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AIM AND SCOPE

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WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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ORIGINAL ARTICLE

Basic Study

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

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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 QG 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 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.

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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 bp and 749 bp long) of the *Rosa26* gene locus. Mice were injected with 2.1 × 10^8 VP of this vector (*rAAV8-ROSA26.HAL-TTR.Fah-ROSA26.HAR*) *via* the tail vein. Mice in the control group were injected with 2.1 × 10^8 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 1 × 10^6 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 d before, 6 out of 6 mice showed long-term survival, 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.

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 *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, Ott M. Homologous recombination mediates stable *Fah* gene integration and phenotypic correction in tyrosinaemia mouse-model. *World J Hepatol* 2018; 10(2): 277-286 Available from: URL: http://www.wjgnet.com/1948-5182/full/v10/i2/277.htm DOI: http://dx.doi.org/10.4254/wjh.v10.i2.277

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 epige-

nomic 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 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, 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 d 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
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 vector
The adeno-associated virus serotype 8 (AAV8) vectors (Figure 1A), *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].

AAV8 vector administration into Fah$^{-/-}$ mice
Mice were injected with 2.1×10^8 VP *rAAV8-ROSA26. HAL-TTR.Fah-ROSA26HAR via* the tail vein. Mice in the control group were injected with 2.1×10^8 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 d, 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 (1×10^6) 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 1 x target retrieval solution

A

B
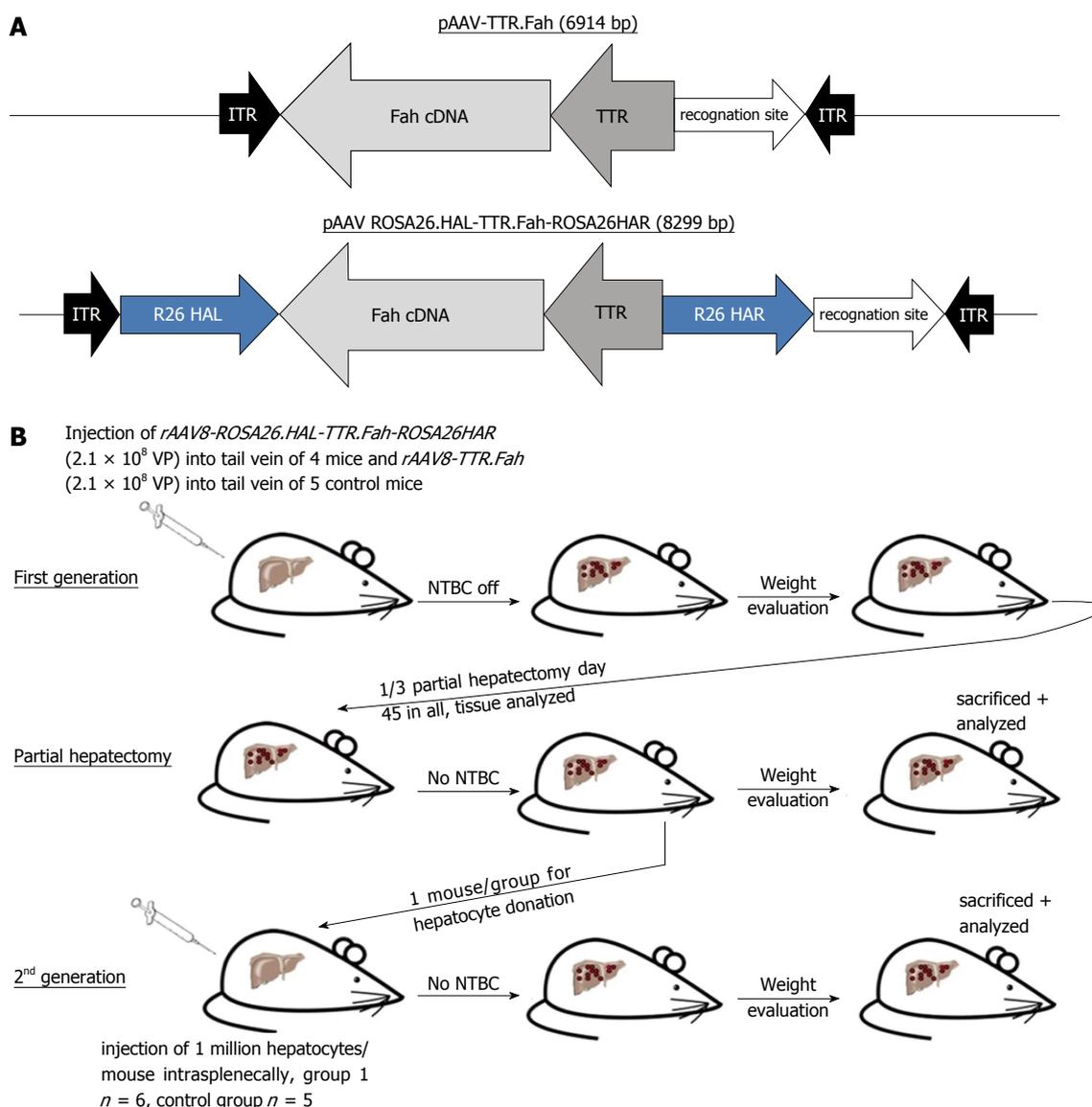
Figure 1 Flowchart of treating mice. A: Vector map for *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and for *rAAV8-TTR.Fah*. *Fah* cDNA is driven by the TTR promotor and for *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* located between the homologous arms of the Rosa26 locus. The vector was cloned into an AAV backbone; B: Scheme for the *in vivo* experiments. First-generation mice (*C57BL/6 FAH^Δexon5* 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 d, a partial hepatectomy was performed. In each group, one mouse was used as the donor for hepatocyte transplantation into *C57BL/6 Fah^Δexon5* mice. These recipients were the second generation of mice in our study. NTBC treatment was discontinued after hepatocyte transplantation. 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.

