Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Point-by-point response

We thank all three reviewers for their comments and suggestions to improve the manuscript !

PEER-REVIEW REPORT

Name of journal: World Journal of Hepatology

Manuscript NO: 37312

Title: Homologous recombination mediates stable transgene integration and phenotypic correction in tyrosinemia mouse-model

Reviewer's code: 02741591

Reviewer's country: Egypt

Science editor: Fang-Fang Ji

Date sent for review: 2017-12-11

Date reviewed: 2017-12-12

Review time: 1 Day

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

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

COMMENTS TO AUTHORS

The title could be refined to to refer to the precise Fah gene

We thank the reviewer for the comment. The reviewer is right, the title would be more precise with his suggestion and therefore we refined the title.

The first 4 lines in the abstract results could better move to the background of the abstract as they do not include results found in this work. The abstract results is superficially presented and values of signficance much be presented.

We revised the abstract according to the comments.

The introduction is nicely written The methodology is adequate but could better be wrapped up. The results should be revised and written in a way to present the reached findings values. Many parts within the results would be more sutiable for the methodology or the discussion.

We revised the result section and tried to focus more on the results.

Discussion: In many in vitro 18 and some in vivo 19 studies.... COMMENT: Using the words 'many' and 'some' 'studies' implies that more than 2 references are cited. Rephrase the sentence to best reflect the single references cited.

We rephrased this.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

....with a point mutation for FAH..... COMMENT: Unify using the abbreviation through out the article 'Fah'.

The reviewer is absolutely right, this was a mistake. We corrected it and specified as FAH for the protein; and *Fah* for the gene.

Almost all the second paragraph of the discussion (17 lines) is not cited by appropriate references.

We have added references here.

In our serial transplantation experiments, ... whereas only 1/5 mice injected with rAAV8-R26.Fah had FAH-positive clusters. COMMENT: The third paragraph is not cited by the required references although it refers to serial studies of the authors that should also have been cited and references presented.

This part just describes the second in vivo experiment of this study, therefore does not require citation. Nevertheless, we have rephrased the paragraph, so that it is clearer that we describe our presented study.

We could not find any tumour formation in any of our mice, ... of cell doubling for the hepatocytes 24. COMMENT: It is unclear from this paragraph if the statements in this short paragraph refer to the present study or that of the reference cited [24].

We agree that this sentence may lead to confusion about this. We have rephrased the sentence. The cell doubling part refers to a reference.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

PEER-REVIEW REPORT

Name of journal: World Journal of Hepatology

Manuscript NO: 37312

Title: Homologous recombination mediates stable transgene integration and phenotypic correction in tyrosinemia mouse-model

Reviewer's code: 00291404

Reviewer's country: United States

Science editor: Fang-Fang Ji

Date sent for review: 2017-12-11

Date reviewed: 2017-12-14

Review time: 3 Days

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

COMMENTS TO AUTHORS

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

This is a very good study, well thought and controlled. The manuscript is also well written.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

PEER-REVIEW REPORT

Name of journal: World Journal of Hepatology

Manuscript NO: 37312

Title: Homologous recombination mediates stable transgene integration and phenotypic correction in tyrosinemia mouse-model

Reviewer's code: 01806391

Reviewer's country: Spain

Science editor: Fang-Fang Ji

Date sent for review: 2017-12-11

Date reviewed: 2017-12-14

Review time: 3 Days

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

COMMENTS TO AUTHORS

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

In this study the authors have tried to demonstrate that in a state of extensive hepatocyte proliferation, targeted integration by homologous recombination would be superior to gene therapy based on episomal AAV gene therapy. Both the promoter and the recombination strategy and locus have been already used by this and other groups. The study is interesting but of limited originality.

We thank thw reviewer for the comment. Indeed parts of our approach have been used be others and by our group, but the combination of AAV8 gene therapy with liver specific promoter and homologous arms for a safe harbor in in-vivo gene addition in a C57BL/6 *Fah*$^{\Delta exon5}$ mouse in state of extensive hepatocyte proliferation was not investigated before. We show first time proof of concept for the gene addition by homologous recombination at a safe harbour (Rosa26) site in the genome. There are two important studies on gene therapy in tyrosinemia mouse model from Paulk Nk et al. [1,2] but the crucial difference of these studies to ours is that they used a mouse model with a point mutation. Therefore, they aimed for gene correction and not for gene addition like we did. A gene correction with homologous arms of the point mutated gene is very elegant but has important disadvantages in comparison to gene addition. (1) The constructed plasmid/virus can only be used for one mutation, for most diseases; however, many different mutations are known to lead to phenotypic disease. (2) Thus, the published approach can only be used for one particular disease. In the case of gene addition the plasmid and vector with the homologous arms and the target safe harbour could be used for various disease entities.

COMMENTS In the primary recipient mice of both experimental groups (rAAV8-TTR.Fah and rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR) survival and phenotypic rescue that would be derived by clonal expansion of corrected hepatocytes (which implies vector integration) were found. The authors explain these findings by a selection advantage for corrected hepatocytes and random integration or another mechanism of integration when using rAAV8-TTR.Fah. To demonstrate the advantage of using AAV gene therapy with homologous recombination they included another set of experiments of serial transplantation, in which they observe an advantage of homologous recombination vs AAV without ROSA26 seq. 1. Why if in primary recipient mice rAAV8-TTR.Fah showed similar long-term efficacy to rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR the authors concluded that there was

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

an advantage of the later in the second recipient mice? Could this be explained by a marked difference in the number of Fah positive hepatocytes isolated in each group of primary recipient animals? Fah immunohistochemistry would be required to calculate the percentage of positive Fah hepatocytes in each case.

Our hypothesis was that the mouse group receiving virus containing the Fah gene with homologous arms, but without nuclease, would also show a survival advantage after hepatocyte transplantation. Homologous recombination of the Fah gene may happen even in the absence of nucleases, although at a very low frequency. Due to the strong selection advantage of gene corrected hepatocytes, the livers of both, primary treated secondary transplant recipients would thus be repopulated. Only those effects, which are not related to homologous recombination (i.e. episomal expression), would be lost in secondary recipients.

We agree with the reviewer's suggestion that difference in the number of FAH positive hepatocyte may be a determining factor. Hence, we determined presence of FAH positive area in the both groups of primary recipients. We did not find significant differences in FAH positive areas indicating similar number of FAH positive hepatocytes in both groups of mice. The FAH immunohistochemistry data is presented in the Figure 2 and 3 of original version of the manuscript. We have added this information in the "Discussion" section on the page 10 and 11 of the revised manuscript.

2. To demonstrate that homologous recombination improves the survival and phenotypic rescue in the second recipient mice a selection of isolated hepatocytes should by develop to inject the same number of Fah positive hepatocytes (from rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR and rAAV8-TTR.Fah mice) in the second recipient mice. If authors wanted to justify that the episomal expression was lost after the implant in the second group of mice, they should have made sure that they have used the same number of Fah-positive hepatocytes in one case and another.

Indeed, we have transplanted equal number of hepatocytes (1 million cells) in both groups of secondary recipient mice. Hepatocyte suspensions were generated from the whole donor liver by the two-step collagenase (Roche) perfusion method, as described previously [3]. From those hepatocyte suspensions 1 million hepatocytes were used for each second generation recipient mouse. Unfortunately, it is

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

experimentally not possible to isolate only the FAH protein expressing hepatocytes after hepatocyte isolation from mouse. However, due to the high number of FAH positive clusters in both groups it is very likely to have incorporated similar numbers of FAH expressing cells in the transplant. Small differences would not affect the results due to the fact that we transplanted more than one mouse per group. That means it is very unlikely that by chance in 4 cases of the rAAV8-TTR.Fah mice 1 million hepatocytes with less FAH expression were used and in 6 of the rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR mice hepatocytes with high amount of FAH expression.

3. In the last part of paper, the authors used specific primers to demonstrate successful targeted integration of Fah in the ROSA26 locus using rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR. As expected no PCR product was observed in rAAV8-TTR.Fah using these primers.

The aim of this experiment was not to show that there is no PCR product in liver tissue of rAAV8-TTR.Fah mice. In fact the aim was to show that we have a PCR product in the liver tissue of rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR mice, since this verifies the integration at the target locus.

However, it would be interesting to analyze the expression of TTR.Fah in samples of both groups of animals in order to compare the level of transgene expression in both cases. We added SYBR green RT qPCR results for second generation mice (hepatocyte transplanted mice) to the manuscript (Figure 5). These results show that FAH mRNA is clearly more expressed in mice treated with rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR.

4. Figures 2a, 2d, 3a and 3d are not clear enough and it should be improved. An explanation of the abbreviations (PH, HcTx) used in the figures should be included.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

We have now added missing explanation of the abbreviations to the figure legend. We also improved quality of the figures. We can also provide individual figures in the form of power point or TIF in addition to embedded figures in the revised manuscript; this should improve the quality further.

5. In Figures 2d and 3d an arrow (or another indicator) should be used to indicate the end of the body weight line of the mouse used to carry out partial hepatectomy and FAH staining.

In Figure 2d we have only one drop out due to analyses (harvest), we added the arrow there. In Figure 3d is no drop out due to experimental design, just due to progressive disease.

6. Untreated controls (injected with sodium chloride) in Figure 2a and 3a are the same animals? Figure 2a includes three control mice and figure 3a includes only two.

The missing control mice in 3a is added know. We apologize for this technical error. Yes we used one control mouse group for the first generation experiments, since all mice have been treated the same time. Due to ethical aspects we aimed to use as few as possible mice for untreated controls since the natural course of this mouse model without treatment is already well know and 100% fatal.

We also used one control mouse group for the second generation experiment (hepatocyte transplant recipients) due to the same reasons.

We added this information to the method part and to the figure legend.

