentence structure. Rewrite for clarity

2) Line 67. This is also true for other rodents, in particular rats, not just mice.

3) Lines 118-120. Specific formulations and any specialized procedures for preparing 4-OHT and 5-EdU should be specified in sufficient detail to allow other researchers to replicate the procedure and reported survival rates.

We have revised these lines as the reviewer has requested.

4) Line 234. Silk sutures are used so the protocol likely needs to include removal of suture.

We do not remove the suture and have found that they fall out from the dermis as the mice grow in size.