(Dako) at 98 ℃ for 20 min. 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).

Integration PCR
Genomic liver DNA was extracted from snap-frozen liver tissue with the DNeasy Blood and 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'-GGAGAGAGGCATTCAT GGGAGTGGAAAGTTAAGC-3' and B: 5'-GCAGCATGG TCCAGTACATGTGCTTAAAGTTAGACC-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 ℃, followed by 50 cycles for 10 s at 98 ℃ and 90 s at 72 ℃, finished by one cycle for 10 min at 72 ℃. The
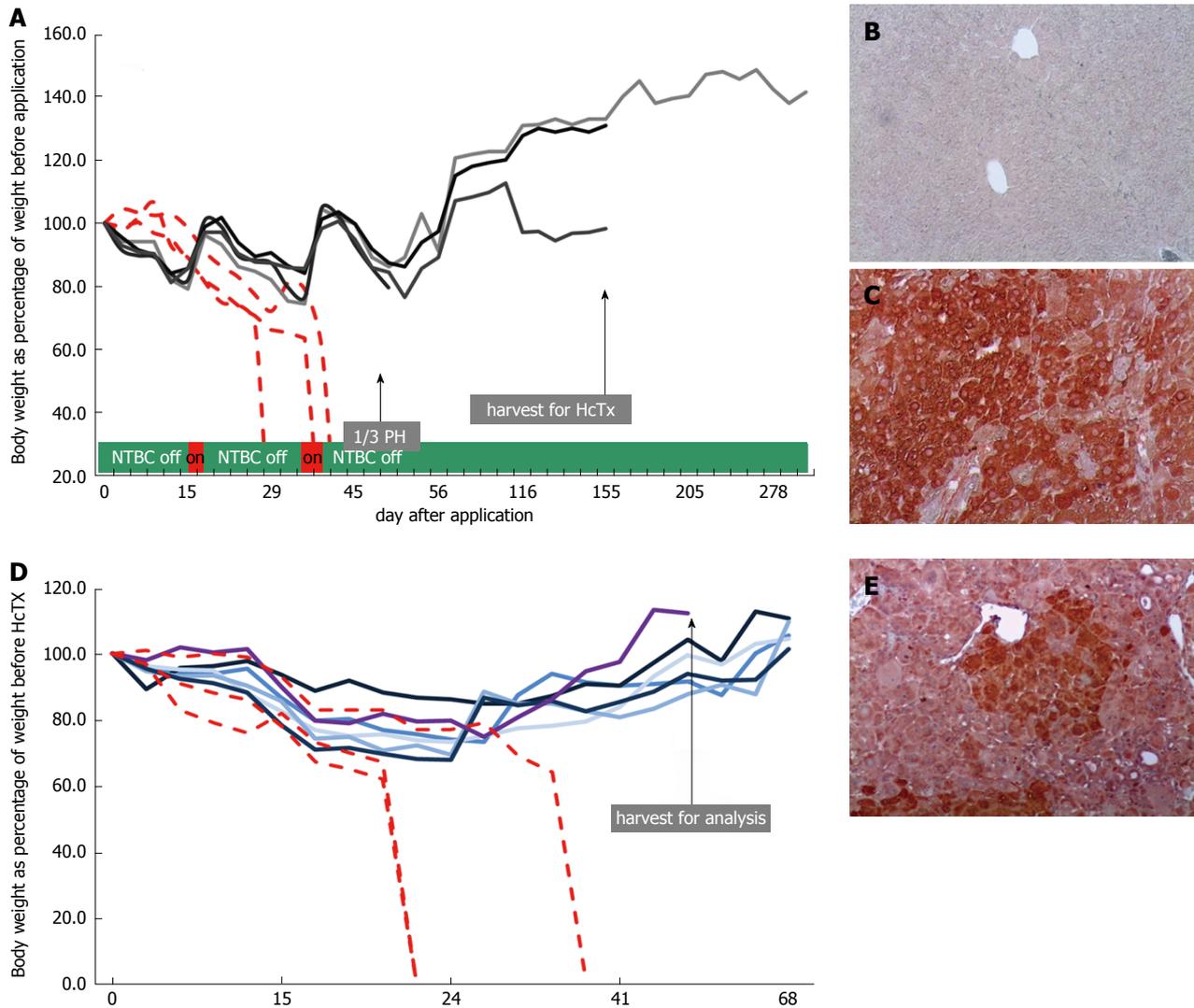
Figure 2 Mice treated with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR*. A: Weight graph and survival for first-generation mice (*n* = 4) injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* and 3 untreated controls (injected with sodium chloride). Continuous line = *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* mice, broken 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; B: FAH staining of liver tissue from controls (mice with sodium chloride injection) after death (100 × magnification); C: FAH staining of liver tissue from partial hepatectomy in mouse injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* (100 × 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* (100 × magnification). 1/3PH: One third partial hepatectomy; HcTx: Hepatocyte transplantation.