7. In results section the title of the paragraph "Absence of long-term in vivo correction of Fah in the absence of homologous sequences" must be corrected because primary recipient mice injected with rAAV8-TTR.Fah survived and showed phenotypic rescue after >280 days of NTBC withdrawal. We specified the title and added "after hepatocyte transplantation".

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

8. Figure 4 should be simplified or any explanation about the numbers of each line must be included. Since the number of animals in each group was very low it would be better if samples of all the animals were shown in the PCR gel electrophoresis (the results could be reinforced if a sample of all the animals was included in the gel, not only two).

The PCR gel electrophoresis shown in the Figure 4 is one of the representative figure of multiple analyses. We have now mentioned "representative gel" in the figure legend of Figure 4.

In addition, there is no information regarding whether the samples used in the PCR were from primary or secondary recipient mice, and in what moment these samples were collected.

We added this information to the figure legend.

References

1 Paulk NK, Wursthorn K, Wang Z, Finegold MJ, Kay MA, Grompe M. Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo. *Hepatology* 2010; **51**: 1200-1208 [PMID: 20162619 DOI:10.1002/hep.23481 [doi]]

2 Paulk NK, Loza LM, Finegold MJ, Grompe M. AAV-mediated gene targeting is significantly enhanced by transient inhibition of nonhomologous end joining or the proteasome in vivo. *Hum Gene Ther* 2012; **23**: 658-665 [PMID: 22486314 DOI:10.1089/hum.2012.038 [doi]]

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Name of Journal: *World Journal of Hepatology*

Manuscript Type: *Basic Study*

Homologous recombination mediates stable Fah gene integration and phenotypic correction in tyrosinaemia mouse-model

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Norman Junge, Qinggong Yuan, Thu Huong Vu, Simon Krooss, Christien Bednarski, Asha Balakrishnan, Toni Cathomen, Michael P. Manns, Ulrich Baumann, Amar Deep Sharma and Michael Ott

Norman Junge, Thu Huong Vu, Ulrich Baumann, Department of Pediatric Gastroenterology and Hepatology, Hannover Medical School, Hannover, Germany

Qinggong Yuan, Asha Balakrishnan, Michael P. Manns, Amar Deep Sharma, Michael Ott, Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

Qinggong Yuan, Simon Krooss, Asha Balakrishnan, Michael Ott, TWINCORE, Centre for Experimental and Clinical Infection Research, Hannover, Germany

Christien Bednarski, Toni Cathomen, Medical Center - University of Freiburg, Institute for Cell and Gene Therapy, Freiburg, Germany

Toni Cathomen, Faculty of Medicine, University of Freiburg, Freiburg, Germany

Amar Deep Sharma, Research Group MicroRNA in Liver Regeneration, Cluster of Excellence REBIRTH, Hannover Medical School, Hannover, Germany

ORCID number: Norman Junge (0000-0002-3099-2667), Qinggong Yuan (0000-0001-8446-8193), Thu Huong Vu (0000-0002-3050-9583), Simon Krooss (0000-0001-6372-395X), Christien Bednarski (0000-0002-4787-3293), Asha Balakrishnan (0000-0002-1366-3386), Toni Cathomen (0000-0002-7757-4630), Michael P. Manns (0000-0002-4485-8856), Ulrich Baumann (0000-0002-0162-0266), Amar Deep Sharma (0000-0003-3599-2372) and Michael Ott (0000-0002-1245-358X)

Author contributions: Junge N was involved in conception and design of the research, performed the majority of the experiments, analysed the data and wrote the manuscript; Yuan Q performed mouse surgery and immunostaining and revised the work critically for important intellectual content; Huong Vu T had substantial contributions to the experiments, animal care and analysis and interpretation of data for the work; Krooss S, Bednarski C, Balakrishnan A and Cathomen T helped with the experiments and design of the research and revised the work critically for

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

important intellectual content; Manns MP and Baumann U revised the work critically for important intellectual content; Sharma AD and Ott M were initiator and supervisor of the work, the developed initial concept and design of the research and conducted important preliminary studies.

Institutional review board statement animal care and use committee statement: all experiments were approved and performed according to guidelines and ethical regulations from Hannover Medical School and local government.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author (Ott.Michael@mh-hannover.de)

Conflict-of-interest statement: There are no conflicts of interest for any of the authors regarding this work

Biostatistics statement: This work is a proof of concept study; therefore statistical analysis was not suitable and not applied.

ARRIVE Guideline Statement: This study was performed according to the ARRIVE guidelines.

Correspondence to: Michael Ott, MD, Professor of Medicine, Head of Division
Cell and Gene Therapy Clinical Research Group; TWINCORE, Centre for Experimental and Clinical Infection Research; Feodor-Lynen-Str. 7; 30625 Hannover; Ott.Michael@mh-hannover.de

Telephone: +49-511-220027-120

Fax: +49-511-220027-178

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Abstract

AIM

To stably correct tyrosinaemia in proliferating livers of fumarylacetoacetate-hydrolase knockout (*Fah*$^{-/-}$) mice by homologous-recombination-mediated targeted addition of the *Fah* gene.

METHODS

C57BL/6 *Fah*$^{\Delta exon5}$ mice served as an animal model for human tyrosinaemia type 1 in our study. The vector was created by amplifying human Fah cDNA including the TTR promoter from a lentivirus plasmid as described. The *Fah* expression cassette was flanked by homologous arms (620 and 749 bp long) of the Rosa26 gene locus. Mice were injected with 2.1x10^8 VP of this vector (*rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR)* via the tail vein. Mice in the control group were injected with 2.1x10^8 VP of a similar vector but missing the homologous arms (*rAAV8-TTR.Fah)*. Primary hepatocytes from *Fah*$^{-/-}$ recipient mice, treated with our vectors, were isolated and 1x10^6 hepatocytes were transplanted into secondary *Fah*$^{-/-}$ recipient mice by injection into the spleen. Upon either vector application or hepatocyte transplantation NTBC treatment was stopped in recipient mice.

RESULTS

Here, we report successful HR-mediated genome editing by integration of a *Fah* gene expression cassette into the "safe harbour locus" Rosa26 by recombinant AAV8. Both groups of mice showed long-term survival, weight gain and FAH positive clusters as determined by immunohistochemistry analysis of liver sections in the absence of NTBC treatment. In the group of C57BL/6 *Fah*$^{\Delta exon5}$ mice, which have been transplanted with hepatocytes from a mouse injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR* 156 days before, 6 out of 6 mice showed long-term survival,

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

weight gain and FAH positive clusters without need for NTBC treatment. In contrast only 1 out 5 mice, who received hepatocytes from *rAAV8-TTR.Fah* treated mice, survived and showed few and smaller FAH positive clusters. These results demonstrate that homologous recombination-mediated *Fah* gene transfer corrects the phenotype in a mouse model of human tyrosinaemia type 1 (*Fah*$^{-/-}$ mice) and is long lasting in a proliferating state of the liver as shown by withdrawal of NTBC treatment and serial transplantation of isolated hepatocytes from primary *Fah*$^{-/-}$ recipient mice into secondary *Fah*$^{-/-}$ recipient mice. This long term therapeutic efficacy is clearly superior to our control mice treated with episomal rAAV8 gene therapy approach.

CONCLUSION

HR-mediated rAAV8 gene therapy provides targeted transgene integration and phenotypic correction in *Fah*$^{-/-}$ mice with superior long-term efficacy compared to episomal rAAV8 therapy in proliferating livers.

Key words: Gene therapy; AAV8; Liver based metabolic disease; Targeted integration; PFIC; ROSA26; Paediatric liver disease

Core tip: Recombinant adeno-associated virus (rAAV) has been explored for gene delivery in various murine models of hereditary liver disease, but in young children transgene expression from AAV-epigenomes diminishes over time. We thus explored, whether homologous recombination-mediated targeted gene addition of the fumarylacetoacetate hydrolase (Fah) gene would stably correct tyrosinaemia in rapidly proliferating livers of Fah-/- mice. Here, we report successful homologous recombination-mediated genome editing of a Fah gene expression cassette at the

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Rosa26 locus by rAAV8. We demonstrate that this approach corrects the phenotype and is long lasting in a proliferating state of the liver, as shown by serial transplantation.

Junge N, Yuan Q, Huong Vu T, Krooss S, Bednarski C, Balakrishnan A, Cathomen T, Manns MP, Baumann U, Sharma AD and Ott M. Homologous recombination mediates stable Fah gene integration and phenotypic correction in tyrosinaemia mouse-model.

INTRODUCTION:

Therapy for many liver-based metabolic diseases (LBMD) is limited to supportive measures and may entail significant side effects, such as organ failure, metabolic crisis, malignancy and impairment of quality of life. Until now, the only established curative treatment is liver organ transplantation (LTX). Although LTX for LBMDs has excellent long-term outcomes, the procedure is associated with significant morbidity and mortality and dependent on limited donor organ availability. Gene therapy could provide a minimally invasive therapeutic alternative to whole organ transplantation.

Recombinant adeno-associated viruses (rAAV) have evolved as promising vehicles for gene therapy to date and shown to produce long-term therapeutic effects in many mouse models of inherited liver diseases as well as in patients with haemophilia B[1-3]. AAV of serotype 8 has been shown to target mainly hepatocytes in the liver and is considered to be safe for clinical application[3-6]. Recombinant AAVs express the transgenes from epigenomic circular DNA with only rare genomic integration events[7]. Insertional mutagenesis resulting from random vector integrations has been observed in only one study[8] and these results remain to be confirmed by other studies[9]. Notably, AAV gene therapy in 77 dogs did not cause tumour formation

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

during an observation period of up to 10 years[10]. Nathwani et al[11] presented a study in non-human primates with no signs of insertional mutagenesis 5 years after AAV application. Further, serotype 8 shows lower seroprevalence of preformed antibodies in humans than other AAV serotypes[3,12] thus minimizing risk of significant immune response.

