Responses to Review of Manuscript 58399\_R0\_050318

**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.  
2. Please upload each Figure individually to your Editorial Manager account as a .png or a .tiff file.  
3. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file.  
4. Figure 2: Please provide a scale bar in the figure.  
5. Figure 3: Please include a space between the number and the units of the scale bar. Please increase the font size of text in scale bar to make it easier to read.  
6. Please provide an email address for each author.

7. Please rephrase the Long Abstract to more clearly state the goal of the protocol.  
8. Please define all abbreviations before use.  
9. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.  
10. 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: Endo Catch, Dermabond, etc.  
11. 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.  
12. Please mention how animals are anesthetized and how proper anesthetization is confirmed throughout the procedure.  
13. Please specify all surgical instruments used.  
14. 1.1: Please provide the gender, age and type of animal.  
15. 2.1: Please provide more details about the open Hasson technique or add references.  
16. 2.2: Please use subscripts in chemical formulae to indicate the number of atoms, e.g., CO2.  
17. 3.9.4: What is used to count cell concentration?  
18. There is a 2.75 page limit for filmable content. 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.  
19. 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.  
20. References: Please do not abbreviate journal titles.  
21. 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.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**  
Manuscript Summary:  
This protocol describes porcine hepatocyte isolation and ex vivo gene delivery to cure metabolic diseases via autologous cell transplantation. The particular model that is described is characterized by unique advantages that favor successful therapy.  
  
Major Concerns:  
None  
  
Minor Concerns:

1. **Line 313: please correct ex vivo**

*Response:* Corrected

2. **Please reduce the number of non-standard abbreviations to increase readability of text.**

*Response:* This has been addressed throughout text

3. **The approach is described for treatment of fumarylacetoacetate hydrolase deficiency. The success is essentially dependent on the selective advantage of transduced cells. What is the percentage of transduced hepatocytes immediately after cell transplantation (percentage of total number of hepatocytes)? What is the absolute number of infused cells (and the percentage of total number of infused cells) that engrafts?**

*Response:* The reviewer makes an excellent observation, and this study was carried out to evaluate the long-term benefits of *ex vivo* transplantation. Due to the expansion of the engrafted cells, the method employed (IHC data several months after transplantation) cannot address actual initial engraftment values, and text has been added to indicate that although 109 cells were transplanted, actual initial engraftment was not evaluated. Related data and some literature accounts suggest this is probably 10-20% of the transplanted cells that engraft, acutely comprising ~0.1% of the hepatocytes in the liver. For indications that do not have the selective advantage for corrected cells, this will need to be carefully considered. However, these efficiency data are not readily attainable, requiring either biopsy or other forms of *in vivo* imaging that require additional cell manipulations.

**Reviewer #2:**  
Manuscript Summary:

In this article the authors describe a protocol to isolate hepatocytes from a liver resection, followed by lentiviral transduction and autologous cell transplantation. The authors perform this procedure in a pig model of tyrosinemia type I, which has a natural high potential of cell repopulation for corrected cells. They highlight the potential benefits of this approach for the treatment of this and potentially other inborn errors of metabolism.  
  
Major Concerns:  
The reviewer does not have any major concerns.  
  
Minor Concerns:  
The following minor concerns should be addressed by the authors.  
**1) Although the repopulation of normal hepatocytes is very high in HT1, there is never a complete repopulation of the liver with normal cells and some Fah-deficient cells will always remain, and these will over time progress to tumours. I believe that the authors should mention this in their article, highlighting that the approach suggested, although very useful as proof of principle in an animal model of HT1, cannot be curative in humans.**

*Response:* The reviewer is correct in pointing out that there may be some un-transduced cells that remain in the liver, and we have removed a reference to “fully” repopulated livers in acknowledgement of this possibility. However, we respectfully disagree that the approach cannot be used to cure humans with HT1. The question of clinical relevance is valid for this indication and our data support that this is an effective cure in pigs. We observe NTBC-free weight gain, biochemical normalization, metabolic integrity, and reversal of the fibrosis, with all indications suggesting translation to humans with FAH deficiency. Furthermore, our long term treated pigs (including an animal up to three years after transplant) show no pockets of uncorrected cells after taking thousands of histological specimens and have never demonstrated HCC, long term fibrosis and certainly no cirrhosis. In HT-1 mice, HCC still develops even after correction, which is an important note. However, mice unlike humans and pigs develop HCC through a fibrosis cirrhosis independent process. With the exception of rare tumors like fiborlamellar HCC, HCC in humans and other higher mammals occurs through a fibrosis, cirrhosis liver injury process. In all of our long-term animals we see no long term fibrosis or liver injury. The fibrosis and liver inflammation and injury we see during NTBC cycling is reversible and by 12 months and progressing to the life of our animals we see no persistent inflammation or injury.

We have added text to the discussion on the point of clinical relevance without deviating from the objective of the manuscript, which is general application of this surgical method to this and other disease models for research purposes.

2) **The authors include two solutions PerI (PerIA and B) in table 1, however they do not mention the use of these two solutions in section 3. It would be good to specify how and when these two solutions are used.**

*Response:* References to PerI and II are made throughout Section 3, and how and when it is used can be found in subheads 3.1.1., 3.4., and 3.5.

3) **The authors should explain which component in PerII is used for the digestion of the tissue. The most commonly used digesting enzymes are collagenases, which, according to table 1 recipe, do not seem to be part of PerII.**

*Response:* Thank you for this important observation. PerII has been updated with the collagenase concentration used

4) **In the results section, the authors should clarify whether figure 2 refers to transduced or native cells. It is expected that it is referred to transduced cells, however it is not clear. The authors should make this clear in figure 2 caption too.**

*Response:* This has been clarified to be transduced cells in this figure

5) **As highlighted by the authors in the discussion, 'a good transgene expression of the functional FAH enzyme is a minimum requirement for liver repopulation'. The authors are advised to provide more information about the efficiency of transduction in cells before transplantation and how this is assessed. Does the virus contain any tags, e.g. GFP, allowing quantitation of transduced cells over total population? Also, the authors should provide more information about which lentiviral plasmid has been used to transduce the cells, as it is important in case other research groups want to perform the same protocol.**

*Response:* The reviewer raises an interesting point, which we have addressed in text added in 4.5.1. Basically, timing of the surgical procedure precludes diagnostic evaluation of the cells for transduction or viability prior to transplantation. However, we suggest evaluation of the leftover cells as a indicator of success of the methods, and thereby a predictor of overall study success. We use untagged cDNA, and expression of Fah in culture was not performed, as the result of these experiments is best evident in subsequent biopsy and immunohistochemistry in the actual tissue. However, this method is intended to be generally applicable to ex vivo gene therapy in pigs, and we have made mention that this step provides an excellent opportunity to gauge success and characterize the initial cells transplanted for those manipulations lending themselves to such interrogation here.