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

Long-term functional correction of the Fah gene defect by homologous recombination at the ROSA26 Locus in mice
We confirmed the correct design of our plasmids (Figure 1A) 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 d, all mice injected with *rAAV8-ROSA26.HAL-TTR.Fah-ROSA26HAR* survived beyond 45 d after injection (Figure 2A). On the 45th-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 1 x 10^6 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 *Fah*^{-/-} mouse showed liver repopulation and survived long-term in the absence of NTBC (Figure 2D and E).

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 (1 × 10^6 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 E). 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 trans-

plantation 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) 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 1 × 10^6 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*[19]. 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.

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
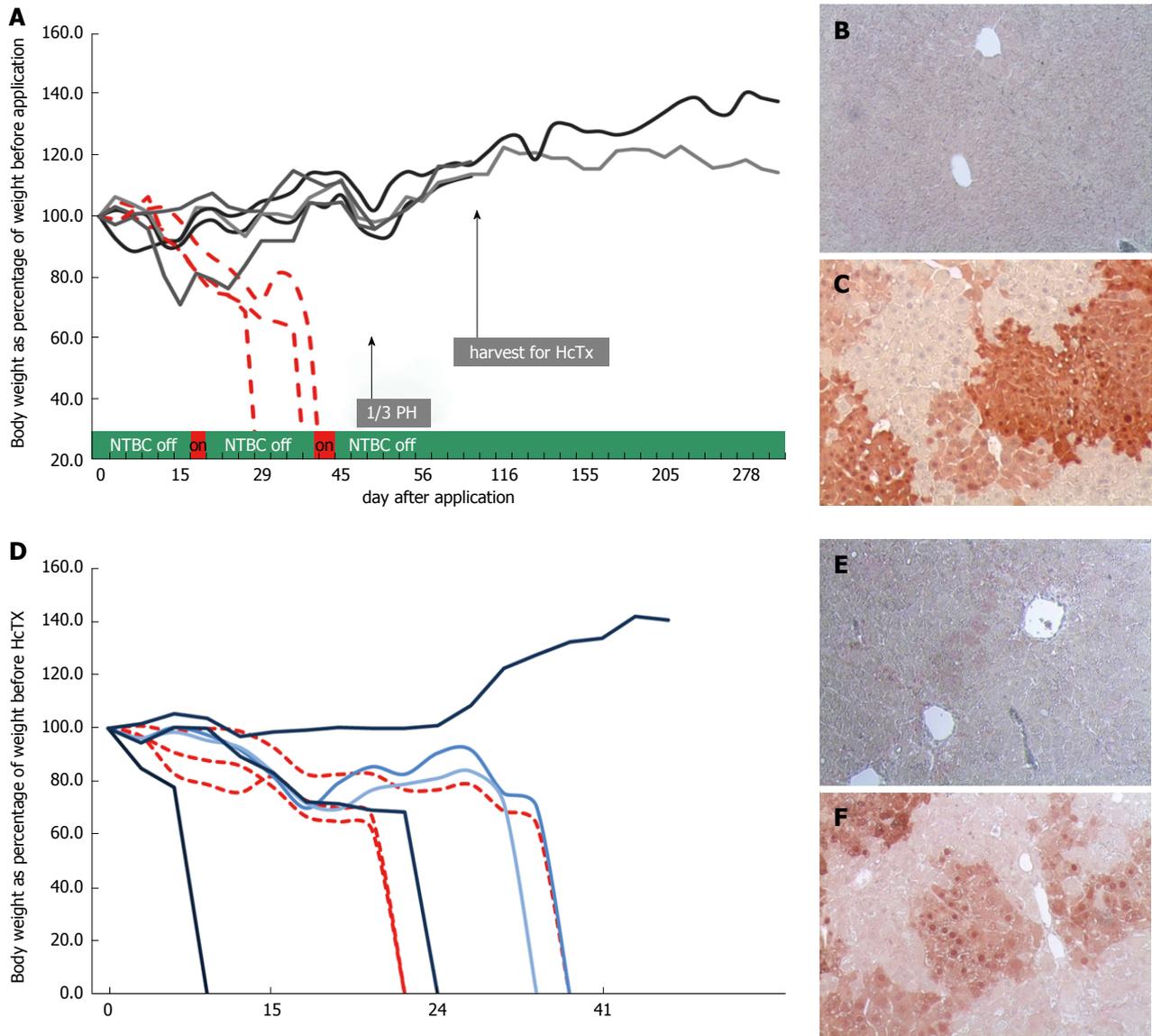
Figure 3 Mice treated with *rAAV8-TTR.Fah*. A: Weight graph and survival for first-generation mice injected with *rAAV8-TTR.Fah* (*n* = 5) and 3 untreated controls (injected with sodium chloride). Continuous line = *rAAV8-TTR.Fah* mice, broken line = controls (same control mice as displayed at Figure 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 (100 × magnification); C: FAH staining of liver tissue from a partial hepatectomy from a mouse injected with *rAAV8-TTR.Fah* (100 × 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-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* (100 × magnification); F: FAH staining of liver tissue from a partial hepatectomy from the single second-generation mouse that showed cluster and weight gain.