Epigenomic expression of the therapeutic transgene from rAAV is thought to gradually decline in tissues with high cell turnover. Therapeutic efficacy of AAV-mediated gene transfer would thus decrease in growing livers of newborns or in diseases with intrinsic stimuli causing hepatocyte turnover. In some studies, gene correction by homologous recombination of rAAV transduced therapeutic genes was shown to result in long-term cellular persistence. Although the feasibility of in vivo gene correction in mice has been demonstrated in several models, therapeutic and superior therapeutic efficacy of gene therapy by gene addition mediated by homologous recombination remains to be demonstrated. Therefore, we examined whether the application of a *Fah* expression cassette flanked by homologous arms for the ROSA 26 Locus improves the efficacy and persistence of *Fah* gene delivery by integration at the *Rosa26* gene locus through homologous recombination in a mouse model of human tyrosinaemia type 1. We used C57BL/6 $Fah^{\Delta exon5}$ mice, which served as an animal model for human tyrosinaemia type 1[13]. Liver physiology and function in these animals can be maintained by providing water that is supplemented with the drug NTBC (2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione). Control mice die 20-45 days after deprivation of NTBC due to liver failure. In the absence of NTBC, gene corrected hepatocytes proliferate and repopulate the liver.

MATERIALS AND METHODS:

Animal Model

Baishideng Publishing Group
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

All mouse experiments were granted permission and were performed according to the guidelines of the Hannover Medical School, Germany and the local government. Mice were kept on standard laboratory chow and free access to drinking water. They were housed in a restricted access room with controlled temperature and a light/dark cycle. We used C57BL/6 *Fah*$^{\Delta exon5}$ mice, which served as an animal model for human tyrosinaemia type 1[13]. Tyrosinaemia type 1 is caused by genetic alterations of the gene coding for FAH. The mutated *Fah* gene produces an unstable protein, which results in deficiency of fumarylacetoacetate hydrolase activity. The mice were provided with water supplemented with 1 mg/100 ml of NTBC (2-[2-nitro-4-(fluoromethyl)benzoyl]cyclohexane-1,3-dione) before performing experiments. Surgery was done under general anaesthesia with 2% isoflurane and 2 litres/min oxygen flow.

Cloning of AAV plasmids

For cloning of the *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR* plasmid, 620 and 749 bp Rosa26 gene locus homologous arms flanking the *Fah* expression cassette were subcloned into a pBlue-Script II plasmid. The entire transgene was further subcloned into the AAV backbone plasmid for virus generation. For the *Fah* expression cassette, we amplified hFah cDNA, including the TTR promoter, from a lentivirus plasmid described earlier from our group[14] by PCR (Phusion® High-Fidelity PCR Kit, Thermo scientific).

For cloning the *rAAV8-TTR.Fah expression cassette,* we created a similar plasmid with the same transgene cassette but not flanked by the homologous arms.

Preparation of adeno-associated virus serotype 8 (AAV8) vector

The AAV8 vectors, *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and *rAAV8-TTR.Fah*, were prepared as described previously[15]. The titre was determined by qRT-PCR using primers spanning the region of the TTR promoter, as published before[16].

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

AAV8 vector administration into Fah$^{-/-}$ mice

Mice were injected with 2.1x10^8 VP *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* via the tail vein. Mice in the control group were injected with 2.1x10^8 VP *rAAV8-TTR.Fah*. Viruses were diluted in sorbitol to a total volume of 220 µl for injection. Non-treated control mice were injected with 0.9% sodium chloride. We used one control mouse group (N=3) for the first generation experiment. Subsequently, the mice were monitored and weighed daily until they reached stable conditions or gained body weight. After 45 to 47 days, a 1/3 hepatectomy was conducted to analyse the presence of FAH protein-positive cell clusters. Tissues were fixed in 4% paraformaldehyde or snap frozen for subsequent analyses.

Serial transplantation of hepatocytes from virus-injected mice

Primary hepatocytes from primary *Fah$^{-/-}$* recipient mice were isolated with the two-step collagenase (Roche) perfusion method, as described previously[4]. Hepatocytes (1x10^6) were transplanted into secondary *Fah$^{-/-}$* recipient mice by injection into the spleen. Control mice were injected with sodium chloride into the spleen. We used one control mouse group (N=3) for the second generation experiment.

Immunohistochemistry

Tissues were embedded in paraffin (ROTH) and cut in 2-µm-thick slices. Immunohistochemistry was carried out as described previously[17]. Briefly, after deparaffinization and blocking for endogenous H_2O_2, the slides were incubated in 1x target retrieval solution (Dako) at 98°C for 20 minutes. For FAH (primary antibody, Abcam, ab81087) staining, tissues were blocked with the Avidin/Biotin blocking kit (Vector laboratories). Goat serum (Abcam) or rabbit serum (Abcam) was then used for blocking. Biotinylated goat anti-rabbit and rabbit anti-goat secondary antibodies (Vectastain, Vector laboratories) were used. Colour development was conducted using AEC substrate chromogen (Dako). Counterstaining was performed using haematoxylin (Merck Millipore, Germany).

Baishideng Publishing Group
Baishideng®

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Integration PCR

Genomic liver DNA was extracted from snap-frozen liver tissue with the DNeasy Blood & Tissue Kit (Qiagen) according to the protocol of the vendor. Two primers were designed, A and B. A was located in the *Rosa26* locus of recipient mouse 5′ to the donor gene. B was located in the *Fah* sequence of the donor DNA. Primer sequences were A: 5′-GGAGAGAGGCATTCATGGGAGTGGAAAGTTAAGC -3′ and B: 5′-GCAGCATGGTCCAGTACATGTGCTTAAAGTTAGACC-3′. The expected length of the PCR amplicon was 1107 bp. PCR amplification was conducted with the Phusion® PCR Kit (New England BioLabs), and 200 ng of liver genomic DNA was used. The amplification was carried out under the following conditions: one cycle for 190 s at 98°C, followed by 50 cycles for 10 s at 98°C and 90 s at 72°C, finished by one cycle for 10 min at 72°C. The PCR product was analysed utilizing gel electrophoresis on a 1% agarose gel (Biozym) for 50 min at 90 V.

qRT-PCR for FAH Expression

RNA was isolated from snap frozen liver tissue of sacrificed mice. RNA was isolated with RNeasy® mini Kit (Qiagen) and QIAshredder® according to manufacturer instructions. After DNase treatment cDNA writing was performed (iScript™ reverse transcriptase supermix, BIO-RAD). SYBR green qRT-PCR (Qiagen QuantiTect Sybr green®) was performed at Stratagene Mx3000P (Aligent) with following primer (forward primer AGAATGCGCTGTTGCCAAA, reverse primer GGAAGCTCGGCCATGGTAT) spanning exon 5-6 and beta actin as housekeeping gene.

RESULTS

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Long-term functional correction of the Fah gene defect by homologous recombination at the ROSA26 Locus in mice

After confirming the correct design of our plasmids by sequencing and by evaluating FAH-Expression in Hepa1.6 cells by RT qPCR, For our experiments, we used *Fah*$^{-/-}$ mice that contain a disruptive insertion in exon 5 of the *Fah* gene[13].

We prepared a high titre AAV8 vector suspension using the aforementioned AAV vector plasmids.

Next, we injected 4 mice with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* via the tail vein (Figure 1b). To stimulate the proliferation of FAH-expressing hepatocytes, protective NTBC-treatment was discontinued immediately after injection. Whereas control mice (injected with saline) died before 45 days, all mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* survived beyond 45 days after injection (Figure 2a). On the 45-47th days, 1/3 of the liver was removed and analysed for the presence of FAH cell clusters by immunohistochemistry. All animals injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* showed robust repopulation of the liver as indicated by survival, weight gain (Figure 2a) and multiple large FAH protein positive cell clusters in immunohistochemistry analyses (Figure 2c). Importantly, these mice survived without NTBC until the end of the study (day 288; Figure 2a).

Due to high selection pressure for gene corrected hepatocytes in the *Fah*$^{-/-}$ model, phenotypic correction of the enzyme deficiency as result of diluted, but still sufficient, FAH protein expression from epigenomic AAV DNA could not be excluded in the first generation. To test whether homologous sequences facilitated targeted integration and increased therapeutic efficacy, we isolated primary hepatocytes from one recipient mouse after recovery from partial hepatectomy and transplanted $1x10^6$ cells each into the spleens of the secondary *Fah*$^{-/-}$ recipient mice (Figure 1b). All recipient animals (6/6) that were transplanted with hepatocytes from repopulated

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Fah$^{-/-}$ mice showed liver repopulation and survived long-term in the absence of NTBC (Figure 2d and 2e).

Missing long-term in vivo correction of Fah in the absence of homologous sequences after hepatocyte transplantation

To establish unequivocally that homologous recombination is indeed capable of long-term stable correction of *Fah* deficiency and superior to non-homologous, episomal gene therapy, we generated a control group with five mice, who were injected with *rAAV8-TTR.Fah*. All five primary recipient mice survived with weight gain (Figure 3a) and showed clusters of FAH-positive cells at partial hepatectomy on day 45 (Figure 3c). To show inferiority of this episomal approach we further increased the proliferation conditions by transplanting hepatocytes (1x10^6 cells for each recipient) from one first generation recipient mouse into 5 secondary *Fah*$^{-/-}$ recipient mice in this group also. Only one of the five secondary recipient mice (hepatocyte recipients) survived NTBC withdrawal and showed few and small FAH-positive cell clusters (Figure 3d and 3e). Hence, these results suggest that in the absence of homologous arms, the observed FAH-positive clusters in the primary recipient *Fah*$^{-/-}$ mice mostly resulted from epigenomic AAVs or an unexplained mechanism of integration/anchorage on cellular DNA, which was lost upon transplantation into secondary *Fah*$^{-/-}$ recipient mice.

Successful targeted integration of Fah cDNA at the Rosa26 locus

So far, our results revealed that mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* had robust liver repopulation and improved survival after secondary transplantation. However, it is important to prove that homologous arms facilitated targeted integration/gene addition of *Fah* cDNA into the *Rosa26* locus. We therefore examined targeted integration by genomic PCR amplifying portions of the *Rosa26* gene locus and the *Fah* transgene cassette. Indeed, we found an expected band of 1071 bp in mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (Figure 4)

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

but not in mice injected with *rAAV8-TTR.Fah*. Our data thus indicate that homologous arms facilitated targeted integration at a frequency sufficient for increased therapeutic outcome and phenotypic correction in *Fah*$^{-/-}$ mice. This is further confirmed by Sybr green qRT-PCR results. These showed a clearly higher expression of FAH in mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* compared to mice treated with *rAAV8-TTR.Fah* alone (Figure 5).

In summary, we can conclude that in the first generation we could not detect a difference for survival, weight gain and FAH positive cell cluster between mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* or *rAAV8-TTR.Fah* but in secondary generation (recipients of 1x10^6 hepatocytes from first generation) we could detect a clear improved survival for the group with homologous arms in the vector. In this group 6 out of 6 mice survived and in the other group 1 out 5 mice survived. Furthermore the detection FAH positive cell clusters showed the same distribution.

DISCUSSION

In in vitro and in vivo studies[18,19], the AAV vector is used as the vector of choice for gene correction approaches by homologous recombination; one important reason is its single-stranded nature. Reports on gene correction or gene addition by homologous recombination for liver-based metabolic diseases are rare and have shown correction frequencies[20] too low for phenotypic correction, except for the study of Paulk et al[21]. However, they used a mouse model with a point mutation for *Fah* gene; therefore, their approach was a gene correction. Here, we provide proof of concept for in vivo targeted gene addition mediated by homologous recombination in a liver-based metabolic disease. Our findings demonstrate that in a state of extensive hepatocyte proliferation, targeted integration by homologous recombination was superior to gene therapy based on episomal AAV gene therapy.

Baishideng Publishing Group
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Primary recipient mice that were injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* or *rAAV8-TTR.Fah* survived and showed phenotypic rescue after NTBC withdrawal. Notably, livers of mice from both groups showed clear FAH-positive cell clusters in immunohistochemistry. We determined the presence of FAH positive area in the both groups of primary recipients. We did not find significant differences in FAH positivity indicating similar number of FAH positive hepatocytes in both groups of mice. So far, cell clusters have always been explained by clonal expansion of corrected hepatocytes, which would implicate the necessity of vector integration. In the tyrosinemia mouse model *Fah* corrected hepatocytes have a strong selective advantage so they grow clonally, form nodules and can repopulate the entire liver at least[22,23]. Therefore, it is reasonable that a small number of hepatocytes with random integrations or another unexplained mechanism such as of integration/anchorage on cellular DNA proliferate preferentially and repopulate the diseased liver, leading to FAH-positive cell clusters. A human liver contains approximately 300 billion hepatocytes, which means, in case of 10% transduction efficiency with an integration rate of 0.1%, a single individual will have ~30 million hepatocytes with at least one integration event[24]. Therefore, one can assume that the phenotypic correction in these mice can be explained by the selective proliferation advantage of a small number of hepatocytes with successfully integrated *Fah* cassettes. A spontaneous reversion of the genetic defect, as the underlying cause for phenotypic correction and FAH-positive cell clusters, as described in humans[25], is not possible in the *Fah*^{exon5} mouse model[26].

Therefore we increased the proliferation conditions by hepatocyte transplantation form one first generation recipient per group into secondary *Fah*$^{-/-}$ recipient mice ($1x10^6$ hepatocytes for each secondary recipient mouse). In this experiment, the advantage of homologous recombination became clearly visible, since phenotypic correction could be achieved in all mice (6/6). In the *rAAV8-TTR.Fah* group, only 1/5

Baishideng Publishing Group
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

mice survived. In accordance with these results, 6/6 mice co-injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* showed clear FAH-positive cell clusters in livers, whereas only 1/5 mice injected with *rAAV8-R26.Fah* had FAH-positive clusters. Furthermore Sybr green qRT-PCR showed higher FAH expression in liver tissue of *ROSA26.HAL-TTR.Fah-ROSA26HAR-mice* than in *rAAV8-R26.Fah-mice.*

Partial hepatectomy and serial transplantation together are supposed to have triggered at least 30 rounds of cell doubling for the hepatocytes[27], nevertheless we could not find any tumour formation in any of our mice. This is in line with other studies showing a good safety profile for rAAV8 gene therapy[5]. Our proof of concept approach demonstrated that the targeted integration/addition of a therapeutic gene allows for safer (compared to random integration) and more efficient (compared to epigenomic) gene therapy, especially for gene therapy of liver-based metabolic diseases in paediatric patients, since the *Rosa26* locus exists in mice[28,29] as well as in humans[30]. In contrast to the assumption that homologous recombination alone is not sufficient for a long-lasting phenotypic correction of a liver-based metabolic disease, we could show the opposite with this study, at least for diseases with selection advantage for corrected hepatocytes, like tyrosinaemia type 1. Further potential target diseases with selection advantage could be Wilson disease or bile-acid transporter defects. Continuing studies should evaluate the efficiency of this approach in liver-based metabolic diseases without selection advantage such as Crigler Najjar Syndrome.

In summary, we demonstrate that targeted in vivo integration of a *Fah* expression cassette mediated by homologous arms is a highly efficient approach to stably correct a metabolic liver disease in an FAH mouse model with extensive hepatocyte proliferation. Since many metabolic disorders must already be treated in children with fast-dividing hepatocytes, targeted transgene integration is an important step to safe and long-lasting gene therapy in the developing liver.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

ARTICLE HIGHLIGHTS

Research background

We describe an important proof of concept in the field of AAV gene therapy for liver based metabolic diseases (LBMD). First gene therapy studies in humans are done (Hemophilia B) or very ready to start (Crigler-Najjar Syndrome); even an EMA approved drug for AAV gene therapy (Glybera) exists already. But all these approaches have a major weakness, the missing permanence of the gene therapy effect, especially in young children. But they are the main target group for gene therapy in LBMD, since early therapy could avoid irreversible damage to the organs of the patient. In these patients the advantage of recombinant AAV gene therapy, the almost missing integration into the host genome turns into a disadvantage since donor cDNA will be lost during cell turn over.

Research motivation

Targeted integration into safe harbors like the ROSA26 locus could overcome the problem of diminishing donor-cDNA in rAAV gene therapy. There are studies, showing proof of concept for targeted integration with nucleases like zinc fingers or CRISP/CAS9, but these approaches contains also new potential sources of side effects. However in our study only natural appearing cellular repair mechanism has been used to generate a targeted integration.

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Research objectives

Up to know it was assumed that the efficiency of gene addition by targeted integration into a safe harbor mediated by homologous recombination would be to low for phenotypic correction of liver based metabolic diseases (LBMD) in growing livers. But we could show in a disease model for LBMD with selection advantage of corrected hepatocytes that this is not the case. This could be transferred to other diseases like the group of familial intrahepatic cholestasis or Wilson disease or even to diseases with less selection advantage.

Research methods

C57BL/6 *Fah*$^{\Delta exon5}$ mice served as an animal model for human tyrosinaemia type 1 in our study. We treated these mice with a rAAV Vector containing human Fah cDNA, a liver specific promotor (TTR) and homologous arms for ROSA26 locus. We compared this group to mice treated with a vector without homologous arms. Hepatocyte proliferation was induced by partial hepatectomy and serial hepatocyte transplantation. Survival of mice without NTBC and existence of FAH positive cell cluster at immunohistochemistry staining on liver tissue of the mice were the main endpoints.

Research results

We could show for the first time proof of concept for phenotypic correction of a LBMD in a mouse model under conditions of extensive hepatocyte proliferation with rAAV mediated gene addition by targeted integration at a safe harbor without the

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

use of nucleases or gene repair. Further studies have to show if this concept is transferable to LBMD with less section advantage of corrected hepatocytes.

Research conclusions

Our study shows that phenotypic correction of a LBMD by rAAV gene therapy under conditions of extensive hepatocyte proliferation is possible with homologous recombination (HR) alone and does not necessarily have the need for nucleases.

In conclusion we showed that HR-mediated rAAV8 gene therapy provides targeted transgene integration and phenotypic correction in *Fah*$^{-/-}$ mice with superior long-term efficacy compared to episomal rAAV8 therapy in proliferating livers.

In opposite to approaches with the aim of point mutation repair on genes of LBMD our system with gene addition into a safe harbour can be easily transferred to other LBMDs and is not mutation specific.

Research perspectives

Our results are an important step into the solution of a main clinical problem for gene therapy of LBMD, since mostly this therapy is mandatory in growing children, where episomal gene therapy is not lasting. In opposite to studies with nucleases our study focus on a natural mechanism for targeted integration which avoids potential side effects of nucleases. A very important question for following studies would be if these results could also be observed in LBMD with less selection advantage for corrected hepatocytes (e.g. Crigler-Najjar Syndrom).