both groups showed clear FAH-positive cell clusters in immunohistochemistry. We determined the presence of FAH positive areas 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[21,22]. 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 approximately 30 million hepatocytes with at least one integration event[23]. Therefore, one can assume that the phenotypic correction in these mice can be explained by the selective proliferation advantage of a small
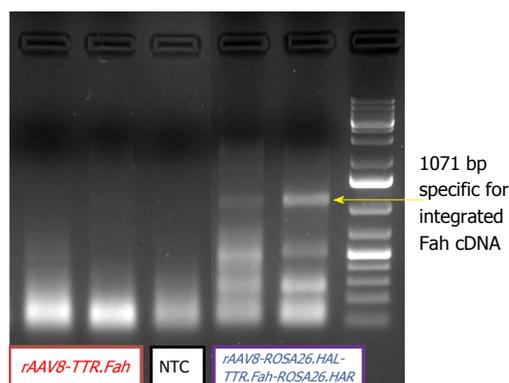
Figure 4 Integration PCR gel electrophoresis. A representative gel picture from the analyses of genomic liver DNA, that was extracted from snap-frozen liver tissue harvested between 60-70 d after hepatocyte transplantation. Primers were located in the *Rosa26* locus and in the FAH sequence of the donor 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.

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.

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[24], is not possible in the *Fah^exon5* mouse model[25].

Therefore we increased the proliferation conditions by hepatocyte transplantation from one first generation recipient per group into secondary *Fah^-/-* recipient mice (1 x 10^6 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 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[26], 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[27,28] as well as in humans[29]. 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
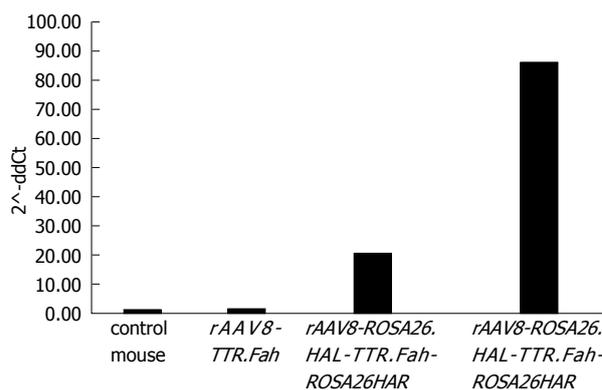
corrected hepatocytes, like tyrosinaemia type 1. Further potential target diseases with selection advantage could be Wilson disease or bile-acid transporter defects. Continuing studies should evaluate the efficiency of this approach in liver-based metabolic diseases without selection advantage such as 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.

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 contain 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.

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 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).

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