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

ACKNOWLEDGEMENTS:

We would like to thank Sabine Brandes and Nico Jäschke for support and Rebirth, SFB 738 and the "Deutsche Forschungsgemeinschaft" (Gerok-Grant) for financial support.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

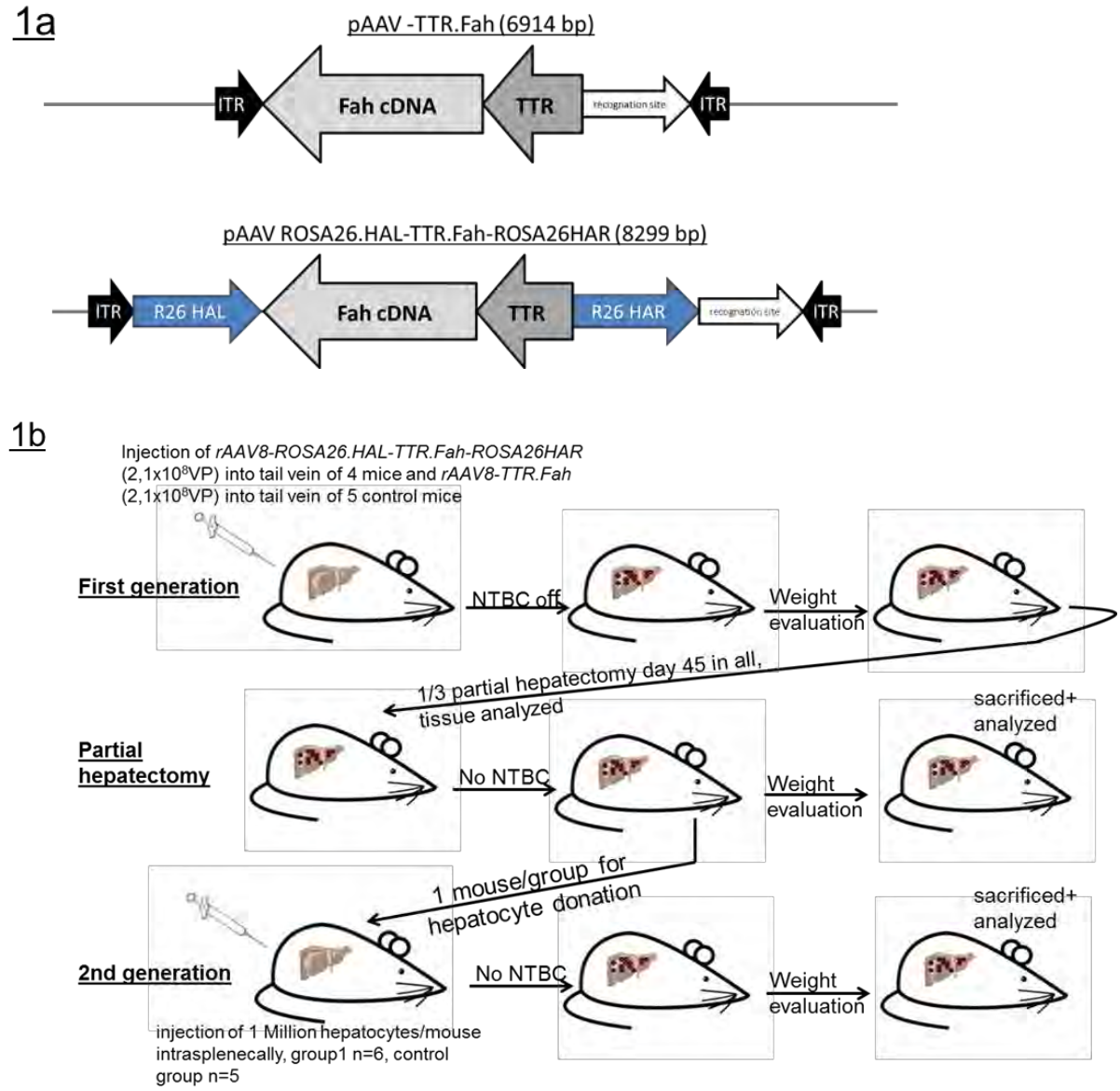


Figure 1:

(a) Vector map for ***rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR*** and for ***rAAV8-TTR.Fah***. Fah cDNA is driven by the TTR promotor and for ***rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR*** located between the homologous arms of the *Rosa26* locus. The vector was cloned into an AAV backbone. TTR=transthyretin promoter (liver specific), R26 HAL=homologous arm left for target locus in *Rosa26*, HAR=homologous arm right for target locus in *Rosa26*, ITR=inverted terminal repeat.

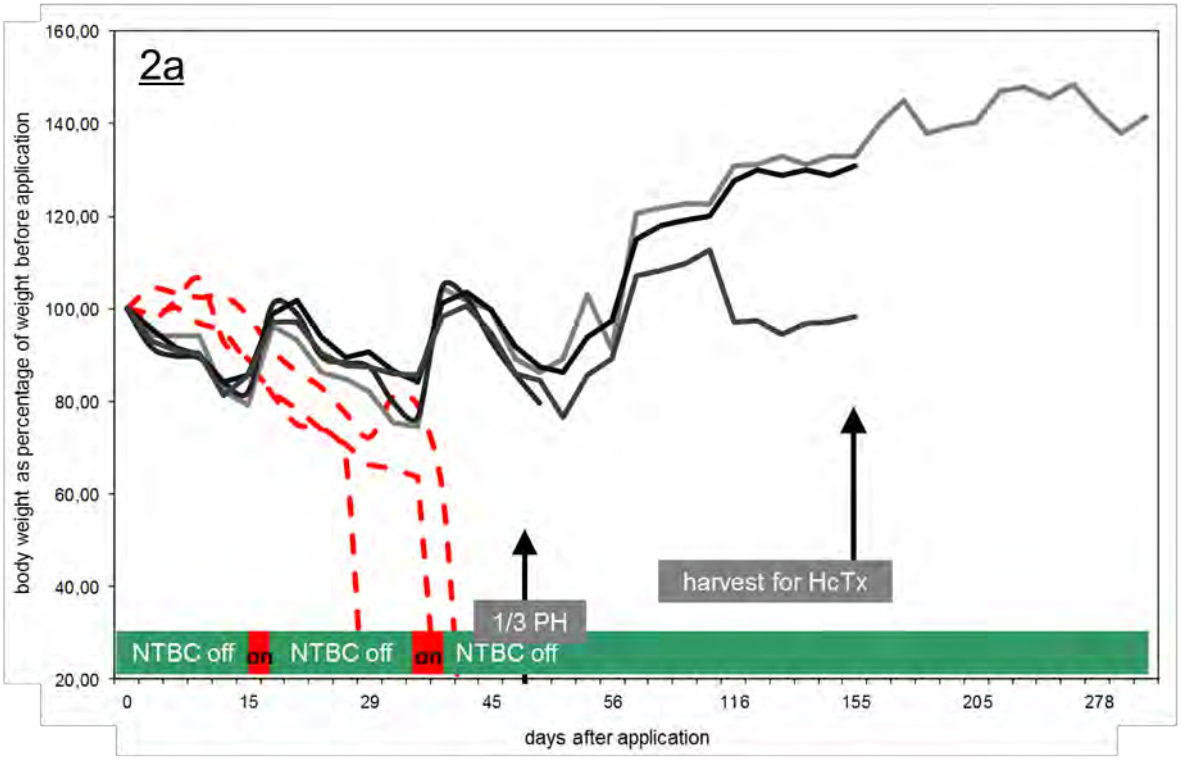
Baishideng Publishing Group

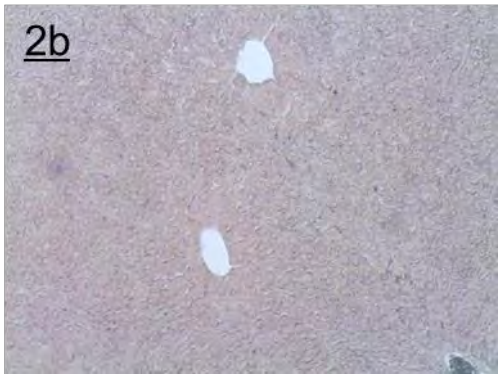
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

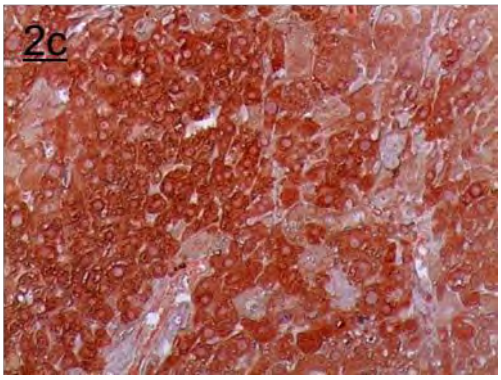
(b) Scheme for the in vivo experiments. First-generation mice (*C57BL/6 FAH*$^{\Delta exon5}$ strain) were injected with ***rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR*** *(group 1, N=4) or* ***rAAV8-TTR.Fah*** *(control group, N=5).* The NTBC treatment was stopped, and after 45 days, a partial hepatectomy was performed. In each group, one mouse was used as the donor for hepatocyte transplantation into *C57BL/6 Fah*$^{\Delta exon5}$ mice. These recipients were the second generation of mice in our study. NTBC treatment was discontinued after hepatocyte transplantation.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com







Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

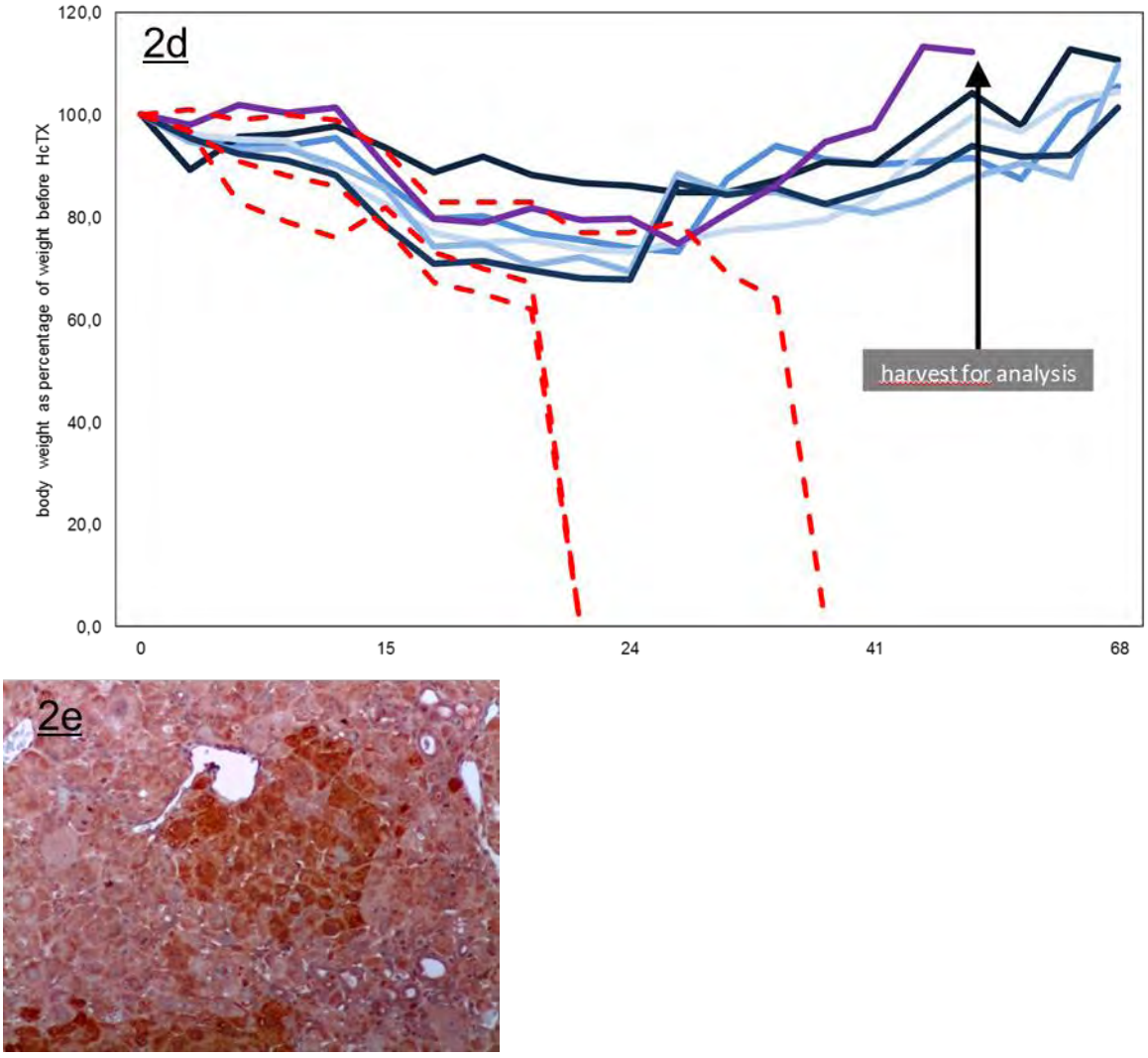


Figure 2: Mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (a) Weight graph and survival for first-generation mice (N=4) injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and 3 untreated controls (injected with sodium chloride). Continuous line=*rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR mice*, broken line=controls (same control mice as displayed at Figure 3a). Body weight is displayed as percentage of body weight at the time of virus injection or sodium chloride injection (controls). The timeline (x-axis) is displayed in days beginning with the day of virus/sodium chloride injection as day zero. 1/3PH=one third partial hepatectomy; HcTx=hepatocyte transplantation.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

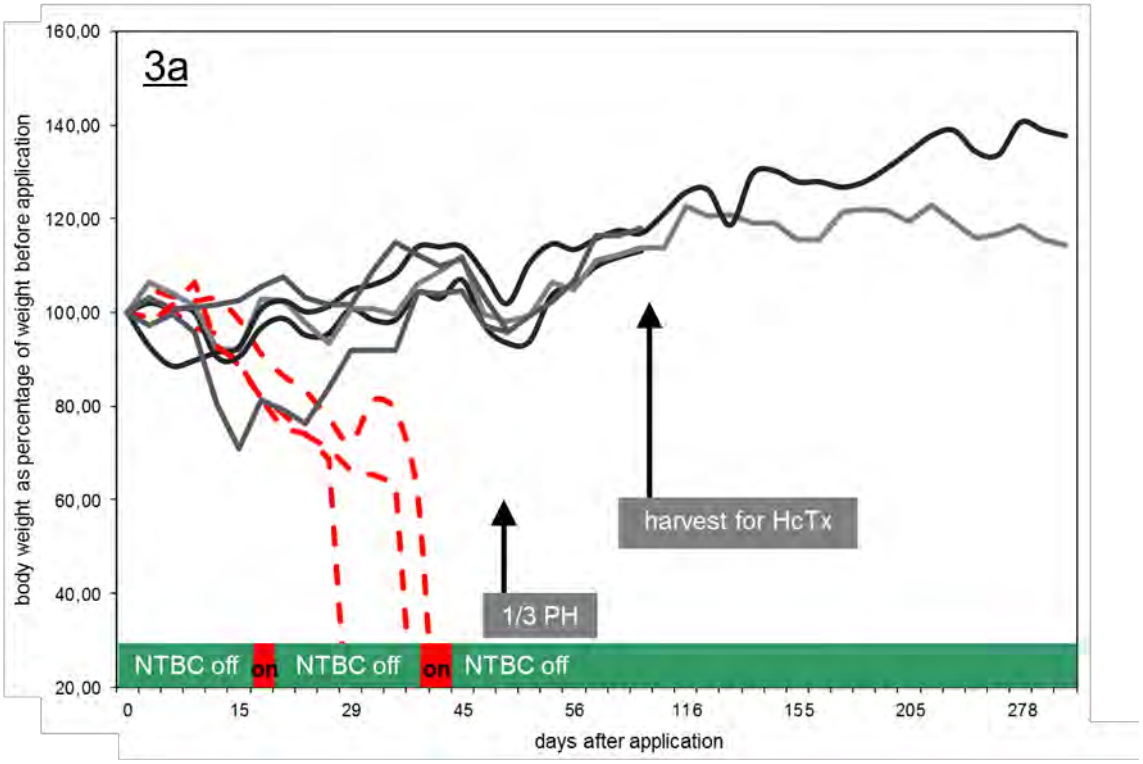
(b) FAH staining of liver tissue from controls (mice with sodium chloride injection) after death (100x magnification). **(c)** FAH staining of liver tissue from partial hepatectomy in mouse injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (100x magnification). **(d)** Weight graph and survival for second-generation mice (continuous line), which were transplanted with one million hepatocytes from mice primarily injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and controls (same control mice as displayed at Figure 3d) without hepatocyte transplantation (broken line). Body weight is displayed as percentage of body weight at time of hepatocyte transplantation. The timeline (x-axis) is displayed in days, beginning with the day of hepatocyte transplantation as day zero. **(e)** FAH staining of liver tissue from a partial hepatectomy from a second-generation mouse, which received one million hepatocytes from mice primarily injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (100x magnification).

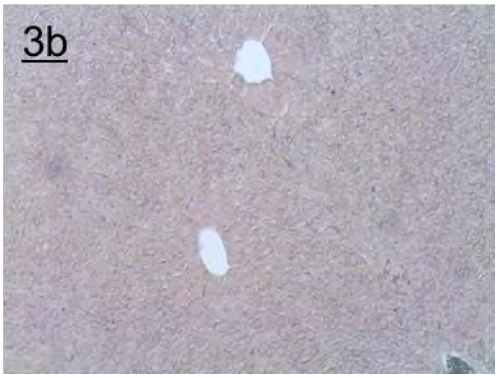
Baishideng Publishing Group

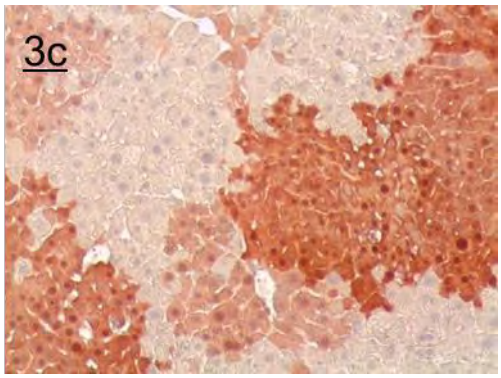
7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com







Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

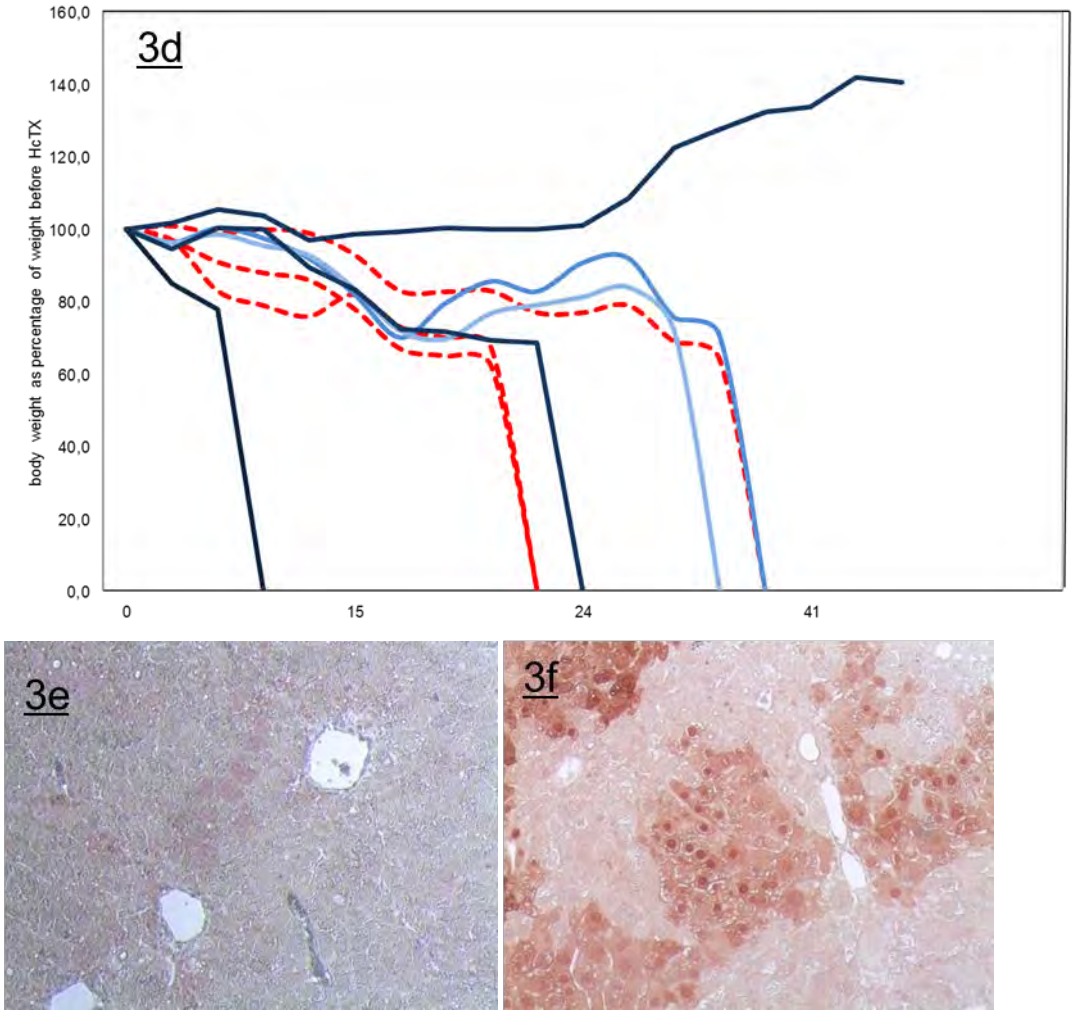


Figure 3: Mice treated with *rAAV8-TTR.Fah* **(a)** Weight graph and survival for first-generation mice injected with *rAAV8-TTR.Fah* (N=5) and 2 untreated controls (injected with sodium chloride). Continuous line=*rAAV8-TTR.Fah mice*, broken line=controls (same control mice as displayed at Figure 2a). Body weight is displayed as percentage of body weight at the time of virus injection or sodium chloride injection (controls). The timeline (x-axis) is displayed in days beginning with the day of virus/sodium chloride injection as day zero. **(b)** FAH staining of liver tissue from controls (mouse with sodium chloride injection) after death (100x magnification). **(c)** FAH staining of liver tissue from a partial hepatectomy from a mouse injected with *rAAV8-TTR.Fah* (100x magnification). **(d)** Weight graph and survival for second-

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

generation mice (continuous line), which were transplanted with one million hepatocytes from mice primarily injected with *rAAV8-TTR.Fah* and controls (same control mice as displayed at Figure 2d) without hepatocyte transplantation (broken line). Body weight is displayed as percentage of body weight at time of hepatocyte transplantation. The timeline (x-axis) is displayed in days, beginning with the day of hepatocyte transplantation as day zero. **(e)** FAH staining of liver tissue from a partial hepatectomy in a second-generation mouse, which received one million hepatocytes from mice primarily injected with *rAAV8-TTR.Fah* (100x magnification). **(f)** FAH staining of liver tissue from a partial hepatectomy from the single second-generation mouse that showed cluster and weight gain.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

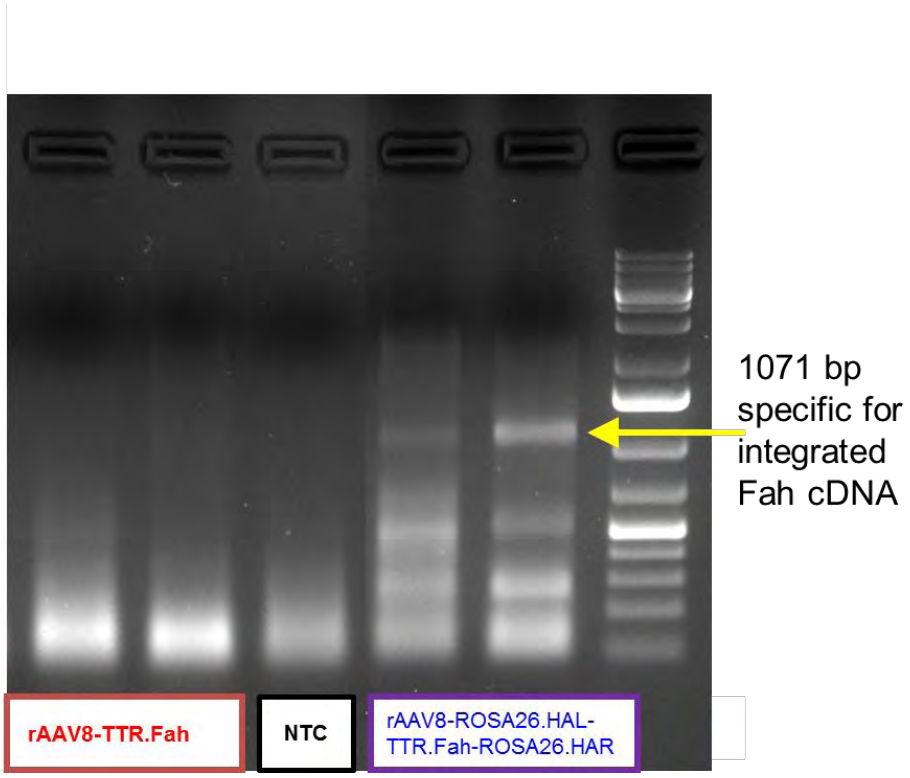


Figure 4: Integration PCR gel electrophoresis. A representative gel picture from the analyses of genomic liver DNA, that was extracted from snap-frozen liver tissue harvested between 60-70 days after hepatocyte transplantation. Primers were located in the *Rosa26* locus and in the FAH sequence of the donor DNA. Product could only be amplified if targeted integration occurred. The expected length of the PCR amplicon was 1107 bp. The PCR product was analysed utilizing agarose gel electrophoresis.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

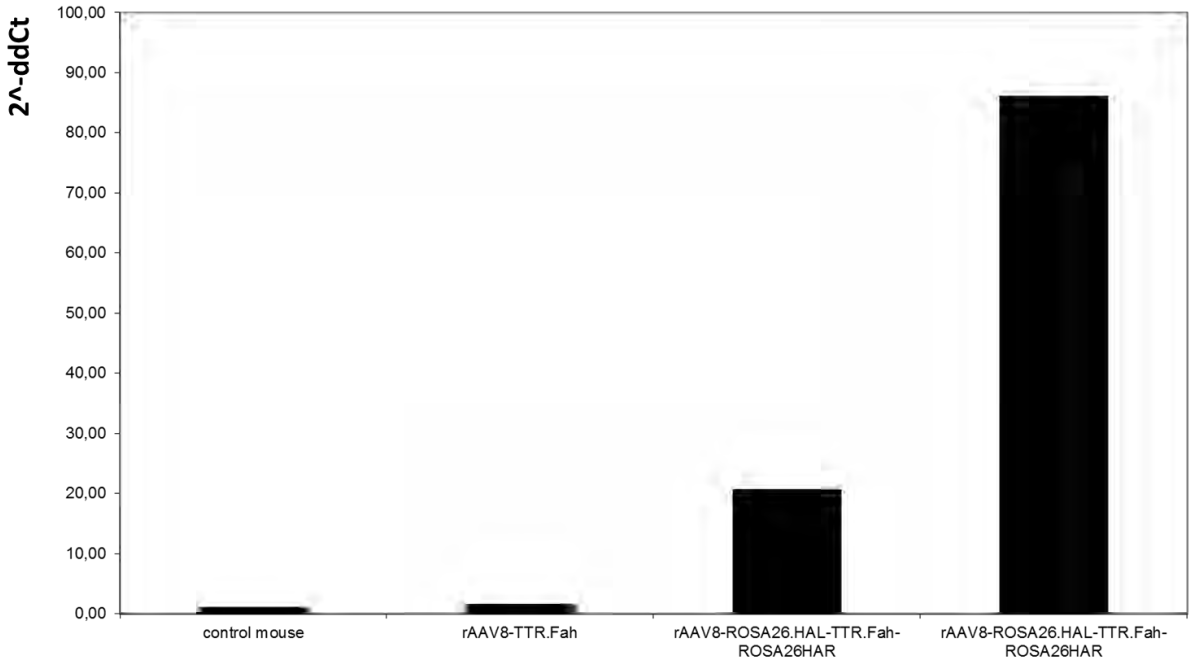


Figure 5: Sybr green qRT- PCR. Shown are the 2^-ddct values of two mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and one mouse treated with *rAAV8-TTR.Fah* calculated on an untreated control mouse.

Baishideng Publishing Group

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

References

1 Nathwani AC, Reiss UM, Tuddenham EG, Rosales C, Chowdary P, McIntosh J, Della Peruta M, Lheriteau E, Patel N, Raj D, Riddell A, Pie J, Rangarajan S, Bevan D, Recht M, Shen YM, Halka KG, Basner-Tschakarjan E, Mingozzi F, High KA, Allay J, Kay MA, Ng CY, Zhou J, Cancio M, Morton CL, Gray JT, Srivastava D, Nienhuis AW, Davidoff AM. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med* 2014; **371**: 1994-2004 [PMID: 25409372 DOI:10.1056/NEJMoa1407309 [doi]]

2 Nathwani AC, Tuddenham EG, Rangarajan S, Rosales C, McIntosh J, Linch DC, Chowdary P, Riddell A, Pie AJ, Harrington C, O'Beirne J, Smith K, Pasi J, Glader B, Rustagi P, Ng CY, Kay MA, Zhou J, Spence Y, Morton CL, Allay J, Coleman J, Sleep S, Cunningham JM, Srivastava D, Basner-Tschakarjan E, Mingozzi F, High KA, Gray JT, Reiss UM, Nienhuis AW, Davidoff AM. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med* 2011; **365**: 2357-2365 [PMID: 22149959 DOI:10.1056/NEJMoa1108046 [doi]]

3 Junge N, Mingozzi F, Ott M, Baumann U. Adeno-associated virus vector-based gene therapy for monogenetic metabolic diseases of the liver. *J Pediatr Gastroenterol Nutr* 2015; **60**: 433-440 [PMID: 25594875 DOI:10.1097/MPG.0000000000000703 [doi]]

4 Paneda A, Vanrell L, Mauleon I, Crettaz JS, Berraondo P, Timmermans EJ, Beattie SG, Twisk J, van Deventer S, Prieto J, Fontanellas A, Rodriguez-Pena MS, Gonzalez-Aseguinolaza G. Effect of adeno-associated virus serotype and genomic structure on liver transduction and biodistribution in mice of both genders. *Hum Gene Ther* 2009; **20**: 908-917 [PMID: 19419275 DOI:10.1089/hum.2009.031 [doi]]

5 Hasbrouck NC, High KA. AAV-mediated gene transfer for the treatment of hemophilia B: problems and prospects. *Gene Ther* 2008; **15**: 870-875 [PMID: 18432276 DOI:10.1038/gt.2008.71 [doi]]

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

6 High KA, Aubourg P. rAAV human trial experience. *Methods Mol Biol* 2011; **807**: 429-457 [PMID: 22034041 DOI:10.1007/978-1-61779-370-7_18 [doi]]

7 Inagaki K, Piao C, Kotchey NM, Wu X, Nakai H. Frequency and spectrum of genomic integration of recombinant adeno-associated virus serotype 8 vector in neonatal mouse liver. *J Virol* 2008; **82**: 9513-9524 [PMID: 18614641 DOI:10.1128/JVI.01001-08 [doi]]

8 Donsante A, Miller DG, Li Y, Vogler C, Brunt EM, Russell DW, Sands MS. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* 2007; **317**: 477 [PMID: 17656716 DOI:317/5837/477 [pii]]

9 Bell P, Wang L, Lebherz C, Flieder DB, Bove MS, Wu D, Gao GP, Wilson JM, Wivel NA. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol Ther* 2005; **12**: 299-306 [PMID: 16043099 DOI:S1525-0016(05)00144-9 [pii]]

10 Niemeyer GP, Herzog RW, Mount J, Arruda VR, Tillson DM, Hathcock J, van Ginkel FW, High KA, Lothrop CD,Jr. Long-term correction of inhibitor-prone hemophilia B dogs treated with liver-directed AAV2-mediated factor IX gene therapy. *Blood* 2009; **113**: 797-806 [PMID: 18957684 DOI:10.1182/blood-2008-10-181479 [doi]]

11 Nathwani AC, Gray JT, McIntosh J, Ng CY, Zhou J, Spence Y, Cochrane M, Gray E, Tuddenham EG, Davidoff AM. Safe and efficient transduction of the liver after peripheral vein infusion of self-complementary AAV vector results in stable therapeutic expression of human FIX in nonhuman primates. *Blood* 2007; **109**: 1414-1421 [PMID: 17090654 DOI:blood-2006-03-010181 [pii]]

12 Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis* 2009; **199**: 381-390 [PMID: 19133809 DOI:10.1086/595830 [doi]]

13 Grompe M, al-Dhalimy M, Finegold M, Ou CN, Burlingame T, Kennaway NG, Soriano P. Loss of fumarylacetoacetate hydrolase is responsible for the neonatal hepatic dysfunction phenotype of lethal albino mice. *Genes Dev* 1993; **7**: 2298-2307 [PMID: 8253378]

14 Rittelmeyer I, Rothe M, Brugman MH, Iken M, Schambach A, Manns MP, Baum C, Modlich U, Ott M. Hepatic lentiviral gene transfer is associated with clonal

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

selection, but not with tumor formation in serially transplanted rodents. *Hepatology* 2013; **58**: 397-408 [PMID: 23258554 DOI:10.1002/hep.26204 [doi]]

15 Yuan Q, Loya K, Rani B, Mobus S, Balakrishnan A, Lamle J, Cathomen T, Vogel A, Manns MP, Ott M, Cantz T, Sharma AD. MicroRNA-221 overexpression accelerates hepatocyte proliferation during liver regeneration. *Hepatology* 2013; **57**: 299-310 [PMID: 22821679 DOI:10.1002/hep.25984 [doi]]

16 Sharma AD, Narain N, Handel EM, Iken M, Singhal N, Cathomen T, Manns MP, Scholer HR, Ott M, Cantz T. MicroRNA-221 regulates FAS-induced fulminant liver failure. *Hepatology* 2011; **53**: 1651-1661 [PMID: 21400558 DOI:10.1002/hep.24243 [doi]]

17 Song G, Pacher M, Balakrishnan A, Yuan Q, Tsay HC, Yang D, Reetz J, Brandes S, Dai Z, Putzer BM, Arauzo-Bravo MJ, Steinemann D, Luedde T, Schwabe RF, Manns MP, Scholer HR, Schambach A, Cantz T, Ott M, Sharma AD. Direct Reprogramming of Hepatic Myofibroblasts into Hepatocytes In Vivo Attenuates Liver Fibrosis. *Cell Stem Cell* 2016; **18**: 797-808 [PMID: 26923201 DOI:10.1016/j.stem.2016.01.010 [doi]]

18 Inoue N, Dong R, Hirata RK, Russell DW. Introduction of single base substitutions at homologous chromosomal sequences by adeno-associated virus vectors. *Mol Ther* 2001; **3**: 526-530 [PMID: 11319913 DOI:10.1006/mthe.2001.0283 [doi]]

19 Paulk NK, Wursthorn K, Wang Z, Finegold MJ, Kay MA, Grompe M. Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo. *Hepatology* 2010; **51**: 1200-1208 [PMID: 20162619 DOI:10.1002/hep.23481 [doi]]

20 Miller DG, Wang PR, Petek LM, Hirata RK, Sands MS, Russell DW. Gene targeting in vivo by adeno-associated virus vectors. *Nat Biotechnol* 2006; **24**: 1022-1026 [PMID: 16878127 DOI:nbt1231 [pii]]

21 Paulk NK, Wursthorn K, Wang Z, Finegold MJ, Kay MA, Grompe M. Adeno-associated virus gene repair corrects a mouse model of hereditary tyrosinemia in vivo. *Hepatology* 2010; **51**: 1200-1208 [PMID: 20162619 DOI:10.1002/hep.23481 [doi]]

7901 Stoneridge Drive, Suite 501,
Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
https://www.wjgnet.com

22 Manning K, Al-Dhalimy M, Finegold M, Grompe M. In vivo suppressor mutations correct a murine model of hereditary tyrosinemia type I. *Proc Natl Acad Sci U S A* 1999; **96**: 11928-11933 [PMID: 10518553]

23 Overturf K, Al-Dhalimy M, Manning K, Ou CN, Finegold M, Grompe M. Ex vivo hepatic gene therapy of a mouse model of Hereditary Tyrosinemia Type I. *Hum Gene Ther* 1998; **9**: 295-304 [PMID: 9508047 DOI:10.1089/hum.1998.9.3-295 [doi]]

24 Valdmanis PN, Lisowski L, Kay MA. rAAV-mediated tumorigenesis: still unresolved after an AAV assault. *Mol Ther* 2012; **20**: 2014-2017 [PMID: 23131853 DOI:10.1038/mt.2012.220 [doi]]

25 Kvittingen EA, Rootwelt H, Brandtzaeg P, Bergan A, Berger R. Hereditary tyrosinemia type I. Self-induced correction of the fumarylacetoacetase defect. *J Clin Invest* 1993; **91**: 1816-1821 [PMID: 8473520 DOI:10.1172/JCI116393 [doi]]

26 Overturf K, Al-Dhalimy M, Tanguay R, Brantly M, Ou CN, Finegold M, Grompe M. Hepatocytes corrected by gene therapy are selected in vivo in a murine model of hereditary tyrosinaemia type I. *Nat Genet* 1996; **12**: 266-273 [PMID: 8589717 DOI:10.1038/ng0396-266 [doi]]

27 Montini E, Held PK, Noll M, Morcinek N, Al-Dhalimy M, Finegold M, Yant SR, Kay MA, Grompe M. In vivo correction of murine tyrosinemia type I by DNA-mediated transposition. *Mol Ther* 2002; **6**: 759-769 [PMID: 12498772 DOI:S1525001602908129 [pii]]

28 Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P. Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A* 1997; **94**: 3789-3794 [PMID: 9108056]

29 Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 1999; **21**: 70-71 [PMID: 9916792 DOI:10.1038/5007 [doi]]

30 Irion S, Luche H, Gadue P, Fehling HJ, Kennedy M, Keller G. Identification and targeting of the ROSA26 locus in human embryonic stem cells. *Nat Biotechnol* 2007; **25**: 1477-1482 [PMID: 18037879 DOI:nbt1362 [pii]]